Dual Role of Transforming Growth Factor β in Mammary Tumorigenesis and Metastatic Progression

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ABSTRACT

It is generally accepted that transforming growth factor β (TGFβ) is both a tumor suppressor and tumor promoter. Whereas loss or attenuation of TGFβ signal transduction is permissive for transformation, introduction of dominant-negative TGFβ receptors into metastatic breast cancer cells has been shown to inhibit epithelial-to-mesenchymal transition, motility, invasiveness, survival, and metastases. In addition, there is evidence that excess production and/or activation of TGFβ by cancer cells can contribute to tumor progression by paracrine mechanisms involving neoangiogenesis, production of stroma and proteases, and subversion of immune surveillance mechanisms in tumor hosts. These data provide a rationale in favor of blockade of autocrine/paracrine TGFβ signaling in human mammary tumors with therapeutic intent. Several treatment approaches are currently in early clinical development and have been the focus of our laboratory. These include (1) ligand antibodies or receptor-containing fusion proteins aimed at blocking ligand binding to cognate receptors and (2) small-molecule inhibitors of the type I TGFβ receptor serine/threonine kinase. Many questions remain about the viability of anti-TGFβ treatment strategies, the best molecular approach (or combinations) for inhibition of TGFβ function in vivo, the biochemical surrogate markers of tumor response, the molecular profiles in tumors for selection into clinical trials, and potential toxicities, among others.

INTRODUCTION

The transforming growth factor β (TGFβ) family comprises a superfamily of ligands that includes the TGFβs, activins, and bone morphogenetic proteins. TGFβ ligands play a role in cell proliferation, functional differentiation, extracellular matrix production, cell motility, and apoptosis (1). The TGFβs are secreted as small latent complexes, which must be activated in order to enable TGFβ binding to receptors. The small latent complex is composed of the active COOH-terminal TGFβ dimer linked noncovalently to a dimer of the latency-associated peptide. The large latent complex is the small latent complex linked via disulfide bonds to the latent TGFβ binding protein. The latent TGFβ binding protein glycoproteins play a central role in the processing and localization of TGFβ complexes in the extracellular matrix where the bulk of TGFβs are sequestered. Release of active TGFβ from matrix-associated latent complexes may require two steps: release of the complex from the extracellular matrix by proteolysis and subsequent activation by disruption of the noncovalent association between TGFβ and the latency-associated peptide. This can be achieved by chemical, enzymatic, proteolytic, and hormonal mechanisms (reviewed in ref. 2).

There are three mammalian TGFβ isoforms, TGFβ1, TGFβ2, and TGFβ3 which, in general, exhibit similar function in vitro, most notably on cell growth regulation, extracellular matrix production, and immune modulation (1). However, each seems to have distinct activities in vivo as evidenced by the phenotypes of mice lacking any one of the TGFβ ligands. Targeted disruption of the Tgfb1 gene leads to hematopoietic and vasculogenic defects that result in death of about half of null embryos at 10 days of gestation (3). The embryos that survive succumb to a wasting syndrome and multiorgan failure due to inflammation after weaning (4). TGFβ2-null mice exhibit perinatal mortality as a result of developmental abnormalities affecting the cardiopulmonary, urogenital, visual, auditory, neural, and skeletal systems (5). Mice lacking TGFβ3 die in the perinatal period and exhibit abnormal lung and palate development (6, 7).

The TGFβs bind to a heteromeric complex of transmembrane serine/threonine kinases, the type I and type II receptors (TβRI and TβRII). These ligands also bind a large transmembrane proteoglycan referred as the type III TGFβ receptor (also called betaglycan) whose role is to present ligand to TβRII (1). Following ligand binding to TβRII, TβRI is recruited to ligand–receptor complex. This allows for the constitutively active TβRII kinase to transphosphorylate and activate the TβRI kinase (8), which, in turn, phosphorylates the receptor-regulated Smad2 and Smad3 (Fig. 1). Smad2 and Smad3 then associate with a common mediator Smad (Smad4) and translocate to the nucleus where they regulate gene transcription (9). By contrast, the inhibitory Smad7 can interact with TβRI and prevent the phosphorylation of effector Smads (10). In addition to Smads, other signaling pathways have been implicated in TGFβ actions. These include the extracellular signal-regulated kinase, c-Jun NH2-terminal kinase, p38 mitogen-activated protein kinase, phosphatidylinositol-3 kinase, and Rho GTPases (reviewed in refs. 11, 12). The roles of these non-Smad pathways in mediating the cellular effects of TGFβ remain to be fully characterized.

