Soluble Type II Transforming Growth Factor-β (TGF-β) Receptor Inhibits TGF-β Signaling in COLO-357 Pancreatic Cancer Cells in Vitro and Attenuates Tumor Formation

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ABSTRACT

Human pancreatic ductal adenocarcinomas overexpress transforming growth factor-β (TGF-βs). This overexpression has been correlated with decreased patient survival. TGF-βs bind to a type II TGF-β receptor (TβRII) dimer, which heterotetramerizes with a type I TGF-β receptor (TβRI) dimer, thereby activating downstream signaling.

Purpose and Experimental Design: To determine whether blocking TGF-β actions would suppress pancreatic cancer cell growth in vivo, we expressed a soluble TβRII, encoding amino acids 1–159 of the extracellular domain in COLO-357 human pancreatic cancer cells. This cell line expresses all of the three mammalian TGF-β isoforms and is growth inhibited by TGF-β in vitro.

Results: COLO-357 clones expressing soluble TβRII were no longer growth inhibited by exogenous TGF-β1 and exhibited a marked decrease in their invasive capacity in vitro. When injected s.c. into athymic mice, these clones exhibited attenuated growth rates and angiogenesis and decreased levels of plasminogen activator inhibitor-1 mRNA as compared with tumors formed by sham-transfected cells.

Conclusions: These results indicate that endogenous TGF-βs can confer a growth advantage in vivo to a pancreatic cancer cell line that is growth inhibited in vitro and suggest that a soluble receptor approach can be used to block these tumorigenic effects of TGF-βs.

INTRODUCTION

Mammalian cells express three TGF-β isoforms that regulate many cellular processes (1). They inhibit the growth of cells of epithelial origin and modulate differentiation, migration, deposition of the extracellular matrix, immunosuppression, motility, and cell death (1). They signal by binding to a TβRII dimer that heterotetramerizes with a TβRI homodimer (1). This leads to the phosphorylation of signaling pathway specific Smad2 and Smad3 molecules that oligomerize with the common mediator, Smad4 (1). The resulting complexes translocate to the nucleus where they regulate gene transcription (1). In contrast, the TβRII is devoid of signaling capabilities and acts to enhance ligand binding to TβRII (1).

Human pancreatic cancer is the fifth leading cause of cancer related mortality in the Western industrialized countries. The mortality rate virtually equals its incidence rate (2). The reasons for this biological aggressiveness are unknown. However, it has been established that these cancers harbor p53 tumor suppressor gene and K-ras oncogene mutations and overexpress multiple mitogenic growth factors and their receptors (3, 4). In addition, 50% of these cancers have Smad4 mutations (5), and many exhibit inactivating mutations or deletions in the p15 and p16 genes (6, 7). These mutations may cause these tumors to be resistant to TGF-β-mediated growth inhibition. However, pancreatic cancers overexpress all of the three TGF-βs, and this overexpression has been correlated with decreased patient survival (8). The mechanisms by which TGF-βs confer a growth advantage to pancreatic cancer cells in vivo have not been elucidated.

In the current study, we used a soluble receptor approach to block the local actions of TGF-β in a s.c. nude mouse model of pancreatic cancer. To this end, we generated a DNA encoding a human soluble TβRII receptor and stably transfected this construct into the human pancreatic cancer cell line, COLO-357. These cells express all of the three TGF-β isoforms and are growth inhibited by TGF-β1 in vitro in conjunction with increased expression of the mammalian cyclin-dependent kinase inhibitors p15, p21, and p27 (9, 10). They also express a functional Smad4 gene, and their TβRI and TβRII genes are not mutated (10–12). Furthermore, COLO-357 cells engineered to overexpress inhibitory Smad6 or Smad7 are no longer growth inhibited by TGF-β1 (13, 14). Surprisingly, overexpression of Smad7 is also associated with enhanced anchorage-independent growth and increased tumorigenicity in nude mice (14), raising...
the possibility that COLO-357 cell-derived TGF-βs promote cancer growth in vivo. It is not known, however, whether inhibiting the biological actions of COLO-357 cell-derived TGF-βs would attenuate the in vivo growth of these cells. We now report that COLO-357 clones expressing the soluble TβRII exhibit attenuated growth inhibition in vitro in response to TGF-β1 when compared with the sham-transfected cells. These clones also demonstrate a decreased invasive capacity in vitro, as well as a decreased capacity to form tumors in nude mice and attenuated angiogenesis in vivo.

