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TUMOR-SUPPRESSIVE AND PROMOTING FUNCTION OF TRANSFORMING GROWTH FACTOR BETA

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1. ABSTRACT

Transforming growth factor beta (TGFβ) is a multifunctional polypeptide. Its role in carcinogenesis can be either suppressive or promoting depending on tumor developmental stages and cellular context. During the early phase of epithelial tumorigenesis, TGFβ inhibits primary tumor development and growth by inducing cell cycle arrest and possibly apoptosis. However, in late stages of progression, as tumor cells evade the growth inhibition by TGFβ due to inactivation of its signaling pathway or aberrant regulation of cell cycle machinery, the role of TGFβ signaling is often switched from tumor suppression to promotion. TGFβ can apparently act in tumor stroma as well as tumor cells to inhibit host immune surveillance and stimulate invasion, angiogenesis, and metastasis. Studies have shown that antagonizing TGFβ activity can inhibit tumor progression, especially metastasis, in certain tumor models. However, the molecular markers that can indicate the feasibility of the use of TGFβ antagonists as cancer therapeutics remain to be determined.

2. INTRODUCTION

Transforming growth factor beta (TGFβ) is a homodimeric polypeptide of 25 kDa. Three TGFβ isoforms termed TGFβ$_1$, β$_2$, and β$_3$ have been identified in mammals. The mature TGFβs are derived from the C-terminal 112 amino acids of their respective precursors by proteolytic cleavage. All three TGFβ isoforms are secreted in a latent form in that the mature TGFβs are noncovalently associated with the N-terminal remainder of their processed precursors. This latent complex cannot interact with the cell surface TGFβ receptors and thus requires activation to release the mature, active TGFβ through its interaction with various extracellular proteins (1). The active TGFβ binds to three different cell surface receptors called type I (RI), type II (RII) and type III (RIII) receptors. RIII (also called betaglycan) has two TGFβ binding sites in its extracellular domain and can sequester and present TGFβs to RII to augment their activity when it is membrane-bound (2-6). RI and RII are serine/threonine kinase receptors. Binding of TGFβ to RII recruits and transphosphorylates RI (7). The activated RI phosphorylates intracellular Smad2 and Smad3, which then interact with Smad4 protein to form an oligomeric complex (8). Once transported into nuclei, Smad2/Smad4 and Smad3/Smad4 complexes bind to specific DNA sequences and can act as transcription activator or repressor depending on DNA sequence and cellular context (1, 9).

TGFβs are multifunctional polypeptides involved in the regulation of cell proliferation, differentiation, extracellular matrix formation, and immune response (8, 10, 11). TGFβ was initially identified as a **transforming** growth factor in that it stimulated anchorage-independent growth of the NRK fibroblasts in the presence of epidermal growth factor (12). Later, it was found to be a potent growth inhibitor in various types of cells including epithelial cells (13). More recently, many studies have shown that TGFβ and its signaling components can act to inhibit or promote tumor progression depending on stages of carcinogenesis or model systems used. This minireview is intended to furnish the readers current information on the research findings of the role of TGFβ signaling in epithelial tumor progression and the utilization of TGFβ antagonists in suppressing TGFβ-induced tumor progression.

3. TUMOR SUPPRESSIVE ACTIVITY OF TGFb

3.1. Disruption of TGFb signaling pathway promotes early stage tumor progression

When TGFβ was shown to act as an autocrine negative growth regulator in various epithelial cell lines (14-16), it was quickly hypothesized that TGFβ isoforms may act as inhibitors of tumor progression. Indeed,

