Energy Balance and Cancer 7

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Obesity, Inflammation and Cancer

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Obesity, Inflammation and Cancer

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Preface: What's Old Is New Again and Now It's Red Hot

As the worldwide obesity pandemic expands, obesity has been associated with an increased risk of more and more cancer types. The original malignancies shown to be associated with obesity included esophageal adenocarcinoma, colon cancer, renal cell cancer, postmenopausal breast cancer, endometrial cancer, and advanced prostate cancer. More recently, obesity has been identified as a risk factor for cancers of the pancreas, gall bladder, and ovary and several hematologic malignancies including leukemia, lymphomas, and myeloma, and the list continues to grow.

From a historical viewpoint, while early studies considered the possibility that inflammation initiated the process of carcinogenesis, this was generally considered to be a local effect associated with tissue injury or chronic infection. With elucidation of DNA structure and function and development of the concept of chemical carcinogens as mutagens, attention turned to identification of activated oncogenes and deactivated tumor suppressor genes in the carcinogenic process. Separate studies demonstrated that inflammation extended beyond the local site, mediated by cellular and humoral components. As noted above, independent epidemiologic studies confirmed an association of obesity with cancer incidence, morbidity, and mortality. Studies to identify the mediators of these processes focused on the effects of obesity on growth factors and hormones and the mechanisms of carcinogenesis they commonly affect. More recently, it has become apparent that adipose tissue, in addition to serving as a fat storage depot, is an intensely active metabolic organ. In obesity, low-grade chronic adipose tissue inflammation occurs, resulting in multiple cellular and humoral inflammatory factors. Seminal studies showing that systemic metabolic disorders, such as insulin resistance, could be mediated, in part, by inflammatory cytokines, synthesized and secreted by adipose tissue, resulted in a whole new approach to understanding and attempting to control obesity-associated comorbidities. Moreover, elucidation of the prostaglandin pathway and its role in inflammation, as well as the observations that anti-inflammatory agents, especially the nonsteroidal anti-inflammatory drugs (NSAIDs), could prevent the development and progression of several forms of neoplasia, provided a major stimulus to the field. A major goal of ongoing research is to inhibit inflammation as an approach to cancer prevention and control.

The above brief description traces the complex transdisciplinary evolution of this area of research endeavor. Not only does it illustrate the impact of sometimes divergent disciplines on the evolution of a concept, but it also indicates the potential value of moving forward in this field with a transdisciplinary approach. Accordingly, the goal of this volume of Energy Balance and Cancer, volume 7 in the series, is to highlight the cutting-edge transdisciplinary science linking obesity, inflammation, and cancer. We are grateful to all the authors listed below for their contributions to this volume and look forward to their collective impact in further advancing this rapidly developing field.

This volume first provides information on inflammation as an important link between obesity and insulin resistance, which is in itself linked to promotion of cancer through hyperinsulinemia. The volume then covers some of the most important mechanisms by which obesity leads to inflammation, including the novel inflammasome concept, alterations in chromatin structure, circulating inflammatory factors, unique cellular interactions between adipocytes and macrophages, and the direct link of dietary fat to inflammation and cancer. Subsequently addressed in this volume are a number of target organs and interventional strategies for disrupting inflammation and their effects on cancer prevention and control.

In Chap. 1, Lesley G. Ellies, Andrew Johnson, and Jerrold M. Olefsky (University of California, San Diego) describe the mechanisms by which obesity stimulates low-grade inflammation leading to insulin resistance. Chapter 2, written by Tuo Deng, Christopher J. Lyon, Nan Zhang, Helen Y. Wang, Rong-fu Wang, and Willa A. Hsueh (Weill Cornell Medical College) and Jun Cui (Sun Yat-sen University), reviews the basis for understanding the emerging concept of the inflammasome and its mechanisms of activation and role in obesity. Gerald V. Denis and Deborah J. Bowen (Boston University School of Public Health) describe in Chap. 3 chromatin-based, transcription co-regulatory mechanisms that may link obesity, inflammation, and cancer. Carey Nien-Kai Lumeng (University of Michigan Medical School), in Chap. 4, describes the important role that adipose tissue macrophages play in breast and ovarian cancer. In Chap. 5, Stephanie K. Doerner and Nathan A. Berger (Case Western Reserve University School of Medicine) discuss the impact of different dietary fatty acids on promoting or suppressing colorectal cancer. In Chap. 6, Anamay Sharma, Ahmed Elebiary, Sonia Chowdhury, and Navtej Buttar (Mayo Clinic) describe the contribution of gastric reflux to inflammation in Barrett's esophagus and esophageal adenocarcinoma and potential interventions. In Chap. 7, Stephanie K. Doerner (Case Western Reserve University School of Medicine) and Jason D. Heaney (Baylor College of Medicine) describe the role of obesity-induced intestinal inflammation on colorectal cancer incidence. In Chap. 8, Neil M. Iyengar, Patrick G. Morris and Clifford A. Hudis (Memorial Sloan-Kettering Cancer Center) and Andrew J. Dannenberg (Weill Cornell Medical College) review the emerging evidence supporting the contribution of adipose tissue and chronic breast inflammation to the development of breast cancer. In Chap. 9, the relation of obesity, inflammation, and hepatocellular cancer is discussed by Naim Alkhouri and Arthur McCullough (Cleveland Clinic Lerner College of Medicine at Case Western Reserve University), and in Chap. 10, Jorge Blando, Achinto Saha, Kaoru Kiguchi, and John

DiGiovanni (University of Texas at Austin) describes the role of obesity and inflammation in prostate cancer. Louise R. Howe (Weill Cornell Medical College), in Chap. 11, describes the central role of cyclooxygenase-derived prostaglandins as potential mediators of obesity-related cancer and outlines how targeting this pathway may be protective against obesity-associated carcinogenesis. In Chap. 12, Harmony F. Turk, Jennifer M. Monk, Tim Y. Hou, and Robert S. Chapkin (Texas A&M University) discuss mechanisms through which n-3 polyunsaturated fatty acids interfere with the inflammatory process to suppress carcinogenesis, and in Chap. 13, Gary Stoner and Li-Shu Wang (Medical College of Wisconsin) describe key mechanisms by which naturally occurring dietary compounds reduce the harmful effects of inflammation and the risk for cancer development. In Chap. 14, Stephen D. Hursting, Nikki A. Ford, Sarah M. Dunlap, and Laura M. Lashinger (University of Texas at Austin) and Marcie J. Hursting (Clinical Science Consulting) describe the modification of inflammatory pathways and their impact on cancer by diet and caloric restriction. Ahmad Salameh and Mikhail G. Kolonin, in Chap. 15, describe an innovative approach to adipose tissue control by vascular targeting. In Chap. 16, Michael Gleeson (Loughborough University) describes the anti-inflammatory effects of exercise.

Overall, this volume on Obesity, Inflammation, and Cancer provides an up-to-date status report on the latest developments and state-of-the-art understanding of the role of inflammation in mediating the effects of obesity on cancer and describes possible strategies for targeting inflammation as an approach to cancer prevention and control. The book should be useful for students, researchers, and clinicians, especially those interested in the role of inflammation and its impact on cancer. It is our expectation that this volume will both stimulate research on the role of inflammation in cancer etiology and progression and lead to new approaches and clinical trials for cancer prevention and control by targeting obesity-related inflammation.

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Chapter 1
Obesity, Inflammation, and Insulin Resistance

Lesley G. Ellies, Andrew Johnson, and Jerrold M. Olefsky

Abstract Obesity is a pressing public health concern as it leads to a collection of abnormalities often termed the metabolic syndrome. Molecular studies are revealing novel pathways by which obesity-associated hormonal, nutrient, and tissue factors can stimulate the chronic low-grade inflammation that leads to insulin resistance. Signaling interactions between proinflammatory immune cells, particularly macrophages and lymphocytes, and insulin target cells in the liver and adipose tissue are key to this process and provide potential opportunities for the development of targeted therapies to improve insulin sensitivity and correct energy imbalance.

Abbreviations

ATM	Adipose tissue macrophage
DIO	Diet-induced obesity
FFA	Free fatty acid
GPCR	G protein-coupled receptor
HFD	High-fat diet
IL	Interleukin
SAT	Subcutaneous adipose tissue
SFA	Saturated fatty acid

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TNF	Tumor necrosis factor
VAT	Visceral adipose tissue
WAT	White adipose tissue

1.1 Introduction

Overnutrition leads to energy imbalance and obesity, a precursor to the metabolic syndrome and type 2 diabetes, with increased risks of cardiovascular disease and certain types of cancer, resulting in a serious global health issue [1–3]. Traits of efficient nutrient storage and responsive immune cell activation that were advantageous during human evolution have become detrimental to human health in times of food excess [4]. In combination with the reduced physical activity and increasingly sedentary lifestyles associated with improved technology, the sequelae associated with obesity are rapidly increasing. More than one third of adults and almost 17 % of youths in the United States are obese as defined by having a body mass index (BMI, kg/m^2) of at least 30 [5]. Moreover, the most recent data from the National Health and Nutrition Examination Survey indicate that 68.3 % of individuals studied were overweight, having a BMI of at least 25 [4]. Thus, there is a pressing need to understand the molecular mechanisms underpinning obesity so that novel therapies can be developed.

Excess nutrients lead to the expansion of adipose tissues throughout the body and it is not surprising that adipocytes play a major role in obesity-induced insulin resistance [6, 7]. The discovery that low-grade, chronic inflammation in adipose tissue activated proinflammatory pathways critical for the development of insulin resistance has had a major impact on our understanding of the pathophysiology of obesity and type 2 diabetes mellitus. Hotamisligil et al. [8] provided the first evidence that proinflammatory tumor necrosis factor-α (TNFα) produced by adipose tissue could impair insulin signaling and that blocking TNFα activity ameliorated insulin resistance. Another milestone in this field was the work done by Xu et al. and Weisberg et al. [9, 10], who showed that in obesity large numbers of proinflammatory adipose tissue macrophages (ATMs) accumulate in various fat depots. The location of adipose tissue is also important in the development of insulin resistance. Adipose tissue that is deposited centrally, visceral adipose tissue (VAT), is more metabolically detrimental than subcutaneous adipose tissue (SAT) [11], and central obesity is more strongly associated with an increased risk of insulin resistance, the metabolic syndrome, and cardiovascular disease than BMI alone [12, 13]. Gender differences in fat distribution can also affect the incidence of obesity-associated diseases as women have relatively more SAT than VAT compared with men [14]. Men have approximately twice as much VAT as women and this correlates with a higher prevalence of the metabolic syndrome [15]. Estrogen may be a key regulator in mediating these effects as postmenopausal women undergo a redistribution of adipose tissue with increased amounts of VAT and an increased risk of obesity-related metabolic disorders [14]. Interestingly, epidemiologic studies suggest that

obesity in premenopausal women is protective against the development of breast cancer, while obesity in postmenopausal women increases the risk for the disease [16]. Regardless of menopausal status, both obesity and type 2 diabetes are associated with breast cancers that are more aggressive at the time of diagnosis and have a poorer prognosis [17, 18].

1.2 Insulin Signaling

Insulin regulates the metabolism of glucose and lipids and insulin signaling is a complex cascade of events downstream of the insulin receptor (IR). There are two main pathways: the phosphatidylinositol 3-kinase (PI3K)-AKT pathway, which mediates glucose uptake and suppresses gluconeogenesis, and the Ras-extracellular signal-related kinase (ERK) pathway, which mediates gene expression and also interacts with the PI3K-AKT pathway to control cell growth and proliferation [19]. Insulin receptor substrates (IRSs) are key mediators of insulin signaling and include four distinct family members, IRS-1–4. IRS-1 and IRS-2 are widely expressed in mammalian tissues, while IRS-3 and IRS-4 have a more restricted distribution. Tyrosine phosphorylation of IRS by the IR generates binding sites for Src homology 2 (SH2) domain proteins, including the p85 regulatory unit of PI3K. The IRS-dependent activation of PI3K is critical for insulin-mediated regulation of metabolism as it leads to the activation of the serine/threonine kinase AKT. AKT phosphorylates key regulatory proteins in multiple tissues, leading to increased glucose transport in muscle and adipocytes, decreased gluconeogenesis and glycogenolysis in hepatocytes, and anti-lipolysis in adipocytes. Shc is another IR substrate, which subsequently engages the Grb21-Sos1-Ras pathway. Although IRS and Shc proteins are the major substrates of the IR and IGFR tyrosine kinases, other substrates such as Grb2-associated binder (GAB) and downstream of kinases (DOKs) can act as tissue- or pathway-specific alternatives to IRS [19].

Insulin resistance can arise when insulin signaling is restricted at any point in this signaling cascade. In inflammation, immune cells release increased levels of obesity-associated inflammatory cytokines which activate serine kinases such as c-Jun amino-terminal kinase (JNK) [20], inhibitor of nuclear factor kappa-B kinase subunit β (IKKβ) [21], and protein kinase Cθ (PKCθ) [22], resulting in serine phosphorylation of IRS-1. This impairs tyrosine phosphorylation and activation of IRS-1, reducing insulin receptor-mediated signaling and leading to insulin resistance.

The importance of immune cells in the etiology of metabolic disease has led to the emergence of a new field termed immunometabolism [23]. While the precise sequence of physiological events initiating inflammation in obesity remains poorly understood, as with other chronic inflammatory conditions, there is a failure in the control mechanisms that rein in overactive immune responses. This review will focus on the immune cells regulating early events in obesity-mediated inflammation and how nutrients and inflammation-related proteins may impact insulin sensitivity.

1.3 Immune Cells

The inflammatory response that occurs during obesity involves multiple immune cell types. Macrophages, neutrophils, $CD4^+$ and $CD8^+$ T cells, B cells, natural killer T (NKT) cells, eosinophils, and mast cells can all be targeted in ways which either positively or negatively regulate inflammation [24]. Thus, the immune response should be considered as a multifaceted, interactive process whereby many cell populations are inter-reliant (Fig. 1.1).

1.3.1 Macrophages

Tissue macrophage populations encompass a heterogeneous group of cells with diverse phenotypes and functions. Canonically, "classically activated" M1 macrophages, expressing the integrin CD11c, are proinflammatory, whereas "alternatively activated" M2 macrophages, which do not express CD11c, are anti-inflammatory [25]. However, such clear distinction into M1 and M2 phenotypes is not always apparent in vivo, and it is likely that a continuum exists between pro- and anti-inflammatory states [25]. Obesity is characterized by a substantial accumulation of $CD11c^+$ macrophages in the VAT and liver and an overall imbalance towards a more proinflammatory phenotype [9, 10, 26, 27].

The central role of macrophages in mediating obesity-associated insulin resistance is best demonstrated by the numerous genetic studies targeting macrophages and macrophage signaling which ameliorate or exacerbate the insulin-resistant state [28]. For example, the depletion of proinflammatory $CD11c^+$ macrophages [29] or the macrophage-intrinsic deletion of the inflammatory mediators JNK1 [30] or IKK-β [31] improves insulin sensitivity, whereas increasing inflammatory macrophage polarization via cell-intrinsic deletion of PPARγ [32] renders mice more insulin resistant [32].

In obesity, adipocytes and endothelial cells secrete chemokines, such as MCP-I and leukotriene B4 (LTB_4), that attract monocytes into the adipose tissue and liver where they differentiate into macrophages [33–36]. Once inflammation is established, the production of chemokines and cytokines by infiltrating immune cells including ATMs provides a feed-forward mechanism exacerbating macrophage recruitment (Fig. 1.1). MCP-1 mediates recruitment by binding to its cognate receptor CCR2 [37, 38]. In diet-induced obesity (DIO) mice, *Ccr2* deficiency reduced ATM accumulation, the inflammatory profile of adipose tissue, and ultimately improved systemic glucose homeostasis and insulin sensitivity [33]. In the same study, short-term treatment of obese mice with a Ccr2 antagonist also reduced ATM content and improved insulin sensitivity. However, these results have not been consistently observed in MCP-I or *Ccr2* knockout (KO) mice [39, 40] suggesting that there is a degree of redundancy in chemoattractant pathways in vivo.

Leukotriene B4 (LTB4) is a proinflammatory lipid mediator generated from arachidonic acid that promotes chemotaxis [41, 42]. Its potent biological actions are

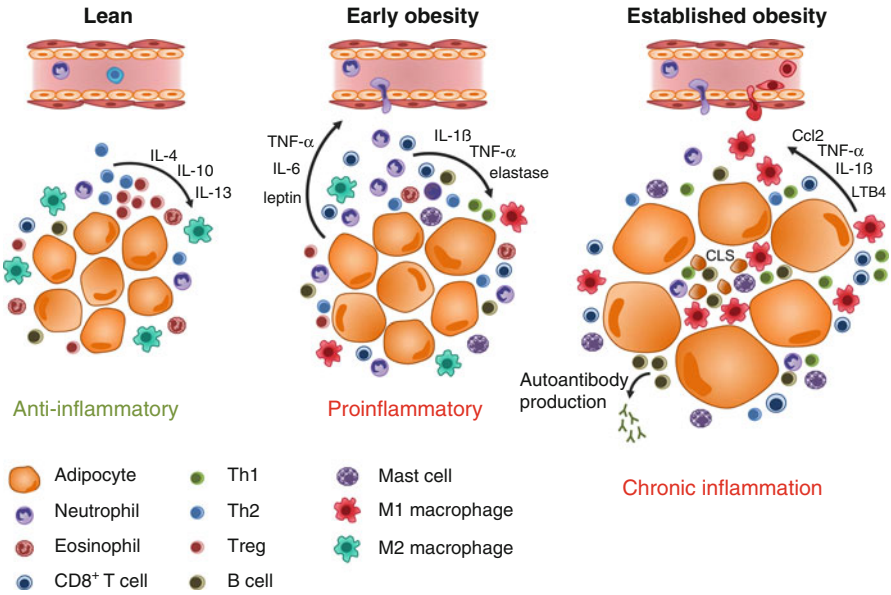

Fig. 1.1 Initiation of adipose tissue inflammation in obesity. In the lean state Th2 T cells, Tregs and eosinophils secrete cytokines such as IL-4, IL-10 and IL-13 that maintain resident macrophages in the M2 state. Early in obesity, there is an influx of neutrophils responding to chemotactic factors released by stressed adipocytes. Neutrophils release neutrophil elastase that promotes proinflammatory cytokine release from $CD8^+$ T cells and Th1 cells that begin to accumulate. This initiates the recruitment of proinflammatory macrophages that are polarized towards the M1 state. In established obesity, M1 macrophages release cyokines that act in a paracrine fashion to maintain the abnormal level of inflammation. Hypoxia and adipocyte cell death fuels the cycle of cytokine and chemokine production that results in a loss of Th2 and Treg cells, while increasing $CD8^+$ T cells, B cells and mast cells. Immune cells congregate in regions of adipocyte lysis forming crown-like structures (CLSs). Cytokines can act locally to cause insulin resistance and can be released into the circulation to promote inflammation in distant tissues

mediated primarily through binding to its G protein-coupled receptor (GPCR), BLT1 [43]. Spite et al. [35] showed that DIO increases the circulating levels of $BLT1^+$ monocytes and genetic ablation of BLT1 reduces ATM accumulation, the expression of proinflammatory cytokines and chemokines and improves insulin sensitivity [35]. Thus, the LTB_4-BLT1 pathway represents a second chemotactic axis promoting macrophage infiltration to adipose tissue and insulin resistance.

Recently, three GPCRs, GPR120, GPR105, and GPR21, have also been implicated in the regulation of macrophage chemotaxis and insulin sensitivity [44–47]. The genetic deletion of GPR105 and GPR21 reduces macrophage chemotaxis to liver or adipose tissue and thus improves insulin sensitivity in DIO mice [44, 46, 47]. The ligand for GPR105 is UDP-glucose, released from injured hepatocytes, and plasma levels of UDP-glucose are elevated in obese mice [44]. Therefore, this is likely to represent a chemotactic pathway whereby macrophages are recruited to

sites of liver damage. In contrast, the ligand for GPR21 at present remains unknown. GPR21 KO macrophages are broadly compromised in their cytoskeletal response to inflammatory stimuli, including MCP-1, suggesting a more general defect in their capacity to migrate [46]. GPR120 is a receptor for anti-inflammatory, omega-3 fatty acids. Omega-3 fatty acids inhibit macrophage chemotaxis, ameliorate adipose tissue inflammation, and enhance insulin sensitivity in a GPR120-dependent manner indicating that this is a potent anti-inflammatory pathway in vivo [45, 48]. Interestingly, a loss of function polymorphism in the human GPR120 gene has recently been associated with obesity emphasizing that this pathway has translational and possibly therapeutic significance [48].

1.3.2 Neutrophils

Among the first cells to arrive at sites of inflammation are neutrophils (Fig. 1.1). Neutrophil deployment is normally tightly regulated since they carry potent cargoes of proteases used to dispose of harmful bacteria. Within 3–7 days of initiating a high-fat diet (HFD), an increase in neutrophil recruitment to adipose tissue occurs [49], suggesting that neutrophils could play a role in initiating the inflammatory cascade in response to excess nutrients. Examination of adipose tissue neutrophils (ATNs) over a longer period of HFD showed that ATN numbers remained elevated for up to 90 days [50]. Expression of neutrophil elastase (NE), a potent serine protease known to have proinflammatory effects [51], was also higher in the obese mice. Pharmacologic or genetic inhibition of the NE improved glucose tolerance of obese mice, while administration of recombinant mouse NE to normal chow-fed mice caused glucose intolerance [50]. In concordance with previous studies demonstrating degradation of IRS by NE in tumor cells [52, 53], NE led to decreased IRS1 levels in mouse and human hepatocytes with subsequent impaired insulin signaling, increased hepatic glucose production, and insulin resistance, indicating that neutrophils may play a previously unsuspected role in the initiation of obesity-induced insulin resistance [50].

1.3.3 CD4⁺ T Cells: T-Helper and T-Regulatory Cell Subsets

$CD4^+$ T cells can be separated into distinct subsets with diverse phenotypes and functions referred to as T-helper (Th1, Th2, and Th17) cells and T-regulatory (Treg) cells, and each of these subsets is present in VAT [24]. Treg and Th2 cells predominate in lean VAT where they play a role in preventing the onset of inflammation [54, 55]. Interestingly, the proportion of Treg cells within the VAT (~40 %) is higher than in any other tissue in the body other than the colon indicating a strong requirement for anti-inflammatory strategies in this tissue [54]. The preferential accumulation of Treg cells in lean VAT is mediated by expression of the transcription factor

PPARγ [56]. Treg cell-specific PPARγ KO mice have reduced Treg cell numbers in VAT and subsequently display enhanced insulin resistance on an HFD [56]. Conversely, treatment of mice with the PPARγ agonist, pioglitazone, enhances Treg cell numbers in obese adipose tissue, and this is part of the mechanism by which pioglitazone increases insulin sensitivity [56]. The precise mechanisms by which Treg and Th2 cells act to limit inflammation in VAT are unknown; however, high expression of the anti-inflammatory cytokine IL-10, especially by Treg cells [54], together with the canonical Th2 cytokines, IL-4 and IL-13, could polarize macrophages towards a less inflammatory state. In contrast, obese adipose tissue is characterized by a specific accumulation of Th1 cells defined by their production of proinflammatory cytokines, such as IFN-γ and unchanged or declining numbers of Th2, Th17, and Treg cells [54, 55, 57]. The accumulation of Th1 cells is subsequent to macrophage and neutrophil infiltration (occurring between 4 and 20 weeks after HFD feeding in mice), and the relatively narrow T cell receptor repertoire of these cells suggests a requirement for antigen presentation [55]. A reversal of this Th1 cell dominance either by anti-CD3 antibody treatment [55] or by pioglitazone-mediated expansion of Treg cells [56] increases insulin sensitivity, and so Th1 cells are thought to contribute to the insulin-resistant state.

1.3.4 CD8⁺ T Cells

$CD8^+$ T cells also accumulate in obese adipose tissue and this begins within 2 weeks of HFD feeding and peaks approximately 9 weeks later [58]. Depletion of $CD8^+$ T cells either prophylactically or after the onset of inflammation improves insulin sensitivity indicating that $CD8^+$ T cells contribute to the insulin-resistant state [58]. $CD8^+$ T cells act to enhance inflammatory macrophage recruitment and differentiation emphasizing the integration of different immune cell pathways during obesity.

1.3.5 B Cells

B cells are recruited to adipose tissues shortly after initiation of an HFD [59], and an absence of B cells protects mice from the development of insulin resistance [60]. Transfer of IgG antibodies from obese WT mice to B cell-deficient mice enhances TNFα production, proinflammatory macrophage polarization and decreases glucose tolerance [60]. Therefore, antibody production is one mechanism by which B cells promote insulin resistance in DIO mice. In human studies, islet cell autoantibodies have been identified in ~10 % of type 2 diabetes patients, and the levels of these antibodies correlate with the need for insulin therapy [61]. Furthermore, autoantibodies against glial fibrillary acid protein (GFAP), one of the antigens most strongly associated with insulin resistance, occur in approximately 30 % of people with type 2 diabetes [62]. Interestingly, B cells deficient in the antigen presentation, major histocompatibility

molecules (MHC-I and MHC-II), do not promote insulin resistance, indicating that an interaction with T cells is a feature of B cell-mediated insulin resistance [60].

1.3.6 Natural Killer T Cells

NKT cells recognize glycolipid antigens and are present in significant proportions in lean VAT and the liver, although their numbers are depleted after the onset of obesity [63–66]. A similar depletion is also observed in obese humans [65]. Studies in mice have shown little or no effect of NKT cell deficiency on the development of insulin resistance on an HFD [64–66]. However, a recent study found that NKT cell-deficient mice display reduced glucose tolerance in lean settings suggesting that NKT cells might be protective during homeostasis [66]. Furthermore, activation of NKT cells with the model ligand, α-Galactosylceramide, can increase glucose tolerance in obese mice by promoting anti-inflammatory macrophage polarization [65]. However, previous studies in younger NKT cell-deficient mice did not observe the same protective effect [63], and therefore, the role for NKT cells in regulating insulin sensitivity remains to be clarified.

1.3.7 Eosinophils

Eosinophils migrate into adipose tissue by an integrin-dependent process and promote M2 macrophage polarization by secretion of IL-4- or IL-13. Eosinophil-deficient mice fed with an HFD develop increased body fat, increased inflammation, impaired glucose tolerance, and insulin resistance, suggesting a role for eosinophils in protecting from DIO [67]. Interestingly, infection by parasitic helminth worms induces an adipose eosinophilia that enhances glucose tolerance, suggesting that targeting eosinophils to increase their numbers or enhance their function could be a useful therapeutic strategy to increase insulin sensitivity [67].

1.3.8 Mast Cells

Mast cells are typically associated with allergic hyperresponsiveness [68]. Increased numbers of mast cells are found in obese adipose tissues of mice and humans compared with their lean counterparts [69–71], and this is accompanied by increased circulating levels of the mast cell protease tryptase [69]. Genetic depletion of mast cells or pharmacologic inhibition of mast cell function reduces body weight gain; reduces the levels of inflammatory cytokines, chemokines, and proteases in serum and adipose tissue; increases energy expenditure; and improves glucose homeostasis [69]. Therefore, mast cells function to promote obesity-associated metabolic changes.

1.4 Signaling Pathways Linking Inflammation and Insulin Resistance

1.4.1 Cytokine Signaling

The inflammatory state of obese adipose tissue leads to increased local cytokine secretion, which directly causes decreased insulin sensitivity. While a number of immune cell types can produce these factors, the macrophage is the major cell type behind the release of proinflammatory cytokines. TNFα is the most well studied and stimulates serine kinases including IKK [72], JNK [20], S6 kinase (S6K) [73, 74], mammalian target of rapamycin (mTOR) [74], and double-stranded RNA-dependent protein kinase (PKR) [75] that can phosphorylate IRS1 on serine residues attenuating downstream insulin signaling (Fig. 1.2).

A variety of interleukins are released during inflammatory responses, and the two most prominent proinflammatory interleukins upregulated in obesity are IL-1β and IL-6 [76, 77]. IL-1β protein levels are increased in mice on HFD and *Il1r1* KO mice are protected from adipose tissue inflammation [78].

1.4.2 Lipid Signaling

The association between increased circulating free fatty acid (FFA) levels and insulin resistance is well known [79]. In the context of obesity, ATMs and other immune cells are exposed to high local concentrations of FFAs released by adipocyte lipolysis. The effects of saturated fatty acids (SFAs) are mediated in part through activation of the pattern recognition receptors (PRRs) Toll-like receptor 4 (Tlr4) and/or Tlr2 [26, 80, 81]. Tlrs normally recognize pathogen-associated molecular patterns (PAMPs) such as bacterial lipopolysaccharides (LPS). SFA-mediated proinflammatory signaling is attenuated in adipocytes deficient in Tlr4 [82] and *Tlr2* or *Tlr4* KO mice are partially protected against HFD-induced insulin resistance [82–85].

Early binding studies indicated that SFAs do not interact directly with Tlr4 [86]. Recent evidence suggests that fetuin A (FetA), a liver-derived circulating glycoprotein, serves as an adaptor molecule presenting FFAs to Tlr4 and activating the Tlr4 inflammatory pathway [87] (Fig. 1.2). *FetA* knockdown in insulin-resistant DIO mice resulted in reduced Tlr4-mediated inflammatory signaling in adipose tissue, whereas administration of FetA restored inflammatory signaling and induced insulin resistance. In addition, fetuin-deficient mice are protected against aging-associated obesity and insulin resistance [88]. FetA levels are elevated in type 2 diabetes and could therefore serve as a biomarker for the inflammatory state, providing an attractive target to improve glucose homeostasis without affecting immune function [89].

SFAs can also activate the Tlr4 pathway by inducing dimerization and recruitment of Tlr4 into lipid rafts, thereby enhancing its association with adaptor

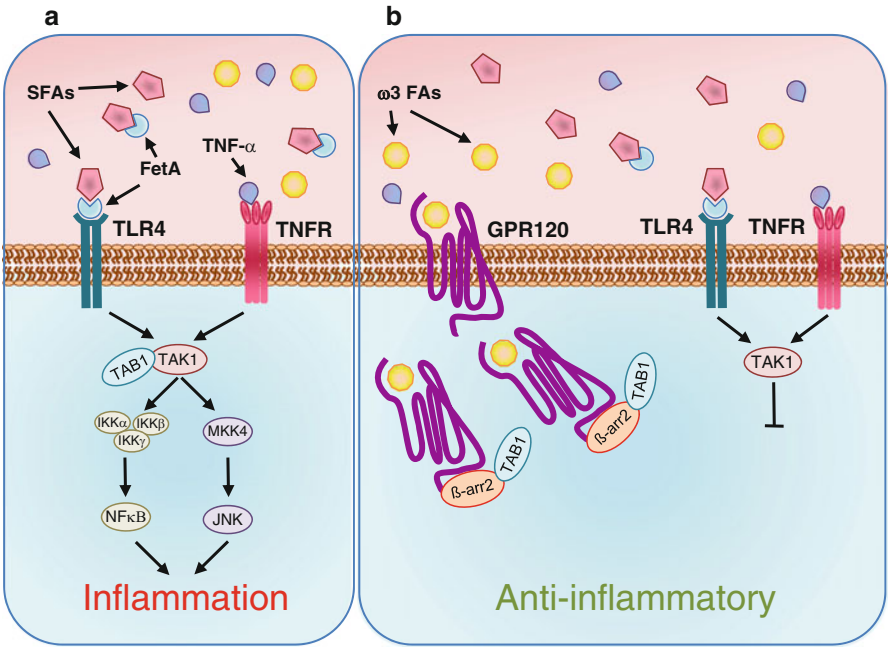

Fig. 1.2 Mechanism for proinflammatory saturated fatty acid (SFA) and anti-inflammatory omega-3 fatty acid (ω3 FA) activity. (**a**) Circulating FetA produced by the liver, functions as an adaptor between SFAs and toll-like receptor 4 (TLR4) signaling. TLR4 activated by SFAs and tumor necrosis factor receptor (TNFR) activated by TNF-α interact with transforming growth factor β (TGF-β) activated kinase 1 (TAK1) with TAK1 binding protein 1(TAB1), initiating a proinflammatory signaling cascade by activating nuclear factor kappa B kinase (NF-κB) and c-Jun N-terminal kinase (JNK). (**b**) Binding of ω3 FAs to GPR120 activates and internalizes the receptor which then binds to β-arrestin 2 (β-arr2) and sequesters TAB1, inhibiting inflammation

molecules TRIF and MyD88 [90]. This process is dependent on the production of reactive oxygen species (ROS) and leads to activation of downstream signaling and increased target gene expression. Other studies have shown that SFAs can alter the membrane distribution of c-Src, partitioning it into lipid rafts where it becomes activated [91]. This leads to signaling via JNK and the transcription of proinflammatory genes. SFAs such as palmitate activate a specialized inflammasome activation pathway in which SFAs inhibit AMP-activated protein kinase (AMPK) leading to defective autophagy, which then activates the inflammasome, resulting in IL-1β cleavage and release [92]. IL-1β stimulates TNFα production and the lack of NLRP3 and its adaptor protein, apoptotic speck protein containing a caspase recruitment domain (ASC), prevents HFD-induced inflammation.

In addition to stimulating inflammatory pathways, metabolites of SFAs, such as ceramide, can directly inhibit insulin signaling by inhibiting Akt. Virtually all stress stimuli increase rates of ceramide synthesis, and numerous studies have

demonstrated a strong association between intracellular ceramide levels and the development of insulin resistance [93–96]. Recent studies have revealed an IKKβ-dependent pathway whereby Tpl2 or Tnf2 induce expression of the genes driving ceramide biosynthesis [93], indicating an additional mechanism by which inflammatory cytokines can act to inhibit insulin signaling [93, 97]. Thus, in addition to acting as ceramide precursors, SFAs stimulate ceramide biosynthesis, amplifying the effects of ceramide on insulin signaling [98].

1.4.3 ER Stress

ER stress activates the unfolded protein response (UPR) to restore ER homeostasis by inhibiting protein synthesis, increasing the degradation of proteins from the ER, and increasing the level of chaperone proteins to assist in protein folding [99]. If these adaptive mechanisms are insufficient to restore ER homeostasis, the cell undergoes programmed cell death [100]. Downstream signaling through regulators of the UPR, PKR-like eukaryotic initiation factor 2α kinase (PERK), inositol-requiring enzyme 1 (IRE-1), and activating transcription factor 6 (ATF-6) can activate both JNK and IKK, leading to the expression of inflammatory cytokines [101, 102]. In addition to protein folding, ER stress response genes play an important role in lipid metabolism as indicated by abnormal lipid processing in the absence of these genes [103–105]. Numerous studies have shown increased ER stress in fatty liver tissues from obese mice.

ER chaperones and folding enzymes such as glucose-regulated protein (GRP) 78, GRP94, protein disulphide isomerase (PDI), calnexin (CNX), and calreticulin (CRT) assist in protein folding and prevent aggregation of unfolded or misfolded proteins [106]. Treating obese or diabetic mice with chemical chaperones [107] or overexpressing GRP78 [108] restores systemic insulin sensitivity and improves hepatic steatosis. Mice deficient in X-box-binding protein-1 (XBP-1), a transcription factor, have an aggravated UPR response, develop insulin resistance and glucose intolerance [109], suggesting that ER stress could be a factor in initiating metabolic inflammation.

1.4.4 Hypoxia

In vivo measurements demonstrate that fat depots in obese animal and human subjects contain distinct regions of microvascularity and exist in a hypoxic state [110, 111]. Hypoxic adipose tissue shows a substantial induction of hypoxia-inducible factor (HIF). Evidence suggests that HIF-1α can activate inflammatory pathways and Krishnan et al. [112] have shown in mice that Hif-1α in VAT is critical for development of glucose intolerance and insulin resistance in HFD mice.

1.5 Anti-inflammatory Therapies

Given the important role of chronic tissue inflammation in the etiology of insulin resistance, anti-inflammatory therapy may prove clinically efficacious in type 2 diabetes. Promising studies have already been reported using high-dose salsalate, an inhibitor of the nuclear factor kappa-B kinase (NFκB) pathway in insulin-resistant type 2 diabetic patients [113, 114]. Treatment with this agent leads to an improvement in insulin sensitivity and a modest decrease in hemoglobin A1c levels. Given the modest nature of these effects, more efficacious treatments will be needed in the future, but these studies provide a potential proof of concept that anti-inflammatory therapy can be a beneficial antidiabetic approach.

TNFα plays a role in insulin resistance [115–117] and TNFα blockade can increase insulin sensitivity in mice [8]. However, in man the process is less clear. There is a range of results reported from clinical TNFα neutralization studies on relatively small patient populations. For example, little to no benefit in terms of insulin sensitivity was reported with anti-TNFα therapy used in the treatment of rheumatoid arthritis patients [118] or obese patients with features of metabolic syndrome [119]. In contrast, there are reports of improved insulin sensitivity in patients receiving long-term treatment with TNF inhibitors [120, 121]. Retrospective analysis of a large cohort of rheumatoid arthritis or psoriasis patients showed that individuals treated with TNF inhibitors had a reduced risk for developing type 2 diabetes [122, 123], suggesting a need for further clinical trials in this area that more directly test the hypothesis that long-term anti-TNFα therapy increases insulin sensitivity.

IL1β is a circulating cytokine and antibody-mediated inactivation of IL-1β improves glycemic control in DIO due largely to increased pancreatic beta cell formation [124]. Humans treated with an IL-1 receptor antagonist also show reduced systemic inflammation, but with only modest effects in improving glycemia and no effects on insulin sensitivity [125, 126]. The lowered glucose levels were accompanied by increased insulin secretion, suggesting that IL-1 blockade inhibits damaging effects of IL-1β on pancreatic islets, improving pancreatic beta cell function and increasing insulin secretion [125, 126].

The thiazolidinedione (TZD) group of drugs are currently used in the treatment of type 2 diabetes, and although their precise mechanism of action is not fully understood, they are agonists for nuclear receptor PPARγ [127]. Although adipocytes have been considered the primary target cells for the anti-inflammatory effects of TZDs [128], additional studies indicate an important role for PPARγ in macrophages [32, 129], muscle [130], and the CNS [131, 132]. In addition to the transactivation of genes, PPARγ can also transrepress target genes by antagonizing signal-dependent activation of transcription factors such as NFκB and AP-1, thereby reducing proinflammatory signaling [133]. However, although anti-inflammatory effects are part of the overall mechanism of TZD-induced insulin sensitization, the contributions of this to the beneficial clinical effects of these drugs remain to be defined [134].

The anti-inflammatory properties of omega-3 FAs have long been known and are associated with protection from cardiovascular disease [135, 136]. Fish oil, a major source of omega-3 FAs, leads to decreased production of TNFα IL-1 and IL-6 by macrophages in mice and man [137–139]. The lipid-sensing receptor GRP120, highly expressed in proinflammatory macrophages, can mediate the effects of omega-3 FAs [45]. Thus, when WT and GPR120 KO mice were placed on an HFD with or without omega-3 FA supplementation, the omega-3 FA treatment inhibited inflammation and enhanced systemic insulin sensitivity in WT mice, but was without effect in *Gpr120* KO mice. Signaling studies demonstrated that after ligand stimulation, GPR120 couples to β-arrestin2, which is followed by receptor endocytosis and inhibition of TAB1-mediated activation of TAK1, providing a mechanism for inhibition of both the TLR and TNFα proinflammatory signaling pathways (Fig. 1.2). Importantly, a loss of function mutation in GPR120 in humans increases the risk for obesity and lends support for the therapeutic targeting of this lipid sensor [48].

1.6 Links with Cancer

The chronic low-grade inflammation in obesity is also associated with cancer progression through a variety of common pathways [140–142]. Mechanisms by which inflammation can contribute to carcinogenesis include induction of genomic instability, increased DNA damage, alterations in epigenetic events and subsequent inappropriate gene expression, enhanced proliferation of initiated cells, and resistance to apoptosis. While there is a clear link between obesity, inflammation, and cancer, there are many areas that remain poorly understood. Whether obesity-related inflammation is more important in early events in cancer initiation and how tumor-associated macrophages interact with ATMs during tumor progression are questions that remain to be answered. Inflammation-induced insulin resistance is known to be tissue selective and the resulting hyperinsulinemia may have direct effects on promoting the growth of tumor cells [143]. A detailed discussion of the relationships between obesity, inflammation, insulin resistance, and cancer will be covered in the following chapters.

1.7 Conclusions

Since the discovery that inflammation was a critical component of the pathway from obesity to insulin resistance, research efforts have been focused on understanding the cellular and molecular basis for these changes. It now appears that there are multiple cell types and signaling molecules that offer potential drug discovery efforts to improve insulin resistance.

Given the important role for immune cells in the initiation of obesity-related disease and our knowledge of their protective role in host defense from studies of individuals with genetic abnormalities in immune cell function, finding the balance between therapies that dampen the deleterious aspects of the immune response while maintaining the ability to combat infections and disease will be challenging. For example, targeting of TNFα, a major cytokine released by activated macrophages, has been an effective therapy for the treatment of inflammatory disorders such as rheumatoid arthritis [144]. Concerns regarding long-term effects such as an increase in cancer risk have been largely dispelled by the results of long-term follow-up; however, the risk of exacerbating infection remains [145, 146]. Whether these compounds can be used in the insulin-sensitizing armamentarium is yet to be determined.

Advances in technology and the application of genome-wide association studies (GWAS) are likely to identify novel pathways and linkages between existing pathways that will aid in our understanding of multifactorial inflammatory diseases. A recent study in mice suggests that less than 10 % of transcripts altered in DIO are shared between the classical insulin target tissues, white adipose tissue (WAT), skeletal muscle, liver, and heart [147]. In humans, GWAS have identified novel loci associated with fasting insulin and insulin resistance [148]. These studies and many others provide fruitful avenues for future research.

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Chapter 2
Inflammasomes and Obesity

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Abstract Inflammasomes are a family of protein complexes that recognize diverse microbial and endogenous danger signals to promote innate immune responses, tissue inflammation and injury, or cell death via pyroptosis. Inflammasome activation results in the recruitment and activation of caspase-1, which is required for the production of the proinflammatory cytokines interleukin-1β (IL-1β) and IL-18 that can modulate both adaptive and innate immune responses through effects on leukocyte survival, proliferation, differentiation, and migration. Recent studies suggest that inflammasome activity may also play important roles in several nonmicrobial disease states associated with chronic inflammation. For example, NLRP3 inflammasome expression and IL-1β production are both increased in obesity, and recent work has implicated NLRP3 inflammasome activation in a variety of obesity-linked conditions including gout, type 2 diabetes mellitus, metabolic liver disease, atherosclerosis, Alzheimer's disease, cancer and rheumatoid arthritis. Further, many of the factors associated with these conditions, including elevated plasma glucose, fatty acids, uric acid and cholesterol crystals, and β-amyloid, have been shown to be elevated during obesity and to stimulate NLRP3 inflammasome expression or activation. Since chronic NLRP3 activation appears to play important roles in several common disease states, better understanding of inflammasome regulation and function may lead to better therapeutic approaches. Several agents that attenuate NLRP3 inflammasome activity or inhibit its primary effector, IL-1β, are currently under development or in early clinical trials as therapeutics to treat these common disease

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conditions. This chapter will review new research on inflammasome activation, its role in obesity and other chronic inflammatory states, and the status of approaches to attenuate NLRP3 inflammasome activity.

2.1 Inflammasomes Are Key Mediators of the Innate Immune System

The innate immune system serves as a first line of defense against invading microbes and other harmful agents by sensing pathogen-associated molecular patterns (PAMPs), such as microbial nucleic acid, lipoproteins, and lipopolysaccharides (LPSs), or danger-associated molecular patterns (DAMPs) released during cell stress, including ATP, uric acid, heat shock proteins, and other cellular components [1]. The initiation and regulation of innate immune responses to these factors are orchestrated by several classes of germline-encoded pattern-recognition receptors (PRRs), including toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs), and AIM2 (absent in melanoma 2)-like receptors (ALRs) [2, 3]. NLRs comprise a large protein family of intracellular sensors, the members of which share a conserved central nucleotide-binding, oligomerization domain (NOD), a leucine-rich repeat (LRR) region, and a variable N-terminal effector domain. NLRs can be classified as receptors (Nod1 and Nod2) or by their different central proteins and their diverse functions: negative regulators include NLRX1, NLRC5, and NLRP4 and inflammasome activators include NLRP1, NLRC4, NLRP3, and NLRC5 [4–7]. Several NLRs and AIM2 can form large multi-protein "inflammasome" complexes upon stimulation by PAMP or DAMP signals.

Following activation, NLRs oligomerize and recruit pro-caspases through direct interaction with the NLR caspase recruitment and activation domain (CARD) and through interaction with the pyrin domain (PYD) of the ASC (apoptosis-associated speck-like protein containing a CARD) adaptor protein. ASC resides in the nucleus, but its movement from the nucleus to the cytosol is required for inflammasome assembly [8]. Caspase zymogen recruitment into an inflammasome complex induces a conformational change leading to caspase autoactivation.

IL-1 and IL-18, generated by capase-1 maturation of pro-IL-1 and pro-IL-18 peptides, are the major functional effectors of inflammasome activation, inducing multiple responses to infectious agents. IL-1β induces fever, T cell survival, B cell proliferation, and leukocyte migration and promotes polarization of CD4+ T helper 1 (Th1) cells [9], while IL-18 synergizes with IL-12 to promote Th1 polarization and IFNγ production and facilitates proinflammatory Th17 responses [10]. Caspase-1 activity is also required for pyroptosis, a proinflammatory cell death mechanism for self-clearance of infected macrophages and dendritic cells thought necessary to prevent intracellular replication of pathogenic microorganisms (Fig. 2.1 from Zitvogel et al. [11]). Both IL-1 and IL-18 production and pyroptosis have implications for many diseases including infections, arthritis, metabolic syndrome, cancer, and inflammatory bowel disease. Caspase-1 also contributes to host defense through

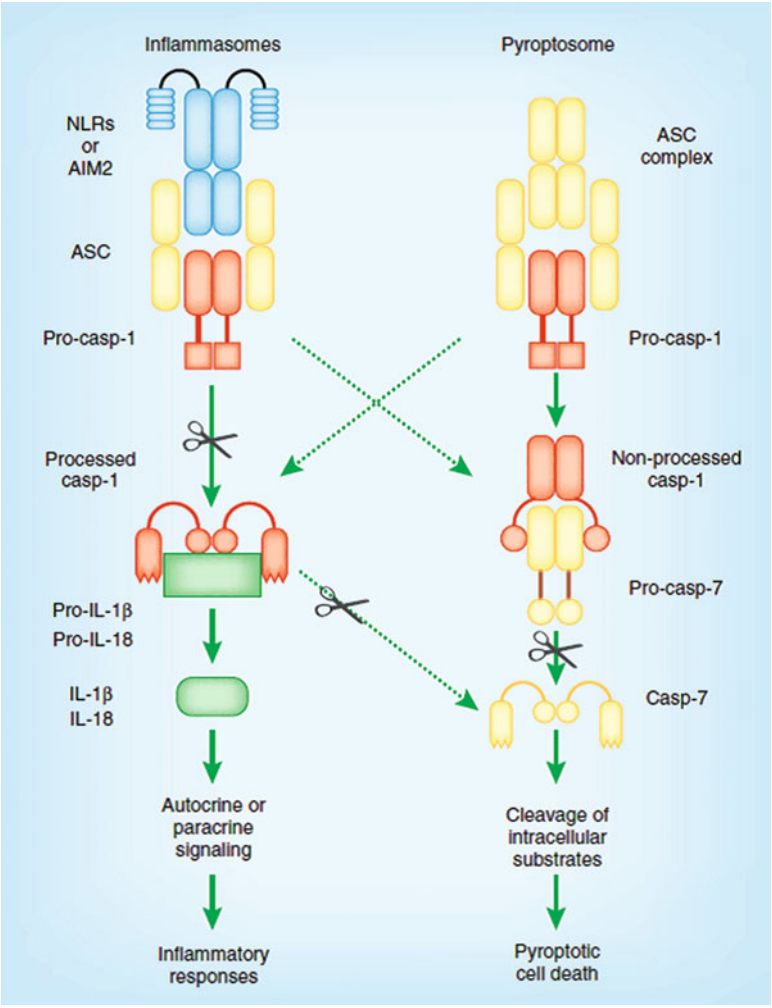

Fig. 2.1 Pyroptosis as a possible outcome of caspase-1 activation. Activation of caspase-1 (casp-1) mediated by the inflammasome or by the supramolecular assembly of ASC dimers known as the "pyroptosome" can derive from the proteolytic cleavage of pro-caspase-1 (pro-casp-1) or follow less well-understood (perhaps conformational) mechanisms. Both examples of caspase-1 activation can lead to the proteolytic maturation of caspase-7, followed by the cleavage of several intracellular substrates and pyroptotic cell death. Nevertheless, cleaved caspase-1 is required for the processing of pro-IL-1β and pro-IL-18, as well as for the release of mature IL-1β and IL-18 into the microenvironment (reprinted with permission from Zitvogel et al. [11])

additional mechanisms, including secretion of leaderless peptides, cleavage of glycolytic pathway enzymes, restriction of bacterial replication, and augmentation of cell repair through control of lipid metabolism (summarized in Lamkanfi and Dixit [12]).

These cytokine- and caspase-1-regulated processes must be tightly controlled balancing the ability to curb infection and develop T and B cell memory to pathogen

exposure vs. host injury and damage. Some invading microbes evade various steps in IL-1 and IL-18 production to overcome this host defense. However, uncontrolled activation of inflammasomes contributes to autoimmune diseases such as familial Mediterranean fever and Muckle-Wells syndrome (reviewed in Ting et al. [13]). Several distinct mechanisms can attenuate inflammasome-mediated responses [14]. The anti-inflammatory cytokine IL-10 can inhibit pro-IL-1 synthesis via the STAT3 signaling pathway, while activation of the STAT1 pathway signaling can inhibit IL-1 maturation by blocking caspase-1 processing through an unknown mechanism specific for NLRP3 and NLRP1 inflammasomes. IFNγ produced by activated CD4+ Th1 cells and CD8+ T cells can also transiently inhibit IL-1 production [15], which has been suggested to serve as negative feedback inhibition upon activation of the adaptive immune system. Effector and memory T cells can also inhibit inflammasome activity through the CD40L, OX40L, and RANKL signaling pathways [16].

2.2 The NLRP3 Inflammasome is a Major Mediator of Human Disease

NLRP3 inflammasomes are among the most highly studied, since *Nlrp3* gene mutations have been linked to the autoinflammatory Muckle-Wells syndrome, familial cold auto-inflammatory syndrome, and neonatal-onset multisystem inflammation [17–19]. Most of these *Nlrp3* genetic mutations produce constitutively active NLRP3 proteins that promote IL-1β secretion to elicit inflammatory responses [13]. Inhibition of the IL-1β pathway using an IL-1 receptor antagonist, anakinra, successfully ameliorates the severity of these inflammation-induced diseases, corroborating the essential role of NLRP3-induced IL-1β their inflammatory pathologies [20, 21]. The NLRP3 inflammasome is composed of the NLRP3 protein, the adaptor molecule apoptosis-associated speck-like protein containing a CARD (ASC), and the cysteine protease caspase-1. Human, but not mouse, NLRP3 inflammasome complexes also contain the Cardinal protein, whose function remains unclear [22].

Similar to other inflammasomes, the NLRP3 inflammasome activates caspase-1 to cleave pro-IL-1β and pro-IL-18 into IL-1β and IL-18. NLRP3 inflammasome activation can also induce pyroptosis and modulate immune responses by regulating the secretion of more than 20 leaderless cytokines and growth factors, including HMGB1, IL-1α, fibroblast growth factor 2 (FGF2), galectin-1, and galectin-3 [23]. NLPR3 inflammasome activity thus plays critical roles in inflammation, host defense, and other related activities.

2.2.1 NLRP3 Inflammasome-Priming Signals

IL-1β, unlike most other cytokines, is regulated by both gene transcription and post-translational modification. Pro-IL-1β expression in myeloid cells, such as macrophages and dendritic cells (DCs), is induced upon exposure to LPS, a bacterial-derived

TLR4 ligand, which activates the NF-κB signaling pathway. Many NF-κB pathway activators, including TNF-α, CpG, Pam3CSK4, poly(I:C), R848, imiquimod, and IL-1β itself, have also been shown to increase pro-IL-1β expression [24–28]. However, while NF-κB-activating stimuli are required to induce pro-IL-1β expression in vitro, it is unclear whether such priming agents are also required to increase in vivo pro-IL-1β expression. For example, intraperitoneal injection with alum or monosodium urate (MSU) crystals triggers IL-1β secretion without an exogenous priming step to induce NF-κB activation [29, 30]. Differences between in vitro and in vivo inflammasome responses could be explained, at least in part, by differences in the sensitivity of the responding cell types and their microenvironment.

Most NF-κB-dependent "priming" signals induce pro-IL-1β and increase NLRP3 protein levels, an essential step for inflammasome formation [24]. NLRP3 expression in bone marrow-derived dendritic cells (BMDCs) and macrophages is markedly increased upon TLR stimulation [24, 28], and signaling through the TLR adaptor proteins MyD88 and TRIF increases NLRP3 expression [24, 31]. In contrast, the inflammasome complex proteins caspase-1 and ASC are widely expressed in most cell types and are not further upregulated upon NF-κB activation.

2.2.2 The Assembly of the NLRP3 Inflammasome

NLRP3 is highly expressed in primary mouse neutrophils, peripheral blood mononuclear cells (PBMCs), and BMDCs and moderately expressed in established Th2 and macrophage cell lines [28, 32]. NLRP3 protein is sequestered in an autoinhibitory conformation by heat shock protein 90 (HSP90) and suppressor of G2 allele of SKP1 (SGT1) in resting cells [33], but NLRP3 can recognize stimulatory signals through its LRR domain and undergo a conformational change, leading to ATP-dependent self-oligomerization via its NOD domain. NLRP3 oligomers then recruit the adaptor protein ASC via PYD-PYD domain interactions, which then recruits caspases-1 via CARD-CARD domain interactions to generate the final ~700 kDa NLRP3 inflammasome complex [34, 35]. This multi-protein complex assembly activates caspase-1, which cleaves pro-IL-1β and pro-IL-18 to generate mature IL-1β and IL-18 cytokines for secretion.

It remains unclear how NLRP3 recognizes the diverse range of stimuli that can activate NLRP3 inflammasome assembly and activation. It is plausible that NLRP3 is not a direct sensor of all the external stimuli and cellular "danger signals" it recognizes. Recent results have identified the non-NLR/ALR protein guanylate-binding protein 5 (GBP5) as a sensor for some NLRP3 inflammasome activators, stimulating NLRP3 inflammasome activation in response to pathogenic bacteria and soluble, but not crystalline, inflammasome-priming agents [36]. Interestingly, GBP5-deficient mice demonstrate impairments in both host defense and NLRP3-dependent inflammatory responses, indicating that proteins other than NLRP3, ASC, and caspase-1 are involved in sensing pathogenic "danger signals" that activate the NLRP3 inflammasome. Moreover, the restricted range of NLRP3 inflammasome stimuli

recognized by GBP5 suggests that more than one sensor is involved in this process. These results, however, do not fully preclude the possibility of different sensor proteins giving rise to a common signaling intermediate that directly regulates the NLRP3 inflammasome complex. Given the ability of the NLRP3 inflammasome to regulate critical innate and adaptive immune responses to induce both protective and pathogenic responses, future studies are required to characterize signaling small molecules and proteins that regulate NLRP3 inflammasome activation.

2.3 The Diverse Regulation of NLRP3 Inflammasome Activation

Notably, the NLRP3 inflammasome is responsive to a wide array of stimuli, ranging from microorganisms of both endogenous and exogenous origin to inorganic agents such as asbestos, silica, and alum (Table 2.1). Specific activators have been identified for the NLRP1, NLRC4, and AIM2 inflammasomes, but much less is known about what triggers the assembly and activation of the NLRP3 inflammasome [37]. Studies indicate that reactive oxygen species (ROS), potassium efflux, and lysosomal damage are involved in NLRP3 inflammasome activation. Indeed, regulation of NLRP3 inflammasome activity by many stimuli can be explained by these three mechanisms; however, no single mechanism accounts for inflammasome activation by all known stimuli. The following sections summarize and discuss these models and the potential metabolic signals that can induce NLRP3 inflammasome responses.

2.3.1 Reactive Oxygen Species

Many NLRP3 agonists induce ROS formation, and reactive oxygen compounds such as hydrogen peroxide can induce inflammasome formation [38]. NADPH oxidase was initially postulated as the cellular ROS source that activated the NLRP3 inflammasome [39–41], since many factors that induce inflammasome activity also induce NADPH oxidase. However, studies performed with cells lacking various NADPH oxidase components indicate that NADPH oxidase activity is not required for NLRP3 inflammasome activation [42–44].

More recent studies have implicated mitochondrial ROS in inflammasome activation. Mitochondria are the main source of cellular ROS and markedly increase mitochondrial ROS production under various stress conditions [45]. Mitochondria also produce ROS during normal respiration, which can eventually alter respiratory chain function and lead to increased mitochondrial ROS production [46]. Such damaged mitochondria are continuously removed by a specialized form of autophagy, known as mitophagy, to avoid cellular damage from excess ROS exposure. Chemical and genetic manipulations that increase mitochondrial dysfunction and ROS production activate NLRP3 inflammasomes [47, 48], as does pharmacologic or genetic inhibition of mitophagy leading to the accumulation of damaged, ROS-producing mitochondria [46, 47, 49]. Both NLRP3 and ASC relocate to the

Table 2.1 Sensing of microbes, pathogen-associated molecular pattern (PAMPs), bacterial toxins, and "danger signals" by NLRP3

Elicitor	References
Microbial motifs	
MurNAc-L-Ala-D-isoGln (muramyldipeptide)	[221]
Bacterial RNA	[26]
Imidazoquinoline compounds (R837, R848)	[26]
Live bacteria	
Staphylococcus aureus	[27]
Listeria monocytogenes	[27]
Shigella flexneri	[222]
Virus	
Sendai virus	[223]
Influenza virus	[223, 224]
Adenovirus	[225]
Microbial toxins	
Aerolysin (*A. hydrophila*)	[226]
Maitotoxin (*Marine dinoflagellates*)	[27]
Nigericin (*Streptomyces hygroscopicus*)	[27]
Listeriolysin O (*L. monocytogenes*)	[226]
Danger-associated host components	
ATP, NAD+ (P2RX7)	[27, 28]
Mitochondrial membrane potential and generation of ROS	[47, 48]
Crystalline or aggregated substances (asbestos, silica, uric acid (MSU), etc.)	[37]
Necrotic cell components	[227]
Low intracellular potassium	[228]
Cell volume change	[56]

perinuclear space upon inflammasome activation to colocalize with endoplasmic reticulum mitochondria, further implying a role for mitochondrial ROS in inflammasome activation [48].

However, while ROS have been repeatedly implicated in NLRP3 inflammasome activation, it is still not clear precisely how ROS alter the inflammasome complex to activate capase-1 activity. ROS are toxic, but can also serve as signals for various physiologic processes. One recent study found that thioredoxin-interacting protein (TXNIP), a binding partner of the antioxidant protein thioredoxin, can directly bind NLRP3 and regulate inflammasome activation in a ROS-inducible manner [50], and similar results were found in a second study, albeit with minor differences [51]. It is possible that NLRP3 is influenced directly by ROS, although there is currently no experimental evidence to support such a mechanism.

2.3.2 Cytoplasmic Potassium

Intracellular potassium is a common initiator of inflammasome activity, as inhibition of potassium efflux by hyperosmotic potassium can block NLRP3 inflammasome activation [39, 52, 53]. Cellular potassium concentration can serve as an

indicator of membrane integrity, due to its high cellular and low extracellular levels. NLRP3 inflammasomes are activated by disruption of cell membrane integrity, but extracellular potassium as low as 20 mM is sufficient to block NLRP3 inflammasome formation [54]. NLRP3 inflammasome activity is thus likely regulated at least in part by potassium efflux during loss of membrane integrity, but many stimuli markedly induce inflammasome activity without significantly destabilizing the cell membrane. Inflammasome responses to several such stimuli, including PAMPs, DAMPs, and crystals, can be markedly attenuated by the potassium channel inhibitor glibenclamide [55], however, suggesting that potassium channels mediate many of these responses by increasing intracellular potassium efflux.

Changes in cell volume in response to low environmental osmolarity can also regulate NLRP3 inflammasome activation [56], at least in part by decreasing intracellular potassium concentrations. However, low intracellular potassium is required but not sufficient to induce NLRP3 inflammasome activation during cell swelling. Cell swelling was also found to alter transient receptor potential (TRP) channel activity to regulate an intracellular calcium increase that was found to stimulate transforming growth factor β-activated kinase 1 (TAK1) to activate the NLRP3 inflammasome.

2.3.3 Phagocytosis and Lysosomal Damage

Phagocytosis of crystalline or particulate structures, such as MSU, silica, asbestos, amyloid-β, and alum, can lead to the release of proteolytic lysosomal contents into the cytosol, resulting in NLRP3 inflammasome activation [42, 57]. Leakage of the lysosomal cysteine protease cathepsin B into the cytoplasm has been proposed to result in the cleavage of a putative factor regulating NLRP3 complex activity, since specific inhibition or genetic ablation of cathepsin B activity impairs inflammasome activation induced by phagocytosis of various particulates [42, 58, 59]. However, either cathepsin B or cathepsin L deficiency attenuated NLRP3 inflammasome activation in one of these studies, and repression was less pronounced at higher doses [58], implying functional redundancy or alternative protease activities. Further, not all studies found cathepsin B deficiency attenuated NLRP3 inflammasome activation by crystalline agonists [60].

2.3.4 The NLRP3 Inflammasome is Negatively Regulated by IFNγ and T Cell Responses

Emerging evidence indicates that cytokine signaling events can inhibit inflammasome activation and IL-1β production [15]. For example, IFNγ transiently inhibits LPS-stimulated IL-1β production in mouse macrophages and dendritic cells, although not in human cells [61], but is limited by LPS-induced SOCS1 expression, a negative

regulator of the IFNγ signaling cascade. Endogenous IFNγ production in *M. tuberculosis-infected* mice also selectively inhibits IL-1α and IL-1β production by monocyte-macrophages and DCs [15]. IFNγ is a signature cytokine of CD4+ Th1 and CD8+ T cells [62, 63], suggesting that T cell IFNγ secretion during adaptive immune responses may act to attenuate inflammasome responses. Similarly, both effector and memory T cells can directly attenuate NLRP3 and NLRP1 inflammasome activity in macrophages and DCs through a cell-to-cell contact-dependent mechanism apparently mediated by TNF superfamily ligand-receptor interactions [16].

2.4 NLRP3 Inflammasome Is a Metabolic Regulator

Excess weight gain is associated with the dysregulation of multiple metabolic factors that increase inflammasome activation.

2.4.1 Fatty Acids

Elevated plasma free fatty acids (FFA) are strongly associated with insulin resistance and type 2 diabetes [64]. Saturated FFAs can activate toll-like receptor 4 (TLR4) signaling to induce proinflammatory responses in adipocytes, macrophages, and pancreatic β-cells to increase insulin resistance in target tissues and reduce pancreatic β-cell function [65, 66]. TLR4-NFκB pathway signaling induces transcription of multiple proinflammatory factors, including IL-1β [67]. Recent work indicates that the saturated FFA palmitate can induce NLRP3-inflammasome activity by suppressing AMP-activated protein kinase activity, resulting in decreased autophagy, accumulation of damaged mitochondria, and increased production of mitochondrial-derived ROS [68]. Thus high FFA levels can provide both the transcription and inflammasome activation signals required for IL-1β production.

Cellular palmitate uptake also induces ceramide accumulation, which can function as a second messenger in several key signaling pathways [69]. Plasma ceramides are increased in obese patients with type 2 diabetes and positively correlate with insulin resistance, implying a potential role for ceramide in obesity-induced insulin resistance [70]. Ceramide has been shown to potently activate NLRP3 inflammasomes in isolated macrophages and adipose tissue explants [71], suggesting a new mechanism for ceramide-mediated insulin resistance in target tissues.

2.4.2 Hyperglycemia

Hyperglycemia induces IL-1β production in multiple cell types, including endothelial cells, monocytes, and pancreatic islet β-cells [72–74], and has been shown to activate the PKCα-NFκB signaling pathway to stimulate IL-1β gene

transcription [75]. More recent work suggests that TXNIP plays an important role in hyperglycemia-induced pancreatic β-cells IL-1β secretion by directly interacting with the NLRP3-inflammasome in a ROS-dependent manner [50]. TXNIP expression is consistently higher in β-cells of subjects with type 2 diabetes [76], and its expression is robustly induced in response to high glucose [77]. Similar results are found in human adipocytes, where high glucose markedly induces TXNIP expression, caspase-1 activation, and IL-1β secretion [78], and TXNIP knockdown reduces hyperglycemia-induced IL-1β production, albeit primarily through IL-1β transcription [78]. Thus, both FFA and hyperglycemia appear to act through the NF-κB signaling pathway to stimulate IL-1β gene transcription, but use alternate mechanisms to provide the second signal required for inflammasome activation, IL-1β processing, and release.

2.4.3 Uric Acid and MSU Crystals

Multiple studies have indicated that uric acid or MSU crystals can stimulate inflammasome activity. For example, mice intraperitoneally injected with MSU crystals demonstrate NLRP3 inflammasome activation and develop IL-1β-dependent peritonitis [79]. MSU crystals are frequently found in patients with gout, but uric acid has also been implicated in inflammasome activation in other acute and chronic proinflammatory disease conditions. For example, during bleomycin-induced acute lung injury leading to pulmonary inflammation and fibrosis, uric acid released from injured cells can activate the NLRP3 inflammasome to induce IL-1β production [80]. Both adipose tissue uric acid levels [81] and inflammasome activity [82] are elevated in obesity, suggesting that uric acid may also promote NLRP3 inflammasome activation and IL-1β production in adipocytes, although there is no direct evidence for such a mechanism. Similarly, since plasma uric acid concentrations were one of the original diagnostic criteria for metabolic syndrome, it is tempting to speculate on the role of uric acid on systemic complications of obesity. Several recent studies have indicated that elevated plasma uric acid confers increased risk for multiple disease states associated with metabolic syndrome, including obesity, insulin resistance, hypertension, and cardiovascular disease [83].

2.4.4 Cholesterol

Plasma cholesterol levels are strongly linked to atherosclerosis, and cholesterol crystals are a recognized hallmark of advanced atherosclerotic lesions [84]. Cholesterol crystals can induce robust, dose-responsive, and caspase-1-dependent macrophage IL-1β secretion by inducing lysosomal damage [58]. Bone marrow NLRP3, ASC, or IL-1α/β deficiency markedly decreases atherosclerosis and inflammasome-dependent IL-18 levels, suggesting that NLRP3 inflammasomes

play major roles in atherosclerosis [58]. Recently, autophagosomes were identified in atherosclerotic plaques, but autophagy was found to be defective [85]. Complete deficiency of autophagy in the apolipoprotein E-deficient mouse led to accelerated atherosclerosis with increased IL-1β levels and ASC protein bodies indicative of inflammasome complex formation and activation. These results are consistent with observations suggesting that lysosomal cholesterol crystals alter autophagic processes [58, 86]. These changes, in addition to increased vascular ROS, contributed to inflammasome activation.

2.4.5 Amyloid

Islet amyloid polypeptide (IAPP), which is co-secreted with insulin, can form amyloid deposits in the pancreas during type 2 diabetes. IAPP aggregates have cytotoxic properties that are believed to play a key role in β-cell loss and type 2 diabetes progression [87]. Recent work now indicates that IAPP crystals can trigger pancreatic NLRP3 inflammasome activation and IL-1β secretion [51].

2.5 Obesity and the NLRP3 Inflammasome

Chronic inflammation and recruitment of macrophages has been recognized as a hallmark of obesity for decades. Recent work indicates that innate and adaptive immune processes in adipose tissue play central roles in the development of obesity-induced inflammatory responses and indicates potential roles for NLRP3 inflammasome activity in these processes.

2.5.1 Chronic Inflammation in Adipose Tissue

Adipose tissue is an important endocrine organ, which can undergo marked changes in both its cellular composition and secretory profile during the onset of obesity. Caloric excess alters adipocyte function and dramatically increases the relative abundance and proinflammatory phenotype of adipose-resident immune cells, resulting in increased serum levels of proinflammatory adipokines [88, 89], while reductions in fat mass strongly correlate with serum adipokine decreases [90], suggesting that adipose tissue plays a central role in the systemic proinflammatory milieu of obesity. Adipocytes are important endocrine cells in this process, and adipocyte secretion of proinflammatory or pathogenic adipokines, such as TNFα, leptin, plasminogen activator inhibitor-1, monocyte chemotactic protein-1, IL-6, resistin, angiotensinogen, and IL-1β, markedly increases with obesity [88, 89]. Obesity also increases the relative adipose abundance of proinflammatory CD4 T cells (Th1) and CD8 T cells, resulting

in increased adipose expression of IFNγ [91, 92]. The polarization of adipose tissue macrophages (ATMs) from an anti-inflammatory (M2) to a proinflammatory (M1) phenotype likely results from this increase in adipose IFNγ [93]. However, other immune cells, including B cells [94], mast cells [95], eosinophils [96], and neutrophils [97], have also been reported to regulate obesity-induced inflammation. The trigger signals for immune cell accumulation and polarization are unclear, although adipocyte death, hypoxia, and adipokines secreted in response to metabolic overload may all direct inflammatory cells to adipose tissue [98].

NLRP3 inflammasome components are highly expressed in ATMs, whose abundance can dramatically increase with the onset of obesity and contribute to the metabolic dysregulation and insulin resistance that give rise to obesity-associated complications. IL-1β directly inhibits adipocyte insulin signaling by decreasing insulin receptor substrate-1 [99], and both IL-1β and IL-18 can induce adipose-resident Th1 and CD8+ T cells [71, 100] to further increase adipose tissue inflammation. NLRP3 inflammasome activation in preadipocytes inhibits adipocyte differentiation and fat accumulation by reducing preadipocyte expression of PPARγ, a key regulator of adipogenesis, while caspase-1-deficient adipocytes are more metabolically active, suggesting that obesity-induced changes in NLRP3 inflammasome activity can negatively impact adipocyte function [82]. High-fat diet (HFD)-fed IL-1R knockout mice have less adipose inflammation and better insulin sensitivity than wild-type control mice [101]. Similarly, HFD-fed $Nlrp3^{-/-}$, $Asc^{-/-}$, and $Casp1^{-/-}$ mice demonstrate decreased weight gain and body fat, reduced inflammation, and improved insulin sensitivity [71, 82, 102], although both IL-1R and IL-18 deficiencies were subsequently found to induce hyperphagia resulting in delayed-onset obesity and insulin resistance in chow-fed mice [103–105].

2.5.2 NLRP3 Inflammasome Expression and Activity in Obese Mice

Several studies have investigated IL-1β/IL-18 production and inflammasome activation in mouse models of diet-induced obesity. Visceral adipose IL-1β and Nlrp3 mRNA expression is reported to positively correlate with body weight and adiposity in mice fed with standard chow diet and to markedly decrease upon caloric restriction [71], while adipose tissue IL-1β protein is increased in obese db/db and HFD-fed C57BL/6 mice relative to their lean controls [71, 82]. Similar to IL-1β, adipose tissue and circulating IL-18 protein levels are increased in HFD-fed or genetically obese mice [71, 82, 106], despite no IL-18 mRNA differences, suggestive of increased adipose inflammasome activity. Caspase-1 mRNA and activity are increased in multiple tissues of HFD-fed and genetically obese mice [71, 82]. NLRP3 ablation attenuates obesity-induced caspase-1 activity in adipose tissue and liver, but not kidney [71], implying that kidney caspase-1 activation is either

NLRP3 independent or complemented by another inflammasome. NLRP3 inflammasome-dependent IL-1β secretion by the pancreas also increases during obesity and likely mediates chronic obesity-induced pancreatic damage [107].

2.5.3 *NLRP3 Inflammasome Expression and Activity in Obese Patients*

Circulating IL-1β concentrations are low in healthy human subjects. Elevated circulating IL-1β levels have been reported in some studies of obese patients [108, 109], but it is questionable whether these concentrations, typically <100 pg/mL, can induce biologically meaningful systemic effects. IL-1β protein concentrations in metabolic tissues, such as adipose tissue and liver, however, are clearly increased in obese human subjects, at levels likely to produce pathological effects, and weight loss reduces adipose and liver IL-1β mRNA expression [71, 110]. IL-1β secretion from human omental fat explants is correlated with the donor's body mass index [111], while LPS-stimulated IL-1β production from primary monocytes of obese alcoholics is correlated with body mass index, percent body fat, abdominal circumference, and total histologic score [112]. Similar to IL-1β, circulating IL-18 concentrations are higher in overweight subjects [113] and are decreased by weight loss [114]. However, unlike IL-1β, weight loss significantly decreases IL-18 mRNA expression in liver, but not in adipose tissue [110].

Few studies have investigated NLRP3 inflammasome genes in obese human subjects. In one study, adipose tissue caspase-1 mRNA expression increased in obese subjects, while NLRP3, IL-1β, and IL-18 mRNA expressions were not significantly different in normal-weight and obese subjects [115]. Results from weight loss studies are contradictory, with weight loss reducing NLRP3 expression in one study [71] but having no effect in another [110], although gender, ethnicity, and adiposity differences between these cohorts may explain this discrepancy.

No comparable data is available for the effect of weight gain and loss on caspase-1 activity in human adipose tissue. However, a recent analysis of paired subcutaneous and visceral adipose tissue biopsies from ten overweight subjects found that IL-1β and IL-18 production as well as caspase-1 activity was dramatically higher in visceral than subcutaneous adipose tissue [100]. Interestingly, caspase-1 activity levels were positively correlated with CD8+ T cell numbers present in both tissues. These findings are consistent with well-established results that visceral adipose tissue is more inflammatory than subcutaneous adipose tissue, and suggest a possible role for caspase-1 activity in the infiltration of CD8+ T cells into human adipose tissue. Further, our recent microarray analysis and RT-PCR analyses of high-purity human and mouse adipocyte fractions from subcutaneous and visceral adipose tissue indicate that the expression of NLR pathway genes, including NLRP3 and ASC, is significantly upregulated during obesity (unpublished data), suggesting that obesity may induce adipocyte inflammasome activity.

2.5.4 *Mechanisms of NLRP3 Inflammasome Activation in Adipose Tissue*

Adipose tissue TLR4 signaling plays an important role in obesity-associated insulin resistance [66]. In obesity, increased FFA levels stimulate adipose tissue TLR4 signaling by increasing adipose expression of fetuin A, an endogenous TLR4 ligand [116]. Since the proinflammatory TLR4-NFκB signaling cascade regulates both NRLP3 and IL-1β transcription, increased TLR4 signaling may provide the priming signal for the increased NLRP3 inflammasome activity observed in obesity. However, while TLR4 signaling may prime the system, it is unclear what triggers NLRP3 inflammasome activation during obesity. Extracellular ATP can active the NLRP3 inflammasome via a P2X purinoceptor 7 (P2RX7)-dependent mechanism [117], but inflammasome activation and adipose and metabolic phenotypes are similar in HFD-fed P2RX7-deficient and wild-type mice [118], excluding it from a role in this process. Obesity-associated metabolic danger signals, such as elevated FFA and ceramide, hyperglycemia, and mitochondrial dysfunction, may trigger NLRP3 inflammasome activation, as discussed above. Due to the central role adipose inflammation plays in obesity-related complications, identification of the responsible signal(s) regulating adipose tissue inflammasome activity is an important challenge for future investigation.

2.6 The NLRP3 Inflammasome in Metabolic Diseases Associated with Obesity

The prevalence of obesity has led to the increased incidence of obesity-associated metabolic diseases and become a serious threat to public health in the developed world. Overweight and obesity are associated with increased risk of atherosclerosis, type 2 diabetes, nonalcoholic fatty liver disease (NAFLD), neurodegenerative disease, and cancer [119]. Chronic inflammation, a key feature of obesity, plays an important role in the development of many metabolic diseases. As discussed above, the NLRP3 inflammasome has been recognized to mediate inflammatory reactions to several metabolic danger signals that are increased in obesity, implying that these stimuli induce NLRP3-driven inflammation to produce tissue injury in obesity. Indeed, the major effectors of inflammasome activation, IL-1β and IL-18, have been linked to inflammatory responses in various metabolic diseases [9, 120]. However, new research has just begun to explain the specific stimuli and molecular mechanisms acting on the NLRP3 inflammasome for individual metabolic diseases.

2.6.1 *Type 1 and Type 2 Diabetes*

Diabetes is characterized by uncontrolled blood glucose elevations resulting from inadequate insulin production due to pancreatic β-cell loss (type 1 diabetes) or

insulin resistance in skeletal muscle and other metabolic tissues associated that is with a slower decline in pancreatic β-cell function (type 2 diabetes). Mounting evidence now indicates that inflammasome activity plays critical roles in the dysregulation of both insulin production and insulin signaling.

IL-1β production by both pancreatic β-cells and infiltrating immune cells is known to regulate β-cell viability and insulin secretion. IL-1β treatment of both rodent and human pancreatic islets potently inhibits pancreatic β-cell-specific transcription factors and insulin secretion [121], while IL-1β secretion from invading immune cells induces pancreatic β-cell death during the development of autoimmune type 1 diabetes [122], partially through increased β-cell iNOS expression and NO production [123, 124]. Chronic hyperglycemia can also stimulate pancreatic β-cells to produce IL-1β, further impairing their viability and insulin secretion [73], through ROS-mediated activation of the NLRP3 inflammasome [50]. Finally, during type 2 diabetes high insulin secretion can result in the formation of cytotoxic IAPP aggregates that induce NLRP3 inflammasome activation and IL-1β secretion to stimulate β-cell apoptosis [51].

2.6.2 Metabolic Liver Disease

NAFLD is strongly associated with abdominal adiposity, and proinflammatory liver gene expression increases with adiposity [125]. NAFLD affects more than one-third of the Western world population, but only about 25 % of cases develop nonalcoholic steatohepatitis (NASH), which is characterized by chronic inflammation. NASH is a major cause of cirrhosis and liver transplantation, but the mechanism(s) underlying NAFLD progression to NASH remains elusive. Liver expression of NLRP3 inflammasome genes, caspase-1 activity, and mature IL-1β is, however, dramatically increased in mouse models of NASH [126]. NAFLD, the precursor to NASH, is also characterized by a marked increase in hepatocyte lipid accumulation (steatosis), which is attenuated by genetic ablation of NLRP3 inflammasome components [71, 82]. Saturated fatty acid accumulation in NAFLD may play an important role in hepatic inflammation, since the saturated fatty acid palmitate can induce inflammasome activation in cultured hepatocytes [126]. Hepatic inflammasome activation appears to require TLR2 signaling, which is induced by saturated fatty acids, since hepatic caspase-1 activation and serum IL-1β levels are suppressed in TLR2$^{-/-}$ mice fed with a NASH-inducing choline-deficient diet [127]. Finally, a gene regulating the elongation of C12- to C16-length saturated and monounsaturated fatty acids, Elovl6, has also been reported to regulate NASH development, hepatic NLRP3 inflammasome activation, and IL-1β release [128]. Taken together these results suggest that fatty acid accumulation directly or indirectly stimulates NLRP3 inflammasome activity. Genetic or pharmaceutical blockade of IL-1R signaling also attenuates NASH progression in mice [129, 130]. However, NAFLD/NASH phenotypes in these studies could result from non-hepatic inflammasome effects on insulin resistance and inflammation. Liver-specific NLRP3 inflammasome knockouts are required to address this argument.

2.6.3 Atherosclerosis

Chronic inflammation is a well-known contributor to atherosclerosis. Macrophage phagocytosis of modified low-density lipoprotein cholesterol present in the vasculature results in the accumulation of cholesterol-laden foam cells, increased oxidative stress, and vascular inflammation, which can ultimately lead to plaque destabilization and thrombosis. Moreover, IL-1β has been shown to participate in both the development and destabilization of atherosclerotic lesions. For example, genetic ablation of IL-1β or the IL-1 receptor in apolipoprotein E-deficient ($Apoe^{-/-}$) mice, a standard mouse model of atherosclerosis, significantly decreased atherosclerosis development [131, 132], as did treatment with an IL-1 receptor antagonist [133, 134]. Results from low-density lipoprotein receptor-deficient ($Ldlr^{-/-}$) mice fed with a high-cholesterol diet also indicated that macrophage-derived NLRP3 and IL-1 were essential for cholesterol-driven atherosclerosis, since these mice revealed significantly less atherosclerosis after receiving bone marrow transplants from NLRP3- or IL-1α/IL-1β-deficient mice [58]. Surprisingly, however, NLRP3 inflammasome activity was not required for normal atherosclerosis progression in $Apoe^{-/-}$ mice, since mice deficient in NLRP3, ASC, or caspase-1 expression revealed no decrease in atherosclerosis [135].

It is not clear why these results differ from those of $Apoe^{-/-}$ mice lacking IL-1β or IL-1 receptor expression or $Ldlr^{-/-}$ mice with NLRP3- or IL-1α/IL-1β-deficient bone marrow. One possible explanation, however, may be that $Apoe^{-/-}$ mice, unlike $Ldlr^{-/-}$ mice fed with a high-cholesterol diet, represent a nonobese mouse model of atherosclerosis and may therefore lack a metabolic stimulus found in obese mice. Furthermore, while this data indicates that inflammasome activity can play an important role in the pathogenesis of atherosclerosis, the agents that trigger NLRP3 inflammasome activation remain unclear. Duewell et al. have reported that cholesterol crystals, detected in early atherosclerotic lesions, induce NLRP3 inflammasome-dependent IL-1β production in murine macrophages [58], with similar results observed for human macrophages [86]. However, several other factors associated with increased atherosclerotic risk or the atherosclerotic microenvironment (hyperglycemia, ROS, uric acid) may also impact inflammasome activation.

2.6.4 Gout

Gout is the accumulation of MSU crystals in the joints, often leading to an inflammatory arthritis associated with elevated plasma uric acid. MSU crystals have long been identified as the causative agent of gout [136], but only recently has progress been made regarding the mechanism underlying their recognition as a proinflammatory danger signal. MSU crystals induce the production of inflammatory cytokines from monocytes and macrophages, particularly IL-1β [137–139], but do not induce IL-1β production in macrophages with ablations of various NRLP3 inflammasome

components [79], indicating that inflammasome activity plays an essential role in MSU recognition. Elevated plasma uric acid was proposed as one of the original criteria for diagnosis of obesity-associated metabolic syndrome [140], and obese patients are more than twice as likely to develop gout [141] and develop it about 11 years earlier than their normal-weight counterparts [142].

2.6.5 Alzheimer's Disease

Cerebral accumulation of amyloid-β plaques is one of the main pathologic features of Alzheimer's disease (AD), the most common form of dementia, and this is believed to be a critical stimulus for many proinflammatory components of AD that induce neuronal death and memory loss. Although the role of IL-1β in the pathogenesis of AD is controversial, numerous studies suggest that IL-1β can induce neuronal death and recruit inflammatory cells into the central nervous system [143]. Amyloid fibrils have been reported to trigger IL-1β release from microglia and monocytes [144, 145], suggesting a potential role for inflammasome activity in AD pathology. In keeping with this hypothesis, microglial NLRP3 inflammasome activity and IL-1β secretion are activated by phagocytosis of amyloid-β protein [57], while microglia with genetic ablation of caspase-1 or inflammasome components revealed lack of attenuated or defective inflammatory responses to amyloid-β exposure. Taken together, this data suggests that the inflammasome may represent a novel therapeutic target for the treatment of AD. Small molecule caspase-1 inhibitors such as those currently in clinical trials (see below) appear to be the best candidates for AD therapeutics, due to the difficulty expected in delivering recombinant protein therapeutics across the blood–brain barrier.

2.7 Cancer, Obesity and Inflammation

Mounting evidence suggests that inflammation promotes cancer development. Inflammatory responses are associated with 15–20 % of all cancer deaths worldwide, and inflammatory cells and factors are present in the microenvironment of most tumors, where they are often associated with metastasis and poor prognosis [146]. Proinflammatory cytokines, including IL-1β, IL-6, and TNFα, implicated to link inflammation and increased cancer risk, are expressed by adipose tissue and demonstrate increased systemic expression in overweight and obese human subjects [90]. Body fat is convincingly associated with colorectal, kidney, esophageal, pancreatic, endometrial, and postmenopausal breast cancer, and a recent meta-analysis of prospective cancer studies has expanded this list to include thyroid cancer, leukemia, multiple myeloma, and non-Hodgkin lymphoma in both men and women and increased risk of malignant melanoma in men [147]. Body weight is also associated with increased cancer mortality, since a prospective analysis of more than 900,000

US adults proposed that overweight and obesity may account for 14 % and 20 % of cancer deaths in men and women ≥50 years of age, respectively [148]. Based on the growing worldwide obesity epidemic, weight gain may represent the largest avoidable cause of cancer in nonsmokers.

2.7.1 Cell Death and Inflammasome Activity

An important hallmark of cancer is the ability of cancer cells to escape immunosurveillance [149]. Cancer cells are often resistant to various types of programmed cell death including apoptosis, programmed necrosis, and mitotic catastrophe [150]. Similarly inflammasome-mediated cell death, or pyroptosis, also appears to be involved in oncogenesis, since dysregulation of pyroptosis is tightly associated with tumor progression [151, 152]. Pyroptosis is stimulated by caspase-1 activity and plays key roles in anti-pathogen inflammatory responses, since bacterially infected macrophages and dendritic cells are usually eliminated through pyroptosis [152]. Caspase-1 activity can be activated by several mechanisms during pyroptosis. First, caspase-1 activity can be induced by classical inflammasomes, which usually contain NLRP3 or the cytoplasmic DNA sensor protein AIM2 [153]. Second, ASC can be recruited to a single subcellular location and aggregate into a polymer called an "ASC focus," "ASC speck," or "pyroptosome" that can then recruit and activate caspase-1 [154–156]. Finally, the NLRC4 inflammasome can also activate caspase-1 via a nonclassical, ASC-independent mechanism [157].

In pyroptosis, activated caspase-1 catalyzes the proteolytic activation of caspase-7, but not caspase-3, caspase-8, or caspase-9, to initiate programmed cell death [158]. However, while pyroptosis shares certain features with classical programmed cell death mediated via apoptosis, recent studies have identified characteristics that distinguish pyroptosis from apoptosis. First, apoptotic and pyroptotic cells demonstrate distinct DNA damage patterns. Both apoptotic and pyroptotic DNA damage can be detected by TUNEL staining [159, 160], but pyroptotic cells have a distinct nuclear morphology and usually lack the DNA laddering pattern characteristic of apoptotic DNA damage [159, 161]. Second, these processes have differential effects on cell membrane composition and integrity. For example, apoptosis triggers phosphatidylserine translocation from the inner to the outer plasma membrane, resulting in a cell surface annexin V-staining pattern. By contrast, during pyroptosis the formation of plasma membrane pores allows annexin V to enter the cell to produce an inner cell membrane staining pattern [162–164]. Finally, apoptotic and pyroptotic cell debris demonstrate different fates. During apoptosis, dying cells are cleaved into spherical membrane-bound structures known as apoptotic bodies that are ultimately cleared by phagocytosis [165], while the cytosolic contents of pyroptotic cells are released into extracellular space [159].

Similar to apoptosis, pyroptosis is thought to contribute to cell-autonomous tumor suppression. In support of this hypothesis, caspase-1 is downregulated in most human prostrate cancers, and caspase-1 overexpression in cultured prostate

cancer cells enhances their sensitivity to radiation-induced killing [166]. One study has also reported that NLRP3-deficient mice are more susceptible to colitis-associated colon cancer [167], while another has reported that NLRC4-deficient mice, but not NLRP3-deficient mice, have more tumor load than wild-type mice after exposure to azoxymethane-dextran sulfate sodium (AOM-DSS) [168]. Thus, while the function of specific inflammasomes in tumor suppression remains a matter of debate, these results appear to support the hypothesis that pyroptosis plays an important regulatory role in cancer development.

2.7.2 The Inflammasomes and Carcinogenesis

Chronic inflammation is often linked with increased cancer risk, and proinflammatory cytokines, such as IL-1β, TNF-α, and IL-6, are frequently associated with tumor progression [169]. However, the effect of inflammasomes on specific proinflammatory processes associated with increased cancer risk, such as colitis and colitis-associated cancer, remains controversial. For example, capase-1 or NLRP3 deficiency is reported to reduce colitis severity in DSS-treated mice [170, 171]. Consistent with these results, high levels of IL-1 in the tumor microenvironment usually correlate with a poor prognosis [172]. However, other authors have reported that mice with NLRP3, ASC, caspase-1, NLRP6, or NLRP12 deficiencies are more susceptible to DSS-induced colitis and death [167, 173–177]. In one case, an IL-18 deficit following AOM-DSS-induced intestinal damage in caspase-1 and ASC-deficient mice caused a systemic spread of commensal bacteria due to the inability of these mice to repair the mucosal barrier [174]. Colitis-associated cancer has also been reported to be significantly increased upon genetic deletion of components of the NLRP3 inflammasome (ASC, NLRP3, or caspase-1) [167, 168, 177]. These contradictory observations for NLRP3 inflammasome effects on AOM-DSS-induced colitis may be due to animal facility-dependent differences in gut microflora [178].

Despite contradictory reports on inflammasome effects on colitis, in vivo experiments suggest that IL-1β and IL-18 play important roles in promoting gastric, hepatic, and breast cancer progression [179–181]. Further, gastric-specific IL-1β expression in transgenic mice induces gastric tumorigenesis [182], while IL-1β-deficient mice have reduced and retarded subcutaneous tumor development in response to transdermal 3-methylcholanthrene [183], suggesting that local IL-1β-induced inflammation is strongly associated with carcinogenesis.

Based on this link, approaches that inhibit NLRP3, caspase-1, IL-1β, and IL-18 have been used to develop novel therapies to that have been assessed in a variety of experimental cancer systems. Small compounds targeting inflammasomes or NLRP3 have not been successful due to off-target effects [184–187]. However, reagents targeting IL-1β and IL-18, including monoclonal antibodies and recombinant derivatives [188, 189], have been more successful. For example, in patients with smoldering or indolent multiple myeloma, treatment with anakinra, a

recombinant non-glycosylated form of the human IL-1 receptor antagonist (IL-1ra), was found to decrease myeloma proliferation rates and high-sensitivity C-reactive protein (hsCRP) levels, leading to a chronic disease state with improved progression-free survival [190]. IL-1 inhibitors have also been shown to reduce the side effects of anticancer therapy [191, 192]. Based on the ability of IL-1R antagonism to reduce tumor burden and metastasis and the relatively low risk of in vivo IL-1 inhibition, future preclinical and clinical trials are needed to examine the effect of IL-1 inhibition on cancer outcomes [193].

2.7.3 Inflammasome-Dependent Anticancer Responses

While the inflammasome has often been linked to innate immunity, chronic inflammation, and carcinogenesis, mounting evidence suggests that the inflammasome is also involved in anticancer responses. Inflammasomes are hypothesized to inhibit tumor progression through several mechanisms, including (1) triggering innate immune reactions against potentially carcinogenic microbiota; (2) inducing the pyroptotic demise of premalignant, infected cells; and (3) facilitating antitumor adaptive immune responses [11].

