Review

Transforming growth factor-β and breast cancer
Tumor promoting effects of transforming growth factor-β

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Received: 3 December 1999
Revisions requested: 3 January 2000
Revisions received: 19 January 2000
Accepted: 26 January 2000
Published: 21 February 2000

Breast Cancer Res 2000, **2**:125–132

Abstract

The transforming growth factor (TGF)-βs are potent growth inhibitors of normal epithelial cells. In established tumor cell systems, however, the preponderant experimental evidence suggests that TGF-βs can foster tumor–host interactions that indirectly support the viability and/or progression of cancer cells. The timing of this 'TGF-β switch' during the progressive transformation of epithelial cells is not clear. More recent evidence also suggests that autocrine TGF-β signaling is operative in some tumor cells, and can also contribute to tumor invasiveness and metastases independent of an effect on nontumor cells. The dissociation of antiproliferative and matrix associated effects of autocrine TGF-β signaling at a transcriptional level provides for a mechanism(s) by which cancer cells can selectively use this signaling pathway for tumor progression. Data in support of the cellular and molecular mechanisms by which TGF-β signaling can accelerate the natural history of tumors will be reviewed in this section.

Keywords: transforming growth factor (TGF)-β, TGF-β receptors, epithelial-to-mesenchymal transition, angiogenesis

Introduction

Although the transforming growth factor (TGF)-βs can be tumor suppressive [1], there is increasing evidence that TGF-β secretion by tumor cells and/or stromal cells within the peritumoral microenvironment can contribute to tumor maintenance and progression. How, then, can TGF-βs be both tumor suppressive and tumor promoting? This apparent paradox is reconciled by a study showing that, in a mouse skin model of chemical carcinogenesis, targeted expression of TGF-β1 in suprabasal keratinocytes appears to have dual effects. It suppresses the formation of benign skin tumors, but once tumors develop, it enhances their progression to a highly invasive spindle cell phenotype [2••]. These results suggest that the effects of TGF-β1 are biphasic: TGF-β1 acts early as a tumor suppressor, probably by inhibiting the proliferation of nontransformed cells, and it acts later as a tumor promoter by eliciting an epithelial-to-mesenchymal transition (EMT). Additional experiments have suggested that upregulation of TGF-β3 in the spindle carcinomas was responsible for maintenance of this invasive phenotype [2••]. This is consistent with TGF-β3 expression at sites in mouse embryos where epithelial–mesenchymal interactions are important, like the lung and palatal shelves [3,4], and also the abnormal lung development and cleft palate observed in TGF-β3 null mice [5]. Also consistent with an early tumor suppressive effect is the recent observation that *tgf-β1*−/− mice develop an accelerated progression of epithelial hyperplasia to

CTL = cytotoxic T lymphocyte; EMT = epithelial-to-mesenchymal transition; JNK = c-Jun *N*-terminal kinase; MMP = matrix metalloproteases; PAI-1 = plasminogen activator inhibitor; PTHrP = parathyroid hormone-related protein; TβRI = TGF-β receptor type I; TβRII = TGF-β receptor type II; TGF-β = transforming growth factor-β.

colonic adenomas and cancers [6•]. The existence of dual effects for TGF-βs in tumor progression follows the observation that TGF-β-induced growth inhibitory responses and extracellular matrix responses may represent distinct processes in certain cell types. For example, overexpression of the antagonistic Smad, *Smad7*, in pancreatic carcinoma cell lines not only suppresses TGF-β1-mediated growth inhibition, but enhances the ability of TGF-β1 to induce matrix associated transcriptional responses [7•].

The progression of epithelial tumors to an invasive metastatic state is often associated with EMT, downregulation of cellular adhesion molecules, elevated expression of metalloproteases, and increased motility and angiogenesis, all of which can be modulated by TGF-βs. It is therefore not surprising that the TGF-βs can also promote tumorigenesis by modulating these critical processes. In support of this view, elevated levels of TGF-β are often observed in advanced carcinomas, and have been correlated with disease progression in several studies [8–13]. This suggests that secreting higher levels of TGF-β may provide an advantage to tumor cells. Both autocrine and paracrine signaling may be involved in conferring this selective advantage. While mutations in various components of the TGF-β signaling pathway have been observed in some carcinomas, particularly colorectal cancers [14,15], an intact TGF-β signaling pathway is often retained in other malignancies as some tumors can exhibit increased invasiveness in response to exogenous TGF-β [16,17•,18,19,20•,21]. Moreover, in a recent study of a large cohort of human breast tumors, loss or low levels of the type II TGF-β receptor (TβRII) correlated with high tumor grade, but 60% of *in situ* and invasive breast carcinomas retained robust levels of TβRII expression by immunohistochemistry [22]. Finally, although *Smad4* is frequently inactivated in pancreatic cancers [23,24], the *Smad* genes, which encode proteins that transduce TGF-β signals, are rarely mutated in most human carcinomas [25–30]. This suggests that after cells lose their sensitivity to TGF-β growth inhibition, autocrine TGF-β signaling may potentially promote tumor progression. In addition, TGF-βs produced in excess by tumor cells may act in a paracrine fashion on the peritumoral stroma, tumor neovessels, or the immune system, indirectly fostering tumor progression.

