Chapter 6

TRANSFORMING GROWTH FACTOR BETA AND BREAST CANCER

Virginia Kaklamani and Boris Pasche

Cancer Center Genetics Program, Division of Hematology/Oncology, Department of Medicine and Robert H. Lurie Comprehensive Cancer Center, Feinberg School of Medicine, Northwestern University, Chicago, IL

1. INTRODUCTION

Cancer research over the past few decades has generated a rich and complex body of knowledge showing that cancer cells acquire numerous features that differentiate them from their normal counterpart. These functional differences arise from the acquisition of multiple genetic changes affecting a variety of cellular pathways. It has been proposed that the diversity of cancer cell features is a manifestation of six essential alterations in cell physiology that collectively control malignant growth: abnormally activated growth signals, insensitivity to growth inhibition, evasion from programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis [1]. Laboratory experiments have demonstrated that, at a minimum, several of these essential alterations are necessary for the direct tumorigenic transformation of normal human epithelial and fibroblast cells[2]. Conversely, one may expect that effective treatment of an established cancer would require simultaneous therapeutic actions on at least several of these essential alterations. The Transforming Growth Factor Beta (TGF-β) signaling pathway is one of the few pathways that either directly or indirectly modulate several of these essential alterations: abnormally activated growth signals, insensitivity to growth inhibition, evasion from programmed cell death, and tissue invasion and metastasis [3]. This explains why the TGF-β signaling pathway plays a central role in cancer development and progression.

Transforming Growth Factor Beta (TGF-ß) is part of a large family of polypeptides that includes more than 30 members. This superfamily is broadly divided into two subfamilies, the TGF-ß/Activin/Nodal subfamily and the BMP (bone morphogenetic protein)/GDF (Growth and Differentiation Factor)/MIS (Muellerian Inhibiting Substance). There are three isoforms of TGF-β, TGFB1 (TGF-β1), TGFB2 (TGF-β2) and TGFB3 (TGF-β3). These isoforms are encoded by different genes but all bind to the same receptor: TGFBR2 [4]. Of the three isoforms, TGFB1 is most frequently upregulated in cancer cells [5,6] and has been more extensively studied.

TGF-β is secreted in a latent form and is activated by plasmin [7,8], thrombospondin[9], MMP-9 and MMP-2 [10]. Interestingly, plasminogen is converted to plasmin at sites of cell migration and invasion, which may result in increased activated TGF-β concentrations at those sites. MMP-9 and MMP-2 are expressed by malignant cells at sites of cell invasion [11,12] providing another mechanism for activation of latent TGF-β.

Once TGF-β becomes activated it can then bind to the type II receptor (TGFBR2), which then phosphorylates the type 1 TGF-β receptor (TGFBR1) leading to phosphorylation of its kinase. The next step in the signal transduction pathway is the phosphorylation of downstream elements. Several intracellular proteins have been shown to interact with the TGF-β receptor complex, including FKBP12 [13-15], STRAP [16] and TRIP-1 [17]. The current model of induction of signaling responses by TGF-ß related factors is a linear signaling pathway initiated by the activated TGFBR1 and resulting in ligand-induced transcription[18,19]. SMAD2 and SMAD3 are phosphorylated by TGFBR1 and form complexes with SMAD4. Activated SMAD complexes enter the nucleus where they regulate transcription of target genes through physical interaction and functional cooperation with DNA-binding transcription factors and CBP or p300 coactivators. SMAD6 and SMAD7 inhibit this pathway by interacting directly with TGFBR1 and preventing SMAD2 and SMAD3 phosphorylation.

The TGF-ß however interacts with other signaling pathways. These pathways regulate SMAD-mediated responses but also induce SMAD-independent responses[20]. The TGF-β signaling pathway is tightly regulated by other cellular elements and pathways. The activation of the epidermal growth factor receptor (EGFR) [21]interferon-γ (IFN-γ) signaling through STATs [22] and tumor necrosis factor α (TNF-α) through activation of NF-κB [23], inhibit the TGF-β signaling pathway by inducing expression of SMAD7. Other pathways that are tightly related to TGF-β include the RAS/MAPK pathway, which is able to inhibit SMAD signaling [24]. Furthermore several studies show a direct interaction between TGF-β and the p38/MAPK pathway indicating that TGF-β can activate the p38 pathway independently

from the SMADs in mammary cells [25]as well as other cell types including prostate [26].