MATERIALS AND METHODS

Materials. The following materials were purchased: FBS, DMEM medium, trypsin solution, penicillin-streptomycin solution, and Geneticin (G418) from Irvine Scientific (Santa Ana, CA), AmpliTAQ DNA Polymerase from Perkin-Elmer (Norwalk, CT), restriction enzymes, pMH vector from Boehringer-Mannheim (Indianapolis, IN), PCR primers from Bio-Synthesis, Inc. (Lewisville, TX), TA cloning pCRRI vector from Invitrogen (Carlsbad, CA), mini-plasmid DNA purification kit from Promega (Madison, WI), maxi-DNA plasmid purification preparation (Carlsbad, CA), mini-plasmid DNA purification kit from Promega (Madison, WI), maxi-DNA plasmid purification preparation kit and DNA gel extraction kit from Qiagen (Thousand Oaks, CA), Sequenase version 1.0 DNA sequencing from USB Specialty Biochemicals (Cleveland, OH), Genescreen membranes from New England Nuclear (Boston, MA), random primed labeling kit from Ambion (Austin, TX), [α-32P]dCTP and [γ-32P]dATP from Amersham (Arlington Heights, IL), TE Select D G50 columns from 5 Prime—3 Prime, Inc. (Boulder, CO), DNA and protein molecular weight markers, Life Technologies, Inc. (Gaithersburg, MD), anti-HA, anti-ERK-2, anti-TβRII antibodies and protein A agarose from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), biotinylated antihuman TβRII polyclonal antibody from R&D Systems, Inc. (Minneapolis, MN), horseradish peroxidase-conjugated antibodies from Bio-Rad (Hercules, CA), PECAM-1 monoclonal antibody (clone C70A) from Oncogene Research Products (Cambridge, MA), enhanced chemiluminescence substrate and Restore Stripping Buffer from Pierce (Rockford, IL), Vectastain Universal Elite ABC kit from Vector Labs (Burlingame, CA), Immobilon-P nitrocellulose membranes from Millipore Corp. (Bedford, MA), streptavidin-peroxidase from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD), centriprep concentrators from Amicon Inc. (Beverly, MA), Lab-Tek chamber slides from Nunc Inc. (Naperville, IL), Transwell chambers from Costar (Cambridge, MA), Matrigel from Becton Dickinson (Bedford, MD), and all of the other reagents from Sigma Chemical Co. (St. Louis, MO). COLO-357 human pancreatic cancer cells were a gift from Dr. Richard S. Metzgar (Duke University, Durham, NC). TGF-B1 was a gift from Genentech, Inc. (South San Francisco, CA). Human dermal microvascular endothelial cells were a gift from Dr. Joyce Bischoff (Children’s Hospital, Harvard University Medical School, Boston, MA) and Dr. Jian Luo (University of California Irvine, Irvine, CA).

Construction of a Mammalian Expression Vector. The complete cDNA of human TβRII was used as the template for PCR amplification of the coding sequence of the extracellular domain of TβRII (nucleotides 1–477 including the signal sequence). PCR was performed using the sense primer, 5’-AAGCTTGGCCGCGCATGGGTCG, and antisense primer, 5’-CTGGAATTCGTCAGGATTTCGTCGG. The sense primer introduced a HindIII restriction site and the consensus Kozak translation initiation start site. The antisense primer introduced an EcoRI site. The PCR fragment was ligated into the PCRZ.1 vector. The soluble TβRII coding fragment was isolated after digestion with HindIII and EcoRV. This gel-purified fragment was subsequently ligated into the HindIII/Eco721-digested pMH expression vector, which is tagged at its COOH terminus with a HA epitope. The constructed vector, pMHsTβRII, contained the open reading frame encoding the human soluble TβRII and nucleotides encoding nine amino acids of HA. The sequence and orientation was confirmed by dideoxy chain termination sequencing. The pMH plasmid containing the G418 resistance gene (neomycin) was used for construction of control clones (sham) expressing the vehicle vector alone.