Autocrine effects

Epithelial-to-mesenchymal transition

Similar to keratinocytes [2••], TGF-β1 can also induce a rapid and reversible EMT in melanoma cells [31], and in both nontumor [32] and Ha-Ras transformed [17•] mammary epithelial cells *in vitro*. In Ha-Ras mammary tumors, EMT appears to be initiated by TGF-β produced by peritumoral host cells and later maintained by autocrine TGF-β1 as the converted tumor cells themselves begin to secrete TGF-β1. The Ha-Ras tumor cells obtained after EMT *in vitro* or *in vivo* display loss of epithelial polarity, downregulation of E-cadherin, disruption of cell–cell adhesion, and invasive properties in several *in vitro* assays [17•]. Supporting the importance of autocrine TGF-β for the tumorigenesis of Ha-Ras mammary cells, introduction of dominant negative TβRII into these cells retarded tumor formation and prevented EMT *in vivo*; moreover, introduction of the same construct into highly invasive murine colon carcinoma cells reconstituted an epithelial phenotype *in vitro*, and inhibited both tumor outgrowth and the establishment of metastases [20•]. In colon cancer cells of low invasive potential and with naturally occurring mutations in the TβRII gene, re-expression of TβRII function restored tumor cell invasiveness [20•]. In another study, expression of dominant negative TβRII in clones derived from a metastatic squamous carcinoma cell line prevented their spontaneous progression to a spindle phenotype *in vivo* [21]. Furthermore, approximately 90% of colon cancers with microsatellite instability have inactivating mutations of TβRII [33], and this instability is significantly correlated with longer patient survival [34], suggesting that complete loss of TβRII in carcinomas may limit systemic metastases. Taken together, these results suggest that EMT, local tumor growth, and metastatic progression can be sustained by autocrine TGF-β signaling.

When tumors are grown in nude mice, TGF-βs made by host cells can induce responses in tumor cells with intact TGF-β signaling, with the net effect of these tumor–host interactions being deleterious to the host. For example, MDA-231 human breast tumor cells secrete parathyroid hormone-related protein (PTHrP) in response to exogenous TGF-β1, metastasize to bone when injected into nude mice, and induce osteolysis and hypercalcemia, resulting in host death. Transfection of these cells with dominant negative TβRII blocks TGF-β1-mediated stimulation of PTHrP production. Mice injected with these cells exhibited less osteolysis, higher body weight, lower serum calcium and PTHrP levels, and longer survival than mice injected with control MDA-231 cells [35•]. On the contrary, accelerated osteolysis and reduced host survival were observed when mice where injected with tumor cells transfected with a constitutively active TβRI, suggesting a possible role for TGF-β-mediated responses in the pathogenesis of some adverse paraneoplastic syndromes.

Several recent studies have contributed to our understanding of the biochemical mechanisms by which transformed cells can lose autocrine growth inhibition but retain TGF-β-mediated responses that contribute to tumor progression. For example, oncogenic activation of the Ras pathway, acting via MAP kinases, causes phosphorylation of Smad2 and Smad3 at specific Erk consensus sites in the linker region between their DNA binding and transcriptional activation domains. This results in loss of nuclear accumulation of Smad2/3 and silencing of TGF-β-mediated

