16 Vitamin A

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Key Points

1. Retinoids are one of the most effective classes of agents for promoting cell differentiation, and therefore are of strong interest for cancer prevention and cancer therapy. Nevertheless, despite a great deal of testing, their use in cancer chemoprevention has been limited by the side effects associated with most compounds.
2. Vitamin A, also known as retinol, and retinoic acid (RA) are widely recognized as important factors in the maintenance of healthy cells and tissues. RA possesses a fundamental ability to regulate cell growth, generally by slowing the rate of the cell cycle, and to induce immature and transformed cells to differentiate toward a more mature phenotype.
3. Retinol is an essential nutrient that serves as the substrate for the production, within various cells, of retinal required for rhodopsin biosynthesis, and for the production of RA, which functions as a critical regulator of cellular functions in essentially all tissues. RA is now recognized as a potent regulator of gene expression.
4. Metabolism is central to the biological basis of vitamin A's actions in cancer prevention. Retinoid metabolism is closely regulated through a variety of homeostatic mechanisms including transport proteins, intracellular chaperone proteins, nuclear receptors, and enzymes. The apparent "goal" of the body's homeostatic mechanisms is to maintain steady levels of plasma retinol and RA, which in turn assure a well-regulated exposure of extrahepatic tissues to these molecules.
5. There is no compelling evidence that changing current recommendations for dietary vitamin A would be helpful in reducing cancer risk. A recent report on the topic of "Multivitamin/Mineral Supplements and Chronic Disease Prevention" concluded that while supplement use has grown and now more than half of the adult population of the USA uses multivitamin/mineral supplements, most of the studies that were reviewed do not provide strong evidence for health-related effects.

Key Words: Vitamin A; retinol; retinoic acid; retinoid; gene expression; cell differentiation; epidemiology; nutrition; dietary recommendations

1. INTRODUCTION

Vitamin A (retinol) and its active metabolite, retinoic acid (RA), are widely recognized as important factors in the maintenance of healthy cells and tissues. RA possesses a fundamental ability to regulate cell growth, generally by slowing the rate of the cell cycle, and to induce immature and transformed cells to differentiate toward a

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more mature phenotype. These intrinsic biological properties appear to be nearly ideal for the chemoprevention of cancer, possibly even for the treatment of established cancers. However, when vitamin A is consumed at elevated levels, it accumulates over time within tissues and can be damaging to the integrity of cellular membranes, culminating in symptoms of hypervitaminosis A. Thus, natural vitamin A is not well suited for long-term systemic therapy. The idea of producing "retinoids" *(1)* – i.e., synthetic analogues structurally related to retinol and RA, that would potentially be beneficial in the fight against cancer while also less toxic, has motivated the field of vitamin A and retinoid research for three decades. Numerous retinoids are significantly growth inhibitory to rapidly proliferating and transformed cells. These results have served as a strong impetus for studies in preclinical models and for clinical trials. As discussed in this chapter, results have been promising in the treatment of some premalignant diseases, such as leukoplakia, but for established cancers, results have generally been disappointing. However, an outstanding success has been achieved with the discovery that all-*trans*-RA (at-RA) is effective in the treatment of acute promyelocytic leukemia (APL) *(2)*. In a high proportion of APL patients, at-RA induces a complete remission. Retinoids have also become widely used in the treatment of diseases of the skin, such as cystic acne, which have proved refractory to other therapies.

Clinical studies have also revealed some unintended consequences of retinoids used for therapy on the metabolism of natural vitamin A. In a trial of fenretinide (4-HPR) for prevention of breast cancer recurrence, 4-HPR-treated women reported problems with dark adaptation (night blindness) *(3)*, a well-known sign of vitamin A deficiency *(4)*. Plasma analysis revealed low levels of retinol, despite no evidence of low-dietary vitamin A, and subsequent studies revealed that 4-HPR disrupted the plasma protein complex that transports retinol *(5)*, which is likely to increase its turnover rate. Such findings serve to underline that the metabolism of dietary vitamin A and synthetic retinoids used for therapy are highly intertwined. Some retinoids used for treatment, such as at-RA, are chemically indistinguishable from their naturally formed biological counterparts, but due to high dosage they still can perturb the normal metabolism of vitamin A. Indeed, at-RA at pharmacological dosage also rapidly reduces the concentration of plasma retinol *(6)*. Thus, to avoid unintended consequences, greater attention must be paid to understanding the interactions of diet-derived vitamin A and retinoids used or proposed for use as therapeutic drugs.

The purpose of this chapter is to review the biological basis for a role of vitamin A in cancer prevention and treatment, the types of evidence and the totality of evidence that this nutrient can reduce cancer risk, and the status of nutritional recommendations for vitamin A intakes that are optimal in terms of reducing the risk of cancer.

2. RATIONALE FOR WHY VITAMIN A CAN AFFECT CANCER PREVENTION AND/OR TREATMENT

Retinol is an essential nutrient that serves as the substrate for the production, within various cells, of retinal required for rhodopsin biosynthesis, and for the production of RA, which functions as a critical regulator of cellular functions in essentially all tissues. RA is now recognized as a potent regulator of gene expression. While the effects of

RA on gene expression are cell-type specific, three general outcomes on cell physiology have been reported for a wide variety of cells: a reduced rate of cell proliferation, enhanced cell differentiation, and in some cells with some retinoids, induction of apoptosis.

Today's understanding of retinoids and their potential role in cancer prevention is based on decades of prior research. The idea that vitamin A may be important for cancer prevention can be traced back to the 1920s, when Wolbach and Howe reported on histopathological changes in the epithelial tissues of rats fed a vitamin A-deficient diet *(7)*. The mucosal linings of various epithelial tissues were squamous, dry, and keratinized in the vitamin A-deficient state. In other studies, vitamin A-deficient animals were more likely to develop metaplasia and spontaneous tumors *(8)*. The field of vitamin A research then advanced to studies of specific metabolites, with the identification of retinal as the form essential for vision and of RA as an important, but quantitatively small, active metabolite of retinol. In 1960, Dowling and Wald *(9)* reported that at-RA can replace retinol for nearly all of the essential functions of vitamin A, except in vision where retinal is required. These studies helped lead to the current understanding of retinol as a precursor molecule from which all of the active forms of vitamin A are produced by metabolism. By the 1970–1980s when cell culture models became increasingly important in biomedical research, seminal studies were reported on the ability of RA to inhibit cell growth and induce cell differentiation. In relatively undifferentiated F9 embryonal carcinoma cells, RA induced formation of parietal endoderm *(10)*, while in HL-60 myeloid leukemia cells, RA induced a myeloid, granulocytic phenotype *(11)*. In 1987, the discovery of two families of nuclear retinoid receptors, RAR and RXR, provided a conceptual understanding of how retinoids may work as transcription factors in the regulation of gene expression *(12, 13)*. In rapid succession, three RAR and three RXR genes were cloned and characterized, and their expression patterns were determined to elucidate where and under what conditions retinoid-activated nuclear receptors might function. These discoveries opened the way to a molecular understanding of retinoid actions. X-ray crystallographic studies of the RAR and RXR ligand-binding domains, with and without bound ligand, provided important information on how retinoid receptors interact with their ligand molecule, and suggested that significant conformational changes are induced by the binding of the retinoid to the ligand-binding domain of the receptor protein *(14)*. This information then facilitated the production of new agonistic and antagonistic ligands, some with significant receptor specificity, such as the "rexinoids" specific for binding to RXRs.