IL-1β plays an essential role in stimulating adaptive immune responses and facilitating anticancer immunosurveillance [194]. Anticancer chemotherapies primarily eliminate tumors by inducing immunogenic cell death [195]. The immunogenic signals secreted by dying tumor cells not only attract innate immune effector cells into the tumor bed but also stimulate P2RX7 receptor-mediated activation of the NLRP3 inflammasome to produce IL-1β [196]. IL-1β, in conjunction with IL-23, then induces IL-17 secretion by γδ T cells [197, 198] and the polarization of CD8+ αβ T cell responses toward increased IFN-γ secretion [199]. Mice lacking P2RX7, NLRP3, ASC, caspase-1, or IL-1R1 do not respond to chemotherapies that elicit immunogenic cell death signals in wild-type mice [196]. Similarly, blockade of IL-1β signaling significantly impairs the growth-inhibitory effects of tumor chemotherapies in a variety of mouse models [197, 200]. Finally, P2RX7-mediated activation of the NLRP3 inflammasome is also required for the efficacy of anticancer therapies in patients [196, 201–203]. Taken together, these results indicate that inflammasome activity contributes to antitumor adaptive immune responses induced during chemotherapy. The study of the precise molecular mechanisms through which inflammasomes modulate anticancer immune responses may therefore yield insight into better anticancer therapeutics.

2.8 Pharmaceutical Interventions Targeting the Inflammasome

Several treatments designed to attenuate complications of metabolic disorders have now been developed using approaches that target NLRP3 inflammasome activity. Most of these strategies have focused on the development of therapeutic agents to

attenuate IL-1β activity, although other agents that directly target inflammasome function and caspase-1 activity are also under development and may provide alternative therapeutic approaches with distinct advantages and disadvantages. One major concern of all these approaches, however, is the potential for reduced tissue repair and immune surveillance, as a result of excessive suppression of NLRP3 inflammasome responses.

2.8.1 Therapeutics Targeting IL-1β Activity

IL-1β can reduce tissue insulin sensitivity and inhibit insulin production by pancreatic β-cell [204, 205], while antagonism of IL-1β signaling, by receptor blockade or cytokine neutralization approaches, has been shown to ameliorate several proinflammatory conditions, including type 2 diabetes. For example, short-term treatment of a small cohort of type 2 diabetic patients with anakinra, a recombinant non-glycosylated form of the human IL-1 receptor antagonist (IL-1ra) that is FDA approved for treatment of rheumatoid arthritis, was found to significantly improve glycemia and β-cell function, while decreasing the plasma level of two surrogate markers of systemic inflammation, hsCRP and IL-6 [206]. These effects occurred within 4 weeks of treatment and were sustained at 13 weeks of treatment. However, no significant improvements were observed in insulin sensitivity, insulin-regulated skeletal muscle gene expression, or serum adipokine levels, indicating anakinra treatment effects were due primarily to improvements in β-cell function rather than enhanced glucose disposal. Similar results were found in a second study performed with a cohort of insulin-resistant but nondiabetic obese human subjects, where anakinra also failed to improve insulin sensitivity [207]. Anakinra-mediated IL-1β antagonism does not, therefore, appear to significantly impact skeletal muscle insulin sensitivity, although the reason for this failure is unclear. Adverse events associated with anakinra usage in these studies were relatively mild, primarily consisting of transient injection-site reactions that most likely result from the solution used to dissolve the recombinant protein, although this is aggravated by a need for daily subcutaneous injections due to the short half-life (<1 h) of anakinra [208].

IL-1 neutralizing agents provide an alternate means to attenuate the deleterious effects of inflammasome activation. Rilonacept, a recombinant therapeutic agent used to neutralize free IL-1α and IL-1β, has been shown to significantly reduce pain scores and plasma hsCRP levels in a small study of ten patients with chronic gouty arthritis, refractive to standard treatment approaches, and to prevent acute gout flares during the initiation of urate-lowering therapy [209, 210]. Rilonacept, however, attenuates both IL-1α and IL-1β activity and thus cannot differentiate between inflammasome-mediated IL-1β effects and those of IL-1α. More recent approaches to specifically inhibit IL-1β bioactivity in the treatment of human inflammatory disorders have resulted in the production of new agents such as canakinumab, a fully human monoclonal antibody that neutralizes IL-1β bioactivity. Canakinumab can be administered intravenously or subcutaneously and is FDA approved for the treatment of cryopyrin-associated periodic syndromes (CAPS), including familial

cold auto-inflammatory syndrome and Muckle-Wells syndrome [211], which are associated with mutations in NLRP3 [13]. Canakinumab is also now being used in clinical trials for the treatment of a number of inflammation-related disorders such as type 2 diabetes and its derived complications and chronic obstructive pulmonary disease, with the CANTOS trial (cardiovascular risk reduction in type 2 diabetes) representing the largest trial of any anti-cytokine drug to date [212].

In addition to these three FDA-approved drugs, a number of agents targeting the IL-1 pathway are under preclinical or clinical development. The majority of these agents are neutralizing antibodies, such as gevokizumab (XOMA-052) and LY2189102, that reduce IL-1β bioavailability [212], although IL-1 receptor blocking antibodies, such as MEDI-8968 and AMG-108 (aka MEDI-78998), are also under study as novel therapeutics for treatment of chronic inflammatory diseases. XOMA-052 has been granted orphan drug status by the FDA for the treatment of Behçet's disease, a rare condition where chronic immune-mediated vascular inflammation can result in severe neurological, pulmonary, gastrointestinal, and cardiovascular complications [213]. XOMA-052, LY2189102, MEDI-8968, and AMG-108 are all now under investigation in phase II clinical trials: XOMA-052 and LY2189102 for the treatment of type 2 diabetes, MDI-8968 for the treatment of chronic obstructive pulmonary disease [212], and AMG-108 for the treatment of rheumatoid arthritis. Both MEDI-8968 and AMG-108 are fully human monoclonal antibodies that selectively bind IL-1R to inhibit the binding and subsequent signaling of both IL-1α and IL-1β, resulting in inhibition that is not restricted to inflammasome-mediated IL-1β responses. However, a clinical trial of AMG-108 effects on patients with rheumatoid arthritis or osteoarthritis of the knee found moderate disease improvement coupled with an excellent safety profile [214, 215], suggesting that an "off-target" suppression of IL-1α may not produce severe side effects. Finally, induction of endogenous antibodies by therapeutic vaccines has proven to be a safe and effective means of attenuating other disease conditions [216]. Recently, this approach has also been extended to cytokine-induced disease conditions, and a vaccine targeting IL-1β (CYT-013) is currently in phase I clinical trial in patients with type 2 diabetes [212].

2.8.2 Therapeutic Approaches Acting on the Inflammasome or Caspase-1

Inflammasomes play important roles in immune surveillance and tissue repair. Thus, approaches designed to directly target pathological inflammasome activity need to maintain a careful balance between attenuating deleterious inflammatory activity and maintaining necessary host defense and tissue repair responses. Relatively little is known about the exact mechanisms that regulate inflammasome assembly and caspase-1 activation, however, and these may differ according to the stimulus and NLR subtype of the inflammasome complex. Caspase-1 blockade thus

appears to be a far more attractive target for treatment of inflammasome-related disorders due to the greater knowledge base available for caspase-1 inhibition.

Two small molecule caspase-1 inhibitors, VX-765 and VX-740 (pralnacasan), have been tested in clinical trials for the treatment of chronic plaque psoriasis, rheumatoid arthritis, and psoriasis [217]. VX-765 has also been tested on six patients with Muckle-Wells syndrome, resulting from mutations in NLRP3, where it was found to significantly reduce inflammatory markers, recurrent fevers, and arthritis [212]. The rheumatoid arthritis clinical trial using VX-740 was discontinued, however, due to liver abnormalities in animal toxicology studies [217]. No caspase-1 inhibitors are currently approved for clinical use, but other approved drugs with known anti-inflammatory activity may function in part by attenuating inflammasome complex activity. For example, treatment with glyburide, a sulfonylurea drug frequently used for the treatment of type 2 diabetes [51, 55], has been shown to attenuate inflammation under diabetic conditions by blocking IAPP-stimulated NLRP3 inflammasome activation and IL-1β secretion [51]. Glyburide has also been shown to prevent NLRP3 inflammasome activation in response to pathogen-associated and DAMP signals and biological crystals [55]. Glyburide inhibits ATP-sensitive potassium channels (K_{ATP}) in pancreatic β-cells to induce insulin secretion, but NLRP3 activation and glyburide-mediated inhibition were preserved in macrophages lacking K_{ATP} subunits and ATP-binding cassette transporter proteins, indicating that glyburide does not inhibit NLRP3 inflammasome activity through attenuating potassium efflux. Moreover, glyburide had no effect on NLRP3 ATPase activity, strongly suggesting that glyburide acts upstream of the NLRP3 inflammasome [55].

2.8.3 Other Therapeutic Targets to Attenuate Inflammasome Activity

Improved knowledge of the signaling networks involved in inflammasome activation has led to the discovery and testing of new therapeutic targets, such as the P2X purinoceptor 7 (P2RX7), an ATP receptor that regulates potassium efflux to induce inflammasome-mediated caspase-1 activation [117]. However, while several studies have now been performed with P2RX7 antagonists, none have shown significant action to reduce inflammation. AZD9056, an oral P2RX7 antagonist, has been evaluated in a phase IIa and subsequent phase IIb clinical trial for treatment of rheumatoid arthritis, but failed to demonstrate any significant efficacy [218]. The P2RX7 antagonist CE-224,535 also failed to reveal efficacy in patients with active rheumatoid arthritis and an inadequate response to methotrexate [219]. Finally, the P2RX7 modulator GSK1482160 was also recently investigated for single-dose safety, tolerability, pharmacokinetics, and pharmacodynamics, in healthy human subjects, but simulations lead to the conclusion that it was not possible to achieve the desired level of pharmacology, resulting in the termination of GSK1482160 development for chronic inflammatory pain [220].

2.9 Summary

Increasing evidence indicates multiple metabolic and cellular factors contribute to NLRP3 inflammasome activation, which plays a key role in adipose tissue inflammation and resultant obesity-associated tissue injury and metabolic derangement. These observations have widespread implications for a variety of diseases that are increased in obesity including diabetes, atherosclerosis, NASH, Alzheimer's disease, and cancer. Better understanding of the roles of NLRP3, IL-1β and IL-18 in mediating specific tissue injuries coupled with new therapeutics that target inflammasome activity may permit the development of novel and more precise interventions to prevent or treat these important disease conditions. Therapeutic control of the inflammasome is in our grasp.

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Chapter 3
Uncoupling Obesity from Cancer: Bromodomain Co-regulators That Control Inflammatory Networks

Gerald V. Denis and Deborah J. Bowen

Abstract As the epidemic of overweight and obesity spreads, the number of individuals at risk for metabolic complications of obesity, including cardiovascular disease, type 2 diabetes, and cancer, is expected to increase. Importantly, the risks of complications are not evenly distributed, because not all obesity is biochemically identical. Here we describe "metabolically healthy obese" humans and animal models that show remarkable protection from insulin resistance and glucose intolerance, despite severe obesity. A hallmark of these patients and animals is their reduced inflammatory profile, which we hypothesize confers protection not only from cardiometabolic risk in obesity but also from obesity-associated cancers. Research is urgently required to investigate the basis for this protection, to identify treatment options and prevention strategies for at-risk populations. We explore novel insights into chromatin-based, transcriptional co-regulator mechanisms that link apparently unrelated diseases, with the idea that certain molecularly targeted strategies could moderate multiple risks in obesity. We voice concern that low socioeconomic status citizens are particularly at risk for cardiometabolic disease and obesity-associated cancer, in part because many such individuals live in inflammatory and obesogenic environments. An integrated and hypothesis-driven approach is needed to study and protect these vulnerable and underserved populations from the rising tide of obesity-associated cancer.

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3.1 The Problem of Obesity-Associated Cancer

Diet-induced overnutrition that causes unhealthy weight gain, defined in humans as *overweight* (Body Mass Index [BMI] 25.0–29.9 kg/m^2), *obesity* (BMI ≥30.0–39.9 kg/m^2), and *morbid obesity* (BMI ≥40.0 kg/m^2), has many medical complications. The realization that obesity had become a serious public health concern was initially driven by projected increases in the prevalence of metabolic complications, such as elevated risk for stroke, cardiovascular disease, and type 2 diabetes. The complications of obesity also include dyslipidemia, hypertension, sleep apnea, hepatosteatosis, and glucose intolerance [1]. However, attention has been recently focused on a particularly worrisome complication: obesity-associated malignancy [2, 3]. Recent epidemiological reports have caused serious disquiet that, despite overall declines in cancer rates, particularly the rates for tobacco-associated cancers, the rates of obesity-associated cancers are climbing. Obesity is now thought to be one of the most important preventable causes of several cancers [4]; these include esophageal adenocarcinoma, colorectal cancer, breast cancer in postmenopausal women (but not premenopausal women), and cancers of the endometrium, kidney, pancreas, liver, and gallbladder [3, 5, 6]. The National Cancer Institute, American Cancer Society, and American Association for Cancer Research have been using their influence and expert opinion in recent years to increase the public profile and research portfolio devoted to this problem and its allied risk factors. Obesity-associated malignancies have been estimated to account for 14 % of male and 20 % of female US cancer mortality, notably colorectal cancer and postmenopausal breast cancer [2]. This chapter will present some of the molecular, cellular, and immunological features that link obesity and its complications to cancer.

In view of recent data from the US Centers for Disease Control and Prevention [7], showing that all US states now report at least 20 % prevalence of obesity among adults, as well as the classification of 1.7 billion people worldwide as overweight [8], obesity-associated cancer is positioned to become one of the defining preventable diseases of our time. Diabetes is also a serious complication among the chronic disease burdens of obesity. In fact, overweight and obesity are now well established to be the direct cause of most cases of type 2 diabetes [9]. Three hundred and sixty-six million cases worldwide was a frequently cited early estimate of the incidence of type 2 diabetes by 2030 [10]. Alarmingly, more recent estimates have adjusted upward the anticipated number of diabetic individuals to 439 million by 2030 [11]. Almost all of the incidence will be driven by overweight and obesity. The anticipated further increases in BMI worldwide [12] predict that the seriousness of the problem of obesity-associated cancer will also deepen in coming decades.

3.2 Molecular Features of Insulin-Resistant Obesity

The molecular mechanisms that explain how obesity contributes to cancer risk are still largely unknown in detail. Early epidemiological investigation of obesity and its comorbidities identified an association between the incidence of

type 2 diabetes and obesity [13]. Some of the key features of insulin-resistant obesity include elevated concentrations of blood glucose in fasted subjects and impaired glucose tolerance, as well as elevated blood concentrations of insulin in fasted subjects and reduced insulin sensitivity. Leptin, which is a critical regulator of appetite, is produced by adipocytes and is elevated in obesity in proportion to adipose tissue mass [14]. This hyperleptinemia in obesity has been frequently described as "leptin resistance," a term now thought to lack clinical utility [15]. In addition, insulin-resistant obesity frequently features reduced serum concentrations of adiponectin [16, 17], an insulin-sensitizing adipokine [18] that exhibits beneficial, antiatherogenic effects. These characteristics are commonly observed together in obese subjects [19–21] and animal models [22] and reflect the growing inability of peripheral tissues of the obese subject, such as skeletal muscle and fat stores, to transport glucose from blood into cells at normal levels of insulin present in the circulation. This state has been described as peripheral insulin resistance. Commonly, the pancreatic β-cells of such a subject are required to secrete ever higher levels of insulin to compensate for the peripheral insulin resistance. In humans and certain rodent models, this chronic hyperinsulinemia and accompanying β-cell dysfunction are two of the defining characteristics of insulin-resistant obesity and are often associated with increased serum levels and bioavailability of the related mitogenic factor, insulin-like growth factor (IGF)-1 [23].

These clinical presentations provoked questions about which biochemical features of insulin resistance and type 2 diabetes were important for carcinogenesis in obesity. Signal transduction through the insulin receptor and IGF-1 receptor [24] is thought to increase cancer risk in obesity [25, 26]. Fasting insulin concentrations have been used convincingly as a prognostic factor for overall survival among breast cancer patients, with the highest hazard ratio associated with the highest insulin concentrations [27]. Leptin also promotes mitogenic [28–31] and invasive [32] effects in a variety of human cancer cell lines [33–35] and tumor models in animals [36]. For example, leptin-deficient mice appear to be protected from mammary carcinogenesis [37–39]. Adiponectin not only protects insulin-sensitive glucose transport but also appears to be inversely correlated with susceptibility to certain obesity-associated cancers [40–42]. Furthermore, the leptin-adiponectin ratio has been proposed to be a critical predictor of cancer risk [38]. These features have been well summarized elsewhere [43–45]. However, the co-occurrence of these multiple factors in obesity has made it difficult to define the relative importance of each. Overall, rodent models have tended to show that alteration of any single factor in isolation affects mitogenesis, tumor progression, or other relevant parameter of the malignancy. Experimental designs that use rodent models in which multiple variables are manipulated simultaneously to influence cancer risk do not permit straightforward interpretation; thus, the field remains divided about which cellular and molecular factors are of paramount importance for specific obesity-associated cancers.

3.3 Insulin-Resistant Obesity Is Also an Inflammatory Disease

One of the earliest described immunological features of insulin-resistant obesity was subclinical, unresolved, chronic inflammation, which occurs both systemically [47, 67] and in white adipose tissue [48], which is infiltrated with pro-inflammatory macrophages [49–52]. Specifically, such patients demonstrate elevated serum concentrations of acute phase proteins and pro-inflammatory cytokines [53], such as interleukin (IL)-1β, IL-6, and C-reactive protein, that improve over time after intentional weight loss [54, 55, 67] or bariatric surgery [56]. Exposure of glucose-transporting cells to the pro-inflammatory cytokine tumor necrosis factor (TNF)-α was demonstrated as long ago as 1993 to promote insulin resistance directly [57]. Adipose tissue depots, composed of white adipocytes, are typically inflamed, that is, infiltrated with Th1- and Th17-polarized T cells [58] and pro-inflammatory macrophages, both in obese humans [59–63] and animal models [50, 51, 59, 64–66] of diet-induced obesity. In insulin-resistant obesity, the pro-inflammatory macrophages that infiltrate these depots secrete significant amounts of pro-inflammatory cytokines, which, in addition to TNF-α [57], include IL-6, IL-8, and monocyte chemoattractant protein (MCP)-1/chemokine (C–C motif) ligand (CCL)2 [46, 67]. Systemic inflammation is also a feature of insulin-resistant obesity, as indicated by elevated serum levels of C-reactive protein [68] and several of the aforementioned and other cytokines [61]. Furthermore, chemokines such as MCP-1/CCL2 also serve to recruit additional leukocytes, such as peripheral blood monocytes that express the C–C motif chemokine receptor (CCR) 2 [50, 51, 60], to infiltrate the insulin-resistant adipose depot in a deepening cycle of unresolved, chronic inflammation. Thus, a feed-forward loop is established that is difficult for homeostatic forces in the immune system to oppose [58]. Failure of the anti-inflammatory balance may also be an independent, critical factor in the emergence of the many comorbidities of obesity.

Moreover, certain specific, histological features define insulin-resistant adipose tissue. The adipocytes frequently become stressed as their storage limits are exceeded, leading to a large number of apoptotic adipocytes, a process that is thought to recruit additional leukocytes [59]. The dead and dying adipocytes of stressed white adipose tissue appear surrounded with a ring of pro-inflammatory macrophages ($CD68^+$ in humans [69]) that are histologically termed “crown-like structures” [59] and are associated with fibrosis [70] and increased metabolic risk [71–74]. How these structures arise and are resolved by weight loss or drug treatments is not well understood. In mouse models, the macrophages that infiltrate metabolically unhealthy white adipose tissue tend to express a surface phenotype, that is, $CD11b^+$ $CD11c^+$ $F4/80^+$ by flow cytometry, that identifies them as pro-inflammatory. These pro-inflammatory macrophages have been directly implicated in the decline of metabolic health of adipocytes in white adipose tissue in different adipose depots in animal models [75, 76] and humans [101]. Early in the kinetics of diet-induced obesity in rodent models, adipocyte death and the development of whole-body insulin resistance [65] also correlate with a switch in macrophage

polarization toward the classically activated, pro-inflammatory (so-called M1) phenotype and away from the alternatively activated, anti-inflammatory ("M2") phenotype [78]. The $CD11c^+$ adipose tissue macrophage populations also transiently remodel the white adipose tissue, which then exhibits activities connected with M2-associated genes, such as increased expression of arginase, IL-10, IL-4, and transforming growth factor (TGF)-β [79]. The net balance of these M1 and M2 inputs defines the profile and magnitude of white adipose tissue inflammation. Relatively high expression of M1 cytokines is associated with metabolic complications of obesity, including insulin resistance. However, in human adipose depots, the molecular details of putative M1 phenotypes and function, and the M1/M2 switch, are less well understood than in animal models.

T cells are also recruited to white adipose tissue in diet-induced obesity through "regulated on normal T cell expressed and secreted" (RANTES/CCL5) and its receptor CCR5 in white adipose tissue [80, 81], where the Th1/Th2 polarization and proliferation of T cells are influenced by macrophage-produced cytokines [82]. T cells play a major role in insulin resistance [62] through macrophage recruitment [83]. T cell polarization between the pro-inflammatory (interleukin-17 producing) subtype (Th17 [84]) and the anti-inflammatory (IL-10 producing) T regulatory subtype ($Foxp3^+$ Treg [85]) also influences metabolism in white adipose tissue. A pro-inflammatory imbalance in $CD4^+$ T cell subsets has been demonstrated both systemically and in adipose depots of type 2 diabetic subjects [58]. The balance of pro-inflammatory and anti-inflammatory cytokines and T cell subsets remains perturbed in insulin-resistant obesity; some investigators hypothesize the adipocyte/T cell cross talk is the critical factor that promotes disease pathogenesis, whereas others hypothesize that macrophages have primary importance. The interpretations have remained controversial. Recent data from human studies also supports a central role for B cells in the pathogenesis of type 2 diabetes in obese subjects [58, 86, 87]. The independent and interdependent roles of T cell subsets, B cell subsets, and monocyte/macrophage polarization, and their specific cross talk with adipocytes that influences risks for obesity-associated cancer and type 2 diabetes, define a central focus of the exciting new field of immunometabolism [58].

Outside the adipose depots, the immune system of the obese and insulin-resistant subject demonstrates systemic, pro-inflammatory changes in T cell, B cell, and myeloid subset differentiation and function that exacerbate the deepening cytokine/chemokine imbalance as metabolism deteriorates in diet-induced obesity. Animal models demonstrate that stoppage of immune cell-mediated inflammatory cascades by any one of several diverse techniques (e.g., genetic, small molecule, or antibody-based) frequently delays or prevents insulin resistance [88, 89]. If metabolic parameters improve through dietary intervention, adipose tissue inflammation also typically improves [90]. The long-established links between chronic, unresolved inflammation and cancer therefore provide a basis to hypothesize that the presence of crown-like structures, for example, or elevation of other local and systemic inflammatory markers, is positively associated with cancer risk for obesity-associated cancers that have an inflammatory component.

3.4 Insight from Unexpected Results

Obesity-associated malignancies are not linked to every type of cancer. Apart from lung cancer, which is associated with cigarette smoking [91, 92] or asbestos inhalation and not with obesity [3], certain other cancers are also clearly not associated with obesity, including but not limited to astrocytoma; glioma; Kaposi's sarcoma; neuroblastoma; head, neck, and oral cancer; pituitary cancer; retinoblastoma; salivary cancer; and testicular cancer. A possibly relevant, shared feature of these cancers is that they do not originate in or near visceral adipose tissue. Compared to subcutaneous depots, the visceral or "central" adipose depot [63, 93, 94] is the most inflamed in obese insulin-resistant patients [95–97] and is independently associated with cardiometabolic risk. In animal models, the epididymal adipose depot of male mice is regarded as a good model for visceral adipose tissue inflammation in diet-induced obesity [59, 65, 66]. Likewise, many (but not all) of the obesity-associated cancers are resident in or likely influenced by inflamed visceral adipose tissue. All female breast carcinomas, for example, are surrounded by significant adipose depots in humans and the mammary fat pad in mice. It is likely that the metabolic and inflammatory properties of this adipose depot are highly relevant to specific aspects of breast cancer progression, invasiveness, metastasis, or recurrence, although this area has not received sufficient attention from investigators.

The observation that insulin-resistant obesity features *systemic* elevations of pro-inflammatory cytokines, as well as systemically elevated glucose, insulin, IGF-1, leptin, and depleted adiponectin, raises a problem. Why are not all cancers obesity-associated? If the argument is made that insulin and IGF-1 signaling cross talk, as well as leptin-promoted, broad-spectrum mitogenesis or diminished protection from adiponectin, are critical factors that explain obesity-associated cancers, why should so many cancers be unrelated to obesity? Presumably these systemic factors affect diverse tissues roughly equally, although different cells of origin of the tumor likely respond differently to the complex endocrine and metabolic microenvironment in each tissue. A recent repeated measures study from the Women's Health Initiative suggests that, at least in the case of colorectal cancer risk in postmenopausal women, the most important association is with elevated glucose, not elevated insulin [98]. It seems likely that additional features of the obese subject influence carcinogenesis and perhaps stratify risk for obesity-associated cancer.

Although insulin-resistant obesity is a chronic inflammatory disease, 20–30 % of adult obese individuals preserve a *reduced* inflammatory profile. The white adipose tissue of these un-inflamed, adult subjects shows lower numbers of infiltrating leukocytes [72], while systemically, serum concentrations of pro-inflammatory cytokine are lower [99, 100] than in insulin-resistant obese adult subjects. These un-inflamed subjects exhibit normal or near-normal glucose tolerance [72] (Fig. 3.1), reduced cardiovascular disease risk [77], and lack metabolic syndrome [72, 102, 169]. They remain relatively "metabolically healthy" with low-inflammatory profiles despite obesity [103, 104, 169] and represent an important off-diagonal population for which cardiovascular risk appears to be uncoupled from obesity [105, 106].

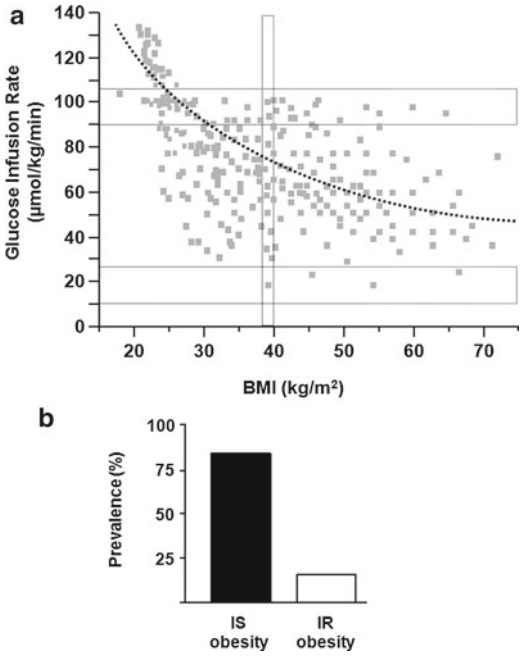

Fig. 3.1 "Metabolically healthy obesity." (**a**) Glucose infusion rates (GIR) in 237 subjects for a broad range of BMI and metabolic health. Insulin sensitivity was determined by GIR during the steady state of a euglycemic-hyperinsulinemic clamp. The highest and lowest quintiles of GIR are marked (*horizontal boxes*) to show that the frequency of insulin resistance is very low in healthy obesity. A regression curve (*dotted line*) of GIR over BMI is based on all available patients. The BMI stratum 39–40 identifies a continuous distribution (*vertical box*) of rates to show that there is no clear separation between insulin-sensitive and insulin-resistant obesity. Note that certain, rare individuals with unusually high BMI (>60) nevertheless display normal, healthy GIR during the clamp. (Subject exclusion criteria were diabetes, hypertension, acute or chronic inflammatory disease with leukocyte count >8,000 Gpt/L, CRP >5.0 mg/dL or clinical signs of infection, and other relevant criteria as detailed in ref. [77].) (**b**) Prevalence of insulin-sensitive (GIR >80 µmol glucose/kg/min) and insulin-resistant (GIR <40 µmol glucose/kg/min) healthy obesity (data from Blüher M (2010). The distinction of metabolically 'healthy' from 'unhealthy' obese individuals. *Curr Opin Lipidol*, 21(1):38–43 are reproduced with permission from M. Blüher and Wolters Kluwer, publishers)

The well-established association between unresolved, chronic inflammation and cancer [107, 108] (e.g., between Crohn's disease and colorectal cancer [109]) suggests that inflamed adipose tissue in insulin-resistant obese adults plays a critical role in obesity-associated carcinogenesis. We have previously hypothesized that the low-inflammatory features and preserved glucose tolerance of these subjects *also protects against risks for obesity-associated cancers* [110].

How do these metabolically healthy obese individuals remain un-inflamed? We have focused on a recently described transcriptional mechanism that may link chronic inflammation, obesity, and cancer [140]. Bromodomain-containing transcriptional co-regulators [112] bind to acetylated histones [113–115] in the

nucleosomal chromatin of mammals and target specific genes for transcriptional activation or repression. In this way, they functionally resemble SWItch/Sucrose NonFermentable (SWI/SNF) nucleosome remodeling complexes, which also contain bromodomain subunits and function to activate or silence coordinated networks of genes [116]. Bromodomains are protein motifs of about 110 amino acids in length [117, 118], comprised of four antiparallel α-helices that are linked by connecting loops, which form a binding pocket that is specific for acetylated lysine [119]. These motifs are found in transcription factors, histone acetylases, and related chromatin-directed proteins that are important for gene regulation [117, 120, 121]. Previous work has shown that the double bromodomain-containing proteins Brd2 [122, 123] and Brd4 [124] couple histone acetylation to transcription [125, 126] and are critical for transcriptional control of cell cycle genes [127–131]. Increased or deregulated expression of either protein is oncogenic. In humans, reciprocal chromosomal translocation of *BRD4* [132] creates a dangerous oncoprotein that promotes an aggressive, poorly differentiated, and incurable carcinoma of the midline, called NUT midline carcinoma, that afflicts relatively young people [133, 134]. Remarkable recent studies with small molecule inhibitors of the binding interface between the acetylated lysines and bromodomain have revealed that the chromatin-bromodomain interaction is "druggable" [135], which came as a surprise to the field. Conventional wisdom had held that interactions with such protein motifs in chromatin were unappealing targets for the development of small molecule inhibitors. In the case of midline carcinoma [136] and other human malignancies [137–139], such drugs appear to have significant anticancer benefit [140]. These developments linked Brd2 and Brd4 with cancer and chromatin-controlled networks of gene expression that are coordinated through shared complexes, analogous to the SWI/SNF-regulated array of genes. But the chromatin-based connections between cancer, obesity, and inflammation remained obscure until unexpected results from a bromodomain-manipulated mouse model appeared.

We initially developed a mouse model for Brd2 transgenic expression [141] and showed that upon B cell-restricted expression of Brd2, mice upregulate B cell mitogenic responses through cyclin A transactivation [131] and eventually develop a B cell malignancy [141]. This cancer exhibits a transcriptional fingerprint most similar to the "activated B cell" (ABC) form of diffuse large B cell lymphoma in humans [142], with an inflammatory signature. Surprisingly, whole-body reduction of Brd2 in "Brd2 hypomorph" mice, by *lacZ* gene disruption, caused the development of a glucose-tolerant type of obesity that features elevated serum adiponectin and a remarkably attenuated inflammatory profile [143]. These results suggested that the Brd2 hypomorphic mouse might represent a useful model for human subjects who are metabolically healthy obese (Fig. 3.1) [144]. These humans share with the Brd2 hypomorphic mice a low-inflammatory profile [77, 103, 105] and less reduction in serum adiponectin concentrations despite obesity [72]. The elevated concentrations of adiponectin measured in adiponectin transgenic mice are also metabolically protective [170], although neither adiponectin expression nor any other loci apart from *Brd2* were directly manipulated in Brd2 hypomorphic mice. More significantly, the chromatin-directed networks that these bromodomain-containing co-regulators

control likely connect cancer, obesity, and inflammation directly, through coordinated co-activation or co-repression of interacting networks of genes; certain diseases likely share the same or overlapping sets of gene expression co-regulators. This topic has been reviewed [140].

In forthcoming work, we show that small molecule inhibitors of these bromodomain proteins or shRNA ablation are effective as anti-inflammatory strategies, acting as global "uncouplers" of signal transduction that normally activate transcription of diverse cytokine genes [111]. Because many cancers have an inflammatory component, it is reasonable to hypothesize that the inflammatory microenvironment of certain tumors will exacerbate carcinogenesis, tumor progression, invasion, metastasis, or recurrence. Targeted uncoupling of signal transduction from transcription through bromodomain protein-specific small molecule inhibitors [140] may prove to be a novel and efficacious therapeutic or preventive approach to reduce the inflammatory cascades that contribute to obesity-associated cancer. The combined anti-inflammatory [145] and anticancer [136–138] effects of bromodomain protein inhibition are already established. Our previous work has shown that reduced expression of Brd2 protein, equivalent to a haploinsufficient phenotype, de-represses specific genes that are important for metabolism. These reduced levels are sufficient *simultaneously* to stimulate insulin gene transcription in β-cells [143, 146], to increase adipogenesis in pre-adipocytes through stimulation of peroxisome proliferator-activated receptor (PPAR)γ-directed transcriptional programs [143, 147]. The hyperadiponectinemia of obese Brd2 hypomorphic mice [143] suggests that Brd2 is also normally required to corepress transcription of the murine adiponectin gene (*Adipoq*), although this hypothesis has not yet been tested. Conversely, Brd2 reduction also ablates the production of pro-inflammatory cytokines in macrophages such as TNFα, IL-1β, IL-6, and MCP-1 [111, 140, 144]. Taken together with the anticancer properties of Brd2 reduction through attenuated cell cycle progression, as discussed above, these coherent transcriptional and metabolic features (stimulated insulin production, increased adipogenesis, and increased adiponectin production; and reduced production of multiple pro-inflammatory cytokines) lead us to propose a Brd2 mechanism for broad protection against obesity-associated malignancy. Small molecule inhibitors that target this family of transcription co-regulators, or naturally occurring single nucleotide polymorphisms in the human *BRD2* locus that reduce Brd2 expression, may therefore confer multiple forms of metabolic and cancer protection to obese patients.

Several phenotypes of unintended weight loss, such as chronic heart failure [148], share a systemic inflammatory profile [149], with marked elevations in serum levels of IL-1β, IL-6, and TNFα. These observations suggest a mechanistic relationship between the immune system, metabolism, and energy balance, reinforcing the aforementioned argument. Furthermore, it has been noted that "unhealthy aging" [150] is often associated with a pro-inflammatory, pro-senescent phenotype in somatic cells, the local production in skeletal muscle and adipose depots of inflammatory cytokines that are associated with muscle wasting syndromes, and frailty in geriatric patients [151–153]. Investigators have been considering the cause and effect relationships among unresolved, chronic inflammation, energy imbalances associated with weight

loss or weight gain, and cancer risk. Many of these relationships may work in both directions. It is reasonable therefore to hypothesize that a chromatin-based therapeutic strategy to treat these connected phenotypes may have broad benefit for more than one type of risk and may be useful for geriatric patients.

3.5 Other Links Between Obesity, Inflammation, and Social Determinants

One social determinant that plays a role in inflammatory disease processes is socioeconomic status (SES). Asthma rates in children are two to three times higher in poor families than in wealthy families; SES shows a dose response relationship with asthma diagnosis and severity [154]. Public housing residents and inner city dwellers, who are among the poorest of urban dwellers in the United States, report higher rates of asthma than do private home and apartment dwellers [155]. This disease arises from allergic reactions to irritants and allergens that are commonly found in public housing, including dust mites, pets, rodents, mold, and cockroaches [156]. Massachusetts public housing has been linked to some of the highest national rates of asthma [157]. The prevalence of asthma is highest among African American families, with overall prevalence of 40 % of adults and 56 % of children [158]. These same populations, that is, poor and low SES individual and public housing residents, report two to three times the obesity rates of other residents who are higher along the SES continuum [159]. According to the American Lung Association, there is no evidence that asthma can cause lung cancer. However, there is evidence that asthma is associated with obesity [155, 160–162]. The risk of asthma has been reported in one study [163] to be up to three times greater for obese subjects compared to lean subjects (odds ratio for obese vs. normal BMI=2.28, 95 % CI: 1.76, 2.96). These observations suggest that socioeconomic factors also influence risk for obesity-associated morbidity, including type 2 diabetes and cancer. Specifically, there may be a rationale to investigate the relationship between asthma, obesity, and obesity-associated cancer. For example, does poverty produce obesity and inflammation, enhancing opportunities for the development of asthma? Are these issues causally related or simply comorbidities of living in high poverty settings? If we are able to reduce one set of comorbid conditions, as is under investigation now in Boston and elsewhere [164–166], will that outcome reduce or alter others?

Several sociological, economic, and behavioral factors have been established to link obesity and type 2 diabetes incidence to cancer incidence. As discussed above, there is strong epidemiological evidence that SES is correlated with both the prevalence of obesity and diabetes and with lung cancer mortality, a malignancy that is associated with smoking (Fig. 3.2a). There is no known molecular association between lung cancer and obesity [3]. However, the use of tobacco in cigarette, cigar, and pipe smoke is strongly associated with lung, tracheal, and oral *cancers* [167]. Furthermore, low SES individuals suffer disproportionately higher health risks due to increased prevalence of smoking [168]. These correlations among chronic diseases that have no downstream molecular connection suggest that the problem of obesity-associated cancer is more complicated in its structural and upstream origins

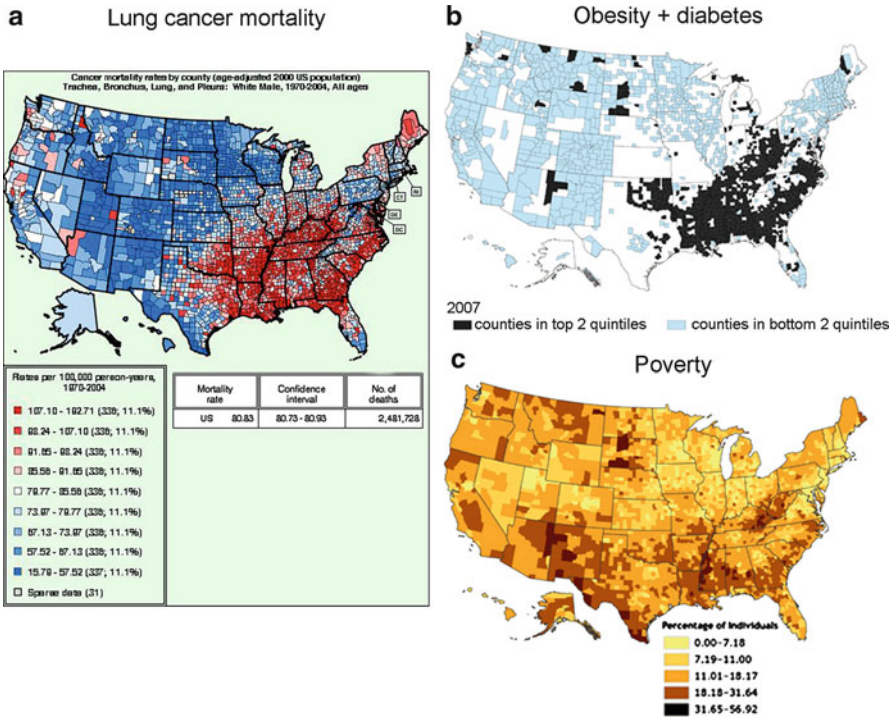

Fig. 3.2 Overlapping morbidities. (**a**) Lung cancer mortality. Mortality among 3,056 United States counties for cancer of the lung, trachea, bronchus, and pleura in white males of all ages, 1970–2004 (age-adjusted 2000 US population). Calculated from *National Cancer Institute data drawn from Atlas of Cancer Mortality in the U.S., 1950–1994*; rates per 100,000 person-years presented here in nine equal intervals with a diverging *red/blue* color scheme. The national rate was 80.83 (CI 80.73–80.93) per 100,000, with the total number of deaths 2,481,728. http://ratecalc.cancer.gov/ratecalc/. (**b**) Diabetes and obesity diagnoses. Estimates among 3,141 United States counties for age-adjusted rates of both diagnosed diabetes and obesity presented together. Estimates were calculated from Census and Behavioral Risk Factor Surveillance System (self-reported) data for 2006–2008. The national proportion of US adults who were obese in 2008 was 26.1 %. In 2007, 8 % of the US population, or 24 million individuals, were diabetic, of which 5.7 million were estimated to be undiagnosed. http://www.cdc.gov/diabetes/pubs/factsheets/countylvlestimates.htm. (**c**) Socioenvironmental map of poverty. County-level data from United States Census Bureau statistics for 2004. Estimated percentage of population living below the poverty threshold as defined by US Census methods is defined by size of family and ages of members and includes information about earnings, unemployment compensation, workers' compensation, Social Security, Supplemental Security Income, public assistance, veterans' payments, survivor benefits, pension or retirement income, interest, dividends, rents, royalties, income from estates, trusts, educational assistance, alimony, child support, assistance from outside the household, and other miscellaneous sources (http://www.cdc.gov/dhdsp/maps/sd_poverty_2004.htm, http://www.census.gov/hhes/www/poverty/about/overview/measure.html)

than a one-to-one correspondence between an obesity exposure and a cancer rate. In other words, the same causal factors that produce increased levels of obesity might also be at work to promote increased rates of lung cancer and lung cancer mortality, as well as type 2 diabetes (Fig. 3.2b). Obesity and cancer are likely to be linked

through their upstream causes: downturns in the economy, the nature of work and labor markets, social stratification and economic inequality (Fig. 3.2c), and lack of opportunity and local infrastructure that set the stage for and contribute to the human biochemical mechanisms at work in carcinogenesis. As research identifies the central role of social determinants in chronic disease development, investigators need to pay closer attention to the common origins, even if not directly biologically linked. The social and structural origins of this problem demand structural solutions beyond the power of the prescribing physician's pen: it is clear that *certain specific environments are both obesogenic/diabetogenic and carcinogenic*. Solutions will require focused political will, participation of corporations and community groups, entrepreneurs, school districts, and local employers, not just obese Americans and their physicians.

3.6 Interactions Among Biological and Social Factors

Ultimately, we need to understand both the biology and the social forces that govern obesity to reduce this burden in modern, industrialized societies. The movement toward tailored or "personalized" medicine may be one way in which both perspectives can be not only accommodated but relied upon as translated intervention tools to reduce obesity. For example, identification of an individual's likelihood of being a metabolically unhealthy obese person may provide additional motivation to engage in healthy behaviors. Alteration of the shape of environments for individuals and groups, such that there are clear and accessible food and activity choices, will help families facing both obesity- and asthma-related health issues. Increasing the opportunity for non-obesogenic activities might be a necessary investment for individuals who become inflamed if they become obese or maintain obesity. It may be helpful for the current conditions to consider obesity as a health problem that, for some, causes clear measurable changes related to a variety of chronic diseases, but that is ultimately preventable. Translating the basic research on vulnerability to inflammation with obesity into usable interventions will require new ways of thinking about environment, motivation, and human behavior. From the discussion in this chapter, it is clear that we have begun to consider the broad, powerful mechanistic connections among inflammation, obesity, and cancer and the need to link the cellular, serological, and dietary environments of obese, at-risk individuals to exposures in the built environment, urban infrastructure, and economic policy. Without transdisciplinary, innovative, "out-of-the-box" thinking, the problem of obesity-associated cancer will prove too difficult to address effectively. We therefore call for additional funding and research to investigate these unexpected connections among important variables, with focused conversation among molecular biologists, immunologists, geneticists, cancer and endocrine clinicians, epidemiologists, sociologists, public housing officials, and public health officials. In view of the increasing seriousness of the obesity epidemic, time is running out for this conversation to plan for research and policy priorities.

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The authors report no conflicts of interest.

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Chapter 4
Adipose Tissue Macrophages in Obesity, Inflammation, and Cancer

Carey Nien-Kai Lumeng

Abstract The chronic low-grade inflammation induced by obesity is a key connection between obesity and disease. It is now understood that the supporting stromal cells in adipose tissue contribute to this inflammatory response in significant ways. Of these, adipose tissue macrophages (ATMs) are major effectors of inflammation in hypertrophic adipose tissue. However, we now know that ATMs are diverse in their phenotypes and have functions that may contribute directly and indirectly to impact cancer risk. This review will summarize our current understanding of the phenotypic diversity in ATMs and how this is altered in obesity. The potential role that ATMs play in breast and ovarian cancer pathogenesis will also be discussed given the close association between adipose tissue and these cancer types.

4.1 Introduction

Adipose tissue has been traditionally thought of as being a static organ composed of only adipocytes, specialized cells designed for the storage of excess nutrients as lipid (primarily triglyceride). By mass, adipocytes make up the bulk of adipose tissue and can drastically enlarge and contract in times of nutrient excess and demand. However, by number, adipocytes may be a minority cell population in adipose tissue as demonstrated in Fig. 4.1. A major advance in the last 5–10 years in obesity research has been the realization that adipose tissue has a complex and dynamic range of cellular components beyond adipocytes that play critical roles in the maintenance of nutrient homeostasis.

Importantly, it is now known that alterations in the non-adipocyte stromal cells in fat form crucial links between obesity and many of its associated diseases. The advancements in understanding obesity-induced inflammation are illustrative of the

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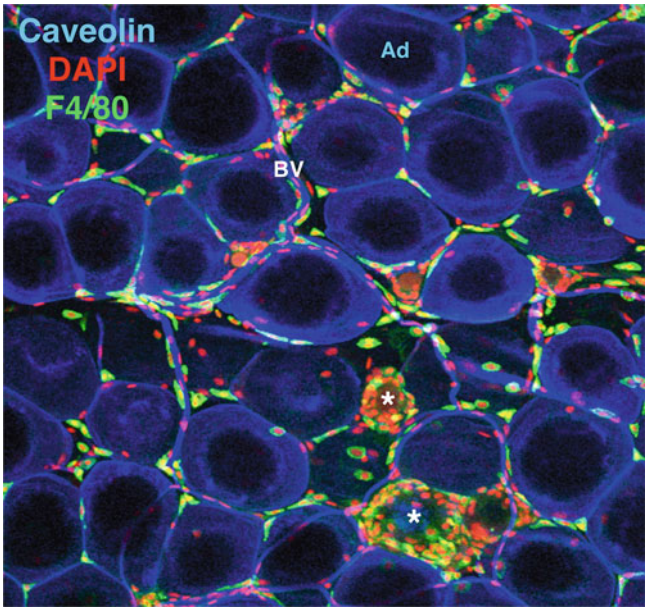

Fig. 4.1 Adipose tissue architecture in obesity. Confocal microscopy image of visceral/gonadal fat depot in C57Bl/6 mice fed with a high-fat diet (60 %) for 16 weeks. Tissue was stained with antibodies against caveolin (*blue*) and F4/80 (macrophages, *green*) and co-stained with DAPI (*red*) to indicate nuclei. Crown-like structure (CLS) of ATMs (*asterisks*) is shown. Adipocytes (Ad) are identified by caveolin staining. Blood vessels (BV) are shown

new directions that are being explored in obesity research. As this field expands, it provides opportunities to identify novel mechanisms by which obesity modifies cancer risk.

This review will summarize the current understanding of adipose tissue macrophages (ATMs), a leukocyte in fat that is fairly well characterized in animal models and in humans. I will review the state of the art view of ATM function and diversity in adipose tissue and how this is altered by obesity. In this framework, I will present the current views of how ATMs promote adipocyte dysfunction and contribute to metabolic dysregulation. This will lead to a better understanding of the ways that ATM activation may both directly and indirectly contribute to the link between obesity and cancer.

4.2 Adipose Tissue Macrophages as Inflammatory Engines in Obesity

When nutrient intake outpaces energy utilization, the only tissue that is designed to efficiently store excess nutrients is adipose tissue. Adipose tissue expands by producing new adipocytes (hyperplasia) and enlarging existing adipocytes (hypertrophy).

For reasons that are still elusive, the chronic and excessive expansion of adipose tissue is associated with the activation of inflammation in fat. Such inflammation was initially identified by the elevated expression of inflammatory cytokines and chemokines such as tumor necrosis factor-α (TNFα) [1], interleukin (IL)-6 [2], monocyte chemoattractant protein 1 (MCP-1) [3, 4], and plasminogen activator inhibitor-1 (PAI-1) [5] from obese adipose tissue. Given the large mass of adipose tissue in obese subjects, such tissue-specific cytokine production likely contributes greatly to the systemic elevations of these cytokines in obesity.

Based on these observations, initial studies focused on how these inflammatory mediators were generated by adipocytes. However, in 2003, two papers demonstrated that most of the inflammatory factors were made not by the adipocytes, but by the non-adipocyte stromal cells which were enriched in macrophages [6, 7]. This led to a series of studies that demonstrated that macrophages accumulate in adipose tissue in obese subjects, are prominent in visceral over subcutaneous fat depots, are associated with measures of metabolic dysfunction, and are decreased with weight loss [8–11].

The evidence for a role of macrophages as a contributor to metabolic disease has been reinforced by many clinical and preclinical studies. In a systems biology study in humans, expression profiling of blood and adipose tissue was combined with genetic dissection of expression quantitative trait loci (eQTL) to find genes associated with obesity and metabolic disease [12, 13]. This unbiased approach identified a gene expression signature in adipose tissue associated with metabolic dysfunction that was enriched for genes typically found in the spleen- and bone marrow-derived macrophages. This macrophage-enriched metabolic network (MEMN) of genes includes *Cd68*, *Emr1* (*F4/80*), *Cd14*, and *C3ar1* and emphasized the link between inflammatory networks in fat and the complex trait of metabolic disease. Follow-up studies validated the importance of many of these genes to metabolic traits using loss of function mouse models [14].

Rodent models of obesity have been instrumental in demonstrating that macrophage activation plays a substantial role in the link between obesity and insulin resistance. Mouse models deficient in receptors critical for inflammatory monocyte/macrophage trafficking have impaired accumulation of ATMs in adipose tissue. These include mice deficient for C-C chemokine receptor type 2 (CCR2) [15], macrophage galactose-type C-type lectin (MGL1) [16], and C-C chemokine receptor type 5 (CCR5) [17]. These models share the similar phenotype when exposed to high-fat diets where adipocyte hypertrophy occurs in the absence of an accumulation of ATMs in crown-like structures (CLS). Despite obesity and increased fat mass in these models, the mice demonstrate an improved metabolic profile (e.g., improved insulin sensitivity and glucose tolerance) and a decrease in hepatic steatosis.

These studies suggest that the ATM activation and inflammation in fat play a major role in impairing the function of fat as an efficient nutrient store (Fig. 4.2). Fat dysfunction promotes lipid and chemokine release as opposed to storage and places pressure on other organs to store the excess nutrient load. Attenuation of ATM accumulation produces “healthier” fat with a sustained capacity to store excess nutrients

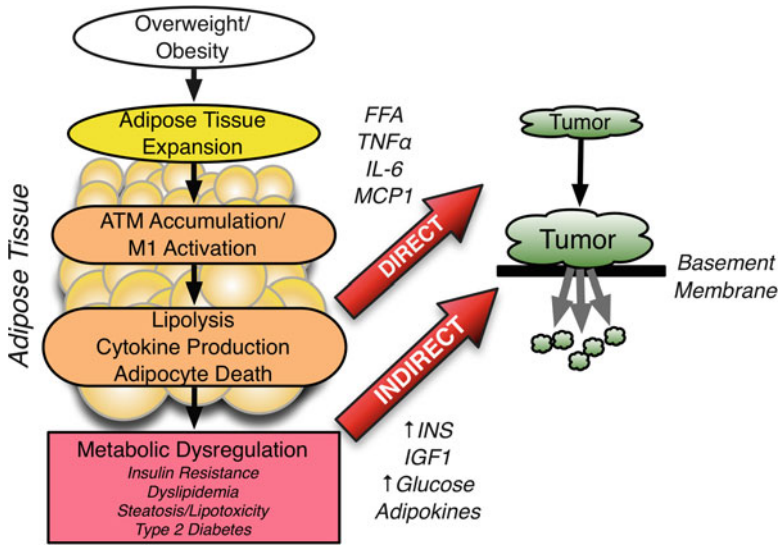

Fig. 4.2 Direct and indirect contributions of ATMs to cancer biology. Overweight and obesity status is associated with expansion of adipose tissue. Triggers concurrent hypertrophy of adipocytes and accumulation of M1-like macrophages in fat. M1 ATMs generate inflammatory cytokines that may have direct effects upon the local tumor environment. ATMs also promote adipocyte dysfunction triggering adipocyte death and release of free fatty acids (FFA). Chronically this process promoted metabolic dysfunction associated with systemic insulin resistance and lipotoxicity. Such metabolic regulation and its associated hormonal changes are downstream of ATM activation and function as a promoter of "sick" adipose tissue

and partition lipids away from other organs such as the liver. A similar phenotype was seen in leptin-deficient mice with transgenic overexpression of adiponectin [18]. Despite morbid obesity, these mice had no evidence of metabolic abnormalities. This was associated with a lack of visceral fat and a preferential expansion of subcutaneous fat depots containing small adipocytes and few ATMs. Overall, these studies have supported the concept that ATMs contribute to the development of insulin resistance and type 2 diabetes with obesity by promoting chronic fat dysfunction.

4.3 Adipose Tissue Macrophage Diversity

A simplified interpretation of the observations above is that all macrophages in fat are deleterious. This generalization is hard to reconcile with the observation that macrophages are found in all fat depots in lean insulin-sensitive subjects [19, 20]. Based on this and other observations, current evidence supports a model where both quantitative and qualitative changes occur in ATMs with obesity. While it is likely that these two events (macrophage accumulation and changes in their activation

profile) are coupled together, there is also growing evidence that ATMs are active sensors of the fat environment and can alter their inflammatory output in situ in response to metabolic and inflammatory cues [21, 22].

4.3.1 Macrophage Activation States

Around the time when ATMs were first described, there was a rapid evolution in the understanding of the different activation states that macrophages can assume dependent on the local context. In vitro these have been classified based on the differential responses of macrophages to Th1 and Th2 signals. M1 or classically activated macrophages are generated upon exposure to lipopolysaccharide (a bacterial component) and interferon-γ (IFNγ) and have the capacity to express high levels of pro-inflammatory cytokines/factors (e.g., iNOS, TNFα) that promote killing of foreign pathogens. The M1 program also induces a variety of metabolic changes in macrophages that include an increase in glucose utilization, decreased fatty acid utilization, and an increase in iron uptake to increase their "killing" capacity [23]. In contrast, when macrophages are stimulated with Th2 cytokines such as IL-4 or IL-13, they assume a very different profile. These alternatively activated or M2 macrophages have low production of inflammatory cytokines and instead generate IL-10 and arginase which dampen inflammatory responses and promote repair. Metabolically these cells are different as well and preferentially utilize fatty acids for energy and promote iron release. These metabolic changes and M2 profile appear to be coupled by nuclear transcription factors peroxisome proliferator-activated receptors (PPAR) γ and δ via signal transducer and activator of transcription 6 (STAT6) activation [24–26].

The M1/M2 dichotomy is an in vitro simplification of a complex range of macrophage activation profiles. While the M1/M2 paradigm is useful in many contexts, including that of ATM biology, it is clear that macrophages in vivo assume states along a continuum between these extreme states. In addition, many aspects of the M1/M2 axis differ substantially between human and murine models as gene profiling has shown that there is only 50 % commonality between human and mouse macrophages treated with M1 or M2 stimuli [27].

4.3.2 The M1/M2 Paradigm in Obesity

Despite the limitations noted above, there is evidence to support the concept that the balance between M1 and M2 states is altered by obesity and can explain many of the features of obesity-associated inflammation [28, 29]. In lean animals and humans, the resident population of ATMs is M2-like based on surface marker and gene expression profiles [9, 20, 30, 31]. Animal models with impaired generation of an alternatively activated M2 state demonstrate an exaggerated inflammatory response

to obesity and have a more pronounced insulin resistance phenotype. Examples of this include macrophage-specific knockout of *Pparg* and *Ppard* [24–26]. In addition, mice deficient in GPR120, a receptor for omega-3 fatty acids on macrophages, have an impaired M2 gene expression in their ATMs and have an exaggerated pro-inflammatory response to obesity [32, 33].

On the other side of the coin, obesity induces many changes in ATMs consistent with an activation of an M1 profile. At the gene expression level, this includes the induction of *Tnfa* and *Nos2* genes in mice [20, 34]. In both mice and humans, obesity induces the expression of receptors that promote the capacity of ATMs to function as antigen-presenting cells (e.g., CD40, CD11c, and MHC class II), a feature of M1 activation [9, 30]. In addition, mice deficient for critical M1 macrophage activation components have attenuated obesity-induced inflammation. Examples of this include knockout mice for toll-like receptors 2 and 4 (TLR2, TLR4) and mice deficient in IFNγ production [35–38]. Attenuation of IκB kinase β (IKKβ) signaling pathways in macrophages leads to a substantial reduction in inflammation and protection of mice from insulin resistance [39, 40]. Based on this, novel anti-inflammatory treatments for type 2 diabetes are under clinical trial to target pathways that lead to NFκB activation, a key component in the generation of an M1 activation profile [41, 42].

4.3.3 The Nature of the Phenotypic Shift from M2-Like to M1-Like ATMs in Obesity

The mechanisms by which the qualitative and quantitative changes in ATMs are induced are under active investigation. What has become clear is that the changes in the inflammatory profile in fat with obesity are driven by alterations in the balance between at least two distinct types of ATMs (Fig. 4.3). A resident ATM population is found between adipocytes and along blood vessels in fat and is established early in adipose tissue development [43]. These have many features of M2 macrophages and appear to interact with eosinophils and other cells in fat that secrete Th2 cytokines such as IL-4 [44, 45]. The recruitment of the resident M2-like ATMs (type 2 [46]) appears to be independent of traditional chemokine pathways as they are present in normal numbers in *Ccr2*- and *Ccl2*-deficient mice [30].

In obesity, this resident ATM population is retained with similar distribution and surface markers; however, a distinct type of ATM is superimposed on this population. The "phenotypic shift" is generated by an increase in the ratio of these recruited M1-like (type 1) ATMs relative to the resident (type 2) ATMs. In mice and humans, the ATMs induced by obesity preferentially express the surface marker CD11c and have lipid-laden features similar to foam cells in atherosclerosis [47, 48]. The recruitment of $CD11c^+$ ATMs to fat is rapid compared to type 2 ATMs and is concentrated in CLS seen on standard histology preparations [30]. CLS are dense accumulations of ATMs that form around lipid remnants of dead adipocytes and are also sites of fibrosis and accumulation of collagen fibers [49, 50].

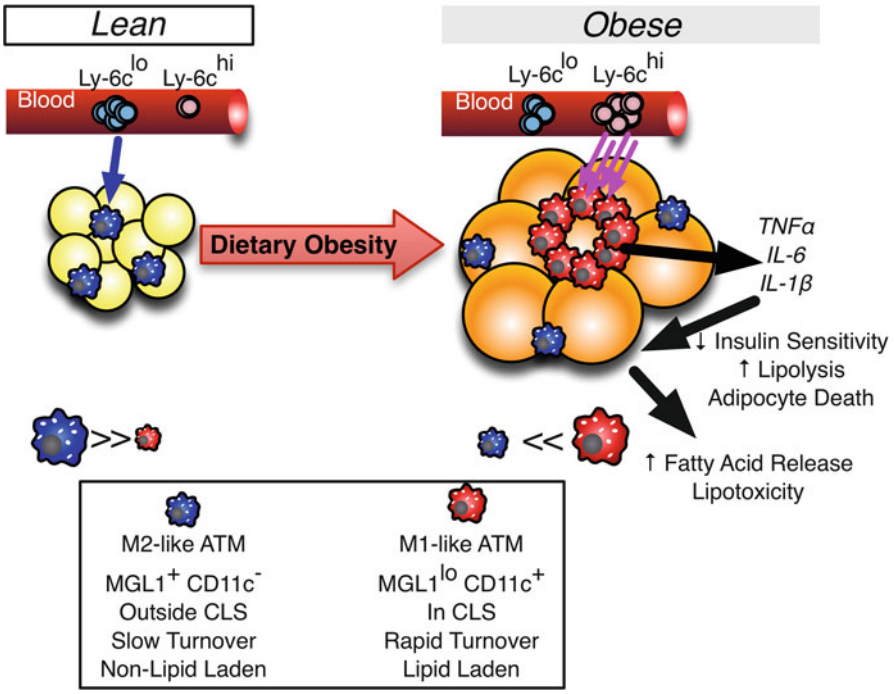

Fig. 4.3 Model of ATM phenotypic changes in obesity. In lean states, M2-like resident ATMs are the dominant ATM population (*blue cells*). These ATMs express M2 surface markers in mice (e.g. MGL1) and are located between adipocytes. With dietary obesity, a distinct type of ATM accumulates with features of M1 macrophages (*red*) that express CD11c and have low MGL1 expression in mice. These ATMs are concentrated in CLS and generate pro-inflammatory cytokines that cause local adipocyte dysfunction which contribute to systemic cytokines. The appearance of M1-like ATMs is coupled to a population of Ly-6c^{hi} monocytes in the circulation that are induced with obesity

The weight of evidence suggests that the accumulation of type 1 CD11c^{+} ATMs is linked to the enhanced trafficking of specific inflammatory monocyte populations from the circulation [51, 52]. In mice, monocytes that express the surface marker Ly6c are induced with obesity and tightly correlate with M1-like ATM accumulation [16, 53]. The specific mediators that promote monocyte trafficking to fat are incompletely understood but include chemokine-dependent (MCP1 [54]) and chemokine-independent (lipolysis) pathways [21].

4.4 ATM Functions

The list of functions of ATMs is incomplete. There is evidence that ATMs contribute to normal physiologic regulation of adipose tissue (e.g., fat development) in addition to their function in pathophysiology (e.g., obesity). In lean subjects, resident ATMs integrate systemic and local fat signals to maintain homeostasis. Macrophage

depletion during development impairs angiogenesis in the developing fat pads [43]. This function may relate to the capacity of ATMs to remodel the extracellular matrix surrounding adipocytes by secretion of proteinases and protease inhibitors [55]. Resident ATMs are also able to rapidly sense and respond to acute changes in nutrient status suggesting a dynamic function for ATMs in metabolism [22].

In obesity, an association between ATMs and adipocyte death exists that supports the notion that ATM accumulation is driven by a response to adipocyte stress/death with hypertrophy [50, 56]. Clearly, an interplay exists between ATMs and adipocytes as there is no increased death observed with hypertrophied fat in models where type 1 ATM accumulation is attenuated. However, there is also evidence to suggest that adipocyte death and ATM accumulation are independent unrelated processes [57].

The active production of M1 cytokines from CLS sends signals to adipocytes that impair their function. In vitro studies have shown that M1 ATMs are capable of decreasing insulin sensitivity of adipocytes by direct and indirect (secreted) mechanisms [58]. In addition, M1 ATMs impair adipogenesis while M2 ATMs have little to no effect on adipogenesis [59]. Therefore, the shift toward an M1 profile impairs two crucial functions of adipocytes that can buffer chronic states of nutrient excess—the ability to take up nutrients and the ability to generate new fat cells. By antagonizing both processes, M1 signals promote adipocyte dysfunction and breakdown, the normal homeostatic mechanisms that permit proper nutrient storage.

Additional postulated functions for ATMs in obesity include the regulation of hypoxic responses [60], the control of adipose tissue fibrosis [61], and the function as dendritic cells to activate T cells in fat [62]. All of these may contribute to alter tissue environments in a way that may impact tumor formation, growth, and metastatic disease.

4.5 Links Between Adipose Tissue Macrophages and Cancer

The link between increased adiposity, high BMI, and cancer likely flows through several overlapping mechanisms related to systemic and local factors [63] (Fig. 4.2). These include impaired fatty acid storage in adipocytes, alterations in adipokine secretions (e.g., leptin, adiponectin), shunting of lipids for storage in non-adipose tissues causing lipotoxicity (e.g., hepatic steatosis), systemic elevations in inflammatory cytokines, local production of inflammatory cytokines in tumor-associated adipose tissue, and local activation of leukocytes in adipose tissue near tumor sites (e.g., mammary gland). ATMs have been shown to partially contribute to the development of all of these processes; therefore, one may think of direct and indirect connections between ATM activation and cancer risk.

The relative contributions of these factors to the links between cancer and obesity are still poorly understood and will likely be tissue specific. The systemic alterations in nutrient metabolism (e.g., insulin resistance) will be reviewed in other chapters in this book. I will next discuss the potential for local ATM involvement in cancer progression and metastatic disease by highlighting recent data regarding breast and ovarian cancer biology that intersects with ATM function.

4.5.1 ATMs and Breast Cancer

As in other adipose tissue depots, ATMs are present in normal breast tissue and are critical for the physiologic remodeling that occurs in and around the mammary epithelial cells. Macrophage depletion blocks post-lactation remodeling in the mouse mammary gland [64]. These remodeling changes are associated with an M2-like polarization of the ATMs that contribute to clearance of apoptotic mammary epithelial cells and may modify cancer risk [65]. Similar M2-like changes have been observed in other mouse models where extensive adipose tissue remodeling is induced [66].

The importance of tumor-associated macrophages (TAMs) in breast cancer progression is well described; however, the importance of local ATMs in this process is incompletely understood. Like in adipose tissue, there is significant heterogeneity in the TAMs seen in breast cancer [67]. Macrophages along the invasive front along the tumor periphery in mouse models demonstrate a high degree of motility and migration suggesting that local ATMs may contribute to TAMs [68]. Work in our lab using intravital microscopy corroborates these observations and finds extensive patrolling behavior of ATMs in multiple fat depots (C.N.L., unpublished observation). The function of ATMs at the invasive front may include remodeling of the extracellular matrix, promotion of angiogenesis, direct enhancement of tumor invasion, and blockade of antitumor inflammatory responses by their M2 polarization [65, 69]. Overall, these observations suggest that ATMs from the surrounding adipose tissue stroma may contribute in critical ways to breast cancer induction and metastasis.

These observations also suggest that alterations in ATM phenotypes with obesity may contribute to the association between obesity and breast cancer risk and prognosis [70, 71]. Recent studies have demonstrated a strong association between the density of CLS in the breast and BMI, adipocyte size, inflammation, and aromatase expression [72]. In mouse models, obesity led to an increase in inflammatory cytokine production in mammary ATMs consistent with an M1 profile that was capable of inducing aromatase expression in preadipocytes [73]. Therefore, the induction of inflammatory ATMs in the breast with obesity may be a component of the link between obesity and breast cancer development. This activation may be related to the role that colony stimulating factor 1 (CSF1) appears to play in macrophage activation and the promotion of metastasis [74]. In addition, hormonal regulation and metabolism within ATMs may be important as macrophages express aromatase and are capable of generating estrogen at physiologic levels [75].

Future studies will be required to understand how resident ATMs contribute to the local tumor environment and the leading edge. The idea that ATM accumulation may be a biomarker for disease risk will also deserve further study. Besides ATMs, the possibility that other leukocyte types in obese tumor-associated adipose tissue will need to be studied as potential modifiers of the mammary epithelial environment.

4.5.2 ATMs and Ovarian Cancer

Epithelial ovarian cancer morbidity and mortality is related to the fact that diagnosis of cancer is often delayed until the disease has reached an advanced stage [76]. Primary tumor cells leave the ovarian capsule and are disseminated via the peritoneal fluid. Within this space, the greater omentum, a major visceral adipose tissue depot, is a preferential site of attachment of tumors and a supportive environment for aggressive tumor growth [77]. The interface between the tumors and the omentum is not diffuse but appears to be localized to structures known as milky spots—immune aggregates on the surface of the omental fat pad. Milky spots are collections of immune cells that include large numbers of macrophages and lymphocytes [78]. These regions are highly vascularized and may provide a rich angiogenic environment to support tumor growth. Work from our lab has demonstrated a significant enlargement in milky spots with aging in mice due to an expansion of $CD4^+$ T cells [79] which may play a role in peritoneal immune surveillance and age-related insulin resistance. Milky spots are not limited to the omentum as we have identified them in multiple visceral adipose tissue depots [80].

The nature and function of milky spot ATMs are poorly understood. Evidence suggests that these ATMs play an important role in immune surveillance of the peritoneal cavity as guardians against pathogens as well as tumor cells [81, 82]. Animal models have demonstrated that tumor cells rapidly accumulate in milky spots and then are cleared presumably by the active innate immune system [83]. Cells that escape this clearance can migrate away from the milky spots where they are capable of forming tumors. In vitro studies suggest that communication between ovarian cancer cells may directly influence the function of milky spot macrophages and T cells to promote a tumorigenic environment [84]. This appears to be linked to the ability of ovarian cancer cells to enhance regulatory T cell function and promote macrophage scavenger receptor expression and cytokine production that enhance tissue repair.

The unique nature of milky spots have led some to hypothesize that enhancement of milky spot ATMs (e.g., enhancement of antigen presentation capacity) may be a future tool in cancer treatment and prevention [82]. It has been speculated that milky spots may provide a supportive environment for cancer stem cells and that enhanced ATM function may promote cancer clearance [85]. Given the involvement of the omentum in metastatic colon and stomach cancer, milky spot ATMs may have a broader function outside of ovarian cancer.

4.6 Conclusions

4.6.1 Current Needs in the Field

While this review has centered around the role of ATMs in the inflammatory response to obesity, it is clear that ATMs are one part of a network of leukocytes in adipose tissue that cooperate to initiate and sustain inflammation in fat with nutrient excess.

Essentially all types of leukocytes have been described in fat including regulatory $CD4^+$ T cells, effector/memory $CD4^+$ T cells, $CD8^+$ T cells, activated B cells, eosinophils, mast cells, neutrophils, and NK cells. Given this, it is unclear if ATMs are initiators and/or effectors of adipose tissue inflammation in obesity. In addition, while we know a reasonable amount about the events that sustain fat inflammation in obesity, little is known about the events that lead to a resolution of this inflammation. Understanding how weight loss resolves inflammatory processes may provide insight into new interventions and prevention strategies in cancer biology.

4.6.2 Future Directions

Currently, the descriptive phase of evaluating adipose tissue leukocyte biology is closing. The current need in the field is to integrate the biology of adipose tissue inflammation and understand how the different types of adipose tissue leukocytes communicate as it is unlikely that they are each operating in a vacuum. This challenge is coupled to the need to increase our understanding of human ATMs which have very different activation profiles than those seen in animal models of obesity.

Part of the challenge in human studies is the limitation of most studies to the examination of subcutaneous fat depots and a limited assessment of visceral fat or tumor-associated adipose tissue. Expanding our understanding of tumor-associated adipose tissue and the contribution of leukocytes derived from adipose tissue is a future need. The conceptual shift that adipose tissue is a dynamic inflammatory organ is still young. Given the importance of inflammatory cytokines and pathways in the initiation and maintenance of tumor biology [86, 87], signals from ATMs around tumor sites may have a significant impact upon cancer biology. Advancements in this concept provide new opportunities in human and animal studies to investigate the contribution of ATMs to tumor biology and to identify new treatment approaches.

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Chapter 5
Dietary Fats as Mediators of Obesity, Inflammation, and Colon Cancer

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Abstract Obesity and associated low-grade inflammation are clearly risk factors for development of diabetes, cardiovascular disease, and cancer; however, the mechanisms and pathways by which obesity and inflammation lead to these disorders are not clearly defined. Since obesity is largely determined by levels of energy expenditure as well as quantities and composition of consumed nutrients, especially fats and carbohydrates, the question exists as to whether obesity and/or dietary components contribute directly to development of inflammation and/or associated comorbidities including diabetes, cancer, and cardiovascular disease. In this chapter, we examine the evidence supporting a role for dietary fats in the development of inflammation and intestinal tumorigenesis. We also compare different fats and different diets for their ability to promote or prevent intestinal tumorigenesis and explore possible mechanisms of action. These considerations are important for the potential prevention and control of intestinal cancer, since overall diet and specific dietary components are modifiable risk factors and increasing numbers of dietary and pharmacologic interventions are becoming available to control both inflammatory and carcinogenic processes.

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5.1 Introduction

Dietary fat was once considered to simply be a source of fatty acids to fuel energy needs and to support development of adipose tissue to serve as an energy reserve, with fatty acid oxidation supplying as much as 2 times the amount of energy that can be derived from equal amounts of carbohydrates or proteins. It is now apparent that dietary fatty acids provide key structural elements for cellular and organelle membrane structure, function and fluidity; support proper growth and development; facilitate absorption of fat-soluble molecules including vitamins A, D, E, and K; and insulate internal organs and neural processes [1, 2]. Fatty acids and their derivatives are critical in regulating and contributing to organogenesis, body temperature, the immune and inflammatory responses, and unique signaling pathways such as those mediated by prostaglandins, phospholipids, sphingolipids, ceramides, and others. Fatty acid consumption and metabolism is regulated by a complex series of ecologic, ethnic and environmental factors, and biobehavioral processes. Disorders of fatty acid intake and metabolism contribute to a wide variety of metabolic, immunologic, and neoplastic disorders including obesity, inflammation, diabetes, and cancer.

Obesity is clearly associated with an increased risk for a number of malignancies including colon cancer, esophageal adenocarcinoma, postmenopausal breast cancer, renal cell carcinoma, uterine cancer, and pancreatic cancer, and the list continues to expand [3, 4].

In addition to its association with metabolic and neoplastic disorders, obesity is now recognized as a chronic low-grade inflammatory disorder, where both dietary fats and the increase in adipose tissue contribute to the inflammatory state and associated comorbidities [5]. Although not consistently confirmed, there is substantial evidence linking dietary fats as contributing factors to a variety of malignancies including breast, pancreatic, hepatic, colon, prostate and others [6–17]. However, because of the complex interactions between diet, obesity, inflammation, and cancer, it is frequently difficult to separate the etiologic contribution of each in the process and their independent role in carcinogenesis. In particular, the fact that high dietary fat leads to both obesity and inflammation makes it difficult to determine which component leads to cancer. Defining these relations is further confounded by the fact that all fats are not equal, in that fatty acids of different lengths and degrees of saturation may have different biologic and pathophysiologic effects, which sometimes may be additive or complimentary, and at others may be competitive or antagonistic. In this regard, it is important to remember that although large studies such as the Women's Health Initiative, in which a total of 48,835 postmenopausal participants were randomized to a diet modification where 19,541 were assigned to consume 20 % lower fat compared to 29,294 women who consumed a usual diet, there was no significant reduction in risk for colorectal cancer (CRC) [18, 19]. In contrast, other studies, outlined below, do show changes in CRC risk in association with different levels of consumption of specific fatty acids.

In this chapter, we focus on the relation of dietary fats to inflammatory processes and to colon cancer. First, we provide a brief outline of fatty acid structure and nomenclature. We next review the epidemiologic and nutritional evidence

supporting a role for dietary fatty acids and selected diets in the promotion and prevention of colon cancer. We subsequently review the pro- and anti-inflammatory effects of specific fatty acids and diets and their possible contribution to colon cancer. Finally, we address potential mechanisms and mediators of this relation and how they may serve as targets for cancer prevention and control.

5.2 Fatty Acid Structure and Nomenclature

Fatty acids are hydrocarbon chains with a methyl group at one terminus and a carboxyl at the other. As indicated in Table 5.1, they vary in chain length of carbon atoms connected by bonds that may be saturated or unsaturated [20]. Those with a single unsaturated bond are designated monounsaturated fatty acids (MUFAs), whereas those with more than one double bond are polyunsaturated fatty acids (PUFA). When multiple double bonds exist, they are never adjacent. The hydrogen atoms on either side of the double bond, in naturally occurring fatty acids, are almost always in the *cis* configuration, whereas in synthetic fatty acids, the hydrogen atoms on either side of the double bond may be in the *trans* configuration providing the basis for trans fats. Fatty acids are numbered with the carbon atom in the carboxyl group designated number one, and subsequent numbers progressing sequentially through consecutive carbons to the methyl group at the other end of the molecule which is designated the omega (ω) carbon. The (ω) carbon is sometimes designated as n for its position at the end. In the case of unsaturated fatty acids, the location of the first double bond is counted from the ω carbon atom of the methyl group at the end of the hydrocarbon chain.

Table 5.1 Structure and nomenclature of dietary fatty acids with important impact on colon cancer

Symbol	Common name	Structure	CRC impact
Saturated fatty acids			
C12:0	Lauric	$CH_3(CH_2)_{10}COOH$	↑
C14:0	Myristic	$CH_3(CH_2)_{12}COOH$	↑
C16:0	Palmitic	$CH_3(CH_2)_{14}COOH$	↑
C18:0	Stearic	$CH_3(CH_2)_{16}COOH$	↑
Monounsaturated fatty acids			
C16:1n-7	Palmitoleic	$CH_3(CH_2)_5CH{=}CH(CH_2)_7COOH$	
C18:1n-9	Oleic	$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$	↓
Polyunsaturated fatty acids			
C18:2n-6	Linoleic	$CH_3(CH_2)_4(CH{=}CHCH_2)_2\ (CH_2)_6COOH$	↑
C18:3n-3	Linolenic	$CH_3CH_2(CH{=}CHCH_2)_3\ (CH_2)_6COOH$	↓
C20:4n-6	Arachidonic	$CH_3(CH_2)_4(CH{=}CHCH_2)_4\ (CH_2)_2COOH$	↑
C20:5n-3	Eicosapentaenoic	$CH_3CH_2(CH{=}CHCH_2)_5\ (CH_2)_2COOH$	↓
C22:6n-3	Docosahexaenoic	$CH_3CH_2(CH{=}CHCH_2)_6\ CH_2COOH$	↓

Symbol provides number of carbon atoms in fatty acid chain: followed by number of double bonds for unsaturated fatty acids, n-x provides position of first double bond relative to the ω carbon at the terminal methyl end [20]. Impact on colorectal cancer (CRC), ↑ = promoter, ↓ = suppressor

Structure and function of fatty acids are determined by the hydrocarbon chain length and the number, location, and geometric configurations of the double bonds. The saturated fatty acids are the most flexible, with unsaturated fatty acids being more rigid and angled at their double bonds. The long-chain fatty acids of biologic significance usually contain an even number of carbon atoms, between 14 and 22, with 16–18 being most abundant. Lauric and myristic acids are medium-chain saturated fatty acids; palmitic and stearic acids are long-chain saturated fatty acids. Among the unsaturated fatty acids, the most abundant are oleic, linoleic, linolenic and arachidonic acids. The biologically important MUFAs, palmitoleic and oleic acids, can be synthesized in animal tissues from the saturated fatty acids, palmitic and stearic acids by process of fatty acid desaturation, whereas other biologically important MUFAs, linoleic or linolenic, cannot be synthesized by mammalian tissues, must be derived from exogenous sources, and are consequently designated essential fatty acids. Polyunsaturated fatty acids (PUFA) contain more than one double bond. Their composition in the cell membrane can be determined by diet, and they drastically affect membrane function and intracellular signaling [21, 22]. There are two general groups of PUFAs, both are important to general health and disease and specifically to colon cancers. They are the ω3 PUFAs and the ω6 PUFAs. They are not interconvertible and dietary substitution of one results in a competitive reduction in the other [21, 23]. Omega 3 fatty acids, containing unsaturated, double bonds after the third carbon from the methyl end, include eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which are common in marine plants and fish, are generally not made in mammalian tissues, and are also considered essential fatty acids. Another ω3 fatty acid, linolenic acid, is derived from a number of plant seeds and green leafy vegetables. The n6 or ω6 fatty acids, in which the first unsaturated double bond is located after the sixth carbon atom from the methyl end, of biologic importance, include arachidonic acid and linoleic acid, commonly found in corn, soybean, sunflower, sesame, and palm oils [24].

5.3 Epidemiology of Dietary Fat and Colon Cancer

Early observations implicating dietary fats in the etiology of colon cancer were based on differences in the incidence of malignancy where significant anthropological and/or ecological differences existed in diet or occurred in association with diet change, particularly in migrating populations [25–27]. Anthropologic studies indicate that our Paleolithic ancestors consumed a diet high in fiber and carbohydrates, with abundant protein from fruit and vegetable sources, fish, and wild game [28, 29]. In contrast, current diets in affluent Western countries are composed of calorie-dense, nutrient-poor foods that are rich in fat, oil, and sugar [30]. This dietary composition is implicated in the increased risk of colon cancer and other malignancies characteristic of many Western countries [31, 32] and, in particular, the increased cancer risk in populations that migrate from countries that consume low amounts of these same detrimental nutrients [33].

Studying geographic differences, Wynder et al. reported the high incidence of colon cancer in the USA compared to the lower incidence in Japan [27]. They noted also that the increase in colon cancer that occurred in Japan in the 20-year post-World War II period occurred in association with relatively high consumption of eggs, milk, meat, fat, and fruit [27]. They also postulated that the difference might be associated with different intestinal bacteria associated with either the different ethnic groups and/or different diets. Also on the basis of geographic differences in food consumption, especially comparing African to Western countries, Burkitt found that CRC incidence was associated with high dietary fat consumption but low dietary fiber [26]. Based on these observations, he introduced the concept that specific foods could be protective against the carcinogenic effects of others and recommended consumption of diets containing reduced fats and increased fiber to protect against colon cancer, especially in Western countries [26].

Comparison of environmental factors and relation to cancer in 30 countries by Armstrong and Doll [25] showed that colon cancer incidence correlated with per capita consumption of meat, animal protein, and total fat, with the United States and New Zealand showing both the highest per capita consumption and the highest colon cancer incidence compared to Japan and Nigeria with the lowest. These observations were subsequently confirmed and refined to demonstrate that correlation of colon cancer with fat was more specifically associated with animal fat [12].

An important example of emigrational epidemiology involving diet change and cancer risk is shown by the observation that Japanese men born in Japan had a reduced risk of CRC compared with US-born white men, whereas Japanese men born in the USA experienced CRC rates twice as high as foreign-born Japanese men [34–36]. US-born Japanese women had higher rates of CRC than Japanese-born Japanese women or US white women. Colon cancer mortality was also increased in US-born Japanese patients [34, 37]. Evidence suggests that the increase in colon cancer development and mortality is due to an increased consumption of Western dietary components, mainly high dietary fat [35]. Similar observations of increased risk for colon cancer in association with diet change have been made in population groups moving to the United States from South Korea, Vietnam, Cuba, Puerto Rico, and Mexico [38–40].

Another example of the impact of dietary composition on cancer incidence is provided by comparison of Pima Indians who have migrated from Mexico to Arizona [41, 42]. The Pima Indians living in Arizona are exposed to energy-dense foods and a decreased need for physical labor. They have a high prevalence of obesity, metabolic syndrome, diabetes, and multiple malignancies including colon cancer, when compared to the Pima Indians that still live in Mexico, who follow a traditional diet and customs and have a lower prevalence of these diseases [43]. Compared to the general population, Pima Indians have a strong genetic susceptibility to obesity, metabolic syndrome, and significantly higher mortality from heart disease and malignancies. The increase in these disorders associated with migration from Mexico to Arizona demonstrates a powerful impact of environment, especially diet on these conditions.

Another series of studies supporting an association of dietary fat with colon cancer comes from comparing the so-called Mediterranean diet to Western diets. In general, Mediterranean countries have lower rates of colon cancer compared to Western countries [44, 45]. As much as 76 % of the intercountry variation in colon cancer incidence rates have been attributed to three dietary factors, meat, fish, and olive oil, with meat and fish showing a positive association and olive oil showing a negative association with colon cancer [46]. Other studies, including three out of six case–control studies in Mediterranean populations, show an inverse association between monounsaturated and saturated fatty acid ratio with CRC [47].

5.4 Animal Studies Supporting Involvement of Fatty Acids in Colorectal Cancer

As detailed in this section and for each fatty acid, multiple studies in animal models now support the association between high-fat diets and intestinal tumor development. These models usually examine the effects of varying dietary fat quantity and/or quality in rodents treated with primary intestinal carcinogens such as azoxymethane (AOM) or dimethylhydrazine (DMH) or in rodents with hereditary genetic mutations predisposing to intestinal tumorigenesis [48]. A common model used in the latter situation is the APC^{Min} mouse containing a mutation in the FAP gene, the same gene that leads to familial adenomatous polyposis in humans and also shows spontaneous mutations in a high percentage of sporadic cancers [49, 50].

Initial studies to define the specific effects of different fats showed that increased amounts of dietary fat increased intestinal tumorigenesis in rats treated with DMH, and that both corn oil, high in unsaturated fats, and lard, high in saturated fats, each promoted increased intestinal tumorigenesis [51]. Subsequent studies showed differential effects of fat on intestinal tumorigenesis based on source, with corn oil, lard, or beef fat causing an increase in intestinal tumorigenesis in DMH-treated rats, whereas supplementation with olive oil reduced tumors in DMH-treated rats. Similarly, supplementation with fish oil (Menhaden oil) reduced tumors in AOM-treated rats [10, 11]. Dietary fats may also influence carcinogen-induced intestinal tumorigenesis by altering intestinal microbiota. Germ-free rats treated with DMH, which is actually a procarcinogen, showed less tumorigenesis than conventionally housed animals. In contrast, tumorigenesis in germ-free rats treated with the primary carcinogen *N*-methyl-*N*-nitro-*N*′-nitrosoguanidine (MNNG) showed slightly increased tumorigenesis, relative to MNNG-treated conventionally housed animals [52]. Since DMH is a procarcinogen, requiring metabolic activation, whereas MNNG is a direct-acting carcinogen, these studies suggest a role for intestinal microflora in carcinogen activation and/or tumor promotion. These and similar results led to an extensive expansion of studies, summarized below, to define the intestinal tumorigenic effects of specific fatty acids from multiple sources.

5.5 Effects of Specific Fatty Acids

5.5.1 Saturated Fatty Acids

5.5.1.1 Lauric and Myristic Acids

Lauric acid (C12.0) and myristic acid (C14.0) are saturated, medium-chain fatty acids (MCFA) found in high concentration in tropical plants such as coconuts and in dairy fats. They appear to promote colon tumorigenesis and significant increases in lauric, and myristic acids have been identified in subjects at high risk for colon cancer [53]. At least one case–control study noted a significant increase in lauric acid intake with colon cancer risk [54]. These two saturated MCFAs have been shown to induce proinflammatory factors in murine and human macrophages and colon cell lines. Treatment of a murine monocyte macrophage cell line, RAW 264.7, with lauric or myristic acid independently increased NF-κB activation, which serves as a transcription factor for multiple proinflammatory cytokines including interleukin-1β (IL-1β), IL-6, TNF-α, and C-reactive protein (CRP) as well as increased expression of cyclooxygenese-2 (COX-2) [55]. COX-2 is a major enzyme involved in synthesis of prostaglandin E_2 (PGE_2), the latter playing an important role in development of colon cancer [56–58]. Many studies show higher PGE_2 levels in human CRC samples compared to paired tissue samples of normal colon mucosa from the same patient [59]. These latter studies suggest that elevated tumor levels of PGE_2 are determined by specific processes within the tumor that increase PGE_2 and auto-promote tumor growth. PGE_2 levels are regulated both by COX-2-mediated synthesis and degradation by 15-prostaglandin dehydrogenase (15-PGDH). 15-PGDH is an NAD+-linked dehydrogenase that oxidizes the hydroxyl group at the 15 position, reducing the level and biologic consequences of PGE_2. 15-PGDH, the rate-limiting enzyme catalyzing degradation of PGE_2, is commonly inactivated in colon tumors providing a partial explanation for elevated levels of PGE_2 in the tumor tissue [60, 61]. PGE_2 is a proinflammatory mediator that decreases production of IL-2 and IFN-α leading to production of proinflammatory T helper cells. PGE_2 also has direct effects on stimulating growth-promoting pathways, increasing cell proliferation and decreasing apoptosis in intestinal epithelial cells [58, 62].

Treatment of cultures of human nonmalignant colon epithelial cells (HCEC) and multiple human colon cancer cell lines, including HCT 116, SW48, SW480, HT29, and HCA-7, with lauric or myristic acids increases NF-κB, COX-2, and prostaglandin production [63–65].

Further studies suggest that the effects of fatty acids on NF-κB activation and COX-2 expression may result from incorporation of saturated fatty acids into lipopolysaccharide (LPS) followed by signaling through stimulation of Toll-like receptors (TLRs) [66]. These studies show that lauric and myristic acid act on both intestinal epithelial and immune cells to promote increases in inflammatory factors, some of which promote growth in normal and malignant colon tissues.

5.5.1.2 Palmitic and Stearic Acids

Palmitic (C16:0) and stearic acids (C18:0) are long-chain saturated fatty acids found in both animal and plant sources. Epidemiologic studies on the association of palmitic and stearic acids with colon cancer are controversial with many reporting no relation with colon cancer risk [67, 68]. However, in a national prospective case–control study in Scotland that included 1,455 incident cases and 1,455 matched controls, palmitic acid was dose-dependently associated with increased colon cancer risk [69]. In the $Apc^{Min/+}$ murine model, levels of erythrocyte membrane palmitic acid were significantly associated with the development of intestinal tumors [70]. Several studies have demonstrated an increase in stearic acid intake and colon cancer risk, increased stearic acid in the plasma of colon cancer patients compared to controls [67], and increased stearic acid in colon cancer specimens compared to normal tissue [53, 71].

Stearic acid has been shown to increase proliferation of HT-29 human colon cancer cell lines [72] and stearic acid has been shown to promote colon carcinogenesis in male Sprague Dawley rats injected with colon carcinogens [73]. Examination of membrane phospholipids in these rodents showed significantly higher amounts of stearic acid in the tumor-bearing mice compared to controls [73]. From a mechanistic viewpoint, at least one study demonstrated that high levels of membrane stearic acid in tumor cells could inhibit the immune response and subsequent apoptosis [74]. Concentrations of fibrinogen, another marker of inflammation, were shown to be elevated after high dietary consumption of stearic acid [75]. Although not specific to palmitic or stearic acids, examination of the 1999–2000 National Health and Nutrition Examination Study (NHANES 1999–2000) showed a modest association of elevated CRP with consumption of saturated fatty acids [76].

5.5.2 Monounsaturated Fatty Acids

5.5.2.1 Palmitoleic Acid

Palmitoleic acid (C16:1n-7), an omega MUFA, commonly found as a minor component of many animal and vegetable oils and in higher concentration in macadamia nuts, has been shown to have anti-inflammatory properties, to reduce hypercholesterolemia, and to have a beneficial effect on muscle insulin resistance; however, its effects on cancer and especially on CRC remain to be investigated [77, 78].

5.5.2.2 Oleic Acid

Oleic acid (C18:1n-9) is a MUFA that is the major component of olive oil and can also be found in meats, nuts, and dairy products. Several studies have suggested that the ratio of oleic acid to stearic acid is important in determining disease outcome. In two human studies, a decreased erythrocyte oleic/stearic acid ratio was reported in patients with CRC, and similar changes were shown in their cancer tissues [71, 79].