TGF-β is a potent growth inhibitor of several cell types including epithelial cells. This inhibition is achieved through the induction of expression of CDKN2B ($p15^{INK4B}$) [27,28] and CDKN1A ($p21^{CIP1}$) [29]. Other mechanisms that lead to cellular growth arrest include the inhibition of MYC expression, CDK4 and CDC25A. The inhibitory signal of TGF-β can also induce apoptosis in several cell types [30-35]. This may be achieved through the DAXX adaptor protein which interacts with TGFBR2 [36] and through increased levels of SMAD3 and SMAD4 [37,38].

2. THE ROLE OF TGF-β IN MAMMARY GLAND DEVELOPMENT

There are several studies that point toward an important role of TGF-β in the development of the mammary gland. The morphologic and functional development of the breast tissue takes place during the postnatal period. During puberty, and with the influence of rising hormone levels, the mammary tree is established within an adipose stroma. During this period, the end-bud develops, which is the morphologic unit. The end-bud functions in extending the ductal epithelial tree. During pregnancy, growth and differentiation results in lobuloalveolar differentiation of the epithelium in order to produce milk.

As in most tissues, TGF-β seems to play a dual role in mammary gland development. One of the first studies evaluating the role of TGF-β in mammary gland development came from Daniel et al [39], who administered exogenous TGF-β via diffusion from miniature inorganic pellets, showing that end-buds undergo reversible regression during puberty, whereas alveolar buds in pregnancy do not.

The role of TGF-β in this process is not fully understood. Several studies have localized TGF-β as well as its type I, II and III receptors to the breast epithelium and stroma [40-42]. Furthermore all three TGF-β isoforms seem to be expressed in the epithelium during all phases of mammary development [43]. TGFB2 (TGF-β2) is less abundant whereas TGFB3 (TGF-β3) is the only isoform present in the myoepithelium. TGFB1 (TGF-β1) transcription decreases during pregnancy but, whereas the expression of the other two isoforms increases.

Another interesting observation is the difference in localization between latent TGF-β (LTGF-β) and active TGF-β. It has been shown that ionizing radiation induces activation of LTGF-β to TGF-β [44]. Furthermore radiation

induces stromal extracellular matrix (ECM) remodeling, which can be blocked by the use of TGF-β neutralizing antibodies [45].

TGF-β has also been shown to suppress the ability of mammary gland explants cultured with lactogenic hormones to secrete casein [46]. It inhibits ductal morphogenesis by mammary epithelial cells and this function can be reversed by the use of neutralizing antibodies, which simulate duct formation [47]. However it seems that this action is dose dependent: picomolar concentrations of TGF-β inhibit branching morphogenesis, whereas fentomolar concentrations stimulate it [48].

TGF-β has also been implicated in tumor progression. Overexpression of TGF-β1 in the mouse mammary gland inhibits tumorigenesis, while interfering with TGF-β receptor function enhances it [49,50]. Furthermore it has been shown that TGF-β receptor levels are diminished in human breast cancer cell lines and some primary tumors [51,52]. However expression of TGF-β is paradoxically increased in late stages of tumor progression especially in association with invasion and metastasis [53,54].

2.1 The role of TGF-β in Breast Cancer

In normal cells TGF-β is a potent growth inhibitor. On the other hand it is now appreciated that TGF-β is prooncogenic and that metastases in most tumor types require TGF-β activity [55,56]. It therefore seems that for every action of TGF-β there is a counteraction that TGF-β is capable of performing [57].

