Targeting the TGFβ signaling network in human neoplasia

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Introduction

The transforming growth factor (TGF) βs are polypeptides within a superfamily of ligands that includes the TGFβs, activins, and bone morphogenetic proteins (BMPs). TGFβ ligands play a role in cell proliferation, functional differentiation, extracellular matrix (ECM) production, cell motility, and apoptosis (Massagué, 1998). Three mammalian TGFβ isoforms, TGFβ1, TGFβ2, and TGFβ3, have been identified. In general, they exhibit similar functions in vitro, most notably on cell growth regulation, ECM production, and immune modulation. However, each appears to have distinct activities in vivo, as evidenced by the distinct phenotypes of mice lacking any one of the TGFβ ligands (Massagué, 1998). The TGFβs bind to a heteromeric complex of transmembrane serine/threonine kinases, the type I and type II receptors (TβRI and TβRII). Following ligand binding to TβRII, TβRI is recruited to the complex. This allows for the constitutively active TβRII kinase to transphosphorylate and activate the TβRI kinase which, in turn, phosphorylates Smad2 and Smad3. Smad2/3 then associate with a common mediator Smad, Smad4, and translocate to the nucleus, where they regulate gene transcription. By contrast, the inhibitory Smad7 can interact with TβRI and prevent the phosphorylation of the effectors Smad2/3 (Massagué et al., 2000). In addition to Smads, more recently, other signaling pathways have been implicated in TGFβ actions. These include the extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK), phosphatidylinositol-3 kinase (PI3K), and Rho GTPases (reviewed in Derynck et al., 2001).

TGFβ is both a tumor suppressor and a tumor promoter

The ability of TGFβ to inhibit epithelial cell proliferation suggests a role in tumor suppression. MMTV/TGFβ1$^{S223/225}$ transgenic mice expressing active TGFβ1 in the mammary gland exhibit a hypoplastic ductal epithelium (Pierce et al., 1993). Conversely, expression of dominant negative TβRII results in accelerated lobulo-alveolar mammary development (Gorska et al., 1998) and enhanced propensity for carcinogen-induced lung, mammary, and skin tumors (Bottinger et al., 1997). Mice with targeted disruption of either the *Tgfb1* or the *Smad3* gene develop colon adenomas and carcinomas (Engle et al., 1999; Zhu et al., 1998). Mice carrying an inactivated allele of *Smad4* develop intestinal polyps that progress to carcinomas (Xu et al., 2000). Finally, mice heterozygous for the *Tgfb1* gene express 10%–30% of the TGFβ1 protein, and this partial loss results in enhanced carcinogen-induced tumors (Tang et al., 1998).

TGFβ function is also compromised in some human cancers. Mutations in the *TGFBR2* gene occur in both sporadic and inherited colon cancers with microsatellite instability, and reconstitution of TβRII expression reverses transformation in colon cancer cell lines (Markowitz et al., 1995). Inactivating mutations in genes encoding Smad2 and Smad4 have been found in cancers of the gastrointestinal tract and pancreas (Hahn et al., 1996). A low level of TβRII protein identifies a cohort of women with mammary epithelial hyperplasia at increased risk for development of breast cancer (Gobbi et al., 1999). Other studies have suggested that an excess of endogenous TGFβ can prevent tumor development. For example, MMTV/TGFβ1 mice are resistant to DMBA-induced mammary tumors, and MMTV/TGFα × MMTV/TGFβ1 bigenic mice fail to develop mammary carcinomas seen in MMTV/TGFα mice (Pierce et al., 1995). Mice overexpressing active TGFβ1 under the control of the K14 promoter are relatively protected from skin tumors induced by chemical carcinogens (Cui et al., 1996). This is consistent with the ability of TGFβ to maintain tissue architecture, inhibit genomic instability, and induce replicative senescence and apoptosis in nontransformed cells and/or tissues (illustrated in Figure 1). These studies support the tumor-suppressive role of endogenous TGFβ. To date, however, administration of exogenous TGFβ has not been reported to prevent cancer formation.