antisense inhibition of TGFβ expression was shown to stimulate tumor formation and growth of two well-differentiated human colon adenocarcinoma xenograft models in mice (17, 18). Cloning of TGFβ receptors and identification of Smad proteins as intracellular TGFβ signaling mediators have dramatically expanded our understanding of the tumor suppressive role of TGFβ signaling pathway. *TGFBR2*, the gene that encodes RII, was found to be mutated in colon carcinoma cells from hereditary non-polyposis colorectal cancer patients (19). These patients are predisposed to frequent insertion or deletion of repeated mono- or di-nucleotide sequences due to germline mutations of their DNA mismatch repair enzymes, a phenomenon called microsatellite instability. The mutation of *TGFBR2* occurs in its coding sequence containing a repeated segment of adenines and is also observed in gastric cancer cells with microsatellite instability (20). In other types of carcinoma with microsatellite instability such as breast, lung, pancreatic, and endometrial carcinoma, RII is not frequently mutated (20-22). However, its expression can be down-regulated during carcinogenesis in some cases (23-25). On the other hand, re-expression of RII in human carcinoma cells with loss of or reduced RII expression can inhibit their malignancy (26-28). Mutation of RI gene has been reported in ovarian cancer with a high frequency (29, 30), whereas its expression is transcriptionally repressed by DNA methylation in gastric cancer (31). Like RII, ectopic expression of RI in human carcinoma cells with a low level of cell surface RI also inhibited tumor progression (32).

Deletions and mutations of the genes of the Smad proteins that mediate TGFβ signal transduction have also been observed in human carcinomas. Most notable is the mutational inactivation of *SMAD4*, also known as *DPC4* (deleted in pancreatic carcinomas), in human pancreatic cancer (33, 34). Mutations of *SMAD4* have also been described in human colorectal cancer, especially in late stage, metastatic colon cancer patients (35-37). In contrast, mutational inactivation of *SMAD3* has not been reported, whereas mutations of *SMAD2* have only been observed in a limited number of human colon and lung carcinomas (38, 39) and are apparently uncommon. These observations suggest a pivotal role of Smad4 in TGFβ-induced tumor suppression.

The tumor suppressive activity of TGFβ signaling pathway during the early stage of tumorigenesis has also been extensively demonstrated in experimental mice. Transgenic expression of active $TGF\beta_1$ in the mammary gland was shown to inhibit TGFα- or chemical carcinogen-induced tumor formation (40). Conversely, $TGF\beta_1$ heterozygous null mice expressed 10-30% of the wild-type $TGF\beta_1$ level and were more susceptible to chemical carcinogen-induced tumorigenesis (41). Similarly, disruption of TGFβ signaling by the expression of a dominant negative RII in the mammary gland or the skin also enhanced chemical carcinogen-induced tumor formation and progression (42-44). Smad4 heterozygous null mice was shown to develop gastric and duodenal polyps, which can progress into invasive tumors with the loss of the other copy of *SMAD4* (45, 46). Compound Smad4 and APC (adenomatous polyposis coli) heterozygous null mice was shown to develop more malignant intestinal tumors than APC heterozygous null mice and the remaining copy of both *SMAD4* and *APC* was found to be lost in the malignant tumors (47). Thus, TGFβ signaling components are necessary to protect epithelial tissues from tumorigenesis.

3.2. The tumor suppressive activity of TGFb is mediated by its effect on cell cycle progression and apoptosis

Although TGFβ is a multifunctional cytokine involved in the regulation of many genes and cellular phenotypes, its tumor suppressive activity is widely attributed to its ability to regulate the expression of a number of key proteins in the control of cell cycle progression from G1 to S phase. One of them is the proto-oncogene, *c-myc*, which is known to promote cell cycle entry into S phase by regulating the transcription of various cell cycle related genes (48). TGFβ treatment can rapidly inhibit the transcription of *c-myc* in epithelial cells (49-51). The inhibition is apparently accomplished by TGFβ-stimulated nuclear translocation of a transcription repression complex containing Smad3, E2F4/5 and the corepressor p107, and subsequent docking of this complex in association with Smad4 on a Smad/E2F binding site of *c-myc* promoter (52). On the other hand, TGFβ can also stimulate the transcription of the cyclin-dependent kinase (CDK) inhibitors, $p15^{ink4b}$ and $p21^{cip1}$ (53, 54). The former interacts with CDK4 and CDK6 to inhibit their kinase activity and association with cyclin D, whereas the latter mainly inhibits the activities of cyclin A-CDK2 and cyclin E-CDK2. Both cyclin D-CDK4/6 and cycline E-CDK2 can phosphorylate retinoblastoma gene product pRB to inactivate its ability to block G1 to S phase transition. The transcriptional activation of $p15^{ink4b}$ and $p21^{cip1}$ by TGFβ is through a synergistic interaction between Smad proteins and the Sp1 transcription factor at the promoter region of $p15^{ink4b}$ or $p21^{cip1}$ genes (54-57). It should also be noted that the binding of $p15^{ink4b}$ can also displace $p21^{cip1}$ and another CDK inhibitor $p27^{kip1}$ associated with cyclin D-CDK4/6 resulting in more $p21^{cip1}$ and $p27^{kip1}$ to inhibit cyclin A/E-CDK2. Thus, the stimulation of $p15^{ink4b}$ by TGFβ can lead to the inhibition of both cyclin D-CDK4/6 and cyclin A/E-CDK2 (58, 59).

