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Thrombospondin-1 Is a Major Activator of TGF-β1 In Vivo

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Summary

The activity of TGF-β1 is regulated primarily extracellularly where the secreted latent form must be modified to expose the active molecule. Here we show that thrombospondin-1 is responsible for a significant proportion of the activation of TGF-β1 in vivo. Histological abnormalities in young TGF-β1 null and thrombospondin-1 null mice were strikingly similar in nine organ systems. Lung and pancreas pathologies similar to those observed in TGF-β1 null animals could be induced in wild-type pups by systemic treatment with a peptide that blocked the activation of TGF-β1 by thrombospondin-1. Although these organs produced little active TGF-β1 in thrombospondin null mice, when pups were treated with a peptide derived from thrombospondin-1 that could activate TGF-β1, active cytokine was detected in situ, and the lung and pancreatic abnormalities reverted toward wild type.

Introduction

Transforming growth factor β (TGF-β) is a versatile cytokine intimately involved in cell growth, differentiation, and immune modulation (reviewed in Roberts and Sporn, 1988; Barnard et al., 1990; Massague, 1990; Lawrence, 1996). Although named originally for its ability to induce a transformed phenotype in cultured cells (Moses et al., 1981; Roberts et al., 1981), TGF-β and its signaling pathway are frequently involved in suppressing the growth of human tumors (Markowitz and Roberts, 1996; Moskaluk and Kern, 1996; Serra and Moses, 1996). TGF-β also has contradictory effects on normal cells. Depending on the cell type and the environment, it may stimulate or inhibit growth, regulate developmental fate in an instructive or a selective manner (Moses and Serra, 1996), and contribute to both the initiation and the resolution of processes involved in inflammation and tissue repair (McCartney-Francis and Wahl, 1994). In mammals, distinct genes encode three closely related isoforms, TGF-β1, -2, and -3, that are expressed in unique, occasionally overlapping patterns and can perform a variety of distinct functions in vivo (Proetzel et al., 1995; Letterio and Roberts, 1996; Sanford et al., 1997).

Accurate regulation of the amount of active TGF-β protein is essential to the health of all mammals. Too little TGF-β is incompatible with life. Mice null for either TGF-β1, or -2 or -3 do not survive beyond a few days or weeks (Shull et al., 1992; Kulkarni et al., 1993; Kaartinen et al., 1995; Proetzel et al., 1995; Sanford et al., 1997). In the case of animals null for TGF-β1, when on a mixed genetic background half of them succumb in midgestation, and half develop normally until 3–4 weeks of age, due in part to a supply of growth factor from heterozygous mothers (Letterio et al., 1994). Surviving pups exhibit dysregulated myelopoeisis (Yaswen et al., 1996) and a wasting syndrome characterized by an inflammatory response targeting the heart, lung, pancreas, stomach, liver, and striated muscle that has been attributed to an autoimmune process (Boivin et al., 1995; Diebold et al., 1995). Too much TGF-β1 can also be catastrophic. Generalized overexpression causes lethality in utero or just after birth, and targeted overexpression results in organ-specific and occasionally generalized pathology (see Border and Ruoslahti, 1992; McCartney-Francis and Wahl, 1994; Wahl, 1994).

The major regulatory step controlling TGF-β1 activity takes place extracellularly (Munger et al., 1997). TGF-β1 is secreted by most cells in an inactive form that is unable to interact with cellular receptors. Both in vitro and in vivo, the activity of the mature TGF-β1 is initially masked by its noncovalent association with a dimer of its N-terminal propeptide, called latency-associated protein, LAP (Gentry et al., 1987; Bottinger et al., 1996; McMahon et al., 1996). The LAP component of this small latent complex is often disulfide-linked to separately encoded latent TGF-β1-binding proteins, LTBPs, resulting in the large latent form of TGF-β1. These latent forms enhance the stability of the cytokine and provide a means of targeting it to matrix and to cell surfaces, insuring a ready source for local activation (Lawrence, 1996).

Active growth factor can be released from either of its latent complexes in vitro by extremes of pH or heat or by chaotropic agents (Brown et al., 1990) and can be

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activated in vivo by low dose gamma irradiation (Ehrhart et al., 1997), but none of these mechanisms seem likely to play a major role in its activation in vivo during normal growth and development. A variety of more physiological treatments can also activate TGF-β1, including deglycosylation, calpain, cathepsin, or plasmin proteolysis (see Miyazono and Heldin, 1991; Abe et al., 1998), and exposure to reactive oxygen species (Barcellos-Hoff and Dix, 1996) or to thrombospondin-1 (Schultz-Cherry and Murphy-Ullrich, 1993). It is not yet clear which, if any, of these mechanisms of activation are utilized in vivo under normal physiological conditions, although plasmin has seemed the most likely (Munger et al., 1997).

Plasmin activates latent TGF-β1 by cleaving LAP (Lyons et al., 1990). A detailed model of plasmin-dependent activation of the large latent complex has been delineated in vitro (Nunes et al., 1997) to explain TGF-β1 activation seen in some cultured cells that involves urokinase plasminogen activator and the mannose-6-phosphate/insulin-like growth factor II receptor. There is correlative in vivo evidence for a plasmin-dependent activation of TGF-β1 in the aortic walls of mice (Grainger et al., 1994) and for mannose-6-phosphate receptor involvement in TGF-β1 activation following liver injury (Jirtle et al., 1991; Saperstein et al., 1994; De Bleser et al., 1996). However, the apparent failure of the plasminogen-null animals to replicate the pathology of the TGF-β1-null animals and the recent observation that the multisystem pathology that these mice do exhibit can be alleviated by removal of fibrinogen (Bugge et al., 1996) suggest that plasmin can not be responsible for the majority of the activation of TGF-β1 in vivo under normal physiological conditions.

Here we consider the possibility that thrombospondin-1 (TSP-1) is an important activator of TGF-β1 in vivo. TSP-1 is a large homotrimeric protein that is secreted by many cell types (see Lahav, 1993; Bornstein, 1995). In cell-free systems, it binds to and activates both small and large latent forms of TGF-β1 (Murphy-Ullrich et al., 1992; Schultz-Cherry et al., 1994a; Souchelnitskiy et al., 1995). This plasmin-independent activation is thought to be the result of a conformational change, possibly in the LAP (Schultz-Cherry and Murphy-Ullrich, 1993; Schultz-Cherry et al., 1994a; S. M. F. R. et al., submitted). It requires the presence of the central type 1 repeats of the TSP-1 protein (Schultz-Cherry et al., 1994b) and can be reproduced by small peptides derived from one of these repeats that contain amino acids KRFK (Schultz-Cherry et al., 1995). In activating TGF-β1, TSP-1 interacts with the N-terminal region of LAP, forming a trimolecular complex. Within the complex, a conformational change takes place that alters the immunoreactivity of TGF-β1 (Schultz-Cherry et al., 1995) and makes it accessible to its receptor. Antibodies raised against LAP can block TSP-1 binding (Yang et al., 1997; S. M. F. R. et al., submitted), and in experiments containing only purified components, peptides derived from LAP containing the amino acids LSKL can prevent both TSP-1 binding and TGF-β1 activation (S. M. F. R. et al., submitted).