Most cancers retain TGFβ receptors but attenuate TGFβ-mediated antimitogenic effects. In contrast to colorectal and pancreatic cancers, Smad2 and Smad4 mutations are rare in other tumor types. Furthermore, the tumors that develop in TGFβ haploid mice do not lose the second TGFβ allele and, in fact, express higher levels of TGFβ protein than TGFβ$^{+/+}$ tumors (Tang et al., 1998). In addition, many tumor cells exhibit increased invasiveness in response to TGFβ (reviewed in Dumont and Arteaga, 2000). TGFβ can also induce an epithelial-to-mesenchymal transition (EMT) in tumor and nontumorigenic immortalized cells (Miettinen et al., 1994). Reexpression of TβRII in colon cancer cells with low invasive potential restores tumor cells invasiveness (Oft et al., 1998), and induction of TGFβ1 in papillomas induces metastatic carcinomas (Weeks et al., 2001). Patients whose pancreatic cancers overexpressed TβRII mRNA had a shorter survival than patients bearing cancers with lower TβRII mRNA (Wagner et al., 1999). Forced expression of active Smad2 in squamous cancer cells results in enhanced tumor cell motility and metastatic dissemination (Oft et al., 2002). Further, introduction of dominant negative TβRII into a variety of metastatic carcinoma cells prevents conversion to a mesenchymal phenotype and inhibits motility, tumorigenicity, and metastases (reviewed in Dumont and Arteaga, 2000). These data suggest that, although partial loss of TGFβ signals is permissive for tumor development, there is no selective pressure in cancer cells to lose TGFβ signaling completely. Secondly, some residual TGFβ might be required for

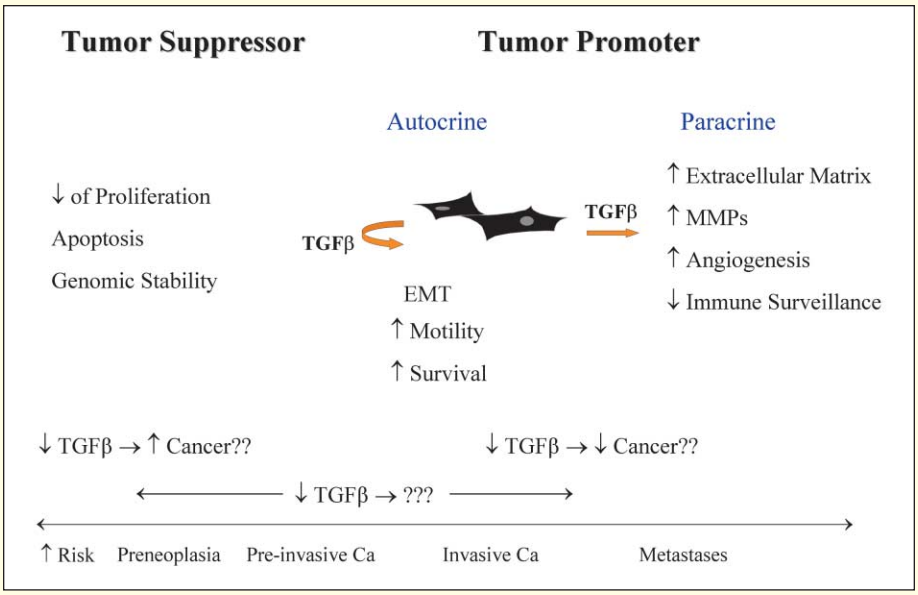

Figure 1. Diagram of tumor suppressor and tumor promoting effects of TGFβ

As a result of oncogenic stimuli or other unknown mechanisms, human cancers attenuate TGFβ-induced suppression but exploit TGFβ-mediated autocrine and paracrine mechanisms conducive to tumor progression. It is likely that in many cancers, both situations are operative, i.e., TGFβ may still attenuate proliferation while inducing cellular events associated with metastatic dissemination. There is experimental evidence that (1) loss of TGFβ signaling will increase the risk of cancer development (tumor suppressor function), and (2) inhibition of autocrine/paracrine TGFβ in tumors will delay metastatic progression (tumor promoter function). In the natural progression of different cancers from preneoplasia to advanced metastatic states, the timing at which the role of TGFβ switches from tumor suppressor to promoter is unclear.