antiproliferative responses [36••]. In nontransformed mammary cells, introduction of mutant Ras not only blocks growth inhibition by TGF-β, but also subverts this pathway into one that can stimulate epithelial-to-mesenchymal transdifferentiation [17•,20•]. In MDCK epithelial cells, transfection of the missense mutations Smad2.D450E and Smad2.P445H, reported in primary colorectal and lung carcinomas, does not abolish TGF-β-mediated growth arrest. Instead, it increases both basal and TGF-β stimulated invasiveness, neither of which is prevented by overexpression of the inhibitory Smad7 [37•]. This suggests the existence of *Smad* 'gain-of-function' mutations that enhance malignant progression by mechanisms independent of TβRI and Smad phosphorylation. Another study has shown that Smad7 mRNA levels are increased in human pancreatic cancers compared with normal pancreas [7•]. Stable transfection of COLO-357 human pancreatic cancer cells with a Smad7 expression vector results in loss of TGF-β1-mediated growth inhibition and p21/Cip1 promoter activity. However, TGF-β1-induced plasminogen activator inhibitor-1 (PAI-1) promoter activity is maintained and, more importantly, basal PAI-1 promoter activity, PAI-1 mRNA levels, anchorage independent colony growth, and tumorigenicity in nude mice, are all increased in the Smad7 transfected clones [7•]. This result suggests another potential mechanism, the overexpression of Smad7, for the segregation between antiproliferative and matrix associated TGF-β responses. In addition, overexpression of Smad4 in colon carcinoma cells does not reconstitute TGF-β-mediated antiproliferative responses [38•,39], but inhibits cell adhesion and spreading, reduces the levels of urokinase plasminogen activator and PAI-1, and prolongs tumor latency [39], suggesting an additional function for Smad4 in restraining genes involved in peritumor proteolysis and invasion. This is further supported by reports of homozygous deletion of *TβRI* or homozygous missense mutations of *TβRII* [40,41], each coexisting with deletions of *Smad4* in individual tumors. The coexistence of these mutations in the same tumors would not be expected if the function of these two gene products (TβRII and Smad4 or TβRI and Smad4) was limited to a single common signal transduction pathway. Taken together, these studies suggest, first, that the threshold for loss of TGF-β antimitogenic effects is lower than that required to lose responses associated with cell adhesion, invasion, and metastases; second, that not one but multiple biochemical mechanisms can contribute to the enhancement or unmasking of the tumor promoting effects of autocrine TGF-β; and, third, that some of these mechanisms may be independent of Smad function or TβRI phosphorylation. The identification of Smad dependent and independent genes causally involved in these TGF-β-mediated tumor promoting effects requires further research. Of note, Hocevar *et al* [42•] recently reported c-Jun *N*-terminal kinase (JNK) dependent TGF-β-induced fibronectin expression in cell lines lacking the *Smad4* gene or protein expression.

Increased motility

TGF-β can stimulate the motility of many cell types *in vitro* [43–45], therefore suggesting that TGF-β production *in vivo* may enhance migration of tumor cells and metastatic potential. Indeed, cyclosporine treatment of lung adenocarcinoma cells results in increased cell motility and anchorage independent growth *in vitro*, as well as increased metastases *in vivo*, all of which can be blocked with neutralizing TGF-β1 antibodies [46]. These results suggest that *in vivo* tumor progression by cyclosporine is dependent on autocrine TGF-β1. In prostate cancer cells, TGF-β1 stimulates motility without affecting cell proliferation, suggesting that the effects on motility and proliferation may occur via different biochemical pathways [43].

Whether blockade of the Smad pathway, critical for TGF-β-mediated antimitogenic effects [47,48], is also critical for the effects of TGF-βs on cell motility is not clear. Some evidence suggests that the latter may follow alternative signaling pathways, perhaps in cooperation with activated oncogenes. Atfi *et al* [49] reported recently that inactivating components of the JNK pathway, which regulates AP-1 activity via c-Jun, inhibits TGF-β-mediated induction of 3TP-Lux, a reporter construct that contains Smad and AP-1 binding elements. Dominant negative mutants of RhoA, Rac1, and Cdc42, GTPases that mediate cell shape, cytoskeletal organization, and motility, abolish TGF-β-mediated transcription of AP-1 [49,50], suggesting that the Rho family of GTPases and the JNK pathway are essential components of TGF-β signaling responses. TGF-β1 can also upregulate integrin linked kinase [31], a protein associated with fibronectin production and increased cell motility. In another study, TGF-β1 treatment of NMuMG mouse mammary epithelial cells increased the expression of *N*-cadherin [51], which has been shown to increase motility of squamous cancer cells [52].

Paracrine effects

Induction of metalloproteases

Matrix metalloproteases (MMPs) play a critical role in the proteolytic degradation of basement membrane that is required for tumor invasion [53]. The expression of several MMPs, including MMP-2 [54] and MMP-9 [18,31,55], can be induced by TGF-β. Moreover, TGF-β1 has been shown to selectively induce MMP-9 activity in a subset of metastatic but not primary mouse prostate tumors, implying that this TGF-β1-induced response may be an important selection step in tumor progression [18]. There is also evidence that TGF-β increases MT-MMP-1 and MMP-9 expression in metastatic melanoma [31]. Although MMPs are listed separately, recent data implicate them strongly in the process of tumor-induced neovascularization [56], thereby suggesting that their upregulation might be an integral component of the TGF-β-mediated angiogenic processes discussed next.