Data presented below show that TSP-1 contributes significantly to the activation of TGF-β1 in vivo. A systematic comparison of the histology of young mouse pups that were null for TSP-1 with pups null for TGF-β1 disclosed a strikingly similar pathology in multiple organ systems, particularly the lung and pancreas. Several of these similarities could be attributed directly to TSP-1 activation of TGF-β1 because the phenotype of the TGF-β1 null mice could be pharmacologically reproduced by treating wild-type animals with a peptide that blocked the activation of TGF-β1 by TSP-1. Moreover, a shift toward the normal phenotype occurred in the TSP-1 null animals in response to treatment with a peptide derived from TSP-1 that activated TGF-β1.

Results

Histological Similarities between TSP-1 Null and TGF-β1 Null Animals

Seeking parallels that would suggest a role for TSP-1 in TGF-β1 activation in vivo, we evaluated the histopathology of a variety of organ systems in young mouse pups that were null for TGF-β1 or for TSP-1 and compared them to one another and to wild-type animals (summarized in Table 1). Mouse pups under 2 1/2 weeks of age were chosen for these experiments in order to make direct comparisons of age-matched animals as TGF-β1 null pups do not survive beyond about 4 weeks. As expected, there were significant differences among these animals, the most dramatic being the subsequent early death of the TGF-β1 null pups. In contrast, the TSP-1 null animals, although they also suffer some fetal wastage, live on to reproduce (Lawler et al., 1998). Not surprisingly, the autoimmune-based diffuse inflammation and wasting syndrome that accompanies the early demise of the TGF-β1 null animals was not seen in TSP-1 nulls where inflammation was maintained at a low level in most organs (lung, liver, and pancreas) and was completely absent from others, such as the heart. Some histological abnormalities such as the inflammation of the heart were confined to the TGF-β1 null animals. Other defects were specific to the TSP-1 nulls, such as the skeletal kyphosis and the excessive vascularity of the pancreatic islets and the dermis.

Despite these important differences, a careful histological analysis revealed that the pups of the two null genotypes had markedly similar abnormalities in many other organs (Table 1). Some of these, such as the 3-fold increase in mitoses seen in the testes and gastric epithelium of both TGF-β1 and TSP-1 null pups, suggested a parallel loss of TGF-β1 inhibitory effects in mice of both genotypes. For practical reasons, we have concentrated our more detailed subsequent analyses on two organ systems, the lung and the pancreas.

In the lung, there were a variety of histopathologic similarities between the TSP-1 null and the TGF-β1 null pups. There were vessel abnormalities, as might be expected from the ability of both TGF-β1 (Majack, 1987; Merwin et al., 1991) and TSP-1 (Majack et al., 1986) to influence vascular smooth muscle growth. Whereas age-matched wild-type mice had thin-walled vessels (Figure 1D) with no evidence of alveolar hemorrhage (Figure 1A), null animals of both genotypes had bronchiolar arteries that were thickened and tortuous with an increased number of vascular smooth muscle cell nuclei (Figures 1E and 1F). Active TGF-βs (antibodies recognize the active form of TGF-βs 1, 2, and 3) could be

Table 1. Comparison of Morphological Features of TSP-1 Null and TGF-β1 Null Animals

Morphological Feature	Wild-Type Animals	TSP-1 Null Animals	TGF-β1 Null Animals
Lung			
Bronchial epithelial hyperplasia	0	++	++/+++
Proximal mucinous metaplasia	0	+/++	+/++
Vascular SMC hyperplasia	0	++	++/+++
Clara cell hyperplasia	0/+	++	++
Inflammation	0	+/++	++/+++
Pancreas			
Islet cell hyperplasia	0	++	++/+++
Acinar hypoplasia	0	+/++	+/++
Inflammation	0	+	++
Islet vessels/10 high-power fields	2.0 ± 1.0	7.7[a] ± 1.5	4.3 ± 1.2
Liver			
Dilatation of bile ducts adjacent to distended gallbladder	0	++	+/++
Periportal acute inflammation	0	+	+/++
Kidney			
Indistinct cortico-medullary junction	N	++	++
Stomach			
Gastric epithelial hyperplasia	N	++	++
Intestinal metaplasia at G–E junction	N	++	+/++
Mitoses/10 high-power fields	3.3 ± 1.5	11[a] ± 1.5	12[a] ± 2.1
Testes			
Nuclear atypia	0	+/++	++
Mitoses/10 high-power fields	7.3 ± 1.5	21[a] ± 2.1	20[a] ± 1.2
Skin			
Paucity of dermal matrix	N	+/++	+/++
Excessive dermal vascular density	N	+/++	N/+
Bone			
Irregular bony spicules in endochondral ossification centers	0	+/++	++
Thoracic kyphosis	0	++	0
Heart			
Enlarged myocytes within the ventricular septum	0	+/++	++
Inflammatory infiltrates	0	0	+++

0/N, normal; +, mild; ++, moderate; +++, severe.
[a]Different from wild type, $p < 0.05$.

detected in the vascular smooth muscle cell layer of wild-type animals (Figure 1G) but were significantly reduced in TSP-1 null mice (Figure 1H). Multifocal capillary leakage and alveolar hemorrhage were also common in TSP-1 null (Figure 1B) and TGF-β1 null (Figure 1C) animals, although they were not seen in wild-type mice (Figure 1A). In the TSP-1 null animals, the lung hemorrhage was seen in areas lacking inflammatory infiltrates and did not directly correlate with the degree of inflammation. Hemorrhage did not resolve with age since hemosiderin-laden macrophages were evident within alveolar spaces in adult mice (Lawler et al., 1998).

Animals null for either TSP-1 or TGF-β1 also had similar morphological disturbances in the proximal airway where TGF-β1 is particularly highly expressed (Jetten et al., 1986; Zhou et al., 1996) and can induce squamous differentiation and inhibit epithelial cell proliferation (Masui et al., 1986). The proximal segments of the lung of both the TGF-β1 (Figures 2C and 2D) and TSP-1 (Figures 2B, 2F, and 2I) null animals showed hyperplasia, pleomorphism, and increased mitoses, which suggested the presence of altered proliferation as well as a possible arrest or disturbance in differentiation. These changes were associated with a shift in the composition of proximal airway cell types to a high number of Clara cells and goblet cells, supporting a metaplastic change. In one third of the TSP-1 null animals, mucinous secretions were so excessive that the larger bronchi were partially occluded (see mucicarmine stain, Figure 2B).