Overall, many studies have led to the current understanding that RA is naturally involved in a great number of biological processes. The "fingerprint" of RA can be found in nearly every metabolic pathway *(15)*.

Retinoid molecules of natural and synthetic origin. Major nutritional and pharmacologic retinoids are listed in Table 1. While the term retinoid once was used to distinguish structurally related synthetic analogues of vitamin A compounds, it now applies to all molecules of this structural group, whether produced in vivo from vitamin A or produced by chemical synthesis. In this chapter, the term vitamin A is used when the nutritional form is specifically meant, including retinyl esters and retinol and their natural metabolites. Vitamin A compounds exist in multiple oxidation states, in different

Table 1
Major Nutritional and Synthetically Derived Retinoids

Nutritional forms and physiologically produced metabolites	*Structurally identical physiological and synthetic forms*	*Synthetic retinoids used in animal and human studies*
Retinol (plasma; precursor to other forms)		
3,4-didehydroretinol (skin)		
Retinyl esters (storage)		Retinyl acetate; retinyl palmitate (RP)
Retinal (vision)		
All-*trans*-retinoic acid (ligand for RAR receptors; regulator of cell growth, cell differentiation, and apoptosis)	Yes	All-*trans*-RA (at-RA)
13-*cis*-RA (metabolite in plasma)	Yes	13-*cis*-RA
9-*cis*-RA (putative metabolite and ligand for RXR receptors)	?	9-*cis*-RA
Retinoyl glucuronides (water soluble; excreted metabolites)	Yes	Retinoyl glucuronides
		Axerophthene, anhydroretinol (hydrocarbons)
		Retinyl ethers
		Polyprenoic acid (acyclic retinoid)
		Arotinoids
		Retinobenzoic acids
		Rexinoids
		Hydroxyphenyl retinamide

isomeric forms, and in unconjugated or conjugated states. The principal oxidation states are alcohol, aldehyde, and carboxylic acid. Natural forms exist in the all-*trans* configuration, while the 11-*cis*-isomer of retinal, formed from retinol in retinal pigment epithelial cells, is an essential component of the visual pigment rhodopsin.

The mode of formation of *cis* isomers of RA in vivo, and even their physiological significance, is unclear. 13-*cis*-RA is a normal metabolite present in plasma *(16)*. Although 13-*cis*-RA as a drug has shown activity in chemoprevention studies, and is often used in treating diseases of the skin, 13-*cis*-RA does not transactivate nuclear receptors as all-*trans*-RA does. Exogenous 13-*cis*-RA might act as a pro-drug that is slowly

converted to at-RA *(17)*. Although exogenous 9-*cis*-RA clearly binds to and transactivates nuclear retinoid receptors (see below), mainly of the RXR family, it is not certain that 9-*cis*-RA is an endogenous metabolite of vitamin A. Doubts have been raised based on finding very low or undetectable levels of this molecule in tissues where it would be expected and the possibility that it is formed artifactually during isolation. To date, no isomerase has been reported that catalyzes the formation of 9-*cis*-RA. This isomer could be generated nonenzymatically from all-*trans*-RA or 13-*cis*-RA, as to some extent all isomers of RA exist in equilibrium mixtures. The binding of at-RA to cellular retinoic acid-binding proteins stabilizes the all-*trans* isomer, but 9-*cis*-RA is not bound by these proteins. Therefore, even if 9-*cis*-RA is formed in vivo it may be quickly degraded. Other possible ligands for the RXR receptors have been suggested, including long-chain polyunsaturated fatty acids and phytanic acid.

The family of synthetic retinoids covers a great variety of forms. Detailed discussions of their synthesis and properties can be found in references *(18, 19)*. These retinoids exist in various oxidation states, as *cis* and all-*trans* isomers, in locked conformations, and with various substitutions and linkages (Table 1, Fig. 1). Some of the synthetic retinoids are chemically identical to natural metabolites of vitamin A, with at-RA as a prime example.

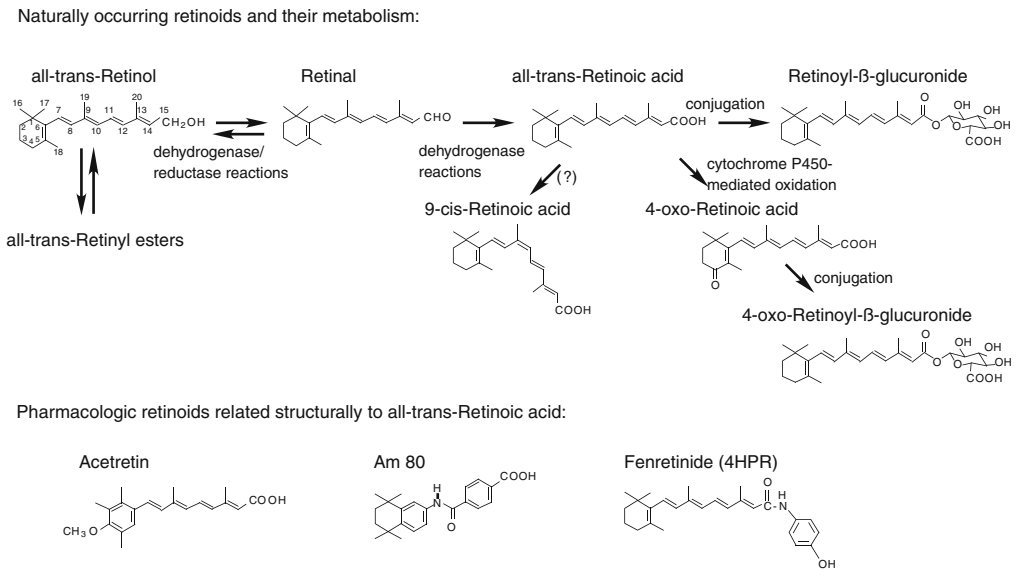

Fig. 1. Examples of naturally occurring and synthetic retinoids. The conversion of retinol to retinal and at-RA constitutes the major pathway of bioactivation for dietary vitamin A. Cytochrome P-450-mediated oxidation and subsequent conjugation contributes to catabolism of RA, whether generated from diet or administered for treatment. Retinol is also eliminated by this route (not shown). The *lower* panel shows three examples of synthetic retinoids, illustrating the substitution of an aromatic ring for the natural β-ionone ring of retinoic acid (acetretin); introduction of a heteroatom and creation of a locked conformation (Am80); and an amide analogue, 4-HPR, which has been tested extensively in cell, animal, and human clinical studies.