Cell Culture. COLO-357 human pancreatic cancer cells were grown in DMEM, supplemented with 8% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml), and 5% fungazone termed complete medium. Cells were maintained in monolayer cultures at 37°C in humidified air with 5% CO2. The selection medium for the cell lines containing the neomycin resistance gene was supplemented with 0.4 mg/ml G418. For TGF-β1 experiments, cells were incubated overnight in serum-free medium (DMEM containing 0.1% BSA, 5 μg/ml transferrin, 5 ng/ml sodium selenite, antibiotics, and fungazone). To generate cells expressing the human soluble TβRII, COLO-357 cells were transfected in a stable manner with the pMHsTβRII plasmid (10 μg) using Lipofectamine as reported previously (15). After reaching confluence, cells were split 1:10 into selection medium, and single clones were isolated after 3–4 weeks. After expansion of each individual clone, cells from each clone were screened for expression of soluble TβRII by Northern and Western blot analysis. Two clones were selected for additional studies.

Immunoblotting and Immunoprecipitation. Exponentially growing human pancreatic and dermal microvascular endothelial cells (50–60% confluent) were washed with ice cold 1× PBS and lysed in buffer containing 1% NP40, 20 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium phosphate, 1 mM β-glycerophosphate, 1 mM sodium vanadate, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Frozen tumor samples derived from COLO-357 sham-transfected or pMHsTβRII clones were homogenized and lysed in the same buffer. Lysates were subjected to SDS-PAGE and electrotransferred to Immobilon-P membranes for 40–80 min. After blocking with 5% milk (1× Tris-buffered saline with 0.1% Tween 20), the membranes were incubated with anti-HA monoclonal antibody (1:1200 dilution), anti-CD31 antibody (1:3000 dilution), washed, and incubated with a secondary horseradish peroxidase-conjugated antibody. After washing, bound antibodies were visualized using enhanced chemiluminescence. To confirm equal loading, membranes were stripped for 20 min at room temperature in either Restore stripping buffer or 30 min at 50°C in buffer containing 2% SDS, 62.5 mM Tris (pH 6.7), and 100 mM 2-mercaptoethanol and blotted with an anti-ERK-2 antibody (1:8000 dilution).

For immunoprecipitation with the anti-HA antibody, cells
transfected with the soluble TbRII expression construct or sham were grown to 80% confluency in complete medium and then incubated for 24 h in serum-free medium. Conditioned medium from the clones or sham-transfected cells was concentrated by using a 10,000 cutoff filtration membrane. The concentrated medium was incubated for 12 h at 4°C with the anti-HA antibody (2 μg/ml), followed by a 2-h incubation with protein A agarose (50 μl) at 4°C. Precipitates were washed three times with ice-cold PBS, resuspended in 2 x loading buffer, and boiled for 5 min at 100°C. After centrifugation, the supernatants were subjected to Western blotting using the biotinylated anti-human TbRII polyclonal antibody (1:5000 dilution).

Immunohistochemistry. COLO-357 cells were plated in chamber slides and grown to 70% confluency for 48 h. The cells were fixed in 1.5% paraformaldehyde for 45 min at room temperature and incubated sequentially for 30 min (room temperature) with 0.1% Triton X-100, 30 min (room temperature) with 0.3% hydrogen peroxide/methanol, 30 min (37°C) with 1 mg/ml hyaluronidase, and 40 min (room temperature) with 10% normal goat serum. Cells were then incubated for 16 h (4°C) with the highly specific anti-HA antibody (0.4 μg/ml) recognizing the HA epitope encoded by pMHsTbRII or with the highly specific anti-TbRII antibody (0.2 μg/ml) recognizing the epitope corresponding to the full-length TbRII. To assess TbRII, HA, and CD31 immunoreactivity, tumors from s.c. lesions were removed and immediately divided. Tissues were fixed in 4% formaldehyde and embedded in paraffin wax. Paraffin-embedded sections (4 μm) from tumor tissue derived from sham-transfected or pMHsTbRII-transfected cells were cut and mounted on poly-L-lysine-coated glass slides and air-dried overnight at room temperature. Representative sections of each case were examined by the streptavidin-peroxidase technique using appropriate positive and negative controls. Endogenous peroxidase activity was blocked by incubation for 30 min with 0.3% hydrogen peroxide in methanol. Tissue sections were incubated for 15 min (room temperature) with 10% normal goat serum and then incubated for 16 h at 4°C with anti-HA antibody (0.4 μg/ml), anti-TbRII antibody (0.2 μg/ml), or anti-platelet endothelial cell adhesion molecule-1 antibody (1:50 dilution) in PBS containing 1% BSA.