Tumor angiogenesis

It is generally accepted that solid tumors require an adequate blood supply in order to grow beyond a few millimeters in size. TGF-βs, particularly TGF-β1, have been shown to regulate new blood vessel formation both *in vitro* and *in vivo* by a combination of responses that include increased production and facilitation of vascular endothelial growth factor, facilitation of basic fibroblast growth factor mediated capillary sprouting, inhibition of endothelial cell migration, and increased production of extracellular matrix, among others (reviewed in [57]). In most cells, TβRI/ALK-5 is the signaling receptor for TGF-β. However, in endothelial cells, it has been suggested that ALK-1 may also function as a type I receptor for TGF-β [58]. In addition to the type I, II, and III TGF-β receptors, endoglin is another integral membrane protein that binds TGF-β1 and TGF-β3, and is highly expressed in endothelial cells [59]. Although TGF-β effects appear to be mediated mostly by the receptor specific Smad2 and Smad3 proteins [47,48], there is evidence that Smad5 is involved in TGF-β signaling in hematopoietic cells [60]. Targeted disruption of genes encoding various components of the TGF-β signaling pathway, including TGF-β1 itself [61], its receptors, TβRII [62], ALK-1 [63], and endoglin [64], and one of its signal transducers, Smad5 [65], has each revealed that these proteins play an important role in vascular development. The phenotype of the TGF-β1 and TβRII knockout mice is virtually indistinguishable and is characterized by defective endothelial differentiation resulting in abnormal capillary tube formation [61,62]. In contrast, disruption of ALK-1, endoglin, or Smad5 does not affect endothelial differentiation or vasculogenesis, but instead they each affect angiogenesis. In addition, *endoglin−/−* and *Smad5−/−* mice exhibit impaired vascular smooth muscle cell development. These results are consistent with previous reports demonstrating that TGF-β can regulate smooth muscle cell differentiation and migration *in vitro* [66•], thus contributing to pericyte recruitment and vessel stabilization. This hypothesis, as it applies to tumor angiogenesis, is somewhat challenged by the notion that the majority of intratumoral neovessels seem to lack periendothelial smooth muscle cells [67], suggesting that there may be additional roles for the TGF-βs in tumor angiogenesis. In that light, Higaki and Shimokado [68] recently reported TGF-β1-mediated stimulation of phosphatidylinositol-3 kinase activity and amino acid uptake in vascular smooth muscle cells, suggesting a direct anti-apoptotic role for TGF-β. Elucidation of the paracrine mechanisms driving TGF-β-mediated tumor angiogenesis requires further investigation.

Further supporting the role of TGF-βs in tumor angiogenesis, administration of a neutralizing TGF-β1 antibody to nude mice harboring CHO cell xenografts transfected with ectopic TGF-β1 inhibits both tumor growth and intratumor microvessel density [69]. In addition, a monoclonal antibody that blocks TGF-β1, TGF-β2, and TGF-β3 has been shown to suppress the growth of TGF-β1-overexpressing renal cancer xenografts [70]. In this study, the TGF-β blocking monoclonal-abrogated factor VIII staining in the xenografts, suggesting an antitumor mechanism that targets endothelial cells [70]. Furthermore, TGF-β1 and PAI-1 have been shown to inhibit the conversion of plasminogen to the anti-angiogenic molecule angiostatin in medium conditioned by human pancreatic cancer cells [71]. This suggests an additional pro-angiogenic mechanism for TGF-β by interfering with the production of endogenous inhibitors of endothelial cell proliferation. Finally, high levels of TGF-β1 mRNA correlate strongly with high microvessel density in breast tumors, and each of these factors is associated with poor patient outcome [72].

Host immunosuppression

TGF-β1 and TGF-β2 are potent immunosuppressants [73]. Thus, elevated levels of TGF-βs secreted by tumors could potentially inhibit immune effector cells and favor tumor progression. In support of this idea, Torre Amione *et al* [74] demonstrated that, unlike parental tumor cells, fibrosarcoma cells transfected to express 10 ng/ml TGF-β1 *in vitro* are unable to induce cytotoxic T lymphocyte (CTL) responses and can escape immune recognition. Likewise, EMT6 mammary tumor cells, which produce high levels of TGF-β1, can inhibit CTLs *in vivo*. Transfection of these cells with interleukin-2, a known T cell growth factor, can reverse this TGF-β1 effect and induce tumor rejection [75]. This result suggests that, by dampening the generation of tumor reactive T cells, TGF-β can promote tumor viability. There is also evidence that overexpression of the soluble TβRII extracellular domain in thymoma cells can prevent the progression of unmodified thymoma cells when injected near the primary tumor inoculation site [76], further suggesting that secretion of soluble TβRII by these cells is sufficient to restore tumor specific cellular immunity and mediate partial tumor rejection. Overall, these results are consistent with the phenotype of *TGF-β1* null mice that die shortly after birth as a result of widespread inflammation and multiorgan T cell infiltration and necrosis [77].