To test the ability of oleic acid to interfere with the procarcinogenic effect of stearic acid, mice that were treated with DMH and fed diets high in stearic acid were supplemented with oleic acid. The latter mice showed reduction in intestinal polyp multiplicity and size relative to those without the oleic acid supplement [80]. In human cell culture, it has been shown that oleic acid can inhibit the proliferative effects of stearic acid as well as inhibit stearic acid-induced NF-κB activation and intercellular adhesion molecule-1 (ICAM-1) expression [81]. Studies conducted with human colon cancer cell lines show that treatment of HT-29 and Caco-2 cells with oleic acid results in decreased proliferation and increased apoptosis. Oleic acid has been shown to decrease factors associated with inflammation and tumorigenesis such as COX-2 and Bcl-2 [82]. Raising dietary levels of oleic acid showed a trend to lower circulating inflammatory markers including CRP, IL-6, and E-selectin compared to diets enriched for saturated fatty acids, trans fatty acids, or stearic acids [75, 76]. Oleic acid has also been shown to inhibit calcium influx pathways important for proliferation and inflammation as well as for calcium-induced apoptosis in HT-29 colon cancer cells [83]. Interestingly, olive-oil-fed mice showed reduced production of nitric oxides and other inflammatory mediators [84].

5.5.3 Polyunsaturated Fatty Acids

PUFA composition of the cell membrane can drastically change intercellular signaling and can be modulated by diet [22]. Two general groups of PUFAs that play critical roles in health and disease are the omega-3 and omega-6 PUFA. They are not interconvertible, and dietary substitution of one results in a competitive reduction in concentration of the other in all tissues [21, 23]. Omega-3 (ω-3) PUFAs are essential fatty acids commonly found in plant and marine oils that include α-linolenic, eicosapentaenoic, and DHAs [21]. Omega 6 (ω-6) PUFA are essential fatty acids commonly found in corn, soybean, sunflower, and palm oils and include arachidonic, linoleic, and γ-linoleic acids [24]. Comparing tumor tissue to normal intestinal mucosa in patients undergoing surgery for colon cancer showed that tumor tissue contained elevated PUFA levels, including arachidonic acid and its derivative PGE_2 which serves to promote both inflammation and proliferation. These studies also showed elevated levels of the lipid peroxidation product, malondialdehyde, which serves as a potential DNA-targeted mutagen [85].

5.5.3.1 Linoleic Acid

Linoleic acid (LA) C18:2n-6 is an essential ω6 fatty acid derived mostly from corn oil but also from safflower and sunflower oils; it is the predominant PUFA of the Western diet common in America [86, 87]. Diets high in corn oil have been shown to significantly increase polyp multiplicity in various rodent studies [88–90]. However, data surrounding the effect of LA on intestinal tumorigenesis is complicated by the fact that early studies did not differentiate between LA and an

alternative set of isomers, designated conjugated linoleic acid (CLA). Without differentiating between specific LA isomers, numerous studies demonstrated that LA had a beneficial effect on colon cancer outcome [67, 68, 91, 92], whereas more recent studies suggest that different LA isomers could have contrasting effects [93].

Supplementation of AOM-treated rats with linoleic acid resulted in a significantly higher incidence of colon tumors, with histology showing greater malignant differentiation [94]. Further studies of dietary supplementation with LA and/or glucose in Fischer 344 rats treated with the carcinogen AOM showed that both LA and glucose each increased intestinal tumor frequency, multiplicity, and metastasis and the greatest increase occurred in rats treated with the combination of LA and glucose. This increase was associated also with greater weight gain in rats [95]. Several meta-analyses in normal individuals have not shown a relation between linoleic acid intake and levels of inflammation except for an increased level of PGE_2 excretion [96, 97].

Levels of LA have been found to decrease with advanced stages of cancer [22]. In contrast, patients with normal colon exhibited high mucosal LA content compared to those with colon adenomas or colon cancer [91]. Circulating LA concentration have been shown to be decreased in colon cancer patients compared to hospital-based controls [67] and decreased in patients with familial adenomatous polyposis compared to healthy controls [98].

The Netherlands Cohort Study, which comprised 123,852 individuals, of which 531 were diagnosed with colon cancer, detected that LA intake was associated with increased colon tumors. Specifically, this group observed a significant association of LA with tumors that contained KRAS mutations [99, 100]. In contrast, there were no significant associations for CRC with dietary differences in total fat, saturated fats, MUFAs, or linoleic acid [99]. These observations could be explained by lipid peroxidation of ω6 PUFAs with the generation of mutagenic by-products leading to procarcinogenic changes in the K-ras oncogene [99]. Human Caco-2 colon cancer cell lines supplemented with LA showed increases in cell growth and proliferation [101]. Similarly, human SW480 colon cancer cells treated with LA showed significant elevations in cell proliferation and prostaglandins, including PGD_2 and PGE_2, none of which were observed in cells treated with CLA [102].

LA serves as a precursor for the biosynthesis of AA and subsequent proinflammatory prostaglandins, such as PGD_2 and PGE_2. Thus, many of the consequences of dietary supplementation with LA are probably mediated through its conversion to arachidonic acid [103].

5.5.3.2 Conjugated Linoleic Acid

CLAs, C18:2, are a series of 28 different isomers of linoleic acid that vary in the location of their unsaturated double bonds and geometric arrangements [104]. Molecular arrangements around these unsaturated double bonds may be either in the *cis* (c) or *trans* (t) configuration. As such, they provide a source of naturally occurring trans fatty acids derived primarily from dairy products and meat from ruminant animals. The different isomers may have similar and/or opposing effects

associated with their unique geometries accounting for conflicting reports of their consequences. Of the various CLA isomers, those most abundant in natural products are the *cis*-9, *trans*-11 isomer, (c9, t11 CLA) and the *trans*-10, *cis*-12 isomer (t10, c12-CLA) [105]. In natural products, the c9, t11 CLA usually exceeds the t10, c12 variety; however, they are commonly present in equal amounts in commercially available CLA supplements used to reduce obesity or build lean body mass [106].

Numerous studies have been conducted in rodent and human cell lines and animal models to examine the outcomes of CLA associated with inflammation and colon cancer development. t10, c12 CLA induces an inflammatory response in cultured adipocytes from Caucasian and African-American women as demonstrated by increased levels of IL-1β, IL-6, IL-8, and COX-2, with the induced inflammatory response being much greater in fully differentiated adipocytes compared to preadipocytes [106]. In contrast, treatment of RAW264.7 murine macrophages with a series of CLAs, both *cis* and *trans*, significantly suppressed IL-1β, TNF-α, INF-γ, COX-2, PGE_2, and iNOS. Many of these effects were eliminated by peroxisome proliferator-activated receptor gamma (PPARγ) mutation, suggesting PPARγ as an important mediator of CLA effect [107].

CLA treatment in tissue culture has also been shown to affect cell growth and other tumor-associated properties. Several studies show that treatment with c9, t11-CLA results in time- and dose-dependent decrease in proliferation in HT-29 and Caco-2 human colon cancer cell lines [101, 108–110]. Treatment of Caco-2 cells with an equal mixture of c9, t11 and t10, c12 reduced their viability, but the effect was blocked by inhibitors of PPARγ, again indicating a role for PPARγ as mediator of the CLA effects [110]. In addition, treatment in tissue culture with c9, t11 CLA but not t10, c12 CLA reduced migration of SW480 human colon cancer cells, and both agents decreased pulmonary metastasis of IV-administered CT-26 mouse colon cancer cells in BalbC mice [111].

Multiple studies show that dietary supplementation with t10, c12 CLA promotes inflammation in murine white adipose tissue [112] whereas c9, t11 CLA reduces inflammation [113] and reduces colonic aberrant crypt formation (ACF) and tumorigenesis in DHM- or AOM-treated murine models [114–116]. The decreased tumor formation in the treated animals was associated with decreased levels of PGE_2, thromboxane A2, arachidonic acid, increased apoptosis, decreased infiltrating macrophages, and increased regulatory T cells in mesenteric lymph nodes [114–116]. CLA supplementation of AOM-treated rats reduced formation of both ACF and tumors and increased PPARγ in tumors and surrounding normal mucosa [117]. Mice, genetically deficient in PPARγ, showed similar polyp numbers and infiltrating immune cells when treated with CLA, demonstrating again that the antitumor effect can be modulated through PPARγ signaling [118].

Similarly, in AOM-treated Sprague Dawley rats, supplementation with CLA containing approximately equal concentrations of the c9, t11 and t10, c12 isomers showed decreased cancer incidence and decreased tumor multiplicity (number of cancers/rat) along with increased apoptosis. The antitumor effect of the CLA supplement was associated with decreased COX-2 and PGE_2. Administration of CLA

as free fatty acids was more effective than when they were administered as conjugated triglycerides [119].

Although evidence for beneficial effects of CLA in humans has been hard to establish, given the complications surrounding the LA isomers, the Swedish Mammography Cohort examined 60,708 women and observed that women who consumed high amounts of CLA-containing foods, such as dairy products, showed a significant reduction in colon cancer risk. Each increment of two servings of high-fat dairy foods corresponded to a 13 % reduction in the risk of CRC [120].

5.5.3.3 Arachidonic Acid

Arachidonic acid (AA), 20:4n-6, contained in a variety of dietary food sources and synthesized in the body by desaturation and elongation of linoleic acid, is an ω6 PUFA associated with detrimental effects on disease outcome. Two independent studies demonstrated significantly increased AA levels in plasma and intestinal tissue of colon cancer patients compared to controls, and AA levels were significantly elevated in patients with FAP [98]. In DMH-treated rats, supplementation with AA resulted in a significant increase in colon cell proliferation and tumors [121]. Studies in $APC^{Min/+}$ mice demonstrate that dietary supplementation with AA can increase intestinal AA content, increase PGE_2 formation, but not increase intestinal tumorigenesis. In contrast, EPA supplementation decreased intestinal AA content and PGE_2 production. Supplementation of dietary EPA with AA restored the intestinal AA content, returned capacity for PGE_2 production, and abolished the antitumorigenic effect of EPA [122]. Thus, dietary supplementation with AA did not increase intestinal tumorigenesis in normal-diet-fed mice, but AA was able to overcome the antitumorigenic effect of EPA.

Arachidonic acid is metabolized to PGE_2 and related eicosanoids by the cyclooxygenase system, including COX-1 and COX-2. As noted earlier, COX-2 is upregulated under inflammatory conditions. PGE_2 is degraded by 15-PGDH whose level is decreased in colon neoplasia [56, 57, 60, 61]. Thus, increased COX-2 and decreased PGDH, each contribute to the elevated PGE_2 in colon cancer.

Elevated levels of intestinal PGE_2 lead to increased inflammatory processes, stimulate increased replication of intestinal epithelial cells, prolong their survival due to decreased apoptosis, and increase tumor-associated angiogenesis and neovascularization [123]. The proinflammatory and procarcinogenic effects of arachidonic acid and PGE_2 are further supported by the demonstration that nonsteroidal anti-inflammatory drugs (NSAIDs), which interfere with PGE_2 synthesis, are effective agents in rodents and humans for suppressing development of colorectal adenomas and carcinomas [124] and reducing mortality in patients being treated for CRC [125]. In addition, n-3 PUFA supplementation, especially with EPA and DHA, interfere with the cancer-promoting effects of PGE_2, and knockdown of PGE_2 receptors block development of intestinal tumors in carcinogen-treated rodents.

Although numerous experimental approaches in rodents have been used to demonstrate that AA supplementation and elevated PGE_2 levels promote both local gastrointestinal and systemic proinflammatory states and increased intestinal

tumorigenesis, all of these models require either a predisposing genetic mutation, such as the $APC^{Min/+}$ model of familial adenomatous polyposis; a carcinogen-induced mutation, induced by treatment with agents such as AOM or DMH; or spontaneous mutation as expected to occur during free radical generation associated with inflammatory bowel disease [48]. These observations indicate that AA and its growth-promoting derivatives such as PGE_2 function to promote progression of tumors that are initiated by primary mutagenic changes. Overall, we are unaware of rodent studies showing that arachidonic acid initiates tumorigenesis in animals without preexisting genetic alterations. Moreover, while some epidemiologic studies have identified an increase in CRC in association with increased arachidonic acid consumption [126], most studies have not supported this association [54, 69, 127–130]. These observations may account for the lack of any significant increase in reports of adverse effects in populations where diets in the young or the elderly are supplemented with AA [103, 131]. The protumorigenic effects of AA and its use as a dietary supplement in infants and adults are an interesting paradox.

5.5.3.4 Alpha Linolenic Acid

Alpha linolenic acid (ALNA) 18:3n-3 is an essential ω-3 PUFA found in high quantities in vegetable oils, seeds, nuts, and dark green leafy vegetables. Common sources include perilla oil, flaxseeds, kiwi fruit seeds, walnuts, and canola oil. As an n-3 fatty acid, ALNA has similar properties to EPA and DHA and can be converted in the body at limited rates to EPA 20:3n-3 and DHA 22:6n-3.

Dietary supplementation in rodents with perilla oil, walnut oil, or flaxseed oil, all high in ALNA, resulted in increased circulating and tissue ω-3 fatty acids compared to control-fed animals and decreased intestinal ACF, tumor incidence, and tumor load in animals treated with *N*-methyl-*N*-nitrosourea or AOM [132–134]. Similar effects were seen in $APC^{Min/+}$ mice where a flaxseed oil supplemented diet decreased small and large intestine tumor number and size [135].

Further studies in rodents and man showed that diets supplemented with walnuts, flaxseed oil, or purified ALNA decreased circulating and tissue levels of proinflammatory factors including IL-1β, IL-6, TNF-α, myeloperoxidase, CRP, NF-κB, COX-2, and urinary PGEM. ALNA supplementation also decreased oxidative stress as measured by decreased urinary 8 isoprostanes [136–141]. Interestingly, flaxseed-dependent lowering of inflammatory factors in rats was further enhanced by exercise [138]. The ALNA lowering of inflammatory factors is similar to those produced by EPA and DHA [141]. While fish oil supplementation increases circulating EPA and DHA, flaxseed supplementation increases ALNA [142]. Flaxseed oil has also been shown to reduce adipocyte size and monocyte chemoattractant protein 1 in obese rats [143]. The combination of flaxseed oil and fish oil in healthy volunteers produced greater suppression of inflammatory factors than either agent alone [144].

In rodents with inflammatory bowel disease, ALNA decreased inflammatory cytokines and intestinal inflammatory disease. Increased intake of ALNA reduced inflammatory death rate in patients with inflammatory disease, whereas dietary

intake of supplementary fish oil was associated with no changes in death from inflammatory diseases [145].

An ALNA-supplemented diet in men and women compared to a linolenic acid-supplemented diet or the average American diet resulted in decreased circulating CRP, decreased intercellular cell adhesion molecule 1 (ICAM-1), and decreased E-selectin which serve as attractants for inflammatory monocytes and macrophages [146]. ALNA caused an increase in apoptosis and dose-dependent decreases in proliferation in human Caco-2 colon cancer cell lines.

5.5.3.5 Eicosapentaenoic Acid and Docosahexaenoic Acid

EPA (20:5n-3) and DHA (22:6n-3) are essential ω-3 long-chain PUFAs found in marine fish and in some seaweeds with multiple health benefits against cardiovascular disease, and with anti-inflammatory, antihypertensive, antiarthritis, antioxidative, and anticancer effects. Numerous studies have demonstrated that EPA and DHA have an inverse relationship with colon cancer [67, 147]. Although EPA and DHA are metabolized differently, nearly all studies which address the association between ω-3 fatty acids and colon cancer in humans use a mixture of EPA and DHA. Because most clinical studies supplement them together, they will be discussed concurrently.

Significantly decreased EPA levels were observed in the mucosa and plasma phospholipid profiles of patients with colon adenomas or colon cancer compared to healthy controls [91]. DHA, but not EPA, was significantly lower in serum from FAP patients compared to controls [98]. Several trials have observed significantly low intake and plasma concentrations of DHA and EPA among colon cancer patients as well as individuals at high risk for developing colon cancer [67, 147]. In a 12-week randomized control trial on the effect of ω-3 fatty acids, specifically EPA, in 20 subjects at risk for colon cancer, a significant reduction was found in the abnormal cell proliferation in the experimental group receiving EPA. EPA supplementation (4.1 g per day for 6 months) increased mucosal composition of this fatty acid and significantly decreased intestinal proliferation in cancer patients [148, 149]. Similar to results with EPA, treatment of colon cancer patients with DHA (1.1 g per day) led to a reduction in cell proliferation in rectal mucosa [148, 149].

The distinct beneficial effects of DHA have been investigated in more detail in animal models and human colon cancer cell lines. DHA decreased aberrant crypt foci (ACF), which are precursors to colon polyps, and decreased polyp number by 40 % in rats treated with DMH and significantly reduced ACF and tumor multiplicity in rats treated with AOM [150, 151].

In human colon cancer cells, DHA was shown to activate many genes involved in apoptosis, such as cytochrome c and caspases 5, 8, 10, and 15, while decreasing expression of anti-apoptotic, Bcl-2 family members as well as components of the prostaglandin synthesis pathway such as COX-2 and PGE_2. In human HT-29 and HCT116 colon cancer cells, DHA reduced the levels of PPARγ, which regulates fatty acid storage and glucose metabolism [152, 153].

Comparing the effect of PUFA treatment on NCM 460 normal colon cells, DHA, but not arachidonic acid, palmitic acid, or oleic acid, selectively stimulated growth, whereas in Caco-2 colon tumor cells, DHA was the only fatty acid that stimulated apoptosis, as demonstrated by cleavage of caspase 3 and poly ADP-ribose polymerase (PARP). Moreover, DHA was the only PUFA tested that reduced viability of Caco-2 cells. This change occurred in association with increased DHA membrane concentration, decreased PI3K, due to decreased concentration of its p85 regulatory subunit, and decreased PTEN phosphorylation, resulting in increasing negative control of PI3K. In addition, DHA and oleic acid significantly decreased Ser^{473} phosphorylation of AkT, thereby interfering with the PI3K/AkT growth-promoting pathway [154]. Additionally, DHA in combination with the saturated fat, butyrate, has been shown to enhance mitochondrial lipid oxidation and calcium-dependent apoptosis in human HCT116 cells [155].

From a mechanistic viewpoint, EPA is a competitive inhibitor of the proinflammatory effects of arachidonic acid mediated through COX-1 and COX-2, yielding PGE 3 which is less inflammatory than PGE_2. EPA and DHA reduce TNF-α, IL-1, IL-6, IL-8, resistin, plasminogen activator inhibitor 1, and monocyte chemoattractant protein 1. The ω-3 PUFAs also increase adiponectin which reduces inflammation [156].

EPA has been independently investigated in more mechanistic detail and in association with its beneficial effects on colon cancer. Prostaglandin synthesis and PPARγ expression were significantly reduced in human HT-29 cells after treatment with EPA. These same studies demonstrated a significant decrease in proliferation and induction of apoptosis in the HT-29 human cancer cell line [157, 158]. Treatment of human HCT116 colon cancer cells with EPA resulted in reduction in DNA polymerase activity and cell cycle arrest at the G1 checkpoint [159]. AOM-treated F344 male rats supplemented with fish oil diets enriched for EPA and DHA decreased intestinal tumor incidence and multiplicity [160].

Studies conducted in $APC^{Min/+}$ mice demonstrated that DHA and EPA supplementation decreased polyp multiplicity and size by 50 % and significantly reduced proinflammatory prostaglandin levels [23, 161, 162]. $Apc^{Min/+}$ mice and AOM-treated rats fed diets supplemented with EPA showed significantly fewer polyp numbers as well as decreased levels of COX-2 and PGE_2 [161, 163, 164]. Interestingly, $APC^{Min/+}$ mice provided with supplemental dietary EPA were protected from the cachexia that usually accompanies intestinal tumorigenesis in these mice [163].

5.5.3.6 Importance of the Omega-3 to Omega-6 Ratio

Many studies have shown that changes in the omega-3/omega-6 (ω-3/ω-6) ratio may contribute to the early development of human colon cancer. Competition occurs between these fatty acids in the COX-signaling pathway which gives rise to a series of prostaglandins, some pro- and others anti-inflammatory. Based on their competitive nature, clinical guidelines recommend a 1:1 ratio between ω-3 and ω-6 fatty acids for optimal health [165]. In contrast to recommendations, it is estimated that Westernized diets are composed of an ω-3/ω-6 ratio of 1:10–25 [166].

A randomized clinical trial examined the plasma phospholipid ω-3/ω-6 ratio as a nutritional marker for the prevention of colonic tumor development and metastasis in 27 patients. A significant increase in the plasma phospholipid content of ω-3 and ω-6 ratio was found in the experimental group which correlated with inhibition of the mucosal neoplastic proliferation, thus associating an increase in an ω-3/ω-6 ratio with decreased cancer risk [167]. In an ecological study in Belgium, ω-3/ω-6 ratios were examined in 11,302 individuals for correlations with mortality associated with colon cancer. A significant inverse relationship was established between colon cancer and high ω-3/ω-6 ratios [168, 169]. Similarly, another study that examined 363 cases of patients with colon adenomas and 498 adenoma-free controls also observed an inverse relationship between an increased ω-3/ω-6 ratio and colon cancer risk [170].

In a prospective study of 73,242 Chinese women participating in the Shanghai Women's Health Study, there was no association of CRC with total ω-6 PUFA or total ω-3 PUFA consumption. There was, however, a dose-dependent association of CRC with arachidonic acid consumption and a strong association of CRC with increasing ratio of ω-6/ω-3 with the highest relative risk of 1.95 comparing highest to lowest quintiles of ω-6 to ω-3 ratios. Interestingly, the risk for rectal cancer was greater than that for colon [126]. In a nested case–control study of 150 cases and 150 controls in women, the ω-6/ω-3 ratio correlated with urinary metabolites of PGE_2 suggesting the possibility that the association between ω-6/ω-3 ratio and CRC is mediated by PGE_2.

5.6 Specific Diets

5.6.1 Animal Fats

Studies supporting an association of CRC with diets containing high intake of red meats and processed meats have been contradictory. A meta-analysis of articles published from 1973 to 1999, examining the relation of meat consumption to CRC, found no significant association for CRC with total meat consumption (red, white, and processed). In contrast, the relative risk of CRC was increased with consumption of red meat and was even greater for processed meat [171, 172]. Results from the Nurses' Health Cohort Study, 121,700 women, provided further support that dietary animal fat, but not vegetable fat consumption, was associated with increased risk for colon cancer. Red meat and processed meats were associated with increased risk, whereas fish and chicken were associated with decreased risk [14]. Some studies show an inverse or no relation between total dietary fat and colon cancer [173, 174], although an increased risk for colon cancer was associated with increased red meat consumption [173].

The presumptive carcinogenic effects associated with meats have been attributed to proteins, fats, pyrolysis products including heterocyclic aromatic amines and polycyclic aromatic hydrocarbons associated with cooking, and *N*-nitroso

compounds used as preservatives in processed meats. An interesting example of the effect of diet and genetic interactions on cancer causation is provided by the observation that the rapid acetylator phenotype, associated with NAT2 genetic polymorphisms, contributes to activation of the mutagenic potential of heterocyclic aromatic amines generated by cooking meat at high temperatures, and increases the association of dietary meat consumption with CRC [175].

Rats treated with the carcinogen DMH and fed beef tallow as primary fat source showed significantly increased intestinal tumor incidence and tumor numbers per rat compared to rats fed fish oil diets. The beef tallow-fed rats showed less apoptosis and more PGE_2 in intestinal mucosa compared to rats on the fish oil diet. Interestingly, supplementation of either diet with CLA increased the percentage of cells undergoing apoptosis, decreased PGE_2, and decreased tumorigenesis in DMH-treated animals [176].

In a case–control study in North Carolina, involving 945 cases and 959 controls, oversampled for African Americans, with diet analyzed retrospectively for the past year, the highest intake of red meat was associated with decreased risk of distal CRC (sigmoid, rectosigmoid, or rectal) in both Caucasians and African Americans that only reached significance in Caucasians. There was no statistical difference in distal CRC with total fat, MUFA or PUFA in either race, although a significant trend was noted for decreased risk in African Americans for saturated fat measured as percent energy but not as total fat. The highest level of PUFA intake in African Americans was associated with lower risk, but this did not reach statistical significance [177]. No estimates were provided of absolute amounts or ratios of ω-3/ω-6 PUFAs.

5.6.2 Mediterranean Diet

The Mediterranean diet has high amounts of whole grains, fruits, vegetables, fish, and olive oil, moderate amounts of alcohol and dairy products, low amounts of red or processed meats, and low glycemic loads. As indicated earlier, Mediterranean countries have lower rates of colon cancer compared with other Western countries. For example, colon cancer mortality in Greece is about 40 % lower than that in the United Kingdom [44, 45]. A large meta-analysis of 12 studies examining the health benefits of the Mediterranean diet showed a decreased risk of all cause mortality, cardiovascular disease mortality, and cancer mortality [178]. Another more recent meta-analysis of 19 observational studies, including patients from both Mediterranean and non-Mediterranean countries, showed that the group with the highest consumption of olive oil compared to the lowest had lower odds of having any cancer and specifically lower odds for breast and digestive cancers [179]. More specifically, some, but not all, studies indicate that adherence to the Mediterranean diet is associated with reduced risk of CRC in both men and women [180]. When an altered Mediterranean diet was compared to the DASH diet (Dietary Approach to Stop Hypertension), with higher intake of whole grains, decreased red and preserved meats, and moderate amounts of low-fat dairy products, in two very large cohort studies, The Nurses' Health Study (NHS), 87,256 women, and The Health

Professionals Follow-Up Study (HPFS), 45,490 men, adherence to the altered Mediterranean diet resulted in a reduction in CRC that did not reach statistical significance, whereas adherence to the DASH diet resulted in a statistically significant reduction in CRC in both men and women [181, 182].

Olive oil has been shown to be an important contributor to the cancer preventative effects of the Mediterranean diet [179]. A case–control study conducted in the Marseilles region of southern France examined 399 patients with colon cancer and observed a reduction in olive oil intake compared to age-and sex-matched controls [183]. The relationship between various seasoning fats and colon carcinoma risk was investigated using data from a second case–control study conducted in six Italian areas. Cases were 1,953 patients with histologically confirmed colon carcinomas, while the controls were 4,154 subjects with no history of cancer. This study found that olive oil intake was negatively associated with risk of colon carcinomas [184].

Rats, chemically treated with DMH, fed a higher olive oil-based diet, developed a significantly lower number of ACF than rats fed a low concentration of olive oil. Olive oil dose-dependently downregulated the expression of both Bcl-2 and COX-2 in colonic mucosa and also abrogated the upregulation of Bcl-2 by DMH [185]. Rats treated with AOM and supplemented with 5 % dietary olive oil showed significant decreases in ACF and PGE_2 [186].

5.6.3 Western Diet

The Western-style diet, high in fat, red meat, sugary deserts, low in fiber, fresh fruits, vegetables, whole grains, seafood, and calcium and vitamin D content [30], is associated with increased risk of CRC in both humans and rodents [33, 187]. The Western diet in rodents is both proinflammatory and procarcinogenic; however, supplementation with calcium and cholecalciferol diminishes tumorigenesis [187]. Mice fed a normal lab chow supplemented with 20 % corn oil to simulate a Western diet showed inflammatory changes in their intestinal mucosa including increased F4/80-staining macrophages in the lamina propria, with increased IgA, CRP, fibrinogen, myeloperoxidase, serum amylase, monocyte chemoattractant protein, and macrophage inflammatory protein-1 [188]. Corn oil-supplemented diets clearly promote intestinal tumorigenesis in $APC^{Min/+}$ mice and in AOM-treated F344 rats [10].

5.7 Summary of Dietary Fatty Acid Effects on Colon Cancer

On the basis of epidemiologic studies conducted in multiple countries, with different dietary patterns and with different populations, especially those undergoing significant dietary change, there is a convincing evidence to support the concept that fatty acids and their concentrations in different tissues have important effects on CRC risk, where specific dietary fatty acids can increase the risk of CRC while

others can suppress it. Studies in tissue culture and animal models as well as those in patients demonstrate that differences in the effects of fatty acids can be related to variations in their structure, intake, biochemical consequences, and their concentrations in different tissues, especially those obtained from patients with colon cancer compared to individuals free of malignant disease. However, before commenting further on mechanistic and differential effects of fatty acids on CRC, it is important to emphasize that almost all studies in animals require the presence of a carcinogenic or hereditary genetic mutation before the effects of fatty acids can be identified. In general, the fatty acids do not produce colorectal tumors in rodents without preexisting mutations. In contrast, diets supplemented with protumorigenic fatty acids, in rodents treated with AOM or DMH or in mice with the APC^{Min} mutation, result in increased tumorigenesis, including earlier time of onset, higher incidence, greater multiplicity, larger size, and higher tumor load, compared to non-diet-supplemented animals. These studies indicate that protumorigenic fatty acids act primarily as tumor promoters contributing to increased tumor growth but not primary carcinogenesis. Conversely, the reduced tumorigenicity or protective effects of other dietary fats appear to be associated with either a direct or indirect effect on suppression of tumor growth as opposed to any anticarcinogen effect. Since human colorectal adenomas and carcinomas are routinely associated with a series of hereditary and/or spontaneous mutations that serve as initiators of the carcinogenic process, it is expected that, as in rodent studies, fatty acids will serve as tumor promoters or suppressors. Some mutations leading to initiation of CRC in humans include DNA alterations leading to inactivation of tumor suppressors such as FAP, TP53, and TGF-β or activation of oncogene pathways including RAS/RAF, PI3K, and PTEN [58]. As detailed previously and summarized below, fatty acids that function as tumor promoters stimulate cell proliferation through inflammatory pathways. The inflammatory pathways enhance cell proliferation which in turn fixes mutagenic DNA alterations in a population of cells establishing the basis for tumor growth and progression [189].

Figure 5.1 summarizes the results discussed in this section, where medium-chain saturated fatty acids, lauric and myristic acid; long-chain saturated fatty acids, palmitic and stearic acids; and ω6 PUFAs including linoleic and arachidonic acids have detrimental proinflammatory, protumorigenic effects as tumor promoters increasing colon cancer risk, development, and outcome. Conversely, oleic, conjugated linoleic, and ω-3 PUFAs including eicosapentaenoic, docosahexaenoic, and linolenic acids serve as tumor suppressors with beneficial effects, mediated by protecting against inflammation, reducing incidence, and retarding growth in colon cancer.

Overall, it is important to note that the fatty acids that promote inflammation also promote intestinal tumorigenesis, whereas those that suppress inflammation also reduce intestinal tumorigenesis. Moreover, in studies, where tested, the fatty acids that promote or protect against inflammatory bowel disease show similar effects for colon cancer. Ongoing studies in our group are focused on defining the separate contributions and mediators of obesity and inflammation on fatty acid-induced intestinal tumorigenesis and identifying the individual mechanisms [190].

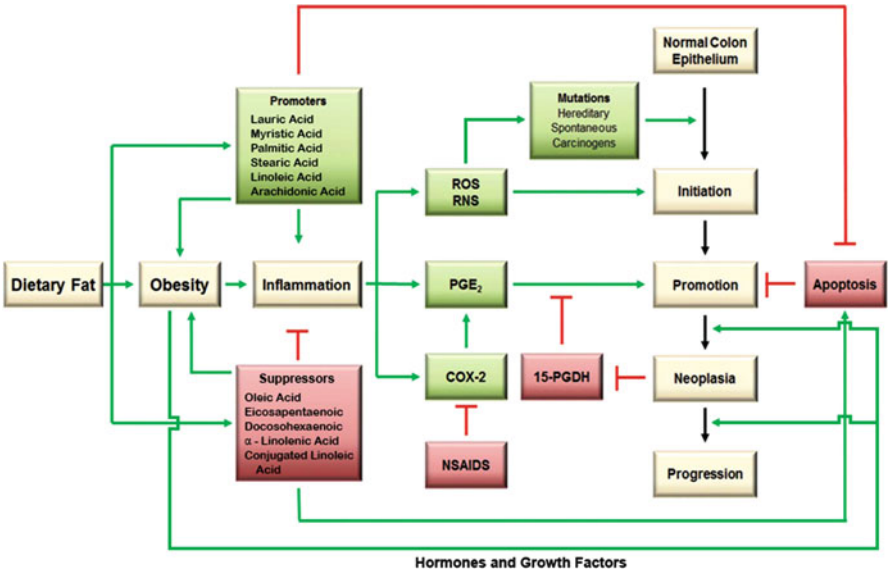

Fig. 5.1 Impact of dietary fatty acids on colorectal cancer. Dietary fatty acids are divided into cancer promoters (*green box*) and cancer suppressors (*red box*). *Green arrows* show positive or stimulatory effects on the indicated process. *Red arrows* show negative or inhibitory effects on the indicated process. *ROS* reactive oxygen species; *RNS* reactive nitrogen species; *PGE_2* prostaglandin E_2; *COX-2* cyclooxygenase 2; *NSAIDs* nonsteroidal anti-inflammatory drugs; *15-PGDH* 15-prostaglandin dehydrogenase

As shown in Fig. 5.1, the specific dietary fatty acids differentially modulate plasma markers and mediators of inflammation in tissue and in circulation. Thus, diets enriched in the fatty acids listed as promoters stimulate synthesis, activation, and release of multiple inflammatory factors including NF-κB, IL-1β, IL-6, TNF-α, E-selectin, fibrinogen, CRP, COX-2, and PGE_2 in both immune system cells and intestinal epithelium. PGE_2 serves as a modulator of inflammation and promotes tumor growth and angiogenesis. PGE_2 levels are determined by its synthetic rate via COX-2 and its catabolic rate determined by 15-PGDH. Elevated levels of PGE_2 in colon cancer are due in part to induction of COX-2 and depression of 15-PGDH in tumor tissue. The increased PGE_2 stimulates proliferation of normal and initiated colon epithelial cells. However, failure of the normal process of apoptosis provides a further basis for unrestricted tumor growth. In addition, fatty acid stimulation of inflammation leads to increased levels of myeloperoxidase indicating increased infiltration by neutrophils and increased synthesis of reactive oxygen species (ROS) and reactive nitrogen species (RNS) which may provide additional opportunities for ongoing mutagenic DNA damage.

The protective fatty acids, including the MUFA, oleic acid, and the ω-3 PUFAs, including EPA, DHA, ALNA, and CLA, inhibit inflammation in tissue culture, murine models, and humans, resulting in decreased COX-2, decreased PGE_2, and, consequently, decreased promotion of cell growth and tumorigenesis. Mechanisms

by which ω3 fatty acids downregulate NF-κB and COX-2 include transcriptional regulation and changes in membrane fluidity leading to alterations in growth-factor sensitivity [191]. In addition to preventing increases in COX-2, these fatty acids serve as competitive inhibitors to block metabolism of AA to PGE_2. Moreover, these fatty acids also stimulate apoptosis in normal and transformed intestinal tissues thereby retarding tumor promotion and possibly tumor growth.

While the effects described above are direct consequences of fatty acids, their consumption in high quantities leads also to obesity, which itself is a low-grade inflammatory disorder [5] and forms the basis for a feed-forward cycle that continues to promote inflammation and tumor development and growth. From the viewpoint of cancer prevention, it is noteworthy that weight loss in obese premenopausal women was associated with significant reduction of circulating and rectosigmoid inflammatory markers [192]. Moreover, obesity is responsible for increased circulating growth factors including insulin and leptin, decreased adiponectin, and other changes which further stimulate tumor promotion and growth through cell surface receptors such as the insulin receptor, the leptin receptor, and the IGF-1 receptor as well as their downstream intracellular pathways [193].

5.8 Dietary Fatty Acid-Targeted Interventions in Colon Cancer

5.8.1 Prevention and Control

While physical activity, sleep, and genetics are all important contributors that need to be addressed to maintain healthy body mass, the observations outlined above indicate the importance of not only controlling quantity of fats but also the quality and composition of dietary fats to prevent cancer, especially CRC. Both saturated and unsaturated fats are needed for comprehensive structure and functional development; however, CRC prevention strategies favor dietary consumption of the unsaturated fats relative to saturated fats. As indicated above, there is a significant relationship between increased ratios of ω3 to ω6 fatty acids and reduced colon cancer risk, indicating the importance of maintaining this ratio, either through natural food stuffs or with dietary supplements. Since tumor promotion and progression progresses over many years, it would be useful to adopt these dietary habits early and maintain them on a lifelong basis. Interestingly, dietary supplementation with ω3 fatty acids is more effective in suppressing inflammatory cytokine production in older than younger women [194]. However, since dietary supplements can change circulating and tissue levels of fatty acids over short periods, days to weeks, it seems advisable to change whenever possible. In this regard, it would be helpful to have a single or few easily measured circulating or excreted metabolites that could be used to judge healthy level, balance, and inflammatory status, demonstrated to be predictive of reduced risk of CRC. CRP is an easily measured component of inflammation that has been considered and rejected as a diagnostic and prognostic marker for

CRC, since it is too nonspecific and is reflective of a broad range of disorders encompassing inflammation and tissue damage [195]. Nonetheless, the criteria for such marker(s) have been described [195], and this remains an ongoing goal.

The demonstration that many of the effects of fatty acids are mediated through inflammatory pathways, particularly through COX-2, provides the basis for the use of NSAIDs for prevention of colon cancer. This approach and the associated cardiovascular risk factors that compromise their use have been detailed in previous reviews [56–58, 60–62]. However, the success of NSAIDs in preventing CRC provides proof of principle and points to the need for better understanding of the pathways connecting fatty acids to inflammation as a focus for developing innovative cancer prevention strategies [196]. For example, studies showing that fatty acid-mediated effects are controlled by G protein receptors [197, 198] and fatty acid-binding proteins [199, 200] that regulate cellular uptake suggest these molecules as important targets for preventing CRC. As indicated above, NAT2 gene genetic variations [175] contribute significantly to interindividual variations in fatty acid-induced synthesis and secretions of cytokines. These genetic polymorphisms may govern pro- and anti-inflammatory responses to fatty acids and may serve as the basis for focused neutriceutical-based interventions [201].

TLRs, proteins that act as LPS and nutrient-sensing regulatory proteins, activate T cell inflammation proteins, including NF-κB. Fatty acids may influence this inflammatory pathway by direct effect on TLRs as well as by altering intestinal microbiota so as to alter synthesis and availability of LPS interacting with TLR [202].

5.8.2 Therapy

While ω3 PUFAs have generally been used to prevent colon cancer, they may also have beneficial effects as adjunctive neutriceutical approaches to enhance more traditional therapeutic interventions with chemo- or radiation therapy [203]. Since the ω3 PUFAs reduce levels of PGE_2 and inflammatory factors, they may be expected to reduce colon cancer growth, increase apoptosis, and reduce tumor angiogenesis. In addition, ω3 PUFAs have been shown to potentiate cytotoxicity of chemotherapeutic agents in multiple human colon cancer cell lines in tissue culture and in tumor-bearing animals [204–206]. Interestingly, n-3 PUFAs have been shown to reduce cachexia in mice with colon adenocarcinoma [206]. In addition, pretreatment with ω-3 PUFAs decreases cancer cell survival following both UV and X irradiation [207]. Clearly, clinical trials of n-3 PUFA supplementation of conventional chemoradiation therapy are warranted for CRC.

5.9 Cholesterol Metabolism and Statin Therapy

To this point, we have focused on the fatty acid components of dietary fats and their contribution to CRC. Cholesterol, a polycyclic steroid molecule, is another important component of dietary fat, derived from animal sources. Originating from both

exogenous, dietary sources, and endogenous synthesis, overall cholesterol levels have long been a public health concern, especially from a cardiovascular viewpoint [208]. Cholesterol synthesis can be interfered with by a group of agents collectively identified as statins, which are competitive inhibitors of the enzyme 3-hydroxyl-3-methylglutaryl coenzyme A reductase (HMGCoA reductase), a key enzyme in the cholesterol synthesis pathway. A recent study, assessing the effect of statin use over a 12-year period (1995–2007), in the entire population of Denmark, showed a significant decrease among statin users in overall cancer-related mortality [209]. An earlier population-based case–control study in Israel showed statin use to be associated with a significant reduction in CRC [210]. Other population-based studies and meta-analysis have found divergent results, with those showing protective results being associated with longer statin use (≥4 years) [211] whereas those with shorter statin use have tended to not show protective effects [212].

By inhibiting HMGCoA reductase, statins block synthesis of mevalonic acid which serves as a precursor for farnesyl pyrophosphate, the immediate precursor for cholesterol. Farnesyl pyrophosphate is also the precursor for geranylgeranyl pyrophosphate which is required for protein prenylation and activation of proteins including Ras, Rho, and nuclear lamins [213]. Thus, by inhibition of farnesylation, statins could interfere with Ras activation and colon cancer growth. Interestingly, in addition to their effect on cholesterol metabolism, statins have been shown to have anti-inflammatory effects preventing activation of NF-κB; lowering TNF-α, CRP, IL-1β, and IL-6; and acting synergistically with NSAIDs [214–219]. Further research is required to define the role of and potential use of statins in CRC prevention and therapy.

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Chapter 6
Inflammation, Obesity, Barrett's Esophagus, and Esophageal Adenocarcinoma

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Abstract Esophageal adenocarcinoma arising in metaplastic Barrett's esophagus is one of the most rapidly increasing cancers in Western countries. Accumulating epidemiological evidence provides support that both chronic reflux injury and being overweight are strongly associated with the risk of esophageal adenocarcinoma. It is proposed that being overweight could contribute to increased predisposition to reflux by mechanically disrupting the physiological mechanisms that prevent reflux injury to the esophagus. Furthermore, mechanistic investigations also provide a link between being overweight to the risk of esophageal adenocarcinoma through increased loco-systemic injury response and metabolic syndrome. Together these observations provide the basis for the hypothesis that being overweight could be a key early trigger for the initiation and an ongoing stimulus for the progression of esophageal adenocarcinoma. In this chapter we will summarize the existing data that supports this hypothesis and discuss ongoing and future investigations to address this hypothesis that links obesity to risk of esophageal adenocarcinoma.

6.1 Introduction, Obesity, and Esophageal Adenocarcinoma: Guilt by Association

The anthropometry of population of Western countries, particularly in the United States, is rapidly changing with an alarming increase in the incidence of obesity or being overweight. Of late there has been a sharp increase in the prevalence of obesity, almost to epidemic proportions, and the rate of increase in esophageal adenocarcinoma has essentially mirrored this rising rate of obesity [1, 2]. Several recent studies found close associations between the risk of esophageal adenocarcinoma

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and being overweight [3–7]. However, the conclusion drawn by these studies does differ depending upon how the obesity is defined or characterized. Interestingly, a study examining the relationship between obesity and Barrett's esophagus, a precursor of esophageal adenocarcinoma, noted that when BMI is used as a continuous variable and adjusted for central adiposity, the BMI did not appear to be associated with increased risk of Barrett's esophagus [8]. According to WHO definition, obesity is defined as BMI of 30 or more but this does not take into account variation in body fat distribution [9]. Several parameters like waist circumference, waist to hip ratio, or imaging techniques like CT and DEXA have been used to assess the body fat distribution [10]. Male preponderance of central obesity as well as esophageal adenocarcinoma suggests that the association between being obese and esophageal adenocarcinoma could be through central adiposity [11, 12]. Indeed, several studies have found stronger relationships between abdominal obesity and esophageal disorders like gastroesophageal reflux disease (GERD) and Barrett's esophagus [3, 4, 10, 13]. Further studies examined and suggested that instead of BMI, there exists a closer association between the risk of esophageal adenocarcinoma and how the fat is distributed in the body [4, 14]. Patients with truncal accumulation of fat, as determined by hip to waist ratio, are more likely to develop esophageal adenocarcinoma [4]. Recent, carefully undertaken studies using CT of the abdomen to examine the relative contribution of visceral vs. subcutaneous fat suggest that the excess visceral fat, compared to subcutaneous fat, more likely predict the risk of esophageal adenocarcinoma [3]. In summary, it appears that being overweight is one of the important pathogenic processes that predispose patients to the development of esophageal adenocarcinoma. The development of esophageal adenocarcinoma is a long protracted process, and its risk can be influenced at several steps. Being obese could facilitate the degree of reflux or the consequence of the reflux by altering local or systemic inflammatory response that could facilitate the development of premalignant Barrett's esophagus. Finally, being overweight could also activate prooncogenic pathways or interfere with tumor-suppressive mechanisms in Barrett's mucosa. In the following paragraphs we will discuss these issues and the putative mechanisms.

6.2 Obesity and Degree of Reflux: Disruption of Mechanical Barrier

Long-standing reflux injury is associated with replacement of normal squamous mucosa of the esophagus by premalignant metaplastic Barrett's columnar mucosa that, in a subset of patients, progress to esophageal adenocarcinoma. It has been postulated that the pressure effect of abdominal obesity could change the relationship between the gastroesophageal junction and diaphragmatic anti-reflux mechanisms. These changes could decrease in LES (lower esophageal sphincter) pressure to below 10 mmHg from the usual 10–35 mmHg that leads to increase in reflux, eventually causing Barrett's esophagus and esophageal adenocarcinoma [10, 15].

This possibility along with the findings that obese patients are known to have an increased incidence of asymptomatic reflux might explain how the neoplastic progression can go undetected in obese patients [16]. Several studies have demonstrated increased association between obesity and drop in LES pressure and increased incidence of hiatal hernia eventually leading to more reflux. Kuper et al. conducted manometric studies on 47 obese patients without reflux symptoms and 15 normal-weight individuals and found that obesity is associated with a significant drop in LES pressure as well as disruption of normal esophageal motility [17]. Similarly, in a large study conducted by Ayazi et al., where 24-h pH monitoring as well as esophageal manometry was performed, obesity was correlated with low esophageal pH and lower LES pressure. This suggests that obesity likely increase the reflux of acidic gastroduodenal contents by altering LES relaxation leading to a drop in LES pressure [18]. Schneider et al. did a manometric and pH monitoring study in patients where they had groups with obesity, GERD without obesity, diffuse esophageal spasm, and normal controls and found that obese patients and GERD patients without obesity showed significantly increased transient LES relaxation [19]. In a novel manometric study Pandolfino et al. found that obesity is positively correlated with the disruption of esophageal gastric junction and higher value of gastroesophageal pressure gradient. This study supports the mechanical basis of increased reflux and hiatal hernia in patients with obesity [20]. Therefore it appears that augmented relaxation of the LES is one of the underlying mechanisms for reflux in the obese. Together these findings support the idea that by increasing the degree of reflux, truncal obesity could play a role in the development of esophageal adenocarcinoma. In addition, being obese could also make the esophageal mucosa more vulnerable to injury as discussed below.

6.3 Obesity and Altered Composition of Reflux: Facilitating Injury and Carcinogenesis

Contents of reflux, particularly bile acids in a pH-dependent manner inflict mucosal injury as well as facilitate neoplastic progression in Barrett's esophagus. Bile acids are derived from hepatic metabolism of cholesterol. Dietary and systemic derangements that are associated with being obese could potentially modify composition and the amount of bile salts in reflux. In a systematic review McQuaid et al. found that obese patients with GERD had higher concentration of bile acid in their esophageal aspirate [21]. Development of Barrett's esophagus is considered a protective mechanism against reflux injury as intestinal columnar cells are more resistant to toxic insults compared to squamous cells. Continued injury leads to release of increased concentration of pro-inflammatory cytokines, prostaglandins, reactive oxygen species (ROS) which through various signaling pathways induce cell proliferation and mutagenesis and inhibit apoptosis that likely facilitate the neoplastic transformation [22]. In various in vivo/in vitro studies it is noted that bile acids stimulate metaplastic and squamous esophageal cells to

produce pro-carcinogenic inflammatory mediators like IL-8/COX-2 and cause oxidative stress and DNA damage [43]. Obesity-associated changes in bile acid composition and subsequent generation of free radicals have been advocated to play an important role both in perpetuation of inflammation as well as induction of various signaling pathways which eventually promote growth, angiogenesis, and tumor development. These free radicals include ROS like superoxide anion, hydrogen peroxide, and hydroxyl radical and reactive nitrogen species like peroxynitrite [22]. In experimental studies, bile acid-induced intracellular ROS mediate proliferation, DNA damage, and apoptosis through activation of tyrosine kinase, ERK1/2, and AKT as well as through protein kinase C signaling pathways [23, 24]. Song et al. in a study on Barrett's and EAC cell lines found that unconjugated bile acids induced COX-2 through ROS-mediated activation of PI3K/AKT and ERK1/2 and their downstream effectors CREB and AP-1 to facilitate neoplastic transformation [45]. These experiments were replicated in vivo in rats where it was found that bile acid-associated COX-2, p-AKT, and p-CREB overexpression induced metaplastic and neoplastic changes in the esophagus [45]. In an in vitro study performed by our group on Barrett's cell lines, we found that COX-2 inhibition significantly decreases proliferation of Barrett's esophageal cells, whereas treatment of these cells with PGE2 (product of COX-2 activity) restored proliferation of the cell lines, suggesting a possible role of COX-2 inhibitors for chemoprevention in Barrett's esophagus [25]. We further tested the role of COX-2 inhibitors in an animal model, where reflux injury was induced by performing esophagojejunostomy, and the animals were then randomized to groups receiving a COX-2 inhibitor vs. placebo [26]. After 28 weeks it was found that COX-2 inhibition significantly decreased the degree of inflammation and PGE2 biosynthesis with a parallel decline in the risk of development of esophageal cancer giving direct evidence for the chemopreventive role of anti-inflammatory agents in Barrett's esophagus [26]. Based on these findings and epidemiological evidence supporting the role of anti-inflammatory agents with reduced risk of esophageal adenocarcinoma, we conducted a multicenter double blind, randomized, placebo-controlled, phase 2 clinical trial where 114 patients with Barrett's esophagus were randomized in three groups [27]. Patients received 40 mg esmoprazole twice daily with either placebo or low-dose aspirin (81 mg) or 325 mg aspirin daily for 28 days, and esophageal endoscopic biopsy was performed pre- and post-intervention to measure PGE2 levels as a biochemical surrogate marker of neoplastic progression [27]. We noted that the higher dose of aspirin in combination with esmoprazole significantly reduced tissue concentrations of PGE2 while low-dose aspirin and placebo did not have a significant effect [27]. No effect with low-dose aspirin might be due to small sample size and a short trial period [27]. Further trials will be needed to establish the role of aspirin and other NSAIDS for chemoprevention of Barrett's esophagus. Epigenetic changes like DNA methylation and histone modifications also occur secondary to pro-inflammatory signals that obesity induces and could facilitate neoplastic transformation [52]. In summary, altered bile acid composition in response to being obese appears to be an attractive hypothesis; however, this premise needs further investigation.

6.4 Obesity and Mucosal Response to Injury: Role of Altered Systemic and Local Cytokine Levels

Inflammation plays a very important role in mounting an immune response against various environmental triggers and when such triggers persists, such as reflux injury, a state of chronic inflammation develops [23]. Virchow proposed the association of inflammation with cancer in 1850s as cancer being a local inflammation resulting from humoral stasis and injury [28]. Inflammation has been linked to various cancers including breast cancer, endometrial cancer, colon cancer, gastric cancer, and esophageal cancer [29]. Inflammation is increasingly being recognized as an important link between obesity, insulin resistance, tissue injury, and carcinogenesis [10].

Two important aspects of esophageal inflammation include the injury-inducing reflux and mucosal response to this injury. Obesity results in systemic and local pro-inflammatory state where adipocytes release high circulating concentrations of inflammatory cytokines like TNF-α, IL6, IL1B, IL10, CRP, etc. [30]. These cytokines create a pro-inflammatory state that has been proposed as an important connecting link between obesity and various cancers including esophageal adenocarcinoma [30]. Several other pro-inflammatory factors like interferon-γ, monocyte chemotactic protein (MCP-1), PAI-1, and fibrinogen have also been found to be associated with adiposity [31]. In a translational study on patients with esophageal adenocarcinoma, Lysaght et al. found higher concentrations of activated inflammatory cells particularly CD8+ cells in the visceral adipose tissue compared to the subcutaneous adipose tissue [32]. Specifically, IFN-γ was the dominant cytokine produced by the activated T cells resulting in upregulation of adipose tissue inflammation and TNF-α production [32]. It is proposed that systemic increase as well as increased esophageal stromal TNF-α promotes esophageal inflammation [32]. We have shown that neutralizing antibodies against TNF-α in the setting of ongoing reflux injury nearly completely mitigate esophageal inflammation [33]. We induced reflux in Sprague–Dawley rats ($n=25$) and randomized animals at 2 months receive to either specific rat anti-TNF-α Ab (1× week, intraperitoneal, monoclonal mouse IgG2a,K Ab 15 mg/kg) or PBS control injection for 8 weeks (time to develop Barrett's). We found that the inhibition of TNF-α with anti-TNF-α Ab for 2 months in rats with chronic reflux significantly reduced PGE2 ($4{,}907 \pm 1{,}200$ vs. 116 ± 18 pg/mg, $p<0.001$) and cPLA2α (0.87 ± 0.2 vs. 0.32 ± 0.02 OD450 $p=0.02$) in Barrett's mucosa compared to controls. The histology showed marked reduction in stromal response to injury. Commonly seen squamous hyperplasia and hyperkeratinization was also absent in anti-TNF-α-treated rats. We further performed in vitro assays that showed that compared to control, exogenous TNF-α treatment increased the number of viable cells by 20 % ($p<0.05$) in primary Barrett's cells. These findings suggest a chemopreventive role of anti-TNF-α Ab on esophageal carcinogenesis. We also show that TNF-α increases prostaglandin biosynthesis in Barrett's mucosa, which increases cell growth and facilitate development of esophageal adenocarcinoma [24].

Emerging evidence supports the hypothesis that TNF-α switches stromal macrophage phenotype to modulate inflammation in target tissues including esophagus. These observations along with increasing expression of both the ligand and the receptor of TNF-α during carcinogenesis in Barrett's esophagus [34] and an increased expression of TNF-α in adipose tissue of obese rats compared to controls [35] suggest that in overweight patients, TNF-α could be one of the mechanistic links where adipocytes in a paracrine manner influence systemic and local inflammatory response and the facilitation of neoplastic transformation. The repertoire of pro-inflammatory cytokines that link obesity-inflammation-carcinogenesis is rapidly expanding. Dvorakova et al. did the tissue analysis of Barrett's esophagus, duodenum, and squamous epithelium distant from metaplastic area and found an increased concentration of IL-6 in metaplastic tissue [36]. Chronic inflammation creates and maintains a tumor microenvironment where there is migration and relative abundance of immune inflammatory cells like neutrophils, dendritic cells, macrophages, and lymphocytes [52]. It has been found that compared to reflux esophagitis, there is a higher concentration of Th1 cells (macrophages and CD8+ T cells) and Th2 cells (plasma cells and mast cells) in the Barrett's mucosa further suggesting the role of inflammation in metaplastic change in the esophagus [37]. Similarly, studies have established higher concentration of cytokines like TNF-α, IL-6, IL1B, IL-4, IL-8, and IL-10 in the Barrett's tissue as well as in esophageal adenocarcinoma [34, 38, 54]. Obesity-associated pro-inflammatory mediators like TNF-α and IL-1 are also known to upregulate inducible nitric oxide synthase (iNOS) leading to increased production of nitric oxide that is typically seen during inflammation and carcinogenesis in Barrett's esophagus [38]. The resulting pro-inflammatory state disrupts the balance between oxidant and antioxidant pathways, and levels of antioxidants are decreased [52]. Congruent with this, biopsy specimens from Barrett's esophagus and esophageal adenocarcinoma have shown low levels of antioxidant enzymes like glutathione S-transferase and glutathione peroxidase. This altered oxidative tissue state favors mutagenesis to promote neoplasia [39], and it is further compounded by impaired anti-oxidative defense in obese patients. There is relative deficiency of antioxidant micronutrients like vitamin C, β carotene, and lycopene in obese patients [40]. Interestingly, consumption of vegetables and fruits rich in natural antioxidant is associated with decreased risk of esophageal cancer [41].

The pro-inflammatory state, due to altered cytokine profile, along with several hormonal mediators as discussed below, together constitute a state named as metabolic syndrome [30], which provide multifaceted link between obesity and cancer progression. Therefore, being obese not only disrupts mechanical barriers that prevent reflux but also makes reflux more pro-inflammatory and oncogenic.

6.5 Obesity and Pro-neoplastic Signaling: Role of Hormonal Mediators in Growth Deregulation

Being obese is a clinical condition of endocrine derangement. Therefore, various hormonal mediators have been studied as possible links between obesity and esophageal disorders. Of particular note are insulin resistance, IGF1, leptin, and adipokines.

As discussed earlier, obesity-induced metabolic state is associated with increased circulating pro-inflammatory cytokines, many of which lead to an insulin resistance state [42]. Specifically, cytokines like TNF alpha, IL6, and IL1B released by adipocytes are thought to contribute to insulin resistance [43]. The IGF-1 axis consists of two ligands, IGF-1 and IGF-2; three cell-membrane receptors, IGF-1R, IGF-2R, and insulin receptor; and six IGF-binding proteins IGFBP-1 to IGFBP-6 [44]. Obesity and metabolic syndrome leads to deregulation of the IGF-1 axis, and it has a key role in cell proliferation, apoptosis, and tumor cell differentiation [45]. Deregulation of the IGF-1 axis leads to a decreased level of IGF-binding proteins and hence a high concentration of free IGF-1, insulin resistance, and eventually hyperinsulinemia [44, 45]. High IGF-1 concentration and overexpression of IGF-1R have been implicated in the transformation of Barrett's metaplasia to esophageal adenocarcinoma [45]. Binding of IGF-1 and IGF-2 to IGF-1R through various signaling pathways leads to cell proliferation and arrest of apoptosis [46]. High insulin level associated with insulin resistance also leads to cancer as insulin has growth-promoting effect, though it is argued that insulin probably mediates its action through IGF-1 receptors as well as insulin receptors. Binding of IGF-1 and insulin to their receptors lead to activation of extracellular-signal-regulated kinase (ERK) and phosphatidylinositol-3 kinase (PI-3K) pathways which in turn result in cell proliferation and arrest of apoptosis [46]. Doyle et al. in a study on esophageal cancer cell lines and tissue from esophageal cancer patients found relative upregulation of IGF-1 levels in esophageal adenocarcinoma compared to squamous cell carcinoma of esophagus [11]. The study also established linear relationship between visceral adiposity and IGF-1 levels; however, relationship with BMI alone was not linear [11]. Similarly in an animal study, Wu et al. found that diet-induced obese mice had increase in local tumor growth as well as metastasis whereas IGF-1-deficient mice did not have increased tumorigenicity [47]. Ounis et al. in their study on the effect of exercise training and diet restriction found that resultant weight loss was also associated with decrease in the level of IGF-1 and inflammatory markers [48].

Obesity and metabolic syndrome also lead to downregulation of adipokines like adiponectin and ghrelin and increased leptin, together these changes result in an increased inflammatory response and abnormal cell survival [46, 49, 50]. Leptin is a hormone controlled by the weight-regulating gene (ob gene) and is increased in obese patients [51]. Leptin is primarily secreted from fat cells and plays an important role in inhibiting weight gain and appetite through action on the hypothalamus [52]. Ogunwobi et al. studied the effect of leptin on Barrett's derived esophageal adenocarcinoma cell lines. They found that leptin stimulates the proliferation of esophageal adenocarcinoma cells and inhibits their apoptosis, which involves COX-2-dependent PGE2-mediated activation of EGFR and activation of c-Jun NH2-terminal kinase [53]. This was further supported by the experiment that leptin deficiency causes extreme obesity in rodents [54]. Several retrospective studies and clinical trials show that leptin levels decrease in response to weight loss achieved by either exercise and/or caloric restriction whereas they increase in response to high-caloric diet [55–59]. Insulin has a positive feedback action on leptin gene expression (whereas IGF-1 has negative control) and hence, in well-fed condition, insulin stimulates leptin release which suppress appetite [60]. The fact that visceral obesity

in patients is associated with very high levels of leptin suggests resistance to its action. Leptins have been found to be mitogenic for various tissues [47]. Somasundar et al. in their study on Barrett's associated esophageal adenocarcinoma cell lines found that exogenous leptin significantly increased cell growth in cancer cell lines [51]. They also found that mitogenic effect of leptin does not affect apoptotic pathways which is in contrast to earlier study by Beales and Ogunwobi where it was found that leptin has synergistic action with acid in suppressing apoptosis and activity of caspase 3 [61].

Adiponectins and resistins are adipokines almost exclusively secreted by mature adipocytes [31]. Adiponectins through their receptors AdipoR1 and AdipoR2 regulate various metabolic processes [62]. Epidemiological studies have also found that adiponectin levels are increased after weight loss in patients either through exercise, caloric restriction, or surgical intervention, and its level is decreased in obese patients [63–66]. Certain dietary modifications without caloric restriction, like the use of fish oil, have also been found to increase the level of adiponectin which indicates these may prove to be anti-inflammatory and carcino-protective [67]. They regulate lipid and glucose metabolism by increasing tissue sensitivity to insulin [62]. Adiponectins also suppress production and action of TNF alpha and are considered anti-inflammatory [68]. By virtue of their anti-proliferative, pro-apoptotic, and anti-angiogenic action, adiponectins are considered protective against malignancies [31, 62]. Studies have suggested that obesity downregulates adiponectin levels, and low adiponectin levels are associated with esophageal cancers [49, 50]. Moreover, higher levels of adiponectins have been associated with low incidence of Barrett's esophagus. The mechanism of cancer protective role of adiponectin has been complex and unclear. However it has been proposed that adiponectins stimulate apoptosis through induction of p53, Bax expression, and suppression of Bcl-2 [69, 70]. Adiponectins also suppress Toll-like receptor-induced activation of nuclear factor κB (NF-κB) and prevent interaction of various growth factors [31]. Adiponectins also exert their tumor-inhibiting action by inactivation of MAPK kinase 1 and 3 and ERK 1 and 2 and concomitant reduced glucose uptake [70]. Anti-angiogenic action of adiponectins occurs through activation of apoptosis in vascular endothelial cells and inhibition of cell migration [31]. Resistins and RELMs (resistin-like molecules) are adipokines which are secreted from adipocytes as well as non-adipocyte sources and are believed to have a role in carcinogenesis of esophageal adenocarcinoma, but the mechanism is unclear [62]. Leptin and adiponectin receptors have also been correlated with esophageal adenocarcinoma, and it was found that upregulation of ObR (leptin receptor) was associated with advanced disease and more aggressive tumor in gastric cancer [71]. Adiponectin receptors, ADIPOR1 and ADIPOR2, were downregulated in Barrett's mucosa compared to normal esophagus [69]. However receptor levels did not correlate significantly with the serum concentrations of their ligands suggesting the complexity of molecular mechanisms involved as well as a possibility that the these adipokines might have paracrine action too [72].

Metabolism and inflammatory pathways converge on many signaling pathways which are important for homeostasis and body functions like immunity. For example, Toll-like receptors which recognize lipoproteins and lipopolysaccharides in

bacterial wall also recognize nutrients like fatty acids [23]. Similarly hormones involved in metabolic pathways like leptins and adiponectins have a role in immunity [23]. Prolonged nutritional imbalances or chronic inflammation can disrupt the equilibrium between metabolism, immunity, and inflammation and lead to metabolic syndrome, obesity, and multiple cancers. The role of nuclear factor kappa B signaling pathway and JNK mitogen-activated protein kinase pathway (JNK MAPK) needs special mention as it has a central role in linking obesity to inflammation, insulin resistance, and also in oncogenesis and cell proliferation [73]. Various metabolic stressors like lipids, oxidative stress, and cytokines activate IKK/NF-κB signaling pathway and JNK MAPK pathway. IKKβ-mediated activation of NF-κB leads to macrophage recruitment, activation, and differentiation which maintains pro-inflammatory status. It also leads to insulin resistance by phosphorylation of insulin receptors. Its activation is also believed to disrupt the central leptin pathway leading to overnutrition and weight gain. It is important to note that activation of IKKβ/NF-κB creates a vicious cycle where inflammation and imbalance in nutrition lead to further inflammation and overnutrition.

Various epidemiological studies have also found that obesity is associated with pro-inflammatory state and weight loss leads to decrease in pro-inflammatory markers. In a recent randomized controlled trial on the effect of caloric restriction diet/exercise on inflammatory biomarkers in obese postmenopausal women, Imayama et al. found that caloric restriction diet and/or exercise led to decrease in hsCRP (high-sensitive C-reactive protein) as well as anthropometric parameters [74]. Cintra et al. also found that CRP level decreased in obese patients after post-bariatric abdominoplasty though the weight remained stable, indicating that diet might have a direct role in causing inflammation [75]. In a longitudinal evaluation of effect of weight loss on cancer-related biomarkers, Linkov et al. found that weight loss leads to decreased level of E-selectin and IL-6 and increased level of GH, adiponectin, and IGFBP-1 [63]. Similarly Ackermann et al. in their study on relationship between metabolic syndrome and inflammatory biomarkers found that waist circumference is positively correlated with inflammatory markers like TNF-α and IL-6 and negatively correlated with anti-inflammatory adiponectin [64]. In another study on obese/overweight breast cancer survivors, it was found that a weight loss intervention led to a fall in the levels of IL-6 and TNF-α [76].

To conclude, obesity in general, and central obesity in particular, leads to enhanced loco-regional inflammatory response in the lower esophagus by both reflux-dependent as well as reflux-independent mechanisms eventually causing Barrett's esophagus and esophageal adenocarcinoma. Adipocytes alter the circulating levels of various cytokines, growth factors, hormonal factors creating a pro-inflammatory state, and increased risk of esophageal adenocarcinoma. Study of various molecular pathways associating obesity and reflux-induced inflammatory response to the neoplastic changes in the lower esophagus is ongoing research which will likely uncover useful targets for screening and therapeutic intervention.

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Chapter 7
Inflammation, Obesity, and Colon Cancer

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Abstract The general focus of this chapter is to overview the contribution of obesity-induced intestinal inflammation to colorectal cancer (CRC) incidence. Using inflammatory bowel disease as a model, the mechanisms by which gastrointestinal microbes and obesity-associated, adipose-derived mediators of inflammatory increase CRC risk will be discussed. Particular emphasis will be placed on the direct impact of these factors on intestinal epithelial cell proliferation, survival, and neoplastic transformation. Additionally, the influence of inflammatory factors on immune cell function and the indirect effect of altered immunity on intestinal epithelial cell proliferation and survival, and CRC risk will be explored.

7.1 Background

Colorectal cancer (CRC) is the third most commonly diagnosed malignancy in both men and women in the United States and is the second leading cause of cancer-related deaths. In the United States alone, 141,380 colon cancer (71,850 in men, 69,360 in women) cases were diagnosed in 2011. The National Cancer Institute estimates that there will be 51,690 CRC-related deaths in 2012, which is 5 % higher than the estimated deaths in 2011 [1, 2]. The lifetime risk for

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individuals to develop colon cancer is approximately 6 %, but the risk increases to 18 % among individuals who have a first-degree relative (parent, sibling, or child) with CRC [1, 3]. Most CRCs begin as foci of intestinal or colonic epithelial cells (IECs) that have accumulated mutations in oncogenes (aberrant crypt foci) [4, 5]. These cells have the capacity to transform into benign adenomas (polyps) with additional mutations or in response to environmental queues [4]. If left untreated, adenomas progress to more invasive and malignant carcinomas [6]. Many factors increase CRC risk, including genetics, diabetes, preexisting adenomas, obesity, diet, microbial profile, and inflammatory diseases [4, 7, 8]. Understanding how these factors influence tumor development will lead to new diagnostic screens, intervention paradigms, and treatment options that may reduce CRC-related deaths.

In this chapter, we will discuss the contribution of two interconnected CRC risk factors, inflammation and obesity, to cancer incidence. We will discuss the contribution of intestinal microbes to chronic inflammation in inflammatory bowel diseases (IBDs) and obesity and their contributions to increased CRC risk. Additionally, we will highlight the inflammatory mediators and mechanisms through which IBD promotes CRC risk and discuss how obesity-associated systemic inflammation similarly increases CRC susceptibility. A model will be proposed whereby obesity promotes a proinflammatory intestinal milieu that directly induces the neoplastic transformation of IECs and indirectly promotes tumor progression through activation and recruitment of immune cells.

7.2 Genetic Risk Factors for CRC

7.2.1 Hereditary Forms of Colon Cancer

It is estimated that about 15 % of colon cancer cases can be attributed to specific inherited gene mutations [9]. Genetic mutations have been identified as the cause of inherited cancer risk in some colon cancer-prone families, and these mutations are estimated to account for only 5–6 % of colon cases overall [10]. It is likely that other undiscovered genes and background genetic factors contribute to the development of familial colon cancer in conjunction with nongenetic and environmental risk factors such as diet [11]. One of several well-described hereditary forms of colon cancer is familial adenomatous polyposis (FAP), which is characterized by the presence of hundreds, sometimes thousands of benign polyps in the colon, rectum, or lower regions of the small intestine [12]. The polyps usually begin to form at puberty, and colon cancer almost always develops later in life. FAP is inherited as an autosomal-dominant trait caused by a mutation in the tumor suppressor, adenomatous polyposis coli (*APC*) [13, 14]. Individuals with FAP have a 90–100 % lifetime risk of developing colon cancer. Surgery is routinely used to remove the colon in order to prevent the development of cancer.

7.2.2 Sporadic Causes of Colon Cancer

Approximately 75–90 % of colon cancer cases are considered sporadic, with no known family history or hereditary genetic mutation [15]. It is now well established that all cancers result from an accumulation of mutations that enhance a tumor cell's ability to proliferate, evade growth suppressors, avoid immune destruction, replicate indefinitely, induce inflammation, metastasize, induce angiogenesis, accumulate additional mutations, resist cell death, and deregulate cellular energetics [16]. For example, somatic mutations can result in the inactivation of tumor suppressors (i.e., *APC*, *P53*, or *SMAD4*) and activation of oncogenes (i.e., *KRAS*, *BRAF*, *PI3KCA*, or *PTEN*) that drive proliferation and progressive transformation of normal IECs to malignant derivatives or inactivate genes necessary for the maintenance of genome stability (i.e., DNA repair factor *MLH1* or *MSH2*) [17, 18].

7.2.3 APC, Wnt Signaling, and CRC

Mutations in the Wnt signaling pathway are common in CRC patients [19, 20]. The Wnt signaling pathway is crucial for an immense number of biological processes during various stages of development [21, 22]. Activation of canonical Wnt signaling results in increased cytosolic levels of β-catenin, which then localizes to the nucleus to initiate transcription of Wnt target genes important for cellular development [21]. In the absence of Wnt activation, β-catenin is degraded to downregulate expression of genes involved in cell growth and proliferation. A complex of proteins (APC, GSK3β, AXIN, and CK1α) forms a "degradation machine" that functions to bind and tag β-catenin for destruction by the proteasome [21, 22]. Mutations in Wnt pathway proteins increase the levels, stability, and nuclear localization of β-catenin protein causing constitutive transcription of Wnt targets. Alterations in direct target genes like c-Myc (*Myc*), cyclin D1 (*CCND1*), *VEGF*, c-Jun (*JUN*), matrix metalloproteinase 7 (*MMP7*), and claudin-1 (*CLDN1*) have all been shown to induce colon cancer in humans [21, 23–25]. The influence of APC on intestinal tumor development can be modeled in mice with inactivating mutations of *Apc* [26]. In particular, the *Min* mutation of *Apc* (Apc^{Min}), which approximates the most common *APC* mutation in humans, has been useful for characterizing the pathogenesis of CRC [26]. Importantly, in both CRC patients and Apc^{Min} mice, CRC risk factors such as intestinal inflammation and obesity interact with genetic mutations in the Wnt signaling pathway to increase cancer susceptibility.

7.3 Intestinal Inflammation: A CRC Risk Factor

When pathogens invade a host and tissues undergo physical trauma, the immune system reacts with a coordinated response involving immune and nonimmune cell types (the inflammatory response). Cross talk between immune and nonimmune

cells eradicates the invading pathogen and initiates a tissue repair response involving the release of cytokines (immunomodulating factors), growth factors, and extracellular matrix (ECM) remodeling proteins, which promote cell proliferation, differentiation, and migration [27]. In some instances, the immune system elicits an exaggerated and chronic response that fails to be resolved, resulting in cellular hyperplasia, disorganization of the surrounding tissue, and eventually fibrosis [27]. IBDs such as Crohn's disease (CD) and ulcerative colitis (UC) are a group of chronic inflammatory disorders of the intestine [28]. Inflammation in CD patients affects all parts of the gastrointestinal tract but usually afflicts the distal small intestine and colon. UC involves inflammation of the colon.

The prevalence for both CD and UC in the United States is greater than 200 cases per 100,000, with the total number of individuals with IBD approaching 1.5 million [29, 30]. Clinical symptoms typically involve diarrhea, abdominal pain, gastrointestinal bleeding, and weight loss [31]. In addition, development of CRC is a serious long-term complication associated with IBD. Epidemiologic and clinical studies indicate that patients affected by UC and CD have an increased risk of developing colon cancer by as much as 18 % and 3 %, respectively, with risk of CRC significantly higher among patients with long-standing disease [32, 33]. How immune responses in IBD patients alter IEC homeostasis and induce tumorigenesis is an area of intensive investigation.

7.4 Obesity: A CRC Risk Factor

In the past 50 years, the worldwide occurrence of obesity in humans has risen at an alarming rate. The World Health Organization (WHO) generally defines obesity as having a body mass index (BMI) equal to or higher than 30, as calculated by weight divided by height squared (kg/m^2). The United States has one of the highest percentages of obese individuals (BMI $\geq$30), with 33.9 % of the adult population (18 years or over) classified as obese [34, 35]. Shockingly, it has been estimated that in the United States, over one-third of children and young adults (age 6–19) and over 40 million children under the age of 5 are obese [36, 37]. Although genetic factors contribute to obesity, increased energy intake and decreased energy expenditure are the major causes of the disease and probably contribute largely to the rapid increase in obesity cases [38, 39].

Obese individuals have an increased risk for developing heart disease, hypertension, infertility, obstructive sleep apnea, dyslipidemia, nonalcoholic fatty liver disease, type 2 diabetes, and metabolic syndrome (MetS) [40, 41]. There is also a strong link between obesity and many malignancies. Several epidemiological studies have linked obesity to cancers of the esophagus, pancreas, prostate, renal, postmenopausal breast, endometrium, kidney, and colon and rectum [40, 42]. For CRC specifically, every 2.4 unit increase in BMI increases cancer risk by 7 % [43]. Additionally, a recent meta-analysis predicted that for every additional inch in waist circumference, there is a 5 % increase in CRC risk [44]. Therefore, given the increased prevalence of and lifetime exposure to obesity, it is imperative to identify the mechanisms by which obesity

increases CRC risk so that effective treatment and preventative options can be delivered to a growing, at-risk population. Similar to intestinal inflammation in IBD patients, inflammation caused by increased adiposity and consumption of a high-fat diet is likely to contribute to increased CRC susceptibility in obese individuals.

7.5 The Intestinal Microbiota Contributes to Inflammation and CRC Risk

7.5.1 The Microbiota and IBD

There are approximately 10^{12} microorganisms in the human gastrointestinal track that belong to greater than 500 different species, which are collectively referred to as the microbiota [45, 46]. In both mice and humans, the majority of the microbiota consists of bacteria belonging to the Firmicutes (gram-positive bacteria) and Bacteriodetes (gram-negative bacteria) phyla [47, 48]. Probacteria (including *Helicobacter* and *Escherichia*) and Actinobacteria are also significant contributors to the gut microflora [31]. The symbiotic relationship between humans and enteric microorganisms is essential to important processes such as digestion, regeneration of the IEC barrier, and immune responses [49, 50]. While the microbiota provides beneficial protective, trophic, nutritional, and metabolic signals for the host, it may become a risk factor for disease depending on context and host susceptibility [50–52].

What were once thought to be diseases involving chronic immune response targeting self-antigens (autoimmunity), IBDs, specifically CD, are now considered to be caused by a loss of tolerance to commensal organisms and enhanced immune response to bacterial antigens [53]. In almost all rodent models of IBD, treatment with antibiotics or elimination of the intestinal microbiota (germfree rodents) significantly alleviates disease pathology [54, 55]. Similarly, in human CD patients, antibiotics and diversion of the fecal stream from the distal colon into an ileostomy facilitate disease remission [56–58]. Therefore, abnormal interactions between bowel constituents, specifically intestinal microbes, and the mucosal immune system appear to initiate CD pathology.

What underlying pathologies lead to the aberrant immune response to the commensal microbes of the gastrointestinal system? One possibility may be alterations to the makeup of the microbiota itself and alterations to intestinal physiology by specific bacterial species. In colon biopsies from CD patients, an increase in Bacteroidetes and Proteobacteria has been observed [59]. However, analysis of the microbial population in the intestines of CD, UC, and non-IBD controls demonstrated that specific flora is not enriched in the small bowel or colon of IBD patients, suggesting that there is not one specific change to the microbiota that contributes to IBD pathology in all patients [60]. Interestingly, a subset of IBD patients do have depletions in certain commensal bacteria, notably members of the phyla Firmicutes and Bacteroidetes, suggesting that, at least in some instances, particular alterations

to the intestinal microflora influences disease progression [31]. Metabolic by-products that are generated from gut bacteria can have beneficial or detrimental effects on host diseases. For example, butyrate is a fermentation product of anaerobic bacteria (Clostridium strains) and has been shown to increase apoptosis, decrease proliferation, and have anti-inflammatory properties in IECs and immune cells [61, 62]. IBD patients have deficiencies in many strains of butyrate-producing Clostridium, such as *Faecalibacterium prausnitzii*, and have impaired butyrate metabolism in the colonic mucosa [63, 64]. Oral and intraperitoneal administration of *F. prausnitzii* in a mouse model of IBD reduced colitis and mortality, respectively, and increased anti-inflammatory cytokines such as IL-10 [63]. Importantly, immune responses and inflammation induced by non-commensal invasive species of *Escherichia coli* and enterotoxigenic subclasses of *Bacteroides fragilis* also enhanced inflammation and disease pathology in IBD [65, 66].

Although evidence suggesting that specific commensal microbes trigger abnormal immune responses in IBD patients is somewhat limited, there is a significant amount of experimental evidence demonstrating that altered sensitivity to the microbiota contributes to IBD. The lamina propria is a thin layer of tissue that lies beneath the intestinal epithelium [67]. Mononuclear immune cells, including macrophages and dendritic cells (antigen-presenting cells or APCs) and T cells, within the lamina propria, which are collectively referred to as LPMNCs, act as a first line of defense against pathogens that penetrate through the epithelial barrier following infection or tissue trauma. Importantly, due to their physical separation from intestinal contents by the epithelial barrier, immune cells normally do not come into contact with or react to commensal microbes. Additionally, under normal physiological conditions, IECs express low levels of toll-like receptors (TLRs), which recognize bacterial antigens to induce proinflammatory responses (e.g., gram-negative lipopolysaccharide [LPS] induction of TLR 4 signaling) [68]. However, when the intestinal epithelium is damaged, microbes can transit through the IEC barrier and come into contact with LPMNCs. Through TLR-mediated activation of APCs, phagocytosis and antigen presentation by APCs, and ultimately T cell activation, a proinflammatory response is induced that promotes wound healing [5, 49, 69]. Once the damage is repaired, the immune response normally subsides.

Importantly, in IBD patients, both genetic and environmental factors increased IEC barrier permeability, which causes chronic inflammation [70–73]. This barrier defect allows for continuous mixing of luminal contents with LPMNCs and chronic proinflammatory immune responses [74]. Interestingly, several of the proinflammatory cytokines, including tumor necrosis factor alpha (TNF-α), interferon gamma (INF-γ), interleukin (IL)-6, IL-1β, and IL-13, that are expressed in the intestines of IBD patients also induce increased barrier permeability by activating nuclear factor kappa B (NF-κB)-mediated transcription and altering the expression of tight junction proteins in IECs (Fig. 7.2) [74–77]. Thus, there is a self-amplifying cycle of barrier dysfunction, immune cell activation, cytokine release, and additional IEC barrier permeability [74]. Adding to this defect, IEC expression of TLR4 is elevated in IBD patients, allowing for LPS stimulation of NF-κB-mediated transcription and alterations in tight junction protein expression (Fig. 7.2) [68, 69, 78, 79].

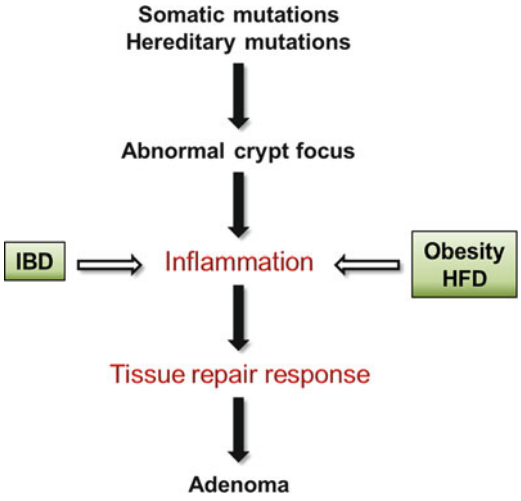

Fig. 7.1 Model for the role of intestinal inflammation in colorectal cancer progression. Colorectal cancer (CRC) susceptibility is induced by the acquisition of genetic mutations (e.g., *APC* mutations) that predispose IECs to neoplastic transformation. Preneoplastic IECs form aberrant crypt foci. Barrier defects caused by the aberrant crypt foci allow luminal contents to cross the epithelial barrier and commensal bacteria to interact with immune cells in the lamina propria to induce inflammation. Inflammation induces tissue repair responses (e.g., proliferation and migration) in IECs that are normally involved in wound healing. In pre-neoplastic IECs, tissue repair responses induced by inflammation interact with genetic predispositions to CRC to induce tumor progression to adenomas. Chronic inflammation induced by inflammatory bowel diseases (IBDs) and obesity/high-fat diets (HFD) causes additional inflammation in the intestine to further increase cancer susceptibility

7.5.2 Inflammatory Responses to the Microbiota Increase CRC Risk

Studies of inflammation and intestinal cancer in humans and mice have led to a model that proposes a proinflammatory intestinal environment increases CRC risk by inducing tissue repair responses (Figs. 7.1 and 7.2) [80–82]. In this model, it is hypothesized that accumulation of mutations in IECs and the formation of aberrant crypt foci damages the surrounding intestinal mucosa. The microbiota penetrates the IEC barrier and induces immune cells to release proinflammatory cytokines that elicit tissue repair responses (e.g., proliferation) in IECs [49, 83]. Normally the tissue repair response fixes damage done to the affected area of the epithelium. However, IECs found within the aberrant crypt foci have developed genetic predispositions to tumor formation, which interact with tissue repair responses to promote the progression of precancerous lesions to adenomas, adenoma growth, and additional tissue damage and inflammatory responses. Secondary conditions such as IBD exacerbate this effect by promoting a chronic, proinflammatory state in the gut that persistently induces IEC hyperplasia.

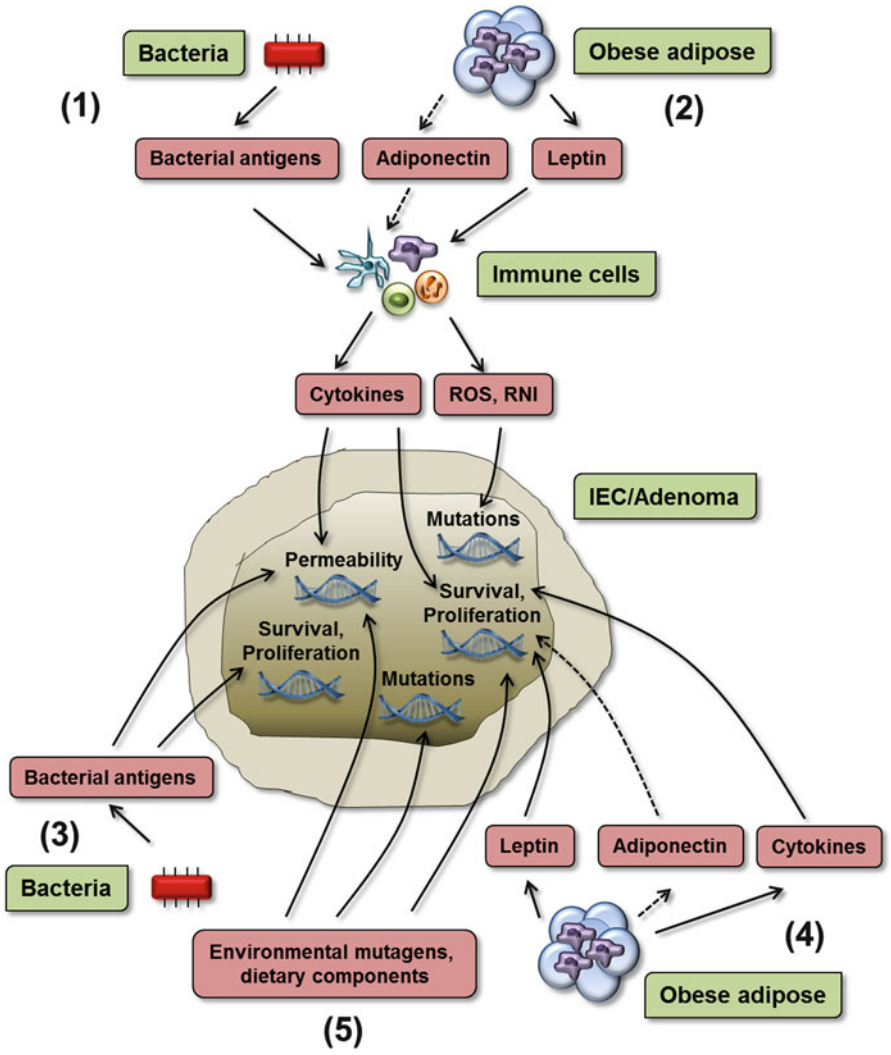

Fig. 7.2 Initiation of colorectal cancer. Susceptibility to CRC initiates from hereditary or somatic mutations that increase tumorigenic potential. Once acquired several factors interact with these genetic predispositions to increase CRC risk. (*1*) Genetic predispositions induce the formation of aberrant crypt foci, which disrupt the epithelial barrier and allow commensal bacteria in the intestinal lumen to interact with immune cells in the lamina propria to induce proinflammatory cytokine production. These cytokines induce gene expression changes in IECs and tumor cells that cause additional barrier permeability and increased survival and proliferation. Additionally, immune cells produce radical oxygen species (ROS) and radical nitrogen intermediates (RNI) that induce additional DNA damage and tumor promoting mutations. Chronic inflammation caused by IBDs increases cytokine production and CRC risk. (*2*) Obese adipose tissue releases proinflammatory leptin and decreases the production of anti-inflammatory adiponectin, resulting in immune cell activation in the intestine, proinflammatory cytokine production, and ROS and RNI release. (*3*) In addition to inducing proinflammatory responses from immune cells, bacterial antigens can interact with toll-like receptors (TLR) on IECs and tumor cells to alter the expression of tight junction

As observed with IBD, particular enterotoxic and invasive species increase CRC risk in humans and mouse models [5, 84]. However, reduced IEC barrier integrity and mixing of commensal bacteria with LMPCs are the most likely mechanism through which intestinal microbes increase CRC susceptibility. Germfree rats and mice provided the first direct evidence that the microbiota contributes to intestinal tumorigenesis. In mice treated with the carcinogen azoxymethane (AOM) to induce genetic mutations and dextran sodium sulfate (DSS) to induce CD-like inflammation, which together cause colitis-associated tumor formation, the absence of the microbiota decreases tumor burden [85]. Similar effects on tumor formation were observed in AOM-DSS-treated rats [86, 87]. Importantly, even in the absence of IBD-like inflammation microbes contribute to tumorigenesis. In the Apc^{Min} mouse model of CRC, which does not develop IBD-like pathologies, the absence of gut microbes reduces intestinal polyp burden [85]. Importantly, new evidence suggests that aberrant crypt foci and adenomas themselves decrease barrier integrity. In mice genetically engineered to be deficient for *Apc* in the colon, loss of IEC barrier integrity at the site of tumor development allowed for penetration of microbial products into lamina propria and induction of T cell-mediated proinflammatory responses that promote tumor growth [83].

7.5.3 The Microbiota, Obesity, and CRC Susceptibility

The intestinal microbiota can be manipulated by a variety of metabolic factors, changes in energy balance, or through the consumption of specific nutritional regimes. Importantly, changes in the gut microbial profile can strongly influence the development of obesity. Germfree mice are resistant to body weight gain induced by a high-fat diet, and transfer of a normal microbiota into germfree mice induced weight gain [46, 88]. Transfer of gut microbiota from an obese mouse to a lean mouse also induced weight gain in mice [45]. Together these studies suggest that the gut microbiota affects energy balance by influencing the efficiency of calorie harvest from the diet and how this harvested energy is used and stored. Human studies of lean or obese monozygotic or dizygotic twins showed that microbial profiles are shared among families on a general level (i.e., phyla), but each individual has a unique profile when examined in more detail (i.e., species) [89, 90]. However, it has been demonstrated that Bacteroidetes are found in lower numbers in obese

Fig. 7.2 (continued) proteins and further increase the permeability of the epithelial barrier, resulting in more inflammation. TLR activation also induces gene expression changes that promote tumor growth. (*4*) Leptin and proinflammatory cytokines released from obese adipose tissue can directly interact with receptors on IECs and tumor cells to promote survival and proliferation. A reduction in anti-proliferative adiponectin production also promotes cell growth. (*5*) Environmental factors, such as saturated fats, interact with IECs and tumor cells to increase barrier permeability (and inflammation) and cell growth and can induce additional genetic mutations that promote tumorigenesis

individuals and that the percentage of Firmicutes is significantly elevated with increased body fatness [91]. A similar finding was observed in obese mice [45]. In contrast, in rats fed with a high-fat diet, Bacteroidetes increased in number [92]. Thus, it is unclear if specific strains modulate obesity (i.e., increase food digestion or absorption) or if obese conditions select distinct families of microbes.

Intestinal bacteria contribute to obesity and cancer susceptibilities in humans and mice, and obesity, diet, and colon cancer can have an effect on the microbial profiles [46]. However, the relationships between host genetics, diet, obesity, susceptibility to colon cancer, and the relative numbers and kinds of intestinal bacteria have not been carefully studied. As discussed above, immune responses to particular bacterial species may be less important than increased immune cell sensitivity or exposure to the microbiota. If this hypothesis is true, a mechanism must exist by which obesity, or diets associated with obesity, increases immune cell–microbiota interactions.

Interestingly, consumption of diets high in saturated fats (HFD) induces low-grade endotoxemia (i.e., increased levels of circulating bacterial-derived LPS), which is indicative of increased IEC barrier permeability [93, 94]. Importantly, saturated fatty acids (SFAs) directly influence barrier integrity by altering the expression of claudin and occludin tight junction proteins in IECs using the same cell signaling pathways as bacterial antigens in IBD (Fig. 7.2) [92, 95]. The activity of the gram-positive and gram-negative bacterial antigens on TLR2 and TLR4 signaling, respectively, is dependent on their saturated fatty acyl moieties [96]. SFAs mimic these moieties and induce both TLR2-depedent and TLR4-depedent responses [96]. SFA stimulation of TLR4 and NF-κB activity alters the expression of tight junction proteins in IECs, resulting in reduce barrier integrity, and induces the expression of proinflammatory cytokines, such as TNF-α, IL-6, and IL-1β, that also decrease IEC barrier integrity [68, 69, 74–79]. Importantly, as discussed with inflammation induced by barrier defects in IBD patients, increased IEC barrier permeability following a meal high in saturated fat allows for the mixing of luminal contents (i.e., the microbiota) with immune cells in the lamina propria. Under normal physiological conditions and diet consumption, an HFD temporarily induces intestinal inflammation, which is resolved with reduced SFA exposure. However, in obese individuals, chronic consumption of HFDs continuously compromises the integrity of the IEC barrier, which in turn induces a chronic, proinflammatory state.