tumor maintenance and, in fact, may select for more metastatic cancers. Perhaps the best demonstration of this hypothesis was provided by Cui et al. (1996). Mice overexpressing active TGFβ1 in keratinocytes develop fewer benign papillomas than controls. However, the transgenic tumors rapidly acquire a spindle cell phenotype, overexpress TGFβ3, and metastasize (Cui et al., 1996). Parenthetically, colon cancers with microsatellite instability and inactivating mutations of the *TGFBR2* gene exhibit favorable survival compared to TβRII-positive colon cancers (Watanabe et al., 2001), suggesting that loss of TGFβ signaling may limit systemic metastases.

There is also abundant evidence to support that excess production and/or activation of TGFβ in tumors can foster cancer progression by paracrine mechanisms (Figure 1). These include an increase in tumor neoangiogenesis and extracellular matrix production, upregulation of peritumor metalloproteases, and the subversion of immune surveillance mechanisms (Derynck et al., 2001). Overexpression of TGFβ ligands has been reported in most tumor types (reviewed in Wojtowicz-Praga, 2003). Most of these reports are immunohistochemical studies utilizing antibodies that do not discriminate between active and latent TGFβ. Although some studies claim excess activation of TGFβ in cancer compared to nontumor tissues, this can only be confirmed by use of TGFβ inhibitors and assessment of their effects against TGFβ-expressing tumors. Nonetheless, elevated levels of TGFβ in tumor tissues correlate with markers of a more metastatic phenotype and/or poor patient outcome (reviewed in Dumont and Arteaga, 2000). In glioblastomas, TGFβ2 overexpression has been associated with immunosuppression in patients (Kuppner et al., 1988). Elevated levels of plasma TGFβ are detected in patients with cancer, and these can predict for early metastatic recurrences (Tsushima et al., 2001).

Is there a role for TGFβ in cancer prevention/treatment?

A recent report indicates that a T29→C polymorphism in the *TGFB1* gene results in increased serum levels of TGFβ1 and is associated with a reduced risk of breast cancer (Ziv et al., 2001). These results plus the ability of TGFβ to inhibit epithelial proliferation and prevent tumor development in transgenic mice suggest that exogenous TGFβ can be utilized for cancer prevention. However, the demonstrations that TGFβ can induce signaling pathways associated with transformations like Erk, p38 MAPK, PI3K/Akt, JNK, and the Rho family of GTPases suggest that exogenous TGFβ might be counterproductive as a therapeutic strategy. Furthermore, overexpression of Ras in nontumor epithelial cells synergizes with TGFβ in the induction of epithelial plasticity and enhanced cell survival (Janda et al., 2002; Oft et al., 1996), suggesting that some oncogenes can potentially synergize with TGFβ in early phases of transformation. Further, MMTV/neu × TGFβ1 and MMTV/PyV mT × TGFβ1 bigenic mice develop mammary tumors with similar latency to MMTV/neu and MMTV/PyV mT mice, respectively (C.L.A., unpublished data), implying that some oncogenes, unlike TGFα, are dominant over TGFβ.