2.1.1 Somatic mutations of the TGF-β pathway

In an effort to explain the dual role of TGF-β in breast carcinogenesis, researchers have tried to find mutations that interfere with its function. Experiments in rodents indicate that increased TGF-β signaling correlates with decreased breast cancer risk. Transgenic mice that express a constitutively active form of Tgfb1 are resistant to DMBA-induced breast tumor formation [49]. Furthermore treatment of *Tgfb1* +/- mice with carcinogens results in enhanced tumorigenesis compared with *Tgfb1* +/+ littermates [58]. TGFBR2 downregulation is observed in breast cancer and seems to be due to a cellular trafficking defect in which most of the TGFBR2 remains in the cytosol [59]. A TGFBR1 tumor specific S387Y mutation was reported in 40% of metastatic breast cancers but in a follow-up study this finding was not reproduced [60,61]. Furthermore, although SMAD4 mutations have not been found in breast cancer, the MDA-MB-468 breast cancer cell line has a homozygous deletion of the gene [62,63]. Overall somatic

mutations in the TGF-β pathway in breast cancer are extremely rare and do not seem to contribute to carcinogenesis.

2.1.2 Germline mutations and polymorphisms of the TGF-β pathway

Recently a *TGFBR1* germline polymorphism was described, which is present in approximately 14% of the population. This common variant results from the deletion of three alanines within a 9-alanine stretch of exon 1 coding sequence and was named *TβR-I(6A)* because it codes for 6 alanines [64,65]. In 2003, it was renamed *TGFBR1**6A in accordance with the HUGO nomenclature. Using a mink lung epithelial cell line devoid of endogenous TGFBR1, transiently and stably transfected *TGFBR1* and *TGFBR1*6A* cell lines were established for functional studies. Compared to *TGFBR1*, *TGFBR1*6A* was moderately impaired as a mediator of TGF-ß antiproliferative signals [65,66]. The additional findings of an overrepresentation of *TGFBR1*6A* heterozygotes and homozygotes among patients with a diagnosis of cancer as compared with the general population suggested that *TGFBR1*6A* might be a new tumor susceptibility allele[65]. Over the past few years several studies have focused on the cancer risk of individuals heterozygous or homozygous for *TGFBR1**6A. A meta-analysis of seven case-control studies showed that *TGFBR1*6A* carriers have a 26% increased risk for cancer. Breast cancer risk was increased by 48%, ovarian cancer risk by 53% and colon cancer risk was increased by 38% [67]. A second meta-analysis of twelve case control studies has added further support to these findings and confirm *TGFBR1**6A as the most common candidate tumor susceptibility allele reported to date that increases the risk of breast, colon and ovarian cancer [68].

Several polymorphisms have been reported within the human *TGFB1* gene. One of them has been extensively studied in relation to breast cancer risk. This polymorphism is represented by the substitution of Leucine to Proline (T→C) at the 10th amino acid position. The Leucine to Proline substitution results in higher TGFB1 secretion [69]. The CC (*TGFB1*CC*) genotype was found by one group of investigators to be associated with a 64% decreased breast cancer risk in a cohort study of 3,075 white American women over age 65 at recruitment [70]. In contrast, in a pooled analysis of three European case-control studies that included 3,987 cases and 3,867 controls, the CC genotype was associated with a 21% increased risk of breast cancer [69]. In a hospital-based study of 232 cases and 172 controls conducted in Japan, there was no significant overall association between the CC genotype and breast cancer. However, the CC genotype was associated with a 65% reduced risk of breast cancer in comparison with the TT

genotype among premenopausal women (OR 0.45, 0.20-0.98)[71]. Most recently, a large multiethnic case control study of 1123 breast cancer cases and 2314 controls from Los Angeles and Hawaii did not find any association between the *TGFB1**CC polymorphism and breast cancer risk [72]. Of major interest is the recent report that patients with a diagnosis of breast cancer that carry the *TGFB1* T to C variant have a significantly decreased survival as compared with non-carriers[73] If confirmed in subsequent studies, this would be the first evidence in humans that increased levels of secreted TGFB1 are associated with more aggressive disease.

3. TGF-β, ESTROGENS AND ANTIESTROGENS

There seems to be a correlation between stage of breast cancer and TGFB1 serum levels. More specifically individuals with more advanced lymph node status, more advanced TNM staging and poorer histologic grade have higher TGFB1 serum levels [74].

TGFB1 serum levels are increased in individuals with metastatic or locally advance breast cancer, compared with healthy donors [75] and there may be a relationship between these levels and patients' response to therapy.

TGF-β has also been implicated in the regulation of *NCOA3*, also named *AIB1* (amplified in breast cancer 1), a nuclear receptor coactivator gene, which is amplified and overexpressed in breast cancer. Experiments with TGF-β and TGF-β neutralizing antibodies have shown that antiestrogens suppress AIB1 gene expression through TGF-β [76].