For both cell-line and tissue immunohistochemistry, bound TbRII and HA antibodies were detected with biotinylated goat antirabbit IgG secondary antibodies and streptavidin-peroxidase complexes, using diaminobenzidine tetrahydrochloride as the substrate. Sections were counterstained with Mayer’s hematoxylin. Sections incubated with nonimmune rabbit IgG or with secondary antibodies alone did not yield positive immunoreactivity. The frequency of blood vessels in the matrix region of the tumor that was positively stained for PECAM-1 was evaluated on September 22, 2017. © 2001 American Association for Cancer Res. clincancerres.aacrjournals.org Downloaded from clincancerres.aacrjournals.org on September 22, 2017. © 2001 American Association for Cancer Research.

In Vivo Tumorigenicity Assay. To assess the effect of the soluble TbRII on tumorigenicity, 2 x 10⁶ or 6 x 10⁵ cells expressing the empty vector alone (sham) or soluble TbRII were injected s.c. into two sites in female athymic (nude) mice. The tumor size was measured weekly until mice were sacrificed 56 days after injection. A portion of the tumor tissue was snap frozen in liquid nitrogen and stored for subsequent RNA and protein analysis. Another portion was prepared for immunohistochemistry studies.

Invasion Assay. The invasive ability of the COLO-357 sham-transfected and soluble TbRII-transfected pancreatic cancer cells were measured as reported previously (20) with some modifications (21). Briefly, polycarbonate membranes (8-μm pore size) of the upper compartment of Transwell chambers were coated with 5% Matrigel. COLO-357 cells that were preincubated for 24 h in serum-free medium containing 0.1% BSA in the absence or presence of 400 μM TGF-β1 were suspended in 100 μl of serum-free medium containing 0.1% BSA and placed onto this upper compartment. The lower compartment was then filled with 600 μl of serum-free medium containing 0.5% FBS. TGF-β1 (400 pg) was added to the lower compartment corresponding to chambers that contained cells previously incubated with TGF-β1. After 16 h, the membranes were fixed in methanol and stained with H&E. Cells on the upper surface of the filter were carefully removed with a cotton swab, and the cells that had migrated through the membrane to the lower surface of the filter were counted in nine different
Soluble TβRII clones and subjected to 13% SDS-PAGE, electrotransferred to a membrane for immunoblotting. Cell lysates (50 μg/lane) were size fractionated, electrotransferred to a nylon Genescreen membrane, and hybridized with the 32P-labeled soluble TβRII cDNA probe (5 × 10^6 cpm/ml 1-h exposure). To confirm equal loading of lanes, the membrane was stripped and reprobed with the 7S cDNA probe (5 × 10^6 cpm/ml 2-h exposure). B, immunoblotting. Cell lysates (50 μg/lane) were prepared from the same clones and subjected to 13% SDS-PAGE, electrotransferred to a membrane, and blotted with anti-HA antibodies (1:1000; 1-min exposure). To confirm equal loading of lanes, the membrane was stripped and reprobed with an anti-ERK-2 antibody (1:8000; 5-s exposure). C, detection of secreted soluble TβRII. Concentrated conditioned medium from either the sham-transfected (S) or soluble TβRII-transfected cells (C1) was immunoprecipitated overnight with 2 μg of anti-HA antibody. The samples were subjected to 13% SDS-PAGE, electrotransferred to a nylon membrane, and blotted with biotinylated antihuman TβRII polyclonal antibody (1:5000 dilution; 1-min exposure).