TGFβ IS BOTH A TUMOR SUPPRESSOR AND A TUMOR PROMOTER

TGFβ was originally reported to induce anchorage-independent growth of mouse fibroblasts (13). Subsequent
studies indicated that TGFβ is a potent inhibitor of epithelial cell proliferation (14). Indeed, overexpression of active TGFβ under the control of tissue-specific promoters in transgenic mice delays mammary gland development (Fig. 2) and has been shown to protect from carcinogen- or oncogene-induced carcinomas (15). Other data indicate that the tumor suppressor role of TGFβ can be explained by its ability to inhibit cell proliferation, maintain tissue architecture (16), inhibit genomic instability (17), and induce replicative senescence and apoptosis (18). A recent report indicates that a T29 → C polymorphism in the TGFBI gene results in increased serum levels of TGFβ1 and is associated with a reduced risk of breast cancer in postmenopausal women (19). A possible cancer-preventive effect of an excess of TGFβ, as indicated by these studies, may relate to its ability to induce stem cell senescence and/or block functional differentiation in some tissues (18).

Loss of TGFβ-mediated growth restraint has been shown to be associated with an increased risk of transformation. For example, mice with complete or partial disruption of Tgfb1 or Smad genes are prone to the development of cancers (16, 20). Attenuation of autocrine TGFβ signaling by expression of a dominant-negative TpRII results in accelerated lobulo-alveolar mammary development (21), enhanced propensity for carcinogen-induced lung, mammary, and skin tumors (22, 23), and spontaneous invasive mammary carcinomas (24). Finally, mutations in the TGFBR2 gene occur in both sporadic and inherited colon cancers with microsatellite instability (25), and restoration of TpRII by transfection reverses transformation in certain colon cancer cell lines (26). However, inactivating mutations in TGFBR2 have not been found in breast cancer (27). Although low levels of TpRII protein, as measured by immunohistochemistry, identifies a cohort of women with mammary epithelial hyperplasia at increased risk for development of breast cancer (28), there is no demonstrable difference in the proportion of invasive breast cancers that express detectable levels of TpRII protein compared with preneoplastic and preinvasive lesions (29). Interestingly, however, Gobbi et al. (29) reported that all low-grade cancers but only 31% of high-grade invasive carcinomas expressed type II receptor (Fig. 3). Although these studies support the tumor suppressive role of endogenous TGFβ, it should be noted that administration of exogenous TGFβ has not been shown to inhibit established neoplasms in vivo, nor has the administration of a TGFβ inhibitor resulted in either spontaneous tumor development or the accelerated growth of an already established cancer.

On the other hand, several reports support a causal association between an excess of endogenous or exogenous TGFβ and tumor progression. Administration of recombinant TGFβ1 to nude mice facilitates tumor formation by estrogen-dependent MCF-7 cells in the absence of estrogen supplementation. In the same report, stable transfection of a TGFβ1 expression vector into MCF-7 human breast cancer cells allows them to form tumors in the absence of estrogen supplementation (30). Overexpression of activated type I TGFβ receptor (31) or active TGFβ1 (32) under the control of the mouse mammary tumor virus promoter were recently shown to accelerate metastases from neu-induced primary mammary tumors in transgenic mice. Furthermore, both exogenous and stably transduced TGFβ1 have been shown to confer motility and invasiveness to MCF-10A nontumorigenic mammary epithelial cells transfected with HER2 (erbB2; refs. 33, 34). We recently developed a triple transgenic mouse model in which expression of active TGFβ1 in mammary tissues also expressing polyomavirus middle T antigen (PyVmT) can be temporally controlled using doxycycline. In these mice, doxycycline-mediated induction of active TGFβ1 for as little as 2 weeks in mice bearing late PyVmT-induced tumors markedly accelerated metastases (Fig. 4; ref. 35).