Another mechanism that is believed to mediate the tumor suppressive activity of TGFβ is its stimulation of programmed cell death called apoptosis. Treatment with exogenous TGFβ has been shown to stimulate apoptosis in various cell types including epithelial cells (60, 61). However, how TGFβ regulate apoptosis at the molecular level is not well defined although several apoptosis-related proteins including Bcl-xL, caspases, Smad7 and p38 MAP kinase have been implicated (60, 62). The extent of apoptosis induced by TGFβ and the effectors involved appears cell context-dependent. Of note, significantly higher concentrations of TGFβ are usually required to induce apoptosis than to inhibit cell proliferation. Furthermore, few reports have shown a regulatory role of autocrine TGFβ signaling in controlling apoptosis. Thus, the role of TGFβ-mediated apoptosis in tumor suppression

remains elusive although it is known to play an important role in tissue development and remodeling (63).

While the inhibition of cell cycle progression is apparently the major mechanism of tumor suppression by TGFβ, recent studies suggest that the inhibition of telomerase activity by TGFβ may also contribute to its tumor suppressive function. TGFβ was shown to inhibit telomerase activity by suppressing the transcription of human telomerase reverse transcriptase (64, 65). The inhibition can lead to attrition of telomere and cell senescence as observed in human lung adenocarcinoma cells (66). These activities of TGFβ should place its signaling pathway as a "gate keeper" in preventing tumorigenesis and inhibiting the growth of primary tumors. However, mutational inactivation of TGFβ signaling components appears to occur at late stages of carcinogenesis. For example, loss of DPC4 expression occurs during the transformation of human pancreatic neoplasm from non-invasive to invasive stage (67). Similarly, mutation of *TGFBR2* is associated with progression of human colon adenomas to malignant carcinomas (68). Thus, TGFβ signaling appears necessary for the suppression of tumor invasion and metastasis. Indeed, targeted expression of a dominant negative RII in mouse prostate was shown to promote metastasis of SV40 large T antigen-induced prostate tumors with little effect on the sizes of the neoplastic prostates (69). Further studies are needed to determine whether loss of control of cell proliferation, apoptosis and senescence by TGFβ is sufficient to promote tumor invasion and metastasis or whether TGFβ signaling specifically regulate a set of metastasis-related genes.

4. SWITCH FROM TUMOR SUPPRESSION TO TUMOR PROMOTION

4.1. Tumor cells often evade the growth inhibition by TGFb

Because of its potent growth inhibitory activity in normal epithelial cell, TGFβ signaling pathway is often disrupted or modulated in tumor cells such that they are resistant to its growth inhibition. In some cases, the loss of TGFβ sensitivity is categorical due to the loss of TGFβ signaling receptors as mentioned above. Loss of Smad proteins, especially Smad4, can also abrogate a majority of TGFβ signaling activities although TGFβ has been shown to inhibit cell proliferation or stimulate apoptosis independent of Smad proteins (70, 71). However, complete inactivation of TGFβ signaling through mutations of RII or Smad proteins is restricted to certain types of cancer and is rare in other types of cancers with or without microsatellite instability. For example, breast, endometrial, pancreatic and lung carcinomas with microsatellite instability showed few or no RII mutation (20-22) and mutation of Smad4 is uncommon in breast and ovarian carcinomas (72). These observations suggest that autocrine TGFβ signaling may be needed for the progression of certain types of cancer.