These pulmonary changes correlated with the loss of TSP-1 and the lack of TGF-β activation. TSP-1 (Figure 2E) and active TGF-βs (Figure 2H) were both highly expressed in the wild-type bronchiolar epithelium, and active TGF-βs were also seen in underlying tissue. Although the localization of active TGF-β may appear diffuse in these and other photos, evaluation under high power confirmed its extracellular location. TSP-1 was expressed well in the TGF-β1 null pups (Figure 2G). In the TSP-1 null animals, although antibodies recognizing all forms of TGF-β showed that latent TGF-βs were clearly present in epithelial cells undergoing proliferative changes (Figure 2F), the active cytokine was no longer detected in this cell layer (Figure 2I). The stain was clearly working properly as underlying tissue stained well for active TGF-βs in TSP-1 null animals (Figure 2I). Despite the ability of antibodies specific for active TGF-βs to recognize all three isoforms, tissue from TGF-β1 null animals was negative (Figure 1J), suggesting that in this tissue, β1 is the dominant active species.

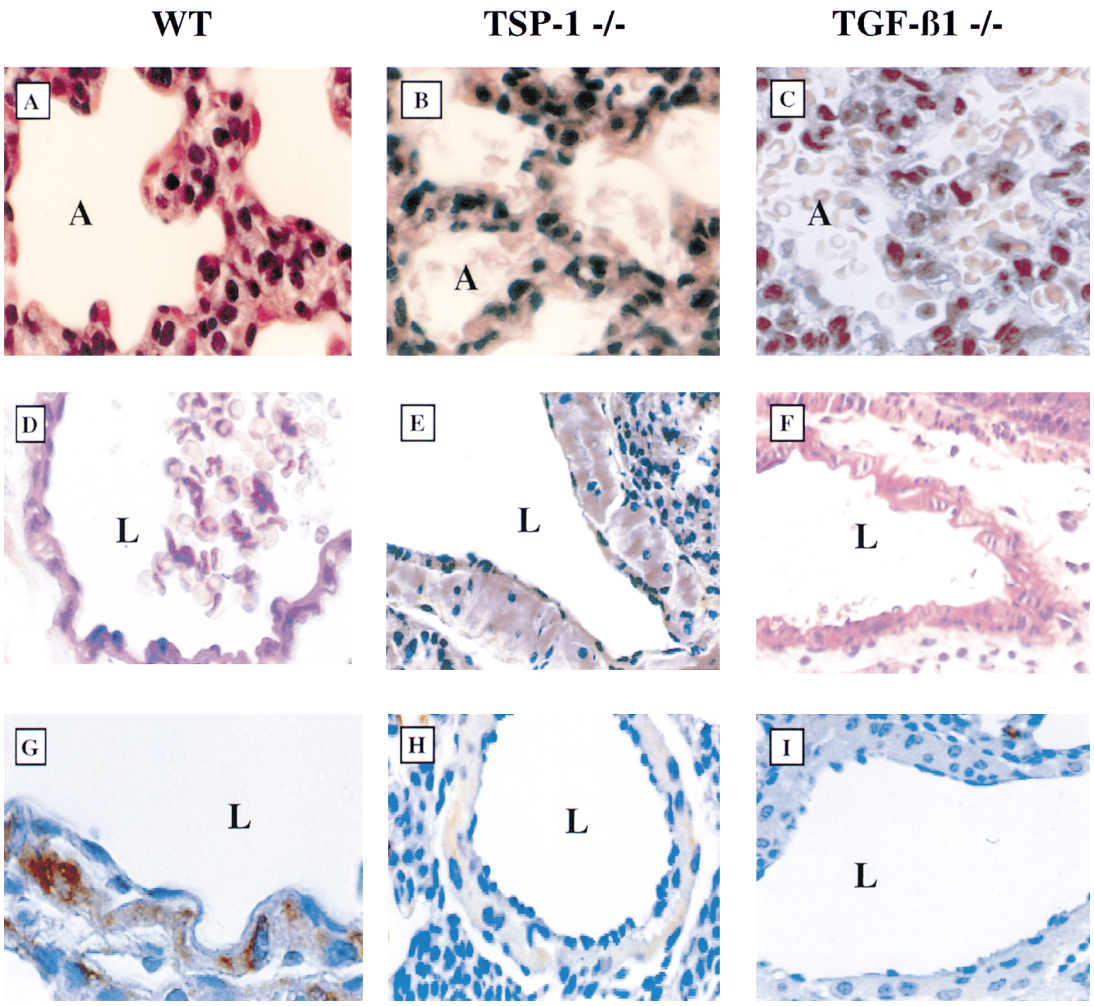


Figure 1. Vascular Smooth Muscle Hyperplasia and Alveolar Hemorrhage in Lungs of TSP-1 Null and TGF-β1 Null Animals

Lung tissue of wild-type mice (A, D, and G), TSP-1 null animals (B, E, and H), and TGF-β1 null animals (C, F, and I) are shown. (A)–(F) stained with HPS and (G)–(I) for active TGF-βs (brown stain). Note the thin-walled vessels (D) with no evidence of alveolar hemorrhage in wild-type mice (A) in contrast to the multifocal alveolar hemorrhage (B and C) and marked vascular smooth muscle hyperplasia (E and F) in both null animals. Unboxed A indicates alveolar space; L, the lumen of blood vessel. (B) and (C), 20×; (G), 100×; others, 40×.

The TSP-1 and TGF-β1 null animals also showed parallel morphological changes in the pancreas, where TGF-β1 is known to be critical in regulating the balance between the acinar and endocrine development (Sanvito et al., 1994). TSP-1 expression has not previously been observed in the pancreas, but immunoreactivity of wild-type mice suggested that it is associated with nearly all islets and a few interstitial cells (Figure 3B) and can be seen in the matrix (evident here in Figure 3H). Comparable staining was evident in pediatric human pancreatic tissue (data not shown). All TSP-1 null animals examined had an increased number of islet cells throughout the organ and exhibited a relative hypoplasia of the exocrine pancreas and interstitial matrix (compare Figure 3A with 3D) that was confirmed by quantitative morphometric analyses (Table 2). The pancreas of TGF-β1 null animals showed similar but even more striking changes in the endocrine and exocrine pancreas with islet cell hyperplasia and moderate to severe inflammation (Figure 3G).

In wild-type animals, TGF-β1 localized to acini (Figure 3C) as has been reported for the human pancreas (Yamanaka et al., 1993). The strong staining for active TGF-βs present in wild-type acini was much reduced in acini of TSP-1 null pups (Figure 3F).