RESULTS

Expression of Soluble TβRII in Transfected COLO-357 Clones. COLO-357 pancreatic cancer cells were stably transfected with a soluble TβRII cDNA construct, encoding the entire extracellular domain (amino acids 1–159) of human TβRII. Transfected clones were selected after 3–4 weeks of growth in medium supplemented with G418, and subsequent experiments were carried out with two independent clones. These clones were selected because they displayed high levels of soluble TβRII mRNA expression by Northern blot analysis (Fig. 1A). COLO-357 cells transfected with the pMH empty vector carrying the G418 resistance gene served as the control (sham) and did not express the soluble TβRII (Fig. 1A). Immunoblotting of cell lysates with the anti-HA antibody, which recognizes the HA epitope at the COOH terminus of the pMH expression vector, confirmed the expression of the soluble TβRII at the protein level (Fig. 1B). To determine whether the soluble TβRII was secreted, conditioned medium from a sham-transfected clone and from clone 1 (C1) was subjected to immunoprecipitation with the anti-HA antibody followed by immunoblotting with the biotinylated anti-human TβRII antibody. A major band (M, 25,000) representing the soluble TβRII protein was visible in conditioned medium from the C1 clone but not from the sham clone. A minor band (M, 35,000) was also present in the C1 conditioned medium (Fig. 1C).

Expression of the soluble TβRII was also confirmed by immunohistochemical analysis. Using the anti-TβRII antibody, there was weak immunostaining for TβRII in the sham-transfected cells (Fig. 2A), representing endogenously expressed TβRII. In contrast, there was strong TβRII immunoreactivity in clone 1 cells (Fig. 2B) and clone 2 (data not shown) expressing the transfected soluble TβRII. When the anti-HA antibody was used, sham-transfected cells were devoid of immunoreactivity (Fig. 2C), whereas positive immunostaining for HA was readily evident in the cells transfected with the pMHsTβRII construct (Fig. 2D).

Effects of TGF-β1 on Cell Growth and Invasion. Sham-transfected COLO-357 cells exhibited a doubling time of approximately 30 h, in agreement with growth characteristics of parental COLO-357 cells published previously (13). Clones expressing the soluble TβRII exhibited similar doubling times ranging from 31 h for clone 1 to 37 h for clone 2. TGF-β1 (10 ps) inhibited the growth of sham-transfected COLO-357 cells (30%; P < 0.002) but was without effect in the soluble TβRII-expressing clones that were used in subsequent experiments (Fig. 3A), as well as in two additional clones (data not shown). TGF-β1 (400 ps) also significantly increased the invasiveness of sham-transfected COLO-357 cells in an in vitro cell invasion assay (Fig. 3B) but was without effect in the clones expressing the soluble TβRII (Fig. 3B). Thus, expression of soluble TβRII effectively blocked the biological actions of exogenous TGF-β1.

Growth Properties of Soluble TβRII-expressing Clones in Vivo. To compare the tumorigenicity of COLO-357 soluble TβRII-expressing cells with sham-transfected cells, 2 × 10⁶ cells/site were s.c. injected in athymic nude mice (two sites/mouse). Transfected clones consistently revealed a significant decrease in tumor volume, as compared with tumors arising from sham-transfected cells. When tumor volumes from three separate experiments were averaged on day 28 after injection, there was significant tumor growth inhibition for both clone 1 (67%) and clone 2 (62%), when compared with the sham controls (Fig. 4A). To determine whether tumor growth was also decreased after injection of a lower number of cells, 6 × 10⁵ COLO-357 cells/site were injected next (two sites/mouse). Three mice received injections with sham-transfected COLO-357 cells, and six mice received injections with pMHsTβRII-transfected COLO-357 clones. There was significant inhibition of tumor growth by the transfected clones at 28 days (70%; P < 0.04) and 35 days (75%; P < 0.005) after injection when compared with the respective sham controls (Fig. 4B). One site that received injections with the pMHsTβRII C1 cells did not form a tumor, even after 56 days. In contrast, all of the sites that received injections with sham-transfected cells always yielded tumors. After day 35, two mice, each bearing two tumors of the sham-transfected cells, had to be sacrificed because the tumor burden reached the maximum allowable limit by our Institu-
tional Animal Care and Use Committee protocol. The remaining mice bearing tumors from either the pMHsTβRII clones or the sham-transfected cells were allowed to grow until day 56. Even at time of sacrifice (day 56), tumors derived from the clones never became as large as the sham did on day 35. At day 56, the tumors derived from sham-transfected cells were still progressively growing, whereas the growth of the tumors derived from pMHsTβRII cells reached a plateau (Fig. 4B).