As mentioned earlier, TGFβ inhibits cell cycle progression mainly by inhibiting the expression of c-Myc, inducing the expression of $p15^{ink4b}$ and $p21^{cip1}$, and consequently causing hyperphosphorylation of pRB. Since tumor progression is often driven by inactivation of growth inhibitory genes such as *pRB* and overexpression of growth promoting genes such as *c-myc*, carcinoma cells are often resistant to TGFβ's growth inhibitory activity while retaining a functional TGFβ signaling pathway. For example, the human carcinoma DU145 cells are resistant to TGFβ's growth inhibition due to mutational inactivation of *pRB*, but are sensitive to its regulation of gene expression (73, 74). Similarly, failure to inhibit c-Myc expression by TGFβ was shown to cause the resistance to its growth inhibition in the human breast carcinoma MDA-MB-231 cells, which are responsive to TGFβ with respect to the regulation of the expression of several other genes (75). Thus, carcinoma cells with an operational TGFβ signaling pathway are invariably less sensitive to the growth inhibition by TGFβ than their normal counterparts.

4.2. TGFb signal promotes late stage tumor progression

The loss of sensitivity to TGFβ's growth inhibition gives tumor cells a selective advantage over normal cells to proliferate. This is further exacerbated by increased TGFβ expression and/or activation that are generally associated with tumorigenesis (76). For example, TGFβ isoforms have been shown to be upregulated during neoplastic development and progression in breast (77-81), colon (82, 83), prostate (84, 85), and bladder cancers (86). Furthermore, a number of studies reported that increased expression of TGFβ could actually promote tumor progression in carcinoma cells. Overexpression of $TGF\beta_1$ in human breast cancer MCF-7 cells led to increased, estrogen-independent tumor formation in athymic nude mice (87). In certain carcinoma cells, overexpression of $TGF\beta_1$ can enhance its growth inhibitory activity *in vitro*, yet stimulate tumor growth and progression when they are inoculated *in vivo* (88-90). In animal models, transgenic expression of $TGF\beta_1$ in the skin was shown to inhibit carcinogen-induced tumor incidence, however it promoted malignant progression to invasive carcinomas (91).

The mechanisms of tumor promotion by TGFβ were initially attributed to its action in tumor stroma (76). Indeed, TGFβ is a potent immune suppressor in that they can inhibit proliferation, activation and differentiation of various types of lymphocytes (92). Overexpression of $TGF\beta_1$ in highly immunogenic murine tumor cells was shown to stimulate tumor growth by escaping immune surveillance (93). TGFβ has also been shown to be angiogenic *in vivo* (94-96). Overexpression of $TGF\beta_1$ in Chinese hamster ovary cells and human prostate cancer cells was shown to significantly stimulate tumor growth and angiogenesis when they are inoculated in mice (97, 98) and the effects could be attenuated by peritumoral injection of a $TGF\beta_1$ neutralizing antibody (98). Thus, tumor stromal cells appear to be major targets of the tumor promoting activity of TGFβ. However, treatment with exogenous TGFβ *in vitro* was shown to stimulate metastatic potential of a mammary adenocarcinoma cell line *in vivo* suggesting that TGFβ can also directly act on tumor cells to enhance their malignancy (99). More recently, several studies have

shown that blockade of TGFβ signaling in late-stage tumor cells can suppress their malignancy. For example, abrogation of TGFβ signaling in mammary and colon cancer cells by the expression of a dominant negative RII was shown to inhibit their *in vivo* growth and metastasis (100). Dominant negative RII expression in human breast carcinoma MDA-MB-231 cell was also shown to inhibit its bone metastatic potential by blocking TGFβ-induced tumor production of parathyroid hormone-related protein, which stimulates osteolytic activity (101). In genetically related progression models of a human mammary epithelial cell line, dominant negative blockade of TGFβ signaling by RII or Smad was shown to promote tumorigenicity of a low-grade pre-malignant cell, but inhibited metastasis of a high-grade tumorigenic cell (102, 103). Interestingly, the blockade of TGFβ signaling did not affect primary tumorigenesis of the high-grade tumorigenic cell (102). These observations demonstrate that TGFβ signaling in late stage, malignant cancer cells is necessary for their metastatic behavior.