It is unclear whether TGF-β levels change significantly with the administration of tamoxifen. A recent study evaluating TGFB1 and TGFB2 levels showed that although TGFB1 levels did not correlate with tamoxifen treatment, TGFB2 levels increased with tamoxifen administration [77]. Antiestrogens have also been shown to inhibit the chemotactic activity of TGF-β in MCF-7 cells [78]. This may point toward the potential benefit of combining antiestrogens with direct TGF-β inhibitors.

There is evidence that the TGF-β pathway interacts with ESR1, also named Estrogen Receptor α (ERα), through crosstalk with SMAD4. More specifically, SMAD4 and ESR1 form a complex when ESR1 binds to the estrogen-responsive element within the estrogen target gene promoter. Furthermore SMAD4 seems to inhibit antiestrogen-induced luciferase activity as well as estrogen downstream target gene transcription in breast cancer cells [79].

4. MECHANISMS OF TGF-β RESISTANCE IN CARCINOGENESIS

Although the growth of normal epithelial and mesenchymal cells is arrested by TGF-β, cancer cells are able to escape this mechanism and become TGF-β unresponsive. The mutations mentioned above provide one such mechanism. More often, however, loss of responsiveness to the TGF-β growth inhibitory effect does not result from inactivating mutations or homozygous deletions of members of the TGF-β signaling pathway. One mechanism involved in acquired TGF-β resistance involves the upregulation of oncogenic expression. One such example is the elevated expression in melanoma of the proto-oncogene *SKI* [80]. This correlates with the decreased responsiveness to TGF-β, probably due to repression of SMAD-mediated transcription [81]. *SKI* as well as *SKIL*, also named *SnoN*, are two protooncogenes that interact in the nucleus with SMADs and negatively regulate them. It has been shown that SMAD2, 3, and 4 bind to different regions of SKI and SKIL. Furthermore mutations in the SMAD-binding regions of these two protooncogenes impair their ability to promote carcinogenesis in chicken embryo fibroblasts [82]. It has been shown that reduced expression of SKIL significantly correlates with longer distant disease-free survival in estrogen receptor-positive breast cancer patients. Furthermore high levels of nuclear SKIL are associated lobular histology and favorable features, whereas high levels of cytoplasmic SKIL are associated with ductal histology and adverse prognostic features [83]. Also, downregulation of *MYC* expression by TGF-β, is lost in several cancer cell lines [84]. Another oncogene, *EWSR1*, represses TGFBR2 expression and may account for decreased responsiveness to TGF-β in cancer cells [85].

5. THE ROLE OF TGF-β IN CELL CYCLE ARREST

Although it has been shown that normal mammary epithelial cells are sensitive to the growth inhibitory effect of TGF-β, human breast cancer cell lines, show a relative resistance to the effect of TGF-β requiring 10 to 100-fold more TGF-β to produce an antimitogenic effect, some show complete loss of response to TGF-β signaling and some are growth stimulated by TGF-β [51,86]. The effect of TGF-β in the cell cycle seems to come in a discrete period in the G1 phase [87,88]. TGF-β has been shown to downregulate *MYC* by inhibiting its transcription [89-91]. *MYC* is needed for the progression from G1 to S phase. This downregulations seems to be important in the cell cycle arrest caused by TGF-β. This is further emphasized by the fact that *MYC* overexpression seems to be one of the mechanisms responsible for

TGF-β resistance [89,92]. TGF-β also causes loss of G1 cyclins [93,94] and regulates CDK2 phosphorylation [93,95].

So what seems to happen during tumorigenesis that causes the loss of TGF-β mediated G1 arrest? One mechanism that seems to contribute to this effect is overexpression of cyclins. It has been shown that cyclin D1 gene is amplified in 40% of breast cancers [96,97]. Furthermore there seems to be overexpression of *CDK4* [98] and activation of *MYC*, which in turn may regulate indirectly the expression of CCND1, CCNE1 and CCNA2 [99,100]. Finally activation of HRAS, which commonly occurs in human malignancies, can increase CCND1 levels, which can provide another mechanism of TGF-β resistance [101-103].