Exogenous TGFβ may also have some negative consequences in patients with cancer. For example, administration of recombinant TGFβ1 to mice bearing MDA-231 xenografts does not inhibit tumor growth, but induces cachexia and generalized interstitial fibrosis in the spleen (Zugmaier et al., 1991). Daily injections of recombinant TGFβ1 facilitate tumor formation by estrogen-dependent MCF-7 human breast cancer cells in the absence of estrogen supplementation (Arteaga et al., 1993a). These data suggest the possibility of significant toxicities and a potential tumor-permissive role for treatment with this cytokine. Thus, the use of TGFβ as a cancer prevention or treatment strategy is highly questionable.

Blockade of TGFβ: Cellular mechanisms of action

The improved outcome of patients bearing cancers with *TGFBR2* mutations suggests an argument in favor of therapeutic blockade of autocrine TGFβ signaling. An additional argument is inferred by the paracrine effects of tumor TGFβs on angiogenesis, stroma formation and remodeling, and immunosuppression. Thus, 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.

Table 1. Current status of development of TGFβ inhibitors

Agent	Class	Development stage	Source	References
CAT-192	Humanized TGFβ1 mAb	Phase II (diffuse systemic sclerosis)	Genzyme/CAT	
CAT-152	Humanized TGFβ2 mAb	Phase III (glaucoma)	Genzyme/CAT	Siriwardena et al., 2002
1D11	TGFβ1, 2, 3 mAb	Preclinical	Genzyme/CAT	Ananth et al., 1999
2G7	TGFβ1, 2, 3 monoclonal IgG_2	Preclinical	Genentech	Arteaga et al., 1993b
sTβRII:Fc	RII/Fc hu IgG_1 fusion protein	Preclinical	Biogen	Muraoka et al., 2002
Betaglycan	Recombinant soluble TβRIII	Preclinical		Bandyopadhyay et al., 1999, 2002
SB-431542	Small molecule TβRI kinase inhibitor	Preclinical	GlaxoSmithKline	Laping et al., 2002
NPC 30345	Small molecule TβRI kinase inhibitor	Preclinical	Scios, Inc.	
LY364947	Small molecule TβRI kinase inhibitor	Preclinical	Lilly Research	Sawyer et al., 2003
AP-12009	Antisense TGFβ2	Phase I/II (glioblastoma)	Antisense Pharma	Marzo et al., 1997

In MMTV/PyV mT transgenic mice, blockade of TGFβ with soluble TβRII:Fc increases apoptosis in primary mammary tumors and inhibits tumor cell motility, intravasation, and metastases (Muraoka et al., 2002). In this report, treatment with sTβRII:Fc inhibited Akt activity in tumors. TGFβ antibodies also inhibit PI3K/Akt activity in 4T1 and EMT-6 mammary cancer cells (Bakin et al., 2000). In transformed cells, TGFβ-induced survival is associated with Akt-induced phosphorylation of FKHRL1, and this effect is blocked by expression of kinase-dead Akt (Shin et al., 2001). These data imply that TGFβ antagonists may need to inhibit PI3K/Akt to induce an antitumor effect. In a recent study, reconstitution of TβRI signaling in MDA-231 human breast cancer cells expressing dominant negative TβRII restored motility and enhanced survival while increasing Akt and ERK activities but not Smad2 phosphorylation (Dumont et al., 2003). This report suggests that, in some metastatic cancer cells, the tumor progressive effects of TGFβ occur at a low threshold of receptor activation and may use Smad-independent mechanisms. The clinical implications of these data are that (1) TGFβ receptor-positive, Smad mutant cancers may still use TGFβ for tumor progression, and (2) therapeutically, a more complete inhibition of TGFβ signaling might be required to block the tumor-permissive effects of TGFβ.