To confirm the expression of the soluble TβRII in vivo, immunostaining and Northern blot analysis were performed next. When using the anti-TβRII antibody, a few cells were weakly positive for endogenous TβRII in the tumors derived from sham-transfected cells (Fig. 5A). As expected, these tumors did not exhibit positive immunostaining when the anti-HA antibody was used (Fig. 5C). In contrast, strong TβRII (Fig. 5B) and HA (Fig. 5D) immunoreactivity was evident in the cancer cells expressing the pMHsTβRII construct. Tumors from both clones expressed high levels of the soluble TβRII mRNA moiety (Fig. 6A). In contrast, tumors from sham-transfected cells did not express the soluble TβRII mRNA transcript (Fig. 6A). Because PAI-1 has been implicated in pancreatic cancer metastasis in humans (22) and is overexpressed in this malignancy (23), its expression was analyzed next. In the tumors derived from soluble TβRII clones, there was a 67% decrease in PAI-1 mRNA levels when compared with the sham tumors (Fig. 6B).

Morphometric analysis (Fig. 7A) revealed a statistically significant decrease in the number of cells that stained positive for anti-PECAM-1 antibody in tumors derived from either clone.

Fig. 2 Immunostaining of COLO-357 cells and transfected clones. An anti-TβRII antibody recognizing the full-length TβRII (A and B) and an anti-HA antibody recognizing the HA epitope of the pMHsTβRII construct (C and D) were used. In the sham-transfected cells, there was weak immunostaining for endogenous TβRII (A) but undetectable HA immunoreactivity (C). In contrast, the cancer cells expressing the pMHsTβRII construct (B and D) exhibited strong TβRII and HA immunoreactivity, respectively. Scale bar, 25 μm.
RII Suppresses COLO-357 Cell Tumorigenicity

Results are expressed as percentage of unstimulated control and are the means (±SE) of eight determinations/experiment from three separate experiments. Error bars for the control groups were exceedingly small. *, P < 0.002 when compared with respective untreated controls. Effects of TGF-β1 on invasion. To assess the ability of TGF-β1 to induce invasion, COLO-357 cells were preincubated for 24 h in serum-free medium containing 0.1% BSA in the absence or presence of 400 pm TGF-β1. The respective groups of cells (1 × 10^5/well) were then added to the upper chambers of a 24-well Transwell unit. Medium devoid of TGF-β1 (●) or containing 400 pm TGF-β1 (■) was then added to the respective lower chambers. Migration across the Matrigel-coated polycarbonate membrane (8-µm pore size) was assessed after 16 h. The number of cells in nine separate high-power fields (magnification, ×200) was counted in triplicate wells. Data are expressed as the means ± SE of triplicate determinations from three separate experiments. *, P < 0.01 when compared with the untreated controls.

1 (6.7 ± 0.35) or clone 2 (5.3 ± 0.37), in comparison with tumors derived from the sham-transfected cells (12.2 ± 0.70). To more clearly assess blood vessel mass, immunoblotting with the anti-PECAM-1 antibody was performed next using frozen tumor tissues derived from both COLO-357 cells. Tumors derived from sham-transfected COLO-357 cells expressed multiple bands of PECAM-1 then those derived from pMHsTβRII-transfected cells (Fig. 7B). Human endothelial cells, which are known to express high levels of PECAM-1, served as a positive control and exhibited a strong PECAM-1 signal.

**DISCUSSION**

In the present study, a cDNA encoding the soluble TβRII was stably transfected into COLO-357 cells in an attempt to suppress the biological actions of cancer cell-derived TGF-β. Both selected clones expressed high levels of the soluble TβRII by Northern blotting and by immunoblotting. Furthermore, the soluble TβRII was secreted into the conditioned medium where it was present as a major band of M_r 25,000 and a minor band of M_r 35,000. These findings are consistent with other studies that showed that the extracellular domain of TβRII was detected as multiple bands of M_r 25,000 to 35,000 in COS cells (24) and EL4 mouse thymoma cells (25). Furthermore, in the present study, HA immunoreactivity was only present in the cancer cells transfected with the pMHsTβRII construct, and TβRII immunoreactivity was only present at high levels in the transfected clones. Together, these observations indicate that both selected clones expressed high levels of the soluble TβRII.