6. INVASION, ANGIOGENESIS AND TUMOR METASTASIS

For a tumor to metastasize, a multistep process has to take place, which requires migration and invasion through the stroma, and then migration in and out of blood and lymphatic vessels. Increased production of TGF-β occurs in several tumor types and frequently correlates with tumor aggressiveness [104]. The contribution of TGF-β to the invasive behavior of tumors has been studied in several mouse models [105-107]. Transgenic expression of activated TGFβ1 in mouse skin epidermis increases the conversion to carcinoma [107]. Also, tumor formation and metastasis to bone was shown that depend on intact TGFBR2 [108]. When the transplanted cells expressed a partially activated TGFBR1, there was acceleration of bone destruction by malignant cells followed by a reduction in survival[108].

Changes in the tumor microenvironment are also an integral part of the process of metastasis. TGF-β seems to play an integrar role in this process. Increase protease expression and plasmin activation by tumor cells [109] promotes activation of TGF-β from its latent form. Furthermore increased levels of activated TGF-β enhance the synthesis of ECM proteins and chemo attraction of fibroblasts, which in turn promote tumor growth, invasion and angiogenesis [1]. Evidence of a crucial role for TGF-β in angiogenesis comes from several observations. Increased expression of TGFB1 in transfected prostate carcinoma or Chinese hamster ovary cells enhances angiogenesis in immunodeficient mice whereas administration of neutralizing antibodies against TGFB1 strongly reduces tumor angiogenesis [110]. Re-expression of SMAD4 in SMAD4-deficient pancreas cancer cells suppresses tumor development primarily by inhibiting angiogenesis [111]. Also in human breast

cancers, high levels of TGFB1 m-RNA are associated with increased microvessel density [112].

TGF-β has also been shown to induce the expression of VEGF, which is a direct stimulant of cell proliferation and migration [113]. TGFB1 is a potent chemoattractant for monocytes, which release angiogenic factors [114-117]. Another mechanism by which TGF-β induces cell migration is the induction of expression of the matrix metalloproteases MMP-2 and MMP-9 and the downregulation of protease inhibitor TIMP in tumor and endothelial cells [118-123]. It was recently shown that TGFB1 works in conjunction with tenascin-c (TN-C) to upregulate MMP-9 expression. Neutralization of TGF-β with a specific TGFB1 antibody results in decreased expression of MMP-9. However, the addition of TN-C upregulates MMP-9 [124].

The role of TGF-β in angiogenesis is further highlighted by the presence of the transmembrane glycoprotein endoglin (ENG; CD105). Endoglin is primarily expressed in endothelial cells and binds TGFB1 and TGFB3, through its association with TGFBR2 [125,126]. It has been shown that endoglin interacts with INHBA (activin-A), BMP7 and BMP2 [126]. Inhibition of endoglin expression in cultured endothelial cells enhances the ability of TGFB1 to suppress their growth and migration [127]. Exogenous TGFB1 has been shown to up-regulate endoglin expression [128]. In fact, it has been suggested that the development of an angiogenic response depends on a balance between levels of TGF-β stimulation and endoglin expression [127]. Furthermore in vivo studies in SCID mice carrying human breast carcinoma showed that anti-endoglin monoclonal antibodies produce anti-tumor effect probably mediated by angiogenesis inhibition and destruction of tumor-associated vasculature [129-131].

Immunohistochemical staining of TGF-β in breast cancer cells from lymph node metastases show that there is preferential staining at the edges of the tumor [132]. TGF-β may play a role in directing metastatic cells to specific sites. It has been shown that TGF-β and MAPK1 (p38) induce expression of PTHLH, a PTH-related protein which directs metastatic cells to the bone [108,133]. Furthermore it has been shown that mRNA levels of Bone Morphogenetic Protein-2 (BMP2), a TGF-β family member with anti-proliferative effects in breast cancer cell lines, are significantly decreased in breast tumor tissue compared with normal breast tissue [134]. This may provide a potential mechanism for the metastatic potential of breast cancers and their capacity to grow in bone.