7.6 Proinflammatory Cytokines Contribute to CRC Susceptibility

7.6.1 T Cell-Mediated Inflammation Drives IBD Pathology

Studies of Crohn's disease patients and CD-like colitis in mice have established that CD is a T helper (Th)1 and Th17 cell-mediated inflammatory disease (Tables 7.1 and 7.2) [97–99]. In CD, APCs that have phagocytosed intestinal

Table 7.1 Intestinal immune cells and function

Cell type	Function
T cells (Th, NK, Tc)	Cytokine/chemokine production: Th1 (INF-γ, TNF-α, IL-2, various chemokines), Th17 (IL-17, IL-23, various chemokines), Th2 (IL-4, IL-13), NK (IL-13). Cytotoxicity and APC activation
Treg cells	Cytokine production: (IL-20, TGF-β). Anti-inflammatory, immunosuppression
APCs (macrophages and dendritic cells)	Cytokine/chemokine production: TNF-α, IL-6, IL-1β, IL-23, IL-12, IL-8, various chemokines. Phagocytosis, antigen presentation, T cell activation, granulocyte recruitment
Neutrophils	Cytokine/chemokine production: IL-6, IL-1β, IL-8. Phagocytosis, nitrogen and oxygen reactive species production, degranulation
Epithelial and tumor cells	Cytokine/chemokine production: TNF-α, IL-6, IL-1β, IL-8, PGE2, various other chemokines

Table 7.2 Intestinal immune cells and function

Cytokine/chemokine	Function and pathways in cancer and immune cells
TNF-α	Survival, growth, epithelial barrier permeability, ISEMF activation, monocyte recruitment and activation, T cell activation. MAPK, NF-κB
IL-6	Proliferation, survival, epithelial barrier permeability, ISEMF activation, Th17 cell differentiation, monocyte and granulocyte recruitment. JAK/STAT, ERK/MAPK, PI3K/AKT, NF-κB
IL-1β	Survival, growth, epithelial barrier permeability, ISEMF activation, Th17 cell activation, cytokine and chemokine production by APCs and IECs. MAPK, NF-κB
IL-23	Th17 cell differentiation, induction of IL-22 and IL-17A/F production, Treg inhibition. JAK/STAT
IL-22	Survival, tissue repair responses in IECs, ISEMF activation. JAK/STAT, MAPK, NF-κB
IL-17A/F	Survival, growth, epithelial barrier permeability, ISEMF activation, cytokine and chemokine production by APCs and IECs, monocyte and granulocyte recruitment. JAK/STAT, MAPK, NF-κB
IL-4	Proliferation, survival, metastasis, wound healing, reduced Th1 and APC production of INF-γ, reduced IL-12 production from dendritic cells, B cell class switching. JAK/STAT, MAPK
IL-13	Similar to IL-4
IL-10	Anti-inflammatory, Treg stimulation. JAK/STAT, MAPK
IL-8	Neutrophil recruitment, angiogenesis. PI3K/AKT, PLC, JAK/STAT, MAPK, NF-κB

microbes induce Th1 cell production of chemokines (immune cell recruiting molecules), such as CCL5, and cytokines, such as INF-γ and TNF-α [100]. Together Th1-derived factors recruit additional APCs and induce APCs to release additional proinflammatory cytokines (e.g., IL-6, IL-1β, TNF-α, IL-12) and chemokines (e.g., IL-8) [101]. These factors enhance APC activity (major histocompatibility class [MHC] molecule expression, release of proinflammatory

cytokines, and oxygen and nitrogen reactive species production) and recruit additional immune cell types, such as granulocytes (neutrophils, basophils, and eosinophils), to the site of inflammation [74]. To control the immune reaction, immunosuppressive regulatory T (Treg) cells produce anti-inflammatory IL-10 and TGF-β to suppress APC and Th1 activation [5, 102]. Importantly, following the Th1 response, TGF-β produced by Treg cells and IL-6 and IL-23 released by APCs induce the differentiation and expansion of Th17 cells [103]. Th17 cells produce IL-17A and F, which induce the production of cytokines (e.g., IL-6, IL-1β, and TNF-α) and several neutrophil (e.g., IL-8 and CXCL1) and monocyte (e.g., CXCL2) chemokines in a variety of immune and nonimmune cell types to promote inflammation [100, 104]. Additionally, Th17 cells produce IL-22, which induces tissue repair responses in IECs [101, 103]. Importantly, transition from Th1- to Th17-mediated immunity has a significant impact on CRC risk and disease prognosis. Expression analysis of CRC specimens revealed that patients with the highest level of Th1-related genes expression had the best disease prognosis, whereas patients with the highest level of Th17-related gene expression had the worst prognosis [105].

The T cell response involved in UC was more difficult to classify because IL-5 and IL-13, two defining effector cytokines produced by Th2 cells, were produced by T cells in the lamina propria, but a third Th2-related cytokine, IL-4, was not (Tables 7.1 and 7.2) [101]. Important evidence for what type of Th cell-mediated immunity is involved in UC came from mice treated with oxazolone (OXA), which causes colitis and initially increases the expression of IL-4 [106]. Importantly, further studies using the OXA model of UC showed that, overtime, IL-13 production is stimulated in the lamina propria [107]. Interestingly, IL-13 originated from natural killer (NK) CD4+ T cells rather than conventional CD4+ T cells [107]. NK T cells have cytotoxic activity and are activated by antigens presented on CD1 molecules rather than MHC molecules [101]. In UC patients, NK T cells also appear to be the source of IL-13 production [108]. Thus, chronic inflammation in UC involves a Th2-like immune response involving IL-13, which may contribute to CRC susceptibility [105]. Importantly, unlike CD, the glycolipid (either bacterial- or self-derived) that induces NK T cell responses and inflammation has not been identified [100].

Bridging the Th1/Th17/Th2 responses in CD and UC is a group of well-known cytokines that contribute to IEC barrier defects and mediate many of the pathologies of IBD. Both APCs and IECs release TNF-α, IL-1β, and IL-6 in response to the cytokines produced by Th1, Th17, and NK T cells (Tables 7.1 and 7.2) [55, 109]. Through the activation of mitogen-activated protein kinase (MAPK) signaling pathways and the stimulation of NF-κB-mediated transcription, TNF-α, IL-1β, and IL-6 activate additional APCs. Furthermore, TNF-α, IL-1β, and IL-6 feedback on T cell function. IL-6 and possibly IL-1β induce Th17 differentiation in CD [110, 111]. Additionally, TNF-α enhances APC production of IL-12, which promotes Th1-mediated responses [101]. Importantly, these cytokines promote additional inflammation and stimulate IEC proliferation and survival, which contributes to the pathology of IBD and CRC susceptibility.

7.6.2 *Proinflammatory Cytokines Directly Influence Epithelial Cell Development and CRC Risk*

Several studies have established that the proinflammatory cytokines elevated in IBD directly interact with their respective receptors on IECs to initiate signaling cascades involved in tumor formation (Table 7.2 and Fig. 7.2). IL-6, IL-1β, and TNF-α, which are produced by APCs and IECs in the intestines of IBD and CRC patients, promote IEC proliferation through several cell signaling pathways involving Wnt, Janus kinase/signal transducer and activator (JAK/STAT), MAPK, and NF-κB [27, 112–118]. Similarly, IL-22, IL-17, and TNF-α produced by Th17 cells in CD and CRC patients induce JAK/STAT, MAPK, and NF-κB signaling in IECs to promote proliferation, survival, and tissue repair responses associated with tumorigenesis [83, 115, 117–119]. The Th2 cell-derived cytokines also modulate IEC function. IL-4 and IL-13 through MAPK-mediated cell signaling induce IEC proliferation and survival [120, 121]. Furthermore, evidence suggests that IL-4-/IL-13-mediated signaling alters colon cancer cell adhesion and contributes to cancer cell invasion and metastasis [122, 123].

Importantly, Th1-/Th17-mediated immune response cytokines found in CD (IL-22, IL-23, IL-17A/F), Th2-mediated immune response cytokines found in UC (IL-4 and IL-13), and cytokines associated with both types of T cell responses and innate immunity in IBD (TNF-α, IL-6) have all been shown to increase CRC risk in humans or tumor burden in mouse models of CRC [83, 113, 114, 120, 121, 123–130]. As discussed previously, these proinflammatory mediators likely interact with preexisting genetic abnormalities to increase cancer susceptibility (Fig. 7.1). Importantly, many of these cytokines influenced tumor burden in *Apc* mutant mice. IBD-like inflammation is not observed in *Apc* mutant mice; however, as discussed in Sect. 7.5.2, precancerous lesions can reduce intestinal barrier integrity, allow microbes to interact with LPMNCs, and induce localized inflammation, which promotes epithelial cell proliferation and survival [83]. Therefore, inflammatory cytokine production in the intestine can contribute to CRC risk even in individuals without preexisting inflammatory diseases.

7.6.3 *Epithelial TLR4 Activation Promotes IEC Proliferation and Tumorigenesis*

Similar to IBD, TLR4 receptor expression increases in CRC patients and promoted intestinal tumorigenesis in mice (Fig. 7.2) [80, 131]. Interestingly, microbial activation of TLR4 receptors on IECs promoted tumor development, in part, by inducing expression of cyclooxygenase-2 (COX-2) [131]. COX-2 is an inducible mediator of proinflammatory prostaglandin E2 (PGE2) synthesis, both of which are increased in both human and experimental models of CRC [132–137]. PGE2 through its receptors (EP family of receptors) induces proinflammatory responses in dendritic cells,

Th17 cells, Th1 cells, and IECs [138]. As with all other activators of immune cells, PGE2 promotes tumorigenesis through its proinflammatory effects. PGE2 induces IECs to express amphiregulin, a member of the epidermal growth factor (EGF) family [131]. Interestingly, amphiregulin interacts with EGFR receptors on IECs to promote proliferation and tumorigenesis [131]. Therefore, IECs can promote their own proliferation and neoplastic transformation through PGE2 synthesis.

7.6.4 Inflammation Induces Proinflammatory Cytokine Production by IECs

In addition to directly promoting IEC proliferation and tumorigenesis, cytokines can also induce proinflammatory gene expression in IECs. Through MAPK-, JAK/STAT-, and NF-κB-mediated signaling, IL-17, TNF-α, IL-6, and IL-1β induce CRC cell lines to express a variety of cytokines and chemokines that recruit and activate APCs and granulocytes [5, 139, 140]. The activation of these cell types to the intestine causes additional cytokine production and inflammation, which promotes IEC proliferation and increased tumor risk. Additionally, chronic accumulation of activated granulocytes, macrophages, and dendritic cells is accompanied by the release of oxygen and nitrogen reactive species (RONS), which promote dysplasia by inducing DNA modifications and genetic mutations in proliferating IECs (Fig. 7.2) [141, 142]. Inflammation-induced DNA damage has been linked to altered expression of genes involved in CRC such as p53, APC, KRAS, and BCL-2 [143, 144]. In the DSS mouse model of CD-like inflammation, deficiency for base-excision repair enzymes induces tumor formation, providing direct evidence that the protumorigenic effects of chronic inflammation are partially mediated through DNA damage [145, 146].

7.6.5 Inflammation Induces Tumorigenesis by Altering the IEC Stem Cell Niche

Cytokine action on cells that regulate IEC homeostasis may also influence tumor development (Fig. 7.3). Intestinal subepithelial myofibroblasts (ISEMFs) are positioned subjacent to the basement membrane in the intestinal mucosa, juxtaposed against the bottom of IECs in intestinal crypts. ISEMFs have a profound influence on the intestinal stem cell niche [147]. Following infection or trauma to the intestinal mucosa and activation of inflammation, ISEMFs release factors that alter IEC homeostasis and induce a tissue repair response [147–149]. Proinflammatory cytokines such as IL-1β, TNF-α, IL-17A/F, and IL-22 induce NF-κB and MAPK signaling in ISEMFs, resulting in ISEMF proliferation and the production and release of ECM components (e.g., type I and IV collagen), basement membrane remodeling

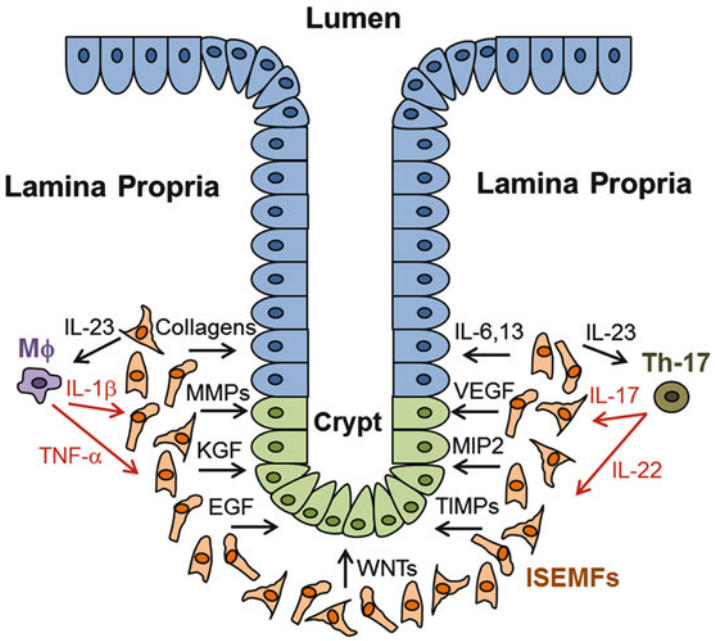

Fig. 7.3 Intestinal subepithelial myofibroblasts (ISEMFs) influence the intestinal stem cell niche during inflammatory responses. In response to proinflammatory cytokines released by macrophages and T cells, ISEMFs express and release additional proinflammatory cytokines (IL-6, IL-13, IL-23), ECM remodeling factors (matrix metalloproteinases [MMPs], tissue-specific inhibitors of MMPs [TIMPs], and collagens) and growth factors (VEGF, KGF, and EGF), and cell signaling ligands (Wnts) into the stem cell niche of the intestinal crypt. Together, the factors released by ISEMFs induce tissue repair responses in IECs that stimulate proliferation and migration. Normally this response repairs tissue damage associated with infection. In CRC-susceptible mice and humans, tissue repair responses interact with genetic predispositions to CRC to further increase cancer risk

factors (matrix metalloproteinases [MMPs] and tissue-specific inhibitors of MMPs (TIMPs)), proinflammatory cytokines (IL-6, IL-13, and IL-23), chemokines (CXCL1 and CXCL2), and growth factors (vascular endothelial growth factor [VEGF], keratinocyte growth factor [KGF], EGF, and Wnt proteins), which induce tissue remodeling and proliferation of IECs [27, 147, 150, 151]. In the chronic proinflammatory state of IBDs, cytokines cause an expansion in ISEMF numbers and induce ISEMFs to continuously release factors that result in IEC proliferation, tissue disorganization, and eventually fibrosis [27, 149]. Importantly, the uncontrolled tissue repair response induced in IECs of IBD patients is the mechanism by which inflammation is proposed to promote adenoma development. Therefore, induction of ISEMFs by inflammatory factors may be an additional mechanism through which inflammation increases CRC risk.

7.6.6 *Adipocytokines: Influence of Obesity on IEC Homeostasis and CRC Risk*

Obesity is characterized by the accumulation of lipid in white adipose tissue (WAT). What was once thought to be a simple storage depot for lipid molecules, adipose tissue is now understood to be a complex endocrine organ consisting of adipocytes, connective and nervous tissue, immune cells (T cells, B cells, and macrophages), chondrocytes, osteocytes, and myocytes [42]. Obese adipose tissue is characterized by immune cell infiltration, with macrophages representing the majority of cell types recruited [152, 153]. A growing body of evidence suggests that adipocyte death resulting from hypertrophy and hypoxia induces the release of chemoattractants that recruit macrophages to adipose tissue (see Chap. 6 for a detailed explanation of these interactions) [154, 155]. The T cell population within obese adipose tissue also changes, potentially due to the influence of leptin on CD8+ cytotoxic T (Tc) cell activation and CD4+ Th1 cell polarization [156]. Tc cell numbers increase, the number of immunosuppressive Treg cells decreases, and production of the Th1 proinflammatory cytokine INFγ increases [157–159]. Together changes in the T cell and cytokine content of obese adipose tissue create an inflammatory milieu conducive to classical (M1) macrophage activation [152, 153]. Once established, the obese adipose tissue-associated M1 macrophages secrete a variety of proinflammatory cytokines (IL-1β, IL-6, and TNF-α) and chemokines (monocyte chemoattractant protein-1 [MCP-1] and IL-8) and reduce expression of anti-inflammatory IL-10, which together maintain an unresolved proinflammatory state in the adipose and a subclinical, chronic inflammatory state systemically [42, 152, 153].

As discussed in the previous sections, the same cytokines released into circulation by obese adipose tissue also modulate intestinal inflammation and CRC susceptibility. Obesity and HFDs interact with DSS and AOM-DSS treatment in mice to increase the severity of intestinal inflammation and CRC incidence, respectively [160, 161]. DSS-mediated inflammation and diet-induced obesity interacted to increase LPMNC expression of proinflammatory cytokines (TNF-α, IL-6, and INF-γ), LPMNC and IEC expression of TLR4, and intestinal helper T cell number [160, 161]. However, the mechanism by which diet-induced obesity increased DSS-mediated inflammation was not clearly demonstrated. In the study of AOM-DSS mice, the increase in intestinal inflammation and CRC incidence was associated with increases in expression of leptin receptor in the colon [161]. As discussed in Sect. 7.7, leptin signaling induces inflammation and IEC proliferation, which may have increased tumor incidence. In the study of DSS-treated mice fed with a high-fat diet, intestinal inflammation was not linked to a specific mechanism. DSS and SFA likely increased the permeability of the intestinal epithelial barrier and allowed LPMNCs to interact with intestinal microbes, which may have induced proinflammatory cytokine production in the intestine. Thus, whether proinflammatory cytokines released into circulation by obese adipose contributed to intestinal inflammation in these studies is an open question. Importantly, TNF-α and IL-1β circulate at very low levels in lean and obese individuals [162]. Therefore, whether these cytokines

function as endocrine factors is debatable. In contrast, circulating levels of IL-6 are substantial in obese individuals and, therefore, may have endocrine effects on distal tissues [162].

7.7 Leptin: A Proinflammatory Adipokine Associated with Increases CRC Risk

In addition to cytokines, adipocytes produce a variety of hormones (adipokines) that regulate several systemic processes, including food intake, metabolism, insulin sensitivity, reproduction, blood pressure, angiogenesis, and inflammation [163]. Leptin (LEP) is an adipokine secreted by white adipocytes in a proportion approximately linear to adipose tissue weight [42]. Leptin plays a key role as a satiety factor and regulator of energy homeostasis through binding to the lepton receptor (LEPR or Ob-R) in the hypothalamus [164]. *LEPR* encodes several splice variants called the long (Ob-Rb), short (Ob-Ra, c, and d), and soluble (Ob-Re) forms [165]. Full-length Ob-Rb retains the intracellular domains necessary to induce downstream signaling events following activation by leptin binding. The short isoform (Ob-Ra) is the most abundant in many tissues and may retain some of intracellular signaling capacities of full-length Ob-Rb. Ob-Re appears to be a carrier molecule for circulating leptin.

As adiposity increases, leptin is released from adipocytes and into circulation to reduce dietary intake and enhance energy expenditure. Interestingly, leptin signaling may have biological functions that reach beyond its normal role in energy homeostasis. Leptin receptor expression and signaling have been reported for a variety of cell and tissue types [165]. Specifically, the leptin receptor is expressed throughout the colonic epithelium, and leptin has been shown to induce proinflammatory innate and adaptive immune cell responses [156, 166, 167]. Epidemiological studies have established that obese individuals have higher circulating levels of leptin compared to normal-weight individuals [168]. Furthermore, several studies have observed an association between higher leptin levels and increased risk of colorectal adenomas [169, 170] and colon cancer [171]. Thus, given the evidence of associations between obesity, chronic inflammation, and CRC risk [172–175], leptin signaling may play an important role in mediating pathophysiological events that lead to intestinal neoplasia.

7.7.1 Leptin and Epithelial Cell Function

Previous reports have established that the leptin receptor is expressed by mouse and human colonic epithelial cells, colon cancer cells, and a variety of human epithelial colon cancer cell lines [166, 167, 176]. In vitro leptin mediates the survival, proliferation, and metastatic capacity of human colon cancer cell lines

through several cell signaling pathways (Fig. 7.2). Stimulation of colon cancer cell lines with leptin induces leptin receptor tyrosine phosphorylation and the activation of a variety of cell signaling cascades [176]. Leptin increased cell number and reduced apoptosis in Caco-2 and HT-29 cultures by inducing extracellular signaling kinases (ERK1/2), PI3-kinase/protein kinase B (PKB or AKT) AKT phosphorylation, c-Jun NH(2)-terminal kinase (JNK), and JAK/STAT-mediated pathways [177, 178]. An additional study of HT-29 response to leptin demonstrated that colon cancer cell proliferation and survival are dependent on NF-κB nuclear translocation and activation [179]. Importantly, the leptin receptor is expressed by both normal and tumorigenic epithelial cells in vivo [176, 180]. However, the proliferative and anti-apoptotic effect of leptin demonstrated in vitro was contradicted by in vivo studies of the $Apc^{Min/+}$ and AOM mouse models of CRC. Curiously, leptin failed to increase intestinal polyp number or size in $Apc^{Min/+}$ mice treated with leptin [181]. Furthermore, leptin-deficient, obese *ob*/*ob* and leptin receptor-deficient, and obese *db*/*db* mice showed increased numbers of aberrant crypt foci (markers of precancerous lesions) relative to controls following AOM treatment despite the absence of leptin signaling [182].

Two studies may have resolved the differences observed in the in vitro and in vivo studies. Endo et al. examined normal and tumorigenic colonic epithelium at various stages of development in leptin-deficient, obese *ob*/*ob*, and wild-type mice treated with the carcinogen AOM [183]. In *ob*/*ob* mice normal epithelial cell proliferation and the number or precancerous aberrant crypt foci were similar to AOM-treated wild-type controls. However, when cancer adenoma polyps were examined, a significant reduction in tumor proliferation and size, but not number, was observed in the AOM-treated *ob*/*ob* mutants. Thus, leptin appears to promote growth subsequent to tumor initiation. This conclusion is in agreement with studies by Fenton et al. who demonstrated that stimulation of a normal mouse IEC line (YAMC), which has two functional copies of *Apc*, with leptin reduced epithelial cell proliferation and induced apoptosis [184]. In stark contrast, the pre-neoplastic mouse IEC line (IMCE), which was derived from mice harboring the Apc^{Min} mutation, exhibited increased proliferation and survival after leptin stimulation [184]. Thus, the influence of leptin on IECs is likely to be dependent on preexisting genetic risk factors that already initiated tumorigenesis.

7.7.2 Leptin and the Modulation of Epithelial Cell-Derived Inflammation

In addition to promoting colon cancer proliferation, survival, and migration, the role of leptin in promoting proinflammatory responses in epithelial cells may significantly contribute to CRC risk. In vitro leptin, through the activation of NF-κB-mediated transcription, induced CRC cells to express several proinflammatory cytokines and chemokines, including IL-1β, which are associated with chronic intestinal inflammation, innate immune cell infiltration, and increased CRC risk

[5, 180]. As previously described in Sects. 7.6.2 and 7.6.5, IL-1β can act directly on epithelial cells or indirectly through ISEMF to promote epithelial cell proliferation and neoplastic transformation. By contrast, leptin reduced CRC cell line expression of anti-inflammatory cytokines, such as IL-10, that downregulate immune responses to gut microbes and reduced tumorigenesis (Sect. 7.5.1) [180]. Furthermore, Fenton et al. used their normal IEC line and preneoplastic IEC line to demonstrate that leptin induces IL-6 expression from precancerous but not normal epithelial cells [184]. These results are in agreement with the postulated role of IL-6 in inducing IEC and promoting CRC risk in individuals with IBDs (Sect. 7.6.2). Therefore, induction of IEC expression of IL-1β and IL-6 by leptin may be a shared mechanism by which obesity and chronic inflammation increase CRC susceptibility.

Leptin also induces the production of chemokines in epithelial cells, which may contribute to CRC incidence. Intraperitoneal injections of leptin induced the expression of the neutrophil chemoattractant CXCL1 in the mouse colonic epithelium [185]. Neutrophils express IL-1β, IL-6, and IL-8, which are associated with IBD and CRC susceptibility, and generate nitrogen and oxygen reactive species that induce DNA damage (Sect. 7.6.4) [141, 156]. Interestingly, CXCL1 and IL-8 are both potent angiogenic compounds that may promote tumor growth through vascularization [186, 187]. Therefore, leptin may increase CRC risk by promoting neutrophil recruitment.

7.7.3 Leptin and the Activation of Immune Cells

Similar to proinflammatory effects on IECs, leptin may induce intestinal tumorigenesis by directly activating and recruiting immune cells (Fig. 7.2). In humans, it has been suggested that reduced leptin levels are associated with impaired immune function [188]. Similarly, leptin-deficient *ob*/*ob* mice are resistant to various experimental models of inflammation, which can be corrected by exogenous administration of leptin [189–191]. Conversely, increased leptin levels may be responsible for the altered T/B cell function, increased monocyte and granulocyte activity, and increased white blood cell counts observed in obese individuals with active leptin signaling [192]. The leptin receptor shares significant similarity to members of the class I cytokine receptor (gp130), which includes the receptor for IL-6 [193]. In fact, Ob-R stimulates many of the same downstream signaling cascades (JAK/STAT, PI3K/AKT, MAPK, and NF-κB) as IL-6, suggesting a potential function for leptin in regulating immune cell function [192, 194]. Importantly, Ob-R is expressed on the surface of several cell types involved in innate immunity [195].

Studies of leptin- and leptin receptor-deficient mice have revealed the effects of leptin on antigen-presenting cell (APC) function. Leptin signaling in macrophage induces activation, increases phagocytosis capacity, promotes proinflammatory cytokine production (e.g., IL-6, TNF-α, and granulocyte chemoattractants), and activates proliferation [192, 196, 197]. Leptin also increases the survival of dendritic cell (DC) and induce DC production of IL-8, IL-6, and TNF-α [156, 198, 199].

Leptin also downregulates DC expression of anti-inflammatory IL-10 [199]. On a functional level, leptin drives DC cells to promote cell-mediated immunity by inducing Th1 differentiation and enhancing Tc cell function [198–200].

Leptin signaling also directly modulates adaptive immune cell function. Mouse and human lymphocytes express the leptin receptor [194, 201]. However, leptin alone cannot activate lymphocytes in vitro [202, 203]. However, leptin enhances proliferation, inhibits apoptosis, and promotes the activation of primed Th cells and Tc cells [204, 205]. Leptin appears to regulate T cell development at two stages. First, immature helper T cells from *ob*/*ob* mice showed impaired development of both Th1 cells and Th2 cells in vitro [206]. Second, evidence suggests that leptin drives helper T cell differentiation toward Th1 cells. In mice, leptin promotes Th1 cytokine production [156, 201, 204].

Importantly, leptin modulates many immune cell function and inflammatory responses in the colon, which could have a profound influence on CRC risk. The leptin receptor is expressed by LPMNCs [207]. Intraperitoneal injections of leptin in mice induced APCs in the lamina propria to express IL-6, IL-1β, and TNF-α [185]. Together, these cytokines represent key inflammatory mediators of IBD and increased CRC risk (Sects. 7.6.2 and 7.6.5). The influence of leptin on T cell function can also promote intestinal tumorigenesis. Using the OXA-induced mouse model of Th2-like UC, Batra et al. demonstrated that leptin-deficient *ob*/*ob* mice are protected against colitis, which was attributed to a decrease in the production of the Th2 cytokine IL-13 in the lamina propria [206]. As discussed in Sect. 7.6.2, the two Th2-related cytokine, IL-4 and IL-13, induce CRC cell survival, proliferation, and metastasis [120–123].

7.8 Adiponectin: An Anti-inflammatory Adipokine Associated with Reduced CRC Risk

Adiponectin (APN) circulates as a low molecular weight (LMW) trimer, a middle molecular weight (MMW) hexamer, and a high molecular weight (HMW) multimer of full-length (fAb) protein [208]. An additional truncated form of APN containing only a C-terminal globular domain (gAb) is produced by proteolytic cleavage and is also found in circulation [208, 209]. All circulating forms of APN are biologically active but have different target tissues and varying biological effects that are not well delineated [210, 211]. APN induces intracellular signaling through its interactions with two receptors (ADIPOR1 and ADIPOR2). ADIPOR1 is predominately located in skeletal muscle whereas ADIPOR2 localizes to the liver [191]. APN regulates a variety of metabolic processes including glucose and fatty acid catabolism and increases glucose uptake by stimulating the translocation of the GLUT4 receptor to plasma membranes [191]. Binding of APN to its receptors activates a variety of cell signaling cascades involving ERK1/2 MAPKs, PI3-K/AKT, NF-κB, and JAK/STAT [209]. However, APN primarily exerts its effects through the activation of the AMP-activated protein kinase (AMPK) signaling cascade [209].

Unlike most other adipose-derived factors, circulating APN levels are reduced in obese individuals and negatively correlate with BMI, waist circumference, and visceral fat mass [212–215]. Although the inverse relationship between circulating APN levels and obesity makes biological sense given its influence on metabolic functions in muscle and liver, chronically low levels of APN may have more far reaching physiological consequences. Both APN receptors are expressed in a variety of tissue including IECs and immune cells [209]. Importantly, epidemiological evidence suggests that CRC risk is inversely associated with circulating APN levels; men with the highest levels of APN had a 60 % reduced CRC risk after adjusting for body size, activity, and waist circumference [216]. Thus, the reduction in APN levels associated with obesity appears to be an important factor in promoting CRC development.

7.8.1 Adiponectin and Epithelial Cell Function

IECs express ADIPOR1 and ADIPOR2 in both mice and humans [166, 217–219]. Benign adenomas and malignant carcinomas in humans and adenomas in $Apc^{Min/+}$ mice also express APN receptors [209, 218, 219]. Thus, APN signaling has the potential to regulate both normal and neoplastic epithelial cell function (Fig. 7.2). Several in vitro studies have demonstrated that APN signaling restricts the proliferation and survival of CRC cell lines. APN decreases proliferation and induces apoptosis in several CRC cell lines [220–224]. Importantly, the effect of APN on CRC cell line growth appeared to be predominately mediated by activation of AMPK signaling, which resulted in the inhibition of the tuberous sclerosis protein 2/mammalian target of rapamycin/S6 kinase (TSC2/mTOR/S6 kinase) cell signaling axis and repression of translation and cell growth [209, 220, 224, 225]. In addition, AMPK activated the G1/S cell cycle control checkpoint by promoting the expression of cell-dependent kinase inhibitors p21 and p27 and the G2/M cell cycle checkpoint by inducing p53 expression [220, 226]. Although most in vitro studies suggest an anti-proliferative and pro-apoptotic role for APN in cancer, the results of two studies imply that APN positively influences CRC cell line proliferation and survival [222, 226]. Interestingly, these contrasting responses appear to be dependent on culture conditions. The pro-proliferative response to APN occurred in the presence of fetal bovine serum (FBS), and the pro-survival effect occurred in glucose-deprived medium [222, 226]. Therefore, the influence of APN on intestinal tumorigenesis may be dependent on the nutritional and hormonal content of the tumor microenvironment.

The preponderance of evidence from mouse models of CRC also suggests that APN signaling negatively regulates intestinal cancer development. Studies of *Apn*-deficient mice harboring the Apc^{Min} mutation and *Apn*-deficient mice treated with AOM or DSS and dehydroheliotridine (DSS-DHH) demonstrated that *Apn* deficiency increases cancer burden, which was associated with reduced AMPK activation, an increase in mTOR and S6 kinase activity, increased JAK/STAT and AKT

activity, and increased cell proliferation [222, 227–229]. Importantly, exogenous administration of APN to *Apc*$^{Min/+}$ mutant mice suppressed intestinal tumorigenesis, providing clear evidence of the anti-tumorigenic properties of APN [219]. Interestingly, one study reported that *Apn* deficiency reduced tumor burden in AOM-treated mice fed with a high-fat, but not a low-fat, diet [228]. As observed in vitro, the nutritional and hormonal milieu of the intestinal mucosa may determine the influence of APN on IEC growth.

7.8.2 Adiponectin and the Modulation of Colonic Epithelial Cell-Derived Inflammation

In addition to inhibiting colon cancer proliferation, survival, and migration, the regulation of inflammatory responses in IECs by APN may contribute to CRC risk. However, unlike the clear proinflammatory influence of leptin on IECs, the contribution of APN to inflammation is less clear. In one in vitro experiment, it was demonstrated that APN inhibits IL-8 production by LPS-stimulated colon cancer cells, suggesting that APN signaling has an anti-inflammatory and anti-angiogenic effect on IECs [217]. In the mouse adenoma cell line IMCE, Fenton et al. demonstrated that APN blocked the ability of leptin to induce IL-6 production [230]. Given the role of cytokines, in particular IL-6, in inducing IEC proliferation and promoting CRC risk, the anti-inflammatory influence of APN on IECs may inhibit tumor development. However, other studies suggest that APN signaling has a proinflammatory effect on IECs. Ogunwobi and Beales demonstrated that APN induces CRC cell line expression and secretion of IL-8 and MCP-1 [222]. In agreement with these findings, APN induced colonic explants from DSS-treated mice to increase IL-6 and granulocyte chemoattractant CXCL2 production [231]. It is currently unclear as to why the reported inflammatory effects of IECs are so variable. Differences in cell culture conditions, such as the isoform of APN (full length or globular) used in the medium, may have influenced results. Therefore, future experiments will need to delineate the effects of APN isoforms on IEC inflammatory responses under different culture conditions.

7.8.3 Adiponectin and the Activation of Immune Cells

In contrast to its ambiguous roles in regulating IEC inflammatory responses, the influence of APN on immune cell function is undoubtedly immunosuppressive (Fig. 7.2) [217, 232, 233]. The ADIPOR1 and ADIPOR2 receptors are expressed on the majority of monocytes, many B cells and natural killer cells, and a small percentage of T cells [234]. Mice deficient for *Apn* present with a heightened immune response in several inflammatory disease models, including microbial sepsis, transplant rejection, and DSS-induced colitis [217, 232, 233, 235]. In the DSS mouse

model of CD, *Apn* deficiency causes a dramatic increase in the number of macrophages that infiltrate the affected colon, resulting in increased fibrosis, breakdown of the intestinal crypts, and destruction of the IEC barrier [217]. As discussed in Sect. 7.5, breakdown of the IEC barrier allows commensal microbes to come into contact with LPMNCs, which induces inflammation and increases CRC susceptibility. Importantly, the expression of several cytokines associated with the progression of IBD and CRC risk, including IL-1β, IL-6, and TNF-α, is higher in *Apn*-deficient mice treated with DSS [217]. Although the cell types producing these factors were not identified in this study, these results clearly demonstrate that *Apn* expression within the intestine is immunosuppressive.

APN has immunosuppressive effects on APCs. Macrophages from *Apn*-deficient mice have increased production of IL-6, TNF-α, and MCP-1 and reduce expression of anti-inflammatory IL-10 [236–239]. In agreement with these findings, several additional studies have shown that APN induces anti-inflammatory IL-10 production and inhibits LPS activation of phagocytosis and production of chemoattractants by macrophages [236–243]. A limited number of studies have identified subtle influences of APN on cell types associated with adaptive immunity. In one study, APN was shown to negatively regulate antigen-activated CD8+ T cell function by reducing INF-γ, TNF-α, and IL-2 production and to promote T cell apoptosis [244]. Supporting these findings, T cells from *Apn*-deficient mice have an increased capacity for activation and expansion [244].

Obesity is associated with lower circulating levels of APN, which may contribute to chronic inflammatory and increased CRC susceptibility in individuals genetically predisposed to tumor development. This model is supported by a study of DSS-DHH-treated, *Apn* knockout mice [227]. Both tumor number and size increased in *Apn* knockout mice exposed to DSS-DHH when compared to wild-type controls treated with DSS-DHH. Importantly, in *Apn*-deficient mice, DSS-DHH increased expression of several proinflammatory and tumorigenic cytokines, including IL-6, TNF-α, IL-1β, and COX-2, and decreased expression of anti-inflammatory and tumor suppressing IL-10 in the intestinal mucosa. Therefore, reduced APN levels are likely to increase CRC risk through the dysregulation of inflammatory responses in the gastrointestinal tract.

7.9 CRC Prevention and Therapeutics: Targeting Inflammation

Although the link between inflammation and CRC risk is well established, this knowledge is just now being translated to the clinic. Results from initial studies using anti-inflammatory agents as a component of CRC treatment are promising. Intestinal inflammation plays an important role in CRC risk and progression. Nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin and sulindac, which inhibit COX-2 production of PGE2, are currently being used to treat CRC or prevent cancer relapse in CRC patients [245–247]. Additionally, several

anti-inflammatory reagents targeting TNF-α, IL-6, IL-12/23, IL-2, and IL-17 have been shown to be effective in treating IBD patients or individuals with autoimmune disorders [5, 248, 249]. These same cytokine targets play central roles in mediating CRC progression and, therefore, may prove to be effective targets for anticancer treatment. However, the immune system possesses antitumor activity, and it will be important to select anti-inflammatory treatment options that block tumor-promoting inflammation without reducing antitumor immune responses. In addition to pharmacological interventions, modulation of the diet might prove as equally effective at reducing intestinal inflammation and CRC risk. High-fat diets increase intestinal inflammation and CRC risk. Importantly, several other dietary components, including carbohydrates, unsaturated n-3 fatty acids, vitamins, minerals, and phytochemicals (e.g., resveratrol), have been shown to reduce CRC susceptibility [250]. Although the molecular mechanisms by which these dietary factors reduce cancer risk are not well established, there is evidence to suggest that reduced intestinal inflammation plays an important role [5, 251]. Therefore, when used with existing chemotherapy and radiotherapy, agents or lifestyle changes that reduce intestinal inflammation may improve treatment outcomes and reduce tumor relapse in CRC patients.

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Chapter 8
Obesity, Inflammation, and Breast Cancer

Neil M. Iyengar, Patrick G. Morris, Clifford A. Hudis, and Andrew J. Dannenberg

Abstract Obesity, which is rising in incidence worldwide, is important with regard to the treatment of breast cancer, disease progression, and carcinogenesis. Obesity is a risk factor for the development of hormone receptor-positive breast cancer in postmenopausal women and is associated with reduced benefits from treatment. Furthermore, irrespective of breast cancer subtype, obesity is associated with worse outcomes after diagnosis. There is increasing evidence of specific biological underpinnings for these observations, including higher circulating estrogen levels, insulin resistance, altered levels of adipokines, and the consequences of chronic in-breast inflammation. Increasing adiposity also has important implications for local therapy including surgery and radiotherapy. This chapter reviews the complex interactions between obesity and breast cancer.

Abbreviations

AMPK	5′-Adenosine monophosphate-activated protein kinase
BCSS	Breast cancer-specific survival
BMI	Body mass index
CEBP-α	CCAAT/enhancer binding protein-α
CI	Confidence interval
CLS	Crown-like structures

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DFS	Disease-free survival
ER	Estrogen receptor
FFA	Free fatty acid
HER2	Human epidermal growth factor receptor-2
HR	Hazard ratio
hsCRP	High-sensitivity C-reactive protein
IGF-1	Insulin-like growth factor-1
IL-1α	Interleukin-1α
IL-1β	Interleukin-1β
IL-6	Interleukin-6
NSAID	Nonsteroidal anti-inflammatory drug
OR	Odds ratio
OS	Overall survival
PGE_2	Prostaglandin E_2
PPAR-γ	Peroxisome proliferator-activated receptor-γ
PR	Progesterone receptor
RR	Relative risk
SERM	Selective estrogen receptor modulator
SHBG	Sex hormone-binding globulin
TLR-2	Toll-like receptor-2
TLR-4	Toll-like receptor-4
TNF-α	Tumor necrosis factor-α
TRAM	Transverse rectus abdominis myocutaneous

8.1 Introduction

Worldwide, the incidence of overweight and obesity are increasing. One widely used metric of adiposity is the body mass index (BMI), calculated using the formula [(weight in kilograms)/(height in meters)^2]. This is a continuous variable but can be divided into categories. According to this scale, underweight is defined as BMI less than 18.5 kg/m^2, normal as 18.5–24.9 kg/m^2, overweight as BMI 25.0–29.9 kg/m^2, and obese as BMI 30 kg/m^2 or greater. Almost 1.5 billion adults in the world are overweight (BMI ≥ 25 kg/m^2), and more than 500 million of these are obese (BMI ≥ 30 kg/m^2) [1, 2]. In the United States, the majority (>60 %) of adults are overweight, and obesity rates are increasing [1, 2]. Recent projections estimate that as much as 65 % of the US population in several states may be obese by 2030 [3].

Obesity has a range of clinical consequences and is a risk factor for the development of several epithelial malignancies, including estrogen receptor (ER)- and progesterone receptor (PR)-positive ("hormone sensitive") breast cancer in postmenopausal women, the most common presentation of the disease [4–8]. Obesity and overweight present a number of challenges for the management of breast cancer, including potential limitations on diagnostic imaging, as well as changes in local and systemic therapeutic options. In addition, obesity is a negative

prognostic variable irrespective of breast cancer subtype. In this chapter, we discuss the complex interactions between obesity and breast cancer in terms of epidemiology, biology, and treatment.

8.2 Epidemiology of Obesity and Breast Cancer

In epidemiologic studies, obesity has been linked to several subtypes of breast cancer. This is most consistently seen with regard to the most common presentation of breast cancer, hormone-sensitive (ER and/or PR positive) disease in postmenopausal women [4–10]. In a pooled analysis of eight prospective studies including over 2,000 women, the relative risk (RR) of developing breast cancer in postmenopausal females associated with each 5 kg/m^2 increase in BMI was 1.19 (95 % confidence interval [CI], 1.05–1.34) [8]. A similar effect was also observed in a larger pooled analysis of several prospective cohort studies including over 300,000 women in which the RR of developing breast cancer was 1.43 (95 % CI, 1.21–1.67) specifically in overweight postmenopausal women with BMI 27–29 kg/m^2 compared to those who were leaner (<21 kg/m^2) (Table 8.1) [6].

Normally, following menopause, circulating estradiol levels decline rapidly as the ovaries no longer produce appreciable amounts of estrogen. Instead, lower levels of estrogen are produced noncyclically from androgen precursors peripherally, primarily in the adipose tissue [11]. The critical step in this process is catalyzed by the enzyme aromatase, a cytochrome P450 enzyme encoded by the *CYP19* gene. Although ER-positive breast cancer makes up the largest proportion of the disease in all ages, the incidence of this subtype increases with age [12]. For example, in a

Table 8.1 Selected epidemiologic studies reporting significant association between overweight/obesity and the development of breast cancer in postmenopausal women

References	Subjects	Number	BMI in "normal" category (kg/m^2)	BMI in "overweight and obese" category (kg/m^2)	Magnitude of effect	95 % confidence interval	*P* value
Trentham-Dietz et al. [7]	Case Control	6,548 9,057	12.9–21.2	27.6–57.1	1.41[a]	1.25–1.60	<0.001
Van den Brandt et al. [6]	Cohort (pooled)	337,819	<21	27 to <29	1.43[b]	1.21–1.67	0.001
Key et al. [8]	Case Control (pooled)	624 1,669	<22.5	≥30	1.36[b]	1.00–1.85	0.004
La Vecchia et al. [10]	Case Control (pooled)	3,108 2,664	<21.8	>28.4	1.4[a]	1.2–1.7	<0.001

[a]Odds ratio
[b]Relative risk

population-based study in the United States, approximately 55 % of breast cancer in women in their 20s was ER positive, rising to almost 85 % in women in their 80s [12]. This leads to a paradox, whereby ER-positive breast cancer incidence increases with age despite the falling circulating levels of estrogen. Increasing adiposity with advancing age has been suggested as one of the underlying mechanisms to explain this phenomenon, but the specifics of this effect are not fully elucidated.

8.3 Obesity and Breast Carcinogenesis

Although the mechanisms remain incompletely understood, the link between obesity and ER-positive breast cancer in postmenopausal women is thought to be partially due to two related factors: increased adipose tissue and elevated aromatase expression in adipose tissue [8, 13]. Given that the incidence of ER-positive breast cancer increases with age as discussed above, it is not surprising that the majority of postmenopausal breast cancers associated with obesity are ER positive [4, 5]. In obesity, growth of hormone-sensitive tumors within the breast is dependent on locally produced estrogens and, possibly, ligand-independent activation of estrogen receptor-α or other paracrine-mediated effects that may drive carcinogenesis. If, as noted, peripheral aromatization in adipose tissue is thought to be largely responsible for estrogen production after menopause, the question remains: where do the estrogens that drive breast cancer in this age group arise? [11]. If they are produced locally, this has important implications for breast carcinogenesis as estrogens can have stimulatory effects on cell growth within the breast but also have genotoxic effects and stimulate angiogenesis (Fig. 8.1). These actions suggest a broader potential role for estrogen than merely stimulating ER in hormone-sensitive breast tumors.

Additional clinical observations suggest other linkages between obesity and breast carcinogenesis. In premenopausal women, BMI > 30 kg/m^2 has also been associated with an increased risk of hormone receptor-negative breast cancers [14, 15]. This association could be related to estrogen-independent pathways such as obesity-associated altered glucose metabolism and increased levels of insulin and bioavailable insulin-like growth factor-1 (IGF-1) (Fig. 8.1). Furthermore, increased leptin and decreased adiponectin can lead to altered cellular proliferation and survival. Finally, obesity is now recognized to be a systemic inflammatory condition with known associations with elevated proinflammatory mediators that promote tumorigenesis and growth [5, 16, 17]. This complex biological interaction between obesity and breast cancer is reviewed in greater detail below.

8.3.1 Dysfunctional Adipose Tissue and Metabolism

Although perhaps not conventionally considered as such, adipose tissue is an active endocrine organ. Adipocytes secrete a variety of factors, known as adipokines, including leptin and adiponectin—which are known to have angiogenic properties—as well as several growth factors [18].

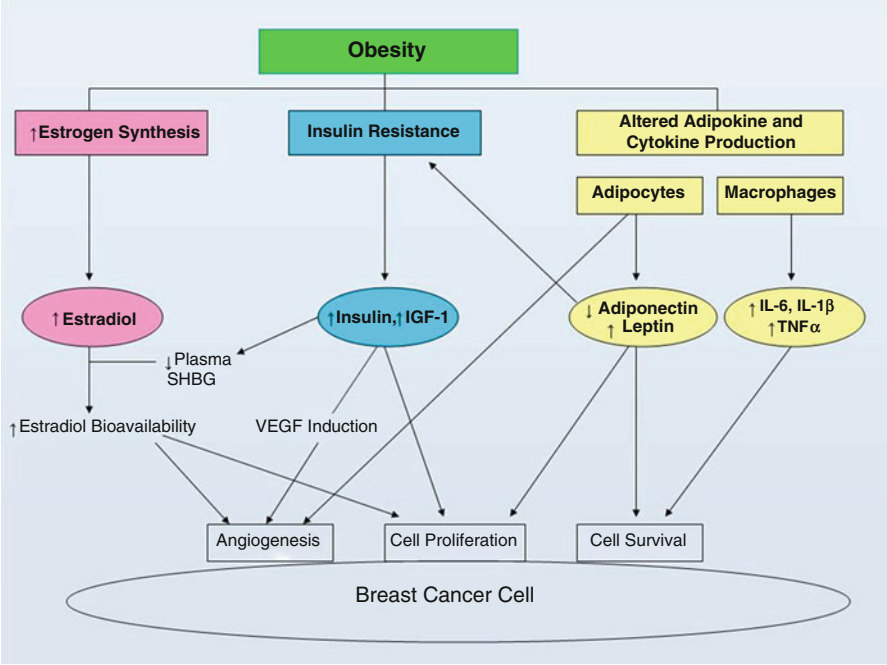

Fig. 8.1 Relationship between obesity and promotion of breast cancer. *IGF-1* insulin-like growth factor-1; *IL-6* interleukin-6; *IL-1*β interleukin-1β; *TNF*-α tumor necrosis factor-α; *SHBG* sex hormone-binding globulin; *VEGF* vascular endothelial growth factor (Sinicrope F et al.: J Clin Oncol Vol. 72(9), 2010:4–7. Reprinted with permission. © 2010 American Society of Clinical Oncology. All rights reserved)

8.3.1.1 Adiponectin

Metabolic conditions, such as obesity, that alter adipose stores modify adipokine production. Increased adiposity is associated with enlarged adipocytes [5] and the enhanced production of chemotactic factors that attract myeloid cells such as macrophages into adipose tissue [19]. This promotes an inflammatory cascade leading to decreased production of adiponectin, release of free fatty acids (FFAs), and the development of insulin resistance, characterized, clinically, by hyperinsulinemia [20]. Decreased levels of adiponectin, an adipocyte-secreted hormone, are associated with hyperinsulinemic states including obesity and type II diabetes mellitus [21]. Furthermore, decreased adiponectin levels have been associated with increasing breast cancer risk [22]. The mechanism underlying this relationship is not completely understood but adiponectin has been shown to inhibit the growth of several breast cancer cell lines in vitro [23]. Additionally, adiponectin activates the 5′-adenosine monophosphate-activated protein kinase (AMPK) pathway leading to upregulation of p53 and p21, important regulators of the cell cycle and apoptosis [5]. Other signaling pathways downstream of the adiponectin receptors, AdipoR1 and AdipoR2, have been implicated in breast cancer cell growth including regulation of aromatase expression, and the PTEN/PI3K/mTOR and MAPK pathways [24].

8.3.1.2 Leptin

Elevated leptin levels have also been associated with increased risk of breast cancer development and progression [25, 26]. Leptin is known to stimulate breast tumor cell proliferation via several different signaling pathways including PI3K/Akt, MAPK, and STAT3 [24]. Additionally, leptin acts via alteration of cell cycle checkpoints to promote proliferation via upregulation of the cdk2 and cyclin D1 genes that advance cells from G1 to S phase [27]. Caldefie-Chezet et al. have demonstrated that leptin is not found in normal breast tissue, whereas it is expressed in the healthy tissue surrounding malignant ductal lesions [28]. Furthermore, these same investigators demonstrated that the leptin receptor, Ob-R, is co-expressed with leptin in human breast cancer tissue [25]. Interestingly, Ob-R expression by the breast carcinoma was positively correlated with expression of the estrogen receptor ($p=0.028$) and tumor size ($p=0.045$). Consistent findings were reported in several other studies [29, 30]. Therefore, leptin exerts both paracrine and autocrine effects on breast carcinoma growth.

8.3.1.3 Insulin Resistance and IGF-1

Obesity is associated with insulin resistance characterized by elevated plasma levels of insulin and IGF-1. The development of insulin resistance is complex and has been implicated, in part, in the relationship between obesity and breast cancer. In a cohort of over 500 women with early-stage breast cancer, elevated fasting insulin was found to be associated with distant breast cancer recurrence (hazard ratio [HR] 2.0; 95 % CI, 1.2–3.3) and death (HR 3.1; 95 % CI, 1.7–5.7) [31]. Additionally, insulin level was correlated with BMI ($p<0.001$). Insulin has important mitogenic effects on breast cancer cells that have not yet been completely elucidated. Binding of insulin and IGF-1 to their respective receptors promotes cell proliferation and inhibits apoptosis via downstream signaling effects of the PI3K/Akt and Ras/Raf/MAPK systems [32]. Interestingly, IGF-1 has been found to be expressed at higher levels in ER-positive tumors than in cancers that do not express the estrogen receptor [33]. Additionally, IGF-1 and insulin, to a lesser degree, have been shown to stimulate aromatase activity in adipose tissue [34]. Beyond this paracrine-mediated effect on aromatase, increased serum insulin levels can stimulate androgen synthesis by the ovaries and reduce hepatic synthesis of sex hormone-binding globulin (SHBG) [5]. As suggested by the name, SHBG is a protein synthesized in the liver that tightly binds testosterone, dihydrotestosterone, and estradiol and transports these hormones in the blood in a biologically inactive state. Changes in SHBG levels affect the amount of hormone available to cells that carry the respective receptors. In a sample of over 1,000 postmenopausal women randomly selected from the Melbourne Collaborative Cohort Study, BMI was positively correlated with increased levels of plasma estradiol and associated with decreased SHBG plasma concentrations [35]. Therefore, IGF-1 and insulin, which are increased in obese patients, are involved in multiple complex systems that are associated with breast carcinogenesis and tumor progression.

8.3.2 *Obesity and Inflammation*

A primary function of white adipose tissue is to store energy as lipid. Excess adipose tissue is associated with elevated serum triglyceride levels. Increased adiposity is accompanied by adipocyte hyperplasia and hypertrophy, which in turn lead to adipocyte stretch and death. Indeed, increasing BMI has been correlated with adipocyte hypertrophy in the breast [36]. It is well known that obesity is associated with chronic, subclinical inflammation [5, 17, 37–39]. There is evidence that adipocyte hypertrophy leads to cell wall stretch, and subsequently, the adipocyte releases several proinflammatory cytokine mediators, including tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6). This attracts and activates immune cells including macrophages [40]. Additionally, lipolysis occurs within the stretched adipocytes resulting in release of FFAs that stimulate multiple inflammatory pathways in the activated immune cells including activation of the transcription factor NF-κB [41]. Activation of NF-κB, a contributor to inflamed adipose tissue, is mediated by Toll-like receptor-4 (TLR-4) [42].

The interactions between adipocytes and immune cells in the stromal vascular fraction of adipose tissue are complex and incompletely understood. Dead and dying adipocytes, described above, interact with macrophages and stimulate production of several proinflammatory cytokines including TNF-α, interleukin-1β (IL-1β), and IL-6 [17, 38]. Increased circulating levels of these cytokines are commonly found in obese women and have been associated with breast cancer development and progression [43–45]. In addition, FFAs are believed to activate macrophages residing in the adipose tissue via a series of receptors including Toll-like receptor 2 (TLR-2) and TLR-4 [46]. Furthermore, macrophage-adipocyte associations are histologically apparent as crown-like structures (CLS) in which macrophages encircle the dead adipocyte [47]. These CLS have been observed in the mammary gland adipose tissue of obese mice, and their presence has been associated with NF-κB-induced production of several proinflammatory mediators including TNF-α, IL-1β, and Cox-2 [48]. In both dietary and genetic mouse models of obesity, the number of CLS has been positively correlated with body weight, in addition to tissue levels of proinflammatory mediators. Importantly, the presence of CLS was associated with increased aromatase levels and activity. These findings have been translated to the human, and CLS of the human breast (CLS-B) have been recently described (Fig. 8.2) [36, 49]. The severity of breast inflammation, manifested as the CLS-B index (proportion of slides with histological evidence of CLS-B), correlated with increasing BMI. Consistent with the mouse models, the presence of CLS-B was associated with activation of NF-κB, and increased levels of proinflammatory mediators including TNF-α, IL-1β, COX-2, and COX-2 derived prostaglandin E_2 (PGE_2). Also mirroring the preclinical findings, the presence of CLS-B and elevated tissue levels of these proinflammatory cytokines paralleled increased transcription of the *CYP19* gene encoding aromatase, leading to elevated aromatase levels and activity [50]. One of the key consequences of this inflammatory pathway was increased expression of the progesterone receptor (PR). Notably,

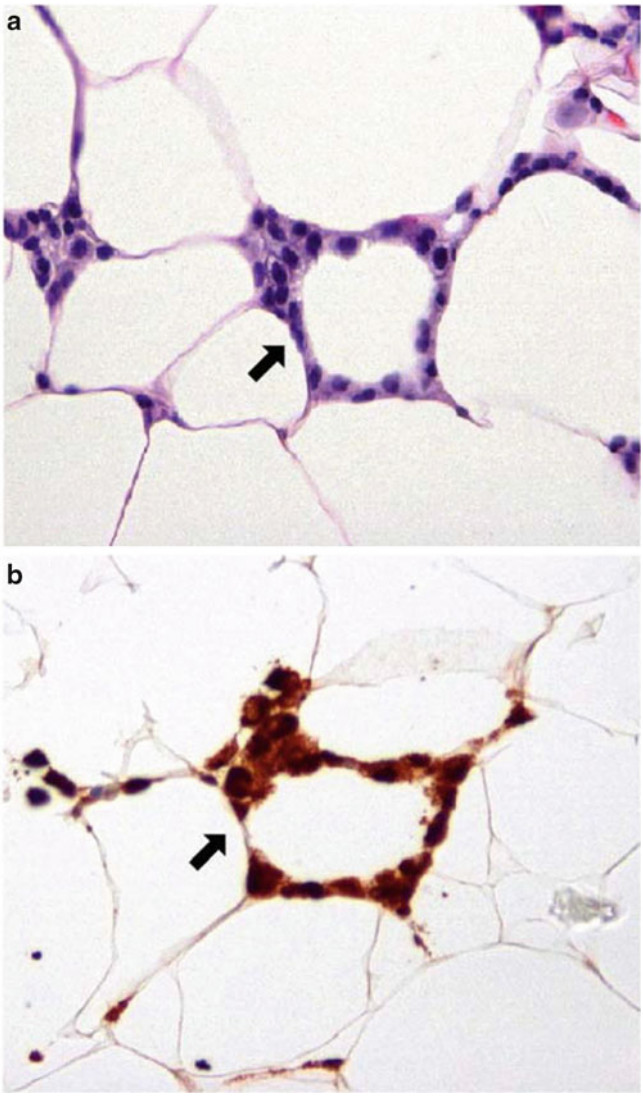

Fig. 8.2 Crown-like structure in human breast adipose tissue (CLS-B). (**a**) Hematoxylin and eosin stain. (**b**) Anti-CD68 immunostain identifies macrophages (reprinted from Morris P et al.: Cancer Prev Res Vol. 4(7), 2011:1021–9)

the extent of CLS-B had a stronger correlation than BMI with aromatase activity, implicating inflammation specifically, rather than obesity alone, as the key inducer of aromatase in the breast. These findings demonstrate an obesity-inflammation-aromatase axis that occurs in the female breast and provide a mechanistic link between obesity and the increased risk of ER-positive breast cancer in

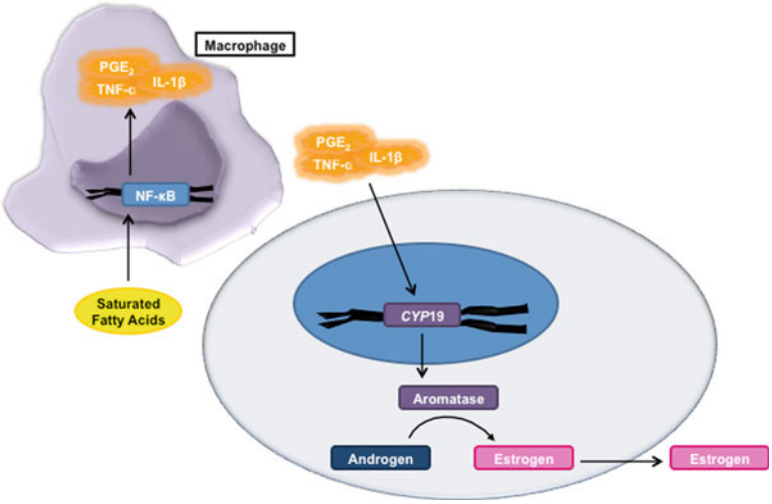

Fig. 8.3 Paracrine interactions between macrophages and other cell types establish an obesity-inflammation-aromatase axis in breast tissue of obese women and mice. *TNF*-α tumor necrosis factor-α; *IL-1*β interleukin-*1*β; PGE_2 prostaglandin E_2

postmenopausal women (Fig. 8.3). In addition, since many of these same proinflammatory mediators have been linked to worse outcome after breast cancer diagnosis, these findings might offer insights into the mechanism underlying this phenomenon (discussed below).

8.3.3 Estrogen

As noted, after menopause, the ovaries are no longer very active in hormone biosynthesis, and peripheral aromatization of adrenal-produced androgens in adipose tissue is largely responsible for estrogen production [11]. In the Health, Eating, Activity and Lifestyle (HEAL) study, which included 505 postmenopausal women with early-stage breast cancer, circulating estradiol levels were found to be 130 % higher in obese women compared to women with BMI < 22 ($p = 0.002$) [51]. As discussed above, in addition to increased adipose mass, obesity-related inflammatory factors appear to induce aromatase in adipose tissue contributing to increased estrogen production. Transcriptional regulation of aromatase expression is complex. Tissue-specific promoters within the *CYP19* gene give rise to unique mRNA species which are translated to the same aromatase enzyme [52]. Each of these promoters is regulated by distinct signaling patterns, and increased activation of promoters I.3, II, and I.7 is observed in breast cancer in contrast to mRNA derived from promoter I.4 in normal breast tissue [53]. Several inflammatory mediators,

including TNF-α, IL-6, and PGE_2, upregulate aromatase expression via specific promotor regions [50, 54]. Collectively, these observations offer critical insights into the mechanisms linking increased adiposity and breast carcinogenesis.

8.4 Clinical Implications

8.4.1 Obesity and Breast Cancer Stage

At the time of breast cancer diagnosis, obesity has been associated with more advanced stage of disease, manifested both by larger primary tumor size and greater propensity for involved lymph nodes [55–58]. In a study of nearly 1,000 women with newly diagnosed breast cancer, compared to underweight/normal weight women, those with BMI of 27.3 or greater were 1.5 times more likely to have stage II or beyond disease at diagnosis, characterized by tumors greater than 2 cm in maximum diameter, and/or those in which cancer has spread to axillary lymph nodes [58]. In a retrospective review of 88,346 consecutive screening mammograms, tumor size was found to be progressively larger with increasing adiposity. Mean tumor diameter was 11 mm in the lowest-weight cohort, compared with 19 mm in the heaviest-weight cohort ($p < 0.02$) [59]. Given the fact that obesity is a risk factor for the development of breast cancer, as expected, increasing body weight was also associated with an increased cancer detection rate. Finally, obesity has also been associated greater incidence of lymph node involvement at the time of diagnosis [55–57]. Consistent with this observation, in the study by Hunt et al., the proportion of patients with stage II disease or higher at diagnosis increased with increasing weight ($p = 0.046$) [59]. Collectively, these findings highlight the challenges in diagnostic imaging in obese and overweight women.

8.4.2 Challenges with Diagnostic Imaging

Increasing BMI presents a series of challenges for the diagnosis and treatment of breast cancer. In the developed world, most breast cancers are diagnosed at an early, and potentially curable, stage. Improvements in the early detection of breast cancer, leading to a decline in breast cancer mortality, have occurred partially as a result of screening mammography [60, 61]. This technique relies on 2-dimensional X-ray technology to detect calcifications or architectural distortion associated with preinvasive and small breast tumors, which may not otherwise be detectable by clinical examination. Obesity is associated with higher mammographic false-positive rates, which may result in unnecessary distress for patients and additional biopsies and other interventions. It has been suggested that greater amounts of adipose tissue within the breast of obese women interfere with the radiologist's interpretation of

mammograms. The largest study to date to examine the diagnostic performance of mammography reported more than a 20 % increased risk of false-positive findings in obese women compared with normal and underweight women [62]. In the study by Hunt et al., discussed above, increasing body weight was associated with increased biopsy and recall rates (the proportion of women undergoing screening who have to return for further assessment due to suspicious findings) [59].

8.4.3 Obesity and Breast Cancer Prognosis

In addition to being a risk factor for breast carcinogenesis and being associated with higher stage at diagnosis, obesity is a poor prognostic factor after breast cancer diagnosis [9, 13, 63–67]. This effect appears to be independent of both menopausal status and of tumor subtype [9, 63, 65–67]. Furthermore, this association cannot simply be explained by the observation that obese women are more likely to present with higher-stage disease at diagnosis as obesity has been shown to be independently associated with an increased risk of developing distant metastases and breast cancer-specific mortality [68].

Seminal observations from key studies are discussed below (Table 8.2). In a study of 3,924 younger women, age 20–54, Whiteman et al. report a significantly higher likelihood of breast cancer-related death in obese women [63]. At a median follow-up of 14.6 years, women with BMI $\geq$ 30 kg/m^2 were more likely to die from breast cancer than lean women (HR 1.34; 95 % CI, 1.09–1.65) [63]. The inclusion of younger women in this study suggests that obesity has broad implications for breast cancer prognosis, irrespective of other factors. Similar results linking obesity to adverse outcomes were seen in the largest cohort study published to date [9]. In this study of nearly half a million women, elevated BMI was associated with increased death rates for all cancers combined as well as for individual tumors including breast cancer [9]. Importantly, Calle et al. report an RR of death from breast cancer in overweight (BMI 25.0–29.9 kg/m^2) and extremely obese women (BMI 35–39.9 kg/m^2) of 1.34 (95 % CI, 1.23–1.46) and 1.70 (95 % CI, 1.33–2.17), respectively, compared to lean individuals ($p < 0.001$). This rigorous analysis provides the evidence in detail that increasing BMI is associated with breast cancer-specific mortality, as opposed to increased death from other obesity-related illnesses such as cardiovascular disease. Second, this important study shows clear evidence of a dose–response effect for increasing BMI on mortality.

In a large retrospective study from the Danish Breast Cancer Cooperative Group database of 53,816 patients, BMI data were available for 18,967 patients. As expected, obese patients (BMI $\geq$ 30 kg/m^2) were more likely to have high-risk disease at the time of breast cancer diagnosis (including larger primary tumors of higher grade and more positive lymph nodes) as compared to women with BMI < 25 kg/m^2. After 10 years, the risk of developing distant metastases was significantly increased by 46 % for patients with BMI > 25 kg/m^2 when compared to women with BMI < 25 kg/m^2 (HR 1.46; 95 % CI, 1.11–1.92; $p = 0.007$) [68]. In a

Table 8.2 Selected studies reporting BMI and outcomes after breast cancer diagnosis

References	Number	Median follow-up (years)	BMI in "normal" group (kg/m^2)	BMI in "overweight and obese" group (kg/m^2)	Outcome	Magnitude of effect	95 % confidence interval	*P* value	Adjustment variables
Whiteman et al. [63]	3,924	14.6	≤22.9	≥30	Breast cancer death	1.34[a]	1.09–1.65	<0.0001	Age, race, radiation therapy, history of benign breast disease, education level, menopausal status, stage
Majed et al. [65]	14,709	8	<25	≥30	Distant metastases	1.17[a]	1.04–1.31	<0.01	Age, tumor size, clinical node involvement, menopausal status, diagnosis year, ER/PR status, clinical tumor extension, nodal status, tumor grade
					OS	1.15[a]	1.02–1.29	<0.05	
Ewertz et al. [68]	18,967	7.1 (estimated potential)	<25	>30	Distant metastases (5–10 years)	1.46[a]	1.11–1.92	0.007	Age, menopausal status, tumor size, nodal status, deep fascia invasion, histologic type and grade, ER/PR status, protocol year, systemic therapy
					Breast cancer death (10+ years)	1.38[a]	1.11–1.71	0.003	

Berclaz et al. [69]	6,370	14	≤24.9	≥30	10-yr DFS 10-yr OS	1.10[a] 1.14[a]	1.10–1.20 1.03–1.27	0.04 <0.01	Menopausal status, nodal status, tumor size, vessel invasion, ER/PR status, tumor grade, treatment
Chen et al. [70]	4,989	3.8	18.5–25	≥30	Relapse and disease-specific mortality	1.44[a]	1.02–2.03	<0.05	Age, education, income, marital status, comorbidity, exercise, meat intake, vegetable intake, soy intake, time from diagnosis to enrollment, menopausal status, menopausal symptoms, treatment, stage, ER/PR status
Kroenke et al. [72]	5,204	9	21–22 21–22	25–29 >30	Breast cancer death (nonsmokers)	1.83[b] 1.96[b]	1.09–3.05 1.30–2.97	<0.001 <0.001	Age, OCP use, birth index, menopausal status, age at menopause, HRT use, protein intake, tumor size, nodal status, chemotherapy, tamoxifen

(continued)

Table 8.2 (continued)

References	Number	Median follow-up (years)	BMI in "normal" group (kg/m^2)	BMI in "overweight and obese" group (kg/m^2)	Outcome	Magnitude of effect	95 % confidence interval	*P* value	Adjustment variables
Calle et al. [9]	147,583	16	18.5–24.9	>40	Breast cancer death	2.12[b]	1.41–3.19	<0.001	Age, education, smoking status, physical activity, alcohol use, marital status, race, aspirin use, HRT, fat consumption, vegetable consumption
Daling et al. [14]	1,177	Up to 17 years	15.8–20.6	25.8–52.6	5-yr survival	1.7[a]	1.0–2.9	<0.05	Age, diagnosis year, tumor size, nodal status, ER/PR status, other tumor characteristics

OCP oral contraceptive pills; *HRT* hormone replacement therapy; *BMI* body mass index; *HR* hazard ratio; *RR* relative risk; *OS* overall survival; *DFS* disease-free survival; *ER* estrogen receptor; *PR* progesterone receptor; *yr* year

[a]Hazard ratio

[b]Relative risk

multivariate analysis adjusted for stage at presentation, obesity emerged as an independent predictor of distant recurrence. At 3 years of follow-up, risk of distant metastases began to show a clear trend of increasing risk with increasing BMI. At 10 years, the cumulative incidence of distant metastases for patients with a BMI of 30 kg/m^2 or more was 24.3 % (95 % CI, 22.1–26.5 %) compared to 20.1 % in patients with a BMI less than 25 kg/m^2 (95 % CI, 19.2–20.9 %). Strikingly, at 30 years of follow-up, the cumulative risks of breast cancer-related death were 57.2 % for patients with BMI of 30 kg/m^2 or more (95 % CI, 51.8–62.2 %) compared with 46.4 % for patients with BMI less than 25 kg/m^2 (95 % CI, 44.8–48.0 %). For overweight women with a BMI of 25–29 kg/m^2, mortality was also higher compared to women with BMI less than 25 kg/m^2 with a 53.4 % (95 % CI, 50.5–56.2 %) cumulative incidence of cancer-related death at 30 years follow-up. Overall the risk of dying as a result of breast cancer after 10 years was significantly increased for obese women compared with women with a BMI of less than 25 kg/m^2 (HR 1.38; 95 % CI, 1.11–1.71). Notably, the risk of dying as a result of other causes not related to breast cancer was also increased after 10 years in obese patients (HR 1.31; 95 % CI, 1.05–1.63).

These findings are consistent with several other population studies. In a French population of over 14,000 patients who were prospectively followed after early-stage breast cancer diagnosis, more advanced tumor at the time of diagnosis was associated with BMI over 30 kg/m^2 [65]. Additionally, obesity was associated with higher rates of distant relapse (HR 1.32; 95 % CI, 1.19–1.48), shorter disease-free interval (HR 1.20; 95 % CI, 1.08–1.32), and shorter OS (HR 1.43; 95 % CI, 1.28–1.60). The International Breast Cancer Study Group reported similar findings in over 6,000 patients with a median follow-up time of 14 years [69]. Women with BMI of 30 kg/m^2 or greater had a 10 % higher risk of distant relapse ($p=0.04$) and 14 % higher risk of death ($p<0.01$) when compared to women with normal BMI. Obesity was associated with worse 10-year disease-free survival (DFS) (HR 1.17; 95 % CI, 1.07–1.28; $p<0.01$) and OS rates (HR 1.25; 95 % CI, 1.13–1.38; $p<0.01$) when compared to women of normal weight. Additionally, elevated BMI was associated with more advanced tumor at the time of breast cancer diagnosis (specifically tumor size of 2 cm or greater), as well as greater incidence of lymph node involvement. Furthermore, Daling et al. report an association between larger tumors in obese women and higher tumor proliferation rate, characterized by the Ki-67 proliferative index [14]. In this population-based study of just over 1,000 women with breast cancer identified from the Cancer Surveillance System of the Surveillance, Epidemiology and End Results (SEER) cancer registry, the heaviest quartile of women (BMI of 25.8 kg/m^2 or greater) were 2.5 times more likely to succumb to breast cancer-related death than women in the lowest quartile of BMI (20.6 kg/m^2 or less) (95 % CI, 1.6–3.9). Women in the heaviest quartile presented with larger tumor size (2 to less than 5 cm odds ratio (OR) 2.2; 95 % CI, 1.5–3.1, and 5 cm or greater OR 2.7; 95 % CI, 1.5–4.8). The authors suggest that this may be related to faster growing tumors in heavier women. Compared to the lowest quartile of body weight, tumors 2 cm or larger in the obese women were more likely to have a Ki-67 proliferative index of 25 % or greater (OR 1.9; 95 % CI, 1.1–3.3) and a high mitotic rate (OR 1.6; 95 % CI, 0.9–3.1). Notably, lymph node status did not vary by BMI in this study.

8.4.4 Impact of Weight Change After Breast Cancer Diagnosis

In addition to the negative effect of pre-morbid obesity on cancer-specific mortality, weight change after breast cancer diagnosis may also have prognostic significance [70–72]. Consistent with other reports, in a study of 5,042 patients identified through the population-based Shanghai Cancer Registry, women who were obese 1 year prior to cancer diagnosis in this study had 1.6 times higher total mortality than women with a normal BMI. As expected, shorter OS and DFS associated with obesity was independent of hormone receptor status. Critically, women who gained 5 kg (approximately 10 lb) or more within 18 months of breast cancer diagnosis had higher breast cancer-specific (HR 1.90; 95 % CI, 1.23–2.93) and total mortality (HR 1.71; 95 % CI, 1.12–2.60) rates compared with women who maintained their weight within 1 kg (approximately 2 lb). Similar results were seen in the Nurses' Health Study, which included 5,204 patients diagnosed with non-metastatic breast cancer at the time of accrual. In this study, nonsmoking women whose BMI increased more than 2.0 kg/m^2 (approximately 11 lb for a woman of an average height of 64 in.) during a median follow-up time of 9 years after diagnosis had an elevated risk of death as a result of breast cancer compared with women whose weight remained stable (RR 1.64; 95 % CI, 1.07–2.51) [72]. Critically, this association was particularly accentuated in premenopausal vs. postmenopausal women. Weight gain after diagnosis was also associated with worse outcomes in premenopausal women undergoing treatment for early breast cancer in a study by Camoriano et al. [71]. In this study of 646 women with node-positive disease, patients who received adjuvant chemotherapy gained more weight compared to those who did not [71]. Among women treated with chemotherapy, weight change at 60 weeks was greater for women who were premenopausal (median gain 5.9 kg) than postmenopausal (median gain 3.6 kg) ($p<0.001$). Among premenopausal women treated with chemotherapy, those who surpassed the median weight gain at 60 weeks had a 1.5 times higher risk of distant relapse (covariate $p=0.17$) and 1.6 times greater risk of death (covariate $p=0.04$) at median follow-up of 6.6 years. In addition, similar trends in increased relapse and shorter OS were seen in postmenopausal women, although these results did not reach statistical significance. Conversely, a stronger association between weight gain and poorer outcomes by menopausal status was not observed in the Shanghai study [70]. Although definitive conclusions from these studies are limited by their design, these findings collectively suggest that weight gain in the first year after breast cancer diagnosis may have a deleterious effect on breast cancer-specific outcomes. In some studies, these effects appear to be independent of the negative effects of preexisting obesity [70, 72]. Although the effect of long-term weight gain cannot be delineated from these studies, it is likely to be similarly detrimental, given the known biological consequences of weight gain.

There are studies that do not confirm any negative effect on breast cancer-specific outcomes associated with weight gain after diagnosis [73, 74]. In a study investigating the psychologic and behavioral functioning related to exercise and eating habits in women undergoing adjuvant chemotherapy for breast cancer, an average weight

gain of approximately 6 kg at 2 years follow-up was reported. However, this weight gain did not translate into a greater risk of breast cancer recurrence, albeit in a very limited sample ($n=32$) [73]. Similarly, weight gain up to 4 years after breast cancer diagnosis was not associated with increased risk of relapse or death in a larger prospective cohort study of 1,692 breast cancer survivors [74]. It is important to note that consistent with other studies, obesity 1 year before diagnosis was associated with increased risk of death from any cause (HR 1.6; 95 % CI, 1.1–2.3) and an increased risk of breast cancer-related death (HR 1.6; 95 % CI, 0.9–2.7), although the latter result was not statistically significant. Therefore, while it is clear that obesity is associated with worse breast cancer-related outcomes, not all studies demonstrate a significant effect for weight gain after breast cancer diagnosis. Nonetheless, available evidence shows consistent trends towards a negative effect of weight gain on breast cancer outcomes.

8.5 Obesity and Breast Cancer Treatment

8.5.1 Breast Surgery

Obesity is associated with greater rates of complications after breast cancer surgery and breast reconstruction [75–85]. In a retrospective analysis of potential factors that affected postoperative hospital stay for 73 patients who underwent modified radical mastectomy, patients with stays under 5 days had a significantly lower BMI than that of patients who required a longer hospitalization, although obesity was not found to be an independent predictor of longer hospital stay in multivariate analysis [75]. In a study conducted by the National Surgical Quality Improvement Program Patient Safety in Surgery, El-Tamer et al. reported morbidity and mortality data for 1,660 women undergoing mastectomy and 1,447 women undergoing lumpectomy with an axillary procedure [76]. Wound infection was the most frequent morbid complication and occurred at a higher rate in patients undergoing mastectomy (4.34 %) vs. lumpectomy (1.97 %). Importantly, BMI greater than 30 kg/m^2 was an independent predictor of wound infection. With specific regard to axillary lymph node procedures, lymphedema (swelling of the arm) is a well-known complication after axillary node dissection and may occur years after the procedure. In a report of 137 patients with breast cancer who underwent sentinel lymph node dissection, of whom 85 underwent immediate completion axillary lymph node dissection, BMI greater than 30 kg/m^2 was associated with a greater risk of developing lymphedema within 2 years of surgery compared to patients with BMI less than 25 (OR 2.93; 95 % CI, 1.03–8.31; $p=0.003$) [77]. Recently, neo-adipogenesis and adipocyte hypertrophy have been implicated in the development of lymphedema in animal models [86, 87]. Using a mouse tail model, Zampell et al. demonstrated a twofold increase in fat thickness ($p<0.01$) in response to lymphatic fluid stasis [86]. Additionally, a marked inflammatory response consisting of a fivefold increase in

CD45+ mononuclear cells ($p<0.001$) and a greater than threefold increase in F4/80-stained monocytes and macrophages ($p<0.001$) in the subcutaneous mouse-tail fat was associated with adipogenesis in response to lymphatic fluid stasis. In part two of this study, Aschen et al. went on to demonstrate concurrent upregulation of several adipogenic factors including adiponectin, CCAAT/enhancer binding protein-α (CEBP-α), and peroxisome proliferator-activated receptor-γ (PPAR-γ) in response to lymphatic fluid stasis [87]. Both CEBP-α and PPAR-γ are key transcription factors involved in adipogenesis and promote adipocyte differentiation. Therefore, adipogenesis, adipocyte hypertrophy, and upregulation of adipogenic factors appear to be integrally involved in the development of lymphedema and in turn promote an inflammatory response within subcutaneous fat.

Breast reconstruction after breast cancer surgery may also be challenging in obese and overweight patients. In a retrospective analysis of 1,195 abdominal flap reconstruction procedures performed in 952 patients, Mehrara et al. reported that obesity, defined as greater than 25 % of ideal body weight, was the major independent predictor of postoperative complications with up to a threefold increased incidence of adverse events [78]. Specifically, in patients who underwent free transverse rectus abdominis myocutaneous (TRAM) flap reconstruction, BMI ≥ 30 kg/m^2 was significantly associated with partial flap loss (OR 2.6, $p<0.03$), donor-site complications (OR 3.0, $p<0.03$), and hernia and/or laxity (OR 2.5, $p=0.05$). In univariate analysis, obesity was associated with fat necrosis, a benign complication of surgery or trauma, but this association was not significant on multivariate analysis. Strikingly, obesity was associated with significantly increased risk of arterial thrombosis (OR 1.7, $p=0.06$) although this did not translate to a higher rate of flap loss. It has been hypothesized that increased rates of arterial thrombosis in obese patients may be related to the increased difficulty of deep microsurgical anastomosis within the axilla. Similar findings were reported by Chang et al. in 718 patients who underwent TRAM flap breast reconstruction [85]. Obese and overweight patients had higher rates of total flap loss, flap hematoma, flap seroma, mastectomy skin flap necrosis, donor-site infection, donor-site seroma, and hernia compared with patients of normal weight. These results suggest that morbidly obese patients are at very high risk of flap failure and other complications and that procedures such as TRAM flap breast reconstruction should be avoided in such patients. In an attempt to minimize these complications, an alternative approach to breast reconstruction in overweight and obese patients might be the use of a deep inferior epigastric perforator flap [88].

Elevated BMI can also complicate other reconstruction surgical techniques. Obesity is an independent risk factor for the development of perioperative complications in patients undergoing expander/implant reconstruction (OR 1.8; 95 % CI, 1.1–3.0; $p=0.02$) [80]. These complications include mastectomy flap necrosis, seroma/hematoma, infection, failed expansion, and expander/implant exposure.

It is important to note, however, that the overall rate of complications associated with reconstructive surgery is low and that the majority of overweight and even obese patients complete reconstruction successfully. Most investigators agree that overweight and obese women should be counseled and informed about risks and benefits of reconstruction, including higher complication rates associated with

elevated BMI despite its overall safety, to aide in their choice of approach [78, 85]. It is useful to note that in a study of patient satisfaction in US patients undergoing breast reconstruction, obese patients with expander/implants were significantly less satisfied with aesthetic outcome than normal weight patients (OR 0.14, $p=0.02$) [83]. There was no significant difference in aesthetic satisfaction between obese and normal weight women undergoing TRAM flap reconstruction. Taken together, these data underscore the importance of careful patient counseling, including a detailed discussion of the risks and benefits involved.

8.5.2 Breast Radiation

In addition to greater rates of postoperative complications, increasing BMI and large breast size have been associated with greater toxicity following adjuvant radiotherapy [89–94]. Pragmatically speaking, very morbid obesity may preclude the use of radiation therapy given the upper weight limit that currently manufactured treatment tables are capable of supporting. For those who do undergo radiation, several studies have demonstrated higher complication rates in overweight and obese patients. Allen et al. report a case control study of women who underwent adjuvant radiotherapy after lumpectomy or mastectomy followed by chemotherapy [89]. In total, 200 patients received radiotherapy to the breast and/or chest wall, with or without inclusion of regional lymph nodes. This analysis identified BMI as the only significant factor associated with the development of radiation pneumonitis (multivariate $p<0.01$). The mean BMI for 14 (7 %) patients who developed radiation pneumonitis was 31.2 vs. 26.3 for patients whose course was not complicated by radiation pneumonitis. These findings could be explained by heterogeneity in dose delivery as a result of increased chest wall separation in obese patients, resulting in higher maximum radiation dose delivered. Indeed, in this study, maximum delivered dose correlated with BMI. Additionally, the known link between obesity, asthma, and other pulmonary comorbidities may predispose this population to development of radiation pneumonitis. It is also possible that circulating proinflammatory mediators associated with asthma and other atopic conditions may contribute to the increased radiation pneumonitis risk. For example, elevated circulating IL-6 and interleukin-1α (IL-1α) levels prior to radiotherapy have been identified as markers of increased radiation pneumonitis risk [95, 96]. As discussed above, elevated circulating IL-6 levels are found in overweight and obese individuals. Therefore, it is possible that obesity-related inflammation may be a key mechanism underlying the increased risk of radiation pneumonitis in obese patients who undergo breast irradiation.

As with breast surgery, elevated BMI increases the risk of ipsilateral arm edema following radiotherapy [90]. In a series of 282 patients with stage I or II breast cancer who underwent breast surgery followed by radiation at Memorial Sloan-Kettering Cancer Center, Werner et al. reported BMI as the most important factor associated with posttreatment arm edema [90]. Larger breast size was also a

significant independent risk factor in the development of this complication. Greater breast volume has also been associated with more fibrosis and poorer cosmetic outcome [91] as well as increased skin toxicity [92] following radiotherapy. Finally, increased radiation dose heterogeneity within larger-volume breasts may result in increased maximum dose, or "hot spots" within the radiated breast, although the incidence of this problem with more modern radiotherapy techniques is less clear [93, 94].

8.5.3 Systemic Therapies

Irrespective of the possible interaction between BMI and cancer biology, overweight and obese patients are more likely to have less favorable outcomes with adjuvant chemotherapy than women of normal weight. A number of contributory factors have been identified and largely fall into two categories—underdosing and decreased efficacy. Most cytotoxic chemotherapy is dosed based on body surface area, which, like BMI, is calculated based on height and weight. Many clinicians limit chemotherapy dosing for some drugs in obese patients, by using a maximum weight, due to concerns regarding an increased risk of toxicity in obese patients if treated with doses based on actual body weight. In a US survey of 1,243 community-based practitioners that extracted data from 20,799 early-stage breast cancer patients, Lyman et al. observed that due to dose reductions and/or treatment delays, 55.5 % of all patients received a relative dose intensity of less than 85 %, a threshold that has been linked to inferior efficacy [97]. In this study, increasing BMI was associated with greater dose reductions ($p<0.001$) although there were no differences in the need for unplanned dose modifications due to toxicity. Importantly, body surface area greater than 2 m^2 emerged as an independent predictor of dose reduction (OR 1.71; 95 % CI, 1.59–1.85; $p<0.001$). Consistent with these observations, another community-based study of 9,672 women treated with anthracycline-based chemotherapy found increasing BMI to be independently associated with first-cycle dose reduction, i.e., the administration of doses below that from the calculated body surface area [98]. First-cycle dose reduction of 10 % or more was seen in 11 %, 20 %, and 37 % of overweight, obese, and severely obese women, respectively, compared with 9 % of normal weight women. After initial dose reduction, it was uncommon (3 % patients) for dose escalation (to a level based on actual weight) to occur in subsequent cycles for obese and overweight patients even if chemotherapy was well tolerated. Contrary to concerns regarding excessive toxicity, an increased hospital admission rate for febrile neutropenia was associated with higher administered dose rather than being overweight or obese. In fact, severe obesity (BMI $\geq$ 35) was associated with lower likelihood of hospitalization for febrile neutropenia (OR 0.61; 95 % CI, 0.38–0.97), although this could have been because the majority of these patients received attenuated doses.

Other studies have also demonstrated that overweight and obese patients who receive doses based on actual body weight do not experience excessive toxicity

Table 8.3 Cytotoxic chemotherapy dosing for obese adult patients with cancer

American Society of Clinical Oncology clinical practice guideline
Use full weight-based chemotherapy dosing for obese patients, particularly when treatment intent is curative
Respond to treatment-related toxicity similarly for both obese and nonobese patients
Dose reductions, if needed, should be done similarly for obese and nonobese patients; consider resuming full weight-based dose on subsequent cycles if feasible
Fixed dosing should only be used for select agents (e.g., carboplatin, bleomycin)

compared with women of normal weight [99–104]. For example, in the Cancer and Leukemia Group B (CALGB) 8541 randomized trial, which examined the schedule and dose of adjuvant anthracycline-based chemotherapy in 1,435 women with node-positive breast cancer, no evidence of increased toxicity was experienced by obese women who received chemotherapy based on actual body weight [100]. In this retrospective analysis, obese women who received cycle 1 doses below 95 % of the calculated weight-based dose had shorter DFS than women who received within 5 % of actual body weight-based doses. The risk of treatment failure for women in the 5 % group was two-thirds the risk of obese women whose dose was reduced below 95 %, although this effect did not reach statistical significance in multivariate analysis (HR 0.67: 95 % CI, 0.38–1.20; $p = 0.25$). Taken together, these findings suggest that dosing of most commonly used drugs based on actual body weight does not increase toxicity and may result in improved outcomes in obese patients. The importance of these observations led to a new guideline from an expert panel convened by the American Society of Clinical Oncology (ASCO) [105]. The main recommendations from this panel include the dosing of chemotherapy by actual body weight in the treatment of obese cancer patients, particularly when prescribed with curative intent (Table 8.3) [105].

8.5.4 Preoperative Chemotherapy in Obese Patients

Apart from the underdosing of chemotherapy in overweight and obese patients, it appears that current preoperative systemic therapies may be less effective in these patients compared with women of normal weight [68, 99, 106–108]. In a study of over 1,000 breast cancer patients who received neoadjuvant (preoperative) chemotherapy at the MD Anderson Cancer Center, overweight and obese patients were less likely to achieve a pathologic complete response (pCR) at the time of breast surgery than normal-weight patients (OR 0.67; 95 % CI, 0.45–0.99) [106]. However, this observation should be interpreted cautiously as chemotherapy dosing for these patients could not be verified in this study. Given the prevalence of underdosing for overweight and obese patients, the lower rates of pCR may be a function of chemotherapy exposure. In a different study, 307 Chinese women with breast cancer

treated at the Shanghai Cancer Hospital all underwent neoadjuvant chemotherapy dosed by actual weight [107]. Women with a BMI of 25 kg/m^2 or greater were less likely to achieve a pCR at the time of breast surgery after neoadjuvant chemotherapy compared to women with BMI less than 25 kg/m^2 (OR 0.45; 95 % CI, 0.22–0.94; $p=0.03$). Notably, BMI was identified as an independent predictor of pCR after neoadjuvant chemotherapy in postmenopausal and hormone receptor-negative subgroups ($p=0.004$ and $p=0.038$, respectively).

8.5.5 Postoperative Chemotherapy in Obese Patients

Less favorable responses to adjuvant (postoperative) breast cancer treatment have also been reported [68, 99, 108]. In the Danish Breast Cancer Cooperative Group study, Ewertz et al. report shorter OS after adjuvant therapy that became evident after 10 years of follow-up in patients with BMI of 30 kg/m^2 [68]. Again, in this study, chemotherapy dosing was not addressed limiting our ability to interpret the results. However, several other studies have reported decreased OS for obese and overweight patients after completing adjuvant therapy [99, 108]. Bastarrachea et al. report a greater risk of disease recurrence after adjuvant chemotherapy dosed by actual body weight in obese vs. nonobese patients (HR 1.33; 95 % CI, 1.05–1.68) [99]. In a post hoc analysis of the Breast International Group (BIG) 02-98 study, which was a randomized phase III trial in which docetaxel was added to adjuvant anthracycline-based chemotherapy, BMI of 30 kg/m^2 or greater was associated with shorter 5-year OS and DFS (HR 1.34, $p=0.013$, and HR 1.20, $p=0.041$) [108]. Taken together, these studies suggest a decreased efficacy of chemotherapy in obese patients; however, underdosing remains a potential confounder. As the ASCO clinical practice guidelines for the dosing of chemotherapy in obese patients become increasingly incorporated into clinical practice, the efficacy of chemotherapy in overweight and obese patients will likely become more apparent.

8.5.6 Obesity and Endocrine-Targeted Therapy

For patients with ER-positive breast cancer, endocrine therapy, which aims to disrupt the tumor supply of estrogen or change the receptor-ligand interactions, is an important aspect of treatment. For obese patients, concerns about the dosing and resultant efficacy have been raised with regard to the use of endocrine therapy [109]. Importantly, in the randomized phase III Arimidex, Tamoxifen, Alone or in Combination (ATAC) trial, proportionately worse outcomes were seen in obese vs. lean women who were treated with the aromatase inhibitor, anastrozole [109]. Treatment with anastrozole was less efficacious in women with BMI $\geq$ 35 kg/m^2 when compared to women with BMI $<$ 23 kg/m^2 in multivariate analysis (HR 1.53; 95 % CI, 1.01–2.32; $p=0.001$). In contrast, tamoxifen appeared to be equally effective across all BMI groups. While recurrence rates were overall lower in the postmenopausal women treated with anastrozole compared to tamoxifen, the benefit of

anastrozole was greater in leaner women. The adjusted HR comparing anastrozole with tamoxifen for distant plus local recurrences in the lowest BMI group was 0.64 (95 % CI, 0.45–0.91) in contrast to 0.84 (95 % CI, 0.61–1.14) for the heaviest women.