COLO-357 clones expressing the soluble TβRII and sham-transfected COLO-357 cells exhibited similar doubling times *in vitro*. However, in the pMHsTβRII-expressing clones, the...
growth inhibitory effect of 10 pM TGF-β1 was completely blocked, indicating that soluble TβRII efficiently bound and sequestered TGF-β1. This conclusion is supported by the observation that the stimulatory effect of 400 pM TGF-β1 on cell invasiveness was also completely blocked by soluble TβRII. The mechanisms by which TGF-β1 promotes cellular invasion are not known. It has been shown that TGF-β1 increases Smad2 expression in COLO-357 cells (23), and elevated levels of Smad2 are known to enhance cellular motility (26). It has also been established that TGF-β1 enhances the expression of PAI-1 (10, 27–29), and increased TGF-β expression correlates with increased PAI-1 levels in pancreatic cancer (23). PAI-1 is the main inhibitor of the urokinase-type plasminogen activator system. It promotes cancer cell migration by preventing excessive extracellular matrix degradation by plasmin proteolysis (22, 30), and its reduced expression correlates with attenuated tumorigenicity in Smad4 reconstituted cancer cells (31). Therefore, it is possible that TGF-β1 acts via these mechanisms to promote the motility of COLO-357 cells across a Matrigel membrane. Because pancreatic cancer is a highly invasive malignancy, these observations suggest that TGF-βs may act directly on pancreatic cancer cells to promote cancer cell invasion.

Multiple s.c. injections of two independent clones expressing soluble TβRII yielded small tumors in nude mice when compared with sham-transfected cells. These tumors expressed the soluble TβRII by Northern analysis and exhibited strong HA and TβRII immunoreactivity, thereby confirming in vivo production of soluble TβRII. These tumors also exhibited attenu-
Expression of soluble TβRII and PAI-1 mRNA transcripts in vivo. Total RNA (20 μg/lane) was prepared from tumors generated in athymic mice after inoculation with sham-transfected COLO-357 cells (S) or clones expressing soluble TβRII (C1-C2). A, expression of soluble TβRII. RNA was size fractionated, electrotransferred to a Genescreen nylon membrane, and hybridized with 32P-labeled soluble TβRII cDNA (2 × 10^6 cpm/ml; 1-day exposure). The membrane was then stripped and probed with 7S cDNA (5 × 10^5 cpm/ml; 3-h exposure). B, expression of PAI-1. Total RNA prepared was pooled in equal portions with three mice/group. RNA was prepared from tumors (35 μg/lane) from three mice that received injections with sham-transfected cells (sham) and three mice that received injections with pMHsTβRII-transfected cells (clones). RNA was size fractionated, electrotransferred to a nylon membrane, and hybridized with the 32P-labeled PAI-1 cDNA probe (5 × 10^6 cpm/ml; 3-day exposure). To confirm equal loading of lanes, the membranes were stripped and reprobed with the 7S cDNA probe (5 × 10^5 cpm/ml; 1-day exposure).

Fig. 6. Expression of soluble TβRII and PAI-1 mRNA transcripts in vivo. Total RNA (20 μg/lane) was prepared from tumors generated in athymic mice after inoculation with sham-transfected COLO-357 cells (S) or clones expressing soluble TβRII (C1-C2). A, expression of soluble TβRII. RNA was size fractionated, electrotransferred to a Genescreen nylon membrane, and hybridized with 32P-labeled soluble TβRII cDNA (2 × 10^6 cpm/ml; 1-day exposure). The membrane was then stripped and probed with 7S cDNA (5 × 10^5 cpm/ml; 3-h exposure). B, expression of PAI-1. Total RNA prepared was pooled in equal portions with three mice/group. RNA was prepared from tumors (35 μg/lane) from three mice that received injections with sham-transfected cells (sham) and three mice that received injections with pMHsTβRII-transfected cells (clones). RNA was size fractionated, electrotransferred to a nylon membrane, and hybridized with the 32P-labeled PAI-1 cDNA probe (5 × 10^6 cpm/ml; 3-day exposure). To confirm equal loading of lanes, the membranes were stripped and reprobed with the 7S cDNA probe (5 × 10^5 cpm/ml; 1-day exposure).