There is also evidence that high production and/or activation of TGFβ in tumors can enhance cancer progression by autocrine and/or paracrine mechanisms (reviewed in refs. 11, 12). Overexpression of TGFβ ligands has been reported in most cancers (reviewed in ref. 36). These high TGFβ levels in tumor tissues correlate with markers of a more metastatic phenotype and/or poor patient outcome, and many tumor cells exhibit increased invasiveness in response to TGFβ (reviewed in ref. 37). TGFβ can also induce an epithelial-to-mesenchymal transition in tumor and nontumor epithelial cells (38, 39). Re-expression of TpRII in colon cancer cells with low invasive potential restores tumor cells invasiveness (40). Forced expression of dominant-active Smad2 in squamous cancer cells also results in enhanced tumor cell motility and metastatic dissemination (41). Further underscoring the tumor-promoting role of autocrine TGFβ, expression of dominant-negative TpRII in metastatic cancer cells prevents epithelial-to-mesenchymal transition and inhibits motility, tumorigenicity, and metastases (reviewed in ref. 42).

These data suggest that TGFβ may select for more metastatic cancers. Indeed, mice overexpressing active TGFβ1 in suprabasal keratinocytes develop less benign papillomas.
compared with controls. However, once tumors develop, the transgenic tumors rapidly acquire a spindle cell phenotype, overexpress TGFβ3, and metastasize (43). More recently, overexpression of active TGFβ1 or activated TβRII in the mammary gland of transgenic mice has been shown to accelerate metastases derived from neu-induced primary mammary tumors (31, 32). Parenthetically, colon cancers with inactivating mutations of the TGFBR2 gene exhibit favorable survival compared with TβRII-positive colon cancers (44), suggesting that loss of autocrine TGFβ signaling may limit systemic metastases.

**CLINICAL DEVELOPMENT OF TGFβ INHIBITORS**

The improved outcome of patients bearing cancers with TGFβ inhibitors suggests that a selective approach to block one of TGFβ signaling with therapeutic intent. An additional rationale can be inferred from the paracrine effects of tumor TGFβs on angiogenesis, stroma formation and remodeling, and immunosuppression. Taken together, these observations suggest that, by blocking TGFβ function, one can interrupt multiple events important for tumor maintenance. Indeed, preclinical studies have proved the principle that inhibition of TGFβ affects these tumor-permissive autocrine and paracrine mechanisms (42).

Several strategies to block TGFβ function are being pursued. One group of strategies is aimed at blocking ligand access to TGFβ receptors. Two humanized monoclonal antibodies: CAT-192, specific to TGFβ1 and CAT-152, against TGFβ2, are in early clinical development (45). The expression of multiple TGFβ isoforms in tumors suggests that a pan-TGFβ antibody might be more effective than isoform-specific antibodies. Two pan-TGFβ monoclonal antibodies, ID11 and 2G7, have been reported (46). The 2G7 pan-TGFβ neutralizing IgG2 suppresses the establishment of MDA-231 tumors and lung metastases in athymic mice and prevents the inhibition of host natural killer cell function induced by tumor inoculation (47). The antibody had no effect against MDA-231 cells in vitro, nor did it exhibit an antitumor effect in natural killer–deficient mice, suggesting that antibody-mediated TGFβ blockade is effective in disrupting tumor-host immunosuppressive interactions that are essential for tumor establishment and metastatic progression. Interestingly, an antibody against the ectodomain of TβRII, which would block ligand binding, has not been reported.

Another approach to prevent binding of TGFβ ligands is the use of recombinant fusion proteins containing the ectodomains of TβRII and TβRIII. Soluble TβRII:Fc has shown efficacy in fibrosis and metastases models (48). In MMTV/PyVmT transgenic mice, blockade of TGFβ with soluble TβRII:Fc increases apoptosis in primary mammary tumors and inhibits tumor cell motility, invasiveness, and metastases (49). In this report, treatment with soluble TβRII:Fc inhibited Akt activity in tumors. Human recombinant TβRIII has shown antitumor and antiendothelial cell activity (50). One attractive feature of recombinant betaglycan over soluble TβRII RII is its greater affinity for TGFβ2.