Metabolism as a major factor in retinoid homeostasis. Metabolism is central to the biological basis of vitamin A's actions in cancer prevention. Retinoid metabolism is closely regulated through a variety of homeostatic mechanisms, discussed below, which include transport proteins, intracellular chaperone proteins, nuclear receptors, and enzymes. The apparent "goal" of the body's homeostatic mechanisms is to maintain steady levels of plasma retinol and RA, which in turn assure a well-regulated exposure of extrahepatic tissues to these molecules. At the molecular level, at-RA itself regulates several genes/enzymes that are central to controlling the levels of retinol and RA, thus acting in an autoregulatory manner *(20, 21)*. Although the retinoid homeostatic system is remarkably efficient over a wide range of dietary vitamin A intakes, it clearly cannot compensate for an inadequate vitamin A intake, and it can be overwhelmed by an excess of vitamin A and by exogenous retinoids. In unbound "free" form, retinoids disrupt cellular membranes *(22)*, while an excess of RA induces gene responses that are inappropriate to the normal functioning of cells. The signs and clinical symptoms of hypervitaminosis A and retinoid toxicity are very similar *(23)*. The maintenance of physiological levels of retinol and RA is essential for normal embryonic development, as both vitamin A deficiency and excess (of either retinol or RA) are teratogenic, causing developmental abnormalities of the head, limbs, heart, and other visceral organs *(24)*. In the postnatal period, a well-regulated supply of vitamin A is necessary for lung maturation, development of immunity to natural pathogens and vaccines, and growth *(25)*. In adults, an adequate intake of vitamin A is essential for maintaining the integrity of the skin, ciliated epithelia, and reproductive organs. These changes are sufficiently reproducible that the ability of retinoids to reverse epithelial metaplasia has been used as a bioassay for retinoid activity. Overall, a diet with an adequate but not excessive level of vitamin A is required across the lifespan for the well-regulated production of its active metabolites. An understanding of vitamin A metabolism is essential both for formulating optimal nutritional recommendations and for predicting how pharmacological retinoids, which at doses currently used significantly alter plasma retinol, are likely to affect the metabolism of natural vitamin A. Moreover, aberrant retinoid signaling appears to be a condition of many transformed cells and tumor tissues (see later). Figure 1 outlines the major pathways of vitamin A metabolism. Specific players in retinoid metabolism and homeostasis are described next.

RBP. Retinol-binding protein is the principal transport protein for plasma retinol. Retinol is released from liver bound to RBP, circulates in plasma within a narrow concentration range of approximately 1–3 μmol/l, and is taken up by target organs *(26)*. In situations of a nutrition deficiency of vitamin A, during inflammation, and as a result of retinoid treatment, plasma retinol is markedly reduced. A recently identified plasma membrane transporter, Stra6, binds RBP and transports the retinol molecule into cells *(27)*. This mechanism appears especially important in the retina. The RBP gene (RBP4) is also expressed in extrahepatic tissues. Kinetic studies have shown that retinol normally circulates several times between liver and extrahepatic tissues before undergoing irreversible oxidation, and thus the synthesis of RBP in extrahepatic tissues is likely to be important for reverse retinol transport back to the liver. RBP has also been shown to have adipokine-like properties and to be a factor in the regulation of glucose metabolism *(28)*, suggesting novel interactions between vitamin A and energy homeostasis.

Cellular retinoid-binding proteins. The cellular retinol-binding proteins known as CRBP-I and CRBP-II are structurally and functionally similar, while CRBP-III is homologous but less well understood. As chaperone proteins, CRBP-I and CRBP-II guide retinol to the esterifying enzyme lecithin:retinol acyltransferase (LRAT) in the liver and intestine, respectively *(29)*, while also playing a role in retinol oxidation–reduction *(30)*. When CRBP-I knock-out mice were stressed by a low-vitamin A diet, they quickly lost retinol from the liver and retina, showing that CRBP is an important "efficiency factor" for the conservation of vitamin A *(31)*.

Intracellular RA is chaperoned by two cellular retinoic acid-binding proteins, CRABP-I and CRABP-II, which are differentially expressed in various tissues. These proteins bind at-RA selectively. They appear to regulate the availability and delivery of at-RA to nuclear receptor proteins. Additionally, in some cell types, CRABP-II appears to function as a transcriptional regulator associated with RAR-alpha-mediated gene expression *(32)*.

Absorption, storage, enzymatic activation, and elimination of retinoids. In the intestine, dietary vitamin A in foods and supplements is digested and absorbed, converted in enterocytes into esterified retinol, and packaged into chylomicrons, which are secreted into the lymphatics and then enter the circulation. Chylomicron remnants deliver most of their vitamin A to the liver, but a small proportion is released to extrahepatic tissues during chylomicron metabolism. The majority of whole-body vitamin A exists in esterified form, stored in the liver, mainly in stellate cells. This form of vitamin A is inactive but readily mobilized, and thus constitutes an important storage form that, when hydrolyzed, provides retinol for delivery throughout the body. Many extrahepatic tissues esterify and store retinyl esters in smaller pools, which may be crucial for the local generation of bioactive retinoids.

In contrast to retinol, retinoids possessing a carboxylic acid moiety are absorbed by the portal route bound to albumin. While diet contains little RA, the majority of retinoids used for cancer prevention and therapy are carboxylic acids (Fig. 1), and thus most clinically important retinoids are absorbed by the portal route. The plasma concentration of physiologically formed RA is normally in the low nanomolar range, but following vitamin A or retinoid treatment concentrations are significantly higher *(33*, *34)*.

Retinol is esterified in the intestine, liver, eye, lung, testis, and other tissues by LRAT. In the liver, LRAT is sensitively regulated according to individual's vitamin A status, and RA is likely to mediate changes in LRAT gene expression. LRAT mRNA and enzyme activity are very low in vitamin A-deficient animals, while LRAT expression is rapidly induced after retinol or RA is administered *(29)*. The feedback regulation of LRAT by RA thus serves to divert retinol into storage, increasing the retinyl ester pool and decreasing the quantity of retinol available for oxidative metabolism (Fig. 2a).

Retinol oxidation is essential for activating the vitamin A molecule into its active forms, retinal and RA *(21*, *30)*. A variety of enzymes with retinol dehydrogenase (RDH) and retinal dehydrogenase (RALDH) activities have been described as capable of forming retinal and at-RA. Multiple enzymes in each of these families exist, with different expression patterns, suggesting that RA formation is regulated in complex manner and by pathways that may differ among tissues. While retinol and retinal can be interconverted, the oxidation of retinal to at-RA is irreversible.