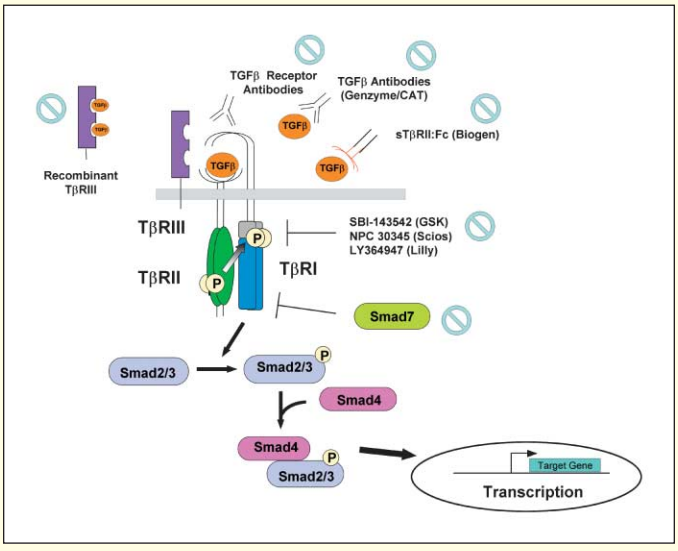

Figure 2. Sites of action of TGFβ antagonists currently in development

Each class of inhibitors is marked with Ø. Smad7 is a negative regulator of TGFβ signaling that has also been proposed as a therapeutic approach. There are no reports of ligand-blocking TGFβ receptor antibodies in clinical development.

A monoclonal pan-TGFβ neutralizing antibody suppresses the growth of TGFβ1-overexpressing renal cancer xenografts while abrogating factor VIII expression (Ananth et al., 1999), suggesting an antiangiogenic effect. Cancer cells stably transfected with soluble forms of TβRII or TβRIII exhibit reduced tumorigenicity and microvessel density compared to control tumors (Bandyopadhyay et al., 1999; Rowland-Goldsmith et al., 2001). There is also evidence that overexpression of sTβRII:Fc in thymoma cells can prevent the progression of unmodified thymoma cells when injected near the primary tumor inoculation site. Stable transduction of breast and glioma tumor cells with antisense TGFβ1 and TGFβ2 retroviruses, respectively, restores their immunogenicity when injected into immunocompetent mice which, in turn, induces partial rejection of unmodified established tumors (reviewed in Dumont and Arteaga, 2000). These examples suggest that presence of TGFβ neutralizing activity within the tumor milieu can restore tumor-specific cellular immunity and mediate tumor rejection. Along those lines, transgenic mice expressing a dominant negative TβRII under the control of a T cell specific promoter eradicate tumors when challenged with live tumor cells (Gorelik and Flavell, 2001). Finally, the 2G7 pan-TGFβ neutralizing IgG_2 suppresses the establishment of MDA-231 tumors and lung metastases in athymic mice and prevents the inhibition of host NK cell function induced by tumor inoculation (Arteaga et al., 1993b). The antibody had no effect against MDA-231 cells in vitro, nor did it inhibit primary and metastatic tumors in NK-deficient mice, suggesting that antibody-mediated TGFβ blockade is effective in disrupting tumor-host immunosuppressive interactions that are essential for cancer progression.

Blockade of TGFβ in normal cells may induce side effects in the tumor host that can be anticipated. Complete loss of TGFβ in mice is associated with a severe inflammatory response. Complete elimination of TGFβ function in T cells leads to autoimmune disorders (Gorelik and Flavell, 2001). A recent experiment, however, suggests that TGFβ antagonists might be well tolerated. Mice expressing soluble TβRII under the regulation of the MMTV/LTR promoter exhibit high levels of the TGFβ antagonist in the circulation without the severe inflammatory phenotype of TGFβ null mice. The circulating levels of sTβRII:Fc were sufficient to inhibit tumor metastases in this model. Mild

lymphocytic infiltration in lungs, kidneys, and pancreas were observed (Yang et al., 2002). 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. Because of the stabilizing effect of TGFβ on blood vessels, TGFβ antagonists may affect wound healing. Interestingly, administration of a TGFβ2 antibody via subconjunctival injections immediately before and after trabeculectomy for glaucoma was not associated with impaired healing (Siriwardena et al., 2002).