Peptides Could Restore the Production of Active TGF-β in Fibroblasts Cultured from TSP-1 Null Mice and Inhibit Its Production by Wild-Type Fibroblasts

If deficiencies in TGF-β1 activation contribute to the morphological changes observed in the TSP-1 null animals, then cells derived from the TSP-1 null animals should be less efficient than those from wild-type animals in activating latent TGF-β1. As it can be quite difficult to measure levels of active TGF-β1 in cell secretions (Munger et al., 1997), to test this prediction we turned to coculturing test cells directly with indicator cells. Strains of fibroblasts derived from the dermis, where

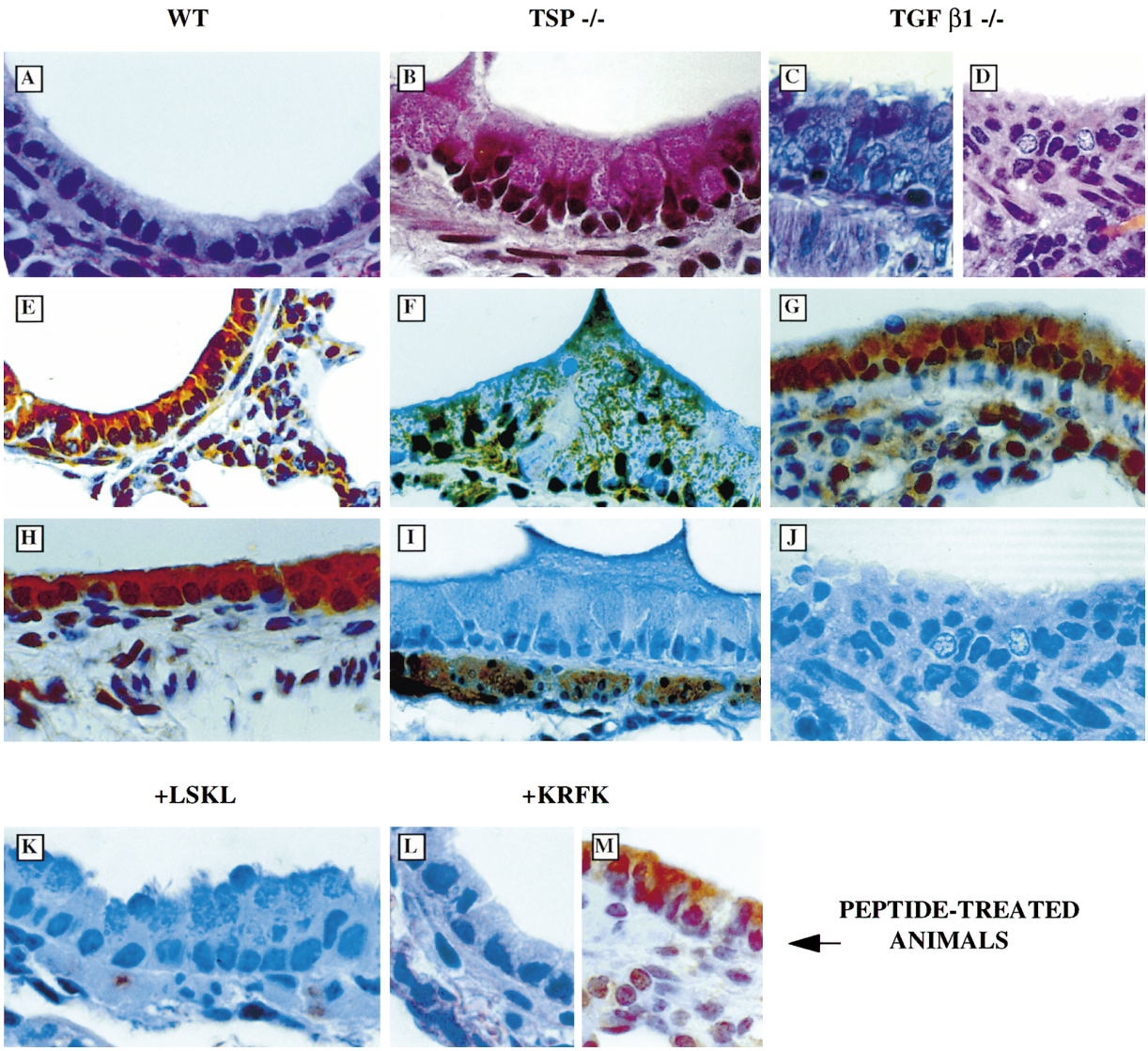


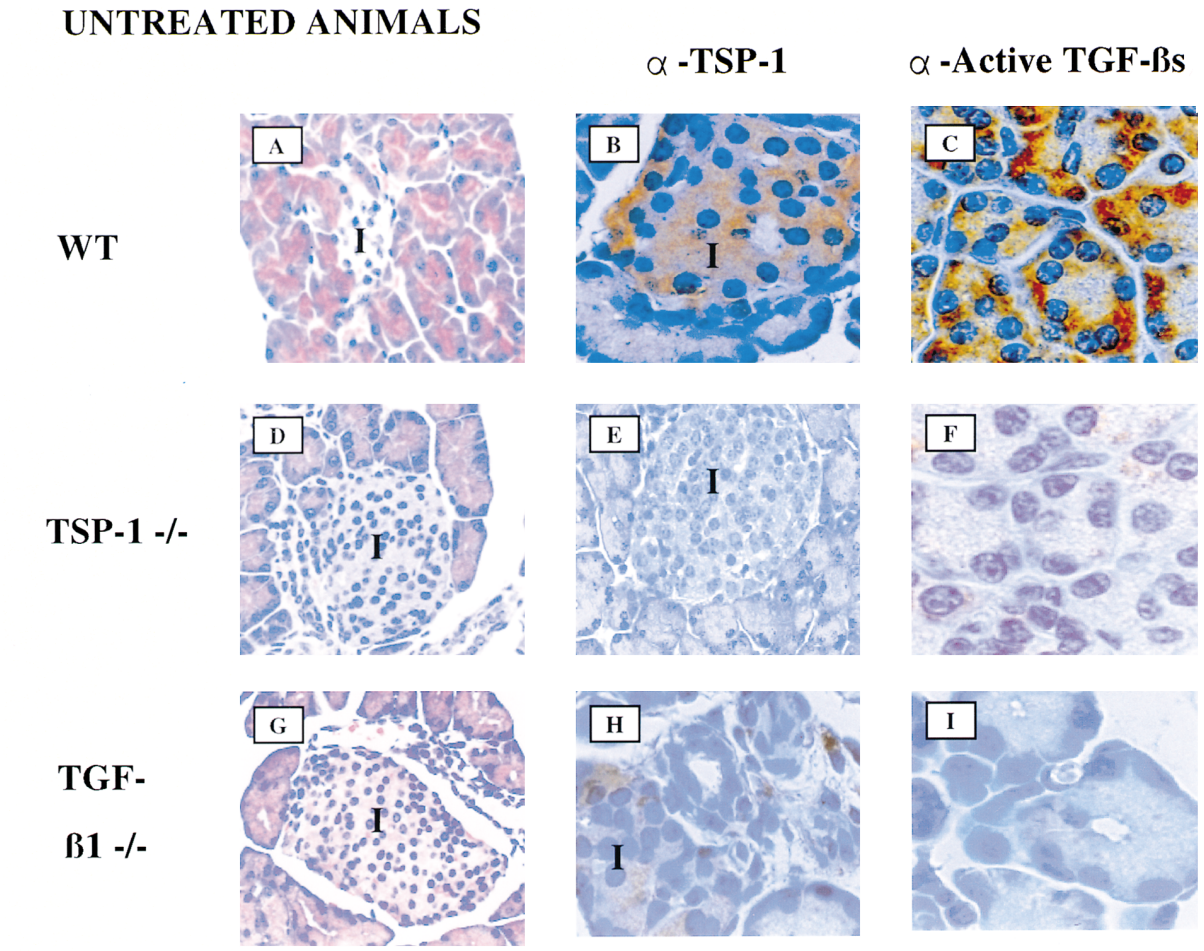
Figure 2. Bronchial Epithelial Hyperplasia and Metaplasia in TSP-1 Null and TGF-β1 Null Animals and in Peptide-Treated Pups