7. ROLE OF TGF-β IN EPITHELIAL-MESENCHYMAL TRANSITION (EMT)

Another important aspect of the contribution of TGF-β to cancer development is its impact on the loss of cell–cell contacts and acquisition of fibroblastic characteristics, a process that is commonly referred to as the epithelial–mesenchymal transition (EMT). Such transitions occur frequently during development and in certain cases are influenced by members of the TGF-β family. Indeed, TGF-β stimulation of both non-transformed and carcinoma-derived cell populations in culture leads to reversible EMT [135-137]. Also, expression of TGFβ1 in the skin of transgenic mice enhances the conversion of benign skin tumors to carcinomas and highly invasive spindle-cell carcinomas[107] and expression of a dominant-negative TGFBR2 prevents squamous carcinoma cells from undergoing EMT in response to TGF-β in vivo [106]. The crucial role of TGF-β as a mediator of stromal cell dependent epithelial carcinogenesis was recently unveiled. Conditional *Tgfbr2* inactivation in mouse fibroblasts resulted in intraepithelial neoplasia in prostate and invasive squamous cell carcinoma of the forestomach [138].

8. ROLE OF TGF-β IN THE IMMUNE SYSTEM

TGF-β plays a direct role in proliferation and differentiation in hematopoiesis [139-142]. TGFB1 influences both proliferation and differentiation of the uncommitted stem cell precursors and of myeloid progenitors [143,144]. Furthermore autocrine production of TGF-β by hematopoietic stem cells acts to maintain their quiescence [145]. TGF-β can also control the expression of the stem cell antigen CD34 [146,147] and under certain circumstances prohibit differentiation [147,148]. Overall, TGF-β preserves self-renewal in primitive stem cells with moderate cell cycle blockade while it favors terminal differentiation of mesenchymal precursors and cell cycle arrest in terminally differentiated immune effectors. Mutations in the TGF-β pathway are very rarely encountered in hematopoietic tumors. There are only anecdotal reports of mutations in TGFBR1 and TGFBR2 occurring in lymphoid malignancies [149 150].

TGF-β can arrest stimulated B cells in G-1 [151], reduce Ig synthesis, and inhibit the switch from membrane-bound to secreted Ig [152]. NK cells lyse appropriate tumor cells in vitro [153,154], are a source of T-cell-cytokines, including IFN-γ [155] and should be effective in surveillance against tumor cells that have lost expression of MHC [156]. In addition, NK cells can secrete

TGF-β [157] which acts by depressing the expansion and generation of cytolytic NK cells [158,159].

Antigen specific CD8+ cells recognize peptides that are presented by MHC class I molecules on target cells. This cell-cell interaction causes destruction of the target cells mediated by perforin released by the cytotoxic CD8+ cells. Therefore any process that causes deactivation of the CD8+ T cells can promote growth and evasion of cancer.

9. ROLE OF TGF-β IN ESCAPING IMMUNOSURVEILLANCE

Tumor escape from immunosurveillance has been demonstrated using syngeneic tumors that grow in nude (T cell-less) and SCID (T and B cell-less mice) mice but grow only for a limited time in normal mice before they are rejected by tumor specific immunity [160,161]. However it seems that if a large enough tumor is inoculated in the normal mice, this tumor progressively grows and the tumor cells no longer expresses the immunodominant epitope of the parent tumor [161].

Tumors have devised several approaches to escape from immunosurveillance. These approaches include: interference with antigen processing and presentation, antigenic variation, lack of costimulatory signals to T cells, induction of apoptosis and secretion of immunosuppressive cytokines. It has been shown that transport associated peptide (TAP), a critical component of antigen presentation is downregulated [162] as is the MHC I complex [163,164]. Also, antigenic peptides expressed on the surface of tumor cells can be downregulated. It has also been shown that B7, a costimulatory molecule, is not present on the surface of tumor cells, contributing to T cell anergy [165,166]. However the mechanism thought to contribute the most to the escape from immunosurveillance is the secretion by tumor cells of cytokines that inhibit immune response. Such factors include prostaglandin E2, interleukin-10 but the most potent immunosuppressor is TGF-β [167].