The switch of TGFβ signaling pathway from a tumor suppressor to a tumor promoter is likely accomplished by an alteration of expression and function of multiple gene products. Phenotypically, the attenuation of sensitivity to the growth inhibition by TGFβ as observed in most malignant tumor cells may be a prerequisite for the switch. The ability of tumor cells to undergo epithelial to mesenchymal transdifferentiation (EMT) in the presence of TGFβ signal activation may also be necessary for the switch since the process of EMT contributes to tumor cell migration and invasion, and TGFβ has been shown to promote EMT in various transformed epithelial cells (104). The aberrant regulation of extracellular proteolytic activity in tumor cells by TGFβ (105) is another important mechanism that can mediate TGFβ-induced invasion and metastasis. Finally, both exogenous and autocrine TGFβ have been shown to generate a cell survival signal in various epithelial cells (106, 107), which is also likely to contribute to the tumor-promoting activity of TGFβ.

5. INHIBITION OF TUMOR PROGRESSION WITH TGFb ANTAGONISTS

The observations that TGFβ can promote malignant progression at late stages of carcinogenesis have stimulated investigations to target TGFβ as a novel therapeutic strategy to suppress tumor progression. Various approaches have been utilized to antagonize the tumor-promoting activity of TGFβ. For example, intraperitoneal injection of an anti-TGFβ antibody that neutralized all three TGFβ isoforms inhibited the tumorigenicity of the human breast carcinoma MDA-MB-231 cell (108). Antisense inhibition of the expression of $TGF\beta_1$ in a murine mammary tumor cell line was shown to abrogate the suppression of cytotoxic T cell by of $TGF\beta_1$ secreted from the tumor cells and resulted in the inhibition of tumor growth in syngeneic mice (109). Administration of TGFβ neutralizing antibodies or $TGF\beta_2$ antisense oligonucleotides stimulated the activity of natural killer cell and restored the inhibition of *in vivo* growth of human breast cancer cells by tamoxifen in mice with proficient natural killer cells (110). Several reports have shown that ectopic expression of TGFβ binding proteins including decorin and extracellular domains of TGFβ RII and RIII can inhibit tumorigenicity, tumor growth, and/or metastasis of xenograft models of glioma, hepatoma, and carcinomas of breast, colon and pancreas (111-116). Furthermore, transgenic expression of a soluble TGFβ RII:Fc fusion protein was shown to reduce metastatic incidence in various organs when the mice were inoculated intravenously with an isogenic melanoma cell line (117). When the RII:Fc transgenic mouse was crossed with the MMTV-neu transgenic mouse that develops metastatic breast cancer, RII:Fc expression also inhibited metastasis from endogenous mammary tumors, but did not enhance primary tumor incidence (117). These observations were consistent with a separate study demonstrating that systemic administration of a recombinant RII:Fc fusion protein inhibited lung metastasis produced by orthotopically inoculated breast cancer cell lines or by mammary tumors in MMTV-Polyomavirus middle T antigen transgenic mice (118). Similarly, systemic administration of a recombinant soluble TGFβ RIII was also shown to inhibit the growth, angiogenesis and lung metastasis of growing tumors formed orthotopically by human breast cancer cells in nude mice (119).

These findings point to potential utility of TGFβ antagonists as a novel class of therapeutic agents for cancer treatment. Indeed, antisense $TGF\beta_2$ is under clinical trial for the treatment of glioblastoma (120). A few small molecules that specifically block the kinase activity of TGFβ RI have also been developed (120). It will be interesting to compare TGFβ receptor blockers and TGFβ binding proteins with respect to their efficacy in blocking malignant progression. While TGFβ binding proteins may neutralize excessive amount of TGFβs associated with tumorigenesis and metastasis, but spare TGFβs at physiological levels in normal tissues (117), one concern of the application of TGFβ receptor blockers is that they may induce toxicity associated with the blockade of TGFβ signaling in normal tissues. Furthermore, since TGFβ signaling can be tumor-suppressive in low-grade, well-differentiated adenocarcinoma cells, a blanket blockade of TGFβ signaling with a TGFβ receptor blocker in a patient with a heterogeneous population of tumor cells may induce the progression of low-grade tumor cells. In contrast, TGFβ binding proteins may be administered at a certain dosage such that they only neutralize those TGFβs acting in paracrine and endocrine fashion to foster a favorable microenvironment for tumor cell growth and metastasis. As such, TGFβ sequesters may be more applicable as cancer therapeutics than TGFβ receptor blockers.