Reassuringly, use of the selective estrogen receptor modulator (SERM), tamoxifen, was also found to be equally efficacious in obese and nonobese women with node-negative ER-positive breast cancer in a cohort of 3,385 women enrolled in the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14 protocol [110]. However, obesity was found to be associated with increased risk of contralateral breast cancer, second primary cancers, and increased overall mortality. Therefore, these findings are consistent with the hypothesis that there is a worsened prognosis for obese patients, but suggest that tamoxifen may be relatively more effective in obese women. To be precise, the efficacy advantages of aromatase inhibition over tamoxifen appear to diminish with increasing weight. However, anastrozole was not inferior to tamoxifen, and, in particular among overweight patients, its toxicity profile may make it preferable despite the lack of superior efficacy in the obese patients.

In terms of overall outcomes, Sparano et al. reported inferior DFS, defined as the length of time after completing cancer treatment that a patient lives without any signs or symptoms of that cancer, and OS, defined as the proportion of patients still alive after starting cancer treatment, in obese vs. lean women with operable breast cancer who received chemotherapy followed by endocrine therapy with tamoxifen [111]. In this pooled outcomes analysis of three adjuvant trials conducted by the Eastern Cooperative Oncology Group (ECOG), all patients received anthracycline-based chemotherapy dosed by actual body weight. There were no significant differences in dose delivered to obese vs. nonobese patients, except in the group that received weekly or every 3-week paclitaxel. In one of the three included ECOG trials (E1199), patients received either tamoxifen or tamoxifen followed by an aromatase inhibitor after adjuvant chemotherapy. In multivariate modeling, BMI > 30 kg/m^2 vs. BMI < 30 kg/m^2 was associated with inferior DFS (HR 1.24; 95 % CI, 1.06–1.46; $p = 0.0079$), inferior OS (HR 1.37; 95 % CI, 1.13–1.67; $p = 0.0015$), and inferior breast cancer-specific survival (BCSS) (HR 1.40; 95 % CI, 1.11–1.76; $p = 0.0042$) in women with hormone receptor-positive, human epidermal growth factor receptor-2 (HER2)-negative (or unknown) disease. Similar findings were observed in the E5188 trial in which premenopausal women with ER-positive breast cancer received no endocrine therapy, ovarian suppression, or ovarian suppression plus tamoxifen after chemotherapy. Survival rates were significantly worse for obese patients (DFS HR 1.41, 95 % CI 1.19–1.67, $p < 0.0001$; OS HR 1.51, 95 % CI 1.24–1.83, $p < 0.0001$; BCSS HR 1.54, 95 % CI 1.26–1.88, $p < 0.0001$). In sum, these findings demonstrate worse survival rates in obese women treated with chemoendocrine therapy, including tamoxifen, for hormone receptor-positive breast cancer, but they do not indicate a superior treatment approach for such patients.

A likely explanation for the reduced efficacy of hormone therapies in obese women is that elevated estrogen levels, which are commonly seen in obese patients, may not be adequately suppressed by aromatase inhibitors such as anastrozole. In support of this, using sensitive estradiol assays, a recent study has demonstrated

that higher levels of circulating estrogens are commonly seen in obese women [112]. In this study, Folkerd et al. reported a positive correlation between circulating estradiol level and increasing BMI ($\rho = 0.57$, $p < 0.001$), noting that values were almost 3 times higher in women with BMI > 35 kg/m^2 compared to women with BMI < 25 kg/m^2. Suppression of estrogens was greater with the newer aromatase inhibitor, letrozole, compared with anastrozole. This raises the question of whether dose escalation or other strategies, such as using more potent aromatase inhibitors, would be useful in these patients. Prospective clinical trials are needed to adequately address these issues and develop a risk-stratified approach for obese patients. Another possible explanation for the reduced efficacy of aromatase inhibitors in the obese is ligand-independent activation of ER-α. This could occur for a variety of reasons including elevated levels of circulating IGF-1 or the effects of proinflammatory mediators and warrants investigation.

Additionally, dosing adequacy remains a concern for obese patients treated with molecular and other targeted therapies (e.g., the monoclonal antibody trastuzumab and the tyrosine kinase inhibitor lapatinib). Several of these agents are delivered as predetermined doses that are not weight based (e.g., lapatinib). Appropriate dosing of these agents is a subject of ongoing investigation and has not yet been addressed by current guidelines [105].

8.6 Interventional Strategies and Future Directions

Having reviewed the pathogenesis, poor outcomes, and treatment implications conferred by increased adiposity on women with breast cancer, we now address potential strategies to reduce these risks. These approaches range from behavioral interventions, including lifestyle modifications such as diet and exercise, to pharmaceutical interventions that target the pathophysiology of dysregulated metabolism and obesity-related inflammation. In terms of breast cancer prevention, there is extensive interest in research to better characterize specific points along the route of carcinogenesis where interventions may be beneficial. Similarly, much work is being done to understand the reasons for poorer prognosis after breast cancer diagnosis, many of which have been discussed above, in order to determine specific interventions that may be used as an adjunct to cancer-directed therapy to improve outcomes.

8.6.1 Weight Loss

Weight loss or maintenance of a normal BMI, achieved by dietary modification and/or physical activity, has been associated with improved outcomes after breast cancer diagnosis [113–121]. Much of these data, however, are observational, which will hopefully set the stage for the development of adequately powered randomized clinical trials. In a prospective observational study reported by Holmes et al., exercise was associated with improved outcome after breast cancer diagnosis [117].

Nearly 3,000 nurses with early breast cancer (stage I to III) who enrolled in the Nurses' Health Study were surveyed about their levels of physical activity and were followed for breast cancer mortality [117]. Women whose physical activity was the equivalent of walking 3–5 h per week at an average pace had the greatest benefit in terms of breast cancer-related death (RR 0.50; 95 % CI, 0.31–0.82). A consistent association between physical activity and reduced breast cancer mortality has been reported in similar observational studies [118–120].

With regard to the effects of dietary interventions on breast cancer-specific outcomes, the Women's Intervention Nutrition Study (WINS) randomly assigned women to a low-fat diet, in which fat accounted for 20 % of total calories, vs. normal diet [121]. Women in the low-fat diet group lost an average of 6 lb and, after 5 years of follow-up, had a 24 % reduction in the risk of relapse (HR 0.76; 95 % CI, 0.60–0.98; $p=0.034$). Although studies have failed to confirm a consistent benefit of diet on breast cancer-specific outcomes, the results of this randomized trial do suggest that diet is one important strategy to reverse the negative effects of obesity on breast cancer mortality. Importantly, reduction in breast cancer recurrence was greater in women with hormone receptor-negative breast cancer than in women with hormone receptor-positive disease. This again suggests that estrogen-independent mechanisms are involved in regulating the growth of breast cancer, such as insulin signaling as discussed above.

Importantly, several studies demonstrate significant associations between weight loss and/or dietary modification with improvements in circulating biomarkers that have been implicated in breast cancer development and progression. A weight loss of 7.1 % after consumption of a hypocaloric diet, defined by a 250–350 kcal/day deficit, for 6 months has been associated with significant decreases in circulating levels of leptin and insulin [122]. In a recent study, weight loss of 5 % or more within 12 months via caloric restriction and/or exercise was associated with significant reductions in circulating markers of inflammation including high-sensitivity C-reactive protein (hsCRP), IL-6, and serum amyloid A [123]. Furthermore, reductions in circulating estradiol levels and elevation of SHBG levels have been associated with diet and exercise [115]. In the Nutrition and Exercise for Women (NEW) trial, circulating estradiol decreased by 20.3 % ($p<0.001$) and SHBG increased by 25.8 % ($p<0.001$) in postmenopausal women with BMI > 25 kg/m^2 who were randomized to reduced-calorie diet and moderate- to vigorous-intensity aerobic exercise [115]. Reduced serum levels of insulin, CRP, and leptin and increased adiponectin were also observed. Additionally, TNF-α levels and insulin release after glucose challenge significantly fell after diet-induced weight loss [44]. The magnitude of this decline is associated with baseline body weight, again suggesting that obese women in particular benefit most from weight loss. Reductions in serum IL-6 and CRP and a rise in adiponectin levels have also been associated with diet-induced weight loss [124, 125]. Additionally, reduced levels of inflammatory markers within subcutaneous white adipose tissue have been demonstrated after weight loss [37]. Collectively, these findings suggest that diet-induced weight loss can have desirable effects on inflammatory mediators, known to be involved in breast cancer development and progression.

With our current understanding of obesity-related inflammation and breast cancer risk and prognosis, these changes induced by weight loss help elucidate specific pathogenic targets for possible intervention. Importantly, the feasibility of long-term weight loss interventions in cancer survivors was recently demonstrated in the Reach Out to Enhance Wellness (RENEW) trial [114]. In this study, 641 overweight and obese patients with a history of locoregional colorectal, breast, and prostate cancer underwent diet and exercise counseling via mailed printed material and telephone. Improvements in diet quality, physical activity, and BMI were demonstrated at 2 years of follow-up. Therefore, the observational data associating weight loss with improved survival and biomarkers of breast cancer prognosis, coupled with the durability of weight loss intervention, set the stage for a prospective randomized trial investigating the effect of dietary and exercise interventions on breast cancer outcomes in selected subsets of patients, using biomarkers known to be dysregulated in overweight and obese women with breast cancer.

8.6.2 Medications

The use of several medications has been associated with attenuation of processes involved in breast cancer development and progression. One attractive investigational agent is metformin, which has beneficial effects on insulin levels. Use of metformin has been suggested to both reduce the risk of breast cancer and improve prognosis. In the preclinical setting, when human breast adipose stromal cells are treated with metformin, aromatase expression is inhibited via activation of AMPK [126]. Furthermore, metformin has been demonstrated to downregulate breast-specific aromatase expression by inhibiting *CYP19* gene promoters I.3 and II [127]. Goodwin et al. reported a small clinical trial of 22 women with early-stage breast cancer in which treatment with 6 months of metformin resulted in significant reduction in fasting insulin level [128]. In addition, diabetic patients treated with metformin were found to achieve pCR at the time of breast surgery more often than diabetic patients who were not treated with metformin after undergoing neoadjuvant chemotherapy for early-stage breast cancer [129]. In a retrospective analysis, 2,529 patients who received neoadjuvant chemotherapy were identified as nondiabetic patients ($n=2{,}374$), diabetic patients taking metformin ($n=68$), and diabetic patients not taking metformin ($n=87$). Patients taking metformin had a significantly higher proportion of pCR at the time of surgery (24 %; 95 % CI, 13–34 %) compared to diabetic patients not taking metformin (8.0 %; 95 % CI, 2.3–14 %) and nondiabetic patients (16 %; 95 % CI, 15–18 %; $p=0.02$). In multivariate analysis adjusting for diabetes status, neoadjuvant metformin use was an independent predictor of pCR (OR 2.95; 95 % CI, 1.07–8.17; $p=0.04$). This clinical evidence in conjunction with the known preclinical antiproliferative effects of metformin has generated significant interest in investigating metformin in ongoing randomized, prospective clinical trials. A significant challenge in determining the optimum use of metformin in breast cancer will be developing biomarkers to select the patients most likely to benefit from this agent.

Use of nonsteroidal anti-inflammatory drugs (NSAIDs) for the prevention of breast cancer is an attractive approach given the role of inflammation in breast carcinogenesis and the reduced risk of other tumors such as colon cancer associated with NSAID use [130–133]. Epidemiologic data, however, are conflicting with regard to the benefits of aspirin and COX-2 inhibitors in breast cancer prevention. In a large cohort analysis of the Nurses' Health Study, Zhang et al. prospectively observed 84,602 postmenopausal women for the development of breast cancer with a follow-up period of 28 years [134]. A total of 4,734 cases of invasive breast cancer were identified, and no statistically significant difference was observed between regular aspirin users and nonusers. The lack of benefit with aspirin consumption was independent of tumor phenotype, dose, or either past or current aspirin use. The authors also examined use of acetaminophen and other NSAIDs and similarly failed to demonstrate any risk reduction. Notably, however, a moderately lower risk of hormone receptor-positive breast cancer was observed in women who consumed six or more tablets of aspirin per week for at least 10 years. While this prospective study provides important information, the question remains as to why a preventative effect associated with NSAIDs is observed in several other epidemiologic studies [135, 136]. Aside from study design limitations, one possible explanation for this inconsistency is the heterogeneity of patients studied. It is possible that a specific population, such as those with breast inflammation and elevated tissue levels of COX-2, is most likely to derive particular benefit from the use of NSAIDs and specific COX-2 inhibitors [137].

A number of other potential therapeutic strategies and targets are at different stages of evaluation. Statins possess anti-inflammatory properties and are the subject of ongoing investigation as data regarding their use and impact on cancer development and evolution are currently conflicting [138–146]. In preclinical ER+ breast cancer cell lines, use of small molecule MEK inhibitors has been shown to counter the anti-apoptotic effects of IGF-1 [147]. Other approaches to IGF-1 inhibition include the use of antibodies or small molecules directed against the IGF-1 receptor [148–150].

8.7 Conclusion

The increasing worldwide problem of overweight and obesity has critical implications for breast cancer. First, increasing adiposity is a risk factor for the development of hormone receptor-positive breast cancer in postmenopausal women. Second, irrespective of breast cancer subtype, obese women have a worse prognosis after breast cancer diagnosis. As obesity rates continue to rise, we are likely to see a concomitant rise in both breast cancer incidence and mortality rates, unless this trend can be reversed. Overweight and obesity have important implications for the local treatment of breast cancer, including surgery and radiotherapy, as well as for the use of systemic therapies such as chemotherapy, endocrine therapy, and novel targeted agents. Critical to the development of effective strategies to prevent

increasing mortality with greater obesity prevalence is an understanding of the biological mechanisms by which rising BMI promotes breast cancer development and progression. As such, a wealth of recent studies have identified several mechanisms, which mediate this link, including dysregulated metabolism, insulin resistance, altered adipokine levels, as well as the emerging role of chronic inflammation of the breast. Tailoring currently available interventions to circumvent the pathways by which inflammation and dysfunctional metabolism cause treatment resistance may be one strategy to improve outcomes in obese and overweight patients.

The development of effective lifestyle modifications may also be important in risk reduction and improving breast cancer-specific outcomes. Finally, identifying specific patient populations that may benefit from pharmaceutical intervention is a subject of ongoing research. It is likely that a comprehensive, multimodality approach that incorporates all of these strategies will prove to provide the greatest benefit and redefine breast cancer treatment in the coming era. Hopefully, in the future, a greater understanding of obesity-breast cancer biology will translate into improvements in patient outcomes. Furthermore, a better understanding of obesity-mediated inflammation may offer broad implications for scientific progress in the many other diseases associated with inflammation and obesity.

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Chapter 9
Obesity, Inflammation, Nonalcoholic Fatty Liver Disease, and Hepatocellular Carcinoma

Naim Alkhouri and Arthur McCullough

Abstract

- Hepatocellular carcinoma (HCC) is a common malignancy worldwide that is increasing in incidence in the United States.
- Viral and alcohol-related liver diseases account for most cases; however, a significant number of patients with HCC do not have a known underlying chronic liver disease.
- New evidence suggests that obesity and type 2 diabetes may play a significant role in the development of HCC.
- Nonalcoholic fatty liver disease (NAFLD) is very common in patients with obesity and diabetes and may progress to cirrhosis and HCC.
- Insulin resistance, adipose tissue inflammation, adipokines, and inflammatory cytokines comprise mechanistic pathways that link obesity with NAFLD and HCC.

9.1 Introduction: The Changing Epidemiologic Pattern of HCC

Primary liver cancer is the sixth most common liver cancer worldwide and the third most common cause of cancer-related death [1]. Hepatocellular carcinoma (HCC) accounts for approximately 90 % of all primary liver cancers, and these two terms are frequently used interchangeably [2]. Each year, 20,000 new cases of HCC are diagnosed in the United States with an incidence that has tripled over the past 2 decades making HCC the fastest growing cause of cancer death in the male population [3].

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Liver cirrhosis is present in approximately 70–90 % of HCC cases. Although chronic hepatitis B virus (HBV) infection is the major risk factor for HCC worldwide, infection with hepatitis C virus (HCV) is the causal factor in the majority of cases in the United States and Japan [4]. Other etiologies of chronic liver disease/cirrhosis associated with HCC include alcoholic liver disease, exposure to aflatoxin, hereditary hemochromatosis, α1-antitrypsin deficiency, primary biliary cirrhosis, and autoimmune hepatitis. However, a significant number of patients with HCC (15–50 %) do not have a known underlying chronic liver disease (cryptogenic) [5].

New evidence suggests that obesity and obesity-related complications, such as type 2 diabetes (DM2), metabolic syndrome (MetS), and nonalcoholic fatty liver disease (NAFLD), play a significant role in the development of HCC in susceptible patients.

In fact, epidemiological data have demonstrated a parallel increase in prevalence of HCC and obesity. In multiple population-based cohort studies from the USA and Europe [6–8], HCC was approximately twice as likely to develop in obese individuals as in those who were not obese, and similar results were found in patients with DM2 compared to nondiabetic subjects [9, 10]. NAFLD is the hepatic manifestation of MetS, and its prevalence (33 % of the adult US population) has been increasing with the growing epidemics of obesity and DM2. In addition, NAFLD appears to be a significant factor in the tumorigenesis of HCC in the setting of obesity [11].

9.2 Mechanistic Pathways Liking Obesity and Inflammation to Chronic Liver Disease and HCC

It has become clear that a state of low-grade chronic inflammation is typically associated with obesity and plays a crucial role in the development of insulin resistance (IR) [12, 13]. An important initiator of this inflammatory response is the adipose tissue, which actively secretes a variety of products such as cytokines, adipokines, and fatty acids into the circulation [14]. Abnormal adipose tissue metabolism has also been identified as a critical mechanistic link between obesity and NAFLD [15, 16]. As shown in Fig. 9.1, the increased release of free fatty acids (FFA) from adipose tissue due to a state of insulin resistance, which is characteristic of obesity, represents the main source of FFA during development of hepatic steatosis and progressive liver injury in NAFLD [17]. A surplus of FFA in non-adipose cells (ectopic fat deposition) may enter deleterious pathways leading to hepatocyte dysfunction (lipotoxicity) and apoptotic cell death (lipoapoptosis) [18, 19] which can occur through death receptors, the mitochondrial-lysosomal pathway, and endoplasmic reticulum (ER) stress [17]. The ensuing responses of cell repair, inflammation, regeneration, and fibrosis may all be triggered by apoptosis of adjacent cells [20]. Of these processes in the liver, hepatic fibrosis has the potential to be the most deleterious, as progressive fibrosis may result in cirrhosis and end-stage liver disease [21, 22].

Liver fibrosis is strongly associated with the development of HCC; in fact, up to 90 % of HCC cases arise in cirrhotic livers [23]. As shown in Fig. 9.2, excess

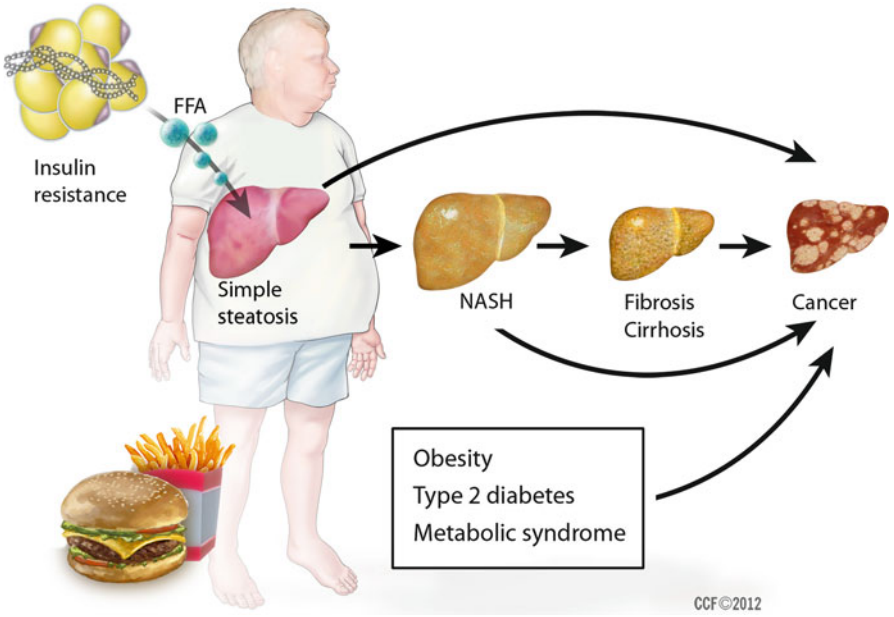

Fig. 9.1 The role of insulin resistance, free fatty acids, obesity, and diabetes in the development and progression of nonalcoholic fatty liver disease (NAFLD)

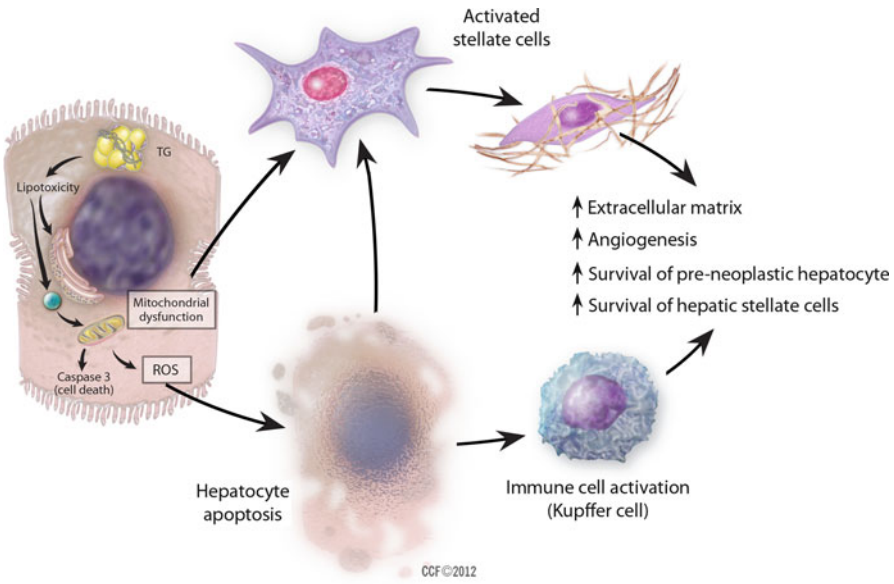

Fig. 9.2 The importance of fibrosis and the immune system for creating an environment in the liver conducive for the development of hepatocellular carcinoma (HCC)

extracellular matrix and stiffness in cirrhotic livers provides a reservoir for bound growth factors, promotes angiogenesis, enhances survival of pre-neoplastic hepatocytes and activated hepatic stellate cells (HSCs), and reduces the activity of natural killer and natural killer T cells that have critical roles in tumor surveillance [24].

9.3 Obesity and Insulin Resistance in the Pathogenesis of HCC

9.3.1 Insulin Resistance/IGF Axis and HCC

IR is frequently present in obese individuals and can lead to a state of compensatory hyperinsulinemia in order to maintain normal metabolic functions. As shown in Fig. 9.3, hyperinsulinemia activates downstream pathways through the insulin receptor substrate-1 (IRS-1), which inhibit apoptosis and increase mitogenesis promoting tumorigenesis. IRS-1 plays an important role in cytokine signaling pathways and is upregulated in HCC [25]. The c-Jun amino-terminal kinase 1 (JNK1) has emerged as a key link between obesity and IR [26]. Obesity is associated with increased release of FFA, pro-inflammatory cytokines, and reactive oxygen species, all potent activators of JNK, which lead to phosphorylation of IRS-1 and IR. JNK1 plays a significant role in the development of nonalcoholic steatohepatitis (NASH) [27] and was found recently to be associated with the development of HCC as more than 50 % of human HCC samples were observed to have increased activation of JNK1 [11, 28, 29].

The liver synthesizes and secretes insulin-like growth factors (IGFs), in response to growth hormone. Insulin upregulates hepatic growth hormone receptors, which in turn increase the activation of IGFs. Accumulating nascent data suggest that dysregulation of the IGF axis, which consists of IGF-1 and IGF-2, their receptors IGF1R and IGF2R, and their binding proteins (IGFBP1–6), plays a role in the development of HCC in animal models and human HCC cell lines [30, 31]. Binding of IGF-1 and IGF-2 to their cell surface receptor results in the activation of the Ras-mitogen-activated protein kinase (MAPK)-ERK pathway and the phosphoinositide 3-kinase (PI3K)/Akt/mTOR pathway, which are major signaling pathways in cellular proliferation and apoptosis [32–34]. Hyperinsulinemia decreases liver production and blood levels of IGFBPs leading to increased bioavailability of IGFs [35]. Indeed, decreased expression of IGFBP-3 in HCC was found to be significantly associated with portal vein invasion and poor prognosis [36], while the treatment of HCC cells with IGFBP-3 in vitro controlled cell proliferation [37].

9.3.2 Inflammatory Cytokines and HCC

Obesity is associated with the upregulation of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6), both of which can stimulate

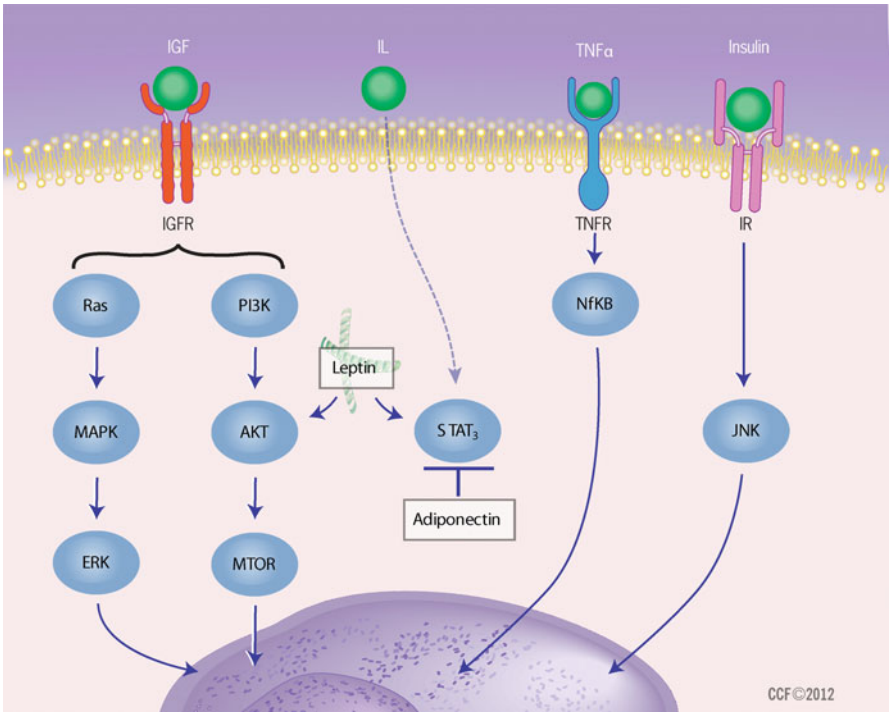

Fig. 9.3 The transcriptional factors and adipokines currently considered the most important carcinogenic in NAFLD

normal and malignant hepatocyte proliferation [38, 39]. The binding of TNF-α to its receptor activates the nuclear factor-κB pathway (NF-κB), which is considered a major factor in determining the ability of pre-neoplastic and malignant cells to avoid apoptosis and therefore providing a link between inflammation and cancer [40]. After activation, NF-κB translocate dimers to the nucleus where they affect transcriptional activation of hundreds of target genes. By using the Mdr-2 knockout mouse model, which spontaneously develops cholestatic hepatitis leading to dysplasia and eventually HCC, Pikarsky et al. demonstrated that TNF-α expression was upregulated in adjacent inflammatory and endothelial cells, triggering NF-κB signaling in hepatocytes, thereby promoting survival and the ultimate formation of HCC [41]. Furthermore, anti-TNF treatment suppressed NF-κB activation resulting in apoptosis of transformed hepatocytes and failure to progress to HCC. In contrast with the tumor-promoting role of NF-κB pathway in the animal model described above, the inactivation of the pathway at an early stage in other models can also promote carcinogenesis because of increased hepatocyte apoptosis which triggers compensatory hyperplasia, regeneration, and fibrosis leading to malignant transformation [42].

Another important inflammation-associated pathway is the signal transducer and activator of transcription 3 (STAT3) which is triggered by IL-6 and inhibited by

SOCS3 (suppressor of cytokine signaling 3) [43]. In a study by He et al., hepatocyte-specific STAT3-deficient mice (*Stat3*$^{\Delta hep}$) exhibit more than sixfold reduction in HCC load relative to *Stat3*$^{F/F}$ in response to the chemical procarcinogen diethylnitrosamine (DEN)-induced liver tumorigenesis [44]. The authors also examined the status of STAT3 activation in a large number of human HCC specimens and found that approximately 60 % exhibited activated nuclear STAT3, with STAT3-positive tumors being more aggressive. Furthermore, hepatocyte-specific SOCS3 deletion in mice with DEN-induced HCC leads to larger and more numerous tumors [45].

9.3.3 Adipokines and HCC

Leptin is a specialized peptide hormone produced mainly by adipocytes (adipokine) and is the product of the obese (ob) gene. Leptin regulates energy intake and expenditure through binding to its receptors in the central nervous system [46, 47]. In overweight and obese subjects, leptin levels are elevated indicating a state of leptin resistance. Using HCC cell lines, Saxena et al. demonstrated that leptin promoted HCC through concomitant activation of the STAT3 and PI3K/Akt pathways and that blocking these signaling pathways can effectively block leptin-mediated migration and invasion of HCC cells [48]. Moreover, leptin can promote tumorigenesis by protecting HCC cells from apoptosis induced by transforming growth factor-β (TGF-β), which occurs by downregulating the pro-apoptotic Bax gene [49].

Adiponectin is another adipokine mainly secreted by visceral adipose tissue. Adiponectin has three receptors, AdipoR1, AdipoR2, and T-cadherin, with AdipoR2 being the one predominantly expressed in the liver [50]. Unlike leptin, adiponectin levels are markedly decreased in obesity, DM2, NAFLD, and atherosclerosis indicating a protective effect of adiponectin against obesity-related disorders [51–53]. Similarly, reduced plasma or hepatic adiponectin correlates closely with the development of HCC with an inverse relationship between adiponectin expression and tumor size [54, 55]. Interestingly, adiponectin may inhibit tumor growth induced by leptin by reducing STAT3 and PI3K/Akt activity and increasing SOCS3 which is a negative regulator of leptin signaling [56].

9.4 HCC and Obesity

9.4.1 Obesity and HCC in the General Population

Obesity has been established as a risk factor for HCC by multiple large cohort studies [57]. This increased risk may be related to the increased prevalence of NAFLD and NASH in obese individuals, and the carcinogenic potential exerted by obesity alone, as discussed above. In a large Danish study of nearly 44,000 obese individuals, Moller et al. found an overall 16 % increased incidence of

cancers and a relative risk of 1.9 for liver cancer [7]. Wolk et al. found that obesity was associated with a threefold increase in HCC risk in a large Swedish study that included more than 28,000 obese patients [8]. A Korean study that followed 781,283 males without a prior diagnosis of cancer for over a 10-year period found a relative risk for HCC of 1.53 in obese patients compared to normal controls after controlling for HBV infection (the most common cause of HCC in Korea) [58]. A mortality study conducted by the American Cancer Society that followed more than 900,000 US adults initially free from cancer for 16 years demonstrated that the relative risk of dying from liver cancer was 1.68 times higher in women and 4.52 times higher in men who had a BMI $\geq$ 35 kg/m^2 compared to lean controls with normal BMI (18.5–24.9 kg/m^2) [6]. Of note, the relative risk of liver cancer mortality among males was the highest of all the cancers studied. A meta-analysis that included 11 cohort studies found that compared to normal-weight individuals, the relative risk of liver cancer was 1.17 (95 % CI, 1.02–1.34) for overweight patients and 1.89 (95 % CI, 1.51–2.36) for obese patients [57].

9.4.2 Obesity and HCC in Patients with Cirrhosis

In addition to the association between obesity and HCC in the general population, obesity is an independent risk factor for HCC in patients with cirrhosis of different etiologies. A review of the United Network of Organ Sharing (UNOS) database that included 19,271 cirrhotic patients who had liver transplantation between 1991 and 2000 revealed that the overall incidence of HCC was higher in obese patients compared to non-obese patients (4.0 % vs. 3.0 %, $p = 0.013$) [59]. In the multivariate analysis of this study, obesity was an independent predictor of HCC in cryptogenic cirrhosis (OR = 11.1, 95 % CI, 1.5–87.4) and alcoholic cirrhosis (OR = 3.2, 95 % CI, 1.5–6.6) but not in cirrhosis due to viral hepatitis or autoimmune liver disease. A prospective 7-year study in France that followed 771 patients with alcohol- or HCV-related cirrhosis demonstrated that a BMI $\geq$ 30 kg/m^2 was associated with a hazard ratio for HCC of 2.8 [60].

Beyond the effect of obesity (assessed by BMI) on the risk of HCC, it appears that visceral fat accumulation per se may play a role in tumor initiation and progression. Visceral adipose tissue is a highly active endocrine organ that produces multiple pro-inflammatory cytokines (such as TNF-α and IL-6), promotes insulin resistance, and causes hepatocyte fat accumulation with ensuing hepatocyte injury and possibly carcinogenesis through FFA toxicity [31]. Ohki et al. studied the effect of visceral fat area, as measured by contrast-enhanced dynamic CT, on the recurrence of HCC after curative ablation [61]. They found that patients with high visceral fat area (defined as >130 cm^2 in male and >90 cm^2 in female) had a cumulative recurrence rates at 1 and 3 years of 15.9 % and 75.1 %, respectively, compared to 9.7 and 43.1 % in the control group ($p = 0.018$).

9.5 HCC and Type 2 Diabetes Mellitus (DM2)

Establishing the causal relationship between diabetes and HCC has proven difficult for multiple reasons. First, cirrhosis and end-stage liver disease can cause impaired glucose tolerance and overt diabetes [62] introducing potential bias in case–control studies. Second, some etiologies of chronic liver disease such as HCV and hemochromatosis are associated with increased diabetes risk [63]. Finally, diabetes is a strong risk factor for NAFLD and NASH which can progress to cirrhosis and HCC [64]. However, several large cohort studies have established the presence of DM2 as an independent risk factor for the development of HCC. Earlier population-based studies from Sweden and Denmark [65, 66] have shown a significant increase in HCC risk among patients with diabetes alone and in the presence of viral hepatitis, alcoholic liver disease, and cirrhosis. A large longitudinal study from the USA followed 173,643 diabetic patients and 650,620 patients without diabetes for 10–15 years and found that diabetes was associated with a hazard ratio for HCC of 2.16 (95 % confidence interval [CI], 1.86–1.09; $p < 0.0001$) even after excluding patients with HCV, HBV, and alcoholic liver disease [67]. Importantly, this study provided evidence for a causal association between DM2 and HCC by demonstrating that diabetes preceded the development of chronic liver disease, as well as an increased risk for HCC in patients with longer duration of diabetes. Similar findings were reported from a hospital-based case–control study with diabetes being present in 87 % of cases before the diagnosis of HCC and patients with diabetes duration >10 years having an adjusted hazard ratio for HCC of 2.2 (95 % CI, 1.2–4.8) compared to those with a diabetes duration of 2–5 years. More robust support for diabetes as a risk factor for HCC came from a meta-analysis of 13 cohort studies that aimed to assess the association between diabetes and HCC. The analysis revealed a statistically significant 2.5-fold increase in HCC incidence among diabetic patients (95 % CI, 1.9–3.2; $p < 0.01$) [9]. A more recent meta-analysis that included a total of 17 case–control studies and 32 cohort studies confirmed the association between diabetes and risk of HCC with a pooled relative risk of 2.31 for HCC among diabetic patients (95 % CI, 1.87–2.84) [68].

9.5.1 Association of Diabetes Treatment with HCC Risk

Several lines of evidence suggest that the type of antidiabetic therapy may increase or decrease HCC risk contingent upon mechanism of action [68].

On one hand, insulin sensitizers such as metformin can improve insulin sensitivity and suppress cell growth by activating the AMP-activated protein kinase yielding a protective effect on cancer risk [69]. A retrospective study from Italy that included 465 patients with HCC, 618 cirrhotics, and 490 controls found a statistically significant reduction in HCC risk for diabetics receiving metformin (OR = 0.33; 95 % CI, 0.1–0.61) [70]. Another study from the University of Texas reported a

similar decrease in the risk for HCC with metformin (OR = 0.3; 95 % CI, 0.2–0.6) [71]. Moreover, a recent study demonstrated that metformin decreased the risk of HCC in diabetic patients in a dose-dependent manner with a 7 % risk reduction of HCC for each incremental year increase in metformin use [72]. Interestingly, metformin inhibited hepatoma cell line proliferation by inducing cell cycle arrest at the G0/G1 phase via AMP-activated protein kinase. Furthermore, metformin had chemosensitizing effect in combination with doxorubicin to accelerate HCC regression in a mouse xenograft model.

On the other hand, exogenous insulin and sulfonylureas lead to an increase in circulating insulin levels promoting carcinogenesis and increasing the risk for HCC. Donadon et al. found a statistically significant increase in HCC risk for diabetics receiving sulfonylureas or insulin (OR = 2.99; 95 % CI, 1.34–6.65), and similar findings were also reported by Hassan et al. (insulin use was associated with HCC OR of 1.9; 95 % CI, 0.8–4.6, and sulfonylurea use was associated with HCC OR of 7.1; 95 % CI, 2.9–16.9) [70, 71].

Overall, in the meta-analysis by Wang et al., the pooled risk estimates for developing HCC were 0.31 (95 % CI, 0.19–0.49) for diabetics treated with metformin and 4.0 (95 % CI, 1.94–8.24) for those treated with sulfonylurea or exogenous insulin [68].

In addition to the effects of antidiabetic therapy on the risk for liver cancer, statin use in diabetics appears to have a protective effect against HCC. Proposed mechanisms include the inhibition of downstream products of the mevalonate pathway which are important for the growth of malignant cells [73] and the inhibition of HSC proliferation and their production of collagen [74]. In a nested, matched, case–control study in diabetics, El-Serag et al. examined 1,303 cases and 5,212 controls and found a risk reduction for the development of HCC that ranged between 25 and 40 % providing evidence of the cancer-preventive effect of statins specific to HCC [75].

9.6 HCC and Nonalcoholic Fatty Liver Disease

9.6.1 The Epidemic of NAFLD

NAFLD is considered the hepatic manifestation of MetS and as such has become the most common form of chronic liver disease in the world [76–78]. NAFLD encompasses a wide histological spectrum of disease ranging from simple steatosis characterized by lipid accumulation in the liver in the form of triglyceride (TG) to NASH characterized by the association of lipid accumulation with evidence of hepatocyte injury, inflammation, and various degrees of fibrosis [79]. NASH is a serious condition that can progress to cirrhosis and its feared complications of portal hypertension and end-stage liver disease requiring liver transplantation. It is estimated that in the United States, NAFLD affects 30–45 % of the general population and as high as 90 % of the morbidly obese [80, 81]. Furthermore, the aggressive

form of NASH affects between 5 and 13 % of Americans, making this entity a rising cause of liver-related morbidity and mortality and potentially the main indication for liver transplantation in the next decade [82]. A recent study found that among diabetic patients, NAFLD was present in 74 % and NASH in 22.2 % [81].

9.6.2 HCC and Cryptogenic Cirrhosis

When NASH progresses to cirrhosis, most of the classic histological features of the disease disappear, making the diagnosis of the underlying etiology difficult [83]. It has been proposed that NASH accounts for the majority of cases of cryptogenic cirrhosis (CC) because these patients have a significantly higher prevalence of conditions associated with NASH including obesity and diabetes compared to patients with cirrhosis of well-defined etiology [84, 85]. Three landmark studies published in 2002 expanded the spectrum of NAFLD from CC to HCC. In the first study, Bugianesi et al. retrospectively identified 646 Italian patients with cirrhosis-associated HCC and found that the prevalence of CC was 6.9 % compared to 55 % for HCV-cirrhosis, 16 % for HBV-cirrhosis, and 13 % for alcoholic cirrhosis [86]. Interestingly, the prevalence of pre-cirrhotic obesity, DM2, and dyslipidemia were more than twice as prevalent in patients who had CC as in the control group (p value <0.05 for all metabolic factors). The second study by Marrero et al. demonstrated that CC was the second most common etiology of underlying liver disease (29 %) after HCV (51 %) in a group of 105 patients with HCC from Michigan likely related to the high prevalence of obesity in the United States [87]. The third study by Ratziu et al. in France found CC in 27 % of HCC patients and corroborated the association between DM2, IR, and dyslipidemia with both CC and HCC [88].

9.6.3 HCC and NASH-Cirrhosis

Several studies have directly examined the incidence of HCC in patients with NAFLD or NASH-cirrhosis (Table 9.1). In a large cohort study that included 7,326 patients discharged with a diagnosis of fatty liver from Danish hospitals over a 16-year period, Sorensen et al. found that the risk for primary liver cancer was significantly elevated in NAFLD patients compared to the Danish general population with a standardized incidence ratio of 4.4 (95 % CI, 1.2–11.4) [89]. A large prospective US study compared 152 patients with NASH-cirrhosis (median age of 55 years) with 150 matched patients with HCV-cirrhosis. Those with NASH-cirrhosis had significantly lower risk of developing HCC over a 10-year follow-up period (6.7 % vs. 17 %, respectively, $p<0.01$) [90]. Hashimoto et al. conducted a case-controlled study that included 34 NASH patients with HCC and 348 patients NASH patients without HCC [91]. Risk factors for HCC in this study included older age, low AST level, low NAFLD activity score, and advanced fibrosis stage. A prospective cohort

Table 9.1 Studies that evaluated the incidence of hepatocellular carcinoma (HCC) in patients with nonalcoholic fatty liver disease (NAFLD)

References	NASH patients (*n*)	Follow-up (years)	HCC incidence (%)	Risk factors
Sanyal et al. [90]	152	10	6.7	None stated
Hashimoto et al. [91]	34 (case-controlled study) 137 (prospective study)	5	7.6	Older age Low AST Low NAFLD activity score Advanced fibrosis
Ascha et al. [92]	195	3.2	12.8	Older age Any alcohol consumption

study of 137 NASH patients with advanced fibrosis was included in that same study; this prospective cohort demonstrated that the 5-year cumulative incidence of HCC was 7.6 % [91]. Finally, a study from the Cleveland Clinic compared 195 patients with NASH-cirrhosis to 315 patients with HCV-cirrhosis that were evaluated for liver transplantation between 2003 and 2007 [92]. The yearly cumulative incidence of HCC in cirrhotic patients with NASH was 2.6 % compared to 4.0 % for those with hepatitis C infection. Interestingly, older age at the time of cirrhosis diagnosis and any alcohol consumption were independently associated with the development of HCC in the multivariate analysis (hazard ratio of 1.08 and 3.8, respectively; $p < 0.005$), supporting the notion that alcohol intake even in small quantities may increase the risk of HCC development in the setting of cirrhosis.

9.6.4 HCC and Non-cirrhotic NAFLD

New data indicate that HCC can arise in steatotic livers in the absence of cirrhosis. In fact, several case series have described the occurrence of HCC in non-cirrhotic NAFLD patients. In a Japanese study that included 9 patients with HCC in the setting of NAFLD, one third of patients only had mild hepatic fibrosis and no evidence of cirrhosis [93]. Hashimoto et al. confirmed these findings by demonstrating that 12 % of their patients with NASH-HCC had mild fibrosis (fibrosis stage 1–2) [91].

In a large nationwide survey of 14,530 HCC patients in Japan, NAFLD-HCC and unknown HCC accounted for 7.1 % of all cases, but only 62 % of NAFLD-HCC had cirrhosis [94]. A European study by Ertle et al. that enrolled 150 patients with HCC (including 36 with NAFLD) intriguingly found that only 58 % of patients with NAFLD-HCC had evidence of cirrhosis [95]. These studies underscore the importance of HCC screening in high-risk NAFLD patients such as those with family history of HCC.

9.7 Summary

NAFLD has emerged as the most prevalent chronic liver disease. Population-based studies in the United States estimate that the prevalence of NAFLD is at least 30 % in adults and as high as 80 % in obese patients and in patients with type 2 diabetes. Of additional concern is the fact that the incidence of NAFLD has increased from 4.5/100,000 to 38/100,000 over the epoch of 1980–1999. Consistent with these data is the recognition that obesity and diabetes are risk factors for both NAFLD and HCC. Although viral and alcohol-related liver disease account for the majority of HCC, the increased incidence of HCC has occurred in parallel with incidences of NAFLD, obesity, and diabetes, with the latter two being risk factors for both the development and progression of NAFLD. This increased incidence of HCC in NAFLD and the fact that there is a predilection for HCC to develop in NAFLD even in the absence of cirrhosis has important clinical implications that may change the current paradigms for cancer surveillance in these patients. Finally, our increased knowledge of the mechanistic pathways that link obesity and diabetes to the pathogenesis of HCC in NAFLD (discussed above) provide strategies for both the clinical management of NAFLD and the development of targeted treatments for HCC associated with NAFLD.

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Chapter 10
Obesity, Inflammation, and Prostate Cancer

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Abstract Obesity and the associated metabolic syndrome produce a complex set of alterations both systemically and locally in tissues that support cancer development and progression. In prostate cancer (PCa), the weight of evidence suggests that obesity is primarily associated with more aggressive disease and increased risk of biochemical failure following prostatectomy or radiation treatment. Inflammation processes and inflammation-associated signaling pathways are upregulated in the obese state, and both human and mouse studies support an important role for inflammation in obesity-driven PCa progression. Inflammation signaling pathways along with other signaling pathways (e.g., growth factor signaling pathways) altered in the obese state represent promising targets for both lifestyle and pharmacologic interventions to prevent or control PCa progression.

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10.1 Introduction

10.1.1 Obesity

The prevalence of obesity, defined as a body mass index (BMI) ≥30 kg/m^2, has increased dramatically in recent decades in the United States, and nearly 35 % of adults and 20 % of children are now obese [1]. Worldwide, an estimated 1.1 billion adults are overweight and 500 million adults are obese (http://www.iaso.org/policy/aboutobesity). The obese state is characterized by an excessive expansion of adipose tissue mass, which manifests as adipocyte hypertrophy (increased size), hyperplasia (increased number), and increased intracellular lipids. Excessive adiposity per se can exert untoward structural and biomechanical effects on organs (such as the lungs, liver, and pancreas), blood vessels, musculoskeletal system, and other tissues [2]. In addition, the resulting adipocyte hyperplasia and hypertrophy are associated with adipocyte dysfunction that can trigger local and systemic changes characteristic of the metabolic syndrome that increase risk and worsen prognosis of several cancers and other chronic diseases [3].

Among obese adults, approximately 60 % meet the criteria for the metabolic syndrome, a state of metabolic dysregulation characterized by insulin resistance, hyperglycemia, dyslipidemias (particularly hypertriglyceridemia), and hypertension [4]. In obesity and/or metabolic syndrome, alterations also occur in circulating levels of insulin, bioavailable insulin-like growth factor (IGF)-1, adipokines (e.g., leptin, adiponectin, monocyte chemotactic factor), inflammatory factors (e.g., cytokines such as interleukin 6 [IL-6]), and angiogenesis factors (e.g., vascular endothelial growth factor [VEGF] and plasminogen activator inhibitor [PAI]-1) [5, 6]. Through these mediators, obesity and metabolic syndrome are linked to various chronic diseases [5, 7] such as cardiovascular disease, type II diabetes, and cancer, including prostate cancer (PCa) that is the focus of this chapter.

10.1.2 Obesity and Cancer

Overall, an estimated 15–20 % of all cancer deaths in the USA are attributable to overweight and obese body types [8]. Obesity is associated with increased mortality from cancer of the prostate and stomach in men; breast (postmenopausal), endometrium, cervix, uterus, and ovaries in women; and kidney (renal cell), colon, esophagus (adenocarcinoma), pancreas, gallbladder, and liver in both genders [8]. While the relationships between metabolic syndrome and specific cancers are less well established, first reports from the Metabolic Syndrome and Cancer Project, a European cohort study of approximately 580,000 adults, confirm associations between obesity (or BMI) in metabolic syndrome and risks of colorectal, thyroid, and cervical cancer [9].

In this chapter, we discuss possible mechanisms underlying the links between obesity, metabolic syndrome, and PCa, with an emphasis on obesity-associated adipocyte dysfunction, inflammation, and growth factor signaling. Specifically, we describe the dysregulation of growth signals (including insulin, IGF-1, and downstream signaling pathways), adipokines (including leptin and adiponectin), inflammatory cytokines (including IL-6 and tumor necrosis factor-α [TNFα]), and angiogenesis factors (including VEGF and PAI-1) in the obese state that may contribute to more aggressive disease and higher mortality in PCa patients.

10.2 Relationship Between Obesity and PCa

10.2.1 Summary of Data from Epidemiologic Studies

Worldwide, PCa is the second most commonly diagnosed non-cutaneous cancer in men and the sixth most common cause of death [10]. In the United States, it is the most frequently diagnosed non-cutaneous cancer and the second leading cause of cancer-related mortality in this population [11, 12]. An estimated 241,740 new cases will be diagnosed in 2012.

Data associating obesity and PCa risk have been inconclusive [13, 14]; however, recent studies have shown increased risk of biochemical failure and metastasis, as well as poorer survival among obese PCa patients with androgen-dependent tumors, especially those who experienced rapid weight gain [15]. Men with low-volume PCa have a lower BMI, less body fat, and a smaller waist-to-hip ratio than men with high-volume PCa, which agrees with other reported findings [16–18]. Fat is the most energy dense component and has been the focus of most PCa dietary epidemiologic investigations. High consumption of energy and fat, especially saturated fat [19–21], is associated with advanced stage PCa and mortality [19, 22]. Several biologic mechanisms have been postulated to explain the role of obesity in PCa progression and these will be discussed in more detail in the sections that follow.

10.3 Mechanisms Associated with Obesity and PCa

As noted above, several biological mechanisms have been postulated to explain the association between obesity and aggressive disease in PCa patients, including increases in circulating levels of growth factors (i.e., IGF-1 and leptin), hyperinsulinemia, and inflammation as well as diet-induced alterations in adiponectin, steroid hormones, and possibly other factors (e.g., angiogenesis-related factors). These mechanisms will be discussed in more detail below.

10.3.1 Growth Factor Signaling

10.3.1.1 Insulin and Insulin-Like Growth Factor 1

Insulin is a peptide hormone, produced by beta cells of the pancreas, and is critical for the regulation of glucose and fat metabolism in the body. Metabolic dysfunction, mainly hyperinsulinemia and insulin resistance, is commonly associated with Western lifestyle and obesity and is considered as one of the risk factors for more aggressive PCa [23–26]. Insulin promotes cell division and proliferation in PCa and facilitates the expression of other growth factors and their regulators and acts as a cell survival factor inhibiting apoptosis [27–32]. Evidence suggests that serum level of C-peptide, a marker of insulin secretion, is positively correlated with obesity, and higher levels of this marker have been associated with high-grade PCa and PCa-specific mortality [33]. In obesity, adipose tissue releases increased amounts of free fatty acids, TNFα, resistin, and reduced amounts of adiponectin, which leads to the development of insulin resistance and chronic hyperinsulinemia. The higher levels of insulin in turn decrease the synthesis of insulin-like growth factor-binding protein 1 (IGFBP1) from the liver and possibly from other tissues and also decrease its blood levels. Hyperinsulinemia is also associated with reduced blood levels of IGFBP2. The IGFBPs normally bind and inhibit the action of IGF-1. So the resultant reduction in IGFBPs leads to increased levels of bioavailable, free IGF-1 in the circulation. Both the insulin and IGF-1 receptors (IGF-1Rs) are expressed in normal and neoplastic prostatic tissue [34–36], and physiological doses of insulin and IGF-1 act as mitogens for a variety of cancer cells, including PCa cells. Insulin and IGF-1 signal through the insulin receptors (IRs) and IGF-1R, respectively, to promote cell proliferation and inhibition of apoptosis, contributing to cancer progression. Binding of IGF-I or insulin to their cognate receptors leads to activation of a number of downstream signaling pathways following phosphorylation of insulin receptor substrates (e.g., IRS-1, IRS-2) and Src homology 2 domain-containing adapter protein (Shc). Phosphorylated Shc activates the Ras/Raf/mitogen-activated protein kinase (MAPK) pathway and ultimately stimulates cell growth and proliferation. Phosphorylated IRS-1 activates the phosphatidylinositol 3′ kinase (PI3K)/Akt pathway, leading to inhibition of apoptosis and stimulation of cell proliferation through downstream mediators including BAD, mTOR, P70S6K, and nuclear factor-κB (NF-κB). Akt is normally inhibited by the tumor suppressor gene phosphatase and tensin homolog (PTEN), which is often lost in advanced PCa [32, 34, 37]. Thus, changes in this signaling pathway associated with obesity may be exacerbated in the presence of PTEN deficiency.

10.3.1.2 Sex Steroids

Androgens are essential for normal development, differentiation, and proliferation of prostatic tissue [38], and it is generally believed that higher concentrations of androgens are associated with increased PCa risk. Testosterone and its metabolite

dihydrotestosterone (DHT) bind to the androgen receptor (AR) which then binds to androgen response elements in the DNA of prostate cells to initiate changes in gene expression [39]. Adipose tissue produces the enzyme aromatase, which catalyzes the conversion of androgens to estrogens (note that DHT is not converted to estrogens by aromatase). Thus, obesity is generally associated with modestly lower serum concentrations of testosterone, lower concentrations of sex hormone-binding globulin, and higher concentrations of estrogens [40–42]. Interestingly, recent data suggest that higher serum testosterone levels are associated with a reduced risk of high-grade PCa but an increased risk of low-grade tumors [43]. It has been hypothesized that lower androgen concentrations may provide a microenvironment, which favors more aggressive and/or androgen-independent tumor growth and ultimately disease progression [32]. Another explanation is that individuals with low serum testosterone levels are at an increased risk of developing metabolic syndrome. However, it is not clear whether low serum testosterone levels associated with obesity and insulin resistance or insulin alone, in the absence of high serum testosterone, is sufficient to trigger progression of PCa to higher-grade tumors. Alternatively, lower testosterone concentrations are associated with a reduced risk of low-grade tumors because low-grade tumors are slow growing and need androgen stimulation for progression [44]. Note that intraprostatic conversion of testosterone to DHT, which is not strongly related to circulating androgen levels, may be more influential in PCa progression than androgens in the circulation.

10.3.2 Adipokines

Two kinds of adipose tissues are present in mammals: white adipose tissue (WAT) and brown adipose tissue. WAT is the most abundant and is considered as the major site of energy storage [45]. In addition to adipocytes, adipose tissue also contains pre-adipocytes, endothelial cells, fibroblasts, leukocytes, and, most importantly, macrophages. Macrophages infiltrate adipose tissue and they remain in increased numbers in association with obesity [46]. Adipose tissue is no longer considered to be an inert tissue functioning only as energy storage, but plays an important role in the regulation of many pathological processes. Polypeptide hormones derived from adipocytes are known as adipokines. Currently more than 50 different types of adipokines are recognized such as leptin, adiponectin, resistin, serpin, lipocalin 2, retinol-binding protein 4 (RBP4), zinc-α2, glycoprotein, vaspin, visfatin, omentin, apelin, TNFα, IL-6, IL-1, CC chemokine ligand 2, and mediators of the clotting process, such as PAI-1 and chemerin [45, 47]. Adipokines may exert their biologic effects either at a local level via autocrine/paracrine pathways or in an endocrine manner by entering the circulation and activating receptors on more distant target cells. Paracrine effects of adipokines are important in cases of PCa progression where extracapsular extension and invasion of the retropubic fat pad occurs [48]. This could result in the exposure of malignant cells to high concentrations of potentially proangiogenic, survival, and proliferative factors, which may enhance their capacity for further growth and metastasis. Adiponectin and leptin are the most abundant and most studied adipokines produced by adipocytes.

10.3.2.1 Leptin

Leptin, first described by Zhang et al. in 1994 [49], is a 16-kD adipokine produced predominantly by adipocytes in WAT. Circulating leptin concentrations exhibit a positive correlation with total body fat, so that serum leptin is elevated in obese individuals compared to lean individuals [50]. Although numerous epidemiologic studies have investigated the relationship between obesity, circulating leptin levels, and PCa [51–57], results from these studies are still inconclusive. The risk for PCa in individuals with higher levels of leptin has not been demonstrated [51–57]. However, it was found that higher leptin levels were linked to tumor progression and advanced disease [51, 52, 54–57]. Collectively, the current data suggest that leptin may be predominantly involved in PCa progression.

Several in vitro studies have shown that leptin induces proliferation, cell migration, and invasion and/or prevents apoptosis in androgen-independent human PCa cell lines [58]. Leptin also induces expression of several angiogenic growth factors including VEGF, transforming growth factor-beta1 (TGF-β1), and basic fibroblast growth factor (bFGF) that may contribute to its action in PCa progression [59, 60]. These responses to activation of the leptin receptor are mediated by several signaling pathways including the Janus kinase-signal transducer and activator of transcription (JAK/STAT) (especially Stat3), phosphatidylinositol 3-kinase (PI3-K)/Akt, and c-Jun NH2-terminal kinase (JNK) pathways [61]. Alterations in these signaling pathways are not only critical in prostate carcinogenesis and malignant transformation, but also important in obesity, diabetes, and insulin resistance [59, 62].

10.3.2.2 Adiponectin

Adiponectin, also known as 30-kD adipocyte complement-related protein (Acrp30), adipoQ, APM-1, or gelatin-binding protein 28 (GBP28), is the most abundant circulating adipokine synthesized mainly by adipocytes but also expressed by skeletal muscle cells, cardiac myocytes, and endothelial cells [45, 48]. In contrast to other adipokines, circulating levels of adiponectin are negatively correlated with central obesity, BMI, visceral fat accumulation, and insulin resistance. Epidemiological studies show more consistent inverse associations between circulating adiponectin levels and cancer [47]. Reductions in the levels of plasma adiponectin have also been observed in obesity-related conditions such as type 2 diabetes, cardiovascular disease, hypertension, and metabolic syndrome [63]. Adiponectin has antiproliferative, anti-angiogenesis, and proapoptotic activity, and the plasma levels are found to be lower in patients with PCa compared to patients with benign prostate hyperplasia (BPH) [47].

Although the exact mechanism of antitumor activity of adiponectin is not clearly understood, it is thought to act by modulation of signaling pathways including AMP-activated protein kinase (AMPK), MAPK, NF-κB, Stat3, and p53 [47, 48, 62]. Two receptor isoforms are known to exist for adiponectin: adiponectin receptor 1 (Adipo-R1) and adiponectin receptor 2 (Adipo-R2). Activation of the receptor(s)

by adiponectin stimulates the activation of AMPK, peroxisome proliferator-activated receptor-α (PPARα), and p38 MAPK. Activation of AMPK is considered to be one of the most important signaling events for metabolic effects of adiponectin and has also been implicated in prostate carcinogenesis [48]. Adiponectin regulates the expression of several pro- and anti-inflammatory cytokines. Its main anti-inflammatory function might be related to the suppression of the synthesis of TNFα and interferon-γ (IFNγ) and to induce the production of anti-inflammatory cytokines such as interleukin-10 (IL-10) and IL-1 receptor antagonist (IL-1RA). Activation of PPARs also exerts anti-inflammatory effects through inhibition of the transcriptional activation of proinflammatory response genes [45]. Recent evidence suggests that the ratio of adiponectin to leptin levels is critical for the overall effects of these two important adipokines [64].

10.3.3 Angiogenesis Factors

10.3.3.1 VEGF

VEGF, a heparin-binding glycoprotein produced by adipocytes and tumor cells, has angiogenic, mitogenic, and vascular permeability-enhancing activities specific for endothelial cells [65]. Circulating levels of VEGF are increased in obese, relative to lean, humans and animals, and increased tumoral expression of VEGF is associated with poor prognosis in several obesity-related cancers [66]. The need for nutrients and oxygen triggers tumor cells to produce VEGF, which leads to the formation of new blood vessels to nourish the rapidly growing tumor and facilitate the metastatic spread of tumor cells [65]. Adipocytes communicate with endothelial cells by producing a variety of proangiogenic and vascular permeability-enhancing factors. These include VEGF, IGF-1, PAI-1, leptin, hepatocyte growth factor, and fibroblast growth factor-2 [67]. In the obese, nontumor setting, these factors stimulate neovascularization in support of the expanding fat mass. These adipose-derived factors may also contribute to obesity-associated enhancement of tumor angiogenesis.

In PCa, VEGF plays a major role in endothelial cell differentiation, proliferation, migration, and vessel formation [68–71]. In addition to adipocytes, VEGF is produced by PCa cells and tumor-infiltrating lymphocytes [72, 73]. Systemic VEGF was found to be significantly elevated in PCa patients [74] and patients with metastatic disease [70]. In a cohort of 50 radical prostatectomy specimens, El-Gohary et al. [75] showed that VEGF expression correlated significantly with angiolymphatic invasion and Gleason score. Clinical studies comparing PCa with BPH revealed that VEGF expression was correlated with increased levels of angiogenesis [76]. The levels of VEGF in serum, plasma, or urine are correlated with patient outcome in both localized and disseminated PCa [70, 77, 78]. In addition, the levels of the VEGFR were correlated with a poorer grade of tumor differentiation and prognosis in PCa [79]. Normal prostate tissue expresses minimal to no VEGF, unlike PCa tissue that stains positively for VEGF in areas of increased microvessel

density (MVD) [73]. PCa cells express VEGF in vitro and in vivo [80]. VEGF expression by PCa specimens [81] and human PCa cell lines (LNCaP, PC-3, and DU145) is far greater than that by stromal cells of the normal prostate [73, 82]. VEGF expression may also contribute to PCa-induced osteoblastic activity in vivo. Using a TRAMP model, Isayeva et al. demonstrated that inhibitors of the VEGFR-2 delayed tumor progression only when administered in the early stages of PCa, before a significant rise in VEGF levels was observed [83]. In a preclinical study using a xenograft model, injecting human DU145 cells in nude mice, inhibition of VEGF resulted in decreased tumor proliferation [84].

10.3.3.2 Plasminogen Activator Inhibitor-1

PAI-1 is a serine protease inhibitor produced by endothelial cells, stromal cells, and adipocytes in visceral WAT [85]. Increased circulating PAI-1 levels, frequently found in obese subjects, are associated with increased risk of atherogenesis and cardiovascular disease, diabetes, and several cancers [6, 85]. PAI-1, through its inhibition of urokinase-type and tissue-type plasminogen activators, regulates fibrinolysis and integrity of the extracellular matrix. PAI-1 is also involved in angiogenesis and thus may contribute to obesity-driven tumor cell growth, invasion, and metastasis [6]. Although PAI-1 levels in obese individuals may be reduced via weight loss or TNFα blockade [86, 87], the role of PAI-1 in tumorigenesis remains controversial [85]. Plasminogen activator activities are elevated in PCa compared with BPH [88]. Swiercz et al. demonstrated that exogenously applied recombinant PAI-1 is a powerful inhibitor of angiogenesis in three different in vitro models and is a powerful anticancer agent in a SCID mice inoculated with LNCaP cells [89]. Therefore, further study is warranted to determine the exact role of PAI-1 in PCa and other cancers.

10.3.4 Inflammation and Inflammatory Mediators

Chronic inflammation is now considered one of the major risk factors linking obesity and the development of PCa [90–92]. The inflammatory response is characterized by increased synthesis of various cytokines, acute-phase reactants such as C-reactive protein (CRP), and the activation of proinflammatory signaling pathways [45]. Adipose tissues in obese individuals and in animal models of obesity are infiltrated by a large number of macrophages, and this is associated with systemic inflammation and insulin resistance as noted above [93]. Macrophages are also the main source of soluble mediators such as TNFα. In addition to macrophages, adipocytes are also responsible for the production of IL-6, CCL2, and other inflammatory mediators that further contribute to macrophage infiltration and proinflammatory cytokine production from the adipose tissue.

IL-6 is a proinflammatory cytokine and is involved in the regulation of various cellular functions including proliferation, apoptosis, angiogenesis, differentiation, and regulation of the immune response [94]. Clinically, plasma IL-6 levels positively correlate with adiposity and insulin resistance in human populations [93]. Although not exclusively secreted by adipocytes, as much as one-third of circulating IL-6 originates from adipose tissue, and expression and secretion of IL-6 is 2–3 times greater in visceral relative to subcutaneous adipose tissue [48].

In PCa patients, serum IL-6 levels are higher in those patients with metastatic disease compared to those with localized disease [46, 95]. It has also been reported that serum IL-6 levels >7 pg/mL are associated with a poor prognosis in men with PCa [96].

Various in vitro studies have shown that IL-6 is secreted by androgen-independent PCa cells suggesting an autocrine effect on tumor cells and paracrine effects on normal cells in the tumor microenvironment [94]. High expression of IL-6 in various organs particularly lung, liver, brain, and bone attracts the circulating tumor cells and therefore promotes metastasis [97]. In one study it was reported that IL-6 levels were significantly higher in PCa patients with bone metastasis compared to patients without metastasis [98]. In another study, serum IL-6 levels were found to be higher in castration-resistant PCa compared to normal controls as well as patients with BPH, prostatitis, and localized or recurrent disease [99]. Higher IL-6 levels in obese individuals also contribute to PCa progression. The periprostatic adipose tissue (adipose tissue surrounding the prostate) is frequently invaded by prostate tumor cells. Studies have suggested that periprostatic adipose tissue produces significantly higher local IL-6 and that may contribute to PCa progression in obese individuals [100, 101].

The biological activity of IL-6 is mediated by binding with the IL-6R/gp130 receptor complex. Dimerization of IL-6/IL-6R/gp130 leads to the initiation of intracellular signaling, through the JAK/STAT, mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase/Akt kinase (PI3-K/AKT) pathways [102]. This leads to the expression of various genes involved in inflammation and cancer development. Binding of IL-6 to its receptor also leads to increased receptor activator of nuclear factor kappa B ligand (RANKL) expression. IL-6 may also activate AKT via increased JAK-dependent PI3K activity resulting in cell survival and anti-apoptosis signaling. Concomitantly, increased MAPK activity downstream of JAK activation can lead to upregulated cell growth, proliferation, and mitosis. In some cases, IL-6 also acts through soluble IL-6R which binds to gp130 and initiates signal transduction [94]. Recent studies have also revealed a role of IL-6 in regulation of the AR [103–105]. In the absence of androgen, IL-6 causes activation of the AR. In low-concentration conditions, androgen action is potentiated by IL-6, leading to synergistic activation of the AR [106]. These observations demonstrate a cross talk between the IL-6 pathway and AR that may play a significant role during PCa progression leading to androgen independence.

TNFα is a cytokine involved in systemic and acute inflammation. TNFα is synthesized as a 26-kD membrane-bound protein and cleaved into a 17-kD soluble

protein by TNF-converting enzyme (TACE) [107]. TNFα is predominantly produced by macrophages, CD4+ T cells, and natural killer (NK) cells, although nonimmune cells such as fibroblasts, smooth muscle, and tumor cells have also been reported to secrete low amounts of this cytokine [108]. TNFα is regulated by the proteolytic activity of stromal metalloproteinases, secreted from the membranes of somatic cells [109], and is directly produced by tumor-associated macrophages (TAMs) [110]. Significantly elevated levels of TNFα are found in patients with metastatic disease compared to those with localized disease [95]. According to Nakashima et al. [111], serum TNFα activity was positive in 76 % of the patients with relapsed disease who had a significantly higher mortality rate than those with undetectable serum TNFα levels. Obesity-related TNFα levels could thus potentially enhance PCa progression.

NF-κB has been shown to be activated by reactive oxygen species and many carcinogens, in addition to proinflammatory chemokines and cytokines such as TNFα [112–114]. Recently, Huerta-Yepez et al. showed that TNFα has a role in conveying resistance to Fas-induced apoptosis through a similar pathway involving NF-κB in human PCa cells [112]. Cyclooxygenase-2 (COX-2), upregulated during the inflammatory response, is thought to be inducible by a number of proinflammatory cytokines, including TNFα [115, 116]. Subbarayan et al. found that TNFα could induce COX-2 expression in both normal prostate and androgen-unresponsive PCa cells where upregulation of COX-2 was related to increases in cellular levels of prostaglandins [117]. COX-2 expression has been suggested to be dependent on inflammatory cytokine/NF-κB pathways in PCa [118], supporting a possible positive feedback between inflammatory mediators, prostaglandin production, and PCa progression. Wang et al. [119] found that foci of chronic inflammation within human samples of BPH were associated with accumulation of inflammatory cells. Moderate to high accumulation of these cells, particularly T lymphocytes and macrophages, was highly correlated with positive staining for COX-2.

10.4 Lessons Learned from Animal Models of PCa

10.4.1 Animal Model Studies on Obesity and PCa

PCa research has been greatly aided by the development and use of relevant animal models, particularly transgenic and knockout mouse models [120–123]. Only a few reports have appeared dealing with the effects of obesity, per se, on PCa development and progression using animal models. In earlier studies using the TRAMP model, mice with higher body weight and body fat content had larger and more aggressive tumors compared to those with lower body weight and body fat content [124]. Although the TRAMP mice in this study were not "obese," the mice with the highest body fat content had higher levels of leptin and lower levels of adiponectin. Additional studies using TRAMP mice fed a "Western-type" diet enriched in both fat and cholesterol showed accelerated prostate tumor growth and increased histological grade

of tumors [125]. This diet also increased lung metastases. It is not clear whether there were differences in body weight between the "Western diet" group and control diet group in this study as body weight and fat mass data were not reported. However, these data would appear to support epidemiological studies showing that fat, and, in particular, saturated fat, is an independent predictor of disease progression [19, 21, 22]. These data are also supported by another study using the HiMyc mouse model of PCa [126]. In this regard, Kobayashi et al. [127] showed that if HiMyc mice were maintained on a low-fat (LF, 12 kcal %) diet vs. a high-fat isocaloric (HF, 42 kcal %) diet, the transition from prostate intraepithelial neoplasia (PIN) to PCa was delayed, and there was a decrease in prostatic Akt activity as well as a decrease in phosphorylation of the downstream targets p-70S6K and GSK3β. Again, there were no differences in body weight between the HF and LF diet groups in this study.

We recently reported a study to evaluate the impact of dietary energy balance on PCa progression using HiMyc mice [126]. In this study, we compared the effect of different body phenotypes from lean to obese using diets of different caloric density. Diet-induced obesity (DIO) enhanced PCa progression in the ventral prostate (VP) of HiMyc mice [126]. Notably, DIO (mice fed a diet containing 60 kcal % fat) increased the incidence of invasive adenocarcinomas as well as the severity of these tumors compared to HiMyc mice on control diet (i.e., modified AIN76 diet containing 10 kcal % fat). In contrast, calorie restriction (30 % CR) completely prevented the formation of invasive adenocarcinomas in these mice. These dietary energy balance-induced alterations in PCa progression in HiMyc mice were associated with differences in circulating levels of insulin, IGF-1, leptin, adiponectin, and resistin. In particular, obese mice had higher levels of insulin, IGF-1, leptin, and resistin and lower levels of adiponectin compared to lean mice on a 30 % CR diet. Further analysis revealed that both growth factor (i.e., Akt/mTOR) and especially inflammatory signaling (i.e., Stat3, NF-κB, IL-1, IL-6, IL-7, IL-23, IL-27, NF-κB1, and TNFα) pathways were upregulated in VP of mice on the obesity-induced diet. Dramatic increases in inflammatory cell infiltrates (macrophages and T cells) were also observed in the VP of HiMyc mice on the obesity-induced diet. In addition to these changes, there were dramatic increases in angiogenesis (increased number and size of blood vessels in the VP) accompanied by dramatic increased in expression of all VEGF family members. These data provide strong support for a role of local inflammation in the effects of obesity on PCa progression using this model system.

Ribeiro et al. [128] evaluated the influence of genetically induced obesity on PCa cell growth and angiogenesis in vivo. In these studies, the androgen-insensitive murine PCa cell line, RM1, when inoculated in obese, hyperleptinemic *db*/*db* mice produced small tumors with a low proliferation index and angiogenesis. In contrast, when these cells were injected into obese, leptin-deficient *ob*/*ob* mice, larger tumors were produced with a higher proliferation index when compared to control mice. These data suggested that high leptin levels are not favorable for the androgen-insensitive PCa cell line and it may limit the growth of these tumor cells. While these studies do not shed light on the role of inflammation in PCa progression, they demonstrate the complexity of genetic models of obesity for such studies. In addition, evaluating serum levels of individual factors such as leptin may not adequately predict local changes in prostate tissues. In a recent study, Zhang et al. [129] reported

that WAT can directly mediate cancer progression by serving as a source of cells (adipose stromal cells, ASCs) that migrate to tumors and promote neovascularization. Recruitment of ASCs by tumors was sufficient to promote tumor growth. In this study, ASCs injected subcutaneously at a distant site migrated to the site of DU145 xenografts promoting tumor growth and angiogenesis.

In other studies, serum obtained from obese Zucker rats that harbor a leptin receptor mutation was shown to stimulate proliferation of human PCa cells (i.e., LNCaP cells) but not rat PCa cells (AT3B1) [130]. This was attributed to higher concentrations of VEGF in the serum of the obese rats. In further studies using C57BL/6 mice on an obesity-induced diet, serum from these mice was found to contain higher levels of leptin, VEGF, PAI-1, and IL-6 and lower levels of testosterone compared to mice fed a control diet [131]. Serum from these obese mice increased proliferation of LNCaP and PacMetUT1 cells but had no effect on PrEc and DU145 cells. Furthermore, serum from obese mice induced increased invasion, migration, and MMP-9 activity in LNCaP, PacMetUT1, and DU145 cells and showed increased expression of EMT-related markers. These later two studies emphasize the potential local effects of circulating factors, including inflammatory cytokines, altered in the obese state on PCa cells localized within the prostate or at other sites.

10.5 Strategies for Reversing the Effects of Obesity on PCa

Obesity is clearly associated with increased morbidity and mortality. Weight loss in overweight and obese individuals reduces risk factors for diabetes and cardiovascular disease. Weight loss and calorie restriction may also be an important strategy for preventing or reducing progression of PCa. A variety of effective options exist for reducing weight including dietary therapies such as low-calorie diets and lower-fat diets, exercise, behavior therapy techniques, medications, surgery, and combinations of these techniques [132]. Losing weight can reduce the risk of many cancers, even a weight reduction of only 5–10 % may be beneficial and improve health [133].

Although the relationship between physical activity and obesity is undisputed, the putative association between physical activity and PCa is inconclusive [134–136]. Postulated mechanisms include the ability of physical activity to modulate testosterone levels and immune function, insulin sensitivity, and reduce obesity [137, 138]. Physical activity increases insulin sensitivity and decreases actual insulin secretion. Increased serum levels of IGFBP-3 are also associated with greater physical activity. Some studies suggested an association between physical activity and leptin levels [139–141]. Exercise appears to lower circulating levels of IGF-1 and insulin and increase levels of sex hormone-binding globulin [142]. In vitro studies have shown that serum from men participating in exercise intervention programs can induce apoptosis and decrease the overall growth of prostate carcinoma (LNCaP) cells [143]. In a study of PCa patients on “watchful waiting,” Ornish et al. reported that those in the experimental group (vegan diet, moderate aerobic

exercise, vitamin supplements, and a stress management program) had lower levels of PSA than did those in the control group, suggesting that intensive lifestyle changes may affect the progression of early, low-grade PCa [144].

Weight loss strategies and increased physical activity could provide a decrease in obesity and parameters associated with metabolic syndrome with potentially beneficial effects on PCa progression in certain individuals. However, it may not be possible for most individuals to undergo extensive dietary manipulation and/or perform rigorous physical exercise. Thus, another important approach to offsetting the effects of obesity on PCa and other cancers is to identify novel agents that can produce some of the same beneficial effects of CR. Such agents or combination of agents could be combined with lifestyle changes as an optimal prevention strategy. An ideal agent for this purpose should produce metabolic, hormonal, and physiological effects similar to CR, without significant reduction in long-term food intake, and activate stress response pathways similar to CR and finally extend life span and reduce or delay the onset of age-related diseases [145]. Several compounds, especially naturally occurring phytochemicals, have been examined as promising agents in this regard. These include resveratrol, curcumin, epigallocatechin gallate (EGCG), quercetin, ursolic acid, rapamycin, and metformin.

Resveratrol, a plant-derived polyphenol in the skin of red grapes, is one of the most studied CR mimetics. Resveratrol has been shown to activate SIRT1 in cells and thus mimic the actions of CR without restriction of food intake. Treatment with resveratrol produces a transcriptional response in cells similar to CR and in obese mice; it has been shown to increase insulin sensitivity [146, 147]. A recent report suggested that dietary resveratrol prevents high-grade PIN development in the PTEN knockout mouse model of PCa by modulating mTORC1 and SIRT1 [148]. Curcumin is a major chemical component of turmeric (*Curcuma longa*) and widely used as a food additive in the Indian subcontinent. Various in vitro and in vivo data have suggested a potential chemopreventive role of curcumin in PCa. For example, curcumin was found to inhibit tumor development in TRAMP model either alone or in combination with phenethyl isothiocyanate (PEITC) [149]. The green tea polyphenol EGCG, another potential CR mimetic agent, decreased PCa progression in the TRAMP mouse model by inhibiting IGF-1 signaling [150]. All these observations indicate that phytochemicals can be considered as potential CR mimetics that might be beneficial in reversing the development and/or progression of obesity-related PCa.

Recently, it has been shown that diabetic patients taking metformin have reduced cancer incidence for a number of cancers [151–153]. Metformin is a biguanide class of antidiabetic agent widely used for the treatment of type II diabetes for its insulin-sensitizing effects. Recent studies also suggest that metformin has beneficial effects on PCa [153]. For example, in one study use of metformin produced 44 % reduction in PCa cases in Caucasian men compared to the control population [152]. Various in vitro and animal studies have also demonstrated the anticancer activity of metformin in PCa [154, 155]. Metformin activates AMPK which is thought to be the primary mechanism through which it exerts its anticancer effects. Thus, metformin may be a promising CR mimetic compound for reversing the effects of obesity on PCa as well as other cancers.

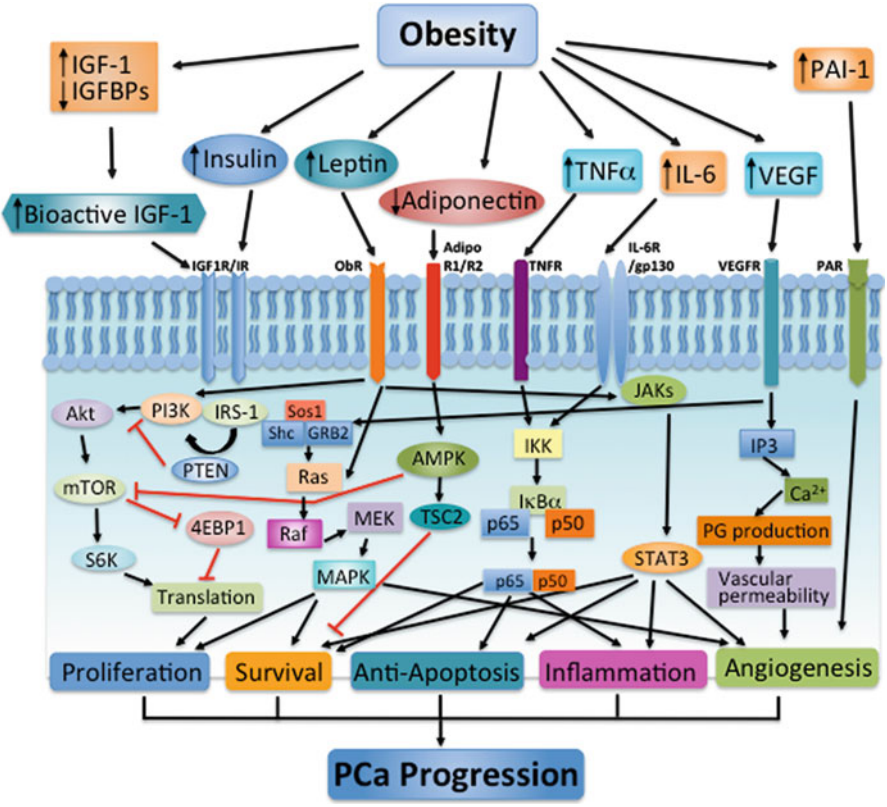

Fig. 10.1 Summary of growth factor and inflammation-associated signaling pathways that may represent targets for reversing the effects of obesity on PCa progression

10.6 Concluding Remarks

Obesity and the associated metabolic syndrome produce a complex set of alterations both systemically and locally in tissues that support cancer development and progression. Adipose tissue dysfunction, along with multiple hormones, growth factors, inflammatory cytokines, and other mediators associated with the obese state, enables cross talk between macrophages, adipocytes, endothelial cells, and epithelial cells that contribute to cancer-related processes (including growth and proliferation signaling, inflammation, and vascular alterations). Inflammation processes and inflammation-associated signaling pathways are upregulated in the obese state, and both human and mouse studies support an important role for inflammation in obesity-driven PCa progression. Inflammation signaling pathways along with other signaling pathways altered in the obese state represent promising targets for both lifestyle and pharmacologic interventions to prevent or control PCa progression. Figure 10.1 summarizes some of these pathways that may represent targets for offsetting the effects of obesity on cancer and in particular PCa progression.

A particular promising approach is the search for, and development of, CR mimetic compounds. Combinatorial approaches (e.g., lifestyle and pharmacologic) may ultimately lead to rational approaches to prevent obesity-driven PCa progression.

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Chapter 11
Pharmacologic Interventions with NSAIDs

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Abstract Obesity as a determinant of increased cancer risk and poorer cancer outcome is well established for cancers of several organ sites, including colorectal and postmenopausal breast cancer. Obesity-associated adipose inflammation leads to local and systemic accumulation of inflammatory mediators and hormones, which have multiple proneoplastic effects. Key among these from a pharmacological perspective are cyclooxygenase (COX)-derived prostaglandins (PGs), since COX enzymes are the primary target for nonsteroidal anti-inflammatory drugs (NSAIDs). Overexpression of the inducible PG synthase COX-2 occurs in the majority of colorectal neoplasias and ~40 % of breast cancers and is also evident in inflamed adipose tissue from obese mice and humans. COX/PG signaling has multiple protumorigenic consequences, which provide at least a partial explanation for epidemiologic and experimental observations of reduced cancer risk associated with NSAID use. Notably, COX/PG-mediated upregulation of estrogen biosynthesis and signaling offers a plausible target for NSAID-mediated risk reduction with respect to breast and other hormone-sensitive cancers. Additionally, "off-target" NSAID effects including modulation of NFκB and AMP kinase activity may be of particular significance in the context of obesity. NSAID-mediated amelioration of obesity-related metabolic dysfunction has been reported, and it seems likely that NSAIDs will be similarly protective for obesity-associated carcinogenesis.

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11.1 Introduction

It is becoming increasingly apparent that the molecular aberrations characterizing chronic inflammation may impact many, if not all, of the hallmark capacities intrinsic to the neoplastic process. Thus, logically, anti-inflammatory approaches may be of benefit in abrogating the increased cancer risk associated with inflammation. This may be of particular relevance in the context of obesity, an established risk factor for cancers of many organ sites [1–3], and now understood to be an inflammatory condition [4].

The classic signs of acute inflammation (pain, heat, redness, and swelling) reflect the ingress and activation of myeloid cells, with consequent release of inflammatory mediators that elicit pain, vasodilatation, and exudation of blood vessel contents into the surrounding tissue. Initial response to injury or pathogenic insult is orchestrated by resident macrophages and recruited antimicrobial granulocytes, whereas persistent inflammation is characterized by monocytic and lymphocytic accumulation. On a molecular level, inflamed tissues exhibit elevated levels of cytokines, chemokines, and eicosanoids. Key among these from a pharmacological perspective are cyclooxygenase (COX)-derived prostaglandins, which can elicit vasodilation, pain, and fever. Inhibition of COX enzymes is the primary mechanism of analgesics of the nonsteroidal anti-inflammatory drug class (NSAIDs).

Significantly, obesity is now recognized as an inflammatory condition, with leukocyte infiltration of white adipose tissue depots resulting in increased production of inflammatory mediators, including adipokines, cytokines, and prostanoids, as well as increased release of fatty acids [4, 5]. Adverse systemic consequences of adipose inflammation include both insulin resistance, leading to type II diabetes, and increased cancer risk. Notably, NSAIDs have been reported to both ameliorate insulin resistance and attenuate cancer risk (discussed below). While intuitively this might appear predictable given the relationship between these diseases and inflammation, the mechanistic basis by which NSAIDs afford protection is likely complex and multifactorial and not predicated solely on COX inhibition. This chapter reviews the evidence for NSAIDs as anticancer agents, considers likely mechanisms, and discusses the relevance of these in the context of obesity-driven neoplasia.

11.2 Cyclooxygenases and Cancer

The established anticancer activity of NSAIDs, with respect to both human disease and experimental models, is best understood in terms of their activity as COX inhibitors. COX enzymes are prostaglandin (PG) synthases, responsible for conversion of arachidonic acid (AA) to the intermediary prostanoid PGH_2, which then serves as substrate for a range of synthases, ultimately resulting in production of numerous eicosanoids, including PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 (prostacyclin), and thromboxanes (TXs) [6]. The complexion of prostanoid production by a given cell type is predicated

by the unique expression pattern of terminal synthases. Thus platelets are a rich source of TXA_2, whereas endothelium is an important site of prostacyclin synthesis. PGE_2, the isoform most strongly implicated in pain, inflammation, and neoplasia, is produced both by inflammatory and epithelial cells. COX expression levels are also important determinants of prostanoid production. COX-1 (encoded by the *PTGS1* gene) is constitutively expressed and believed to serve an important function in protecting the gastrointestinal (GI) mucosa, NSAID-mediated impairment of which is associated with GI bleeds. In contrast, the *COX-2* (or *PTGS2*) gene exhibits a highly restricted pattern of constitutive expression, notably in specific regions of kidney and brain, but induction is elicited by a wide variety of stimuli including cytokines, growth factors, and oncogenes [7–9]. COX-2-derived eicosanoids play key roles in the inflammatory response and thus provide a major focus for pharmacological intervention.

A driving role for COX-2 in neoplasia was initially suggested by parallel findings of COX-2 overexpression in cancers and epidemiological data linking analgesic use with reduced cancer incidence. Notably, the most consistent associations between NSAID use and diminished cancer risk have been identified for colorectal cancer both in epidemiological and interventional studies. Thus this chapter focuses predominantly on two main cancer sites, colorectal, based on the epidemiological evidence, and breast, based on plausible mechanisms for NSAID-mediated protection in the context of obesity for this latter disease.

11.2.1 Cyclooxygenase Expression in Intestinal and Breast Tissues

A general theme with respect to COX expression and epithelial cancers has emerged: *COX-1* expression tends to be relatively constant in normal and cancerous tissues, while *COX-2* is frequently upregulated in precancers and cancers relative to benign epithelium. For example, evaluation of *COX* expression in human colorectal neoplasms revealed unaltered expression levels of *COX-1* in colorectal carcinomas relative to adjacent normal mucosa [10–12], whereas *COX-2* is virtually undetectable in normal colonic mucosa, but highly expressed in adenocarcinomas, with detectable expression in epithelial, endothelial, and stromal components [10–13]. Consistent with human data, *COX-2* upregulation is also evident in intestinal tumors from both genetic (*Apc* mutant) and carcinogen-driven rodent models [14–18]. Given the present focus on inflammation as a driver of carcinogenesis, it is noteworthy that, while in adenocarcinomas *COX-2* expression is predominantly epithelial, in the precursor polyps COX-2 is primarily localized in inflammatory cells in the lamina propria [15, 19, 20]. These data suggest potential paracrine interactions between *COX-2*-expressing macrophages and enterocytes as early drivers of intestinal neoplasia. Intriguingly, a single study has identified an association between rectal mucosal PGE_2 levels and body mass index (BMI) in patients with a history of polyps,

although the cell type from which PGE_2 derived was not identified [21]. Nevertheless, these data provide provocative evidence for a link between BMI and upregulated prostaglandin production in the intestine, potentially mediated via increased inflammation.

In breast carcinomas, *COX-2* overexpression is less prevalent than in CRC, although elevated PG levels were first reported in human breast tumors over 30 years ago [22–24]. In aggregate, immunohistochemical (IHC) studies have established that approximately 40 % of breast carcinomas exhibit epithelial *COX-2* overexpression, with negligible COX-2 in benign epithelium [25–38]. Increased frequencies of *COX-2* overexpression (63–85 %) are evident in precursor ductal carcinoma in situ (DCIS) lesions relative to invasive disease [25, 29, 33, 34, 36, 39, 40], and COX-2 has also been observed in normal-looking epithelium adjacent to neoplastic tissue [28, 29, 34, 36, 39–41]. Additionally, COX-2 is expressed in morphologically normal breast epithelium in association with $p16^{INK4a}$ hypermethylation, perhaps identifying very early foci of precancerous cells [42], and is associated with high mammographic density [43], an index of increased breast cancer risk.

Several studies identify COX-2 overexpression in breast cancer epithelium as a poor prognostic indicator, and COX-2 is associated with large tumor size, high grade, and the presence of nodal metastases [26, 28, 32, 35, 44]. COX-2 overexpression in DCIS precancers also has prognostic significance in association with $p16^{INK4a}$ and Ki67 status [45]. Additional correlations have been observed with overexpression of HER2/neu (human epidermal growth factor receptor 2) and with hormone receptor-negative status of carcinomas, both of which are independently associated with poor prognosis [25, 28, 32, 37, 46], although one recent study identified selective expression of COX-2 in estrogen receptor (ER)-positive breast cancers [44]. In contrast, COX-1 is ubiquitously expressed in mammary tissue and is not of prognostic significance [28, 34, 38]. Rodent models provide parallel observations with respect to COX expression in breast epithelium, including ubiquitous COX-1 expression [47], and upregulated COX-2 expression in mammary tumors from both transgenic and carcinogen-induced models [48–52].

Most recently, attention has focused on COX-2 expression in normal breast tissue as a function of obesity. Specifically, both in human and murine breast adipose tissue, PGE_2 and COX-2 levels increase with increasing BMI [53, 54]. Striking correlations are evident between COX-2/PGE_2 levels and the presence of inflammatory foci, comprising adipocytes encircled by macrophages [54]. These crown-like structures (CLS), first identified in white adipose tissues of non-mammary origin from obese mice and humans, have emerged as hallmark lesions of obesity-associated inflammation [55–57]. In both human and rodent breast tissue, CLS abundance increases as a function of body mass [53, 58, 59]. Simplistically, macrophages can be divided into two classes. Classically activated, or M1-polarized, macrophages secrete proinflammatory mediators including PGE_2, interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNFα), whereas alternatively activated (M2) macrophages triggered by Th2 cytokines are associated with tissue remodeling and immunosuppression. While resident macrophages in lean adipose tend to be M2-polarized, inflammatory M1 macrophages predominate in CLS. Importantly,

these morphologic entities likely correspond to the functional unit responsible for a significant component of obesity-related pathologies. Paracrine interaction between necrotic adipocytes and associated M1-polarized macrophages results in increased synthesis of PGE_2, IL-1β, IL-6, and TNFα in the macrophage compartment [53]. These proinflammatory mediators can have both local and systemic actions, which likely contribute both to insulin resistance and to the increased cancer incidence associated with overweight/obesity [1, 2].