TGF-β inhibits T-cell, NK cells, neutrophils, macrophages and B-cells [117-123,140,168]. It has also been shown that TGF-β downregulates the expression of MHC class II antigen, which makes cell surface less immunogenic [169-171]. More evidence of the role of TGF-β in as a modulator of NK cell activity came from the observation that TGF-β antibodies only suppress tumor growth in mice with intact NK function [172]. This observation together with the findings that TGF-β may be a mediator of tamoxifen's antitumor effect [167,173] suggests a new explanation for tamoxifen resistance: the rise of tamoxifen-induced TGF-β secretion may contribute to the

emergence of tamoxifen resistance by altering NK cell antitumor cytotoxic effects. This hypothesis is supported by the observation that in patients with breast cancer and in experimental models, tamoxifen enhances NK function [167,174-177]. But with prolonged exposure to tamoxifen, inhibition of NK cells has been observed [178].

Due to the apparent role of TGF-β in regulating the immune system, several investigators have used TGF-β targeted vaccine approaches to stimulate the immune system against the tumor cells. In one such approach, a TGF-β-targeted vaccine in rat glioma has been reported to result in the complete eradication of tumors when an antisense TGF-β construct was introduced into resected tumor cells ex vivo and then locally reintroduced into the tumor-bearing host [179]. Furthermore in a mouse thymoma model, tumor cells engineered to secrete soluble TGFBR2, resulted in a suppression of tumorigenicity [180]. Although so far these approaches have not been successfully introduced to clinical practice, they point to the emergence of a new concept in cancer immunotherapy, in which leukocytes, insensitive to TGF-β signals can be genetically engineered and may provide one approach against the "tumor firewall" [181].

10. IMMUNOTHERAPEUTIC APPROACHES TARGETING THE TGF-β PATHWAY

TGF-β is probably a major cytokine responsible for evading the response of the host's immune system. Establishing a population of leukocytes insensitive to TGF-β, which would localize at the site of the tumor and exert their tumoricidal properties is an appealing approach. Such an approach was recently attempted with very encouraging results. Murine melanoma cells were transplanted into mice that had hematopoietic precursors rendered insensitive to TGF-β via retroviral-mediated gene therapy. Survival of the genetically engineered mice at 45 day survival was 70% compared with 0% for vector-controlled treated mice [182]. Similar experiments using ex vivo transfer of an antisense TGF-β construct into isolated tumor cells followed by reimplantation into the brain of rats with established glioma has been shown to result in complete eradication of the tumors in vivo [179]. These preliminary results are encouraging. This approach will be tested soon in clinical trials to determine its potential usefulness in human cancer.

10.1 Soluble protein inhibitors of the TGF-β pathway

A soluble chimeric protein composed of the extracellular domain of TGFBR2 and the Fc portion of the murine IgG1 heavy chain (Fc:TGFBR2) has been found to interfere with the binding of endogenous TGF-β with its receptor. Other cytokine antagonists that use this soluble receptor:Fc fusion protein class include Etanercept, the anti-TNF-α antibody which has received FDA approval for the treatment of rheumatoid arthritis. This fusion protein has shown protection against development of distant metastases in animal studies. In one study investigators used mice transplanted with breast cancer that were systemically given Fc:TGFBR2. It was shown that soluble Fc:TGFBR2 inhibits distant metastases in that experimental model. This was achieved not by alterations in cellular proliferation of tumor cells but through decreased tumor cell motility and intravasation, inhibition of MMP activity and increase in cancer cell apoptosis. Injection of this fusion protein for a total of 12 weeks in mice was not accompanied by any obvious toxicity [183]. In another study investigators exposed MMTV-neu transgenic mice (a commonly used breast cancer mouse model) to lifelong Fc:TGFBR2. The concern was that lifetime exposure to this antibody would have deleterious effects in the immune system similar to what was observed in *Tgfb1* null mice that develop lethal multifocal inflammatory syndrome with features consistent with autoimmune disease [184,185]. However, prolonged exposure to Fc:TGFBR2 conferred protection against metastasis arising from either an endogenous primary tumor or from injection of metastatic melanoma cells. Furthermore when studying the immune function of these mice the only difference observed was a small, clinically insignificant increase with age of memory T cell lymphocytes and a higher incidence of benign lymphocytic infiltrates in the lung, pancreas and kidney [186]. These two studies can lead to certain conclusions: 1) The use of a neutralizing antibody against the TGFBR2 does not spontaneously induce tumors, a phenomenon which had been observed in *Tgfb1* +/- and *Tgfbr2* +/- mice [58]; 2) Administration of Fc:TGFBR2 significantly reduces the incidence of metastases; 3) There doesn't seem to be any obvious toxicity with either short-term or long-term administration of Fc:TGFBR2.