A second group of strategies is aimed at directly blocking the receptors’ catalytic activity. SBI-14352, NPC 30345, and LY364947 (51–53) are ATP competitive inhibitors of the ATP binding site of the TβRII kinase. This approach spares the TβRII kinase and, therefore, may not inhibit TGFβ function completely. If complete inhibition of TGFβ was required for antitumor action, this selectivity could compromise anticancer activity but at the same time ameliorate potential toxicities. These two possibilities are theoretical, because there are no known TβRII functions that do not require TβRI. Nonetheless, the development of bifunctional TβR kinase inhibitors should help in resolving these questions. Vectors encoding the
inhibitory Smad (Smad7) have been used to bind TβRI and interfere with Smad2 and Smad3 phosphorylation (54). This strategy should be viewed with caution in that it will not block Smad-independent TGFβ-induced responses conducive to tumor progression. Indeed, Smad7 mRNA is overexpressed in pancreatic cancers and its forced expression in pancreatic cancer cells results in loss of TGFβ-mediated growth inhibition but facilitates anchorage-independent growth and tumorigenicity (55). Moreover, blockade of Smad4 has been shown to facilitate TGFβ-dependent and TGFβ-independent activation of Erk in squamous cancer cells and promote their motility and transmesenchymal differentiation (56). The TGFβ antagonists discussed above are summarized in Fig. 5.

A potential risk of therapy with TGFβ antagonists is the acceleration of preneoplastic lesions or cancers in which TGFβ still exerts growth restraint. In addition to having an antitumor effect, blockade of TGFβ in normal cells may induce side effects in the tumor host. Complete loss of TGFβ in mice is associated with a severe inflammatory response (4). Complete elimination of TGFβ function in T cells leads to autoimmune disorders (57). A recent experiment, however, suggests that TGFβ antagonists might be well tolerated. Mice expressing soluble TβRII under the regulation of the mouse mammary tumor virus/long terminal repeat promoter exhibit high levels of the TGFβ antagonist in the circulation without the severe inflammatory phenotype of TGFβ-null mice. Interestingly, the circulating levels of sTβRII:Fc were enough to inhibit tumor metastases in this model. Mild lymphocytic infiltration in lungs, kidneys, and pancreas were observed but no spontaneous tumors developed (58). It is likely that the severe inflammatory and autoimmune phenotype observed in genetically engineered mice reflects the complete loss of TGFβ function, a level of inhibition that is unlikely to be achieved with exogenous inhibitors.
CONCLUSIONS

Significant experimental evidence suggests that TGFβ can foster tumor-host interactions that indirectly support the viability and progression of neoplastic cells. Furthermore, autocrine TGFβ signaling is operative in some tumor cells and can contribute to tumor invasion, survival, and metastases. This possibility is likely in a cohort of women with breast cancers in which loss of TGFβ receptors and/or signal transducers is uncommon. The multiple tumor-permissive effects of TGFβ provide a therapeutic opportunity, in that blocking this signaling network interrupts several autocrine and paracrine mechanisms that are essential for tumor maintenance.

OPEN DISCUSSION

Dr. Kent Osborne: One of the things TGFβ does is activate MMPs. So one of the mechanisms, then, of activation of MAP kinase and Akt may be by activation of MMP2, cleavage of heparin-binding EGF and autocrine activation of EGF receptor. Have you tried blocking the EGF receptor and seeing if you can activate that?

Dr. Carlos Arteaga: Yes, we have. Blockade of the EGF receptor tyrosine kinase with gefitinib blocks TGFβ-induced motility completely. In A431 and NMuMG cells, TGFβ can activate MMP2 and MMP9 while the levels of secreted TGFα and amphiregulin go way up. We can block this response by using inhibitors of TACE, TNFα-converting enzyme, which cleaves membrane-tethered TGFα and amphiregulin. So, TGFβ receptors are functioning just like G-protein coupled receptors, in that they activate TACE, which cleaves membrane-tethered ligands, which then activate the EGFR.

Dr. Osborne: It’s like the story with estrogen treatment of those cells in the setting of high HER2 or high EGFR: you get Src activation, MMP2 activation, and the same kind of autocrine-mediated effects.