11.2.2 Animal Models: Genetic Evidence for COX Contributions to Cancer

The fact of COX-2 overexpression in colorectal, breast, and other cancers, and the observed correlations between epithelial COX-2 overexpression and prognosis, argue strongly for a protumorigenic role of COX-2 in neoplasia. This thesis is supported by a wealth of data from animal models including several definitive genetic studies, as well as pharmacological studies (described below; Sect. 11.3.1). The Taketo lab pioneered the genetic approach, demonstrating substantial reduction (86 %) in intestinal tumor incidence in an *Apc* mutant mouse strain ($Apc^{\Delta 716}$) consequent on homozygous inactivation of the *COX-2* gene [15]. Of note, *Apc* mutant strains are widely used to model human colorectal neoplasia because mutational inactivation of the *APC* tumor suppressor gene is the initiating event in the vast majority of human sporadic colorectal neoplasms, as well as in some familial polyposis syndromes.

We subsequently employed a genetic strategy modeled on the Taketo approach to explore the contribution of COX-2 to breast cancer, using a mouse strain with mammary-targeted *HER2/neu* transgene expression that develops multiple DCIS lesions in each mammary gland to model the HER2-COX-2 relationship observed in the human disease. Decreased mammary tumor multiplicity was observed in the context of *COX-2* deficiency in our study [60]. *COX-2* nullizyogosity was associated with reduced DCIS tumor formation and growth, and with substantial diminution in mammary gland vascularization, unequivocally demonstrating a role for COX-2 in mammary neoplasia.

Further knockout studies have implicated both COX isoforms in experimental neoplasia. Similar magnitudes of intestinal and epidermal tumor abrogation can be achieved by disruption of either *COX-1* or *COX-2*, suggesting that perhaps the sum total of COX activity is the key determinant of tumorigenesis, rather than unique contributions of either enzyme [61, 62].

Hla and colleagues adopted a genetic overexpression approach to provide elegant proof of the in vivo oncogenicity of COX-2 through generation of a *COX-2* transgenic mouse strain. Mammary-targeted expression of a *COX-2* transgene drives tumor formation in multiparous mice [63]. Increased microvessel density and angiogenic gene expression are evident prior to discernible tumor formation, and

COX-2 overexpression retards mammary involution in post-weaning dams via suppression of apoptosis, providing mechanistic insights into COX-driven neoplasia [63, 64]. Furthermore, transgenic *COX-2* overexpression in skin increases sensitivity to carcinogen-induced neoplasia, again providing evidence for a proneoplastic role of COX-2 [65]. In aggregate, genetic knockout and overexpression approaches, together with pharmacological data discussed below, firmly substantiate the role of COXs and COX-derived prostanoids in cancer.

Follow-up studies have focused on evaluating alternative molecular targets for anti-inflammatory intervention that retain the efficacy of NSAIDs while minimizing corollary toxicity. A central role for PGE_2 as an effector of COX-mediated tumorigenesis has been established. PGE_2 administration reverses aspirin-induced adenoma regression and enhances carcinogen-induced tumor incidence, whereas genetic deletion of PGE_2 receptors (EPs) confers resistance to formation of aberrant crypt foci, polyps, and cancers in the intestinal tract [66]. Importantly, genetic deletion of the terminal enzyme responsible for PGE_2 synthesis (microsomal PGE_2 synthase-1, mPGES-1) suppresses the growth of both intestinal [67] and mammary tumors (Howe et al., manuscript in preparation), identifying mPGES-1 as a candidate target for antineoplastic intervention.

11.2.3 Cyclooxygenases and Cancer: Mechanisms

COX/PG signaling impacts many of the capabilities highlighted by Hanahan and Weinberg as essential for tumor growth and metastatic dissemination [68], including increased proliferative potential, resistance to apoptosis, enhanced invasiveness, immune suppression, and angiogenesis (Fig. 11.1). Self-evidently, as a key component of the inflammatory response, the "enabling characteristic" of tumor-promoting inflammation is also encompassed by COX-2 overexpression.

11.2.3.1 Prostanoid Effects on Proliferation and Immune Suppression

Prostaglandin stimulation of proliferation is well established for multiple cell types, such as mammary epithelial cells and fibroblasts [69–71]. PGE_2 can also stimulate growth of human colorectal adenoma cells, although it has been suggested that response may vary as a function of EP4 receptor expression level [72]. Notably, reported PG-mediated stimulation of stem cells [73] may have important ramifications for cancer, and indeed recent studies suggest that PGE_2 released from stromal fibroblasts plays a key role in paracrine regulation of cancer stem cells [74, 75]. Numerous pro-proliferative signaling pathways are implicated in mediating PG effects including activation of phosphatidylinositol 3-kinase (PI3K)/AKT and Wnt/β-catenin signaling pathways [76] and activation of epidermal growth factor receptor (EGFR) signaling [77, 78].

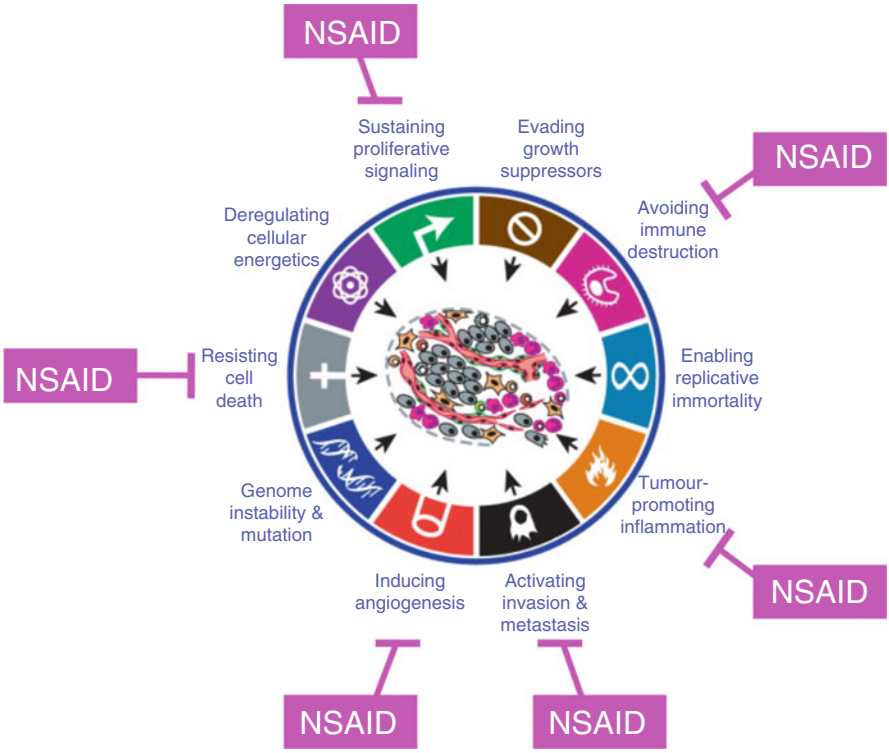

Fig. 11.1 Nonsteroidal anti-inflammatory drug (NSAID) effects on cancer hallmarks. Cyclooxygenase/prostaglandin signaling impacts many of the capabilities suggested by Hanahan and Weinberg to be essential for tumor growth and metastatic dissemination [68]. In turn, NSAID-mediated COX inhibition affords the possibility of reversing the majority of these protumorigenic traits. Persuasive evidence supports positive effects of COX/PG signaling and hence reciprocal NSAID-mediated suppression on the indicated parameters. Additional data suggest the possibility that NSAIDs may suppress genome instability via reducing microsatellite instability [316] and may also antagonize replicative immortality through downregulating telomerase expression [317]

In contrast to these pro-proliferative PG effects, certain immune cells respond to PGs with attenuated proliferative responses, which may be parlayed into an immune evasion strategy in the neoplastic setting [79–81]. Both B and T lymphocytes are subject to PGE_2-mediated growth suppression. PGE_2 also induces biased cytokine production from T cells towards Th2 cytokine release, downregulates Th1-associated cytokine synthesis, and promotes IL-10 expression, all of which may favor immune suppression. Furthermore, COX-2 is implicated in skewing macrophages towards a tumor-promoting M2 phenotype and averting the potential protection afforded by M1 polarization [82, 83]. Of note, while macrophages comprising CLS in inflamed adipose tend towards M1 polarization, tumor-associated macrophages are M2-like and are thought to favor cancer progression by promoting tissue remodeling and immune suppression [84]. Prostanoids also impact the function and development of dendritic cells [85, 86] and enhance accumulation and activity of

myeloid-derived suppressor cells (MDSCs), which can inhibit both innate and adaptive immunity [80]. Thus, undermining the host immune response is likely to be a key protumorigenic property of COX-derived prostanoids.

11.2.3.2 Suppression of Apoptosis

In addition to eliciting tumor-favoring pro-proliferative effects in epithelial cells, COX overexpression may also tip the balance towards cell survival through suppressing apoptotic cell death. Forced overexpression of *COX-2* in epithelial cells suppresses apoptosis both in vitro and in vivo [63, 87]. Consistent with these observations, NSAIDs commonly induce apoptosis of cultured cells, although both COX-mediated and COX-independent mechanisms have been proposed to account for NSAID proapoptotic effects (see Sect. 11.5.2). The dual effects of COX overexpression on proliferation and apoptosis may be important for promoting survival of damaged cells with acquired mutations that would otherwise trigger cytoprotective cell death machinery.

11.2.3.3 Invasion and Metastasis

COX signaling is also strongly implicated in invasiveness and hence metastatic potential. In vitro studies have established increased cellular invasiveness as a consequence of *COX-2* overexpression, with corollary upregulation of matrix metalloproteinases which can promote extracellular matrix remodeling and hence tumor invasion [88, 89]. Consistent with a prometastatic function, correlations are evident between *COX-2* expression and both lymph node metastases and prognosis in human breast cancer [26, 28, 32, 35], and pulmonary metastasis of breast cancer xenografts and allografts can be suppressed by COX inhibitors [90–92]. Furthermore, COX-2 is also implicated in osteoclast differentiation, a key step in bone metastasis, which requires osteoclast-mediated bone resorption to provide a niche for tumor cells. PGE_2 stimulates both osteoclast differentiation and bone resorption, and osteoclastogenesis is impaired in bone marrow from *COX-2*-deficient mice [93–95]. Additionally, in an experimental model of sarcoma bone invasion, COX-2 inhibition reduces osteoclast proliferation and hypertrophy, tumor-induced bone resorption, and tumor burden [96]. These data are consistent with early observations from Powles et al. that aspirin treatment reduced the in vitro osteolysis-inducing activity of human breast carcinomas [97]. In aggregate, these data suggest that COX-derived PGs may regulate cancer cell invasion and metastasis.

11.2.3.4 Angiogenesis

Angiogenesis, the process of tumor neovascularization that ensures adequate blood supply, is another intrinsic feature of neoplasia in which COX/PG signaling plays a role. *COX-2*-deficient mice exhibit substantial reductions in mammary gland

vascularization and expression of proangiogenic genes, with reduced vessel density evident not only in mammary tumors but also in morphologically normal mammary gland, implying a broad role for COX-2 in vascularization of the mammary gland [60]. In contrast, knockout studies using intestinal cancer models suggest that in the intestine *COX-2* contributes primarily to growth and vascularization of tumors beyond 1 mm in diameter [98, 99]. Contribution of stromal *COX-2* is well illustrated by the impaired growth and diminished vascular density of Lewis lung carcinoma xenografts when implanted into *COX-2*-null hosts [100]. Nevertheless, epithelial COX-2 can also promote angiogenesis: transgenic *COX-2* overexpression in mammary epithelium induces substantial remodeling of mammary gland vascular architecture [64]. Together these genetic approaches demonstrate a clear role for COX-2 in angiogenesis. Pharmacological approaches yield similar data: in vivo angiogenesis is reduced by administration of non-selective NSAIDs or selective COX-2 inhibitors (COXibs) in multiple models [64, 90, 91, 101–107], while in vitro COXibs decrease endothelial tube formation [108, 109]. COX-1 is also implicated, since NSAIDs can have similar effects in experimental systems lacking COX-2 expression [106, 108, 109]. COX-regulated production of proangiogenic factors such as vascular endothelial growth factor (VEGF) provides a clear mechanistic basis for the COX-angiogenesis relationship [110, 111] and likely explains observed correlations between COX-2 and microvessel density/VEGF expression in human breast cancers [26, 27, 31, 41].

The COX-2/VEGF interrelationship might also provide insight into the increased cancer incidence and poorer outcome associated with obesity. Human studies have identified positive correlations between circulating levels of VEGF and BMI [112–114]. This could reflect increased adiposity, given that adipose tissue is a rich source of VEGF. Additionally, based on the increased COX-2 levels observed in visceral and mammary adipose depots from obese subjects [53, 54], COX-dependent upregulation of VEGF could induce elevated adipose VEGF expression per wet tissue weight in obese vs. lean adipose. Indeed both adipose and circulating VEGF levels are elevated in a mouse model of postmenopausal obesity [115]. Furthermore, Hung and colleagues recently reported that murine mammary tumor sensitivity to the VEGF antagonist bevacizumab is increased in the context of obesity [116]. Thus, obesity-associated disproportionate increases in adipose VEGF synthesis may contribute to tumor angiogenesis.

Evidence is also available to support a role for COX-2 in regulation of lymphangiogenesis, the formation of new lymphatic vessels. COX-2 expression correlates with that of VEGF-C, the primary driver of lymphangiogenesis, in breast cancer cell lines and tissues, and COXib treatment suppresses tumor expression of a lymphangiogenic marker in vivo [117, 118].

11.2.3.5 Regulation of Peripheral Estrogen Biosynthesis

COX-2 overexpression may have consequences of particular significance for breast neoplasia, based on the ability of prostaglandins to regulate transcription of the *CYP19* gene, which encodes the estrogen synthase cytochrome P-450 aromatase.

Approximately two-thirds of human breast carcinomas express estrogen receptor-α (ERα), and the importance of estrogen in breast neoplasia is well illustrated by the demonstrated clinical efficacy of antiestrogen approaches including selective estrogen receptor modulators (SERMs) and aromatase inhibitors (AIs) as breast cancer therapeutics.

Prior to menopause, the ovary is the primary site of estrogen synthesis from androgens. However, post climacteric, peripheral sources assume increased importance, particularly adipose tissue as well as breast cancer epithelium [119–123]. Notably, a switch in *CYP19* promoter usage occurs in breast cancers and cancer-proximal stromal tissue, involving selective utilization of cAMP-sensitive promoters [124–127]. Consistent with the ability of PGE_2 to elicit cAMP accumulation [128], PGE_2 signaling stimulates aromatase transcription [129–133]. Furthermore, positive correlations between *COX* and *CYP19* expression have been identified in human breast cancers [134–136]. Definitive in vivo evidence for a causal basis for these findings is provided by transgenic and knockdown approaches: aromatase activity is increased in the mammary glands of *COX-2* transgenic mice and, conversely, decreased in *HER2/neu*-overexpressing mammary tissues that are *Cox-2* nullizygous [137]. Together these findings establish COX-2 as an important determinant of aromatase expression in mammary tissues and hence likely to regulate neoplastic growth through increasing tumor-proximal estrogen tissue levels. A further prediction from these data is that COX-inhibiting NSAIDs may regulate circulating hormone levels in postmenopausal women, and indeed some (though not all) studies have identified correlations between NSAID use and serum estradiol in postmenopausal women [138, 139].

Importantly, the COX-aromatase-estrogen pathway may not only be relevant for driving breast cancer progression but may also be a component of the increased breast cancer risk associated with obesity. Based on the importance of adipose as an estrogen source in postmenopausal women, it has long been assumed that increased adiposity should be associated with elevated estrogen synthesis, and indeed BMI is an established determinant of serum estradiol in postmenopausal women [140–142]. However, it is becomingly increasingly apparent that not only the quantity but also the quality of fat is altered in obesity, and this has important ramifications for adipose estrogen production. As described above, white adipose tissue from obese mice and humans is distinguished by the presence of inflammatory foci, or CLS, consisting of macrophage-encircled adipocytes [55–57]. These are characteristic not only in visceral fat but also in breast adipose tissue [53, 54, 58, 59]. Activated M1-polarized macrophages in these foci provide a rich source of proinflammatory factors, including PGE_2 as well as TNFα and IL-1β, which can induce increased aromatase transcription in the surrounding adipose tissue (Fig. 11.2). Thus, obesity drives adipose inflammation leading to local induction of aromatase expression in breast tissue. Delineation of this pathway provides a clear explanation for the earlier observation that BMI is a determinant of aromatase transcript levels in visceral fat [143] and offers mechanistic insight into the positive correlation between postmenopausal obesity and breast cancer risk.

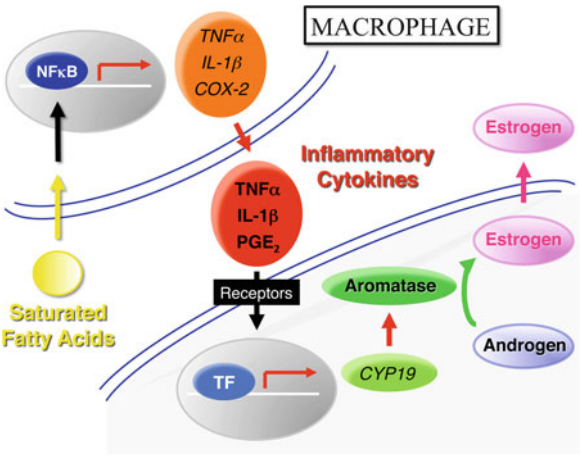

Fig. 11.2 Paracrine interactions between macrophages and other cell types establish an inflammatory milieu in obese breast adipose tissue, resulting in elevated estrogen biosynthesis. Saturated fatty acids, liberated from adipocytes as a result of obesity-associated lipolysis, stimulate NFκB-driven induction of inflammatory mediators in macrophages including TNFα, IL-1β, and COX-2. Additionally, bacterial toxins entering systemic circulation as a consequence of obesity-induced impairment of gut mucosal integrity may elicit NFκB activation through TLR4 ligation. Cytokines and COX-2-derived PGE_2 activate transcription of the *CYP19* gene encoding aromatase in neighboring cells, including preadipocytes, leading to elevated expression and activity of aromatase. Consequently, estrogen biosynthesis is enhanced, leading to increased expression of ER target genes, including PR

11.2.3.6 Mutagen Synthesis

A final procarcinogenic consequence of COX-2 overexpression, independent of prostanoid production, hinges on the ability of COX-2 to generate mutagens. For example, malondialdehyde produced from PGH_2 can induce frameshifts and base-pair substitutions through adduct formation with deoxynucleosides and thus drive mutagenesis [144]. COX-2-mediated oxidation of aromatic amines, heterocyclic amines, and dihydrodiol derivatives of polycyclic hydrocarbons also generates carcinogens. Thus, COX-2-dependent DNA damage may play a role at the earliest stages of neoplasia.

11.3 NSAID-Mediated Suppression of Neoplasia

NSAIDs can be divided into broad functional classes based on their relative selectivity for COX-1 vs. COX-2. Conventional NSAIDs (e.g., indomethacin, ibuprofen) inhibit both COX isoforms, although relative potencies for each enzyme vary between individual drugs. COX-2-selective inhibitors (celecoxib, rofecoxib,

nimesulide, etc.) were developed with the goal of circumventing GI toxicity associated with prototypic NSAIDs. By contrast, the marked activity of aspirin towards COX-1 provides the basis for widespread use for antiplatelet prophylaxis. Intriguingly, some NSAID metabolites such as sulindac sulfone, the hepatic oxidation product of the prodrug sulindac, have anticancer activity in the absence of discernible COX inhibition, highlighting the functional significance of NSAID off-target effects (discussed below, Sect. 11.5).

Myriad studies have evaluated NSAID-mediated tumor suppression in animal models, epidemiological analyses, and clinical interventions. Consensus findings include convincing inhibition of experimental intestinal and breast neoplasia by multiple NSAID classes, strong epidemiological support for NSAID-mediated reduction in colorectal cancer risk, similar but less robust epidemiological evidence for a protective effect with respect to breast neoplasia, and compelling evidence from interventional studies focusing on both neoplasia and cardiovascular disease as primary endpoints. These data are reviewed in the following sections.

11.3.1 NSAID-Mediated Tumor Suppression in Animal Models

COX inhibitor efficacy for suppression of experimental neoplasia has been evaluated in a variety of animal models (for detailed reviews, see [9, 145–147]). For assessing intestinal cancer prophylaxis, the range of model systems encompasses carcinogen-treated rodents (AOM, azoxymethane; DMH, dimethylhydrazine), *Apc* mutant mouse strains (Apc^{Min}, $Apc^{\Delta 716}$, $Apc^{\Delta 474}$, and *Apc1638*), and mismatch repair-deficient mice (*Msh2*-deficient). Experimental endpoints include aberrant crypt foci, adenomas, and colon carcinomas in carcinogen-treated rodents, and predominantly small intestinal polyps in *Apc* mutant mice. Early studies of aspirin and non-selective NSAIDs (e.g., sulindac, piroxicam) were succeeded by analyses of selective COX-2 inhibitors (nimesulide, MF tricyclic, celecoxib, NS-398, JTE-522, rofecoxib) as these became available and most recently NSAID phospho- and NO derivatives. Substantial chemopreventive efficacy with respect to experimental intestinal neoplasia has been reported for all NSAID classes, consistent with human epidemiologic observations (see Sect. 11.3.2.1), although dosing issues in some cases preclude direct interspecies comparisons [8].

Increasing interest has also focused on combination drug approaches, with dual goals of maximizing anticancer efficacy and minimizing NSAID cardiovascular and GI toxicity. COXibs have been tested in combination with EGFR inhibitors [148, 149], the HER2/neu-neutralizing antibody trastuzumab [150], fish oil [151], atorvastatin [152, 153], as well as the ornithine decarboxylase (ODC) inhibitor difluoromethylornithine (DFMO) [154], with generally promising combinatorial efficacy.

Comparable analyses have been performed in rodent breast cancer models, again with a prominent emphasis on chemoprevention, although therapeutic efficacy has also been tested [9, 147]. Protective effects of conventional NSAIDs (e.g.,

indomethacin, flurbiprofen) with respect to carcinogen-induced breast cancer were first established 30 years ago, with subsequent validation of selective COX-2 inhibitors in carcinogen models. Additionally, using *HER2/neu* transgenic rodents to model the correlation between HER2/neu and COX-2 overexpression observed in the human disease, celecoxib has been shown to effect modest retardation of HER2/neu-driven tumor formation [50, 155, 156]. As in intestinal cancer models, combination approaches have been explored. For example, coadministration of celecoxib with the retinoid X receptor ligand LGD1069 achieved a greater degree of tumor retardation than either agent singly [157]. The demonstrated chemopreventive efficacy of COX inhibitors in breast cancer models is consistent with general findings from human epidemiologic analyses (see Sect. 11.3.3.1).

Increasing appreciation of the relationship between obesity and elevated cancer risk is likely to prompt additional evaluation of NSAIDs with respect to obesity-associated cancer, both experimentally and epidemiologically. This is particularly germane given that COX-2/PG levels are elevated in breast adipose tissue of obese mice and humans in association with inflammation [53, 54]. To date, no reports of NSAID efficacy in obesity-driven intestinal cancer models are available. However, emerging data are starting to address this issue in breast cancer. The accelerated growth rate of E0771 mammary tumors implanted into diet-induced obese mice, relative to that in lean mice, is reversed by aspirin treatment [116], implying a selective anti-inflammatory action of aspirin in the obese cohort. Future studies will no doubt substantiate this finding in other models and provide mechanistic insight.

11.3.2 NSAIDs and Human Colorectal Neoplasia

Epidemiological analyses of NSAID use and colorectal cancer incidence yield findings consonant with those from rodent studies. Numerous analyses of both large prospective cohorts and smaller case–control studies have been performed, which variously consider adenoma or carcinoma as an endpoint. Inverse associations between NSAID use and risk of CRC have been fairly consistently identified, with the most abundant datasets for aspirin, based in part on widespread aspirin use for cardiovascular prophylaxis [158]. Randomized clinical trials also provide compelling evidence of suppression of colorectal neoplasia by NSAIDs.

11.3.2.1 Epidemiologic Findings

Bosetti et al.'s recent meta-analysis of aspirin and cancer risk provides an updated overview of observational studies [159]. Thirty studies which considered aspirin use and CRC were evaluated: 15 case–control studies (21,414 total cases) and 15 cohort studies (16,105 cases). Overall, regular aspirin use was associated with a 27 % reduced risk of CRC (relative risk [RR], 0.73; 95 % confidence interval [CI],

0.67–0.79; $P<0.001$). In general, stronger protective effects were observed in case–control studies (RR, 0.63; 95 % CI, 0.56–0.70) than in cohort studies (RR, 0.82; 95 % CI, 0.75–0.89). Similar protection was afforded by aspirin with respect to colon and rectal cancer. Bosetti et al. concluded from their analysis that use of regular/high-strength aspirin for at least 5 years is necessary to confer protection against colorectal cancer [159]. Similar or greater risk reductions have also been identified in aspirin users considering adenomas as an endpoint. Of note, although many observational studies to date have focused solely on aspirin, others have considered combined aspirin and NSAID use. Comparative analyses suggest that non-aspirin NSAIDs afford similar protection [160–163]. Furthermore, use of selective COX-2 inhibitors (rofecoxib, celecoxib) is also associated with reduced risk of colorectal neoplasia [162, 164, 165].

11.3.2.2 Findings from Randomized Clinical Trials

Complementing the epidemiological findings, interventional approaches have also identified reduced colorectal neoplasia in groups assigned to NSAID. Aspirin has been evaluated on colorectal adenoma and carcinoma endpoints in a variety of clinical settings, including patients with prior adenoma, prior carcinoma, and Lynch syndrome patients (i.e., individuals with hereditary predisposition to colorectal cancer due to mutations in DNA repair genes) [158]. Additionally, cancer outcome data have recently become available from several large-scale clinical trials of aspirin primarily intended to evaluate cardiovascular (CV) disease prevention [166–168]. Effects on colorectal adenoma/carcinoma endpoints of several non-aspirin NSAIDs have also been evaluated, most notably sulindac and selective COX-2 inhibitors (see Sect. 11.3.2.5).

Early proof-of-principle data for NSAID-mediated suppression of colorectal neoplasia were provided by demonstration of sulindac-mediated reduction in polyp burden in familial adenomatous polyposis (FAP) patients over a 9 month treatment period [169]. Importantly in mechanistic terms, sulindac treatment caused significant reductions in rectal mucosal prostanoid levels (PGE_2, $PGF_{2\alpha}$, TXB_2) [170]. More recently, substantial suppression of adenoma recurrence has been achieved using sulindac in combination with the ODC inhibitor DFMO for 3 years [171].

Aspirin efficacy for suppression of colorectal adenomas has been evaluated in four placebo-controlled, randomized trials in patients with a history of colorectal adenoma or carcinoma [172–175]. Meta-analysis of these trials showed that aspirin at daily doses ranging from 81 to 325 mg reduced the risk of colorectal adenoma by 17 % (RR, 0.83; 95 % CI, 0.72–0.96) over a median post-randomization follow-up of 33 months [176], although one of the four trials subsequently reported negative findings with 4 years follow-up [177] contrasting with the positive 1-year findings included in the meta-analysis [176]. Notably, aspirin is capable of suppressing mucosal PGE_2 levels over this dose range, when administered for 2 weeks to 3 months [178–182].

In contrast to studies of colorectal adenoma recurrence, few trials have evaluated aspirin with carcinoma as the primary endpoint. One notable exception is the CAPP2 trial, in which 600 mg/day aspirin has been shown to reduce CRC incidence in Lynch syndrome patients who completed 2 years of intervention (hazard ratio (HR), 0.41; 95 % CI, 0.19–0.86; $P = 0.02$) [183]. Importantly, recent meta-analyses of several randomized trials of aspirin for CV disease reduction have identified reduced risk in aspirin cohorts not only of colon cancer incidence but also of metastasis and mortality [166–168]. Initial analysis of four trials (Thrombosis Prevention Trial, British Doctors Aspirin Trial, Swedish Aspirin Low-Dose Trial, and UK-TIA Aspirin Trial) identified a reduced 20-year risk of colon cancer incidence and mortality (incidence: HR, 0.76; 95 % CI, 0.60–0.96; $P = 0.02$; mortality: HR, 0.65; 95 % CI, 0.48–0.88; $P = 0.005$), with no evidence for increased benefit at doses greater than 75 mg/day [167]. Increasing benefit was associated with longer durations of scheduled treatment. Consistent data for death due to several common cancers were obtained through analysis of eight aspirin trials [166]. Further analysis of five UK-based trials of aspirin for CV disease prevention revealed aspirin-associated reduction in adenocarcinoma metastasis, both at initial diagnosis and on subsequent follow-up, particularly in patients with CRC [168].

At odds with data from the CV prevention trials, two large US-based intervention studies of aspirin failed to see reduced CRC risk [184, 185]. The basis for the discrepant observations in the Physicians' Health Study and Women's Health Study relative to other studies remains unclear, but may be a function of insufficient exposure. Both studies used an alternate day dosing schedule. Additionally, insufficient duration of treatment or follow-up may have limited the capacity to detect protective effects. This possibility is supported by the clear signal of increased efficacy as a function of increased duration of aspirin use in both observational and intervention studies [159, 166, 167, 186–189].

11.3.2.3 NSAIDs and CRC Mortality: Determinants of Sensitivity

As mentioned above, it is increasingly apparent that aspirin/NSAID use not only decreases colorectal adenoma and carcinoma occurrence but may also be associated with reduced CRC mortality. In the Nurses' Health Study, regular aspirin use was associated with reduced risk of death from CRC (RR, 0.72; 95 % CI, 0.56–0.92), although greater protection was afforded for death from CV disease (RR, 0.62; 95 % CI, 0.55–0.71) [190]. Several analyses have identified an association specifically between prediagnosis NSAID use and CRC-specific survival [191, 192], although findings are not consistent in all studies [191, 193]. Associations between postdiagnosis aspirin use and reduced CRC-specific mortality have also been observed in several cohorts [194–196]. Consistent with the protective effect of aspirin/NSAIDs observed in epidemiologic analyses, the recent Rothwell meta-analyses of multiple randomized clinical trials with a primary CV endpoint have also revealed reduced colon cancer mortality in patients allocated to aspirin [166, 167].

Tumor COX-2 expression is emerging as a molecular determinant of CRC sensitivity to aspirin. In the Health Professionals Follow-Up and Nurses' Health Study cohorts, aspirin-mediated risk reduction was apparent for COX-2-overexpressing cancers, but not for those with weak or absent COX-2 expression [197]. Subsequent analysis of these cohorts was designed to determine the relationship between aspirin use post diagnosis, CRC-specific mortality, and tumor COX-2 expression. Strikingly, regular aspirin use after diagnosis was associated with a lower risk of CRC-specific mortality among patients whose primary tumors overexpressed COX-2 (HR, 0.39; 95 % CI, 0.20–0.76), but not in patients with primary tumors with weak or absent expression (HR, 1.22; 95 % CI, 0.36–4.18) [194]. Stromal COX-2 expression has also been identified as predictive of adenoma recurrence, although in this study adenoma COX-2 expression did not correlate with aspirin responsiveness [198].

Contrasting with the CRC data, COX-2 expression in breast carcinoma has not been found to be a determinant of aspirin sensitivity. In the Nurses' Health Study, tumor COX-2 expression was associated with higher diagnostic stage [44], consistent with earlier studies in which COX-2 expression in breast cancers was identified as an indicator of poor prognosis [26, 28, 32, 35]. However, the relative risk of breast cancer death for current aspirin use was similar for patients with COX-2-positive and COX-2-negative disease [44].

A recently reported analysis of the Health Professionals Follow-Up and Nurses' Health Study cohorts suggests that mutational activation of PI3K signaling may also confer aspirin sensitivity [199]. Specifically, mutation analysis of the gene encoding the catalytic α subunit (*PIK3CA*) was performed on colorectal cancers from 964 patients, and survival was compared with aspirin use as a function of mutational status. Use of postdiagnosis aspirin was found to selectively confer a survival advantage for patients with *PIK3CA*-mutant tumors. Thus, in aggregate, molecular analyses from the Health Professionals Follow-Up and Nurses' Health Study suggest both COX-2 expression and *PI3KCA* mutation in colorectal cancers as determinants of sensitivity to aspirin [194, 197, 199].

11.3.2.4 Obesity and NSAID-Mediated Suppression of Human Colorectal Neoplasia

To date, little epidemiologic data exists addressing obesity as a modifier of, or sensitizer to, NSAID action in human colorectal neoplasia, although, as discussed below (Sect. 11.5.4), there are abundant potential functional intersections between NSAID actions and obesity-related procarcinogenic mechanisms. Furthermore, an association between BMI and rectal mucosal PGE_2 levels has been identified in patients with a history of polyps, implying increased activation of COX/PG signaling, the primary target of NSAIDs, in association with obesity [21]. Nevertheless, the relationship between BMI and NSAID efficacy remains largely unaddressed in observational studies. Specifically, stratification by BMI with comparative analysis of relative risk in each BMI category has rarely been reported. One puzzling report

from the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial identified protective NSAID effects with respect to polyp formation in women limited to those with BMI < 25 [200]. Furthermore, reductions in colon cancer risk associated with aspirin use were not found to be modified by BMI in the Health Professionals Follow-Up Study or the Nurses' Health Study [201]. In contrast, the Aspirin/Folate Polyp Prevention Study suggested that aspirin may be more effective in preventing colorectal adenomas in patients with higher BMI [202]. Among obese subjects (i.e., BMI ≥ 30), the risk for advanced adenomas in aspirin users (325 mg/day) compared with placebo was 0.44 (95 % CI, 0.17–1.10) vs. RR = 1.23 (95 % CI 0.55–2.77) among those with normal weight (BMI < 25). These data suggest a selective protective effect of aspirin in obese individuals and offer a possible path forward for amelioration of elevated cancer risk in association with obesity.

11.3.2.5 Clinical Evaluation of COXibs for Suppression of Colorectal Neoplasia

The associated GI toxicity of aspirin and conventional NSAIDs represents a clear limitation and provided rationale for development of selective COX-2 inhibitors for anti-inflammatory applications. Evaluation of these latter agents for cancer prophylaxis was logical, based on the substantial epidemiologic evidence for anti-CRC efficacy of NSAIDs. Initial evaluation of celecoxib in FAP patients demonstrated a substantial reduction in polyp multiplicity with 400 mg twice daily for 6 months (28 % vs. 4.5 % reduction in placebo cohort) [203]. Three subsequent large-scale trials, the Adenoma Prevention with Celecoxib (APC), PRevention of Colorectal Sporadic Adenomatous Polyps (PreSAP), and Adenomatous Polyp Prevention On Vioxx (APPROVe) trials, all demonstrated COXib efficacy (both celecoxib and rofecoxib) for suppression of recurrent adenomas [204–206]. Relative risks achieved with 3 years treatment were 0.55 (95 % CI, 0.48–0.64; $P < 0.001$) with 400 mg celecoxib twice daily in the APC trial [206], 0.64 (95 % CI, 0.56–0.75; $P < 0.001$) with 400 mg celecoxib once daily in the PreSAP trial [204], and 0.76 (95 % CI, 0.69–0.83; $P < 0.0001$) with 25 mg rofecoxib daily in the APPROVe trial [205]. Suppression of advanced adenomas was also observed.

Analysis of patient tissue samples from the APC trial revealed a novel determinant of sensitivity to celecoxib—expression of the 15-hydroxyprostaglandin dehydrogenase (15-PGDH) enzyme [207]. 15-PGDH catalyzes conversion of PGE_2 to a relatively inactive keto derivative, 15-keto-PGE_2, and hence terminates PGE_2 signaling. In the APC trial, in all cases tested, those individuals who developed new adenomas while taking celecoxib uniformly exhibited low colonic mucosal *15-PGDH* transcript levels. These data suggest that both adequate *15-PGDH* expression and pharmacological COX inhibition are required to achieve sufficient PGE_2 suppression to effectively prevent polyp formation. Intriguingly, several NSAIDs reportedly elevate *15-PGDH* expression [208], which may be an integral component of their anticancer action.

Excitement generated by positive findings in the COXib polyp prevention trials was attenuated by the revelations of CV toxicity associated with the use of selective COX-2 inhibitors [209–213]. In aggregate these data led the US Preventive Services Task Force to conclude that the risk-benefit ratio did not favor chemoprevention in average-risk individuals [214]. Nevertheless, it may be possible to identify patients with a low CV risk profile for whom COXib-based prophylaxis of colorectal neoplasia is associated with a favorable risk-benefit ratio. Post hoc analysis of the APC trial identified the circulating inflammatory marker high-sensitivity C-reactive protein (hsCRP) as a potential predictor of celecoxib-associated CV toxicity [215]. Among patients with high hsCRP, the RR of CV events compared with placebo was 2.27 (95 % CI, 0.72–7.14) for those in the 200 mg celecoxib cohort and 3.28 (95 % CI, 1.09–9.91) in the 400 mg cohort, whereas no increase in CV risk was associated with assignment to celecoxib in patients with low hsCRP. Thus, it may be possible to identify patients for whom COXib use for chemoprevention is associated with minimal increase in CV risk.

11.3.3 NSAIDs and Human Breast Cancer

Broadly similar epidemiologic findings have been made with respect to NSAIDs and breast cancer as those discussed above for colorectal neoplasia. One important caveat is that the magnitude of risk reduction tends to be less, which could reflect the reduced prevalence of COX-2 overexpression in breast cancer vs. CRC. Furthermore, few data are available from interventional studies. Nevertheless, several recent meta-analyses of observational studies have identified a consistent signal of protection associated with NSAID use [159, 216, 217].

11.3.3.1 Epidemiologic Findings

Bosetti et al.'s recent meta-analysis of aspirin and cancer risk considered 10 case–control studies and 22 cohort studies of breast cancer, including a total of 25,835 and 27,091 breast cancer cases, respectively, and observed a modest but highly significant RR of 0.90 (95 % CI, 0.85–0.95; $P<0.001$) associated with aspirin use [159]. A similar magnitude of aspirin protection was identified in a separate meta-analysis (odds ratio (OR), 0.86; 95 % CI, 0.81–0.92) which included 13 case–control studies, 19 cohort studies, and 1 randomized clinical trial [216]. The findings of both these studies were consistent with an earlier report from Takkouche et al., in which not only aspirin but other NSAIDs were considered [217]. Use of any NSAID was associated with reduced breast cancer risk (RR, 0.88; 95 % CI, 0.84–0.93), with protective effects for both aspirin and ibuprofen when considered individually (RR, 0.87, and RR, 0.79, respectively). In contrast, the vast majority of studies have reported null associations for breast cancer risk and acetaminophen, consistent with this drug having a distinct analgesic mechanism from aspirin and commonly used

NSAIDs [218–222], although a couple of studies have identified acetaminophen-associated protection [223, 224].

Several studies have identified reduced breast cancer risk associated with selective COX-2 inhibitor use [218, 220, 225], although null associations have also been reported, and a single study reported increased risk in COXib users [226]. Consistent with preclinical studies, Valsecchi et al. observed a reduced frequency of bone metastasis in users of COXibs [227]. In high-risk patients, the calculated OR for bone metastases was 0.10 (95 % CI, 0.01–0.78).

Of note, although meta-analyses reveal a consistent protective NSAID effect, several individual studies have reported negative findings, or even increased breast cancer risk associated with NSAID use [228–230]. A population-based analysis of Danish women failed to observe associations between breast cancer incidence and NSAID use [231]. Furthermore, no NSAID-mediated protection was observed in the Nurses' Health Study [232–234]. Interestingly, the Nurses' Health Study cohorts included predominantly premenopausal women, suggesting that NSAIDs could mediate protection selectively in the postmenopausal setting. Mechanistically, this could relate to the ability of COX-derived prostaglandins to regulate estrogen biosynthesis. As discussed above (Sect. 11.2.3.5), PG-mediated upregulation of aromatase expression likely assumes greater significance in postmenopausal women after cessation of ovarian estrogen synthesis. Thus, if suppression of COX-dependent estrogen synthesis is a key mechanism for NSAID-mediated protection with respect to breast cancer, superior NSAID efficacy in postmenopausal women would be anticipated.

The hypothesis that NSAID-mediated suppression of postmenopausal estrogen synthesis is central to NSAID efficacy in the context of breast cancer provides the basis for several further predictions. Most notably, one might expect that NSAID protection would be abrogated by postmenopausal hormone replacement therapy (HRT). Consistent with this notion, neither aspirin use nor total NSAID use was associated with breast cancer incidence in the Cancer Prevention Study II Nutrition Cohort, of which over half were users of hormone therapy [235]. Similarly, in the Danish Diet, Cancer and Health Cohort, NSAID protection was also not detected, but almost 50 % of women were HRT users, with higher hormone use in women using NSAIDs [229]. These data support a model in which NSAIDs are selectively effective in postmenopausal women who are not users of hormonal therapy, although clearly further data are required to validate this concept.

If NSAID-mediated decreases in estrogen biosynthesis are genuinely mechanistically important, one might also anticipate selective suppression of those tumors that express hormone receptors (HRs). This possibility has been addressed in several epidemiologic studies to date, which have yielded mixed results. In the Long Island Breast Cancer Study, aspirin-associated breast cancer risk reduction was selectively observed in women with HR-positive tumors (OR, 0.74; 95 % CI, 0.60–0.93), whereas no benefit was detected for HR-negative tumors (OR, 0.97; 95 % CI, 0.67–1.40) [222]. A similar selective benefit of daily aspirin use for risk reduction of ER-positive breast cancer was identified in the NIH-AARP Diet and Health study (RR, 0.84; 95 % CI, 0.71–0.98) [236]. Additionally, although overall protective

efficacy of aspirin was not observed in the Nurses' Health Study, there was some signal of response of HR-positive breast cancers to high-dose, long-term aspirin exposure [234]. Other studies have also provided suggestive evidence for HR status as a determinant of aspirin sensitivity [237, 238], although aspirin/NSAIDs have not been uniformly observed to confer selective protection against hormone receptor-positive tumors [228, 239–242]. Nevertheless, in aggregate the data suggest that NSAIDs may afford increased protection with respect to HR-expressing tumors, which could reflect NSAID-mediated decreases in estrogen biosynthesis.

Finally, given the observation that breast adipose tissue exhibits inflammation in the context of obesity leading to increased estrogenic signaling [53, 54, 58, 59], it seems likely that NSAIDs might afford increased breast cancer risk reduction in obese vs. lean postmenopausal women. BMI is an established determinant of serum estradiol in postmenopausal women [140–142]. Thus, obesity-associated increases in aromatase expression and hence estrogen signaling in breast tissue might provide a target for NSAIDs. Additional inflammation-associated pathways may also be subject to NSAID-mediated amelioration (discussed below in Sect. 11.5.4). Stratification of NSAID effects according to BMI in cohort and case–control studies may furnish insights into the relative importance of obesity as a determinant of NSAID sensitivity in breast cancer.

11.3.3.2 Interventional Studies

A consensus finding from the epidemiologic studies discussed above is that NSAID use is associated with reduced breast cancer risk, albeit with lesser magnitude of protection than observed for colorectal neoplasia. However, in contrast to CRC, substantial randomized clinical trial data are not available to support these findings. As discussed above (Sect. 11.3.2.2), recent meta-analyses of trials with the primary goal of evaluating aspirin for CV disease prevention have yielded exciting data concerning aspirin protective effects on CRC incidence, mortality, and metastasis [166–168]. However, too few women were included in these trials with long-term follow-up to determine the effect of aspirin on breast or gynecological cancers in the Rothwell analyses. Thus, the major prospective trial of aspirin with breast cancer as a measured endpoint remains the Women's Health Study, a prospective trial of 100 mg aspirin every other day vs. placebo for an average of 10 years. In this trial, no effect of aspirin was observed on total cancer (RR, 1.01; 95 % CI, 0.94–1.08; $P = 0.87$), colorectal cancer (RR, 0.97; 95 % CI, 0.77–1.24; $P = 0.83$), or breast cancer (RR, 0.98; 95 % CI, 0.87–1.09; $P = 0.68$) [185]. Null results in this study have been widely interpreted as indicating that 100 mg aspirin on alternate days is an insufficient dose to achieve meaningful risk reduction. Consistent with this interpretation, several epidemiologic analyses have failed to observe protective effects with respect to breast cancer of low-dose (<100 mg/day) aspirin [220, 221, 225]. Also worth noting with respect to the Women's Health Study was the high proportion of HRT use, approximately 55 %, which could also have obscured protective effects contingent on modulation of estrogenic signals. Nevertheless, at present there is a

dearth of clinical trial data to substantiate epidemiologic findings of NSAID-mediated breast cancer risk reduction.

11.3.3.3 NSAIDs and Breast Cancer Recurrence/Mortality

Associations have been identified between NSAID use and not only decreased breast cancer risk but also improved outcome following breast cancer diagnosis. Several studies have identified decreased breast cancer recurrence and/or mortality in breast cancer patients who used NSAIDs following diagnosis [243–245]. In the Life After Cancer Epidemiology study, use of ibuprofen and non-aspirin NSAIDs was associated with decreased breast cancer recurrence, although aspirin did not afford protection [245]. In contrast, in the Nurses' Health Study, aspirin use was associated with a decreased risk of breast cancer mortality [244]. Interestingly, the association was not modified by menopausal status, BMI, or tumor ER status, and as discussed above, tumor COX-2 expression was also not a determinant of aspirin sensitivity [44, 244], providing few clues as to the responsible mechanisms underlying aspirin effects. Of note, conflicting data were provided by the Collaborative Women's Longevity Study and the Western New York Exposures and Breast Cancer study, neither of which identified a relationship between NSAID use and either all-cause or breast cancer-specific mortality [246, 247]. Further studies will undoubtedly provide greater clarification concerning NSAID use and breast cancer outcome.

11.3.3.4 Therapeutic Interventions with COX-2 Inhibitors

A handful of trials have tested COXibs as therapeutics or performed biomarker studies. Celecoxib has been evaluated in early phase tolerability/efficacy trials in combination with the HER2/neu-directed monoclonal antibody trastuzumab, the aromatase inhibitor exemestane, or the antimetabolite capecitabine in patients with advanced breast cancer [248–252]. Where comparator arms were included, no increase in time to progression (TTP) resulted from addition of celecoxib [250]; however, one study identified a longer TTP in celecoxib/capecitabine-treated patients whose tumors overexpressed COX-2 [251]. Window trials comparing molecular endpoints pre and post intervention have been relatively uninformative to date. Celecoxib-mediated decreases in nipple aspirate fluid PGE_2 levels have been reported in postmenopausal women and in women with newly diagnosed breast cancer [253]. However, neither proliferation nor apoptosis in ER-positive DCIS was altered by 14-day celecoxib treatment, whereas the AI exemestane significantly suppressed proliferation [254]. Similarly, 14-day celecoxib treatment did not effect a significant alteration in proliferation (Ki67 staining) in breast carcinoma tissue relative to that observed in the no-treatment group and also did not significantly affect apoptosis, COX-2, ER, or progesterone receptor (PR) expression [255]. However, statistically significant decreases in carcinoma ER, PR, Ki-67, and COX-2 expression have been achieved by coadministration of celecoxib with exemestane,

although aromatase protein levels were unaltered by exemestane or combination therapy [256]. Given the link between obesity, inflammation, and neoplasia, it would obviously be of interest to determine if BMI is a determinant of clinical response of breast cancers to anti-inflammatory drugs.

11.4 Dysregulated Signaling in Obesity: Implications for Neoplasia

Many of the signaling pathways dysregulated in obesity likely contribute to the associated increase in cancer risk and moreover provide targets for NSAID-mediated intervention. Thus, this section provides a simplified overview of signaling abnormalities associated with obesity and inflammation to generate a framework for considering obesity-related mechanisms of NSAID anticancer action. For increased detail, the reader is directed to accompanying chapters in this book. Since mechanistic contributions of COX/PG signaling to neoplasia are extensively discussed above (Sect. 11.2.3), these are largely not reiterated in this section.

Obesity is defined by increased BMI (≥30) and characterized by disproportionate adipose accumulation. Importantly, as discussed above, adipose tissue from obese individuals exhibits marked inflammation, defined by infiltration of leucocytes, including macrophages, as well as CD8-positive T lymphocytes and mast cells [4]. Inflammatory foci consisting of M1-like macrophages surrounding necrotic adipocytes, the so-called CLS, are observed in breast and other adipose depots from obese mice and humans and increase as a function of body mass [53, 55–59]. Adipose tissue macrophages can comprise up to 40 % of cells in obese adipose tissue and represent a rich source of cytokines, chemokines, and fatty acids, which are key mediators of the increased risk of insulin resistance and neoplasia associated with obesity [4]. Proinflammatory molecules released from activated macrophages include COX-2-derived PGE_2, TNFα, and IL-1β (Fig. 11.2), synthesis of which is driven by increased NFκB (nuclear factor of kappa light polypeptide gene enhancer in B cells) signaling induced by ligation of cell-surface toll-like receptor 4 (TLR4). These cytokines may have both local and systemic effects. Key consequences include attenuation of insulin signaling through kinase-mediated covalent modification of insulin receptor substrate-1 (IRS-1) and decreased expression of the GLUT4 glucose transporter. Thus, obesity-associated cytokine overproduction can drive insulin resistance, hyperinsulinemia, and ultimately type II diabetes. Importantly, tissue inflammation is evident not only in adipose but in all classical insulin target tissues, including liver and muscle.

Obesity-induced cytokine overproduction has important proneoplastic sequelae [257]. Cytokines and chemokines can induce DNA damage via reactive oxygen species and inhibit DNA repair. Furthermore, they may act as paracrine growth and survival factors for malignant cells, as well as inducing angiogenesis via microenvironment remodeling and upregulation of proangiogenic factors. Tissue remodeling may also contribute to cancer cell migration and invasion. Additionally,

obesity-induced cytokines may be important in subverting host immune responses and hence optimizing tumor cell survival. Thus, excessive levels of proinflammatory mediators may impact both tumor initiation and progression.

Key signal transduction pathways activated by PGE_2, TNFα, and IL-1β include protein kinase A, mitogen-activated protein kinase (MAPK), and NFκB signaling, all of which are implicated in carcinogenesis. Additionally, as previously discussed, an important local effect in obese adipose tissue is paracrine induction of aromatase expression in neighboring cells (Fig. 11.2), likely increasing breast cancer risk via upregulated estrogenic signaling. Increased circulating estradiol levels in obesity may also promote neoplasia in other hormone-responsive tissues including uterine endometrium.

In parallel with hyperinsulinemia in obesity, increased production of other growth factors and endocrine agents is observed, including the satiety hormone leptin and insulin-like growth factor 1 (IGF-1) [258]. Increased IGF-1 synthesis and decreased expression of IGF-binding proteins lead to a net increase in bioavailable IGF-1. Both insulin and IGF-1 can induce cell growth and proliferation via PI3K/AKT signaling, a pathway that is of central importance in neoplasia [259, 260]. Additionally, both ligands can induce β-catenin/TCF signaling [261], another highly important pathway in cancer [262].

The peptide hormone leptin emanates from adipocytes and thus is positively correlated with adipose stores. In lean animals, leptin functions as a satiety factor, but leptin overproduction in obesity is accompanied by diminished hypothalamic responsiveness to the leptin signal. In contrast, leptin-regulated JAK/STAT, MAP kinase, and PI3K signaling in other tissues can promote cancer progression. Animal studies have identified leptin signaling as important for mammary tumorigenesis, and it appears that the ratio of leptin to adiponectin (which decreases with increasing BMI) may be a key determinant of breast cancer risk [258, 263].

Several signaling pathways are thus emerging as key mediators of obesity-associated inflammation, both for insulin resistance and cancer risk. NFκB is a central regulator of adipose cytokine synthesis. Saturated fatty acids, and potentially bacterial toxins ingressing via leaky gut mucosa, induce NFκB activation via TLR4, resulting in increased production of cytokines, many of which in turn signal through NFκB. Central importance of NFκB in systemic insulin resistance has been demonstrated via genetic manipulation in rodents, which has further established myeloid NFκB as the key determinant [264–267]. Hyperactivation of PI3K/AKT signaling to mTOR (mammalian target of rapamycin) is apparent in obesity, consequent on overproduction of inflammatory cytokines and hormones (e.g., insulin, IGF-1, leptin). mTOR is a central regulator of cell growth, responsible for integrating growth factor signaling, nutrient, energy, and oxygen availability and translating the net signal to provide the appropriate level of translational activity within the cell [268]. Balancing the progrowth signals initiated by hormones and growth factors, AMP kinase (AMPK) serves as an energy sensor activated by increased AMP:ATP ratio that can signal the need for decreased metabolic activity by providing critical input to mTOR. AMPK activation initiates a switch from glucose to fat utilization and shuts down energy-intense processes. Of note, antidiabetic agents including

metformin function in part through activation of AMPK via decreasing oxidative phosphorylation and hence ATP production. Altered balance between PI3K and AMPK signaling in obesity may prove to be a key regulator of the neoplastic process via control of tumor cell growth.

Thus, obesity is associated with activation of multiple key proneoplastic pathways including NFκB, PI3K/AKT/mTOR, Wnt/β-catenin, JAK/STAT, and MAP kinase signaling and may also effect protumorigenic changes by skewing the cellular energy balance in favor of continued cell growth. Notably these processes are deemed most likely to contribute to cancer progression after acquisition of precursor mutations that induce cell transformation, although inflammatory cytokines may also regulate tumor initiation via increasing DNA damage.

11.5 NSAID Anticancer Mechanisms

11.5.1 NSAIDs Suppress Hallmark Characteristics of Cancer

Consideration of the key signaling abnormalities associated with obesity provides a foundation for reviewing NSAID anticancer mechanisms, although clearly many of these mechanisms are generalizable to the carcinogenic process independent of BMI. As discussed above, COX/PG signaling regulates a number of the intrinsic, or "hallmark", capabilities suggested as essential for tumor growth and metastatic dissemination; thus, NSAID-mediated PG suppression can impact neoplasia via multiple effector pathways (Fig. 11.1). Intriguingly, NSAIDs can decrease PG levels not only via modulation of their synthesis (i.e., COX inhibition) but also through increased catabolism achieved via *15-PGDH* upregulation [208]. Nevertheless, considerable controversy has focused on the mechanistic basis for the protective efficacy of NSAIDs with respect to cancer, based in part on the existence of NSAID derivatives such as sulindac sulfone that lack COX inhibitory activity but are still active in animal tumor models [269], as well as demonstrated activity of NSAIDs towards COX-2-null cells [270–272]. This has been resolved by genetic approaches which provide incontrovertible evidence for the importance of COX/PG signaling in rodent neoplasia (Sect. 11.2.2). Facets of tumorigenesis that can be regulated by prostaglandins and are thus susceptible to NSAID-mediated attenuation include cell proliferation and apoptosis avoidance, invasiveness, angiogenesis, immune suppression, and regulation of estrogen biosynthesis (Sect. 11.2.3; Fig. 11.1). Additionally, recent data identify a key role for stromal fibroblast-derived PGE_2 in regulating cancer stem cells [74, 75]. Nevertheless, it is evident that NSAIDs suppress tumorigenesis through a combination of both COX-dependent and COX-independent effects. NSAID regulation of programmed cell death provides a key illustration of utilization of parallel COX-dependent and COX-independent pathways to effect a response.

11.5.2 NSAID-Mediated Suppression of Apoptosis

COX-2 overexpression in epithelial cells suppresses apoptosis both in vitro and in vivo [63, 87], providing a clear mechanism for NSAID-mediated apoptosis via COX inhibition. Antiapoptotic consequences of elevated COX-2/PG include increased expression of the antiapoptotic gene *Bcl-2* [273] and decreased arachidonate-driven apoptosis consequent on increased conversion of arachidonic acid to PGs [274, 275]. Furthermore, COX-derived prostacyclin may act to suppress apoptosis via direct interaction with the nuclear receptor peroxisome proliferator-activated receptor (PPAR) δ [276, 277]. However, a multiplicity of additional COX-2-independent NSAID-regulated pathways can also impact apoptosis, as illustrated by NSAID induction of cell death in *COX-2* null cells [270–272] and by non-COX-inhibiting drugs [278]. NSAIDs can stimulate induction of multiple proapoptotic effectors, including 15-lipoxygenase-1 (15-LOX-1) [279, 280], NSAID-activated gene-1 (NAG-1) [281], and prostate apoptosis response 4 (Par-4) [282]. NSAID-mediated abrogation of antiapoptotic pathways via inhibition of PPARδ and 3-phosphoinositide-dependent protein kinase-1 (PDPK1) signaling has also been described [283, 284]. Several mechanisms have been advanced to explain PPARδ suppression, including disruption of PPARδ binding to target sequences, and down-regulated PPARδ expression, which can be a consequence of increased synthesis of the 15-LOX-1 product 13-S-HODE [283, 285–287]. NSAID-elicited suppression of apoptosis likely occurs through combination effects on these pathways. Intriguingly, a selective proapoptotic effect of sulindac on intestinal stem cells has been suggested, implying that NSAIDs could selectively impact the biology of cancer stem cells with activation of Wnt/β-catenin signaling [288], which could relate to the previously reported ability of PGE_2 to regulate stem cells [73–75, 289].

11.5.3 Cyclooxygenase-Independent NSAID Effects

The Wnt/β-catenin pathway has itself been identified as an NSAID target: suppression of β-catenin/TCF transcription can be elicited by multiple NSAIDs including aspirin, indomethacin, and sulindac [290–292]. Strikingly, reduced nuclear β-catenin is evident in adenomas from NSAID users [290, 293]. Aspirin-mediated inhibition of phosphatase 2A has been posited as one mechanism for attenuating Wnt/β-catenin signaling [291]. However, regulation of β-catenin signaling by PGE_2 via modulation of glycogen synthase kinase 3β has also been described [76, 294], suggesting that NSAIDs may regulate this pathway via both COX-dependent and COX-independent routes.

An emerging target of interest in the NSAID field is cGMP phosphodiesterase (PDE). Sulindac metabolites inhibit cGMP PDE, resulting in increased cGMP and protein kinase G activity in CRC and breast cancer cell lines [295–297]. Selective expression of cGMP PDE5 in cancer cell lines and tissues suggests this isoform as

the target for sulindac and other NSAIDs [296, 297]. Parallel NSAID effects on cGMP PDE5 activity, apoptosis, and suppression of β-catenin/TCF signaling suggest that cGMP PDE5 regulates these latter responses, and causal relationships have been established using knockdown approaches.

The ability of COX inhibition to directly impact carcinogenesis through diminished COX-mediated activation of procarcinogens was discussed above (Sect. 11.2.3.6). However, NSAIDs can also regulate carcinogen metabolism independent of COX enzymes. For example, the major aspirin metabolite salicylic acid inhibits P phenolsulfotransferase, which mediates sulfation activation of carcinogens [298]. Additional COX-independent effects of NSAIDs of particular relevance with respect to obesity are described in the following section.

11.5.4 Key COX-Independent NSAID Effects in the Context of Obesity

Of the numerous "off-target" NSAID effects described to date, suppression of the NFκB pathway is strikingly relevant to obesity. Inhibition of NFκB transcriptional activity has been described for both aspirin and other NSAIDs, with a variety of proposed mechanisms that dictate NFκB protein localization such that it remains transcriptionally inactive [299–304]. NSAID-mediated suppression of NFκB signaling is likely to have profound consequences for obesity and neoplasia, given the key role of NFκB in both the upregulation and signaling of macrophage-derived inflammatory mediators in obese adipose.

Notably, salicylate treatment attenuates experimental insulin resistance in rodent models, and this has been attributed to NFκB suppression [265, 267]. Furthermore, selective COX-2 inhibitors (celecoxib, nimesulide) reduce parameters of metabolic disease in rats fed high-fat diets [305]. Clinically, improved insulin sensitivity and glycemia has been achieved through administration of aspirin, salicylate, salsalate (a non-acetylated prodrug of salicylate), and COXibs [306–310]. Intriguingly however, NSAID modulation of NFκB may only represent part of the explanation for these findings. Emerging data suggest probable involvement of AMPK as an additional mediator of aspirin/salicylate improvement of metabolic dysfunction. Salicylate has recently been shown to activate AMPK, and salicylate-induced decreases in plasma fatty acids and increased fat utilization are abrogated in *AMPK*-deficient mice [311, 312]. Thus, salicylate-elicited amelioration is likely to reflect modulation of both NFκB and AMPK.

Several other pathways that are altered in obesity-associated inflammation are also targeted by NSAIDs. Multiple lines of evidence support the ability of NSAIDs to attenuate PI3K/AKT signaling and growth control. NSAIDs can suppress PI3K/AKT signaling via COX inhibition, since PGE_2 induces activation of PI3K/AKT through G protein-β/γ subunits [76]. Additionally, NSAID-mediated inhibition of the upstream AKT activator 3-phosphoinositide-dependent protein kinase-1

(PDPK1) has been reported [284]. Reduced phosphorylation levels of ribosomal protein S6 and S6 kinase in rectal mucosa from aspirin-treated patients are consistent with attenuation of PI3K/AKT signaling [312], these proteins being key downstream mediators that regulate protein synthesis and hence cell growth. Furthermore, indomethacin activates the eIF2a kinase PKR in CRC cells, inducing a translation block [313]. Notably, a recent survey of cellular proteins acetylated by aspirin identified 33 substrates including metabolic enzymes, histones, and ribosomal and mitochondrial proteins [314]. These provocative findings suggest that aspirin-mediated acetylation of cellular proteins could have far-reaching metabolic consequences. Of note, aspirin-acetylated COX-2 synthesizes a novel class of lipid mediators, lipoxins, which actively promote resolution of acute inflammation [315], although the role of these lipid moieties in chronic inflammation is less well-defined.

The available evidence suggests that aspirin compounds can favorably impact pleiotropic pathways to improve metabolic health. Thus, NSAID-mediated attenuation of systemic inflammation might also be expected to translate into reduced risk of neoplasia through reducing the parameters of inflammation and thereby modulating the biology of the neoplastic cell.

11.6 Conclusions

Obesity as a determinant of increased cancer risk and poorer cancer outcome is now well established for cancers of multiple organ sites, including CRC and postmenopausal breast cancer. Obesity-associated systemic inflammation is likely to be a key driver of elevated neoplastic risk, with numerous plausible mechanisms consequent on elevated levels of inflammatory mediators and hormones. Both circulating and local tissue levels of these molecules can drive cancer progression and may also contribute to tumor initiation via increased DNA damage. Induction of numerous key protumorigenic signaling pathways may be elicited as a consequence of obesity-associated inflammation, including NFκB, Wnt/β-catenin, MAPK, JAK/STAT, as well as the PI3K/AKT/mTOR axis which is central to cell growth and homeostasis. Strikingly, many of these pathways are targeted by NSAIDs, and this drug class has demonstrated clinical efficacy for ameliorating metabolic dysfunction. Importantly, clinical findings support the antineoplastic activity of NSAIDs with respect to both colorectal and breast cancer, and consistent data have been obtained using animal models. To date there is limited epidemiologic evidence for selective protective effects of NSAIDs in obese individuals. Clinical evidence for a selective signal of NSAID utility for cancer prophylaxis in the context of obesity is mixed for CRC and completely absent for breast cancer. This may reflect the pleiotropic mechanisms, both COX-dependent and COX-independent, through which NSAIDs can attenuate the cancer process, which may confer protection in both lean and obese individuals. Nevertheless, NSAID-mediated suppression of COX/PG signaling, NFκB activity,

and the PI3K/AKT/mTOR axis, as well as activation of AMPK signaling, are likely to have profound consequences for obesity-associated neoplasia. Furthermore, COX/PG-mediated upregulation of estrogen biosynthesis and signaling offers a plausible target for NSAID-mediated risk reduction with respect to breast and other hormone-sensitive cancers in the context of obesity.

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Chapter 12
Omega-3 Fatty Acids in Cancer Prevention and Control: A Membrane Perspective

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Abstract Long-chain n-3 polyunsaturated fatty acids (PUFA) have been shown to provide health benefits in a number of diseases including several forms of cancer. In this chapter we will discuss in detail some of the prominent mechanisms through which n-3 PUFA and its metabolites are believed to function in the prevention of colon tumorigenesis. At the plasma membrane, n-3 PUFA antagonize the production of inflammatory and procarcinogenic n-6 PUFA (i.e., arachidonic acid)-derived metabolites. Additionally, the highly unsaturated nature of n-3 PUFA impacts cell membrane properties and dynamics thereby altering numerous cellular functions, including intracellular signaling, cell growth, survival, and proliferation. Due to the sterically incompatible relationship between docosahexaenoic acid, sphingolipids, and cholesterol, the major constituents of lipid rafts, n-3 PUFA modulate these crucial membrane microdomains and perturb efficient signal transduction thereby eliciting the same biological effects exploited by some anti-cancer therapies. Moreover, we discuss how alterations in lipid rafts and downstream signaling impact both epithelial cells and the activation of immune cells. This is noteworthy, because chronic inflammation plays a critical role in tumorigenesis. Therefore, we also present an overview regarding the anti-inflammatory and immunomodulatory mechanisms

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through which n-3 PUFA perturb the tumor microenvironment and downregulate the activation of critical transcription factors and target genes with an established role in cancer development. Finally, we discuss recent evidence suggesting that n-3 PUFA in combination with other dietary bioactive nutrients, such as soluble fiber and curcumin, could be beneficial in cancer prevention. Collectively, we demonstrate that dietary n-3 PUFA have utility in the prevention of cancer development through mechanisms centered at both the molecular, cellular (plasma membrane), and tissue level.

12.1 n-3 PUFA

Numerous health benefits have been attributed to fish oil. The most notable bioactive components of fish oil are the long-chain omega-3 polyunsaturated fatty acids (n-3 PUFA), eicosapentaenoic acid (EPA, $20:5^{\Delta 5,8,11,14,17}$), and docosahexaenoic acid (DHA, $22:6^{\Delta 4,7,10,13,16,19}$). Fatty acids that have the last double-bond three carbons from the terminal methyl group are characterized as n-3 fatty acids. Mammals lack the desaturases that are required to synthesize n-3 PUFA, so they must be acquired through the diet. The major dietary source of long-chain n-3 PUFA is fatty, cold-water fish, which ingest zooplankton and phytoplankton rich in EPA and DHA [1]. Another shorter-chain n-3 PUFA, α-linolenic acid (α-LNA, $18:3^{\Delta 9,12,15}$), can be obtained from plant sources, including walnuts, seeds (flaxseed), dark green leafy vegetables, and soybeans. Humans express the enzymes required for conversion of α-LNA to EPA and EPA to DHA; however, conversion rates are extremely low. Kinetic analyses suggest that conversion of α-LNA to EPA to be as low as 0.2 %. Further conversion to DHA is calculated to be as low as 0.05 % [2]. Therefore, the optimal way to increase tissue levels of long-chain n-3 PUFA is by directly ingesting foods or supplements high in both EPA and DHA.

12.2 n-3 PUFA in Cancer

As mentioned above, EPA and DHA have been shown to provide health benefits in a number of diseases. Of interest, there is growing evidence that n-3 PUFA may prevent multiple forms of cancer, including some of the top causes of cancer mortality: breast, lung, prostate, and colon cancer [3–6]. In this chapter, we will focus on the evidence for the role of n-3 PUFA in the prevention and treatment of colon cancer. Importantly, many of the mechanisms of action of n-3 PUFA that we will discuss are common between multiple cancer types.

12.3 Human Data

12.3.1 Observational Studies

It is important to first appreciate the mounting human data documenting the role on n-3 PUFA in colon cancer prevention. Numerous epidemiological studies have demonstrated distinct trends in which an increase in consumption of n-3 PUFA is associated with reduced risk of colon cancer. Studies have established a significant risk reduction of colorectal cancer (CRC) with increased consumption of fish, n-3 PUFA, and DHA or EPA [7–9]. A recent study found that dietary intake of marine n-3 PUFA was associated with a decreased risk of adenomatous polyps in women [10], and another described an inverse relationship between the intake of n-3 PUFA and the risk of colon cancer in the proximal colon in both men and women [11]. Additionally, a Scottish study described a dose-dependent reduction in colon cancer risk with increased intake of total n-3 PUFA, EPA alone, and DHA alone [12]. A 22-year prospective study established that intake of fish and fatty acids from fish may decrease the risk of colon cancer [13]. Similarly, a meta-analysis of prospective cohort studies additionally found that fish consumption reduces colon cancer risk [14]. Furthermore, data from 24 European countries established an inverse correlation between fish and fish oil consumption with CRC mortality [4]. Other studies have shown that individuals with the highest percentage of circulating n-3 PUFA display the lowest risk of colon cancer [15–17]. Historically, a very low prevalence of colon cancer existed in Asian countries, which was attributed to a high consumption of fish. However, recent lifestyle changes of these populations have resulted in similar rates of colon cancer to the population of the United States [18]. These trends indicate a role of diet in colon cancer risk.

12.3.2 Clinical Studies

Due to the abundance of data suggesting a protective role of n-3 PUFA, numerous clinical trials have been undertaken to further clarify these effects. A short-term trial demonstrated that fish oil reduces mucosal proliferation, which may protect high-risk subjects from colon cancer [19]. Additionally, a double-blind, cross-over trial further indicated a reduction in mucosal proliferation as well as reduced PGE_2 synthesis in test subjects receiving fish oil [20]. Another study conducted by Anti et al. demonstrated that fish oil supplementation has both short-term and long-term normalizing effects on the abnormal proliferation patterns associated with increased colon cancer risk [21]. Very few studies have focused specifically on purified DHA or EPA. However, two studies have shown a positive effect of purified EPA. One of these investigations found that highly purified EPA reduces crypt cell proliferation

and increases apoptosis in subjects with a history of colorectal adenomas [22]. In a complementary study, the administration of EPA to a population of genetically predisposed subjects for colon cancer exhibited a reduction in the number and diameter of colonic polyps, which can serve as precursors to colon cancer [23]. Overall, human clinical trials have demonstrated a potential use of n-3 PUFA in colon cancer prevention.

12.3.3 Nutrigenetics

Although many studies have demonstrated the beneficial effect of n-3 PUFA in regard to colon tumorigenesis, a subset of studies have failed to show any effect. The answer to some of these differences may lie in the field of nutrigenetics. Nutrigenetics is the study of how certain genes are affected by nutrients, especially with regard to disease. Recently, it has been reported that a single nucleotide polymorphisms (SNPs) within a DNA repair gene modified the effect of marine n-3 PUFA on colon cancer risk [24]. Further developments in this field of research will likely enhance our understanding of the role of n-3 PUFA in colon cancer prevention.

In cancer, many different abnormalities can contribute to the formation of a tumor. Therefore, not all patients will respond to the same type of therapy. If a nutrient is going to be effective for prevention or treatment of cancer, the mechanism of action will elucidate the patients most likely to respond to a given therapy. Therefore, we will now discuss the mechanisms by which n-3 PUFA are believed to function in the prevention of colon tumorigenesis.

12.4 Membrane Effects of n-3 PUFA

Multiple studies have been undertaken to elucidate the mechanisms of action of n-3 PUFA, and the current literature contains evidence of various interactions to explain the antineoplastic activity of DHA and EPA. Of interest, long-chain n-3 PUFA have been shown to alter membrane dynamics, which could explain the pleiotropic effects of these fatty acids. DHA and EPA are both long, highly unsaturated fatty acids, which can directly affect many of the properties of the plasma membrane. Modification to cell membranes can extensively alter numerous cellular functions, including cellular signaling, growth, survival, and proliferation. For example, both EPA and DHA have been shown to increase membrane fluidity [25, 26], and membrane fluidity can directly affect protein diffusion within the membrane. This can then modify multiple cell signaling events which require specific spatiotemporal regulation of protein–protein interactions. n-3 PUFA have additionally been shown to alter permeability, phase behavior, fusion, and flip-flop of the plasma membrane [26, 27]. These effects on the membrane directly contribute to the ability of n-3 PUFA to alter important membrane microdomains. In fact, recent evidence clearly

demonstrates that EPA and DHA can perturb highly crucial microdomains known as lipid rafts [28–31].

Lipid rafts are extremely small membrane microdomains that can vary between 5 and 200 nm [32]. Lipid rafts are further characterized as dynamic plasma membrane domains enriched with cholesterol and sphingolipids [33]. These domains contain highly ordered lipid assemblies and exhibit liquid-ordered characteristics due to the interaction between cholesterol and sphingolipids. Furthermore, lipids that are localized to these domains comprise mostly saturated, long hydrocarbon chains and hydroxylated ceramide backbones [34, 35]. The composition of lipid rafts imparts two distinguishing characteristics: detergent-resistance and low-buoyant density [36]. These qualities have been exploited by researchers to explore the protein composition of lipid rafts. It is important to note that lipid rafts are highly heterogeneous [37]. Additionally, the structure of lipid rafts is decidedly dynamic, and lipid rafts constantly alter their composition of both lipids and proteins. These properties of rafts facilitate the ability of cells to respond to external stimuli and activate intracellular signaling pathways that originate at the cell surface.

The dynamics and heterogeneity of lipid rafts allow for a large number of signals to be transduced from the extracellular environment to intracellular domains. Membrane rafts play a fundamental role in mediating multiple cell functions, including signal transduction [38–42]. Many proteins that function as signaling partners accumulate in lipid rafts, which serve as a platform to support highly efficient signal transduction. Although lipid rafts are small, they can be stimulated to coalesce and form large, stable platforms for signaling [43, 44]. Many important signaling proteins have been shown to be highly enriched in lipid raft domains, including epidermal growth factor receptor (EGFR), G-protein-coupled receptors (GPCR), platelet-derived growth factor receptor (PDGFR), nerve growth factor receptor (NGFR), vascular endothelial growth factor receptor (VEGFR), H-Ras, and many more [33, 45–47]. In many cases, the function of proteins depends greatly on their localization within lipid rafts [48]. These lipid raft-mediated signaling events are essential for a plethora of cell functions.

Lipid rafts have been found to participate in a variety of cellular events. For instance, extensive studies have been performed to elucidate the role of lipid rafts in the activation of T lymphocytes, which requires clustering of signaling components in a large, stable lipid raft domain at the immunological synapse (IS, [49, 50]). Furthermore, extrinsic induction of apoptosis relies on signaling through lipid rafts, and the composition of lipid rafts has been shown to affect apoptotic responses [48, 51, 52]. Of interest, recent evidence suggests that lipid rafts may modulate the malignant transformation process. In fact, the levels of lipid rafts are increased in many types of cancer [53–55]. Additionally, lipid rafts mediate many of the cell signaling events that are often constitutively or hyperactivated in cancer [56–61], including EGFR, Akt, Ras, Src, and HER2. Research suggests that disruption of lipid rafts may enhance responsiveness to anti-cancer therapies [62]. Furthermore, some anti-cancer drugs have beneficial effects through alteration of the protein content of lipid rafts [48]. In colon cancer, lipid rafts have been shown to function in cell death-mediated signaling [63, 64], entry of bioactive compounds [65], and localization of key proteins involved in immune response [66].

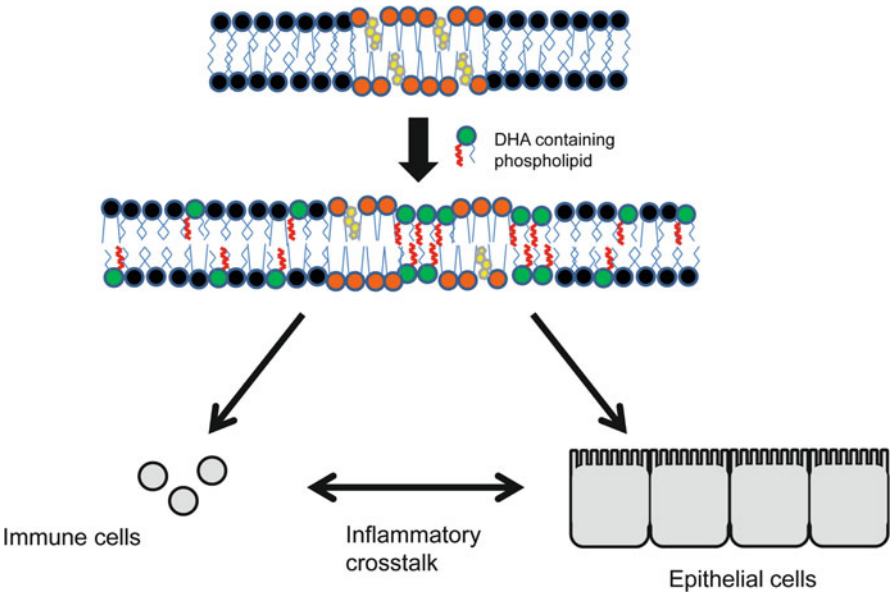

Fig. 12.1 Cross-talk between immune and epithelial cells in inflammation and cancer. Immune cells, e.g., T lymphocytes and epithelial cells engage in constant cross-talk to monitor and survey the physiological environment for foreign antigens and protect the host. Since EPA- and DHA-containing phospholipids are incorporated into membranes of many cell types and affect their signaling, understanding the molecular mechanisms by which n-3 PUFA impact signaling in both immune and epithelial cells will serve to elucidate the effects of n-3 PUFA on inflammation and carcinogenesis

Extensive studies have demonstrated that DHA, due to its high degree of unsaturation, is sterically incompatible with cholesterol [27], a major constituent of rafts. Additionally, DHA displays nonideal mixing with sphingomyelin, another major component of lipid rafts, in model membranes [31]. Conceivably, disruption of lipid rafts could be caused by the lack of affinity of n-3 PUFA for lipid raft components. As mentioned above, several critical processes involve lipid rafts, including T cell activation, signal transduction, and protein and lipid trafficking [33]. Many of these lipid raft-mediated processes play an integral role in colon tumorigenesis. Signaling pathways emanating from lipid rafts, frequently exacerbated in cancer, mediate a variety of tumor-promoting activities, including cell proliferation, migration, and invasion [55]. Additionally, chronic inflammation, central to the process of tumorigenesis [67], involves excessive lipid raft-mediated T cell activation. Numerous recent discoveries highlight the role of n-3 PUFA in the regulation of lipid rafts and lipid raft-mediated signaling in both immune cells and epithelial cells. Due to the extensive cross-talk between immune and epithelial cells, the ability of n-3 PUFA to alter signaling in both cell types likely contributes to a greater effect with respect to cancer prevention than targeting either cell type on its own (Fig. 12.1).

12.5 Effect of n-3 PUFA on Epithelial Cell Lipid Rafts

12.5.1 Epithelial Raft Size

Lipid raft size is an important feature in lipid raft function [68] and has been shown to be altered by n-3 PUFA [28]. Lipid raft size is integral for dynamic lateral segregation of signaling proteins into microdomains. Partitioning of proteins into rafts can increase specific protein–protein collision rates to facilitate efficient signaling. However, to maximize this essential, biologically relevant function, rafts must be mobile and small, with a diameter up to 14 nm [68]. Therefore, altering the size of a lipid raft would likely have adverse effects on signaling. In HeLa cells and T lymphocytes, treatment with DHA resulted in enhanced clustering of lipid raft domains compared to untreated cells [28]. Additionally, DHA treatment was found to increase the height of lipid rafts, while reducing the overall number of lipid rafts [69]. Altering lipid raft size has implications with regard to the regulation of multiple signaling events associated with these microdomains.

12.5.2 Lipid Raft Composition

In addition to the size of lipid rafts, the composition of rafts is important for their function. Interestingly, n-3 PUFA have been found to alter the composition of lipid rafts. Upon feeding mice a diet enriched in n-3 PUFA, the cholesterol content of lipid rafts in colonocytes was reduced by 46 % compared to mice fed a diet enriched in n-6 PUFA [70]. Furthermore, treatment of both endothelial and breast cancer cells with DHA was also found to reduce raft sphingomyelin and cholesterol content [71, 72]. These n-3 PUFA-induced modifications of lipid raft composition are significant because cholesterol and sphingomyelin are major building blocks of lipid rafts that promote the formation of hydrophobic liquid-ordered molecular packing.

In addition to altering the lipid composition of rafts, n-3 PUFA have been shown to modulate raft protein composition. This effect of DHA on Ras is highly important due to the central role that Ras signaling plays in colon carcinogenesis [73]. Feeding mice a diet enriched in n-3 PUFA was found to reduce the localization of H-Ras to colonocyte lipid rafts [70]. Moreover, treating immortalized young adult mouse colonocytes (YAMC) with DHA was also found to decrease the localization of H-Ras to lipid raft domains [70]. DHA was then further shown to not only reduce the localization of H-Ras to lipid rafts but also to inhibit the plasma membrane targeting of H-Ras [74].

EGFR is a transmembrane receptor tyrosine kinase that mediates multiple oncogenic signaling pathways and is enriched in lipid raft domains. Localization of EGFR to lipid rafts is essential for efficient signal transduction. Interestingly, treatment of breast, lung, and colon cell lines with DHA or EPA was found to reduce the

localization of EGFR to lipid rafts [71, 75, 76]. In addition to Ras, Src and Fyn are two other important lipid raft localized signaling mediators. Treatment of the colon cancer cell line, Caco-2, with DHA led to a delocalization of these proteins from rafts [77]. The effect of n-3 PUFA on localization of these signaling proteins to lipid rafts directly altered cell signaling.

12.5.3 Lipid Raft-Mediated Cell Functions

The n-3 PUFA-induced alterations to lipid raft size and composition culminate in the modification to lipid raft-mediated cell functions. For example, n-3 PUFA have been found to alter protein function and signaling. In the case of EGFR, n-3 PUFA increase EGFR phosphorylation [71, 75, 76]. EGFR phosphorylation is a canonical precursor to EGFR signaling. However, n-3 PUFA have been found to paradoxically disrupt EGFR signal transduction [75, 76]. n-3 PUFA have also been shown to reduce activation of Ras [76, 78], which is a lipid raft-mediated process [79–81]. Lipid rafts have also been shown to play a central role in the activation of STAT3 [82], another important signaling mediator that is often hyperactivated in colon cancer. Both in vivo and in vitro activation of STAT3 have been shown to be significantly reduced in colonocytes by n-3 PUFA [76, 83]. Collectively, these signaling events are important for mediating cell proliferation and apoptosis. The altered lipid raft-mediated cell signaling caused by n-3 PUFA has been shown to reduce cell proliferation and increase apoptosis in multiple epithelial cell lines [69, 76, 84]. Interestingly, calcium influx is also a lipid raft-mediated process. It has been shown that DHA decreases oxidative-stress-induced calcium influx [72]. These data signify the implicit role of n-3 PUFA in the regulation of lipid raft-mediated cell processes.

12.6 Additional Mechanisms of Action

Although there is significant evidence for the role that n-3 PUFA plays in altering the plasma membrane, there are likely multiple mechanisms by which these fatty acids function. Research has identified additional mechanisms of n-3 PUFA action. These mechanisms may be dependent or independent of the effects of n-3 PUFA on lipid rafts (described below).

12.7 The Role of Inflammation and Cancer

Inflammation plays a critical role in tumorigenesis, and an inflammatory microenvironment is an essential component of all tumors, including those in which a direct causal relationship with inflammation has not been elucidated [85]. It is

well accepted that inflammation facilitates the initiation and progression of normal cells to malignancy through production of inflammatory cytokines (i.e., IL-1, TNFα, and IL-6) and an array of reactive oxygen and nitrogen species [86, 87]. Downstream, these mediators subsequently activate transcription factors (i.e., NF-κB), inducible nitrogen synthase, and cyclooxygenase-2-related signaling pathways, which generally delay or suppress apoptosis in intestinal epithelial cells and modulate angiogenesis and drug-metabolizing enzymes, especially phase II enzyme induction [87–89]. Additionally, arachidonic acid metabolites are also capable of stimulating oncogenic pathways which favor tumor growth, invasiveness, and metastasis. Therefore, anti-inflammatory agents hold promise for decreasing the incidence of colon and other epithelial cancers [90]. A prominent hallmark of cancer-related inflammation is the presence of various types of immune cells and inflammatory mediators (cytokines, chemokines, growth factors, eicosanoids, reactive oxygen, and nitrogen species) which interact with each other by direct contact or by cytokine and chemokine production (autocrine or paracrine signaling), ultimately promoting tumor cell growth, progression, and metastasis [90].

The interrelationship between chronic inflammation and carcinogenesis is perhaps best documented in the colon. CRC is the third most common malignancy and fourth most common cause of cancer mortality worldwide [91]. Approximately only 20 % of CRC cases have a familial basis, i.e., familial adenomatous polyposis and hereditary nonpolyposis CRC [92]; therefore, environmental causes rather than heritable genetic changes represent the largest contributor and modifiable risk factor for CRC development. Chronic intestinal inflammation both precedes and promotes tumor development. Chronic inflammatory conditions such as inflammatory bowel disease (IBD) are associated with highly enhanced colon mucosa carcinogenesis [93]. Colitis-associated cancer represents a CRC subtype that is associated with a high mortality rate [94] and develops over time in more than 20 % of IBD patients following disease onset [95]. Typically colitis-associated chronic inflammation promotes cancer development by inducing oxidative damage to DNA, contributing to p53 mutations in both tumor cells and the inflamed but nondysplastic epithelium [96, 97]. Experimentally, this condition is recapitulated by the combination of a procarcinogen (azoxymethane, AOM) preceding repeated cycles of dextran sodium sulfate (DSS) administration to induce chronic colonic inflammation. Importantly, n-3 PUFA exert anti-inflammatory and immunomodulatory actions through several molecular pathways crucial for the development of inflammatory processes [93].

12.7.1 COX-2-Mediated Effects

A number of studies have addressed the role that n-3 PUFA plays in regulating cyclooxygenase (COX)-2, a key enzyme involved in production of prostaglandins. Arachidonic acid (AA; 20:4 n-6) serves as the precursor to 2-series prostaglandins

which are generally considered to be proinflammatory. Importantly, PGE_2, one of the most well-studied prostaglandins synthesized from AA, is directly linked to colon cancer risk (described below). Besides antagonizing AA, n-3 PUFA can serve as precursors for prostaglandin synthesis; however, the 3-series prostaglandins are anti-inflammatory [98–100]. For example, PGE_3 produced from EPA mediates multiple anti-inflammatory actions. As mentioned above, inflammation is intimately linked to colon cancer risk; therefore, formation of anti-inflammatory compounds is of critical importance in colon cancer prevention. Consumption and subsequent incorporation of fish oil (FO)-derived n-3 PUFA into inflammatory cell phospholipids occur at the expense of n-6 PUFA, thereby decreasing the amount of AA substrate available for synthesis of AA-derived eicosanoids resulting in an altered eicosanoid profile with reduced levels of 2- and 4-series prostaglandins and leukotrienes in inflammatory/immune cells [101–105]. Therefore, one anti-inflammatory mechanism of n-3 PUFA is to inhibit the production of AA-derived eicosanoids.

In several types of cancer, including most colon tumors, COX-2 is overexpressed, and subsequently nonsteroidal anti-inflammatory drugs (NSAIDs) and selective inhibitors of COX-2 (celecoxib) have been demonstrated to be highly effective colon cancer chemopreventive agents [106, 107]. Aberrant expression of COX-2 leads to an increase in PGE_2 levels, which activates the *Wnt* signaling pathway in CRC and plays a well-established role by enhancing intestinal cell proliferation, angiogenesis, cell migration, and invasion while inhibiting protective apoptotic signaling pathways [108–111]. Additionally, PGE_2 levels are elevated in human colon carcinomas compared to normal mucosa [112] and in the colonic mucosa of rats injected with AOM during the initiation and post initiation stages of carcinogenesis [113]. Interestingly, a novel null mouse model, in which tissue AA mass is reduced to near undetectable levels via systemic disruption of the *Fads1* (Δ5 desaturase) gene, exhibits reduced colon cell proliferation and suppressed PGE_2 levels in the colonic mucosa in null mice compared to wild-type littermates [114]. Future work is needed in order to determine if these mice are resistant to the induction of colon cancer.

n-3 PUFA decrease expression of COX-2 both in vitro and in vivo. In rodents, dietary n-3 PUFA has been shown to reduce COX-2 expression in the colonic mucosa [115, 116]. Moreover, n-3 PUFA have been shown to suppress inflammatory eicosanoid production by suppressing COX-2 activity [117, 118]. Additionally, *Fat-1* (omega-3 desaturase) transgenic mice have lower expression of COX-2 than wild-type littermates [119]. In two distinct human colon cancer cell lines, EPA and DHA have been shown to reduce COX-2 expression [120, 121]. Furthermore, nude mice injected with human colon cancer cells and fed either DHA or EPA exhibit a decrease in both COX-2 expression, PGE_2 synthesis, and tumor growth [120]. Additionally, a combination of DHA with celecoxib, a selective COX-2 inhibitor, was shown to decrease COX-2 expression in a human colon cancer cell line, and the combination was more effective than either treatment alone [122]. COX-2 inhibitors have been found to have beneficial effects for colon cancer, but they can have detrimental side effects that limit their applicability. Therefore, utilization of n-3 PUFA, i.e., innocuous dietary lipids with very minor side effects, could provide the benefits of COX-2 inhibitors without the medical complications.

12.7.2 Effects of n-3 PUFA Metabolites

In addition to the anti-inflammatory metabolites produced by COX-2-mediated metabolism, other enzymes can produce additional anti-inflammatory and pro-resolving compounds from n-3 PUFA. Included among these anti-inflammation and pro-resolution metabolites are resolvins and protectins, which have been shown to elicit potent anti-inflammatory effects on inflammatory/immune cells [123–125]. DHA is the precursor for the D-series resolvins and protectin D1 (PD1), whereas EPA serves as the origin for resolvins E1 (RvE1) and E2 (RvE2) [125–127]. These novel DHA- and EPA-derived metabolites were first observed during the resolution phase of acute inflammation [123, 125]. The resolvins can stimulate the resolution phase of inflammation to begin at an earlier time point, which reduces the overall exposure to inflammation [128]. Generally, resolvins actively contribute to the resolution of inflammation via the removal of inflammatory cells and the restoration of tissue integrity, and these processes can be further facilitated by the presence of aspirin [90]. RvE1 has been found to reduce inflammation, dendritic cell migration, and the production of inflammatory cytokines [129]. Furthermore, PD1 protects tissues from oxidative damage [128]. PD1 also exhibits anti-inflammatory properties by reducing neutrophil infiltration and production of proinflammatory cytokines, specifically during the resolution phase of inflammation [130]. These recently discovered metabolites of DHA and EPA serve as an additional mechanism by which dietary fatty acids can facilitate reduction of inflammation, which is an important aspect of cancer prevention.