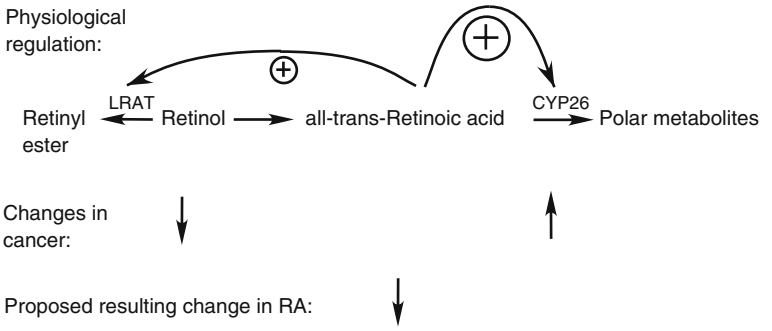

Fig. 2. Regulation of retinol esterification and oxidation by at-RA and proposed changes in cancer. Based on nutritional studies *(29)*, LRAT is kept in a tonic active state when RA is adequate, thus diverting retinol into storage, while CYP26 expression is rapidly and transiently up-regulated when the concentration of RA becomes elevated. In cancer, aberrant expression or the absence of LRAT leads to a lack of retinyl esters, from which bioactive retinoids are formed, and may induce overexpression of CYP26, leading to excessive degradation of RA *(56)*.

RA is catabolized in a sequential phase 1–phase 2 manner, with hydroxylation at C-4 or C-18 preceding conjugation, yielding metabolites such as RA-β-glucuronide *(21)*. A significant advance in understanding the oxidation of at-RA was made when the CYP26 gene family of RA-4-hydroxylases was cloned. at-RA was already known to induce its own metabolism. The CYP26A1 gene promoter has been shown to respond directly to all-*trans*-RA (see gene transactivation, see below). The isoform known as CYP26A1 is expressed in the liver and is highly inducible by RA *(35)*. It is also expressed at lower levels in extrahepatic tissues of the adult *(35)* and in the embryo where its expression pattern is consistent with a role in limiting signaling by all-*trans*-RA *(36)*. CYP26B1 is expressed in a pattern that overlaps with but still differs from CYP26A1. Other members of the cytochrome P-450 gene family have been suggested to play roles in the catabolism of RA, but less is known of their physiological significance. Once RA has been oxidized, it is conjugated to form water-soluble metabolites, which are readily excreted *(37)*. The expression of CYP26A1 and the efficiency of RA oxidation and formation of water-soluble metabolites in the liver was found to be significantly increased when rats were fed a vitamin A-supplemented diet *(37)* (Fig. 2b).

It has been suggested that at least some of the reactions of RA production and catabolism occur in a "cassette" manner, in which metabolic products formed by one enzyme are immediately channeled to become the substrates of other enzymes, resulting in further processing *(30)*. This may contribute to the overall efficiency of metabolism, and may explain why certain metabolites, such as all-*trans*-retinal, do not accumulate in most tissues, although the rate of flux through the pathway may be significant.

Retinoid receptors. Retinoic acid and its analogues are high-affinity ligands for nuclear hormone receptors of the retinoid receptor family. This family is comprised of two subfamilies, RAR (α, β, and γ) and RXR (α, β, and γ), which serve as transcription factors in the regulation of a large number of genes (see *(15*, *38*, *39)* for reviews). All-*trans*-RA is the principal natural ligand for the RAR. While exogenous 9-*cis*-RA, as mentioned earlier, binds effectively to the RXR its physiological role is not certain.

In some studies, the RAR–RXR complex with an RAR ligand is required for transactivation, but apparently a ligand for the RXR is not required. RXR proteins also form heterodimeric partners with several other nuclear receptors of the steroid hormone superfamily, including the vitamin D receptor, thyroid hormone receptor, receptors involved in the metabolism and regulatory functions of fatty acids, sterols and bile acids, and certain xenobiotics, and orphan receptors. One possible mode of regulation among different nuclear hormone receptors that interact with RXR may be the competition for a limited pool of RXR protein.

The RAR and RXR proteins are modular in structure, comprised of six defined regions, named A–F, and intermediate hinge regions, present in all of the receptors *(40)*. While the proteins in each receptor subfamily are well conserved overall, each subtype (α, β, and γ) of RAR and RXR differs somewhat, and isoforms, generated by the use of alternative promoters and/or differential RNA splicing, result in a further diversification of the receptors in different cells. For example, the RARβ receptor is expressed in four isoforms, where the mRNA for RARβ1 and RARβ3 is transcribed from an upstream 5′ promoter, P1, while RARβ2 and RARβ4 mRNA are transcribed from another promoter, P2, located 20 mb downstream. Isoforms 1 vs. 3, and 2 vs. 4, are then formed by alternative splicing, resulting in proteins with different A regions but identical B–F regions in the 4 RARβ isoforms *(41)*. Other RAR protein and the RXR proteins also exist in isoforms. These differences are well conserved suggesting that the individual subtypes could have important but differential roles in gene regulation.

Mode of action of retinoids as regulators of gene transcription. Retinoids similar to at-RA, having a carboxylic acid functional group and an extended conformation, are potent ligands for the RAR family of nuclear retinoid receptors and often regulate gene expression when added to cells at concentrations in the low nanomolar range. Biochemical and structural studies have shown that a single retinoid molecule binds with high affinity to the ligand-binding domain (LBD) of the RAR, with the ligand's negatively charged carboxyl group coordinated with specific amino acids within the LBD pocket, so that the retinoid is positioned in a precise manner. X-ray crystallographic studies of the apo (with RA ligand) and holo (empty) forms of the LBD have shown that particular regions, especially alpha helix 12, are mobile. Helix 12 assumes an "open flap" position in the apo protein, but the flap is closed when RA is bound, forming an interior pocket *(40)*. Each RAR protein interacts with an RXR protein to form a heterodimeric complex of RAR–RXR, and the complex binds to specific DNA sequences through zinc fingers in the DNA-binding domain of each RAR and RXR. The ligand induced change in the position of RAR helix-12 results in an overall change in protein conformation that is critical for altering the surface interactions of the RAR–RXR complex with other proteins of the transcription regulatory complex, including coactivator/corepressor proteins or "mediators," that in turn regulate the basal transcriptional machinery including DNA-dependent RNA polymerase II (Pol-II) *(40)*. In the current general model, the RAR–RXR complex in the nonliganded state is already bound to DNA response elements (below) in a repressive state, associated with corepressors (e.g., N-CoR and SMRT) which recruit histone deacetylases, and thus result in a more compact and transcriptionally inactive form of chromatin. Ligand-dependent factors such as the receptor interacting protein RIP-140 may also be involved in keeping genes in a repressed state *(42)*. The

binding of ligand(s) to the RAR–RXR complex is then thought to alter the conformation of the RAR–RXR complex in such a way that coactivator proteins are recruited. With the binding of coactivators (N-CoA, p300, or CBP) histone acetyl transferase activity is recruited to the complex resulting in the modification of histones, changes in chromatin structure, and recruitment of basal transcriptional factors, including Pol-II, which form the preinitiation complex required for the start of gene transcription *(40)*.