(A–J) Representative sections of bronchial tissue from wild-type animals (A, E, and H), TSP-1 null animals (B, F, and I), and TGF-β1 null animals (C, D, G, and J) are shown with (B) stained for mucicarmine, (E) and (G) stained for TSP-1, (F) stained for total TGF-β, and (H), (I), and (J) immunostained specifically for active TGF-βs. In wild-type tissue, note the single layer of uniform bronchial epithelial cells, which stain positively for both TSP-1 (E) and active TGFβ (H). In TSP-1 null and TGF-β1 null animals, bronchial epithelial hyperplasia and focal mucinous metaplasia are evident. In TSP-1 null animals, the metaplastic epithelial cells failed to stain for active TGF-βs (I), whereas focal positivity is present in bronchiolar smooth muscle cells and infiltrating macrophages. In comparison, antibodies that also recognize latent TGF-β1 stain this tissue well (F). In TGF-β1 null animals, TSP-1 staining remained strong (G) and staining for active TGF-βs was absent (J).

(K–M) Proximal airway bronchial epithelium is shown from a wild-type mouse that was treated with the LSKL peptide that blocked TSP-1 activation of TGF-β1 (K) and from a TSP-1 null mouse treated with the TSP-1-derived peptide KRFK that activated TGF-β1 (L and M). (K) and (M) were stained for active TGF-βs. Similarly stained wild-type tissue is shown in (H). Note changes in the epithelium from a single layer in wild-type animals (A, E, and F) to a metaplastic epithelial lining with enlarged nuclei displaced by goblet-like cells in wild-type animals treated with the blocking peptide (K). Animals lacking TSP-1 that were treated with the activating peptide reverted toward a wild-type bronchial epithelial morphology (L) and showed strong staining for active TGF-βs (M).

All panels, 40×, except (E), 20×.

similar tissue changes were seen in both null animals, were isolated from three wild-type pups and three TSP-1 nulls and cocultured for 16 hr with a reporter cell line harboring a TGF-β1-responsive promoter driving the expression of a luciferase reporter gene (Abe et al., 1994). Active TGF-β was produced by the wild-type fibroblasts in low amounts that were similar to those produced by other cultured cells (Table 3; Sato et al., 1990; Kojima and Rifkin, 1993; Souchelnitskiy et al., 1995). The TSP-1 null cells activated only one eighth the amount of TGF-β that was activated by cells derived from wild-type animals. The deficit in TGF-β activation was apparently due


UNTREATED ANIMALS
α -TSP-1
α -Active TGF-ßs
WT
TSP-1 -/-
TGF-
ß1 -/-
A
B
C
D
E
F
G
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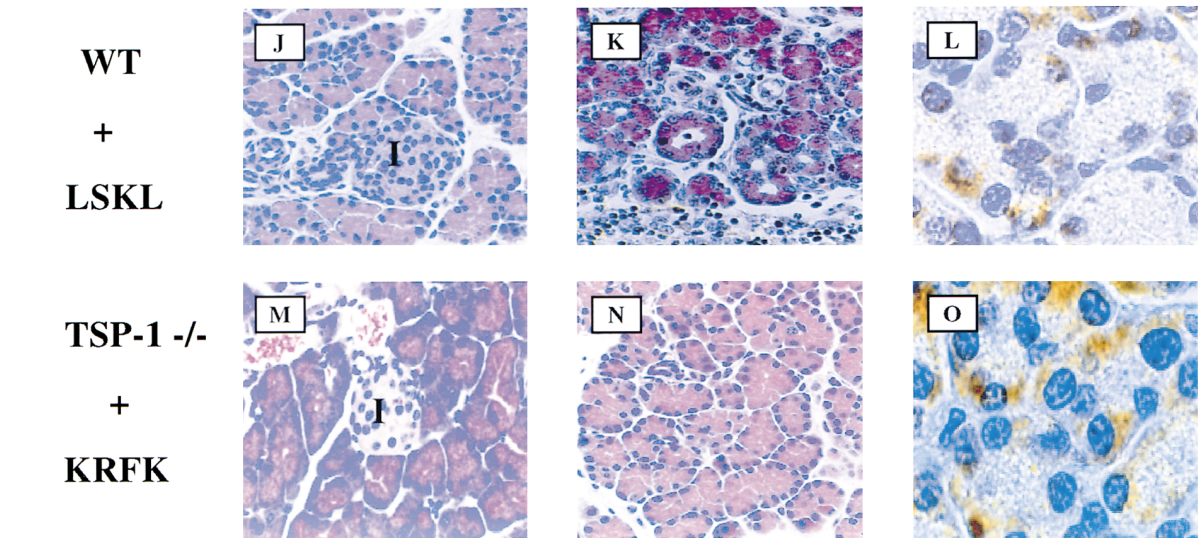
PEPTIDE-TREATED ANIMALS
α -Active TGF-ßs
WT
+
LSKL
TSP-1 -/-
+
KRFK
J
K
L
M
N
O
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Table 2. Effect of Blocking and Activating Peptides on Pancreatic Morphometry

Mouse	Islet Area (μ^2)	Acinar Area (μ^2)	Ratio (Islet/Acinar ×100)
Wild type			
No treatment	1684	64,380	2.6[a]
+ control peptide SLLK	1398	59,110	2.4[a]
+ blocking peptide LSKL	2890	15,322	19
TSP-1 null			
No treatment	6689	30,855	22[a]
+ control peptide KQFK	5688	27,892	20[a]
+ activating peptide KRFK	1125	17,491	6.4

Mice were treated with indicated peptide from day 3 to day 16 and areas measured from histological slides.
[a]These values differ significantly from the corresponding peptide treatment, $p < 0.017$.

to a deficit in TSP-1 because production of active TGF-β could be restored to these cultures by a short peptide derived from TSP-1 (Table 3) that has been shown to activate latent TGF-β1 in biochemically pure systems (Schultz-Cherry et al., 1995). To verify that the TSP-1 molecule could activate the latent TGF-β produced by TSP-1 null cells, human thrombospondin purified from platelets and stripped of bound TGF-β1 activity was tested. At concentrations from 20 to 80 nM, TSP-1 was able to activate up to 15% of the total latent TGF-β present in this media conditioned by TSP-1 null fibroblasts in a dose-dependent manner (data not shown).