6. PERSPECTIVES

The dogma that TGFβ signaling can be either tumor suppressive or tumor promoting has been realized for sometime. However, the molecular markers that can be used to distinguish a tumor that is suppressed by TGFβ from a tumor that is promoted by TGFβ remains unclear.

Published findings are mostly circumstantial and often contradictory. Clearly, the role of TGFβ signaling in tumorigenesis is context and stage dependent. TGFβ is generally believed to suppress the development and early-stage progression of epithelial tumors. However, why the expression or administration of the TGFβ binding protein RII:Fc did not enhance primary mammary tumorigenesis in transgenic mice with endogenous tumors (117, 118) is intriguing. It will be interesting to determine whether the transgenic expression of neu or Polyomavirus middle T antigen in the two studies attenuated the tumor suppressive ability of TGFβ in the mammary epithelial cells. TGFβ signaling is also generally believed to promote metastasis during malignant transformation. However, TGFβ signaling may also be necessary for inhibition of metastasis. For example, loss of TGFβ signaling is associated with adenoma to carcinoma transition in colorectal cancer (68) and abrogation of TGFβ signaling promoted prostate tumor metastasis (69). Future studies will need to elucidate molecular signature(s) in tumor cells that can reveal whether TGFβ signaling has switched from tumor suppression to tumor promotion for successful therapeutic intervention with TGFβ antagonists.

Because TGFβ inhibits host immune surveillance and stimulates tumor angiogenesis, its action in tumor stroma is generally believed to be tumor-promoting. As such, TGFβ action would be solely tumor-promoting in carcinomas with mutational inactivation of TGFβ signaling receptors. Thus, loss of TGFβ RI or RII expression in carcinoma cells should constitute a molecular signature for the use of TGFβ antagonists to inhibit or prevent local or distance metastasis after resection of primary tumor. In fact, since the inhibition of cell cycle progression appears to be the major mechanism of tumor suppression by TGFβ, loss of TGFβ-induced regulation of gene products that control cell proliferation should also indicate a potential favorable outcome with the use of TGFβ antagonist. For example, loss of *c-myc* repression resulted in a significant loss of cell cycle arrest by TGFβ in the human breast carcinoma MDA-MB-231 cell (75). Neutralization of TGFβ isoforms with administration of an antibody or a recombinant soluble RIII protein inhibited tumor growth and metastasis of this cell line *in vivo* (108, 119). Another potential utility of TGFβ sequesters is to inhibit cancer bone metastasis. Active TGFβ isoforms are produced not only by carcinoma cells metastasizing in the bone, but also from bone matrix during osteolysis as observed in breast cancer bone metastasis. These active TGFβ isoforms can stimulate proliferation of osteoblasts as well as differentiation of osteoclasts through various signaling pathways (121). As such, TGFβ is implicated in promoting both osteolytic and osteoblastic bone metastasis. Since active TGFβ isoforms released from bone matrix should be readily accessible by its binding proteins, it appears highly feasible that TGFβ sequesters such as its soluble receptors or neutralizing antibodies will inhibit cancer-induced bone lesions.

The observations that life-long exposure to the TGFβ sequester RII:Fc protein showed little deleterious effect on development and spontaneous tumorigenesis (117) and that systemic administration of a TGFβ soluble RIII did not induce any noticeable side effect (119) point to the feasibility of *in vivo* application of TGFβ sequesters. It is expected that future research will identify more TGFβ binding molecules for experimental trials and specific carcinomas and/or processes of carcinogenesis that can be intervened by TGFβ binding molecules.

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Abbreviations: APC: adenomatous polyposis coli, CDK: cyclin-dependent kinase, DPC4: deleted in pancreatic carcinomas, EMT: epithelial to mesenchymal transdifferentiation, pRB: retinoblastoma gene product, TGFα: transforming growth factor alpha, TGFβ: transforming growth factor beta, RI: the type I receptor of TGFβ, RII: the type II receptor of TGFβ, RIII: the type III receptor of TGFβ

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