Although there have not been any reports of tumor formation with the use of antibodies against the TGF-β pathway, there are some concerns given the "two faces" of TGF-β in carcinogenesis. In a recent study it was shown that TGF-β signaling impairs Neu-induced mammary tumorigenesis while at the same time promoting pulmonary metastasis [3]. When investigators crossed mice expressing activated forms of Neu receptor tyrosine kinase that selectively couple to Grb2 or Shc signaling pathways the activated type I receptor increased the latency of mammary tumor formation but also

enhanced the frequency of extravascular lung metastases. Furthermore expression of the dominant negative type II receptor decreased the latency of Neu-induced mammary tumor formation while significantly reducing the incidence of extravascular lung metastases. Maybe one way to avoid these effects would be to couple an antibody against the TGF-β pathway with a cytotoxic agent. These results, although encouraging, need to be validated in clinical trials to show whether in vivo alteration of TGF-β signaling is a feasible approach for the treatment of human malignancies.

10.2 Small molecule inhibitors of TGF-β

The first specific inhibitor of the TGF-β pathway is the compound SB-431542 [187]. This compound acts as a competitive inhibitor in the TGFBR1 ATP binding site and inhibits in vitro phosphorylation. TGFBR1 phosphorylation of SMAD2 and SMAD3 is inhibited by the administration of SB-431542. Furthermore it has been shown that this small molecule kinase inhibitor is specific the only other weakly inhibited kinase was MAP kinase p38a [188]. Due to its similarity a p38 MAPK inhibitor (SB-203580) has also been shown to inhibit TGFBR1 at high concentrations [189]. SB-431542 has also been shown to inhibit TGFB1-induced generation of collagen Iα1 (col Iα1), a matrix marker [190].

11. CONCLUSIONS

The role of TGF-β in breast cancer development is complex. In early carcinogenesis TGF-β acts as a growth inhibitor. However, later on, TGF-β acts as a prooncogenic cytokine promoting metastasis and escape from immunosurveillance. So far therapeutic approaches using the TGF-β pathway have been met with great enthusiasm. The use of monoclonal antibodies, small molecule kinase inhibitors or gene therapy to block the TGF-β signal has lead to delayed development of metastatic disease and prolonged survival in murine models of carcinogenesis. These observations, together with the fact that there was no observed toxicity give us hope that in the future we will be able to test these molecules in clinical trials. For the time being, however, understanding the mechanisms behind the dual role of TGF-β in cancer development, as well as the potential role of TGF-β in prevention or delaying of cancer development need to be elucidated.

Epidemiologic data indicate that naturally occurring common variants of the TGF-β signaling pathway modulate breast cancer risk and outcome. There is growing evidence that *TGFBR1*6A* may contribute to the

development of a sizeable proportion of breast cancers. Ongoing studies that assess TGF-β signaling through the prism of its functionally relevant common variants, *TGFBR1*6A* and *TGFB1*CC*, will identify subgroups of individuals with increased or decreased breast cancer risk based on the expected level of signaling. It is anticipated that these variants, in particular *TGFBR1*6A*, will become part of the overall breast cancer risk assessment. We foresee that these TGF-β pathway variants will account for a proportion of familial breast cancer cases. While we predict that individuals with overall decreased TGF-β signaling will be more prone to develop certain forms of cancer, we believe that the tumors of these individuals will behave less aggressively because they will not benefit as much from the prooncogenic properties of the TGF-β signaling pathway. On the other hand, individuals with higher baseline TGF-β signaling may have more aggressive tumors.

TGF-β signaling will become a target for cancer therapies. Candidates for these therapies will include patients with aggressive tumors exhibiting intact TGF-β signaling. Small inhibitory molecules and anti-TGF-β antibodies will enter the clinical arena either as adjuvant, second or third line therapies in metastatic cancers. TGF-β will become a bona fide molecular target in the next five years.

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