12.8 n-3 PUFA and Transcriptional Regulation

Since the majority of factors perturbed in cancer are signaling molecules, their downstream effectors, transcription factors, hold promise for cancer therapy [131]. Moreover, oncogenic transcription factors represent a useful class of therapeutic targets since they often have increased expression and activity in a variety of cancer types. One mechanism through which n-3 PUFA exert their anti-inflammatory and anti-cancer effects is through modulation of transcription factor activity and/or expression, thereby altering the downstream gene expression profile as discussed below. Aberrant NF-κB activation has been detected in more than 50 % of colorectal and colitis-associated tumors using animal models, establishing a role for NF-κB in CRC development [132, 133]. NF-κB activation promotes tumorigenesis by inhibiting apoptosis (via activation of Bcl2, Bcl-xL cFLIP, and other genes), increasing cell proliferation, angiogenesis, and promoting cell invasion and metastasis [134, 135]. Moreover, aberrant NF-κB signaling has been proposed to be one of the mechanisms through which chronic inflammation leads to cancer [135].

Most tumor-promoting cytokines are activated by NF-κB directly or in conjunction with other inflammatory stimuli in premalignant cells and immune/inflammatory cells [136]. In this context, deletion of IKKβ within myeloid cells (i.e., dendritic

cells, macrophages, and neutrophils) resulted in a 50 % decrease in tumor incidence and a substantial reduction in tumor size [137], thereby inhibiting inflammation-induced enterocyte proliferation and inflammation-stimulated growth of colitis-associated tumors. Therefore, activation of NF-κB in lamina propria-residing myeloid cells results in the production of cytokines that can act as growth factors for premalignant enterocytes, since several proinflammatory cytokines and chemokines (such as TNF, IL-1, IL-6, and CXCL8) are encoded by target genes of the IKKβ-dependent NF-κB activation pathway and are associated with tumor development and progression [132]. Inflammatory cytokines play crucial roles in colon cancer. For example, TNFα and IL-6 serum levels are associated with increased risk of colorectal adenoma development [138]. Additionally, injection of neutralizing antibodies specific for the IL-6 receptor has been shown to decrease both tumor number and size, thereby demonstrating the critical role of the IL-6 signaling pathway in colon cancer development [139].

In vitro studies have shown that n-3 PUFA exert inhibitory effects on NF-κB by decreasing IκB phosphorylation and activation, subsequently reducing inflammatory cytokine production (IL-1β, IL-6, and TNFα) [140–143]. In a colitis-associated cancer model, *Fat-1* transgenic mice, which endogenously produce long-chain n-3 PUFA de novo, exhibited a suppression of tumorigenesis compared to wild-type mice. This was associated with a reduction in tumor incidence and coincided with lower colonic NF-κB activity and increased mRNA expression of the antiproliferative cytokine TGF-β [144]. The suppression of NF-κB in the chronic colitis/malignant transformation model mirrored the anti-inflammatory effect of n-3 PUFA during acute colitis, wherein NF-κB activation and mRNA expression of key inflammatory makers (inducible nitric oxide synthase [iNOS], IL-1β, and TNFα) were suppressed in the *Fat-1* mouse [145].

Following splenic $CD4^+$ T cell activation, either dietary FO or a diet enriched in DHA ethyl esters inhibited the DNA-binding activity of both NF-κB and AP-1 [146]. Similarly, both NF-κB and AP-1 DNA-binding activity were modestly affected in human fibroblasts by EPA treatment in comparison to AA [147]. AP-1 is overexpressed and has been shown to have increased activity in several types of cancer including breast, ovarian, cervical, and colorectal, consistent with its fundamental role in oncogenesis [148–151]. Functionally, AP-1 is implicated in biological processes integral to cellular transformation including proliferation, apoptosis, differentiation, invasion, and motility [152]. Collectively, these data suggest that n-3 PUFA can potentially impact AP-1-mediated early and late events in carcinogenesis [153].

Another inflammatory transcription factor whose colonic activation and expression is reduced by n-3 PUFA is STAT3 [83, 154]. STAT3 is constitutively activated in biopsies from cancer patients and cell lines. Numerous cytokines, growth factors, and oncogenic proteins activate STAT3, leading to cell transformation and tumorigenesis, as STAT3 target genes are involved in multiple steps of the metastatic process, including invasion, cell survival, self-renewal, angiogenesis, and tumor cell immune evasion [155, 156]. Moreover, STAT3 activation functionally links inflammation and cancer wherein aberrant phosphorylated STAT3 expression in inflammatory cells residing within the tumor microenvironment triggers an

epithelial cell survival and growth response that promotes the overgrowth of neoplastic cells [156]. In summary, STAT3-mediated reciprocal interactions between tumor, inflammatory, and stromal cells collectively make up and prime the inflammatory tumor cellular microenvironment. Therefore, suppressed STAT3 activation by n-3 PUFA represents a potential therapeutic target for the purpose of transcriptional repression.

12.8.1 Role of n-3 PUFA as Receptor Ligands

DHA and EPA have been shown to act as ligands for peroxisome proliferator-activated receptors (PPARs) and retinoid X receptor alpha, which are both nuclear receptors [157, 158]. Binding of ligand to these nuclear receptors stimulates their activity as transcription factors, which alters gene expression. Collectively, these outcomes mediate numerous biological functions, including lipid metabolism, cell differentiation, and cell death. In this context, PPARγ, one of the isoforms of PPAR, was found to mediate DHA-induced apoptosis [159]. The ability of PPARγ to promote differentiation and maturation of epithelial cells has led to studies of its potential role in the cause of CRC, and animal studies suggest both pro- and anti-cancer properties in the colon [160–162]; however, the majority of studies point to PPARγ ligands as chemopreventive agents [163].

PPARs are also involved in the regulation of the production of inflammatory mediators, thereby exerting anti-inflammatory effects [164]. PPARs can physically interact and thereby interfere with other transcription factors involved in proinflammatory signal transduction pathways including AP-1, STAT-1, NF-κB, and NFAT [164, 165]. The ability of activated PPARs to transrepress inflammatory responses mediated by NF-κB represents an important functional mechanism for n-3 PUFA, since NF-κB links inflammation and immunity to cancer development and progression [132, 166, 167]. PPARs further regulate inflammatory processes by suppressing the expression of iNOS thereby inhibiting NO production, inflammatory cytokines (TNFα, IL-6, IL-1β, and IL-12), reducing macrophage recruitment to inflammatory sites by repressing transcription of MCP-1 and its receptor CCR2, inducing T cell anergy, and increasing the formation and suppressive function of regulatory T cells [164, 165]. Therefore, it has been proposed that n-3 PUFA exert their anti-inflammatory and immune suppressive effects through binding to PPARs [168–170]. However, with respect to ligand-binding specificity, this class of nuclear receptor binds n-3 and n-6 PUFA with equal affinity and lacks fatty acid class (n-3 vs. n-6) specificity [157, 171–173]. Therefore, the anti-inflammatory/chemoprotective effects of n-3 PUFA are likely not mediated directly via PPARs alone.

n-3 fatty acids have additionally been found to serve as ligands for the G-protein-coupled receptor GPR120. Binding of n-3 PUFA to GPR120 results in multiple anti-inflammatory effects suppressing NF-κB activation and reducing inflammatory cytokine production in monocytes and macrophages [174]. Moreover, in GPR120 knockdown cells, the inhibitory effect of DHA on the response to endotoxin was

abolished, thereby indicating that the inhibition of NF-κB by n-3 PUFA occurs via a GPR120-dependent mechanism [174]. However, there is concern that the effects of fatty acids on GPR120 are not restricted to n-3 PUFA [175]. Therefore, its activation in vivo using biologically relevant delivery models requires further investigation.

Metabolites of DHA and EPA have been found to function as ligands for some receptors. The PGE_2 EP4 receptor is involved in signaling events that are integral in colonic tumorigenesis [176, 177]. Interestingly, PGE_3 (an EPA-derived metabolite) can also bind to this receptor but with reduced affinity and efficacy compared to PGE_2 [178]. Furthermore, PGE_3 can act as an antagonist to EP4 signaling in human CRC cells [178], and DHA and EPA can reduce EP4 signaling due to the reduced production of COX-2-derived PGE_2 as discussed above. Likewise, RvE1 can serve as a ligand for the ChemR23 receptor. Binding of RvE1 to this receptor has been shown to attenuate NF-κB [129]. RvE1 can also bind to the leukotriene B4 receptor 1, where it serves as a partial agonist and hinders receptor signaling [179]. RvD1 can interact with the lipoxin A_4 receptor ALX and the orphan receptor GPR32 on phagocytes [180]. This interaction stimulates phagocytosis to aid in the resolution of acute inflammation. Overall, n-3 PUFA can directly serve as ligands, as well as precursors of ligands (DHA- and EPA-derived protectins and resolvins), which can directly regulate cell signaling and inflammation biology.

12.8.2 n-3 PUFA and T Cell Activation

Understanding how T cell activation is modulated by n-3 PUFA, specifically with respect to the dampening of uncontrolled T cell-mediated inflammatory responses, could beneficially impact the carcinogenic process. T cells isolated from mice fed an n-3 PUFA-enriched fish oil diet exhibit an altered phospholipid distribution in the liquid-ordered phase compared to mice fed an n-6 PUFA-enriched control diet. Specifically, the percentage of sphingomyelin in the liquid-ordered phase of T cells isolated from the n-3 PUFA-enriched fish oil group exhibited a 50 % decrease compared to the n-6 PUFA-enriched corn oil group. Furthermore, n-3 PUFA such as EPA and DHA are readily incorporated into the phosphatidylserine and glycerophosphoethanolamine in the liquid-ordered phase [181]. These results are consistent with fatty acid changes seen in the aforementioned cell types. Since lipid–lipid interaction and the formation of liquid-ordered and disordered phases are dependent on the composition of not only the fatty acid species but also the phospholipid composition [182], the perturbation of plasma membrane composition by n-3 PUFA could directly influence T cell activation.

T cell activation depends on the formation of an immunological synapse (IS) which requires lipid raft (i.e., cholesterol, sphingomyelin, and saturated phosphatidylcholine) accumulation at the IS [183]. Interestingly, disruption of the liquid-ordered phase of the plasma membrane by oxysterol 7-ketocholesterol (7KC)

resulted in suppressed lipid condensation upon T cell activation [184]. Since dietary n-3 PUFA such as EPA and DHA can be incorporated and disrupt lipid-ordered phase of the plasma membrane [185], it is not surprising that n-3 PUFA impact the lipidomic rearrangement upon $CD4^+$ T cell activation. In $CD4^+$ T cells isolated from transgenic mice that generate n-3 fatty acids de novo (*Fat-1* mice), the lipid-ordered phase at the IS was increased [29], demonstrating how n-3 PUFA are capable of disrupting the lipidomic rearrangement of the IS, impacting downstream T cell signaling and activation.

Another lipidomic impact exerted by n-3 PUFA is observed at the level of phosphatidylinositol-(4,5)-bisphosphate [$PI(4,5)P_2$], a "phospholipid" in the inner leaflet of the plasma membrane [186]. Hydrolysis by phospholipase C generates the second messengers inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), which mediate calcium signaling and protein kinase C (PKC) recruitment, respectively. Recent research has focused on the role of $PI(4,5)P_2$ as a second messenger itself, recruiting and/or activating effector proteins to various $PI(4,5)P_2$-enriched compartments (i.e., plasma membrane, nucleus). The presence of n-3 PUFA such as EPA and DHA is correlated to a decrease in the n-6 PUFA arachidonic acid ($20:4^{\Delta 5,8,11,14}$) content, which is the predominant fatty acid species at the *sn-2* position of $PI(4,5)P_2$ [187]. Thus, it is expected that n-3 PUFA can antagonize the synthesis of $PI(4,5)P_2$. Indeed, the platelet 1-acyl-glycero 3-phosphoinositol acyltransferase that remodels $PI(4,5)P_2$ at the *sn*-2 position prefers n-6 PUFA over n-3 PUFA, suggesting a competitive inhibition by EPA and DHA [188]. Consistent with the acyltransferase specificity, in the *Fat-1* $CD4^+$ T cells, $PI(4,5)P_2$ was decreased by 50 % compared to wild-type control, leading to defects in $PI(4,5)P_2$ metabolism upon T cell activation [189]. This phenotype was recapitulated in a dietary model, in which $CD4^+$ T cells isolated from animals fed a 4 % DHA-triglyceride diet exhibited a 25 % decrease in basal $PI(4,5)P_2$. Defects in $PI(4,5)P_2$ metabolism were also observed in DHA-triglyceride-enriched $CD4^+$ T cells upon activation. Interestingly, the kinetics of DAG, the hydrolysis product of $PI(4,5)P_2$, was suppressed upon activation by concanavalin A in lymphocyte population from mice fed an EPA- or DHA-enriched diet [190].

Proteomic rearrangements and their contribution to the formation of the IS begin with the activation of the tyrosine kinases lymphocyte-specific protein tyrosine kinase (LCK) and zeta-chain-associated protein kinase 70 (ZAP70). Their activation results in the phosphorylation of linker for activation of T cells (LAT) and the assembly of the signalsome composed of effector proteins such as GADS, SLP76, NCK, ITK, VAV, PAK, and PLC-γ1 [191, 192]. Protein localization to specific membrane compartments (i.e., lipid raft-enriched IS) can be affected by two non-mutually exclusive mechanisms: (1) alterations in posttranslational modifications and (2) disruption of membrane microdomains. Proteins may be posttranslationally modified to incorporate fatty acids into various amino acid residues to stabilize their association to specific membrane microdomains. Therefore, it is of interest to note that in Jurkat T cells incubated with EPA, src kinase Fyn failed to localize to the detergent-resistant membrane by inhibiting its palmitoylation [193]. This was correlated with the presence of posttranslational modification by monounsaturated and

polyunsaturated fatty acids [194], demonstrating that n-3 PUFA are capable of modulating posttranslational fatty acid modification and therefore displace proteins from appropriate membrane compartments. Disruption of membrane microdomains by n-3 PUFA can also affect protein localization. Indeed, LAT, an important protein recruited early to the signalsome, was displaced from the detergent-resistant membrane after treatment with n-3 PUFA [195]. This appeared to be the first proteomic perturbation induced by n-3 PUFA, as PUFA had no effect on the phosphorylation of the CD3ζ by LCK or the binding and phosphorylation of ZAP-70 [196]. The localization of PLC-γ1 and subsequent phosphorylation was also suppressed in $CD4^+$ T cells enriched in n-3 PUFA, demonstrating additional proteomic effects induced by n-3 PUFA [29].

Actin reorganization is required for trafficking of the T cell receptor to the site of contact with antigen-presenting cells in order to survey antigens presented in MHCII. Upon antigen recognition by the TCR, actin stabilizes the IS to allow for protein complex formation and downstream signaling [197–199]. Actin-regulatory proteins that are important for T cell activation and are also known to be regulated by $PI(4,5)P_2$ include the Wiskoff-Aldrich syndrome protein (WASP), ERM proteins, talin, WAVE, and ADF/cofilin [200–204]. The decrease of $PI(4,5)P_2$ by n-3 PUFA has been correlated with suppressed actin remodeling upon T cell activation in n-3 PUFA-enriched $CD4^+$ T cells [29, 189]. It was further shown that the recruitment of WASP to the IS was suppressed in *Fat-1* $CD4^+$ T cells, elucidating one pathway in which the effects of n-3 PUFA on actin remodeling could be mediated. These results indicate that n-3 PUFA such as DHA suppress early T cell activation events by antagonizing $PI(4,5)P_2$ and subsequent $PI(4,5)P_2$-dependent signaling processes such as actin remodeling [189]. This is consistent with results seen in the human Jurkat T cell line E6-1 incubated with either EPA or DHA, where the localization of F-actin upon IS formation was suppressed in cells exposed to individual n-3 PUFA [205]. Actin cytoskeleton can also be regulated by the nucleotide exchange factor VAV, which is recruited to the IS upon T cell activation. EPA treatment of T cells showed an inhibition of VAV phosphorylation, leading to instability of the IS and antigen-presenting T cell conjugates [205]. These observations demonstrate the immunosuppressive pleiotropic effects of n-3 PUFA with respect to the actin cytoskeleton, cell lipidomics, and proteomics.

The assembly of the signalsome allows for further downstream signaling and the activation of transcription factors for sustained T cell activation. Signal transduction from the plasma membrane to the nucleus includes the translocation and activation of PKCθ [206], translocation of the mitochondria to the IS [207, 208], Ca^{2+} signaling [209], and ultimately the activation of transcription factors such as NFAT, NF-κB, and AP-1 to induce specific gene expression required for cell proliferation and differentiation [210–212]. Similar to LAT, which was displaced from detergent-resistant membranes, the colocalization of PKCθ to GM1-enriched lipid rafts was decreased in splenic T cells isolated from mice fed an n-3 PUFA-enriched diet, leading to the suppression of PKCθ to the IS [146]. In parallel, downstream signaling events such as mitochondrial translocation and Ca^{2+} signaling were also suppressed

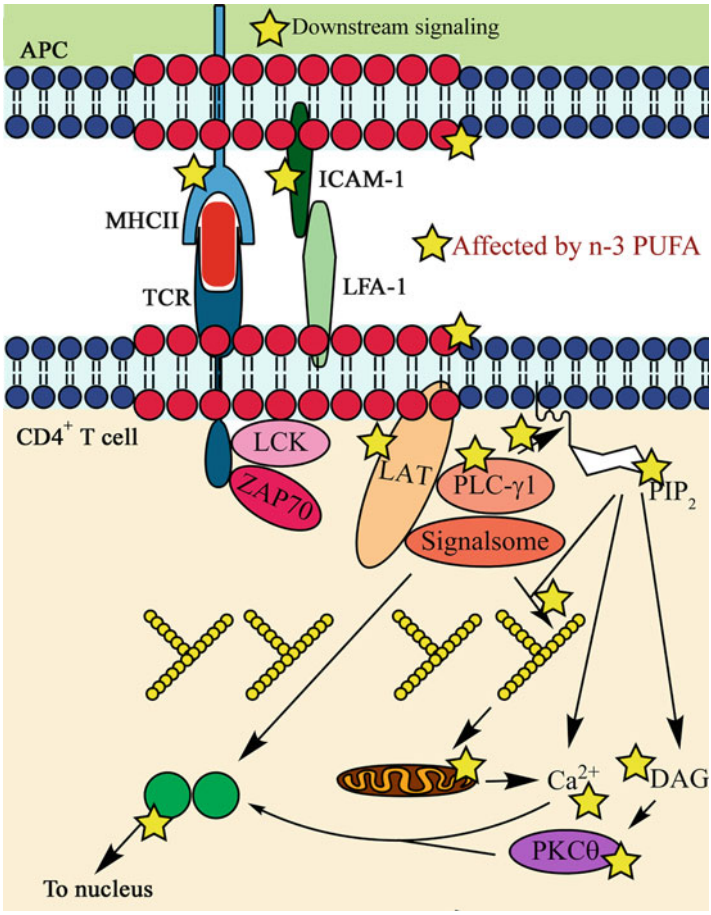

Fig. 12.2 Pleiotropic effects of n-3 PUFA on $CD4^+$ T cell and APC activation. Upon the formation of the immunological synapse, several early signaling events are perturbed in both the $CD4^+$ T cell and the APC. For the APC, n-3 PUFA perturbs the organization of the lipid microdomains, leading to changes in the conformation, lateral organization, and trafficking of MHCII and adhesion proteins such as ICAM-1. This results in alterations in the downstream signaling of APC. For the $CD4^+$ T cells, incorporation of n-3 PUFA leads to changes in the lipidomic (i.e., suppression of sphingomyelin and $PI(4,5)P_2$, changes in lipid microdomains) and proteomic (i.e., decreased LAT and PLC-γ1 localization) rearrangements that occur upon immunological synapse formation. These effects lead to decreased actin remodeling and downstream events such as (1) mitochondrial translocation and Ca^{2+} signaling, (2) DAG metabolism and PKCθ recruitment, (3) activation of transcription factors, and (4) cellular processes such as proliferation, differentiation, and inflammatory cytokine secretion

in $CD4^+$ T cells enriched in n-3 PUFA. Importantly, the upstream suppression by n-3 PUFA has been linked to a decrease in AP-1 and NF-κB activation and ultimately the IL-2 secretion and proliferation of $CD4^+$ T cells following activation [29, 146] (Fig. 12.2).

12.9 n-3 PUFA, Th17 Cells, and Cancer

Th17 cells, an inflammatory T cell subset identified to be the major cellular source of IL-17, play a critical role in the pathogenesis of many inflammatory and autoimmune diseases including IBD [213]. Since tumor development involves chronic inflammatory processes [214], determining the contribution of Th17 cells to this process represents an active area of research. Assessment of tumor-infiltrating lymphocytes isolated from skin (melanoma), breast, ovarian, and colon cancer tumors revealed that the proportion of $CD4^+$ $IL17^+$ T cells was markedly elevated, suggesting that the development of tumor-infiltrating Th17 cells is a general feature of cancer [215, 216]. Additionally, increased expression of Th17 cells has also been documented in patients with gastric, prostate, ovarian, renal cell, and pancreatic cancers [217–219], and IL-17 mRNA expression is high in tumor samples from prostate and ovarian cancer patients [220, 221]. Interestingly, the cells comprising the tumor microenvironment (tumor cell and tumor-derived fibroblasts) were shown to mediate the recruitment of Th17 cells and to support both the generation and expansion of Th17 cells by secreting both chemotactic factors and proinflammatory cytokines [216]. A study conducted in gastric cancer patients revealed that the increased number of Th17 cells was correlated with cancer stage, thereby suggesting that Th17 cells contribute to cancer pathogenesis [219]. Moreover, both human cancer patients and experimental tumor models revealed that IL-17 favors tumor growth and exhibits a significant angiogenic effect [220, 222, 223]. Despite conflicting reports surrounding the functional role of IL-17 as either an antitumor or tumor-promoting factor in different types of human cancers [224], recent evidence supports a role for IL-17A in colorectal carcinogenesis. Human CRC tissue exhibits increased IL-17A expression, which was found to adversely impact the clinical outcome and patient prognosis [225–227]. In animal models, IL-17A was shown to promote colon cancer development [228–230] and functionally blocking IL-17A resulted in decreased formation of hyperplasia and cancer [228]. Overexpression of IL-17 in tumors leads to increased angiogenesis and tumor growth [222], and $IL\text{-}17^{-/-}$ and $IL\text{-}17R^{-/-}$ mice exhibit reduced tumor growth [231, 232]. Additionally, in a mouse model of colon cancer (AOM + DSS), both tumor size and number of colonic tumors per mouse were reduced in IL-17A-deficient mice [233]. Interestingly, in the same cancer model, dietary fish oil reduced the total number of colonic tumor entities (adenomas + adenocarcinomas) per mouse and exhibited a greater percentage of animals that were nonresponsive in terms of tumor formation (48 % vs. 18 %), as compared to mice fed a control diet (5 % corn oil) [83]. Moreover, colonic mucosal mRNA expression of both the Th17 cell signature cytokines (IL-17A and IL-17F) and inflammatory cytokines supporting Th17 cell differentiation (IL-6) and phenotype maintenance (IL-23) was significantly reduced by dietary FO [83]. Lastly, activated (i.e., phosphorylated) STAT3 colonic protein levels were reduced by dietary FO [83], which is significant because T cell expression of STAT3 plays a critical role in Th17 cell differentiation [234] and promotes a procarcinogenic Th17 cell response [230, 235]. Interestingly, the tumorigenic effects of IL-17 are mediated, at least in part, by IL-6 via a STAT3-dependent mechanism [232], and all three of these mediators were reduced in the colon by dietary fish oil [83]. n-3 PUFA also

exhibited a suppressive effect on colonic IL-23 and IL-23R mRNA expression, a key cytokine that not only promotes pathogenic Th1 and Th17 responses in the intestine [236] but also represents an important molecular link between chronic inflammation and carcinogenesis as demonstrated by IL-23p19$^{-/-}$ mice which are resistant to tumor induction [237]. Therefore, this research demonstrates that within the colonic tumor microenvironment, expression of critical factors impacting aspects of Th17 cell polarization and function, which have independent procarcinogenic roles, was collectively suppressed by dietary fish oil, resulting in reduced colonic tumor formation. Although the molecular mechanisms of n-3 PUFA action on Th17 cells have not been elucidated, these data provide insight into the underlying anti-inflammatory and chemopreventive actions of n-3 PUFA.

12.9.1 Oxidative Stress and the Induction of Apoptosis

Studies in several types of cancer, including breast, prostate, and colon, have established that oxidative stress modulates both cancer initiation and progression [238]. In cancer cells, high levels of reactive oxygen species (ROS) can result from increased metabolic activity, mitochondrial dysfunction due to hypoxia or mitophagy, peroxisome activity, uncontrolled growth factor or cytokine signaling, and oncogene activity in addition to enhanced activity of known ROS sources (e.g., NADPH oxidase, COX, and LOX) [238–241]. Moreover, ROS can promote many aspects of tumor onset and progression towards a malignant phenotype [238]. Thus, if the cellular oxidative state increases, ROS form and can cause both cellular and DNA damage. n-3 PUFA, due to their high levels of unsaturation, are oxidatively susceptible lipids [242, 243]. Interestingly, formation of lipid peroxidation products can activate the "intrinsic" mitochondrial apoptosis pathway, which serves as a sensor for damage and oxidative stress [244]. This is noteworthy, because the balance between proliferation and apoptosis is critical for the maintenance of steady-state number for cell populations, particularly in the colon. Feeding a diet containing fish oil can increase oxidative stress and activate apoptosis [243]. In cell culture, treatment with DHA increased lipid oxidation and reduced mitochondrial membrane potential, which resulted in increased apoptosis [245]. Furthermore, in genetically oxidatively stressed mice, feeding fish oil enhanced apoptosis and altered mitochondrial metabolic activity [246]. These findings support the contention that n-3 PUFA alters cellular oxidation to enhance apoptosis and thereby prevent colon cancer.

12.10 Combined Bioactive Nutrients

There is also a wealth of data suggesting a synergistic effect of n-3 PUFA with other dietary compounds. It is important to note that no nutrient is ever consumed in isolation. Therefore, it is imperative to understand how nutrients function in combination with each other.

12.10.1 n-3 PUFA and Fiber

In addition to n-3 PUFA, fiber is another commonly studied nutrient in the context of colonic health. In colonocytes and colon cancer cells, treatment with the combination of DHA and butyrate, a fiber fermentation product, has been shown to induce apoptosis and alter mitochondrial-to-cytosolic Ca^{2+} levels [247, 248]. This effect was specific to the combination of DHA and butyrate, i.e., other fatty acids combined with butyrate did not exert the same effects. DHA and butyrate have also been shown to synergistically increase lipid oxidation in colonocytes [245]. Feeding carcinogen-injected rats a diet of fish oil in combination with enterically coated butyrate, elevated apoptosis and decreased the formation of aberrant crypts foci, which can serve as precursors for colonic tumors [249]. Combination of fish oil and the fermentable fiber pectin was additionally found to reduce oxidative DNA damage and increase apoptosis [250]. Overall, these data indicate that the combination of fish oil and fiber could provide more protection against colon cancer than either nutrient alone.

12.10.2 n-3 PUFA and Curcumin

Another nutrient that has attracted a great deal of interest is curcumin. Curcumin is a curcumoid found in the Indian spice turmeric. Treatment of macrophages with a combination of curcumin and DHA or EPA was found to synergistically suppress nitric oxide levels, PGE_2 synthesis, and the levels of enzymes involved in formation of proinflammatory mediators [251]. The combination of fish oil and curcumin has been shown to modulate colonocyte gene expression and enhance resolution of chronic inflammation, in part, by suppressing a key inflammatory mediator, NF-κB [154]. The combination of fish oil and curcumin was also found to reduce proliferation of $CD4^+$ T cells to favorably modulate inflammation [252]. Together, these studies suggest that the combination of fish oil and curcumin could have potent anti-inflammatory effects, which is beneficial for colon cancer prevention.

12.10.3 Ratio of n-6 to n-3 PUFA

In a pivotal study, Simopoulos identified the ratio of n-6 to n-3 PUFA as an important indicator of the overall health outcome [253]. Currently, the n-6:n-3 ratio in the average Western diet is calculated to be between 10:1 and 20:1 [254–256]. One of the reasons that the ratio of these two classes of dietary lipid is important is the competition between n-3 and n-6 PUFA for many enzymes. These fatty acids utilize many of the same elongases and desaturases required for production of the longer-chain fatty acids in each series. Therefore, if levels of α-LNA are high enough, this

essential fatty acid can compete with linoleic acid (LA 18:2 n-6) and thereby reduce the formation of AA. Likewise, as mentioned above, both EPA and AA can be metabolized by COX-2 to produce anti-inflammatory and proinflammatory prostaglandins, respectively. Therefore, if levels of n-6 PUFA are substantially higher than n-3 PUFA, more prostaglandins will be produced from AA as opposed to EPA. This could then hinder the anti-inflammatory activities of EPA and DHA. Additionally, these PUFA will compete for esterification into phospholipids and, therefore, insertion into the plasma membrane. Due to the important actions of n-3 PUFA in the plasma membrane, it is imperative that the ratio of n-6 to n-3 PUFA be sufficiently low in order for fatty acyl incorporation to occur. Furthermore, whereas n-3 PUFA is associated with reduced colon cancer risk, n-6 PUFA has been found to increase the risk of colorectal adenomas [17]. Therefore, understanding the roles of all dietary lipids, as well as the ways they interact and compete with each, is required in order to make informed recommendations for overall human health.

12.11 Differential Effects of DHA and EPA

A significant proportion of the published studies describing the effects of n-3 PUFA on cellular functions utilizes either fish oil, purified DHA, or a combination of EPA and DHA [28, 71, 146]. This can likely be a source of some of the inconsistencies in the literature. Specifically, although many commercially available fish oils contain approximately a 2:1 ratio of EPA to DHA, fish oils from different sources contain variable mixtures of EPA and DHA. This can make it difficult or impossible to compare results from different studies. Furthermore, whether the effects of n-3 PUFA supplementation are due to EPA, DHA, or both is often undetermined and unappreciated. EPA is both two carbons shorter and less unsaturated than DHA, and the structural differences between these two fatty acids are enough to result in functional differences [69]. A recent biophysical study in model membranes demonstrated differential efficacies of DHA and EPA to modify lipid raft composition and organization [185]. Studies on the effects of DHA compared to EPA on immune cell lipid rafts have observed differential effects of these fatty acids on membrane order [30]. Comparisons of the anti-inflammatory profiles of macrophages treated with DHA or EPA indicated that DHA may be more effective than EPA in alleviating inflammation [142]. Additionally, a study compared the effects of DHA or EPA on EGFR signaling found that DHA but not EPA altered EGFR activation status and signaling [76]. These data highlight the need to better understand the mechanisms by which EPA and DHA differentially modulate cell membranes.

In summary, cogent evidence indicates that n-3 PUFA confer protection against several forms of cancer. We propose that n-3 fatty acids represent a class of *novel innocuous dietary bioactives for lipid raft targeted therapy* and cancer prevention. So why have clinical practitioners currently searching for toxicologically *innocuous cancer chemoprevention* approaches not embraced n-3 PUFA? Perhaps many studies looking for n-3 PUFA-related effects in cancer populations have errored by

intervening too late in the disease process [257]. Undoubtedly, additional work is needed in order to fully elucidate the chemopreventive actions of n-3 PUFA at the molecular, cellular, animal model, and clinical level.

We propose that it is time to get serious about cancer prevention. Unfortunately, only a small fraction of current funding in the USA for cancer research is targeted to early detection and prevention. In addition, less than 1.5 % of total biomedical research funding is devoted to implementation of cancer prevention programs [258]. According to Vogelstein and colleagues [259], the only sure way to reduce cancer risk is through screening and a healthy lifestyle.

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Chapter 13
Natural Products as Anti-inflammatory Agents

Gary Stoner and Li-Shu Wang

Abstract Inflammation is triggered by numerous factors including oxidative stress, environmental pollutants, microbial agents, and physical damage to tissues. Chronic inflammation, characterized by a prevalence of macrophages and lymphocytes in the affected tissues and the overexpression of a host of cellular cytokines, chemokines, and inflammatory enzymes, promotes all stages of cancer development including initiation, promotion, cell transformation, angiogenesis, invasion, and metastasis. This chapter describes some key mechanisms by which naturally occurring dietary compounds, either alone or in combination, reduce the harmful effects of inflammation and the risk for cancer development. The most extensively studied compounds are a series of polyphenols which influence the inflammatory process in multiple ways including their ability to scavenge oxidative radicals, influence carcinogen activation and detoxification, and regulate expression levels of numerous transcription activators and their associated cytokines, chemokines, and inflammatory enzymes. In the past, the inhibitory effects of naturally occurring compounds, especially the polyphenols, have been attributed mainly to their intrinsic antioxidant capacity; however, it is likely that their direct binding to cellular macromolecules and the associated effects on gene transcription and translation, as well as on enzyme activity, may be equally as important.

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13.1 Introduction

It has been known for decades that the cellular components of the inflammatory response are critical for their role in the killing of cancer cells and, hopefully, curing the disease. In recent years, however, it has become apparent that inflammation, especially chronic inflammation, is also a significant risk factor for the development of cancer. Chronic inflammation has been associated with the various stages of tumorigenesis including initiation, promotion, progression, invasion, and metastasis [1, 2]. One of the many cancer types in which there is a strong association between chronic inflammation and tumor development is colon cancer. Meta-analysis has shown that patients suffering from inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis, have a 33-fold increase in risk for colon cancer relative to the general population [3]. Inflammatory bowel diseases affect approximately 1–2 of every 1,000 people in developed countries and are on the rise worldwide [4]. Links between inflammation and cancer have also been confirmed in a number of animal models, including cancers of the colon [5], liver [6], pancreas [7], prostate [8], and lung [9].

One mechanism through which chronic inflammation and carcinogenesis are linked is via the activities of reactive oxygen (ROS) and nitrogen (RNS) species (i.e., RONS). In normal tissues and cells, RONS are important for regulating signaling cascades that govern multiple cellular functions. However, in an inflammatory microenvironment, infiltrating leukocytes raise the level of RONS to amounts that exceed the ability of cells to eliminate them and the cells become "stressed." Stress occurs because the activities of antioxidant phase II enzymes such as superoxide dismutase, catalase, glutathione peroxidase, quinone oxidoreductase, epoxide hydrolase, and others are not sufficient to remove the excess RONS. The infiltrating leukocytes that produce RONS include macrophages, monocytes, neutrophils, T and B lymphocytes, mast cells, and natural killer cells [1, 2]. Some of these cell types produce growth factors, cytokines, and chemokines that stimulate cell proliferation, angiogenesis, invasion, and metastasis. These factors also chemo-attract additional lymphocytes, monocytes, and macrophages leading to a chronically activated microenvironment that becomes a relentless source of genetic and histopathologic damage to the surrounding epithelium. Ultimately, this damage can lead to the development of cancer in all of its stages.

Epidemiological studies provide evidence that persons who consume a diet rich in vegetables and fruit have a reduced risk of cancer at multiple organ sites [10–12]. Because fruits and vegetables are major sources of antioxidants, it is thought that the antioxidants are largely responsible for their cancer-preventive effects. This hypothesis is supported by epidemiological data, implicating micronutrient antioxidants in cancer risk reduction, and by experimental data from in vitro and in vivo studies [13, 14]. The antioxidant effects of vegetables and fruits are due, in part, to their content of flavonoids and phenolic acids [15–17]. Some of the polyphenolic compounds with demonstrated cancer-preventive effects include the catechins in green tea, theaflavins in black tea, curcumin in turmeric, resveratrol in red wine,

quercetin in apples, and ellagic acid and the anthocyanins in berries. Collectively, these antioxidants exhibit a significant ability to scavenge RONS [15, 18, 19], and this is associated, at least in part, with their inhibitory effects on multiple cell-signaling pathways and inflammatory processes. For example, one of the most extensively investigated polyphenols is curcumin, present in the Indian spice, turmeric. Cell-signaling pathways that are inhibited by curcumin alone include activator protein-1 (AP-1), nuclear factor-kappa B (NF-κB), signal transducer and activator of transcription 3 (STAT3), mitogen-activated protein kinase (MAPK), protein kinase B (Akt), cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-LOX), c-Jun N terminal kinase (JNK), human growth factor receptor 2 (HER2), IκB kinase (IKK), epidermal growth factor receptor (EGFR), B-cell lymphoma 2 (Bcl-2), B-cell lymphoma extra large (Bcl-X_L), poly ADP ribose polymerase (PARP), and proapoptotic caspases [20]. Curcumin and other anticancer compounds are also effective in reducing the activities of inflammatory cytokines and chemokines as well as in influencing the trafficking of leukocytes that participate in the inflammatory process.

In the remaining portion of this chapter, we summarize some of the known molecular mechanisms by which naturally occurring compounds, foods, and food extracts elicit anti-inflammatory effects. We refer to whole foods and food extracts as "mixtures" of bioactive compounds. Mechanisms not discussed in this chapter include the effects of naturally occurring compounds on inflammation-associated phase II enzyme induction and angiogenesis; these mechanisms will be discussed elsewhere in this series.

13.2 Inflammation-Related Molecular Targets of Natural Products

13.2.1 Transcription Activators

Transcription activators (factors) are proteins that bind to specific DNA sequences, thereby controlling the transcription of genetic information from DNA to mRNA. Transcription factors perform this function alone or with other proteins in a complex by promoting or blocking the recruitment of RNA polymerase to specific genes. Abnormal expression of these factors can lead to many of the hallmarks of cancer including increased proliferation, reduced apoptosis, malignant conversion, inflammation, angiogenesis, tissue invasion, and metastasis. A large number of transcription factors have been identified in the past few decades and several of these are associated with inflammatory processes and cancer [1, 20]. The following section describes two of the most investigated transcription factors in inflammation and tumor development and mechanisms by which they are influenced by dietary agents. For a more complete discussion on this topic, the reader is referred to the review of Aggarwal and Shishodia [20].

13.2.1.1 Nuclear Factor-Kappa B

NF-κB is a protein complex that is present in nearly all animal cell types [21, 22]. It is important in regulating cellular responses to various stimuli because it belongs to a family of "rapid acting" primary transcription factors, i.e., transcription factors present in cells in an inactive state that do not require new protein synthesis to be activated. Known inducers of NF-κB activity include reactive oxygen species, inflammatory factors such as tumor necrosis factor-alpha (TNFα) and interleukin 1-beta (IL-1β), bacterial endotoxins, chemical carcinogens and toxins, ultraviolet light, and X-rays [20]. Activation of the NF-κB complex is initiated by signal-induced degradation of I kappa B (IκB) proteins. This occurs primarily through activation of a kinase termed IKK. With the degradation of IκB, the NF-κB complex translocates from the cytoplasm to the nucleus where, ultimately, it induces the expression of an ever-increasing number of target genes, including genes that suppress apoptosis (Bcl-2 and Bcl-XL), induce proliferation (cyclin D1, c-myc) and angiogenesis (vascular endothelial growth factor [VEGF]), and promote tissue invasion and metastasis (matrix metalloproteases [MMP]) and inflammation (COX-2, 5-LOX, inducible nitric oxide synthase [iNOS], TNF-α, NADPH oxidase [NOX], and others). Many of these activated target genes are crucial to the development of cancer at multiple tissue sites.

Because of its central role in the control of several cancer-associated cellular functions, numerous investigations have been conducted to identify dietary inhibitors of NF-κB [20]. Among those identified include resveratrol [21], curcumin [22], ursolic acid [23], lycopene [24], apigenin [25], silymarin [26], ellagic acid [27], cyanidin-3-glucoside [28], caffeic acid phenethyl ester (CAPE) [29], epigallocatechin-3-gallate (EGCG) [30], [6]-gingerol [31], S-allyl cysteine [32], flavopiridol [33], benzyl and phenylethyl isothiocyanates [34, 35], indole-3-carbinol [36], sulforaphane [37], quercetin [38], anethole [39], and alpha-lipoic acid [40]. In addition to pure compounds, various "mixtures" of naturally occurring compounds, including soy isoflavones (genistein, daidzein, and glycitein) [41], black tea extract (BTE) [42], lyophilized whole black raspberries (BRBs) and strawberries [43, 44], anthocyanin extracts of black raspberries [43], ginger extract [45], grape seed proanthocyanidins [46, 47], and cranberry proanthocyanidins [48, 49], have been shown to exhibit inhibitory effects on NF-κB activation in vitro and in vivo.

Aggarwal and Shishodia [20] discussed mechanisms by which naturally occurring compounds inhibit NF-κB activation. These include the blocking of one or more steps in the NF-κB signaling pathway, the translocation of NF-κB into the nucleus, binding of the NF-κB dimers to DNA, or interactions with the basal transcriptional machinery. Caffeine, for example, is a potent inhibitor of ultraviolet (UV)-induced NF-κB activation by its inhibitory action on protein kinase C (PKC), resulting in the inhibition of p38-induced activation of the MAPK pathway [50]. Resveratrol suppresses tumor necrosis factor (TNF)-induced phosphorylation and nuclear translocation of the p65 subunit of NF-κB and NF-κB reporter gene transcription [21]. Diosgenin suppresses TNF-induced activation of IKK leading to inhibition of TNF-dependent phosphorylation and degradation of IκBα and nuclear

translocation of the p65 subunit of NF-κB [51]. CAPE suppresses NF-κB activation by interfering with the binding of the p50-p65 complex to DNA [29]. BTE inhibits the nuclear translocation of NF-κB/p65 and the DNA-binding activity of NF-κB [42]. Cranberry proanthocyanidins inhibit the production of MMP by reducing the phosphorylation of at least five intracellular kinases and NF-κB p65 activity [48]. These represent only a few mechanisms by which dietary factors inhibit NFκB activation, and ultimately, these actions reduce the expression of numerous NF-κB-related proteins such as COX-2, iNOS, TNF, and various cytokines and chemokines associated with inflammation.

13.2.1.2 Activator Protein-1

Activator protein-1 (AP-1) is a transcriptional regulator composed of members of the Fos and Jun families of DNA-binding proteins. AP-1 regulates gene expression in response to a variety of stimuli including growth factors, cytokines, environmental stresses such as UV radiation, and bacterial and viral infections. AP-1 activation is linked to increased cell proliferation, cell transformation, innate immune response, and inflammation. AP-1 appears to enhance cell proliferation by activating cyclin D1 and repressing suppressor genes such as p53, p16, and p21cip1/waf1 [52]. Importantly, AP-1 promotes the transition of tumor cells from an epithelial to a mesenchymal morphology which is one of the early steps in metastasis. Both AP-1 and NF-κB are inducible by many of the same stimuli, and in mouse epidermal JB-6 cells, both are required for maintaining the transformed phenotype [53]. With respect to inflammation, many of the cytokine genes and other factors that drive inflammation are regulated cooperatively by a transcription factor complex consisting of AP-1 and nuclear factor of activated T cells (NFAT). AP-1/NFAT-dependent gene regulation has been demonstrated for the proinflammatory cytokines: interleukins 1, 2, and 6 (IL-1, IL-2, IL-6), TNFα, TNFβ, interferon gamma (IFNγ), Fas ligand (FasL), CD40 ligand (CD40L), and granulocyte-macrophage colony-stimulating factor (GM-CSF) [54]. Overexpression of AP-1, therefore, contributes significantly to the inflammatory process.

Several dietary factors have been shown to suppress AP-1 activation and function. These include resveratrol [55], curcumin [56], EGCG from green tea [57], capsaicin [58], oleandrin [59], alpha-mangostin [60], theaflavin-3,3′-digallate [61], anthocyanidins (delphinidin, petunidin, cyanidin) [62], and chlorogenic acid [63]. Mixtures of naturally occurring compounds that inhibit AP-1 include blackberry extracts [64], whole lyophilized BRBs [65] and BRB extracts [66], and apple peel extract [67]. As for NF-κB, the mechanisms though which these agents influence AP-1 activation and function are varied. Resveratrol inhibited 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and UV-induced AP-1 activation in cultured HeLa cells by diminishing PKC and various protein tyrosine kinases upstream of the MAPK pathway [55]. Delphinidin and blackberry extracts inhibited TPA- or UV-induced activation of AP-1 in JB-6 mouse epidermal cells by reducing phosphorylation of protein kinases in the JNK and extracellular signal-related protein kinase (ERK) pathways [64].

EGCG and theaflavin-3,3-digallate inhibited TPA-induced transformation of JB-6 mouse epidermal cells and NIH3T3 cells, respectively, by inhibiting TPA-induced PKC and by reducing the binding of AP-1 to DNA [57, 61]. The remaining dietary compounds and mixtures influence the activation and functions of AP-1 in similar ways leading to reduced cell proliferation, inflammation, and the risk for cancer development.

13.2.2 Tumor Necrosis Factor

TNF, originally discovered in 1968 by Kolb and Granger [68], is a key inflammatory cytokine produced chiefly by activated macrophages and monocytes. The primary role of TNF is the regulation of different immune cell types. A local increase in TNF in tissues will cause the cardinal signs of inflammation to occur, i.e., heat, edema, redness, pain, and loss of function. Dysregulation of TNF has been implicated in a variety of human diseases including inflammatory bowel disease [69] and cancer [70]. The induction of proinflammatory genes by TNF is primarily due to the ability of TNF to activate NF-κB in multiple different cell types [20]. As stated above, NF-κB activation leads to the expression of inflammation-associated COX-2, iNOS, and various chemokines and cytokines. TNF promotes cell proliferation, in part, by activation of JNK and p38 genes in the MAPK cascade, thereby serving as a growth factor for multiple tumor types.

Due to its pivotal role in inflammation and in mediating tumor development, there has been a vigorous search for agents that suppress TNF activity. Some of the known dietary inhibitors of TNF production act by inhibiting NF-κB activation and the subsequent release of TNF from cells. These include resveratrol [71, 72], quercetin [71, 72], EGCG [73], curcumin [74], capsaicin [75], and nordihydroguaiaretic acid [76]. Other dietary compounds inhibit TNF production from monocytes and macrophages [77]. Among these are the terpenoid, cynaropicrin [78], silymarin [79], epicatechin [80], narginin [81], and lignans [82], to name a few. These agents function by either inhibiting the expression of a series of kinase enzymes or exhibiting inhibitory effects on cyclic AMP. Several long-chain fatty acids, including docosahexaenoic acid (DHA) and eicosahexaenoic acid (EHA) in fish oil, have been shown to suppress the serum level of TNF in vivo [83]. Numerous dietary phenols have been shown to inhibit TNF-induced cytotoxicity in vitro. For example, a series of catechols were found to be effective in reducing TNF-induced cytotoxicity, presumably due to their iron-chelating activity and subsequent inhibition of lipoxygenase enzymes [84]. The flavonols, kaempherol, quercetin, myricetin, morin, and rutin also inhibit TNF cytotoxicity, and the C-3 free hydroxyl group on these compounds appears to play a pivotal role in their protective effect. In contrast to the protective effect of flavonols, no protective activity was found for the flavones (chrysin, apigenin, and luteolin), and in fact, apigenin and chrysin which possess one (C-4′) hydroxyl group or none on the B-ring, respectively, enhanced TNF cytotoxicity [85]. These results indicate that relatively minor changes in the structure of phenolic

compounds can have a profound effect on their ability to influence TNF cytotoxicity, an important consideration in choosing agents for clinical trials.

In summary, several target sites have been identified for affecting TNF production and the ensuing inflammation associated with TNF. Of these targets, NF-κB is the most interesting as it regulates TNF production and, in turn, TNF regulates NF-κB reciprocally to produce its biological effects. There are very few natural products that inhibit TNF at the posttranslational level. For a more extensive discussion of natural inhibitors of TNF production, secretion, and function, the reader is referred to the review by Habtemariam [77].

13.2.3 Cyclooxygenase-2

Cyclooxygenase (*COX*), or prostaglandin H synthase, is an enzyme responsible for the conversion of arachidonic acid (AA) into the prostanoids: prostaglandins, prostacyclin, and thromboxane [86]. Overproduction of prostanoids can result in increased cell proliferation, inflammation, and angiogenesis and reduced apoptosis. There are two major isoforms of *COX*: COX-1 and COX-2. COX-1 is a constitutive enzyme found in most mammalian cells, whereas COX-2 is undetectable in most normal tissues. COX-2 can be induced in tissues by various toxins and carcinogens, tumor promoters, proinflammatory cytokines including TNF, and growth factors and is found in abundance in activated macrophages and in other cells at sites of inflammation. Depending upon the inducer and the cell type, several transcription factors including AP-1, NF-κB, and STAT-3 are capable of stimulating COX-2 transcription [87, 88]. Importantly, COX-2 is overexpressed in nearly all premalignant lesions and tumors of the skin, head and neck, esophagus, stomach, colon, breast, lung, pancreas, liver, prostate, bladder, cervix, and uterus [20]. In view of its ubiquitous presence in premalignant lesions and in tumors of many types, COX-2 is an important target for cancer chemoprevention and therapy.

As might be expected, the dietary factors mentioned above that suppress the expression of NF-κB and AP-1 have the potential of inhibiting COX-2 expression. A partial list of these includes luteolin [89], resveratrol [90], curcumin [91], genistein [92], and EGCG [93]. In addition, several studies have demonstrated the ability of mixtures of naturally occurring compounds to suppress the expression of COX-2. Our laboratory observed the ability of diets containing 5 % and 10 % lyophilized BRBs to suppress the expression of NF-κB in carcinogen-treated rat esophagus, and this correlated with inhibition of COX-2 expression, reduced prostaglandin E_2 levels in esophageal tissues, and about a 55 % reduction in tumorigenesis [43]. Montrose et al. [94] evaluated the effects of dietary intervention of 10 % lyophilized BRBs on disease severity in an experimental mouse model of ulcerative colitis. C57BL/6J mice were administered with 3 % dextran sodium sulfate (DSS) in drinking water for 7 days along with either control diet or a 10 % lyophilized BRB diet. Dietary BRBs reduced DSS-induced injury to the colonic epithelium and this was associated with a significant reduction in COX-2 protein levels in the colon and PGE_2

levels in plasma. BRB treatment also suppressed mRNA levels of the proinflammatory cytokines TNF-α and IL-1β in colon tissues. These effects on COX-2, TNF-α, and IL-1β were correlated with reduced mRNA levels of phosphorylated IκBα (PIκBα) in the colon which might be expected to result in an inhibition of NF-κB activity. Other mixtures of naturally occurring compounds that have been shown to suppress the expression of COX-2 include cranberry and blueberry extracts [95, 96], pomegranate juice [97], green tea extracts [98], grape seed proanthocyanidins [99], and soy isoflavones [100]. Because these dietary compounds and mixtures are predominately polyphenols, it is likely that their antioxidant effects play a significant role in their ability to inhibit COX-2 expression and COX-2-associated inflammation.

Mixtures of naturally occurring compounds have also been shown to suppress COX-2 expression in human tissues in vivo. In a phase 1b clinical trial, Shumway et al. [101] observed that the topical application of a 10 % black raspberry gel to oral dysplastic lesions (0.5 g 4 times a day for 6 weeks) resulted in an approximate 41 % reduction in histopathologic grade of the lesions and about a 50 % reduction in loss of heterozygosity (LOH) at three tumor suppressor gene loci. In a companion article, Mallery et al. [102] reported significant reductions in COX-2 and iNOS protein levels in the surface epithelium of the berry gel-treated oral lesions by quantitative immunohistochemistry, but only the reduction in COX-2 was significant. More recently, Chen et al. [44] reported that the oral administration of lyophilized strawberries (60 g total/day) in a slurry of water to Chinese subjects at high risk for the development of esophageal squamous cell carcinoma reduced the histologic grade of dysplastic esophageal lesions in 29 (80.6 %) ($p<0.0001$) of 36 subjects. This observation was associated with reduced protein expression levels of iNOS by 79.5 % ($p<0.001$), COX-2 by 62.9 % ($p<0.001$), pNF-κB-p65 by 62.6 % ($p<0.001$), and phospho-S6 (pS6) by 73.2 %. Measurement of pS6 by Western blot is used to assess the activity of the mammalian target of rapamycin (mTOR) gene which plays a central role in cell proliferation [103]. For a more extensive discussion of the mechanisms of inhibition of COX-2 by naturally occurring compounds, the reader is referred to the review of Aggarwal and Shishodia [20].

13.2.4 Lipoxygenase

Lipoxygenases (LOX) are the key enzymes for the conversion of AA to biologically active leukotrienes. There are three types of LOX: 5-LOX, 12-LOX, and 15-LOX. Several studies suggest a link between 5-LOX and cancer development in animals and humans. 5-LOX catalyzes the oxidation of AA at the 5 position to yield 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and then converts 5-HPETE to leukotriene A_4 (LTA_4). Depending upon the cell type, LTA_4 is converted to a series of additional leukotrienes which are released from leukocytes and mast cells to provoke inflammation. LTB_4, in particular, has potent chemokinetic and chemotactic activity towards leukocytes and is considered a potent mediator of inflammation. Both of the AA metabolizing enzymes, COX-2 and 5-LOX, are commonly overexpressed in

tumors, and blocking both enzymes simultaneously is considered to be a promising approach to treat inflammatory diseases including cancer.

The plant kingdom is a valuable source for 5-LOX and dual 5-LOX/COX inhibitors. Importantly, natural inhibitors of 5-LOX also tend to be effective in reducing the expression levels of COX. Schneider and Bucar [104] provide a list of over 180 different plant extracts and natural compounds isolated from different plant species that exhibit inhibitory effects against 5-LOX and/or 5-LOX/COX in cultured leukocytes and macrophages. Some of the more commonly known inhibitors were found to be linoleic, oleic, and palmitic acids; silibinin; β-sitosterol; luteolin; quercetin; rosmarinic acid; allicin; resveratrol; ferulic acid; EGCG; gingerol; and curcumin. The IC_{50} values for most of these compounds were between 1 and 50 μM although several were effective at doses below 1 μM. Thus, it is apparent that many of these inhibitors are likely to exhibit inhibitory effects in vivo at doses that are achievable pharmacologically.

13.2.5 Inducible Nitric Oxide Synthase

Nitric oxide synthases are a family of three enzymes that catalyze the production of nitric oxide (NO) from L-arginine. NO plays an important role as a cellular signaling molecule as well as a cytotoxic or regulatory molecule of the innate immune response [105]. NO is synthesized for short periods of time (seconds to minutes) following activation of constitutively expressed endothelial NO synthase (eNOS) or neuronal NO synthase (nNOS). In contrast, inducible NO synthase (iNOS) is expressed after cell activation only and it produces NO for relatively long periods of time (hours to days). The principal inducers of iNOS in many different cell types are the proinflammatory cytokines, TNF-α, IL-1β, and IFN-γ, and lipopolysaccharides (LPS) [106], and the expression of iNOS is dependent, in part, upon activation of the NF-κB signaling pathway and the Janus kinase/signal transducer and activator of transcription (JAK-STAT) pathway [107]. iNOS-derived NO plays a critical role in the pathophysiology of several human diseases including Crohn's disease, ulcerative colitis and cancer. iNOS is expressed in a variety of human tumor types including esophagus, lung, breast, bladder, prostate, colorectal, and melanoma [20].

Several investigations have been conducted to identify naturally occurring inhibitors of iNOS expression in vitro and in vivo. Among the individual compounds that have been shown to inhibit iNOS expression in vitro are the polyphenols, EGCG [108], [6]-gingerol [109], resveratrol [110], curcumin [111], indole-3-carbinol [112], quercetin and rutin [113], and chlorogenic acid [114]. This inhibition most likely occurs through the suppression of NF-κB and/or other signaling pathways (JAK-STAT, MAPK, etc.). For example, Lin and Lin [108] reported that EGCG and other tea polyphenols inhibited iNOS mRNA and protein expression in LPS-activated murine macrophages through suppression of the binding of NF-κB to the iNOS promoter, thereby inhibiting the induction of iNOS. Resveratrol was found to inhibit iNOS expression in beta-amyloid-treated C6 glioma cells by downregulation of

NF-κB [110]. Tedeschi et al. [115] reported that green tea inhibits cytokine-induced iNOS expression in human lung adenocarcinoma A549 cells and human colon DLD-1 cells by downregulation of the DNA-binding activity of STAT-1α. Some of these compounds are also effective in inhibiting iNOS expression in vivo. For example, chlorogenic acid protected mice against LPS-induced lung injury by markedly downregulating iNOS expression, myeloperoxidase activity, and the migration of polymorphonuclear neutrophils into bronchiolar lavage fluid [114].

Numerous mixtures of naturally occurring compounds have also been shown to be effective in downregulating the expression of iNOS, usually in combination with COX-2. Kim et al. [116] evaluated a series of plant extracts for their ability to inhibit NO generation in murine macrophages stimulated with LPS and INF-α. Extracts from avocado, basil, Chinese mustard, mitsuba, and red turnip were particularly effective in inhibiting NO production. The roots of *Rhododendron mucronulatum* (RM) have been used in Asian traditional medicine for the treatment of dysuria and fever for centuries [117]. An alcohol extract of RM, found to contain numerous polyphenolic compounds, was highly effective in inhibiting protein levels of iNOS and COX-2 in LPS-stimulated HaCaT cells. Chitosomes (liposomes in which chitosan is bound to soy lecithin) "loaded with" cranberry proanthocyanidins were found to be effective in inhibiting iNOS and COX-2 expression in murine macrophages activated with LPS [118]. Anthocyanin-rich extracts from the acai berry were found to be potent inhibitors of iNOS and COX-2 expression in mouse brain microglial cells, and this correlated with reductions in p38-MAPK, TNF-α, and NF-κB [119]. Our laboratory observed an inhibition of iNOS mRNA and protein expression in carcinogen-treated rat esophageal tissues by diets containing either 5 % whole BRB powder or an anthocyanin-rich fraction of BRBs [43, 65]. These effects of BRBs and their component anthocyanins were correlated with downregulation of NF-κB and COX-2. We stated above that the oral administration of strawberry powder (60 g/day) in a slurry of water for 6 months to Chinese subjects at high risk for esophageal squamous cell carcinoma resulted in about an 80 % regression of mildly dysplastic lesions [44]. This observation was associated with significant reductions in protein expression levels of iNOS and COX-2 which may have been due to downregulation of the phosphorylated form of NF-κB-p65. Collectively, these observations indicate that iNOS is an important target for dietary intervention in the treatment of inflammation and in the prevention of cancer.

13.2.6 Proinflammatory Cytokines

The tumor microenvironment contains innate immune cells (including macrophages, neutrophils, mast cells, myeloid-derived suppressor cells, dendritic cells, and natural killer cells) and adaptive immune cells (T and B lymphocytes) in addition to the cancer cells and their surrounding stroma. These cells communicate with each other by direct contact or by cytokine and chemokine production that act in autocrine and paracrine manners to control tumor growth. The most abundant immune cells within

the tumor microenvironment are the tumor-associated macrophages (TAMs) and T lymphocytes. TAMs are one of the most important players in inflammation and cancer as they promote tumor growth and are obligatory for angiogenesis, invasion, and metastasis [120]. TAMs are classified into M1 and M2 types, analogous to Th1 and Th2 T lymphocytes. M1 macrophages, when activated by various stimuli such as INFγ and LPS, express high levels of the proinflammatory cytokines TNF-α, IL-1β, IL-6, IL-12, and IL-23 as well as iNOS [1]. In contrast, M2 macrophages, induced by IL-4, IL-10, and IL-13, downregulate IL-12 expression and show increased expression of the anti-inflammatory cytokine, IL-10. The tumor-promoting cytokines are the M1 cytokines (TNF-α, IL-1β, IL-6, IL-12, or IL-23), whereas IL-10, the M2 cytokine, has been shown to be tumor suppressive [121].

The proinflammatory cytokines activate various transcription factors such as NF-κB, AP-1, and STAT3 in premalignant cells to induce genes that stimulate cell proliferation, survival, and tumor development. As discussed above, TNF-α activates both NF-κB and AP-1 transcription factors, but in the skin, its tumor-promoting effects are mediated by AP-1 [122]. STAT3 activation in cancer cells is dependent upon a number of growth factors and cytokines, including IL-6, IL-11, and epidermal growth factor (EGF) [123]. The development of specific tumor types tends to be associated with individual cytokines [2]. For example, autocrine production of IL-1β promotes growth of pancreatic carcinoma cell lines [124]. IL-6 acts as a paracrine growth factor for colorectal cancer, non-Hodgkin's lymphoma, renal cell carcinoma, and bladder cancer [125–128]. IL-8 has been detected in multiple cancer types and its expression in human melanomas and ovarian cancers correlates with their metastatic potential [129].

Given the importance of the proinflammatory cytokines in the various stages of tumor development, numerous studies have been conducted to identify naturally occurring compounds and mixtures that inhibit cytokine expression. Typically, cytokine production is induced in cultured cells of various types by treatment with TNF-α, INFγ, LPS, or other inducers, and the individual compounds or mixtures are evaluated for their ability to reduce cytokine expression. Among the numerous compounds that downregulate IL-6 expression in vitro include resveratrol [130], curcumin [131], ellagic acid [132], genistein [133], lycopene [134], capsaicin [135], and EGCG [136]. To varying extents, these compounds also downregulate the expression of IL-12 and IL-1α or IL-1β in cultured cells. Downregulation of these cytokines is often associated with inhibition of MAPKs such as ERK1/2, p38, or JNK as well as the transcription factors, NF-κB, AP-1, or STAT3.

In vivo, green tea polyphenols (GTP) have been tested for inhibition of TPA-induced cytokine expression in mouse skin and in transgenic mouse models that overexpress specific cytokines [136, 137]. Topical application of GTP to the skin of mice previously treated with TPA resulted in reduced expression of TPA-induced IL1-α in the skin [136]. Administration of GTP in the drinking water to TNF-α transgenic mice which are susceptible to idiopathic pulmonary fibrosis led to reduced expressions of TNF-α and IL-6 [137]. In addition to GTP, other mixtures shown to inhibit cytokine expression include grape seed proanthocyanidins, an ethanol extract of pomegranate, tart cherry anthocyanins, and black tea theaflavins.

Grape seed proanthocyanidins inhibited IL-17-induced IL-6 production in cultured human pulmonary epithelial cells by reducing the expressions of MAPK and NF-κB [138]. Pomegranate extract inhibited TNF-α induced production of IL-6 and NO in cultured human osteoblasts [139]. Tart cherry anthocyanins showed an additive effect along with atorvastatin in inhibiting LPS-induced IL-6 production by adipose stem cells [140]. Black tea theaflavins inhibited TPA-induced inflammation in the ears of CD-1 mice, and this correlated with reduced levels of IL-1β, IL-6, PGE_2, and leukotriene B_4 suggesting that the anti-inflammatory activity of the theaflavins may have been due to their ability to inhibit AA metabolism via lipoxygenase and COX pathways [141]. These results suggest that inflammatory cytokines are desirable targets for the anti-inflammatory effects of naturally occurring compounds and mixtures. In addition, cytokines may be excellent biomarkers of effect when measured both in tissues and in serum/plasma.

13.3 Summary and Conclusions

Inflammation is a very complex process, initiated by a host of injurious factors, and characterized by leukocyte infiltration into the affected tissues and the aberrant expression of multiple genes that drive the inflammatory process. The conventional wisdom is that the injurious agents induce one or more transcription activators (e.g., Nf-κB, AP-1, STAT3) and these activators, in turn, cause the overexpression of inflammatory enzymes (e.g., COX-2, iNOS, 5-LOX), cytokines, and chemokines (e.g., TNF, IL-1β, IL-6, IL-12) (Fig. 13.1). Cytokines and chemokines then chemo-attract additional inflammatory leukocytes into the tissues leading ultimately to a chronic state of inflammation and persistent tissue damage. One of the

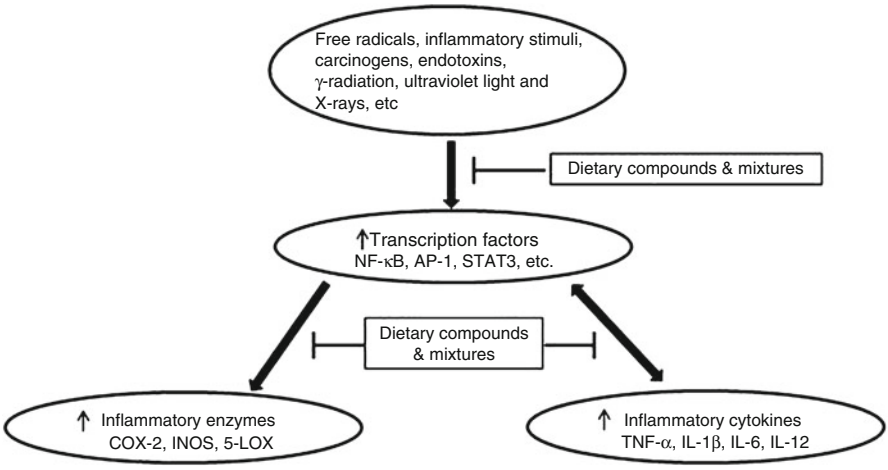

Fig. 13.1 Scheme for effects of dietary factors on inflammation-related molecular targets

outcomes of chronic inflammation is cancer, and indeed, inflammation is associated with essentially all stages of tumor development, i.e., initiation, promotion, malignant conversion, angiogenesis, invasion, and metastasis. Given this, there has been an active search in the past few decades for both synthetic and naturally occurring inhibitors of the inflammatory process to reduce the risk for cancer development.

This chapter lists individual dietary compounds that influence molecular targets involved in the inflammatory process. It is clear that some of the most effective dietary inhibitors of carcinogenesis (e.g., resveratrol, curcumin, EGCG, isothiocyanates, triterpenoids, silymarin, [6]-gingerol) positively influence multiple molecular targets associated with inflammation, providing additional evidence of the close relationship between inflammation and carcinogenesis. The wide-ranging effects of these inhibitors on inflammation targets may be due, at least in part, to the fact that many of them are polyphenols that exhibit antioxidant activity and might be expected to inhibit multiple RONS-driven signaling pathways. Interestingly, most of these agents are not well absorbed and, as such, can be consumed for long periods without producing harmful side effects. In many cases, it is not clear whether the parent compounds themselves or one or more of their metabolites are responsible for the observed biological effects. In that regard, it is important to determine the role, if any, of the enteric microbiome in the metabolism of these compounds. It is also essential to examine the pharmacokinetics of uptake and distribution of these compounds into blood and tissues to provide clues as to how they might be formulated for optimal delivery to target tissues. Finally, studies should be undertaken to determine if the compounds themselves, or their metabolites, interact directly with promoter sequences of inflammatory genes to influence gene expression and/or bind directly to inflammatory proteins (e.g., COX-2, iNOS,5-LOX, TNF, IL-6) to influence their activities.

Whole lyophilized foods and food extracts also exhibit a wide range of inhibitory effects on the expression of inflammation-associated genes. The removal of water from foods by lyophilization and the grinding of dried foods into a powder can result in a significant concentration of their bioactive constituents. For example, we reported that the active constituents in black raspberries are concentrated tenfold by the lyophilization process and that BRB powder, in different formulations, is chemopreventive for oral, esophageal, and colon cancers in rodents and in humans [43, 44, 94, 101, 102, 142, 143]. The inhibitory effects of berry powder on inflammation-associated genes are an important component of the overall chemopreventive effects of BRBs in rodents [143] and, likely, in humans. For example, we reported that the oral administration of BRB powder at a total dose of 60 g/day to colon cancer patients for periods of 1–9 weeks resulted in reduced plasma levels of GM-CSF and IL-8 [144]. Similarly, in obese individuals given a high-fat, high-caloric meal, the oral administration of 45 g/day of BRB powder for 4 days resulted in a significant reduction in plasma levels of IL-6 [145]. It is likely that the other foods and food extracts mentioned in this chapter have similar effects on inflammatory biomarkers suggesting that the routine consumption of anti-inflammatory agents in the human diet in the form of food concentrates and extracts could well reduce the overall risk of humans to cancer.

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Chapter 14
Calorie Restriction and Cancer Prevention: Established and Emerging Mechanisms

Stephen D. Hursting, Nikki A. Ford, Sarah M. Dunlap, Marcie J. Hursting, and Laura M. Lashinger

Abstract Calorie restriction (CR) is one of the most potent, broadly acting dietary interventions for inducing weight loss and for inhibiting cancer in experimental models. Translation of the mechanistic lessons learned from research on CR to cancer prevention strategies in humans is important given the high prevalence of excess energy intake, obesity, and metabolic syndrome in many parts of the world and also given the established links between obesity-associated metabolic perturbations and increased risk and/or progression of many types of cancers. This chapter synthesizes findings on the biological mechanisms underlying many of the anticancer effects of CR, with emphasis on the role of inflammatory processes and growth factor signaling (well-established mechanisms) as well as vascular perturbations, autophagy, and sirtuins (emerging mechanisms). These CR-responsive pathways and processes represent targets for translating CR research into effective cancer prevention strategies in humans.

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14.1 Introduction

Calorie restriction (CR), a dietary regimen in which subjects (typically test animals) receive a reduced energy diet (typically 20–40 % reduction in total energy intake relative to an unrestricted comparison group), is one of the most potent and broadly acting dietary interventions for preventing or reversing weight gain and inhibiting cancer in experimental tumor models [1]. Recent reports of decreased risk of diabetes and cancer in response to CR in rhesus monkeys [2, 3], and observations that CR decreases inflammatory and endocrine markers associated with increased breast cancer risk in women [4–6], suggest that the beneficial effects of CR on metabolism and chronic disease risk observed in rodent models may extend to nonhuman primates and humans.

Observational epidemiologic studies provide further evidence that CR exerts beneficial effects on longevity and cancer risk in humans [1]. For example, inhabitants of Okinawa, Japan, have for centuries consumed significantly fewer calories than residents of the main Japanese islands and have always had lower death rates from cancer and other chronic diseases than inhabitants of the Japanese mainland [7]. It will be interesting to see if the recent westernization of the diet of Okinawans results in increased cancer rates in future. Another example involves patients with early-onset anorexia nervosa and hence periods of energy restriction; these patients have reduced risk of breast cancer [8]. Furthermore, surveillance data from Norwegian women during World War II showed reduced breast cancer risk later in life in association with acute (<1 year) energy restriction (~50 % reduction in calorie intake without significant changes in diet quality) [9]. However, populations with more severe restriction than experienced in Norway, such as survivors of the 1944 Dutch "Hunger Winter," the Jewish Holocaust, and the Siege of Leningrad, actually displayed higher breast cancer rates [10–12]. This indicates a possible threshold beyond which energy restriction (especially when combined with other stressors) may be cancer promoting. This is particularly true for those born around the time of the severe deprivation and stress, suggesting an important perinatal window of susceptibility to metabolic reprogramming in response to energy restriction [13].

These stressful conditions, particularly the confounding effects of severe physical and psychosocial stress, malnutrition, infection, and other factors associated with war conditions in severely affected countries, make many of these wartime surveillance studies a challenge to interpret. These conditions are also in contrast to the controlled conditions characteristic of most CR studies in animal models that consistently show anticancer effects. CR regimens are often referred to as "CR with optimal nutrition" or "undernutrition without malnutrition," and CR experiments typically involve 20–40 % reductions in total energy relative to ad libitum-fed controls but with adequate nutrition and a controlled physical environment [1]. In rodent models, CR regimens administered throughout life are generally more effective against cancer than CR regimens initiated in adulthood, although both early-onset and adult-onset CR, relative to control diet regimens, are protective against a variety of cancer types [1]. In rhesus monkey studies, CR begun in young adults is more protective against cancer than CR initiated later in life [3].

There are several National Institute of Aging-funded clinical trials underway to address the question of whether the observed health benefits of CR in rodents and nonhuman primates translate to humans. One of these trials, called the Comprehensive Assessment of Long-Term Effects of Reducing Intake of Energy (CALERIE) Study, is evaluating the effects of a 2-year CR regimen (25 % less energy than controls) in healthy, nonobese individuals. Preliminary reports on CALERIE indicate that many of the same metabolic and endocrine changes observed in rodents and monkeys are also occurring in humans in response to CR [14, 15]. These findings are consistent with recent studies in women at high risk for breast cancer showing that inflammatory and growth factor signaling pathways are reduced by total CR or 2 days/week of restricted carbohydrate calories [4–6]. The observed metabolic effects of 2-days/week of restricted carbohydrate calories [4] are of particular interest, since it is likely easier and more sustainable for most people to periodically restrict a single macronutrient, such as carbohydrates, than to chronically restrict total energy.

In this chapter we discuss possible mechanisms underlying the anticancer effects of CR, with emphasis on CR-associated changes in inflammation and growth factor signaling, as well as emerging evidence suggesting that vascular perturbations (including angiogenesis) and modulation of sirtuin and autophagy pathways may also play roles in the effects of CR on tumor development and progression. As summarized in Fig. 14.1, we specifically describe the impact of CR on (a) macrophages, cytokines, and other inflammatory mediators; (b) growth factors, including insulin, insulin-like growth factor (IGF)-1, adipokines, and their downstream signaling pathways; (c) vascular integrity factors, including angiogenic regulators; (d) autophagy regulators; and (e) sirtuin pathway components. We discuss how these multifactorial CR-induced changes combine to suppress tumor development and/or progression. Components of these interrelated pathways offer possible mechanism-based targets for the prevention and control of cancers, particularly the estimated 20–25 % [16] of human cancers related to, or caused by, excess body weight and lack of physical activity.

14.2 Calorie Restriction Decreases Chronic Inflammation

Chronic inflammation is characterized by increased circulating cytokines and chemokines that attract immune cells (such as macrophages that also produce inflammatory mediators) into the local microenvironment [17–19]. The inflammatory cascade is further amplified by the release of inflammatory cytokines such as interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and monocyte chemoattractant protein (MCP)-1, primarily from macrophages, into the local and systemic circulation. Adipocytes can enlarge past the point of effective oxygen diffusion, which results in hypoxia and eventually necrosis. Free fatty acids escape the engorged/necrotic adipocytes and deposit in other tissues, which in turn promotes insulin resistance, diabetes (through downregulation of insulin receptors and glucose transporters), hepatic steatosis, and pancreatic steatosis, and also activates signaling molecules involved in

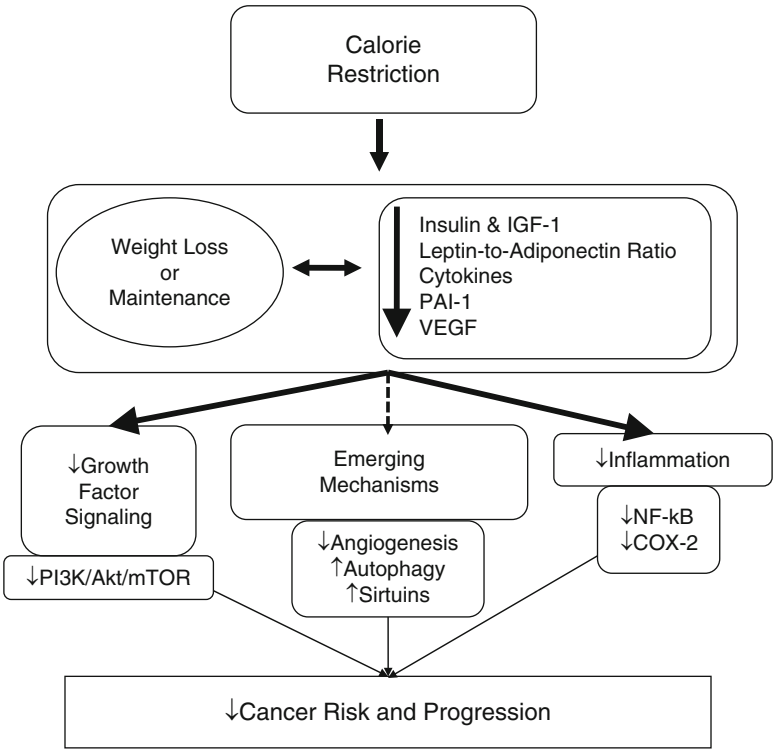

Fig. 14.1 Calorie restriction and cancer prevention: summary of established and emerging mechanisms. An *arrow* preceding text denotes a directional effect (e.g., activity or concentration). *Solid arrow* indicates established effects; *dashed arrows* indicate emerging mechanisms requiring additional study. *IGF-1* insulin-like growth factor-1; *PAI-1* plasminogen activator inhibitor-1; *VEGF* vascular endothelial growth factor; *PI3K* phosphoinositide 3-kinase; *NF-κB* nuclear factor kappa-light-chain-enhancer of activated B-cells; *COX-2* cyclooxygenase-2

epithelial carcinogenesis, such as nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) and cyclooxygenase (COX)-2 [20]. NF-κB is a transcription factor that is activated in response to bacterial and viral stimuli, growth factors, and inflammatory molecules (e.g., TNF-α, IL-6, and IL-1β) and is responsible for inducing gene expression associated with cell proliferation, apoptosis, inflammation, metastasis, and angiogenesis. Activation of NF-κB is a common characteristic of many tumors and is associated with insulin resistance and elevated circulating levels of leptin, insulin, and/or IGF-1 [17, 21, 22].

A connection between chronic inflammation and cancer development was observed 150 years ago when Rudolph Virchow noted an abundance of leukocytes in neoplastic tissue [23]. Inflammation is now considered a hallmark of cancer, and the evidence is accumulating that chronic, "smoldering" inflammation is associated with increased cancer risk [24–26]. Indeed, several tissue-specific inflammatory lesions are established neoplastic precursors for invasive cancer, including

inflammatory bowel disease for colon cancer, pancreatitis for pancreatic cancer, dermatitis for certain forms of skin cancer, prostatitis for prostate cancer, and gastritis for gastric cancer [27, 28]. Tumor and preneoplastic microenvironments are composed of multiple cell types including epithelial cells, fibroblasts, mast cells, and cells of the innate and adaptive immune system [29]. As discussed previously, macrophages, which are activated in the obese state, infiltrate tumors and amplify the inflammatory tumor microenvironment, often through NF-κB-dependent production of cytokines and angiogenic factors [29]. COX-2 is another important cancer-related inflammatory mediator that is upregulated in most tumors and catalyzes the synthesis of the potent inflammatory lipid metabolite, prostaglandin E_2. COX-2 expression, an indicator of poor prognosis in multiple cancer types, is increased in response to obesity [30].

CR can prevent much of the chronic inflammation associated with preneoplasia or neoplasia [17, 31–33]. Specifically, CR decreases the number of tumor-associated macrophages (TAMs), NF-κB signaling, circulating and tissue cytokines, and COX-2 expression, in many tissues and tumor types [17, 32, 33]. A major contributor to the proinflammatory tumor environment is the presence of TAMs. The recruitment of TAMs to the tumor microenvironment is largely dependent on the MCP-1, circulating levels of which are consistently decreased by CR [32, 33]. Levels of MCP-1 in tumor tissue are highly correlated with the accumulation of TAMs [34]. Macrophages are capable of polarizing into what is known as an M1, or classically activated cytotoxic macrophage, or an M2, or immunosuppressive macrophage. The cytokines produced by each type of macrophage are what distinguish an M1 from an M2, and tumor tissue typically contains a larger quantity of M2-type macrophages [34]. In addition to producing cytokines and chemokines, TAMs also produce growth factors that enhance proliferation and angiogenesis and contribute to deposition and dissolution of connective tissue [34]. There is also evidence to suggest that NF-κB plays a role in mediating TAM transcriptional programs and, by extension, protumorigenic effects of TAMs [17, 32, 34].

14.3 Calorie Restriction Impacts Growth Signals

14.3.1 Insulin and IGF-1

The peptide hormone insulin is produced by beta cells in the pancreas and released in response to chronic hyperglycemia, which is associated with insulin resistance, aberrant glucose metabolism, chronic inflammation, and the production of other metabolic hormones such as IGF-1, leptin, and adiponectin [35]. Clinical and epidemiologic evidence suggests that elevated levels of circulating insulin or the cleavage product of proinsulin (C-peptide) are associated with increased risk and/or progression of cancers of the breast (pre- and postmenopausal), endometrium, colon, kidney, and pancreas [35, 36]. Insulin exerts tumor-enhancing effects directly via the insulin receptor or via IGF-1 receptor (IGF-1R)/insulin receptor hybrids on

preneoplastic and neoplastic cells [37]. High circulating levels of insulin also upregulate hepatic synthesis of IGF-1 essential for growth and development of many tissues, particularly during the prenatal period [35, 37]. In the circulation IGF-1 is typically bound to IGF-binding proteins (IGFBPs) that regulate the amount of free IGF-1 bioavailable to bind to the IGF-1R and elicit growth or survival signaling [35, 37]. Elevated circulating IGF-1 is an established risk factor for many cancer types [38, 39], and in states of obesity/metabolic syndrome, the amount of bioavailable IGF-1 increases, possibly via hyperglycemia-induced suppression of IGFBP synthesis and/or hyperinsulinemia-induced promotion of hepatic growth hormone receptor expression and IGF-1 synthesis [35–38]. In contrast to obesity, CR prevents hyperinsulinemia, enhances insulin sensitivity, increases serum levels and tissue mRNA expression of several IGFBPs, and decreases total and bioavailable circulating IGF-1 levels [1].

The phospatidylinositol-3 kinase (PI3K)/Akt pathway, downstream of both the insulin receptor and IGF-1R, is one of the most commonly activated pathways in epithelial cancers [40]. This pathway integrates intracellular and environmental cues, such as growth factor concentrations and nutrient availability, to regulate cellular survival, proliferation, protein translation, and metabolism. Akt regulates the mammalian target of rapamycin (mTOR) [41], which regulates cell growth, cell proliferation, and survival through downstream mediators. Increased activation of mTOR is common in tumors and many normal tissues from obese and/or diabetic mice, while CR decreases mTOR signaling in these same tumors and normal tissues [42]. mTOR activation is inhibited by increased AMP-activated kinase (AMPK) under low-nutrient conditions [43]. Specific mTOR inhibitors block the tumor-enhancing effects of obesity in mouse models [44, 45].

14.3.2 Adiponectin, Leptin, and the Leptin-to-Adiponectin Ratio

Adiponectin is a peptide hormone primarily secreted from visceral white adipose tissue. In contrast to leptin and other adipokines, circulating levels of adiponectin negatively correlate with adiposity and are thus increased by CR and decreased by obesity [46]. Adiponectin functions to counter obesity-related metabolic perturbations, such as insulin resistance and leptin resistance, that impact glucose and fatty acid metabolism, alter insulin responses and increase production of inflammatory cytokines [46]. Thus, possible mechanisms through which adiponectin exerts anticancer effects may include increasing insulin sensitivity and decreasing insulin/IGF-1 and mTOR signaling via activation of AMPK [47]. Adiponectin also reduces proinflammatory cytokine expression via inhibition of NF-κB [47, 48].

Leptin is a peptide hormone produced by white adipose tissue, and the leptin receptor is a member of the class I cytokine receptor family that signals through the Janus kinase and signal transducer activator of transcription (JAK/STAT) pathway commonly dysregulated in inflammatory conditions and many cancers [49, 50]. Circulating leptin levels positively correlate with adipose stores and nutritional status

and function as an energy sensor to signal the brain to reduce appetite. Leptin has direct effects on peripheral tissues and indirect effects on neuroendocrine regulators of appetite and energy expenditure in the hypothalamus and impacts carcinogenesis, angiogenesis, immune responses, cytokine production, and other biological processes [50]. In the obese state, adipose tissue overproduces leptin, and the brain no longer responds to the signal, resulting in leptin resistance. Insulin, glucocorticoids, TNF-α, and estrogens all stimulate leptin release [50]. CR consistently and robustly decreases systemic leptin levels in a manner dependent on the extent of the adiposity loss [1].

In vitro, animal, and epidemiologic evidence linking adiponectin [51–55] or leptin [56–58] individually to cancer risk is mixed. Intermittent CR suppresses murine mammary tumor incidence in association with decreased leptin-to-adiponectin ratio [51]. Associations between the leptin-to-adiponectin ratio and the metabolic syndrome [59–61] and some cancers [62–64] have also been reported.

14.4 Emerging Mechanisms Underlying the Anticancer Effects of Calorie Restriction

14.4.1 Vascular Effects

Perturbations in the production and/or interactions of several factors that influence key functions of the endothelium, including its roles in regulating angiogenesis, hemostasis, vascular density, inflammation, and vascular-wall integrity, have been linked to cancer. One such vascular-related factor is plasminogen-activated inhibitor (PAI)-1, a serine protease inhibitor produced by endothelial cells, stromal cells, and adipocytes in visceral white adipose tissue [65]. PAI-1, through its inhibition of urokinase-type and tissue-type plasminogen activators, regulates fibrinolysis and integrity of the extracellular matrix [66]. Increased circulating PAI-1 levels, frequently found in obese subjects, are associated with increased risk of atherogenesis and cardiovascular disease, diabetes, and several cancers [65–68]. PAI-1 is also involved in angiogenesis and thus may contribute to obesity-driven tumor cell growth, invasion, and metastasis [68]. Circulating levels of PAI-1 are consistently decreased in response to CR [1], although the mechanistic link between PAI-1 and cancer requires further study.

Another important mediator of vascular integrity is the heparin-binding glycoprotein vascular endothelial growth factor (VEGF) produced by adipocytes and tumor cells. VEGF has mitogenic, angiogenic, and vascular permeability-enhancing activities specific for endothelial cells [69]. The need for nutrients and oxygen triggers tumor cells to produce VEGF, which leads to the formation of new blood vessels to nourish the rapidly growing tumor. VEGF may also facilitate the metastatic spread of tumor cells [70]. Adipocytes communicate with endothelial cells by producing a variety of proangiogenic and vascular permeability-enhancing factors, including VEGF and PAI-1 [71]. In the obese, nontumor setting, these factors

stimulate neovascularization in support of the expanding fat mass [71]. Circulating levels of VEGF are increased in obese, relative to lean, humans and animals, and increased tumoral expression of VEGF is associated with poor prognosis in several obesity-related cancers [72–75]. Data to date suggest that CR decreases systemic and tissue VEGF and has antiangiogenic effects in multiple experimental tumor models [73–75].

14.4.2 Autophagy

Autophagy is a cellular degradation pathway involved in the clearance of damaged or unnecessary proteins and organelles. It also provides an alternative source of energy and substrates during periods of restricted dietary intake (such as CR) or metabolic stress to enhance survival. In response to CR, plasma glucose levels (relative to controls) are low, insulin secretion is suppressed, and glucagon is released from the alpha-cells of the pancreas, resulting in increased autophagy in the liver, beta-cells of the pancreas, skeletal muscle, and heart [76, 77]. One of the proposed mechanisms of CR is that under conditions of nutrient limitation, survival is promoted by a shift in metabolic investment from cell replication and growth to maintenance [78]. This tightly regulated process is driven by a group of autophagy-related proteins and is suppressed by the conserved nutrient sensor target of rapamycin (TOR, referred to as mTOR in mammalian species) [79]. CR regulates TOR complex 1, and to a lesser extent TOR complex 2, in many species including flies, worms, yeast, and mammals. TOR complex 1 signaling regulates protein translation and many cellular processes including metabolism and autophagy [79]. Additionally, suppression of nutrient-activated TOR signaling is sufficient to trigger an energy stress response that is coordinated by AMPK, and this metabolic program blunts the growth responses to nutrient availability and promotes autophagy [80].

Several longevity-promoting regimens, including inhibition of TOR with rapamycin, resveratrol, or the natural polyamine spermidine, may require autophagy for their effects [81]. Autophagy activation is essential for clearing cellular damage and preventing disease in normal cells. Tumor cells also utilize autophagy to maintain a favorable metabolic state for daughter cell production, especially under limiting nutrient conditions [82]. However, little is known about what role autophagy plays in CR-mediated effects on tumor development or progression. In particular, the links between autophagy, apoptosis, and energy metabolism in normal vs. cancer cells may provide important insights into the anticancer effects of CR.

14.4.3 Sirtuins

The Sirtuin family of proteins has been implicated in the regulation of endocrine signaling, stress-induced apoptosis, and the metabolic changes associated with

energy balance modulation and aging [83–85]. Sirtuins were originally studied in yeast and nematodes, where CR increases lifespan in association with the levels and activity of the Sir2 protein [86–88]. The levels of Sir2, or its mammalian homologue SIRT1, rise in response to CR [84–88]. SIRT1 is an NAD-dependent deacetylase that inhibits stress-induced apoptotic cell death and modulates IGF-1, adiponectin and insulin production, and insulin sensitivity, in some tissues [88–90].

The specific roles of sirtuins in cancer development and/or progression are not yet clear. SIRT1 is upregulated in several tumor types and can inhibit apoptosis and downregulate the expression of tumor suppressor genes (such as p53, which is frequently mutated in many human cancers) to enhance survival of epithelial cancer cells [91–94]. In addition, the SIRT1 activator SRT1720 promotes tumor cell migration and lung metastases in a murine breast cancer model [95]. In contrast, there is also evidence that SIRT1 can act to suppress polyp formation in the APC^{Min} intestinal tumor model [96]. Additionally, in preclinical studies the phytochemical resveratrol activates SIRT1 and reduces cancer development in multiple models [97]. SIRT1 overexpression does not influence the anticancer effects of an every-other-day fasting regimen (a variation of CR) in a p53-deficient mouse model of cancer, suggesting that SIRT1 may have a limited role in the effects of CR on cancer [98]. Given the conflicting data to date regarding the tumor-enhancing, vs. inhibitory, effects of SIRT1 activation and the apparently limited role of SIRT1 in the response to CR, it remains unclear if SIRT1 or other sirtuins represent mechanistic targets for cancer prevention.

14.5 Targets and Strategies for Mimicking the Effects of Calorie Restriction

The identification and development of natural or synthetic agents that mimic some of the protective effects of CR may facilitate new strategies for cancer prevention. Given how difficult it is for many people to adopt a low calorie diet for an extended period, the identification of drugs or other agents that could either complement or even reproduce the anticancer effects of CR without drastic changes in diet and lifestyle is a goal for many pharmaceutical companies. Numerous studies have used microarray analyses to profile the molecular targets responding to CR and other dietary energy balance modulations [99–103]. Most of these studies were focused on understanding CR effects related to aging, and they revealed that the extent to which CR modulates the transcriptome is species specific, tissue specific, and dependent on the duration and intensity of CR. Nonetheless, some emerging patterns from these studies suggest that transcripts involved in inflammation, growth factor signaling (particularly related to the insulin and IGF-1 pathways), oxidative stress, and nutrient metabolism are commonly altered by CR. Application of the emerging field of metabolomics to this question should accelerate the identification of additional targets.

The IGF-1 and Akt/mTOR pathways, and possibly the sirtuin pathway, have emerged as potential key mediators of CR's anticancer effects and are initial targets for possible CR mimetics. Agents or interventions that safely reduce IGF-1, or inhibit one or more components of the signaling pathways downstream of IGF-1 and other growth factors (including Akt and mTOR), even without dietary changes, may provide an effective physiological or pharmacological mimetic of those effects. The hope is that these agents or interventions could be readily adopted by a large proportion of the population, particularly those unable to lose weight and at high risk for cancer or other chronic diseases associated with obesity. Small-molecule inhibitors of IGF-1, antisense inhibitor approaches, anti-IGF-1 antibody therapies, and microRNA-based approaches are under development [104, 105]. In addition, a wide variety of natural agents with demonstrated cancer chemopreventive or chemotherapeutic activity have recently been reported to inhibit the IGF-1 pathway [106].

Pharmacological mTOR inhibitors have emerged as the lead candidates for CR mimetics. Rapamycin treatment extends lifespan and delays cancer in mice, providing additional support for mTOR as a target for mimicking the effects of CR [107]. We have shown that rapamycin or its analogue, RAD001 (everolimus), offsets the obesity-associated increase in growth of mammary or pancreatic tumors [33, 44]. Rapamycin is a potent inhibitor of the mTOR complex 1, but chronic rapamycin exposure has been linked in some studies to disruption of mTOR complex 2 signaling, resulting in impaired glucose tolerance and insulin action [108]. Thus, while mTOR complex 1 inhibition appears to be a good strategy for mimicking many of the anticancer effects of CR, the search for agents that can do so without disrupting mTOR complex 2 signaling is ongoing.