The canonical retinoic acid response element (RARE) motif is composed of two direct repeats of the hexameric nucleotide sequences, a/gggtca, separated by a spacer (n) of 2 or 5 nucleotides: a/gggtca$(n)_{2,5}$-a/ggtca *(40)*. Some RARE are located in the 5′ regulatory region of target genes near to the transcription start site, but others have also been found within introns or in regions quite distal from the basal promoter. However, while this general model is now well established, it is very likely that regulation in vivo is much more complex. Many more genes have been described to respond physiologically to RA than have been shown to contain an RARE and respond directly through ligand binding to RAR–RXR *(15)*. Moreover, there are undoubtedly additional layers of regulation that affect receptor function, including receptor protein phosphorylation, degradation, and shuttling of receptor proteins between the nuclear and cytoplasmic compartments *(39)*. Furthermore, RAR proteins sometime interact with other families of transcription factors. The antiproliferative activity demonstrated for RA may involve competition of the liganded RAR/RXR complex with the Jun-Fos (AP-1) transcription factor complex for binding to specific DNA sequences. Protein–protein interactions with a number of transcription factors of other gene families have also been noted *(39)*.

3. IN VITRO STUDIES IN CELLS AND ANIMALS – PREVENTION AND TREATMENT

Retinoids exert many of their effects on three major cellular processes: cell proliferation and growth regulation; cell differentiation, characterized by programs of cell-type specific gene expression; and, in some cells and with some retinoids, the induction of apoptosis. Most experiments have been conducted with cycling cells, often either transformed or malignant cells, and have been designed to test whether cell growth is inhibited and/or cell differentiation is promoted by retinoids. A wide variety of cell models have been studied. A few examples are given in Table 2, as the literature is too extensive to review comprehensively, and the principal findings of growth arrest and improved differentiation have been quite consistent regardless of the cell model studied.

In dividing cells, retinoids almost always reduce the rate of cell proliferation. The decision of cells to proliferate or differentiate is typically made in G1/G0 of the cell cycle. Mechanistic studies have shown reduced expression of genes and proteins encoding cyclins and cyclin-dependent kinases, increased expression of cyclin-dependent kinase inhibitory proteins, and increased expression of the retinoblastoma tumor suppressor protein (pRb), accompanied by a reduction in its phosphorylation status. In different cell models, the specific cyclin most affected has differed, for example, cyclins of the D family in some cells, cyclin E in others, etc., and, similarly, different cyclin inhibitory proteins have been the predominant ones increased, e.g., p27, p57, p21, p16, or p19. In nearly all cell models in which the cell cycle is inhibited, pRb expression

Table 2
Representative Cell Culture Studies Demonstrating Mechanisms Through Which Retinoids May Inhibit Human Cancer Cells

Cell type	*Major finding in RA-treated cells*	*References*
HL-60 myeloblastic leukemia cells	Activation of extracellular signal-regulated kinase (ERK)-2 mitogen-activated protein kinase (MAPK) as an early event, required for growth arrest and induction of differentiation	Yen et al. *(43)*
THP-1 leukemia cell line	Increased Rb mRNA and protein levels while reducing Rb phosphorylation state; cyclin E reduced; p27 increased; functional differentiation of cells into macrophage-like phenotype	Chen and Ross *(44)*
CA-OV3 RA-sensitive ovarian carcinoma cells	Increased the levels of p27 and S10 phospho-p27, needed for RA-induced growth inhibition	Radu et al. *(45)*
Nt2/D1 embryonal carcinoma, germ cell cancer cells	Reduced cyclin D1/D2 transcription rates and protein levels, reduced Ki-67 staining and cell number	Freemantle et al. *(46)*
BEAS-2B human bronchial epithelial cells, and cells transformed by tobacco carcinogen	Inhibited transformation of carcinogen-treated cells and induced proteosome-mediated degradation of members of cyclin D family	Boyle et al. *(47*, *48)*
CHP126 neuroblastoma cells	Cell cycle arrest and induced neurite outgrowth induced by 9-*cis*-RA, concomitant with reduction in cyclin-dependent kinase-activating kinase (CAK) phosphorylation of pRb and RXRα proteins	Zhang et al. *(49)*
NB4 APL leukemia cells	Inhibited the action of cyclin-dependent kinase-activating kinase (CAK) on PML–RARα protein and induced degradation of the CAK complex protein MAT1	Wang et al. *(50)*

has been increased, and/or its phosphorylation reduced (hypophosphorylation). Reduced phosphorylation enhances the ability of this "pocket" protein to sequester factors such as E2F required for progression of the cell cycle from the G1/G0 phase through the restriction point and into the S phase of the cell cycle (Fig. 3). These changes result in a prolongation of the G1 phase of the cell cycle and a reduced rate of entry into the S phase of the cycle. The results of many studies are consistent in showing a reduction in cell cycle progression and in cell counts in retinoid-treated cells as compared to untreated cells.

Retinoic acid has also been shown to regulate the cyclin-dependent kinase-activating kinase (CAK), which regulates exit from the G1 phase of the cell cycle (Table 2). The CAK complex can interact with and phosphorylate pRb and at least certain retinoid receptors. When neuroblastoma cells were treated with 1–5 μM 9-*cis*-RA, CAK activity was reduced, as shown by decreased CAK activity, hypophosphorylation of Rb and RXRα, reduced proliferation and morphological evidence of neuronal cell differentiation *(49)*. In NB4 cells, a model of APL leukemia, at-RA reduced CAK abundance and activity, the level of the aberrant receptor PML–RARα, which these cells express, and these changes were associated with a reduction in cell proliferation and induction of myeloid cell-type differentiation *(50)*. An RA-induced induction of proteolysis was observed in these studies and in others reviewed by Dragnev et al. *(51)*.