In addition to inhibiting CTL responses, TGF-βs can modulate other immune functions that may favor tumor progression. For example, CHO cells transfected with an expression vector encoding latent TGF-β1, when injected into nude mice, can decrease mouse spleen natural killer activity and rapidly form tumors [78]. Antagonizing TGF-βs by intraperitoneal injection of an antibody that neutralizes TGF-β1, TGF-β2, and TGF-β3 has the opposite effect. It prevents tumor and metastases formation by MDA-231 human breast carcinoma cells, and markedly increases natural killer activity of mouse splenocytes [79]. Consistent with this TGF-β-mediated immunosuppressive effect, reduced immune function has been observed in animals

bearing TGF-β overexpressing tumors [80] as well as in patients with glioblastoma, a common type of brain tumor that frequently overexpresses TGF-β2 [81].

The cited studies suggest that tumor cell secreted TGF-βs may block the efferent function of immune effectors at sites of tumor implantation. Other reports, however, suggest tumor cell TGF-βs may modify the afferent component of the immune response and confer antitumor immunity. Stable infection of breast and glioma tumor cells with antisense TGF-β1 and antisense TGF-β2 retroviruses, respectively, has been shown to restore the immunogenicity of these tumor cells when injected into immunocompetent animals. Furthermore, they induce a partial rejection of unmodified, less immunogenic established wild type tumor cells [82,83]. In both of these studies, *in vitro* and *in vivo* CTL activity was markedly increased in medium conditioned by antisense TGF-β-infected cells and/or in mice injected with tumor cells bearing the antisense compared with tumor cells infected with a control vector. These studies have therapeutic implications for the use of an antisense TGF-β based approach as a means of adoptive immunotherapy against TGF-β overproducing tumors.

Alternative views and conclusions

A tumor permissive role for the TGF-βs may not apply to all solid tumors. Indeed, transfection of an antisense TGF-β1 expression vector into FET and CBS well-differentiated human colon cancer cells has been shown to enhance tumor formation in nude mice [84,85], supporting the notion that, in some fully transformed cells, endogenous TGF-β1 can continue to mediate a tumor suppressor function. In a recent report, mice bearing transplanted gallbladder Mz-Cha-2 tumors showed inhibition of angiogenesis and leukocyte–endothelial cell interactions at a distant cranial site and threefold higher levels of circulating TGF-β1 compared with tumor free mice [86]. This reduction in microvessel density and leukocyte rolling were reversed by systemic administration of a TGF-β1 neutralizing antibody, suggesting a negative role for TGF-β1 in early neovascularization. Moreover, in a recent survey of 104 *in situ* and invasive primary breast carcinomas, 40/45 (89%) tumors with low invasive potential and low proliferation rate exhibited high levels of TβRII by immunohistochemistry [22]. Whether autocrine TGF-β signaling is causally associated with the observed low proliferation and invasiveness in this subset of breast tumors is a question that remains unclear.

Nonetheless, the potential tumor promoting effects of TGF-β provide novel molecular targets for interventions aimed at altering the natural history of solid tumors. The lack of an obvious physiological role for TGF-β signaling in postdevelopmental normal physiological states suggests that these interventions may in fact be tumor specific and spare the tumor host from undue toxicity. Several approaches have been proposed, including the use of blocking antibodies against TGF-β1, TGF-β2, and TGF-β3, using the soluble ectodomains of the type II and III TGF-β receptors, which would sequester TGF-β isoforms at tumor sites and prevent binding to cognate receptors [87,88], and, finally, using adenovirus encoding inhibitors of TGF-β signaling [89], to name a few. The theoretical and logistical strengths and limitations of these approaches are beyond the scope of this review. Nonetheless, these represent tools that, if effective in blocking TGF-β action, will allow us to address the net effect of autocrine/paracrine TGF-β signaling at early and late stages of transformation and cancer progression.

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Sponsorship: This review was supported by US Army DAMD17-98-1-8263 Pre-doctoral Training Award (to ND), PHS grant R01 CA62212, US Army DAMD17-98-1-8262 IDEA grant, a Department of Veteran Affairs Clinical Investigator Award (to CLA), and a Vanderbilt-Ingram Cancer Center support grant CA68485

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