Fibroblasts cultured from wild-type animals were also examined to determine if blocking TSP-1 activation of TGF-β would significantly decrease active TGF-β present in their conditioned media. TSP-1 activates TGF-β1 by interacting with the latency-associated protein, and in purified systems this interaction is prevented by a peptide derived from the latency-associated protein, LSKL (S. M. F. R. et al., submitted). When this peptide was added to cultured wild-type mouse fibroblasts that were secreting TSP-1, the active TGF-β that accumulated in their media over a 24 hr period was significantly reduced (Table 4).

Treatment of TSP-1 Null Animals with the TSP-1-Derived Peptide that Activated TGF-β1 Normalized the Pathology of the Pancreas and the Proximal Airway

To link more closely the pathology of the TSP-1 null animals to the ability of TSP-1 to activate TGF-β1, three TSP-1 null mice were treated daily from 3 to 16 days of age with the TSP-1-derived peptide KRFK, which activates TGF-β1 in biochemically pure systems (Schultz-Cherry et al., 1995) and in secretions of TSP-1 null cells (Table 3). The treatment caused no apparent toxicity in that pups all gained weight at a similar rate whether treated with KRFK, with a cognate control peptide, KQFK, or with PBS. Histological examination revealed that the lungs and the pancreas of all of the KRFK-treated pups, but none of the control animals, now had a morphology similar to that of wild-type pups.

Each pancreas of animals treated with the activating peptide acquired a more normal phenotype in that their islet area decreased relative to acinar area (Table 2; compare Figures 3M and 3N with 3A and 3B). The acini of the treated pups showed stronger immunoreactivity for active TGF-β1 (Figure 3O) than those from an untreated TSP-1 null pup (Figure 3F). The lung and pancreatic tissues of the three pups with control peptide, KQFK, and of another three pups treated with vehicle PBS were indistinguishable from those of untreated animals.

The lungs of the TSP-1 null pups treated with KRFK (Figures 2L and 2M) were morphologically more similar to those of wild-type animals (Figures 2A, 2E, and 2H) than to untreated TSP-1 null animals (Figures 2B, 2F, and 2I). The epithelial cells of all KRFK-treated pups were less pleomorphic than those of the control peptide–treated animals, and their bronchial epithelium had fewer stratified regions and in many areas was a single cell layer thick as it is in wild-type pups. In contrast to the bronchial epithelial cells of TSP-1 null animals, which were negative for active TGF-β staining (Figure 2I), epithelial cells of the KRFK-treated TSP-1 null animals stained positively for active TGF-βs (Figure 2M). Overall the inflammation in the lungs in KRFK-treated animals improved, and aggregates of acute inflammatory cells were no longer evident. A mild chronic inflammatory infiltrate did persist in the peribronchial regions of the lungs, but it was not consistently found in regions of altered epithelial phenotype.

Figure 3. Comparison of Pancreatic Morphology of Wild-Type, TSP-1 Null, and TGF-β1 Null Mice and the Effects of Specific Peptide Treatments

(Top) Pancreatic tissue from animals that were wild type (A, B, and C), TSP-1 null (D, E, and F), or TGF-β1 null (G, H, and I) stained with HPS(A, D, and G), anti-TSP-1 (B, E, and H), or anti-active TGF-βs (C, F, and I). Islet cell hyperplasia, paucity of acinar structures, and mild chronic inflammation are present in tissue from both null genotypes (D and G). Note expression of TSP-1 in islets of wild-type (B) and TGF-β1 null (H) tissue and the presence of active TGF-β1 in the acini of wild-type tissue (C) that is reduced in tissue from TSP-1 nulls (F) and absent in that from TGF-β1 nulls (I).

(Bottom) Pancreatic tissue from wild-type pups treated with the LSKL peptide that blocked TSP-1 activation of TGF-β1 (J, K, and L) and from TSP-1 null pups treated with the KRFK peptide that activated TGF-β1 (M, N, and O), stained with HPS (J, K, M, and N) and for active TGF-βs (L and O). In LSKL peptide-treated wild-type pups, note reduced amounts of active cytokine and abnormalities characteristic of the untreated null animals. In tissue from KRFK peptide–treated TSP-1 null pups, note positive staining for active TGF-βs and lessening of abnormalities. Unboxed letter I marks islets of Langerhans. (B), (C), and (H), 40×; (F), (I), (L), and (O), 100×; others, 20×.

Table 3. Restoration of Deficits in Production of Active TGF-β Using TSP-1-Derived Peptides on Cells Cultured from TSP-1 Null Animals

Test cells	Active TGF-β (pg/ml)
1. Wild-type fibroblasts	8.8 ± 2.0
2. TSP-1 null fibroblasts	1.0 ± 0.7
3. TSP-1 null fibroblasts + activating peptide KRFK	8.3 ± 1.5
4. TSP-1 null fibroblasts + inactive peptide KQFK	1.5 ± 0
5. No fibroblasts + activating peptide KRFK	1.3 ± 1.3
6. No fibroblasts + inactive peptide KQFK	0.8 ± 0.8

Dermal fibroblasts were cocultured in the presence or absence of peptides at 50 μM with indicator cells containing luciferase under a TGF-β-sensitive promoter, and luciferase production that was sensitive to TGF-β-neutralizing antibodies was measured after 16 hr. Standard errors are indicated. 1 different from 2, $p < 0.008$; 3 different from 4, $p < 0.03$.

Treatment of Wild-Type Animals with a LAP Peptide that Blocks TSP-1 Activation of TGF-β1 Produced Lung and Pancreatic Phenotypes Similar to Those Seen in Null Animals

In an experiment parallel to that described above, three wild-type animals were treated with a peptide, LSKL, derived from that portion of the of TGF-β1 latency-associated protein (LAP) that interacts with TSP-1 and curtails TSP-1 activation of TGF-β1 in pure systems (S. M. F. R. et al., submitted) as well as in complex-conditioned media (Table 4). When examined histologically, the proximal airway epithelium of the LSKL-treated wild-type pups resembled that of the TSP-1 null and TGF-β1 null pups (Figure 2K). It showed focal metaplasia with enlarged nuclei displaced by goblet-like cells, an increased number of Clara cells, and multifocal acute and chronic inflammatory infiltrates. Staining for active TGF-βs that was seen clearly in untreated wild-type animals (Figure 2H) was lost in the bronchial epithelial cells of treated pups (Figure 2K), although areas around underlying smooth muscle and macrophages remained positive.