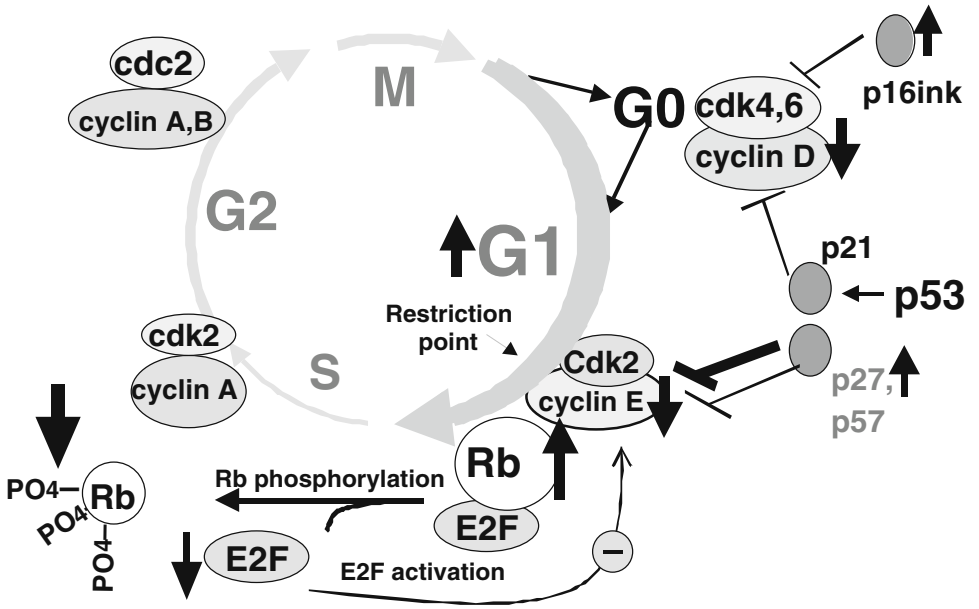

Fig. 3. Prominent effects of RA on the cell division cycle. A schematic of the cell cycle, with frequently observed effects in cells treated with RA shown as *up and down arrows*. Cdk, cyclin-dependent kinase; Rb, retinoblastoma protein; E2FE, transcription factor involved in G1 to M (mitosis)-transition. Generally consistent effects of RA include reduction in expression of G0 and G1 cyclins; increase in cyclin-dependent kinase inhibitors (p16, p21, p27, p57); increased Rb and decreased hyperphosphorylated form of Rb; increased fraction of G1 and decreased fraction of S (synthesis) and M phase cells.

Retinoids almost always either induce cell differentiation or they drive cells into apoptosis. Cell differentiation may be the consequence of the increased time in the G1 phase of the cell cycle when cell cycling is slowed, allowing some cells to "escape" the cell cycle and enter a program of differentiation. The features of cell differentiation observed depend, unsurprisingly, on the cell model studied, but they nearly always include alteration in gene expression as well as phenotypic changes consistent with a more mature cell type. The induction of apoptosis is a more varied finding and may depend on the retinoid used, type of cell, stage of differentiation, or presence of other regulatory factors. CD437, a retinoid with selectivity for RAR-γ, and fenretinide (4-HPR), a retinoid that appears to function independently of receptor binding, have been shown to induce apoptosis in several cancer cell models *(52*, *53)*. Recently, new rexinoids were tested. In B lymphoma cells, certain features of the RXR domain structure and RXR signaling were found to be essential for the induction of caspase activation and apoptosis *(54)*.

The interpretation of results from cell culture studies needs to be made cautiously, and especially so if the retinoid concentrations employed have exceeded those normally found in vivo. Additionally, due to the lipophilic nature of most retinoids, an organic solvent such as ethanol or dimethylsulfoxide is required to dissolve the retinoids prior to their dispersion into tissue culture medium. Whereas the concentration of RA in human plasma is on the order of 10–50 nM, many experiments with culture cells have utilized RA concentrations of 1 μM or higher, with a serum concentration of 5–10%. Nevertheless, cell studies have been important for identifying alterations in retinoid signaling in tumor cells, some of which have been confirmed in cancer tissue specimens.

Aberrant retinoid signaling in cancer cells and tissues. A number of cancer cell lines, compared to nontransformed cells, have exhibited aberrant retinoid signaling *(55–57)*. One of the most often affected genes is RAR-β, especially the isoform RARβ2. Loss of expression of RAR-β2 during cancer development is often associated with tumorigenesis and with retinoid resistance, while conversely, induction of its expression can suppress carcinogenesis *(57)*. Expression of another isoform, RAR-β4, which includes only a short four amino acid section of the N-terminal A-domain that is important for protein–protein interactions and phosphorylation *(39*, *41)*, is increased in various types of cancer *(56)*. RAR-β4 transgenic mice developed hyperplasia and neoplasia in various tissues, and induction of RAR-β4 expression increased the growth of tumor cells that do not express RAR-β2. In patients during breast cancer progression, RAR-β2 is reduced or lost *(58)* and hypermethylation of the 5′-region is thought to be responsible for the silencing of the RARβ gene. Silencing of RAR-β2 by gene methylation was reported to be an early event in head and neck carcinogenesis *(59)* and in other cancers *(56)*.

Other receptors, enzymes, and retinoid-binding proteins have been reported to be aberrantly expressed or silenced in cancer cells and cancer tissue specimens *(56)*. Besides RAR-β, other retinoid-related factors that are often reduced include LRAT, CRBP-I, RALDH2, and ALDH genes, required for retinoid storage and activation. Conversely, the catabolism of RA may be increased by overexpression of CYP26, as has been observed in colorectal cancer cells *(56)*.

The totality of evidence thus suggests that the abnormal biology of cancer cells is often associated with abnormalities in vitamin A-related metabolism. However, whether

these changes are consequence of transformation or whether impaired retinoid storage is part of cancer induction must still be determined.

Animal studies. A number of animal models of carcinogenesis have been used to determine if vitamin A or retinoids can reduce the onset or severity of tumors. Numerous models were employed in the 1970–1980s as previously reviewed *(60)*, and several results were promising enough to serve as an impetus for human clinical trials. Most animal studies used chemical carcinogens, either direct acting or requiring activation to induce tumors, while retinoids were supplied at levels of 1–2 mmol retinoid per kg of diet, sometimes starting before carcinogen exposure. Retinoid treatment did not abrogate tumor formation but several retinoids reproducibly reduced the number, multiplicity, or size of tumors. A few of the early studies investigated dietary vitamin A (retinol, retinyl palmitate (RP), or retinyl acetate) for chemoprevention, but at the doses tested the growth of the animals was significantly impaired or signs of vitamin A toxicity were observed, and so few later studies investigated these forms. Studies then turned to diets supplemented with at-RA, 13-*cis*-RA, or 9-*cis*-RA, or novel retinoids such as fenretinide/4-HPR. Several of these compounds were reported to inhibit the growth of bladder, mammary gland, and other tumors *(60)*. 4-HPR proved to be much better tolerated.