Perhaps the greatest risk of TGFβ antagonists is the potential acceleration of preneoplastic lesions or cancers in which TGFβ still exerts growth restraint (Figure 1). In support of this possibility, expression of an antisense TGFβ1 expression vector in well-differentiated colon cancer cells enhances their tumorigenicity in nude mice (Wu et al., 1993). In other reports, administration of a TGFβ neutralizing antibody or expression of soluble TβRII results in enhanced angiogenesis and/or larger primary tumor size (Gohongi et al., 1999). The possibility that blockade of TGFβ with exogenous antagonists can accelerate transformation, tumor onset, and/or cancer progression requires rigorous investigation in preclinical models.

Clinical development of TGFβ inhibitors

The development of anticancer molecular therapeutics requires the identification of a measurable molecular target that can aid in the selection of appropriate patients for clinical trials. This issue is not clear with TGFβ antagonists, since the target network includes three ubiquitous ligands in an unknown state of activation and receptors present in both tumor and nontumor cells. The ubiquituous expression of all three TGFβ ligands in human tumors, plus the inability to accurately ascertain their level of a possibly dynamic and spatially regulated activation in situ, argue against TGFβ expression being such predictive marker for patient selection. One possible exception are patients with glioblastoma in whom TGFβ2 overexpression is associated with immune suppression. Several arguments suggest that expression of TβRII might be a good marker to use. First, most tumors retain TβRII. Second, interference of TβRII function in mesenchymally differentiated carcinomas has been shown to reverse their mesenchymal phenotype and/or inhibit metastases. Third, expression of TβRII in tumors has been associated with shorter patient survival (Wagner et al., 1999; Watanabe et al., 2001). Therefore, we propose that patients with poorly differentiated carcinomas (i.e., with mesenchymal features) that express TβRII should be appropriate candidates for the testing of TGFβ antagonists. In these tumors, TGFβ blockade could potentially inhibit both autocrine TGFβ signaling in tumor cells and the paracrine/endocrine effects of TGFβ.

Several approaches to block TGFβ function are being pursued (Table 1 and Figure 2). One approach 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. 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, 1D11 and 2G7, have been reported. One 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. Human recombinant TβRIII has shown antimetastatic and antiendothelial cell activity. One attractive feature of recombinant betaglycan over sTβRII 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 are ATP competitive inhibitors of the ATP binding site of the TβRI 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, since there are no known TβRII functions that do not require TβRI. Nonetheless, the development of bifunctional receptor inhibitors should help in resolving these questions. Vectors encoding the inhibitory Smad, Smad7, have been used to bind TβRI and interfere with Smad2/3 phosphorylation (Nakao et al., 1997). 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 (Kleeff et al., 1999). Moreover, blockade of Smad4 has been shown to facilitate TGFβ-dependent and -independent activation of Erk in squamous cancer cells and promote their motility and transmesenchymal differentiation (Iglesias et al., 2000), suggesting that postreceptor interference of Smads as an anticancer strategy may be conceptually weak. The TGFβ antagonists discussed above are summarized in Table 1.

Conclusions

There is preponderant experimental evidence to suggest that TGFβ signaling can foster tumor-host interactions that indirectly support neoplastic cell viability and progression. Further evidence also suggests that autocrine TGFβ signaling is operative in some tumor cells and can also contribute to tumor invasion, survival, and metastases. These multiple tumor-permissive effects of TGFβ provide for a therapeutic opportunity in that by blocking this signaling network, one can interrupt multiple autocrine and paracrine mechanisms that are essential for tumor maintenance. Many questions exist about the viability of this treatment strategy as it applies to patient and tumor selection, molecular predictors of response to therapy, biochemical surrogates for determination of optimal dosing, potential toxicities, and the possible inclusion of inhibitors of TGFβ activation to the strategies summarized above. Nonetheless, the plethora of TGFβ antagonists in development suggest that these questions will be addressed in the near future.

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