Metformin, a biguanide commonly used to treat type 2 diabetes, is another promising CR mimetic that inhibits mTOR signaling and circumvents the side effects of hyperglycemia/insulin resistance that occur with rapamycin [109]. Metformin inhibits gluconeogenesis through indirect activation of AMPK in the liver and possibly cancer cells and may also exert direct effects on AMPK in cancer cells to decrease mTOR activation [109]. Administration of metformin suppresses tumor development and/or growth in multiple experimental models, including colon, mammary, and hematopoietic cancer models [109]. Epidemiological studies have suggested that type 2 diabetic patients treated with metformin have a lower cancer risk and mortality relative to diabetic patients receiving sulfonylurea, insulin or other therapies [110–112]. A randomized trial is now underway to evaluate the effect of metformin on breast cancer recurrence [113]. Phenformin, another biguanide that has been abandoned for diabetes therapy due to its toxicity from lactic acidosis, is a more potent AMPK inhibitor than metformin and may also have some potential as a CR mimetic for cancer prevention at lower, nontoxic doses [109].

Genetic induction of the Sir2/SIRT1 family of NAD-dependent deacetylases mimics some of the effects of CR [84, 86, 87, 96], although the role of SIRT1 in the anticancer effects of CR is unclear and may be minimal [98]. Sirtuin modulators, including resveratrol and its analogues, and pharmacologic modulators of SIRT1 [97, 114] exert some anticancer activity, although much of this work has been limited to in vitro systems and awaits verification in vivo.

14.6 Conclusions

As summarized in Fig. 14.1, this review considers lessons learned from CR and cancer research to discuss promising molecular targets for cancer prevention, particularly for breaking the obesity-cancer link. Potential targets include components of energy-responsive growth factor and adipokine signaling pathways, inflammatory pathways, vascular regulators, autophagy regulators, and the sirtuin pathway. Clearly, no single pathway accounts for all of the anticancer effects of CR. As with most chronic disease intervention strategies, combination approaches involving lifestyle (including diet and physical activity) and pharmacological interventions that target multiple pathways (and that maximize efficacy and minimize adverse effects) will likely be most successful for preventing cancer. Future studies aimed at further elucidating the mechanisms underlying the anticancer effects of CR and that exploit this mechanistic information to target CR-responsive pathways will facilitate the translation of CR research into effective cancer prevention strategies in humans.

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Chapter 15
Vascular Targeting of Adipose Tissue

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Abstract Obesity is a rapidly increasing worldwide threat to health due to its association with cardiovascular disease, diabetes, cancer, and a number of other medical conditions. Considerable efforts are underway to develop drugs for obesity prevention and treatment. Like tumors, white adipose tissue (WAT) overgrowing in obesity depends on functional blood vessels for its expansion and maintenance. Recent findings indicate the apparent vasculogenic role of WAT-derived cells recruited by tumors. Based on these notions, endothelial and perivascular cell populations in WAT have been considered as potential therapy targets in the context of obesity and cancer. In this chapter, we discuss studies aimed at inactivation of WAT vasculature and evaluate it as a prospective approach to treating obesity and its potential implications for cancer and other diseases.

Obesity, defined as a body mass index (BMI) of 30 kg/m^2 or more, is a result of white adipose tissue (WAT) overgrowth [1–4]. Over the past few decades, profound changes in nutrition and lifestyle reaching epidemic proportion in most industrialized countries have led to a sharp increase in the prevalence of obesity and its complications. Decreasing physical activity and increasing food accessibility has resulted in a severe alteration of the balance between intake and expenditure of energy. According to a recent report by the World Health Organization, there are >1 billion overweight adults worldwide, and at least 300 million of these individuals are clinically obese [5].

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15.1 Obesity as a Health Problem

More than 2,400 years ago, Hippocrates observed: "Sudden death is more common in those who are naturally fat than in the lean." This astute observation has remained incompletely explained for many centuries to come. Obesity is associated with the metabolic syndrome, manifestations of which are low-grade inflammation, dyslipidemia, and type-2 diabetes [6]. The risk of cardiovascular disease is highly elevated in obese individuals [7]. In addition, obesity complicates numerous other medical conditions [8]. Finally, over the past decade, the link between obesity and a number of cancers has been revealed [9, 10]. While originally colorectal and endometrial and breast cancers were identified as obesity linked [11], recent reports have added pancreatic, kidney, and esophageal cancers to the list [12]. As more evidence linking excess BMI and increased cancer risk/progression is accumulating, research is turning its focus to uncover the pathophysiology behind this correlation [10]. The underlying mechanisms discussed elsewhere in this book are complex and incompletely understood [13]. Until now, it has been unclear if excess adipose tissue itself affects cancer progression or if this link is predominantly due to diet and lifestyle of obese individuals [14]. Recent animal studies show that the state of obesity can accelerate tumor growth irrespective of diet [15], which explains clinical observations [16]. Based on the increased progression of cancers surrounded by adipose tissue (e.g., prostate, breast, and uterine) in obese individuals, it has been proposed that adipose tissue has a direct effect on tumor growth [10]. An emerging body of evidence confirms that this cross talk indeed takes place at several levels.

15.2 Adipose Tissue as an Organ

In humans, subcutaneous WAT is present as layers between muscle and dermis, while intraperitoneal WAT is located around the gut, kidneys, and other internal organs [17]. Accumulating evidence indicates that different depots of WAT in the body are regulated independently and have different implications in disease. Specifically, it is overgrowth of intra-abdominal WAT, which encompasses omental and mesenteric (visceral), retroperitoneal, and perigonadal depots, that is associated with inflammation and the metabolic syndrome. Abdominal adiposity is believed to predominantly account for the poor prognosis of obese patients with cancer and cardiovascular disease.

The ability of multicellular organisms to store and release energy on demand has been crucial for their survival throughout evolution. In mammals, this function is primarily executed by adipocytes, the large cells of WAT that store fatty acids esterified to triglycerides in lipid droplets. Efficient management of energy in the body is regulated by intricate communication between multiple organs [18]. In response to signals from the central nervous system (CNS) and the digestive tract, the stored lipids can become mobilized, metabolized, and either released as fatty acids or used

to increment thermogenesis by oxidation [19, 20]. As opposed to WAT, brown adipose tissue (BAT) is responsible for energy dissipation in the form of heat [21, 22]. In humans, BAT is clearly present and functional in newborns, whereas adults had been thought to lack BAT until recently. However, recent studies have demonstrated that adults also have BAT, although it is conditionally functional [23].

Adipose tissue is not merely an organ designed to manage energy. It is wired with the immune, cardiovascular, and reproductive systems through the exchange of cells and soluble factors that signal in endocrine and paracrine ways [24, 25]. Mature adipocytes synthesize and secrete numerous enzymes, growth factors, cytokines, and hormones that are involved in overall energy homeostasis [26]. Collectively, factors secreted by cells of adipose tissue are called adipokines [27]. These molecules secreted by adipocytes and other cells of adipose tissue play important roles not only in adipogenesis but also in diverse physiological processes and metabolic pathways in the body including lipid homeostasis and modulation of inflammatory responses. Collectively, adipokines modulate a range of global physiological responses including energy balance, inflammation, angiogenesis (blood vessel formation), hemostasis (regulation of blood coagulation), and blood pressure [28].

15.3 Adipose Tissue Composition

Adipose tissue development and expansion are controlled by the concerted action of extracellular and intracellular signals [4, 29]. Environmental, genetic, and epigenetic stimuli regulate preadipocyte differentiation into lipid-laden adipocytes through a process called adipogenesis (Fig. 15.1). Adipogenic differentiation takes place around the neurovascular bundles. In addition to adipocytes, WAT is composed of other cell types, interactions among which orchestrate obesity progression [30]. As shown in Fig. 15.1, these populations include stromal cells, vascular endothelial cells (EC), perivascular cells (pericytes/mural cells/adventitial cells), and infiltrating blood cells including monocytes/macrophages, lymphocytes, and mast cells [17, 31]. Blood vessels are essential for adipose tissue growth and maintenance as the route for delivery of oxygen, nutrients, and factors modulating differentiation [32]. Another important function of blood vasculature in adipose tissue is serving as a niche for progenitor cells that differentiate into adipocytes [33, 34]. Expansion of WAT in obesity results not only from adipocyte hypertrophy (increase in cell size) but also from hyperplasia (increase in cell number), which relies on progenitor cell proliferation [35]. Adipose progenitors have originally been identified in the stromal/vascular fraction (SVF) of WAT and termed adipose stromal cells (ASC) [36]. Recently it has become apparent that the ASC population appears to contain several distinct subpopulations with different differentiation potentials [37, 38].

The origins of vascular cells in adipose tissue are not completely understood. Adipose tissue is highly vascularized: each adipocyte is encircled by capillaries. Angiogenesis, the process of blood vessel sprouting from existing vasculature well characterized in tumors [39], also has a crucial role in WAT [40]. In the past few

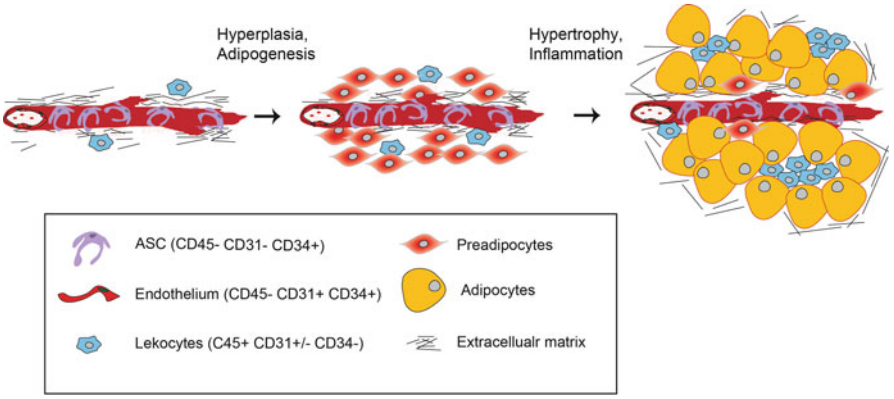

Fig. 15.1 Adipose tissue expansion in obesity. Development of adipose tissue involves proliferation and differentiation of perivascular adipose progenitors residing within the population of adipose stromal cells (ASC) into preadipocytes and finally into lipid-laden adipocytes. Adipogenesis and hypertrophic adipocyte growth are coordinated with angiogenesis and recruitment of leukocytes that are responsible for inflammation onset. Surface markers useful for identification of adipose cell populations are indicated

years, it has become apparent that recruitment of circulating vasculogenic progenitors into blood vessels may also play an important role in adult vascularization [41, 42]. Studies in cancer models have demonstrated that endothelial progenitor cells (EPCs) derived from the bone marrow contribute to neovasculature [41, 43]. In addition, a small percentage of WAT endothelium appears to be derived from transdifferentiating adipocyte progenitors [44]. Therefore, vascular cells of WAT appear to be derived from distinct origins with individual endothelial cell subpopulations distinct in their properties. An important component of adipose vasculature is comprised by adipose progenitor cells residing in the perivascular cell compartment of the blood vessel wall [33, 34].

ASC comprise the majority of cells in the SVF and display multipotency and self-renewal capacity comparable to those reported for mesenchymal stromal cells (MSC) originally characterized in the bone marrow [45]. Mesenchymal progenitors currently occupy the central arena of stem cell biology and regenerative medicine [36, 46]. The ability of MSC to differentiate into cells of mesenchymal lineage, such as adipocytes, osteocytes, and chondrocytes, has coined the term "mesenchymal stem cells" [47]. Although there are reports suggesting that adipocytes can be derived from hematopoietic stem cells [48], there is predominant evidence that bone marrow-derived precursors contribute only to endothelial cells and infiltrating hematopoietic cells but not to mesenchymal stroma and adipocytes [49, 50]. The transition from the adipose progenitors to preadipocytes is still poorly defined, and understanding of intercellular interactions within WAT is incomplete despite recent advancements [51, 52]. In addition to serving as adipocyte progenitors, ASC cooperate with the endothelium and support vascularization [33, 34]. Recent studies show that ASC are expanded in obesity [15]. ASC promote endothelial cell

proliferation and blood vessel formation at least in part via trophic effects of secreted vascular endothelial growth factor (VEGF) and other angiogenic molecules [33, 51, 53]. The molecular mechanisms of interactions between adipose EC, ASC, and adipocytes that dictate the commitment of the progenitor cells toward differentiation or mobilization are being investigated [54].

15.4 Adipose Tissue and Cancer

Composition and function of WAT has pointed to the mechanisms through which obesity may be linked with cancer [55]. Inflammatory and endocrine signaling by adipose tissue-derived molecules (adipokines) has been proposed to account for cancer promotion in obesity [10]. Results from animal models, as well as clinical associations, support this hypothesis [28, 56]. In addition, recent studies have shown that cells from adipose tissue are capable of trafficking to tumors, thus enabling paracrine action of adipokines from within the tumor microenvironment [57, 58]. Investigation of the molecular pathways through which adipose cells traffic to tumors and execute their functions is underway [59]. Extracellular matrix (ECM) modulation, immune system suppression, and direct effects on malignant cell survival and proliferation have been pointed to as potential activities of systemic and locally produced adipokines within the tumor [55]. However, accumulating evidence indicates that cells from WAT promote tumor growth to a large extent through supporting tumor vasculature [57, 58]. The ability of WAT-derived cells to stimulate tumor neovascularization has been supported by a number of recent studies [58–60]. When transplanted into host animals, adipose endothelial cells can directly incorporate into the lumen of tumor vasculature, whereas ASC acquire perivascular localization in tumors [58]. Recent comparison of circulating cell populations in lean and obese mice revealed that cancer and obesity result in ASC exodus into the peripheral blood [15]. Clinical studies also demonstrate association of obesity and cancer with increased circulation of endothelial cells and ASC in patients, suggesting that endogenous WAT serves as a source of cells contributing to tumor vasculature [61–63]. The link between cancer relapse and lipotransfer procedures is consistent with this conclusion [64]. Recent studies by our group have provided evidence that ASC traffic from endogenous WAT to tumors where they become incorporated into blood vessels as pericytes [15]. Other groups have confirmed our findings [60, 65, 66]. In addition, we found that, upon recruitment by tumors, ASC can differentiate into adipocytes in an obesity-dependent manner, thus identifying an origin of intratumoral adipocytes previously implicated in cancer progression [67–69]. Extending this evidence, we found that increased tumor vascular patency is associated with elevated proliferation of neighboring malignant cells. Taken together, these findings indicate that cells recruited from endogenous adipose tissue can be recruited by tumors to potentiate the supportive properties of the tumor microenvironment. This previously overlooked phenomenon appears to at least partially account for the association between obesity and cancer.

15.5 Approaches to Obesity Treatment

Physical activity and diet are the logical measures against obesity; however, modern lifestyle complicates their implementation. Surgical interventions to reduce adipose tissue mass, such as gastric banding, are effective and prevent the risk of obesity-associated diseases [70]. However these highly invasive practices are typically applied to severe obesity cases. Pharmacological treatments for obesity available today are inadequate. Those approved by the FDA achieve ~5 % weight loss per year even when combined with diet- and exercise-based behavioral adjustments [71, 72]. The majority of anti-obesity agents tested clinically target the CNS to alter neuronal signals regulating appetite and the gastrointestinal tract to alter nutrient adsorption. There are two new recently approved drugs, Belviq and Qsymia, that act through the brain to promote satiety; however, their marginal efficacy and concerns over their safety limit enthusiasm [73]. Another drug approved in conjunction with reduced fat diet is Orlistat (Alli, Xenical), which inhibits gastrointestinal lipases and hence blocks lipid digestion. Side effects, such as incontinence and reduced vitamin absorption, have limited its popularity. Gut hormones, such as glucagon-like peptide and ghrelin, provide hope as prospective anti-obesity biologics [74]; however, the need for new approaches to obesity prevention and treatment is clearly pressing.

Directly modulating the content or function of adipose tissue is a potential alternative approach to treat obesity and the related diseases. A number of groups have focused on developing anti-obesity therapies through targeting the mature adipocytes and modulation of their differentiation [75]. An attractive idea related to this pursuit is converting energy-storing WAT to energy-burning BAT. In addition to brown adipocytes derived from common skeletal muscle progenitors, brown-like (beige or "brite") adipocytes can arise from white adipocyte progenitors (ASC) both in cell culture and in vivo [76]. This conversion is driven by sympathetic nervous system stimuli, such as cold temperature, and signal transduction cascades triggered by activation of β3-adrenergic receptors in WAT. In mice, expansion of these brown adipocytes within WAT can lead to virtually all adipose depots becoming BAT-like at the expense of WAT [77]. Targeting adipokines, the bioactive products of adipocytes, has also been pursued as a prospective therapeutic approach [18]. However, current pharmaceutical approaches to adipose tissue targeting remain at preclinical stages. Thus, while many potential adipocyte targets have been identified, they are yet to be translated into drugs.

15.6 Adipose Vasculature Targeting as a New Approach to Obesity Treatment

Blood vessels supply oxygen, nutrients, growth factors, and progenitor cells to all organs. Inhibition of neovascularization has arisen as a powerful approach to controlling tissue expansion. Over the past decade, vasculature targeting as a cancer

Table 15.1 Anti-obesity vascular-targeting agents in animal models

Molecule	Targeted tissue	Experimental model	Body mass	References
VEGF	Angiogenic vessels	Inhibitor administration	↓	[30, 88]
		Overexpression	↓	
VEGFR	Angiogenic vessels	Inhibitor administration	↓	[85]
PIGF	Angiogenic vessels	Inhibitor administration	↓	[90]
		Genetic deficiency	↑	
SPARC	Angiogenic vessels	Genetic deficiency	↑	[92]
Leptin	Angiogenic vessels	Administration	↓	[96]
		Genetic deficiency	↑	[97]
Plasminogen	Angiogenic vessels	Administration	↓	[90] [138]
Tiplaxtinin		Genetic deficiency	↑	[139]
Angiostatin	Angiogenic vessels	Administration	↓	[81]
Endostatin	Angiogenic vessels	Administration	↓	[81]
TNP-470 CKD-732	Angiogenic vessels	Administration	↓	[81]
MMP inhibitors: Galardin, Bay-129566, Ro20-2653	Angiogenic vessels	Inhibitor administration	↓	[40, 140, 141]
Thalidomide	Angiogenic vessels	Administration	↓	[81]
Adiponectin	Angiogenic vessels	Administration	↓	[83]
		Genetic deficiency	↑	
Curcumin (polyphenol)	Angiogenic vessels	Administration	↓	[104]
Ob-x and fumagillin	Angiogenic vessels	Administration	↓	[101, 102]
EGCG (catechin in tea)	Angiogenic vessels	Administration	↓	[40, 142]
Adipotide	Mature vessels	Administration	↓	[117]

EGCG epigallocatechin-3-gallate; *MMPs* metalloproteinases; *PLGF* placental growth factor; *VEGFR* vascular endothelial growth factor receptor

therapy has evolved resulting in clinically approved drugs that have considerably improved the clinical outcome [78, 79]. Like tumors, WAT is a highly dynamic organ, growth and maintenance of which requires continuous remodeling of the capillary networks [32]. Emerging evidence shows that modulators of angiogenesis affect the expansion of WAT mass by regulating the development of adipose vasculature [31]. Pharmaceutical manipulation of adipose tissue neovascularization by angiogenic stimulators and inhibitors might therefore offer novel therapeutic options for the treatment of obesity and related metabolic disorders. Further, recent advances in the development of agents aimed to disrupt the mature vascular components (namely, endothelial and perivascular) represent an alternative direction in anti-obesity drug design. Table 15.1 summarizes studies reporting changes in WAT

vasculature, most of which have been performed in animal models. A detailed discussion of some of the individual molecular mechanisms probed in these publications is provided below.

15.6.1 Targeting Immature WAT Vasculature with Angiogenesis Inhibitors

Recently, it has been appreciated that vasculature is critical for WAT development as the gatekeeper of blood access to adipocytes [4, 30, 80]. The pioneering studies from the Folkman group have demonstrated that inhibition of angiogenesis has the potential to prevent not only tumor growth but also obesity onset [39]. Both angiogenesis and vasculogenesis (progenitor cell recruitment from remote organs) appear to play an important role in WAT vascularization. Because WAT growth-underlying obesity proceeds into adulthood, it has been proposed that adipogenesis and angiogenesis feed onto each other in obesity [81, 82]. VEGFs and their receptors (VEGFRs) comprise the system highly active in adipose tissue [80, 83–86]. VEGF-A is a major angiogenic factor that controls proliferation, migration, and permeability of the endothelium [53, 87]. Administration of anti-VEGF antibodies inhibiting adipose angiogenesis affects adipocyte differentiation and systemic lipid metabolism, indicating VEGF as a key factor linking the interplay between angiogenesis and adipogenesis in WAT with physiological responses in the rest of the body [30, 88]. Interestingly, a recent report from the Scherer group shows that consequences of VEGF modulation in WAT are context dependent, highlighting the complexity of adipose tissue biology and emphasizing the point that broad implications of WAT vasculature targeting are yet to be fully understood [88].

A close recent connection between VEGF-B and endothelial fatty acid uptake [89] also indicates the direct dependence of adipogenesis on angiogenesis.

Placental growth factor (PlGF), a homolog of VEGF, enhances angiogenesis in pathological conditions. Loss of PlGF impairs angiogenesis in the ischemic retina, limb, and heart, in wounded skin, and in tumors, without affecting physiological angiogenesis. Administration of a PlGF-neutralizing monoclonal antibody inhibits angiogenesis and fat pad formation, indicating its function in WAT [90]. Another component of the system, matricellular protein SPARC (secreted protein, acidic, and rich in cysteine), also known as osteonectin, binds to VEGF-A, impairs VEGFR-1 activation, and inhibits FGF-2, resulting in inhibition of endothelial cell proliferation. SPARC is produced by adipose tissue and its expression is upregulated in obesity [91]. SPARC-deficient mice on high-fat diet develop larger fat pads as compared to wild-type mice [92]. The apelin/APJ signaling system [93] and angiopoietins along with their receptors, Tie1 and Tie2, are likely to play a role in WAT angiogenesis [94]. Many other components directly or indirectly regulating angiogenesis have been identified in adipose tissue. These include leptin, neuropilin-1, fibroblast growth factors (FGF-2), thrombospondin-1, adiponectin,

tissue factor, tumor necrosis factor (TNF-α), transforming growth factor beta (TGF-β), insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF), and various components of the ECM [40, 95]. Leptin, adiponectin, and ECM-related mechanisms have drawn particular attention in respect to the link between obesity and cancer.

Leptin is a hormone/cytokine/growth factor secreted by adipocytes, and its inactivation (in ob/ob mice) or loss of its receptor (in db/db mice) causes morbid obesity due to the role of this system in appetite control [96]. Leptin can, however, induce angiogenesis, suggesting that it might contribute positively to the development of adipose tissue [97]. In genetically obese human individuals carrying homozygous mutations in the leptin gene, administration of recombinant leptin successfully reduces body weight; however, the majority of obese humans have unusually high levels of circulating leptin and are leptin resistant [71, 98]. The potential implication of the leptin role in angiogenesis, which could contribute to obesity in these patients, warrants further investigation. Interestingly, while obese cancer patients have increased levels of leptin in circulation, the opposite trend is observed for adiponectin [56]. One of the functions proposed for adiponectin is inhibition of angiogenesis, which may be implicated in both obesity and cancer [99]. However, both antiangiogenic and pro-angiogenic functions have been reported for adiponectin.

Angiogenesis is mechanistically linked with ECM remodeling [100]. Proteolysis is required not only for the expansion of the adipocyte basement membrane in hypertrophic WAT, but also for cell migration during the development of blood vessels and peripheral nerves in the growing adipose tissue. Thus, both adipogenesis and angiogenesis require proteolytic activity, which is mainly regulated via the plasminogen/plasmin (fibrinolytic) and matrix metalloproteinase (MMP) systems. Recently, some evidence has emerged that proteins of the ADAM (A Disintegrin And Metalloproteinase) and ADAMTS (ADAM with TSP motif) families may also be implicated. The named proteinases are collectively able to cleave a wide variety of substrates, including ECM components, other proteinases and their inhibitors, and matrix receptors, thus fine-tuning adipose tissue remodeling. MMPs and plasmin can also release, activate, or degrade several growth factors and cytokines that play major roles in angiogenesis and are implicated in obesity. Several nutritionally induced obesity models in transgenic mice have been used to study the role of the fibrinolytic system in the development of obesity. Mice deficient in plasminogen, the substrate for both plasminogen activators, showed reduced fat accumulation associated with reduced differentiation of progenitor cells [101]. Indeed, tiplaxtinin, designed as a synthetic inhibitor of PAI-1, reduces body weight in WT mice kept on HFD [90].

A number of preclinical studies using angiogenesis inhibitors for obesity intervention, with many reviewed elsewhere [40], are summarized in Table 15.1. In the pioneering study, treatment with angiostatin (kringle 1–4 domains of plasminogen), endostatin (a C-terminal fragment of collagen XVIII), TNP-470, Bay-129566 (a MMP inhibitor), and thalidomide was tested in mouse models. It has been demonstrated that inhibition of angiogenesis is potent enough to impede WAT expansion

even in genetically obese leptin-deficient mice [81]. In addition, agents blocking VEGFR-2 have been shown to prevent the development of obesity in genetic mouse models and studies based on high-fat diets [85]. Inhibition of angiogenesis in the growing adipose tissue associated with a reduction in vascular density and endothelial cell apoptosis caused a decrease in the body weight of obese mice [81, 85, 102, 103]. Although agents such as TNP-470 and its analog CKD-732 are considered to be selective angiogenesis inhibitors, many have off-target effects such as food aversion, which may partially account for weight loss [31]. Therefore, it remains to be determined to which extent WAT is affected through specific targeting of endothelial cells, as opposed to affecting systemic energy balance.

A number of recent reports confirm the initial observation that disrupting WAT neovascularization can prevent the onset of obesity in both genetic and diet-induced obesity models (Table 15.1). For example, curcumin, the major polyphenol in turmeric spice, has been shown to suppress WAT accumulation through its effects on angiogenesis and adipogenesis [104]. Similar results have been obtained with other herbal and synthetic antiangiogenic compounds, such as Ob-x, EGCG (catechin in tea), and fumagillin [105, 106]. However, for most studies, it remains questionable to which extent the effect of these agents directly on the vasculature, as opposed to their effect on other cells in WAT or other organs, affects food intake and adipose tissue levels. Notably, while mice reduce their body weight while on agents such as TNP-470, they regain weight when off schedule [81]. These treatment-regulated cycles can occur several times without causing resistance or other obvious side effects. These findings suggest that antiangiogenic agents can be used repeatedly for the treatment of obesity without encountering drug resistance, which has been observed for cancer [107].

It should indeed be kept in mind that the angiogenic circuits involved in adipose tissue development also are critical to many other biological processes. Although animal treatment with antiangiogenic agents has consistently resulted in inhibition of WAT accumulation, there is much preclinical work to be done before this approach can be considered clinically feasible. Although generally mild, adverse effects of angiogenesis inhibition have been reported in clinical trials [108]. Off-target effects of antiangiogenesis drugs illustrate the fact that neovascularization does occur, although at levels much lower than in development, outside pathological tissues in adulthood [39]. New blood vessel formation generally accompanies tissue remodeling that takes place either upon injury or during certain normal physiological processes. For example, because VEGF/VEGFR signaling is not WAT specific, its inhibition is expected to cause systemic effects. Indeed, VEGF inhibition can result in off-site proteinuria, hypertension, and internal bleeding [109]. Moreover, studies in mouse models show that chronic blockade of certain endothelial pathways can backfire with pathological activation of endothelial cells, perturbed organ physiology, and even vascular tumorigenesis, further emphasizing safety concerns [110]. Little is known regarding the signaling between the angiogenic components of adipose tissues and the CNS as well as other organs [111, 112]. Also, because cell death in WAT results in the recruitment of leukocytes resolving the tissue damage [113], the resulting local and systemic activation in secretion of inflammatory

cell-derived cytokines, such as TNF-α and several interleukins (e.g., IL-6 and IL-8), may have systemic implications. A better understanding of the WAT targeting consequences is instrumental in the development of therapeutic approaches.

15.6.2 Targeting "Mature" WAT Vasculature

Studies on tumor models illustrate the notion that vascular disruption can serve as an effective complementary tool to constrain tissue expansion [114, 115]. It has been proposed that the mature vasculature of WAT could be similarly targeted and that depleting the supply of nutrients and oxygen essential for the maintenance of adipocytes could result in obesity reversal after its onset. Based on the notion that tumor vasculature features differential expression of markers [116], our group had proposed the existence of cell surface molecules selectively upregulated in adult WAT blood vessels that could be therapeutically targeted in pathologically expanded WAT. In a proof-of-principle study aiming to identify and use WAT vascular targets for experimental obesity therapy, we screened a combinatorial library in mice for peptides systemically homing to WAT. As a result, we isolated a peptide with the sequence CKGGRAKDC that selectively accumulated in WAT [117]. The uptake of CKGGRAKDC by adipose endothelial cells suggested that the internalizing receptor could serve as a target of therapies directed to WAT. By using WAT membrane protein extracts, we biochemically isolated the vascular receptor of the CKGGRAKDC peptide and identified it as prohibitin (Phb). Follow-up studies have validated the specificity of the CKGGRAKDC peptide for Phb for WAT endothelium cells using nanocarriers [118]. We used a mouse diet-induced obesity model to test in vivo the capacity of the CKGGRAKDC peptide to deliver cytotoxic (proapoptotic) peptide KLAKLAKKLAKLAK to WAT. Daily subcutaneous injections of the CKGGRAKDC-KLAKLAKKLAKLAK fusion peptide (Adipotide) caused rapid obesity reversal [117]. Another study reproduced these results in the rat model [111] and suggested that reduced food consumption in WAT-targeted animals explains the lack of apparent lipodystrophic effects. These observations uncover a previously unappreciated cross talk between the status of WAT vasculature and central regulation of food intake [111]. Recently, Adipotide, which was validated in three monkey models [119], has been shown to have antidiabetic effects [120], and the first-in-human Adipotide clinical trial is ongoing.

In follow-up studies, we identified annexin A2 (annexin II, Anx2) as a Phb-binding protein mimicked by the CKGGRAKDC peptide [121]. Consistent with Phb/Anx2 interaction in WAT endothelium, Anx2 has been previously shown to have a pro-angiogenic function [122]. In addition, a function of Phb in controlling adipogenesis has been uncovered [123]. Our recent report shows that Phb and Anx2 form a complex in lipid rafts isolated from the cell membranes of WAT cells and identify the domains mediating Phb/Anx2 interaction [121]. Currently, our group is characterizing the function of Phb/Anx2 in adipose tissue in order to better establish this complex as a prospective anti-obesity therapy target. Delivery of other therapeutic agents to this molecular "zip code" is also being pursued [118].

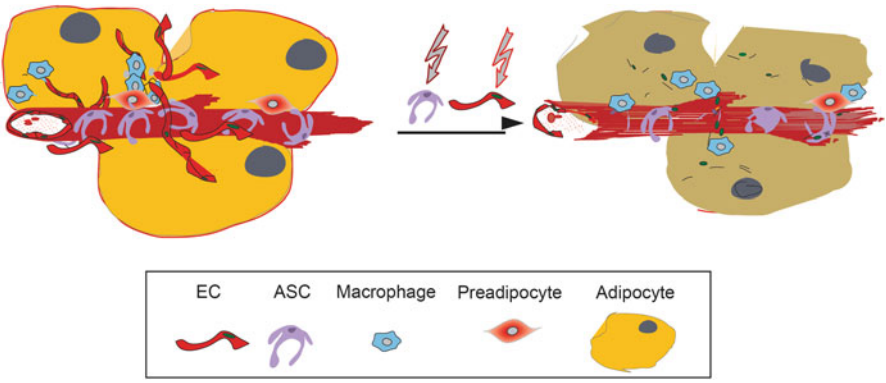

Fig. 15.2 Vascular targeting of adipose tissue as an anti-obesity approach. Targeted depletion of adipose endothelium, the integral component of the vasculature delivering nutrients and oxygen to adipocytes, results in quick tissue resorption in rodent models. Cytoablation of pro-angiogenic adipocyte progenitors (ASC), in parallel with the endothelium, could represent a potential complimentary approach to prevention of adipose tissue regrowth upon discontinuation of anti-vascular therapy

15.6.3 Targeting Perivascular Adipose Cells

Despite the short-term effectiveness in experimental animal models, obesity relapses due to WAT regrowth upon the cessation of treatment with endothelium-targeting agents [117]. Although proliferating endothelial cells serve as building blocks for growing vessels, this process relies on the trophic contribution and mechanical support from stromal cells [33, 54]. Indeed, while endothelial cells and adipocytes undergo cell death under ischemic conditions, ASC are resistant to hypoxia [124]. Therefore, resistance to vascular-targeting agents appears to result from the pro-angiogenic action of surviving ASC that quickly rewire the tissue. We propose that in the future, ASC targeting in parallel with endothelial cell targeting in WAT might provide an approach to long-term control of WAT mass (Fig. 15.2). Accumulating body of evidence indicates that WAT contributes to the pool of progenitor cells mobilized in obesity and cancer [62, 63] and serving as perivascular cells in tumors [15, 58]. Therefore, targeting WAT-derived cells in circulation or upon homing to cancer sites could offer a new direct approach to complementary cancer therapy.

Studies from our research team have contributed to the understanding of how blood vessel formation might be coordinated with ASC differentiation and migration by interaction between SPARC and β1 integrin [125]. SPARC-integrin binding also plays a role in pericyte recruitment during cancer progression [126]. Approaches to SPARC targeting could, therefore, have a two-pronged effect. Another potentially interesting target is aminopeptidase N (CD13), which plays a role in tumor angiogenesis and is also expressed by both endothelial and stromal adipose [127]. Recently, by combinatorial phage display approaches [54, 128, 129], a new decorin isoform (delta-DCN) as marker of ASC with progenitor capacity and a peptide specifically

binding to delta-DCN have been identified [31]. Derivatives of this peptide could potentially be developed for directed delivery of therapy depleting ASC serving as perivascular adipocyte progenitors in WAT and possibly directly in tumors. Approaches to targeting mature adipocytes are also being developed [130].

15.7 Unresolved Issues in Adipose Vascular Targeting

It remains to be determined whether targeting individual adipose tissue depots will be possible and if sparing specific depots may be beneficial. Visceral WAT is susceptible to inflammation in severely obese individuals as a result of insufficient oxygenation of grossly enlarged adipocytes, which is the underlying cause of lipotoxicity and the associated pathological consequences [131]. By contrast, subcutaneous WAT, typically remaining sufficiently vascularized, has the potential to benefit metabolism by improving glucose homeostasis and increasing energy consumption [132]. The content and properties of cells differ between visceral and subcutaneous WAT. A recent comparison of angiogenesis occurring in human visceral and subcutaneous WAT did not detect significant differences [95]; however, separately assessing the physiological consequences of targeting vasculature in distinct depots might be important. Although Adipotide appears to not discriminate between WAT depots [117], future studies may uncover agents useful for targeting of vasculature selectively in a WAT depot of interest. Another important issue is the potential importance of vessel types affected by treatment. For example, at this point it is unclear whether Adipotide has a preference toward arterial or venous vasculature and whether vessel size is a predeterminant of peptide binding and effect. In addition, the unclear role of lymphatic vasculature in WAT has to be taken into consideration. Clearly, WAT contains well-vascularized lymph nodes; however, lymphatic capillaries in WAT have not been reported [133].

It is perhaps even more important to foresee the potential effects of targeted therapies on BAT vasculature. A number of results from the rodent models indicate that BAT has a protective effect against the pathological consequences of obesity. The significance of discovering BAT in adults [134] lies in possible new approaches to treatment of obesity and of the associated disorders [52]. Because angiogenesis accompanies BAT formation [135], inhibiting adipose vascularization in a nonspecific manner is expected to have a negative impact on BAT and, therefore, on energy expenditure, potentially defeating the purpose of the treatment. Thus, development of approaches to targeting specifically blood vessels of WAT might be critical. The apparent dependence of adipocyte physiology on the status of the vasculature raises the possibility that vascular-targeting agents could be designed to convert WAT into BAT, rather than destroying tissue altogether, as a more physiological and safer anti-obesity treatment [31]. Development of pharmacological approaches to activate proliferation, vascularization, and/or metabolism of the existing residual BAT could, in theory, tilt the WAT/BAT balance and be used to treat obesity.

15.8 Adipose Vascular Targeting in Cancer Patients?

According to some studies, reduction of WAT mass has protective effects against cancer and other obesity-linked diseases [70], although this has not been consistently confirmed [9]. It is also possible that reduction of adiposity will benefit obese patients who have been diagnosed with cancer. As new vascular-targeting agents are characterized, new options to treat obesity and cancer become available. However, since the composition and function of adipose tissue are complex and multifactorial, it is questionable whether every anti-obesity strategy will benefit patients [8]. Future therapeutic strategies must take into account the biological role of adipose tissue and the well-described pathologies associated with its deficiency [136, 137]. Specifically, the risk of lipotoxicity is noteworthy. WAT lipid storage defects can lead to increased circulating fatty acids that become ectopically deposited in muscle, liver, and other tissues, leading to insulin resistance and metabolic disease, as seen in genetic or pharmacologically induced lipodystrophies [31]. Therefore, aggressive therapies directly or indirectly affecting adipocytes, in the absence of decreased caloric intake, could result in lipotoxicity, which also applies to vascular-targeting approaches. Lack of apparent steatosis in response to the Adipotide is likely due to decreased food consumption and the metronomic regimen of the reported treatments [117]. The extent to which the anorexigenic effects of this and other experimental compounds targeted at vasculature are responsible for weight loss is not easy to assess. But it is certain that antiangiogenesis drugs, to be used for amelioration of obesity without pathological consequences, must be accompanied by concomitant reduction in food intake. The fine balance between WAT and BAT is another important issue to take into consideration in designing vascular-targeting therapies.

It should be noted that adipose tissue targeting might not be an ideal option in particular for cancer patients [8]. Patients undergoing conventional cancer therapies, with chemotherapy or radiotherapy being the most common, tend to experience nausea, upset stomach, and loss of appetite, which results in nutrient deprivation and body weakening. Therefore, the potential adverse side effects of future anti-obesity drugs are a serious issue for cancer patients. An additional concern is the possibility of WAT cell mobilization as a result of certain approaches to weight loss and the hijacking of these cells by tumors. For example, ablation of adipose endothelium and the resulting WAT resorption could represent the setting when intact ASC and infiltrating macrophages become “homeless” and thereby encouraged to execute their trophic functions as components of tumor microenvironment. Although purely speculative at this point, these potential pitfalls are to be tested in animal models.

15.9 Summary

Systemic deregulation of angiogenesis is a hallmark of obesity-associated pathologies including cardiovascular disease, diabetes, and cancer. Therefore, it is possible that targeting neovasculature not only in WAT but also at other sites of ectopic

angiogenesis may have a combined therapeutic benefit. Considering the body of evidence demonstrating a link between obesity and cancer, it is clear that new approaches to modulate WAT content and activity could be beneficial for patients. There is an apparent need for noninvasive treatment of obesity compatible with the health status of cancer patients. There may be a big advantage to minimizing the effects of anti-obesity treatment outside of WAT in order to prevent adverse effects on CNS and gut physiology. New obesity therapeutic prototypes that enable WAT-specific targeting hold a potential promise. However, recent findings indicate that systemic and even WAT-specific inhibition of vascular function could be dangerous. Therefore, careful analysis of preclinical models will be necessary to establish whether vascular targeting does indeed represent a viable approach for the treatment of obesity, in particular in the cancer setting. As the field of vascular targeting and its application to obesity and cancer treatment is evolving, a number of questions remain to be answered. Continued efforts in unraveling the mechanisms through which obesity supports cancer development and progression might open other new avenues for therapy.

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Chapter 16
Anti-inflammatory Effects of Exercise

Michael Gleeson

Abstract Chronic low-level inflammation is associated with obesity and increased incidence of cancer and chronic disease states. Regular exercise reduces the risk of some cancers as well as chronic metabolic and cardiorespiratory diseases, in part because exercise exerts anti-inflammatory effects. The anti-inflammatory effects of regular exercise may be mediated via both a reduction in visceral fat mass (with a subsequent decreased release of adipokines) and the induction of an anti-inflammatory environment with each bout of exercise via the release of IL-6 from working skeletal muscle, reduced expression of TLRs on monocytes/macrophages, inhibition of monocyte/macrophage infiltration into adipose tissue, phenotypic switching of macrophages within adipose tissue, a reduction in the circulating numbers of pro-inflammatory monocytes and an increase in the circulating numbers of regulatory T cells.

16.1 Exercise in Cancer Prevention and Therapy

There is consensus that exercise training protects against some types of cancers and can have a beneficial role in therapy for cancer patients. Exercise training enhances aspects of anti-tumour immunity and reduces inflammatory mediators. However, the data linking immunological and inflammatory mechanisms, physical activity and cancer risk reduction remains tentative. Determining if regular physical activity reduces cancer risk through immunological mechanisms is of public health relevance and could lead to tailored and novel exercise prescriptions. Comprehensive

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reviews by the International Agency for Research on Cancer [8] and the World Cancer Research Fund [104] identified an independent protective effect of physical activity on colon and postmenopausal breast cancer risk. Evidence is also mounting for risk reduction by physical activity for endometrial, lung and pancreatic cancers.

Physical activity has a therapeutic effect in cancer patients by reducing cancer recurrence, enhancing health outcomes and increasing survival. Women who exercised moderately prior to [28] and after a breast cancer diagnosis had significant improvements in overall and disease-specific survival and quality of life compared to sedentary counterparts [82, 100]. Similarly, studies with colorectal [55] and prostate [45, 75] cancer survivors have suggested that mortality and disease progression are approximately 50 % lower in physically active than sedentary individuals.

Numerous cohort studies have shown a link between obesity and cancer incidence overall and for selected cancer sites including endometrial, breast, colon and oesophageal adenocarcinoma [4]. There is also data showing that individuals who lose weight and maintain the loss have a reduced cancer incidence and mortality, providing some hope that weight loss in obese individuals may help them prevent cancer. Several possible mechanisms have been suggested to explain the association of obesity with increased risk of certain cancers: (1) Fat tissue produces excess amounts of oestrogen, high levels of which have been associated with the risk of breast, endometrial and some other cancers; (2) Obese people often have increased circulating levels of insulin and insulin-like growth factor-1, which may promote the development of certain tumours; (3) Fat cells produce adipokines that may stimulate or inhibit cell growth. For example, leptin, which is more abundant in obese people, seems to promote cell proliferation, whereas adiponectin, which is less abundant in obese people, may have antiproliferative effects; (4) Fat cells may also have direct and indirect effects on other tumour growth regulators, including mammalian target of rapamycin (mTOR) and AMP-activated protein kinase; (5) Obese people often have chronic low-level inflammation, which has been associated with increased cancer risk. Other possible mechanisms include altered immune responses, effects on intracellular signalling pathways involving nuclear factor kappa beta and oxidative stress.

Currently, the biological mechanisms relating exercise and cancer are not well understood. Potential mediators include prevention of obesity, reduction in body weight and/or adiposity, decreases in reproductive hormone levels, altered growth factor milieu, enhanced antioxidant defence mechanisms and changes in immune function including reduced inflammation and enhanced anti-tumour immunity. The relative contribution of these mechanisms in specific cancer types remains unknown. With respect to the hypothesis that exercise induces alterations in immune mediators, more is known about exercise-induced changes in inflammatory mediators than about changes in specific anti-tumour mechanisms.

The association between chronic inflammation and cancer is well established [14]. Human cross-sectional studies demonstrate an inverse relationship between regular physical activity and inflammatory biomarkers including C-reactive protein

(CRP), tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) [42, 70]. Reductions in CRP levels with exercise training have been reported [42]. Although exercise may reduce inflammatory biomarkers, clinical trials indicate variable outcomes, with an effect of exercise on CRP in some but not all studies [74]. Less work has been done with IL-6 in humans, but again there are conflicting results [101]. There can be little doubt that regular physical activity is beneficial in preventing some cancers, as well as in decreasing recurrence, increasing survival and improving quality of life for cancer patients. Animal studies (e.g. [17, 18, 39]) indicate that multiple biological pathways may be involved including a reduction in inflammation and an enhancement of anti-tumour immunity. To date, neither of these mechanisms has been studied in adequate detail to fully understand their role in cancer prevention and therapy with respect to exercise.

The focus of this chapter is to explain the various mechanisms by which exercise exerts its anti-inflammatory effects. Additional research is needed to determine which inflammatory mediators and anti-tumour immune mechanisms are most sensitive to exercise and the dose, duration and frequency of exercise needed to achieve the most potent anti-inflammatory or anti-tumour effects.

16.2 The Links Between Sedentary Behaviour, Chronic Inflammation and Chronic Disease

The prevalence of obesity continues to rise worldwide and is being accompanied by proportional increases in a host of other medical conditions associated with derangements of immunometabolism [50] such as type 2 diabetes (T2DM), cardiovascular diseases, chronic obstructive pulmonary disease, dementia, depression and cancer. Inflammation appears to be aetiologically linked to the pathogenesis of all these conditions [65], and the development of a chronic low-grade inflammatory state (as indicated by elevated levels of circulating inflammation markers such as IL-6, TNF-α and CRP) has been established as a predictor of risk for several of them [73]. Importantly, physical inactivity and sedentary behaviour increase the risk of all these conditions [36, 69, 99]. An inactive lifestyle leads to the accumulation of visceral fat and consequently the activation of a network of inflammatory pathways that results in inflammation in adipose tissue, increased release of adipokines (peptides and proteins including some cytokines that are secreted from white adipose tissue) and the development of a low-grade systemic inflammatory state [65]. Chronic inflammation promotes the development of insulin resistance, atherosclerosis, neurodegeneration and tumour growth and subsequently the development of several diseases associated with physical inactivity (Fig. 16.1). Exercise has anti-inflammatory effects, and therefore, in the long term, regular physical activity can protect against the development of these chronic diseases as well as having other benefits for health, functional capacity and quality of life [36, 69] which are summarised in Table 16.1. Furthermore, exercise can be used as a treatment for (or to

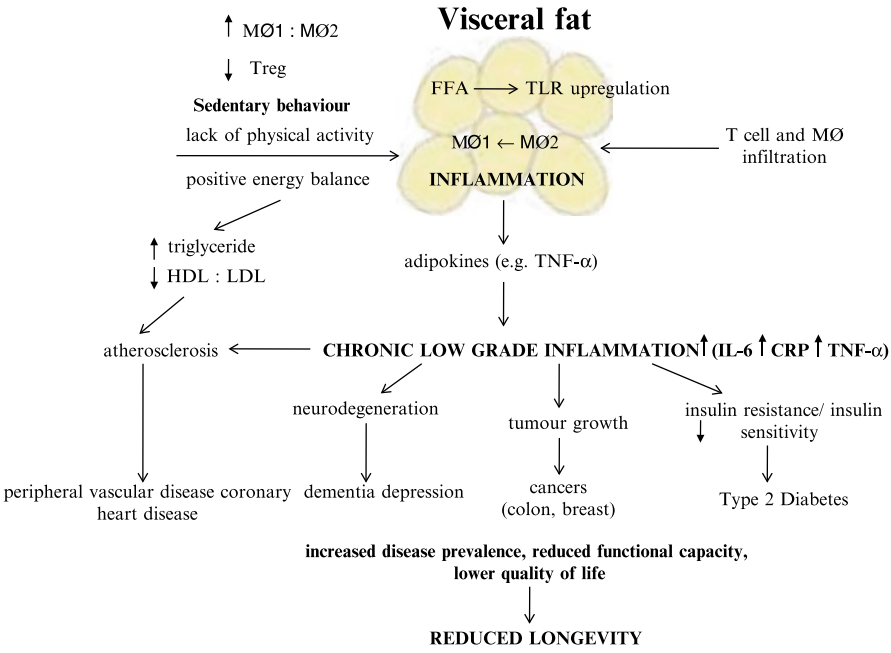

Fig. 16.1 Physical inactivity and positive energy balance lead to an accumulation of visceral fat which becomes infiltrated by pro-inflammatory macrophages and T cells. The pro-inflammatory M1 macrophage phenotype predominates and inflamed adipose tissue releases adipokines and TNF-α that lead to a state of persistent low-grade systemic inflammation. This promotes the development of insulin resistance, tumour growth, neurodegeneration and atherosclerosis. The latter is exacerbated by the deleterious changes in the blood lipid profile associated with a lack of physical activity

ameliorate the symptoms of) many of these conditions which is increasingly being promoted as the concept that "exercise is medicine".

Obviously, exercise increases energy expenditure and burns off some of the body fat that would otherwise accumulate when individuals eat more dietary energy than they need. In that simple sense, exercise reduces the risk of developing obesity and excessive adiposity. Regular exercise also imbues cardiovascular health benefits by improving the blood lipid profile by decreasing the concentration of plasma triglycerides and small LDL particles and by increasing the concentration of protective HDL cholesterol [46]. These beneficial alterations in plasma lipids are presumed to limit the development of atherosclerosis. However, the protective effect of a physically active lifestyle against chronic inflammation associated diseases may, to some extent, be ascribed to an anti-inflammatory effect of exercise [42]. This may be mediated not only via a reduction in visceral fat mass (with a subsequent decreased production and release of adipokines) but also by induction of an anti-inflammatory environment with each bout of exercise [70]. The remainder of this chapter will explain the possible mechanisms by which exercise exerts its anti-inflammatory effect.

Table 16.1 Summary of the interaction between physical activity and major diseases assessing evidence that exercise may (a) lower disease risk and (b) have therapeutic value in treating disease (see Pedersen and Saltin [69] and Hardman and Stensel [36] for further detail)

Disease	Evidence that physical activity may lower disease risk and/or have therapeutic value in treating disease
Cancer	High levels of PA are associated with lower risk of colon and breast cancer. PA may lower cancer risk by systemic (reduced body fat and insulin levels, enhanced immune function) and site-specific (reduced sex steroid hormone levels for breast cancer, decreased bowel transit time for colon cancer) mechanisms. Some observational and RCT evidence supports a therapeutic role for PA in preserving mobility and function in cancer patients
CHD	A large body of epidemiological evidence demonstrates that high levels of PA and PF are associated with a lower risk of developing CHD. RCTs show that regular PA can favourably modify CHD risk factors including (but not limited to) dyslipidaemia, hypertension and obesity. RCTs also show that PA improves survival in CHD patients
Stroke	Evidence that high levels of PA and PF reduce the risk of stroke is suggestive but not as compelling as that for CHD. RCTs show that PA can lower, but not necessarily normalise, blood pressure in hypertensive individuals
T2D	Observational epidemiological evidence consistently demonstrates an association between high levels of PA/PF and a reduced risk of developing T2D. RCTs show that lifestyle intervention (diet and PA) can lower body mass, improve glucose tolerance and reduce the risk of developing T2D in high-risk patients. In patients with T2D, high levels of PA and PF are associated with a reduced risk of CHD and all-cause mortality
Dementia	Observational epidemiological studies indicate that higher levels of PA are associated with a lower risk of cognitive decline and dementia in older adults. Some limited evidence is available from RCTs to suggest that PA induces modest improvements in cognition in people who are at increased risk of dementia/Alzheimer's disease
Other	There is some evidence from observational and intervention studies to support a role for PA for enhancing physical function and improving quality of life in those suffering from chronic heart failure, chronic obstructive pulmonary disease, depression, intermittent claudication, osteoarthritis and osteoporosis

CHD coronary heart disease; *PA* physical activity; *PF* physical fitness; *RCT* randomised controlled trial; *T2D* type 2 diabetes mellitus

16.3 Anti-inflammatory Effects of Exercise

The anti-inflammatory effects of exercise have mostly been ascribed to two possible mechanisms: (1) increased production and release of anti-inflammatory cytokines from contracting skeletal muscle [70] and (2) reduced expression of toll-like receptors (TLRs) on monocytes and macrophages [26] with subsequent inhibition of downstream responses such as pro-inflammatory cytokine production, antigen presentation and costimulatory molecule expression [33]. However, the anti-inflammatory effects of exercise arise not only from these two mechanisms but also other effects of exercise that recently have been established such as the inhibition of monocyte/macrophage infiltration into adipose tissue [43], phenotypic switching of

macrophages within adipose tissue [43], a reduction in the circulating numbers of pro-inflammatory monocytes [95] and an increase in the circulating numbers of IL-10-secreting regulatory T cells [98, 107]. The major focus of this part of the chapter is to explain these various mechanisms.

16.3.1 Reduction in Visceral Fat Mass

The accumulation of body fat, particularly in the abdomen, liver and muscles, is associated with increased all-cause mortality [71], the development of T2DM [5], cardiovascular disease [35], dementia [102] and several cancers [105]. The production of pro-inflammatory adipokines is increased with adipose tissue expansion whereas the amounts of anti-inflammatory cytokines produced are reduced. This leads to the development of a state of persistent system low-grade inflammation [108]. Regular exercise can reduce waist circumference and cause considerable reductions in abdominal/visceral fat, even in the absence of any loss of body weight, in both men and women regardless of age [77]. Therefore, increased physical activity can bring about a reduction in systemic inflammation [108] via a reduction in pro-inflammatory adipokine secretion as a direct result of lowering the amount of fat stored in abdominal depots.

16.3.2 Release of IL-6 from Contracting Muscle

One of the earliest reports on the effects of exercise on cytokines observed an elevation in the circulating levels of several cytokines following the completion of a marathon [60]. Some years later, several reports on the effects of exercise on cytokines began to appear in the scientific literature—the impetus behind this increase was probably the development of sensitive, specific, commercially available, assays for the detection of a large number of cytokines. One of the earliest and most consistent findings has been that of an elevation in the circulating level of IL-6 following prolonged strenuous exercise. In an important study, Nehlsen-Cannarella et al. [58] demonstrated that the plasma IL-6 concentration was dramatically increased following 2.5 h of high-intensity running. Furthermore, it was shown that when subjects consumed a carbohydrate beverage during exercise, the increase in the circulating IL-6 concentration was decreased compared with subjects who consumed a placebo. While these studies provide no mechanistic insight into the source of the exercise-induced increase in the circulating IL-6 concentration or its biological purpose, this study acted as a stimulus for subsequent investigations into the effects of exercise on cytokines. Indeed, many subsequent studies have demonstrated an increase in the circulating concentration of several cytokines following prolonged strenuous exercise. Increases in the circulating concentrations of both pro-inflammatory cytokines (e.g. IL-1β, TNF-α) and anti-inflammatory cytokines

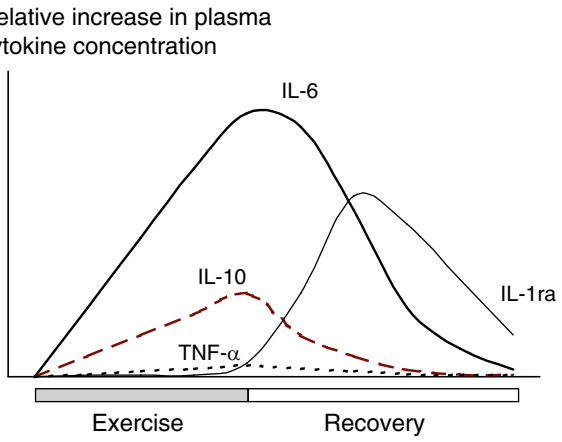

Fig. 16.2 The cytokine response to prolonged strenuous exercise (modified from Febbraio and Pedersen [21])

(e.g. IL-6 and IL-10) [62, 63], cytokine inhibitors (e.g. IL-1 receptor antagonist and soluble TNF receptors) [62], chemokines (e.g. IL-8, macrophage inflammatory protein (MIP) and monocyte chemotactic protein-1 (MCP-1)) [64, 91, 92] and colony-stimulating factors [92] have been reported following endurance exercise. However, the finding of an increase in the circulating IL-6 concentration following prolonged exercise is the most marked and consistent exercise-induced response of any cytokine so far examined (see Fig. 16.2).

At rest, approximately 30 % of circulating IL-6 arises from the adipose tissue but only about 10 % of this can be attributed to the adipocytes with the remainder coming mostly from adipose tissue resident macrophages. Other sources of circulating IL-6 at rest include blood leukocytes (predominantly monocytes), the brain and the liver. An early study indicated that circulating monocytes were unlikely to be the source of the exercise-induced increase in the plasma IL-6 concentration [97] as 1 h of strenuous exercise caused no changes in the amount of IL-6 mRNA detected in peripheral blood mononuclear cells (PBMCs) despite an elevation in the plasma IL-6 level. This finding was later confirmed by Starkie et al. [84] who demonstrated that monocyte intracellular IL-6 protein expression was unchanged following a bout of prolonged strenuous exercise; importantly, Starkie et al. [85] also demonstrated that exercise had no significant effects on TNF-α or IL-1β production from monocytes. Several possible sites of origin where suggested for the exercise-induced increase in the circulating level of IL-6, with contracting skeletal muscle receiving the most attention following the observation by Steensberg et al. [89] that IL-6 released from the exercising leg could account for the rise in the circulating IL-6 concentration during prolonged exercise.

Although initial studies supported the hypothesis that an increase in the plasma level of IL-6 was related to exercise-induced muscle damage [12], it soon became apparent that muscle damage per se was only a minor contributor to the exercise-induced rise in the circulating level of IL-6. Firstly, although many of the initial studies examining the effects of exercise on cytokines used running as an exercise model, several studies have also examined the cytokine response to prolonged

bicycle exercise which induces only a minimal degree of muscle damage (if any) and consequently does not trigger an inflammatory response. However, cycling exercise does result in a considerable elevation (typically 3–10-fold higher than resting values) in the circulating IL-6 concentration comparable to that of running at the same mode-specific relative intensity [86]. However, perhaps the strongest evidence that muscle damage is not a prerequisite for an increase in the systemic IL-6 concentration in response to exercise came from Croisier et al. [15]. In this study, subjects performed two bouts of eccentric muscle contractions separated by a period of 3 weeks. After the initial exercise bout, the expected elevation in serum myoglobin (a marker of muscle damage) and delayed onset muscle soreness was observed, in addition to a rise in the circulating IL-6 concentration. Importantly, it is well known that following a period of recovery from an initial bout of muscle damaging exercise, a second exercise bout identical to the first causes a much lower level of muscle damage. Therefore, and as expected, the second exercise bout resulted in minimal increases in serum myoglobin and muscle soreness, yet the increase in the circulating IL-6 concentration was very similar to that observed in response to the initial bout of exercise. These studies provide compelling evidence that the increase in the circulating IL-6 concentration following exercise is not primarily related to muscle damage.

During and following exercise of sufficient load, the active skeletal muscle markedly increases both cellular and circulating levels of IL-6 [66]. With prolonged exercise (>2.5 h), plasma IL-6 levels can increase over 100-fold although more modest increases are reported with shorter duration exercise [24]. Increases have also been noted using intermittent exercise protocols of relatively short duration [54]. With exercise, the increase in circulating IL-6 is transient, normally returning to resting levels within 1 h after exercise. The plasma IL-6 concentration increases exponentially with exercise duration [89], and a major stimulus of its synthesis and release appears to be a fall in muscle glycogen content ([44, 67]; Fig. 16.3). Increases in intracellular calcium and increased formation of reactive oxygen species are also capable of activating transcription factors known to regulate IL-6 synthesis [24].

IL-6 appears responsible for the subsequent rise in circulating levels of the anti-inflammatory cytokines IL-10 and IL-1 receptor antagonist (IL-1ra) and also stimulates the release of cortisol from the adrenal glands [88]. The causal role of IL-6 in stimulating IL-10, IL-1ra and cortisol secretion is substantiated by the observation that intravenous infusion of IL-6 totally mimics the acute anti-inflammatory effects of a bout of exercise both with regard to elevations of plasma IL-10, IL-1ra and cortisol [88] and with regard to suppression of endotoxin-stimulated increases in TNF-α levels [83]. These actions and possible associated health effects are summarised in Fig. 16.4. The IL-6 half-life is prolonged by combining with the soluble IL-6 receptor (sIL-6R), and it is this complex which is crucial in determining the biological activity of IL-6. The expression of IL-6R in tissues is limited to hepatocytes, leukocytes and adipocytes [76] with a relatively low expression in resting skeletal muscle [44]. Exercise training increases the expression of IL-6R on the muscle membrane [44] removing some of the dependency on the circulating sIL-6 receptor.

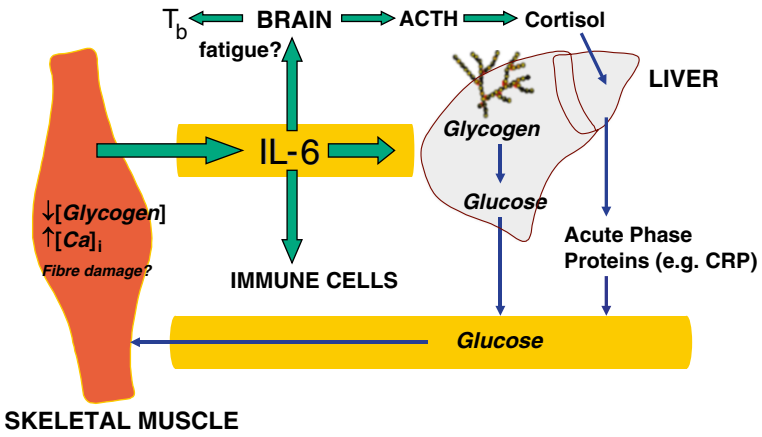

Fig. 16.3 Rises in intracellular calcium ion concentration with contraction and glycogen depletion stimulate the production of IL-6 by the working muscles. IL-6 is then released from the muscle resulting in an elevation in the systemic IL-6 concentration. IL-6 also stimulates liver glycogen breakdown in the liver, lipolysis in adipose tissue and the secretion of ACTH, cortisol, IL-10, IL1ra and CRP and may also be involved in the development of central fatigue

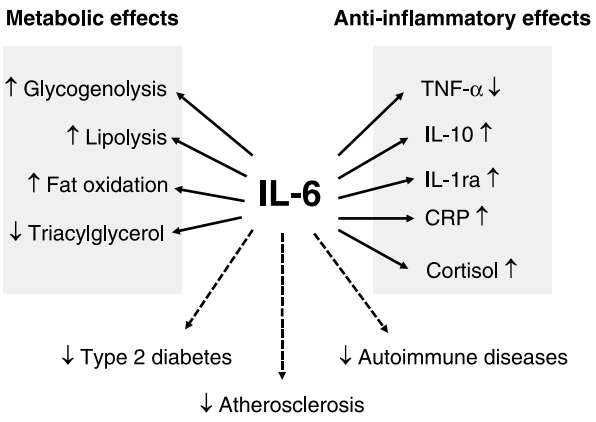

Fig. 16.4 Biological role of muscle-derived IL-6 during exercise. *Full lines* represent acute effects of IL-6 infusion or exercise-induced elevations of circulating IL-6. *Broken lines* represent possible health effects of IL-6 (modified from Steensberg [87])

IL-1ra is secreted mainly by monocytes and macrophages and inhibits the pro-inflammatory actions of IL-1β [27]. IL-10 is known to be produced primarily by regulatory T cells and monocytes but also by Th2 cells, macrophages, dendritic cells, B cells, CD8+ T cells, Th1 cells and Th17 cells [52]. Irrespective of the cellular source, the principal role of IL-10 appears to be containment and suppression of inflammatory responses so as to downregulate adaptive immune effector responses [57] and minimise tissue damage in response to microbial challenges. Accordingly, IL-10 induces downregulation of MHC antigens, the intercellular adhesion molecule-1 and the costimulatory molecules CD80 and CD86 on antigen

presenting cells, and it has been shown to promote differentiation of dendritic cells expressing low levels of MHC class II, CD80 and CD86 [52]. In addition, IL-10 downregulates or completely inhibits the expression of several pro-inflammatory cytokines and other soluble mediators, thereby further compromising the capacity of effector T cells to sustain inflammatory responses.

Thus, IL-10 is a potent promoter of an anti-inflammatory state. Circulating levels of IL-10 are lower in obese subjects, and acute treatment with IL-10 prevents lipid-induced insulin resistance [37]. IL-10 increases insulin sensitivity and protects skeletal muscle from obesity-associated macrophage infiltration, increases in inflammatory cytokines and their deleterious effects on insulin signalling and glucose metabolism [37].

A limitation of the hypothesis that exercise-induced elevations of IL-6 are mostly responsible for the anti-inflammatory and long-term health benefits of regular exercise is that substantial increases in circulating IL-6 do not occur with short durations of low-/moderate-intensity exercise [24] despite the known health benefits (e.g. reduced risk of heart disease) associated with only very moderate increases in physical activity above that of a totally sedentary lifestyle [56].

16.3.3 Increased Levels of Circulating Cortisol and Adrenaline

Secretion of the adrenal hormones cortisol and adrenaline into the circulation is increased during exercise due to activation of the hypothalamic-pituitary-adrenal axis (HPA) and the sympathetic nervous system (SNS), respectively. Impulses from the motor centres in the brain as well as afferent impulses from working muscles elicit an intensity-dependent increase in sympathoadrenal activity and in release of some pituitary hormones including ACTH [29]. Increased SNS activity stimulates adrenaline and noradrenaline release from the adrenal medulla within seconds of the onset of exercise and ACTH stimulates cortisol secretion from the adrenal cortex within a matter of minutes. These hormonal responses usually precede the rises in circulating concentrations of cytokines. Thus, the magnitude of the elevations in plasma cortisol and adrenaline is related to the intensity and duration of exercise [29]. Cortisol is known to have potent anti-inflammatory effects [16], and catecholamines downregulate the lipopolysaccharide (LPS)-induced production of TNF-α, IL-6 and IL-1β by immune cells [7]. Cortisol secretion is also augmented by the aforementioned rise in circulating IL-6 [88]. Thus, hormones, myokines and cytokines all contribute to the anti-inflammatory effect of exercise (Fig. 16.5).

16.3.4 Inhibition of Macrophage Infiltration into Adipose Tissue

The expression of pro-inflammatory cytokines, chemokines and cell adhesion molecules in adipose tissue is increased in obese mice [38]. Macrophages and also

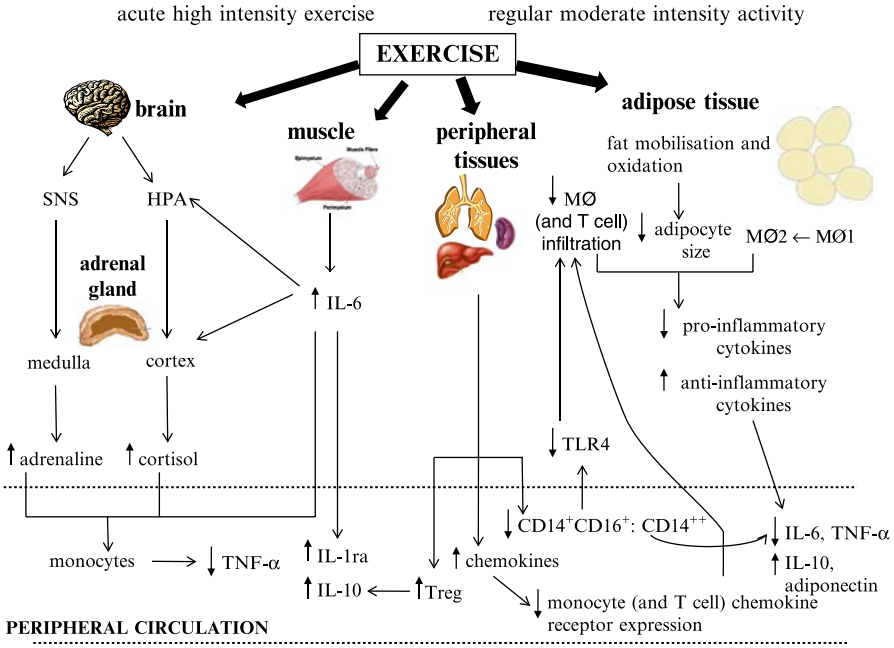

Fig. 16.5 Potential mechanisms contributing to the anti-inflammatory effects of exercise. Activation of the HPA and sympathetic nervous system (SNS) leads to the release of cortisol and adrenaline from the adrenal cortex and medulla, respectively. These hormones inhibit the release of TNF-α by monocytes. IL-6 produced by contracting skeletal muscle also downregulates the production of TNF-α by monocytes and may stimulate further cortisol release. Acute elevations in IL-6 stimulate release of IL-1 receptor antagonist (IL-1ra) from monocytes and macrophages, thus increasing the circulating concentrations of this anti-inflammatory cytokine. Exercise training mobilises T_{Reg} cells, a major source of the anti-inflammatory cytokine IL-10, and decreases the proportion of inflammatory (CD14+CD16+) monocytes, compared with classical (CD14++) monocytes. Following exercise, CD14++ monocytes express less TLR4 and thereby induce a reduced inflammatory response, marked by lower levels of pro-inflammatory cytokines and reduced adipose tissue infiltration. Exercise also increases plasma concentrations of key inflammatory immune cell chemokines; repeated elevations of such chemokines may lead to a downregulation of their cellular receptors, resulting in reduced tissue infiltration. A reduction in adipose tissue mass and adipocyte size, along with reduced macrophage infiltration, and a switch from a M1 to M2 phenotype all may contribute to a reduction in pro-inflammatory cytokine release from adipose tissue, such as IL-6 and TNF-α, and an increase in anti-inflammatory cytokine release, such as adiponectin and IL-10

T cells, which infiltrate adipose tissue in obesity, are known to regulate the inflammatory state of adipose tissue [41]. Thus, the migration of PBMCs towards sites of inflammation, including adipose tissue and damaged vascular endothelium, is central to the development of sustained tissue inflammation [109]. It is thought that the size of the adipocytes triggers macrophage infiltration rather than overall obesity and that recruitment of the macrophages may be stimulated by the chemokines, MCP-1 and MIP [11].

Exercise might limit the movement of PBMCs into inflamed adipose tissue in a similar manner to its effect of reducing PBMC migration towards a virus-infected human bronchial epithelial cell line [9]. Migration of PBMCs from the circulation into the tissues is a tightly regulated process involving a gradient of release of chemokines from the inflamed tissue (including from immune cells residing within), the expression of complimentary chemokine receptors on PBMCs and the expression of adhesion molecules on both immune and endothelial cells. Acute bouts of exercise reduce T cell migration towards the supernatants of human rhinovirus-infected human BEAS-2B airway epithelial cells in a manner that is independent of any involvement of adhesion molecules or exercise-induced elevations of cortisol or catecholamines [9]. However, it is known that acute exercise stress results in the release of chemokines into the circulation from multiple sources, and sustained exposure of PBMCs to physiological concentrations of chemokines including MCP-1 results in chemokine receptor internalisation [48]. This is thought to serve as a negative feedback mechanism to reduce migration and thereby terminate accumulation of PBMCs in inflamed tissue. It is therefore possible that an active lifestyle creates an environment of repeated short-lasting elevations in plasma chemokines that act over time to downregulate expression of their receptors on PBMCs and restrict migration of these cells towards adipose tissue. However, this potential mechanism needs to be explored further in humans.

Whether exercise acts to inhibit the release of chemokines from human adipose tissue and in this way reduce macrophage infiltration is not clear. Certainly, evidence from murine studies has shown that obese mice deficient in the macrophage chemokines MCP-1 and CXCL14 do not exhibit inflammatory responses such as macrophage infiltration, increased tissue expression of IL-6 and insulin resistance [41]. However, while exercise training reduces macrophage infiltration into adipose tissue in obese mice it has little effect on adipose tissue MCP-1 and CXCL14 expression [43], perhaps suggesting that these chemokines are not key to the exercise-induced restriction of macrophage infiltration of adipose tissue. Recent findings in humans also do not report any independent effect of 12 weeks of exercise training on adipose tissue expression of MCP-1 (or MIP, TNF-α and IL-6), despite falls in circulating concentrations of MCP-1 with exercise. Diet-induced weight loss or weight loss in combination with exercise was associated with non-significant falls in adipokine mRNA expression in adipose tissue, and significant decreases in CD14 expression in adipose tissue were only found with diet alone.

In mice, training is reported to decrease the tissue expression of intercellular adhesion molecule-1 (ICAM-1) [43], the expression of which is known to be increased in obesity in humans [10]. Furthermore, antagonism of ICAM-1 in obese mice prevents macrophage infiltration into adipose tissue [13], and circulating ICAM-1 levels were reduced by 6 months of progressive aerobic exercise training in patients with T2DM without changes in body mass and waist circumference [110]. Obviously, further studies in humans are required to ascertain the role of exercise training on ICAM-1 in adipose tissue, but ICAM-I might also play a role in the exercise-induced reduction of macrophage infiltration into adipose tissue.

Macrophage activation has been defined into two separate polarisation states, M1 and M2 [49]. The M1 macrophage produces TNF-α, IL-6 and nitric oxide, while the M2 macrophage produces anti-inflammatory cytokines and arginase. Therefore, M1 macrophages induce a chronic inflammatory state, and M2 macrophages subdue inflammation in adipose tissue. The inflammation state of adipose tissue also appears to be associated with a preferential recruitment of M1 macrophages and/or a phenotypic switch of macrophage polarisation in adipose tissue towards the M1 phenotype. Therefore, it is possible that the attenuated inflammatory state of adipose tissue associated with chronic exercise training occurs by both suppression of macrophage infiltration and acceleration of phenotypic switching from M1 to M2 macrophages. A recent study in mice fed with a high-fat diet to induce obesity provided some evidence that exercise training induces the phenotypic switching from M1 to M2 macrophages in adipose tissue as well as inhibiting M1 macrophage infiltration into adipose tissue [43], although studies in humans are lacking.

16.3.5 Down-Regulation of Monocyte/Macrophage TLR Expression

TLRs are highly conserved transmembrane proteins that play an important role in the detection and recognition of microbial pathogens, and they can also be activated by endogenous danger signals of tissue damage such as heat shock proteins [40]. The key product of TLR signalling in antigen presenting cells is the production of pro-inflammatory cytokines and proteins, and thus, the TLR pathway plays an important role in mediating whole body inflammation [93]. Following a prolonged bout of strenuous exercise, the expression of TLRs 1, 2 and 4 on monocytes is decreased for at least several hours ([33, 47, 61]; Fig. 16.6). Prolonged exercise also results in a decreased induction of MHCII (Fig. 16.7), costimulatory molecules CD80 and CD86 and cytokines following stimulation with known TLR ligands [47]. Whether this reduction in cell-surface expression of TLRs is due to a down-regulation of TLR gene expression, a shedding of TLRs from the cell surface or re-internalisation by the cell remains to be established.

Evidence is now emerging that TLRs may be involved in the link between a sedentary lifestyle, inflammation and disease. Exercise training studies and cross-sectional comparisons between physically active and inactive subjects have shown a reduced inflammatory response of blood monocytes to endotoxin stimulation in vitro and a lowered TLR4 expression at both the cell surface and mRNA level in physically active individuals ([26, 33]; Fig. 16.8) which is associated with decreased inflammatory cytokine production [90] and has been shown to occur in both young and elderly adults (Fig. 16.9). Over the long term, a decrease in TLR expression may represent a beneficial effect for health because it decreases the inflammatory capacity of leukocytes, thus altering whole body chronic inflammation and possibly reducing the risk of developing chronic disease. The precise physiological stimulus

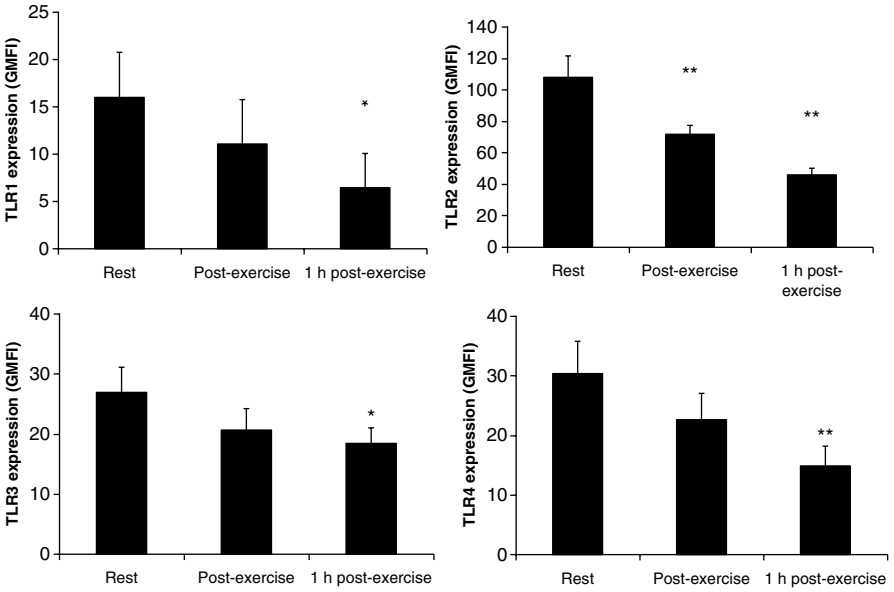

Fig. 16.6 Effect of 2.5-h cycling at 60 %VO_2 max in temperate (20 °C) conditions on monocyte toll-like receptor (TLR expression) (data from Oliveira and Gleeson [61])

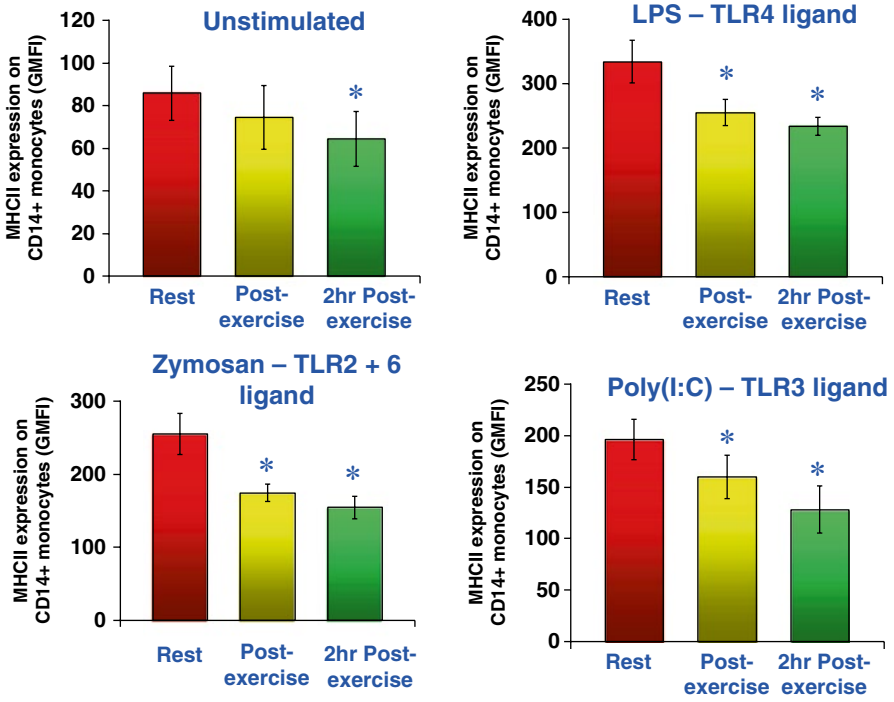

Fig. 16.7 Effects of exercise on TLR activation by TLR ligands—upregulation of MHCII. *Asterisk* indicates significantly different from pre-exercise (data from Lancaster et al. [47])

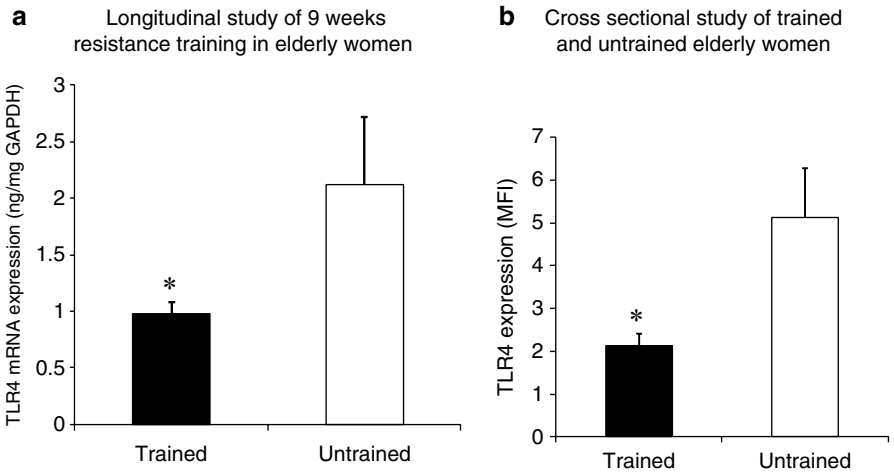

Fig. 16.8 Effect of exercise training and TLR4-mRNA and TLR4 expression. (**a**) A longitudinal study of 9 weeks resistance training in elderly women (data from Flynn et al. [25]) and (**b**) a cross-sectional study of trained and untrained elderly women (data from McFarlin et al. [53])

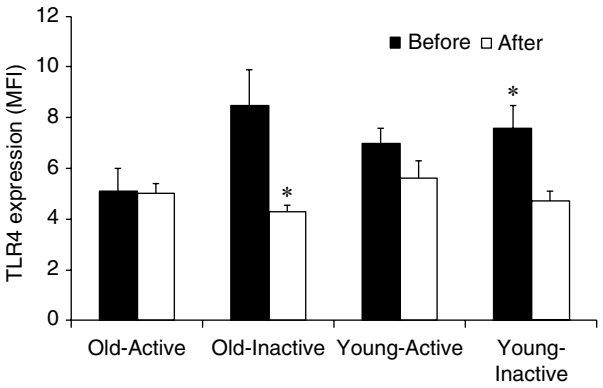

Fig. 16.9 Effects of moderate exercise training and age on monocyte TLR4 expression. Data are for old and young subjects before and after a 12-week training intervention in the inactive groups or maintained activity level in the active groups (modified from Stewart et al. [90])

mediating an exercise-induced decrease in cell-surface TLR expression is not known; however, several possible signals have been implicated including anti-inflammatory cytokines, stress hormones and heat shock proteins [33].

16.3.6 Reduced Numbers of Pro-inflammatory Monocytes in Blood

There are two main populations of monocytes, classical (CD14++) and inflammatory (CD14+CD16+), that differentially express cell-surface TLR4, with the

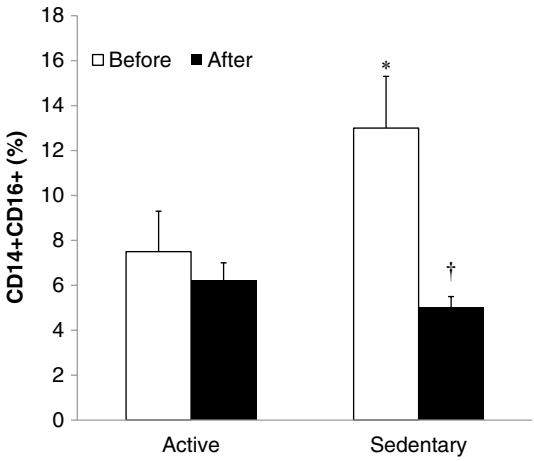

Fig. 16.10 Inflammatory monocyte subset (CD14+CD16+) percentage in physically active and sedentary subjects before and after 12 weeks of exercise training (for sedentary subjects) or continued physical activity (for active subjects). *Baseline group effect ($P<0.05$). **Significant group×time effect ($P<0.05$) (modified from Timmerman et al. [95])

inflammatory monocytes expressing 2.5 times more cell-surface TLR4 [81]. Despite constituting only 10 % of the total monocyte population, inflammatory monocytes contribute significantly to the inflammatory potential of the monocyte pool as a whole [6]. The circulating, inflammatory monocyte percentage is elevated in patients with rheumatoid arthritis [2], cardiovascular disease [79] and T2DM [34], and it has been suggested that inflammatory monocytes play a significant role in the pathogenesis of several diseases linked to inflammation and obesity [72]. Transient increases in the inflammatory monocyte percentage after a single, acute bout of intense exercise have been observed [80] followed by a rapid return to baseline during recovery, but regular exercise appears to reduce the proportion of inflammatory monocytes in the circulation in the resting state. For example, a cross-sectional comparison of healthy physically inactive elderly men and women with an age-matched physically active group indicated that sedentary people have a twofold higher percentage of circulating, inflammatory monocytes [95]. Furthermore, just 12 weeks of regular exercise training significantly reduced the percentage of inflammatory monocytes in the inactive group to the level of the active group (Fig. 16.10), and endotoxin-stimulated TNF-α production was reduced significantly after the training intervention. Based on previous reports that glucocorticoid therapy selectively depletes CD14+CD16+ monocytes [23], it is interesting to speculate that exercise-induced transient spikes in cortisol may have played a role in reducing CD14+CD16+ monocytes with exercise training.

16.3.7 Increased Circulating Numbers of Regulatory T Cells

CD4+CD25+ regulatory T cells specifically express the gene encoding forkhead/winged helix transcription factor (Foxp3) [78] and suppress immune responses via cell contact-dependent mechanisms. Studies show that the depletion of these cells

causes autoimmune disease and enhances the immune response to foreign antigens [22]. Interestingly, a recent study showed that a 12-week programme of tai chi chuan exercise induced a significant increase in regulatory T cells [107]. Production of the regulatory T cell mediators transforming growth factor α (TGF-α) and IL-10 in response to specific antigen stimulation (varicella zoster virus) was also significantly increased after this exercise programme. Furthermore, a study of patients with T2DM showed that regular tai chi chuan exercise altered the Th1/Th2/Treg balance by increasing Foxp3 but not TGF-α expression [106].

In a study that used a running mouse model, the responses of circulating regulatory T cells to moderate and high-intensity exercise training were examined. Only the high-intensity training resulted in increases in regulatory T cell numbers and activation and was also associated with reduced pro-inflammatory and increased anti-inflammatory cytokine expression [98]. Intriguingly, the logical conclusion from these findings is that high-intensity exercise training might be more beneficial than moderate intensity in reducing risk of chronic cardiovascular and metabolic diseases via its anti-inflammatory effects. This notion is supported by another recent study that showed that the combination of high-intensity aerobic plus resistance exercise training, in addition to daily physical activity, is required to achieve a significant anti-inflammatory effect in T2DM patients [3].

16.3.8 Other Factors

During acute exercise, there is also a marked increase in growth hormone, prolactin, heat shock proteins, and other factors that have immunomodulatory effects [68]. Taken together, it appears that each bout of exercise induces an anti-inflammatory environment. Various mechanisms can contribute to this (Fig. 16.5), and it seems likely that their relative importance will vary dependent on the frequency, intensity and duration of the exercise performed. Intuitively, we might expect IL-6 to assume greater relative importance when the exercise is prolonged and glycogen-depleting whereas catecholamine-mediated effects are likely to assume greater importance with shorter duration, high-intensity exercise.

16.4 Exercise Is Medicine

In view of the anti-inflammatory effects of exercise described above and the role of inflammation in the pathogenesis of disease, it is not surprising that exercise is now considered a prophylactic for preventing several major diseases as well as an effective therapy for many conditions/diseases (Table 16.1). Perhaps the strongest evidence for the role of exercise in disease prevention comes from randomised controlled trials evaluating the effectiveness of lifestyle intervention in preventing T2DM (for a review, see [30]). These studies have demonstrated conclusively that

lifestyle intervention (combined diet and exercise) is effective in preventing T2DM in groups of individuals who are at high risk of the disease by virtue of having impaired fasting glucose and/or impaired glucose tolerance as well as being overweight/obese. A limitation of these studies is that they did not isolate the independent effects of exercise and diet in preventing T2DM, but the effectiveness of exercise is supported by the finding in the Finnish Diabetes Prevention Study [96] that among those in the intervention group who did not reach the goal of losing 5 % of their initial body mass but who achieved the goal of exercising for more than 4 h per week, the odds ratio of diabetes was 0.2 (i.e. 80 % lower) than in intervention participants who remained sedentary. Thus, although more needs to be learnt about the role of exercise in preventing T2DM, it is clear that exercise makes a valuable contribution to an overall lifestyle package for preventing this disease.

In addition, exercise appears to have major benefits for the treatment of T2DM. The findings of one non-randomised study (the Malmö feasibility study) showed that 54 % of participants with early stage T2DM were in remission by the end of a 5-year diet and exercise intervention [20]. Moreover, prospective observational studies indicate that high levels of physical activity and/or physical fitness are effective in reducing the risk of cardiovascular disease and/or all-cause mortality and there is evidence implicating inflammation in the pathogenesis of T2DM [50], and it is therefore likely that the therapeutic benefits of exercise for those with T2DM are due, at least in part, to the well-established anti-inflammatory effects of regular exercise [42].

Aside from its role in preventing and treating T2DM, there is a good evidence that exercise is effective in preventing several other major diseases particularly cardiovascular disease [94], breast cancer [19] and colon cancer [103], and there is some evidence to support a role of exercise in preventing dementia [1]. Moreover, while exercise should not be considered a panacea there is evidence to support a role for exercise as a therapy for many diseases/conditions beyond those mentioned above including chronic obstructive pulmonary disease, chronic kidney disease, asthma and osteoporosis [69].

16.5 The Elite Athlete Paradox

Although regular moderate-intensity exercise is associated with a reduced incidence of upper respiratory tract infection (URTI) compared with a completely sedentary state [51, 59], the long hours of hard training that elite athletes undertake appears to make them more susceptible to URTIs [31]. This is also likely attributable to the anti-inflammatory effects of exercise inducing a degree of immunodepression. An increased risk of minor infections may be the (small) price to be paid for the long-term health benefits of regular exercise at high dosage.

A recent murine study indicated that intensive exercise training results in an increased anti-inflammatory cytokine (IL-10) response to antigen exposure [98], and a study on human endurance athletes revealed that those who were illness-prone during a 4-month period of winter training had fourfold higher IL-10

production by antigen-stimulated whole blood culture compared with athletes who remained illness-free during the same period [32]. There is now extensive evidence from both murine and human studies that IL-10 production usually imposes some limits on the effectiveness of antipathogen immune responses, especially innate immunity and adaptive Th1 responses. These studies suggest that very high training loads induce a large enough anti-inflammatory state to increase the risk of picking up minor infections.

16.6 Conclusions

Regular exercise reduces the risk of some cancers as well as chronic metabolic and cardiorespiratory diseases, in part because exercise exerts anti-inflammatory effects. The anti-inflammatory effects of regular exercise may be mediated via both a reduction in visceral fat mass and the induction of an anti-inflammatory environment with each bout of exercise. Various mechanisms may contribute to the anti-inflammatory effects of exercise including increased release of IL-6 from working skeletal muscle, increased release of cortisol and adrenaline from the adrenal glands, reduced expression of TLRs on monocytes/macrophages, inhibition of monocyte/macrophage infiltration into adipose tissue, phenotypic switching of macrophages within adipose tissue, a reduction in the circulating numbers of pro-inflammatory monocytes and an increase the circulating numbers of regulatory T cells. At present, we do not know what the relative importance of the different anti-inflammatory mechanisms that have thus far been identified are, though it seems likely that this will depend on the modes, frequencies, intensities and durations of exercise performed. The anti-inflammatory effects of exercise are also likely to be responsible for depressed immunity that makes the elite athlete more susceptible to common infections.

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