A recent study represents a different approach. McDaniel et al. *(61)* developed a human food-based rodent diet, composed with fruits and vegetables that give rise to vitamin A during digestion, to investigate the effects of vitamin A on adolescent rat mammary gland development and the subsequent risk for mammary carcinogenesis, induced by treatment with methylnitrosourea (MNU). A diet enriched in RP was used for comparison. The adolescent growth period was studied, as it is characterized by rapid body growth, sexual maturation, and mammary gland development, correlating with puberty in humans. Compared with adolescent rats that consumed an adequate diet, rats that consumed the natural foods diet and the RP diet had a reduced multiplicity of mammary cancers, associated with a reduction in alveolar gland development. The food-based diet also suppressed the onset of sexual maturation and inhibited markers of mammary alveoli formation more than the RP diet. Six months after treatment with MNU, latency and incidence of mammary tumors did not differ among dietary groups, but tumor multiplicity was reduced in rats fed either natural food-based or RP diets during adolescence. The results of this study suggest that the amount and source of vitamin A consumed in the adolescent period can influence the onset of puberty, mammary gland alveolar development, and risk of breast cancer. Since it has been suggested that a diet rich in fruits and vegetables may reduce breast cancer risk, these experimental results appear congruent. It is possible that components of the diet besides vitamin A contributed to the observed effects, although the results with the RP-enriched diet were similar to those for the whole foods-based diet.

Another recent approach has been to focus on RXR-selective agonists (rexinoids). Liby et al. *(62)* reported that in the A/J mouse model of lung cancer, a rexinoid known as NRX194204 significantly reduced the number and size of lung surface tumors and total tumor volume by 64–81%. This compound also delayed tumorigenesis in mouse mammary tumor virus-*neu* mice. The latter finding is encouraging, as in a study of *neu* mice treated long term with an implanted RA pellet, tumor growth was reported to be increased *(32)*.

Overall, animal studies of retinoids are continuing, with focus on newer diets approaches and new retinoids that seem promising at lower doses. The majority of evidence from animal studies supports a reduction in tumorigenesis with retinoid treatment, with a minority of studies showing no effect or an increase.

4. EPIDEMIOLOGICAL AND INTERVENTIONAL STUDIES

Hong and Itri *(63)* have thoroughly reviewed epidemiological, therapeutic, and prevention trials of human cancers involving vitamin A and a number of synthetic retinoids through the mid-1990s. Ross *(64)* has reviewed the epidemiology literature on dietary vitamin A and cancer risk. While epidemiological studies have long pointed to an inverse association between vitamin A and cancer risk, many of these studies concerned carotenoids. The preponderance of data supported reduced cancer risk from the consumption of a diet rich in fruits and vegetables, and therefore carotene, but there was little evidence for a relationship with dietary retinol. Both reviews noted strengths and weaknesses of epidemiological studies with vitamin A. Survey studies of vitamin A intake are inherently complicated due to vitamin A being consumed in part as carotenoids, often grouped with other antioxidant vitamins, and in part as preformed retinol in foods of animal origin and supplements. Food frequency questionnaires are imperfect tools for capturing vitamin A intake. Most are only qualitative or semiquantitative, and thus the inferred data on vitamin A consumption is likely to be weak. Associations of cancer risk with serum retinol were also weak *(63*, *64)*. In fact, a weak relationship could be anticipated because serum retinol concentrations are maintained at a relatively constant level over a wide range of vitamin A status *(21)*. Moreover, if a lower retinol level is measured after disease has developed it could be a consequence of cancer rather than a predictor. More recent reports from epidemiological studies on the prevention of prostate *(65)* and breast cancers *(66)* concluded that higher dietary intakes of fruits and vegetables and vitamin A were not associated with prevention of these cancers. Thus, overall the epidemiological evidence for reducing cancer risk by consuming foods containing provitamin A carotenoids or preformed vitamin A is quite weak.

Interventional studies of retinoids for cancer chemoprevention or therapy have been conducted for over three decades. For established cancers, most clinical trials have been disappointing. A review of phase III clinical trials of lung cancer prevention concluded that β-carotene, retinol, and 13-*cis*-RA (as well as other agents tested) did not demonstrate beneficial, reproducible results *(67)*. Retinol combined with zinc did not reduce liver cancer mortality in a randomized double-blind study of hepatocellular cancer in Linxian, China *(68)*. A Cochrane review of trials in which at-RA and 4-HPR were tested for prevention of progression of cervical neoplasia concluded the retinoids were not effective *(69)*.

For premalignancies, vitamin A and retinoids may have a benefit. A Cochrane review of various interventions for treating oral leukoplakia concluded that treatment with β-carotene, lycopene, and vitamin A or retinoids, was associated with significant rates of clinical resolution, compared with placebo or no treatment, although there was no evidence that any of the treatments prevented malignant transformation of leukoplakia *(70)*. For premalignant actinic keratoses, topical retinoids have provided some benefit *(71)*.

However, in a chemoprevention study using biomarkers to assess proliferative changes in the bronchial epithelium of former smokers, 13-*cis*-RA and α-tocopherol, but not 9-*cis*-RA, reduced the intermediate marker of cell proliferation, Ki-67 *(72)*. In another study of current and former smokers, 50,000 IU/d of retinol for 6 months did not up-regulate RAR-β, which as noted above is frequently silenced in cancer, or improve surrogate biomarkers of bronchial dysplasia *(73)*.

One type of cancer stands out for being a remarkable success. The demonstration that APL patients treated with at-RA can result in a high rate of response and complete remission was first demonstrated in a clinical study in Shanghai, China *(2)*, and has since been replicated in several trials in the USA, Europe, and Asia *(74)*. This unanticipated result has led to the use of at-RA for "differentiation therapy," as an important clinical treatment for this disease. APL is a unique form of cancer because a high proportion of patients have a specific chromosomal translocation that results in a break in the RARα gene, located on chromosome 17, and its fusion with another gene, often the PML gene on chromosome 15. In most cases, two abnormal fusion products, RARα–PML and PML–RARα, are formed from this t(15:17) translocation. PML is also a transcription factor. The PML–RARα protein binds to DNA but functions as a dominant negative receptor, interfering with normal RARα signaling *(75)*. Treatment with at-RA is thought to induce the differentiation of precursor cells that have retained a normal copy of the RAR gene, facilitating their progressive differentiation to more mature granulocytic cells, while leukemic cells containing the mutation are induced to undergo apoptosis. Despite the success of at-RA in inducing of cell differentiation, prolonged treatment with at-RA is not well tolerated. About 15% of patients treated with at-RA developed a severe and sometimes fatal thromboembolytic condition, referred to as retinoic acid syndrome *(76)*. Other patients have relapsed after treatment and become refractory to at-RA *(77)*. Clinical protocols have been revised to shorten the length of treatment with at-RA while combining it with other forms of chemotherapy, such as with arsenic trioxide or cisplatin.