In the pancreas of LSKL-treated animals, qualitative and quantitative increases in the number of islet cells were seen (Figure 3J; Table 2). In all three animals, there was focal dropout of acinar structures (Figure 3K), although it did not approach the degree of hypoplasia or inflammatory-related destruction noted in the pancreatic tissue of the TGF-β1 null animals. In 2 of the 3 peptide-treated animals, a mild chronic inflammatory infiltrate was found in the pancreas that was composed of predominantly lymphocytes (Figure 3K) and was similar to that found in both null animals. Reactivity with active TGF-βs, prominent in wild-type acinar tissue (Figure 3C), was reduced in the treated pups (Figure 3L). The pancreas and the bronchial epithelium of the three pups treated with a control peptide, SLLK, and an additional three treated with vehicle PBS did not differ morphologically or histologically from the untreated animals.

Discussion

Data presented above demonstrate that TSP-1 is an important natural activator of TGF-β1 in the lung and in the pancreas where both molecules were expressed and where parallel abnormal pathologies were seen in animals null for either one of the two proteins. The histological changes seen in these null mice can be attributed to lack of activation of TGF-β1 by TSP-1, for they could be reproduced in wild-type pups with a peptide that blocked TSP-1 activation of TGF-β1 in vitro and reduced the presence of active cytokine in vivo, and they were ameliorated in TSP-1 null pups by a TSP-1-derived peptide that activated TGF-β1 in vitro and produced active cytokine in vivo. As the peptides were injected IP, it is possible that TGF-β1 was activated IP and circulated from there to the lung and pancreas, although its short half-life and our ability to stain the active cytokine in situ in peptide-treated animals are more compatible with its being activated locally in individual organs. The similarities between the two null pups extended over multiple organs, suggesting that TGF-β1 activated by TSP-1 also contributes to the normal development of the testes, bone, kidney, liver, heart, skin, and stomach. TSP-1 also may influence the hematopoeitic systems as both null animals also have increased white blood cell counts

Table 4. Effect of Inhibitory Peptide on Media Conditioned by TSP-1-Producing Cells

Cell Strains	Agar Colonies/0.5 ml Conditioned Media		
	Untreated Control	+ Inhibitory Peptide LSKL	+ Control Peptide SLLK
A1	63	70	108
A2	102	78	117
A6	119	74	104
A18	104	69	84
Mean	97 ± 12	73 ± 2[a]	103 ± 7

Serum-free media conditioned for 24 hr in the presence or absence of 1 μM peptide by four independently derived wild-type fibroblast cultures were assayed in triplicate for active TGFβ using the NRK agar colony formation assay. Heat activation of untreated controls resulted in total TGF-β1 sufficient to induce 191 ± 13 agar colonies/0.5 ml conditioned media. Control of 35 for media plus BSA has been subtracted from all values. Standard error of the mean is indicated.

[a] Different from control peptide, $p < 0.02$.

skewed toward monocytes, a smaller thymus, an abnormal spleen, and hypercellular bone marrow (data not shown; Shull et al., 1992; Boivin et al., 1995; Lawler et al., 1998).

Although the antibodies used to stain for active TGF-β1 recognize all three TGF-βs, and TSP-1 is able to activate both TGF-β1 and TGF-β2 in a manner inhibitable by the inactivating peptide LSKL (S. M. F. R. et al., submitted), most of the abnormalities we observed in the TSP-1 null animals and in wild-type animals treated with the inactivating peptide appeared to be specific for TGF-β1, for they were present in the TGF-β1 null animals and have not been reported in the TGF-β2 or TGF-β3 null mice (Kaartinen et al., 1995; Proetzel et al., 1995; Sanford et al., 1997), and no active cytokine staining was seen in the lung or pancreas of TGF-β1 null pups (Figures 2J and 3I). It is likely that TSP-1, but not other TSP family members, is activating TGF-β1. TSP-2 is the family member most similar to TSP-1, but despite one report that a preparation enriched for TSP-2 can activate TGF-β1 (Souchelnitskiy et al., 1995), TSP-2 lacks the essential KRFK sequences, and when recombinantly produced, it has no such activity (Schultz-Cherry et al., 1995).

TSP-1 is the first activator of TGF-β1 shown to function in natural, untreated, nondiseased tissues in vivo, yet mice must have additional ways to produce the active cytokine, for TSP-1 null pups avoid the autoimmune destruction and the early death of the TGF-β1 nulls. Even in the lungs of TSP-1 null pups, light focal staining for active cytokine was seen, although it was much less intense than in comparable tissue from wild-type animals. Active TGF-β1 in TSP-1 null mice could be the result of an alternate activation pathway or of the direct release of active cytokine by some cells, as has been suggested for B cells and plasma cells (Caver et al., 1996). The pathology of the TGF-β1 null pups suggests that their early death is related to local TGF-β1 deficits in specific organs, most likely the heart. It was severely compromised by inflammation in the TGF-β1 nulls whereas the hearts of the TSP-1 nulls who live on to reproduce were free of inflammation.

Just as TGF-β1 has other activators, TSP-1 has a variety of other functions that do not seem to depend on its ability to activate TGF-β1 (Bornstein, 1995). It is one of the most potent natural inhibitors of angiogenesis identified to date (Dawson and Bouck, 1998). Its ability to block the migration of endothelial cells in vitro and to inhibit experimentally induced neovascularization in vivo have been shown to be independent of the activation of TGF-β1 (Tolsma et al., 1993; Dawson et al., 1997). This independence can now be extended to its negative influence on the natural development of small vessels, for the TSP-1 null animals had increased vessel densities in the dermis and in the pancreatic islets that were not seen in the TGF-β1 null pups.

TSP-1 activation of TGF-β1 seems well suited to supply the low but persistent levels of active TGF-β1 important in normal growth and development. TSP-1 activated only a portion of the latent TGF-β1 available either in cell secretions as shown here or in pure systems (Schultz-Cherry and Murphy-Ullrich, 1993), yet it has the opportunity to form an amplifying loop. TGF-β1 can induce TSP-1 in cultured cells derived from vascular smooth muscle and lung epithelium (Penttinen et al., 1988; Majack et al., 1990; Laiho et al., 1991; RayChaudhury et al., 1994), both tissues that showed in vivo abnormalities.

The animal studies presented above suggest several parallels to human disease. In the pancreas, TGF-β1 is known to stimulate insulin secretion and to block the mitogenic response of pancreatic β cells to glucose (Sjoholm and Hellerstrom, 1991), raising the possibility that the pancreatic changes seen in the null mice may represent precursor lesions to insulin-dependent diabetes mellitus or glucose instability. In the lung, the abnormalities seen in both null mice are very reminiscent of the persistent inflammation, mucinous metaplasia, and vascular smooth muscle cell hyperplasia that to varying degrees characterize a variety of pulmonary diseases including asthma and cystic fibrosis. The identification of TSP-1 as a particularly effective activator of TGF-β1 in the lung and the pancreas and the ability of small peptides to modify its effects in vivo suggest new routes that might be considered to interfere with the progression of such diseases.