Dr. Richard Santer: The EGF receptor when activated can bind to focal adhesion kinase, which then turns on downstream PKA and Rac, which are involved in invasiveness. In follow-up to Dr. Osborne’s question, if the heparin-binding EGF is freed up and binds to the EGF receptor, under those circumstances can you demonstrate either EGF receptor binding to focal adhesion kinase or the tyrosine 397 phosphorylation on focal adhesion kinase? Have you looked at that?

Dr. Arteaga: We have not looked specifically at what you are asking but we have a recent paper that addresses a related issue [J Biol Chem 2004;279:24505–13]. In HER2-transfected MCF-10A mammary epithelial cells, but not in control cells with low levels of HER2, TGFβ induces motility and invasiveness. In the control cells it just inhibits their proliferation. So here we have a proto-oncogene, HER2, unmasking the ability of TGFβ to induce motility and potentially accelerate metastatic progression. In the transfected cells, the Rac1 GTPase is very potently activated within 15 minutes but, interestingly, Rac1 is also activated in the control cells. What is different is that the control cells do not move, whereas the HER2-overexpressing cells do. In addition, we see an association between Rac1, PAK, HER2, actin, actinin, and the type II TGFβ receptor at the leading edges of TGFβ-stimulated cells. However, we have not looked at FAK, at focal adhesions, yet as you suggest.

Dr. Santen: How do you explain two cells that have increased Rac, one of which is motile whereas the other isn’t?

Dr. Arteaga: We speculate that, in addition to Rac, there are other cellular determinants activated by HER2 signaling in the proto-oncogene overexpressing cells that are required for cell motility. The recruitment of additional transducers to a GTPase-HER2 complex may be very important for transformed cell motility.

Dr. Jose Russo: Could it have been that the observations in transgenic mice might be an artifact and is not a reflection of the human situation? It could be that we are developing a biology and understanding of TGFβ exclusively for the mouse that is not applicable to humans.

Dr. Arteaga: I presume you are skeptical of the increasing number of studies in transgenic mice supporting a role for TGFβ in metastatic progression. Your presumption is certainly plausible. However, there is a recent interesting report by Knabbe et al. [Clin Cancer Res 2004;10:491–8] that shows that ER-negative tumors that lack the type II TGFβ receptor have a good prognosis, similar to ER+ tumors, further supporting the association between autoctone TGFβ signaling and a more metastatic phenotype. Then there are the data with microsatellite unstable colon cancers that go along the same lines. In my opinion, the soon-to-be-initiated therapeutic trials with TGFβ inhibitors will determine in the end if some tumors are driven by autocrine/paracrine TGFβ, as the preclinical data suggest.

Dr. Douglas Yee: You mentioned a study reporting a polymorphism that reduces the amount of β1. So it’s detectable in the serum? How much circulating TGFβ is there?

Dr. Arteaga: Yes, it is detectable in serum. The paper by Ziv et al. in JAMA [2001;285:2859–63] indicates a polymorphism associated with increased levels of serum TGFβ and a markedly reduced risk of breast cancer. It is difficult to know if that circulating pool reflects what is happening in tumor and nontumor tissues. TGFβs are ubiquitous and made as latent proteins. These latent ligands accumulate in very high levels in the extracellular space where...
they can be activated in a temporally and spatially regulated manner. So, how the circulating levels reflect local activation and function is unclear. I should add that we don’t know much about the physiologic and pathologic determinants of TGFβ activity in situ.

**Dr. Yee:** So no one has attempted to look at TGFβ levels and cancer risk in the big serum banks like the Nurses’ Health Study, for example?

**Dr. Arteaga:** To my knowledge, nobody has looked at this specific cohort, perhaps in part because of the limitations of the methods and the difficulty in the interpretation of any results. Another issue is that TGFβ activation could have occurred in *vitro* but not in the patient. On the other hand, if you measure TGFβ ligands, how do you know they are active in *vivo*? We don’t have good reagents that will look specifically at “active” TGFβ or activation of TGFβ signal transduction in *vivo* and, therefore, offer some assurances that the serum level correlates with TGFβ functions at tissue sites.

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