Tissue specificity. Essentially all nucleated cells express RAR and RXR proteins. It is usual for one or more of these receptor forms to be predominant within a cell type; however, two or more forms may be co-expressed. For example, RARγ is more highly expressed in skin, while RARα has a broader tissue distribution. Although there are hints that the individual receptor types may differ somewhat in their functions, no single receptor has been shown to be indispensable, and the general mechanism of RA action does not appear to be tissue specific. Rather, the differences in tissue response to RA are likely to depend on factors such as ligand availability and the state of chromatin accessibility. Ligand availability is closely related to nutritional status, as an adequate supply of retinol substrate is a prerequisite for the endogenous production of RA. Substrate uptake could depend on the state of vascularization of the tissue or tumor and on the expression of enzymes required for RA production. Thus, *metabolic factors* leading to retinoid availability, as well as *cell-intrinsic properties* like nuclear receptor subtypes and relative amounts, coactivator/corepressor expression levels, and chromatin availability, are likely to interact in ways that determine tissue-specific patterns of retinoid-regulated gene expression.

Totality of the evidence. The totality of evidence suggests that production of RA from its natural nutritional precursor, retinol, is under sensitive control by retinoid-binding

proteins, LRAT, CYP26, and other enzymes. Together, the actions of these proteins serve to maintain a consistent level and tissue distribution of at-RA that, presumably, is appropriate for the cell type and the organism as a whole.

Good nutrition plays a key role in providing adequate, but not excessive, amounts of substrate (retinol stored as retinyl ester) for bioactivation, thus regulating the production of retinal and RA. From these overall features of retinoid metabolism it can be inferred that maintaining a one's dietary intake of vitamin A in a healthful range is a key component for maintaining tissues in an optimally differentiated state.

As noted above, exogenous RA or other retinoids may be helpful in the chemoprevention of premalignancies, but agents like at-RA are not acceptable for long-term treatment. In the case of fenretinide/4-HPR, which is relatively well tolerated, another problem has surfaced, namely low plasma retinol due to impairment of the RBP–retinol transport system *(3*, *78)*. This illustrates that retinoids have the potential to alter the metabolism of diet-derived vitamin A in ways that were not anticipated. It is quite possible that the "best" retinoids are those formed in situ, in a well-regulated process from diet-derived substrates. Thus maintaining an adequate but not excessive dietary intake, and thus an adequate but not excessive pool of retinol substrate in tissues, should be a first principle of good nutrition.

5. FUTURE RESEARCH DIRECTIONS

The differentiation promoting activity of at-RA and other retinoids is still very attractive for cancer treatment. It can be confidently concluded that retinoids do "work" in isolated cells, so how can they be made more effective in vivo? A major challenge is to harness and direct the power of these agents into effective dietary and/or clinical interventions. Several novel ideas for targeting retinoids to tissues are under investigation in preclinical models. Patches, biofilms, the delivery of retinoid on nanoparticles, aerosolized retinoids, etc., all are based on the concept of maintaining a local exposure, in a targeted manner, while minimizing the diffuse spread and total-body exposure to retinoids that occurs when they are administered systemically. New combinations of retinoids with other therapies, similar to the combined use of conventional chemotherapeutic drugs or arsenic trioxide with at-RA in the treatment of APL, also could be promising. Recently, retinoids have gained new interest from the perspective of stem cell biology and regenerative medicine, due to their strong differentiation-promoting actions on immature progenitor cells. For example, retinol promoted differentiation in progenitor cells of mouse pancreas, increasing β-cell differentiation and insulin production *(79)*. The generally positive effects of retinoids that have been observed in cell culture models suggest that they could be used effectively to promote the differentiation of stem, precursor, or progenitor cells ex vivo, prior to the transfer of cells to the patient.

6. CONCLUSIONS AND RECOMMENDATIONS FOR INTAKE/DIETARY CHANGES

The current nutritional recommendations for dietary vitamin A (retinol) are well supported by biological evidence that consumption of retinol at the recommended levels is both safe and adequate for supporting vision and maintaining the other systemic

functions of vitamin A. The Recommended Dietary Allowance (RDA) for vitamin A is based on a factorial model that includes intake of enough vitamin A to build up adequate, but not excessive, tissue vitamin A reserves *(80)*. The Tolerable Upper Intake Level (UL) was established by the Institute of Medicine in 2002 *(80)* as a way to inform the public that excessive intakes of certain nutrients (above ~ 3,000 μg/day of preformed retinol) could lead over time to adverse effects. For vitamin A, the UL for adults was set at 3,000 μg/day of preformed retinol because higher amounts may cause liver damage, increase the risk of birth defects in women of child-bearing age, and possibly increase the risk of osteoporosis. To eliminate these risks, a person could chose to consume more of his or her vitamin A intake in the form of β-carotene, instead of preformed retinol, since consumption of β-carotene at nutritional levels poses no risk of toxicity. Based on the cancer literature reviewed in this chapter, there is no compelling evidence to suggest that increasing the intake of dietary vitamin A above currently recommended levels (i.e., RDA levels) will reduce the risk of developing cancer. Many epidemiological studies have, however, shown that cancer risk is reduced in individuals who consume a diet high in fruits and vegetables, which contains vitamin A as β-carotene, is relatively low in fats, and also contains a mixture of phytonutrients, some of which may have anticancer properties of their own. This type of diet provides most if not all of the vitamin A therein in the form of carotenoids. The 2002 IOM report on vitamin A included sample calculations to show that an RDA level of vitamin A can be obtained from a well-selected vegetarian or vegan diet, as well as from a mixed-type omnivorous diet. The published examples provide evidence that consumption of retinol per se is not required to meet the RDA for vitamin A.

Conversely, high intakes of retinol are potentially deleterious. Case reports and experimental studies have thoroughly documented adverse effects of hypervitaminosis A, while new and accumulating data from epidemiological studies suggest that intakes of retinol from diet and supplements at levels even moderately above the RDA may increase bone loss *(81)*. Based on research in animals, high dietary vitamin A and treatment with RA are expected to affect retinoid homeostasis by inducing genes in the cytochrome P-450 family that result in increased retinoid catabolism.

Overall, there is no compelling evidence that changing current recommendations for dietary vitamin A would be helpful in reducing cancer risk. A report of an NIH State-of-the-Science Conference on the topic of "Multivitamin/Mineral Supplements and Chronic Disease Prevention" (which was not specific to vitamin A or to cancer prevention) concluded that while supplement use has grown and now more than half of the adult population of the USA uses multivitamin/mineral supplements, most of the studies that were reviewed do not provide strong evidence for health-related effects. The report also noted weaknesses in the methodologies for assessing nutrient intake from supplements and from fortified foods *(82)*. At present there is no scientific rationale to suggest that personalized nutritional recommendations for vitamin A would be beneficial. Thus it seems prudent to follow the Dietary Guidelines, which stress obtaining nutrients from whole foods in a mixed diet, at levels near the RDA. The "5-a-Day" recommendation for including five servings of fruits and vegetables, provided these servings include green leafy and yellow vegetables, should supply all the dietary vitamin A now known to be required to meet the body's requirement for this micronutrient.

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