Fig. 7. PECAM-1 expression in tumors derived from COLO-357 cells. A, blood vessel frequency in tumors formed by COLO-357 cells. Fifty different high-power fields (0.25 mm^2/field) were randomly selected for determination of blood vessel frequency after immunostaining with an anti-PECAM-1 antibody. Data are the means ± SE of three separate tumors/group. *, P < 0.002 and **, P < 0.0009 when compared with the tumors derived from sham-transfected cells. B, PECAM-1 immunoblotting in lysates derived from COLO-357 cells. Lysates (20 μg/lane) were prepared from tumor tissues derived from sham or pMHsTβRII COLO-357 cells (clones C1 or C2). Lysates were then subjected to 7.5% SDS-PAGE, electrotransferred to a membrane, and blotted with an anti-PECAM-1 antibody (1:3000; 5-s exposure). Human endothelial (E) cell lysates (1.0 μg) served as a positive control. To confirm equal loading of lanes, the membrane was stripped and reprobed with an anti-ERK-2 antibody (1:8000; 2-s exposure). Exposure time was increased to 10 min to detect ERK-2 in the case of the endothelial cell lysate.

Several different types of experiments have suggested that there is a dissociation between the signaling pathways that mediate the growth suppressive effects of TGF-βs and their effects on the expression of genes that modulate the extracellular matrix. Thus, in mink lung epithelial cells, expression of a truncated TβRII renders these cells resistant to TGF-β1-mediated growth inhibitory effects without altering TGF-β1-mediated induction of PAI-1 (27). Transfection of these cells with a mutant TβRI that lacks the juxtamembrane region preceding the GS domain leads to TGF-β1-mediated PAI-1 production but not to growth inhibition (38). Overexpression of inhibitory Smad7 makes COLO-357 cells resistant to TGF-β1-mediated growth inhibitory effects without altering TGF-β1-mediated induction of PAI-1 (14). Cell lines derived from pulmonary metastases lose TGF-β1-mediated growth inhibitory responses but still exhibit TGF-β1-mediated induction of matrix metalloproteinase-9 (29). In this context, the overexpression of TGF-βs in pancreatic cancer cells that frequently harbor Smad4 mutations and overexpress inhibitory Smad molecules may provide a mechanism for the activation of autocrine and paracrine pathways that lead to the expression of genes that promote cancer spread and angiogenesis. Indeed, it has been shown recently (39) that the angiogenic potential of TGF-βs may be enhanced by the presence of Smad4 mutations. Together with the current findings, these observations suggest that soluble TβRII may act to block both paracrine and autocrine pathways that promote the growth of COLO-357 cells in vivo.

Several approaches have been used to suppress the biological actions of TGF-βs in vivo. These include the use of neutralizing anti-TGF-β antibodies (34, 40, 41) or antisense stra-
egies to inhibit TGF-β synthesis (33, 42, 43), expression of a mutated TGF-β1 precursor to inhibit the processing of all of the three TGF-βs (44), expression of TβRII, soluble TβRII, or soluble TβRII to neutralize TGF-β activity (25, 45–47), or expression of a dominant-negative version of TβRII to interfere with TGF-β signaling (32, 48, 49). In the present study, we have used a soluble TβRII strategy to attenuate the invasive and tumorigenic capacity of COLO-357 pancreatic cancer cells, suppress their angiogenic potential, and down-regulate their ability to overexpress PAI-1. Therefore, our findings indicate that this approach can efficiently interfere with multiple TGF-β-dependent biological activities and paracrine actions in vivo. Inasmuch as PAI-1 is overexpressed in human pancreatic cancer and its overexpression may contribute to tumor invasion and tumor spread (22), our findings also raise the possibility that soluble TβRII may ultimately have a multifaceted therapeutic role in pancreatic cancer.

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