Experimental Procedures

Animals

TSP-1 null animals (Lawler et al., 1998) and TGF-β1 null animals (Shull et al., 1992) were both generated by homologous recombination in 129/Sv-derived ES cells implanted into C57/BL6 blastocysts followed by backcrossing with C57/BL6 animals and inbreeding the F1. For morphological studies, we examined 9 TSP-1 null animals that were 17 days old and 4 TGF-β1 null mice aged 10–21 days, both on a mixed C57/Bl6/Sv129 background, 6 TSP-1 null animals on a Sv129 background, 4 wild-type Sv129 mice, 9 wild-type C57/BL6, and 4 C57/Bl6/Sv129 F1 hybrids within these age brackets. Five adult wild-type, TSP-1 null, and TGF-β1 heterozygous animals and six 1-month-old individuals were also evaluated. Histology was consistent among all wild-type animals, and abnormalities seen in null animals were observed in all individuals examined within a single genotype.

For peptide treatments, 3-day-old TSP-1 null animals were randomized into three groups of three animals each, to receive 14 consecutive days of treatment consisting of intraperitoneal injection with 100 μg peptide/day of either KRFK, a TSP-1-derived TGF-β1-activating peptide (Schultz-Cherry et al., 1995); KQFK, an inactive control peptide (Schultz-Cherry et al., 1995); or vehicle alone (PBS). Three-day-old wild-type mice were treated identically with either LSKL, a peptide that blocks TSP-1 activation of TGF-β (S. M. F. R. et al., submitted); SLLK, an inactive control peptide (S. M. F. R. et al., submitted); or vehicle alone (PBS).

Animals were monitored daily, and no differences were observed in general appearance, coat consistency, or body weight between peptide-treated and vehicle-treated animals. Animals sacrificed with an overdose of metafane underwent gross and microscopic examinations. Peptides were synthesized by the University of Alabama Comprehensive Cancer Center/Peptide Synthesis and Analysis facility and purified by HPLC as described (Lioubin et al., 1991).

Microscopic Examination

Organs were individually dissected, fixed in 10% buffered formalin overnight, processed, and embedded in paraffin. Livers were evaluated using a section that included the gall bladder, hearts with sections taken immediately below the atrioventricular valves, and kidneys with sections that included the renal pelvis and a small portion of the ureter, and ossification centers were observed at the sternocostal junction and in one limb. Slides were prepared and stained with either H and E (hematoxylin and eosin), HPS (hematoxylin-phloxine-saffarin for fibrous tissue), PAS (periodic acid–Schiff), or mucicarmine (for mucin-secreting cells). Although TGF-β1 null

pups begin to fall behind in weight at day 5, the total body weights of wild-type, TSP-1 null, and peptide-treated animals of the same ages did not differ significantly.

For immunohistochemical studies, 5 μm sections of formalin-fixed, paraffin-embedded tissue sections were placed on poly-L-lysine-coated slides, deparaffinized in xylene, rehydrated in descending concentrations of ethanol, washed, and incubated with specific primary antibodies recognizing TSP-1 (clone A4.1, GIBCO BRL, Gaithersburg, MD), total TGF-β1 (reactive with both latent and active TGF-β1 from Anogen, Mississauga, Ontario, Canada), or active TGF-β1,2,3 (clone 1D11.16, cat. #80-1835-03, Genzyme, Cambridge, MA). The D11.16 antibody neutralizes TGF-βs 1, 2, and 3 and recognizes active but not inactive TGF-β1 (and possibly other isoforms) in fixed mouse tissue (Barral-Netto et al., 1992; Caver et al., 1996). Sections were washed with PBS, incubated with a biotinylated anti-human IgG (TGF-β1), anti-mouse IgG (active TGF-β), or anti-mouse IgM (TSP-1), followed by avidin–biotin peroxidase complex (Vectastain kit; Vector Laboratories, Burlingame, CA), and counterstained with hematoxylin. Positive and negative control slides were run in parallel for TSP-1 and TGF-β1 staining procedures to verify the specificity of the immunoreactivity. Immunostaining specificity was also checked by omitting the primary antibody. Positive immunoreactivity was indicated by the intensity of staining graded as strong (uniform, intense staining) or weak (only focal staining). For determination of mitoses and vessels in individual organs, ten nonoverlapping high-power fields were evaluated and the total numbers recorded in a blinded fashion.

Morphometric Analysis of Tissue Sections

To compare the area of the islet cells to the area of acinar structures, glass slides containing tissue sections of the pancreas from wild-type, TSP-1 null, and peptide-treated animals were coded and, in a blinded fashion, scanned, and morphometric measurements (in microns) were analyzed using a Zeiss SEM-IPS Image Processing system. Ten random high-power fields of each cell type were traced on the computer screen from each slide and statistical analyses performed using a paired Student's t test.

Cell Cultures and Conditioned Media Collection

Murine dermal fibroblasts were isolated from 3- to 10-day-old pups as previously described (Yuspa and Harris, 1974) and used prior to passage 6. To collect conditioned media, wild-type fibroblasts were plated at 2.5×10^5 cells/10 cm plate, and 24 hr later growth media were aspirated, and cells were washed three times with Dulbecco's modified Eagle's medium (DMEM) and incubated 4 hr in DMEM. Fresh serum-free DMEM containing 0.1% BSA with or without 1 μM peptide was added, collected 24 hr later, and centrifuged to remove debris. Mink lung epithelial cells (MLEC) (a gift from D. Rifkin, New York University Medical Center) were maintained in DMEM supplemented with 10% FCS, 2 mM glutamine, 200 μg/ml G418.

TGF-β Assays

TGF-β activity was determined using either the NRK colony formation assay (as modified in Schultz-Cherry and Murphy-Ullrich, 1993) or a bioassay that utilized MLEC permanently transfected with a plasmid containing a TGF-β-responsive promoter driving the expression of a luciferase reporter gene (Abe et al., 1994) with the following modifications. Either conditioned media or 100 μl DMEM/0.1% BSA containing $0.75–1.0 \times 10^4$ dermal fibroblast explant cells was incubated overnight with 1.6×10^4 MLEC cells and then processed, and luciferase activity was measured using an automated EG and G Berthold Microlumat LP 96 P luminometer (Gaithersburg, MD). The amount of TGF-β activity was determined by extrapolation from a standard curve generated in parallel using recombinant TGF-β (R and D Systems, Minneapolis, MN). The signal attributable to TGF-β was determined by comparing luciferase activity for each sample in the presence and absence of 15 μg/ml of neutralizing TGF-β antibody (Genzyme, Cambridge, MA). Total TGF-β activity present in conditioned media was determined following heat activation at 80°C for 5 min. TSP-1 was purified from human platelets and stripped of associated TGF-β as previously described (Murphy-Ullrich et al., 1992).

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