Production of Gastrointestinal Tumors in Mice by Modulating Latent TGF-β1 Activation

Kotaro Shibahara1, Mitsuhiko Ota1, Masahito Horiguchi1, Keiji Yoshinaga1, Jonathan Melamed2, and Daniel B. Rifkin1,3

Abstract

TGF-β and its signaling pathways are important mediators in the suppression of cancers of the gastrointestinal tract. TGF-β is released from cells in a latent complex consisting of TGF-β, the TGF-β propeptide [latency associated protein (LAP)], and a latent TGF-β binding protein (LTBP). We previously generated mice in which the LTBP-binding cysteine residues in LAP TGF-β1 were mutated to serine precluding covalent interactions with LTBP. These Tgfb1C33S/C33S mice develop multiorgan inflammation and tumors consistent with reduced TGF-β1 activity. To test whether further reduction in active TGF-β levels would yield additional tumors and a phenotype more similar to Tgfb1−/− mice, we generated mice that express TGF-β1C33S and are deficient in either integrin β8 or TSP-1, known activators of latent TGF-β1. In addition, we generated mice that have one mutant allele and one null allele at the Tgfb1 locus, reasoning that these mice should synthesize half the total amount of TGF-β1 as Tgfb1−/− mice, and the amount of active TGF-β1 would be correspondingly decreased compared with Tgfb1C33S/C33S mice. These compound-mutant mice displayed more severe inflammation and higher tumor numbers than the parental Tgfb1C33S/C33S animals. The level of active TGF-β1 in compound mutant mice seemed to be decreased compared with Tgfb1−/− mice as determined from analyses of surrogate markers of active TGF-β1. In addition, we showed that the expression of TGF-β1 in tumors from these mice was significantly decreased compared with tumors from Tgfb1−/− mice. These results suggest that further reduction in active TGF-β levels yields additional tumors and a phenotype more similar to Tgfb1−/− mice.

Introduction

TGF-β has divergent effects on tumor generation and progression (1, 2). During the early stages of tumorigenesis, TGF-β acts as a tumor suppressor presumably because TGF-β is a powerful inhibitor of the growth of epithelial cells (3, 4). As tumors progress in their evolution, the growth-suppressive property of TGF-β can be lost allowing a growth enhancing effect of TGF-β to predominate (5–8). Thus, the participation of TGF-β in tumorigenesis is complicated and dependent upon multiple factors including cell type, location, and the phase of tumor development.

After synthesis and secretion, TGF-β undergoes a series of modifications to produce the active growth factor (9). Mature TGF-β is a 25 kDa homodimer derived from a larger precursor by intracellular prolyl-tide processing (9). The cleaved propeptide, the latency associated protein (LAP), remains noncovalently bound to the mature growth factor, rendering the TGF-β latent, even after secretion. Dissociation from LAP is required for TGF-β receptor binding. LAP is usually covalently bound to a second protein, a latent TGF-β-binding protein (LTBP), through cysteine residues at the amino terminal region of the LAP monomers and a pair of cysteines in the LTBP. There are 4 Ltbp genes (Ltbp1, 2, 3, and 4), but only Ltbp1, -3, and -4 bind to LAP (10, 11). The complex of TGF-β, LAP, and LTBP is called the large latent complex (LLC), whereas the complex of TGF-β and LAP is called the small latent complex (SLC). There are 3 TGF-β genes—Tgfb1, b2, and b3—and all 3 proteins are secreted as latent complexes (12). The conversion of LLC to active TGF-β is mediated by several mechanisms, including proteolytic processing of LTBP and/or LAP to release active cytokine, the interaction of competitive LAP-binding sequences of thrombospondin-1 (TSP-1) or F-spondin with the latent complex, and the binding of integrins to Arg-Gly-Asp sequences in TGF-β1 and TGF-β3 LAPs (9, 13). Several different β-integrins including β3, β5, β6, and β8 bind LAP and may activate the latent complex, but 2 different mechanisms of activation seem to be used. β5 and β6 integrin-mediated activation uses force supplied by the cell cytoskeleton acting through the integrin upon the LLC
tethered to the matrix via the LTBP (14, 15). The application of force distorts the LLC thereby exposing or liberating the TGF-β. SLC binding to LTBP is required for latent TGF-β activation by the integrins αvβ3 and αvβ6 (15, 16). The integrin β3 activates SLC, but activation requires a metalloprotease in addition to the integrin (17). In this activation process, the integrin serves to colocalize the latent complex and the protease, thereby enhancing the rate of protease-mediated activation. TSP-1 also activates both LLC and SLC (18).

Elucidating the role of LTBP in TGF-β biology has been the focus of a number of investigations (10, 19). Mice and/or people missing LTBP-1, -3, or -4 have phenotypes consistent with alterations in TGF-β levels indicating the importance of LTBP in TGF-β biology (20–24). However, because binding to LTBP facilitates SLC secretion, the cause of LTBP-related phenotypes is unclear; that is, are they related to decreased SLC secretion or impaired latent TGF-β activation. To address this question, we generated mice in which the cysteines at position 33 in the TGF-β1 LAP, which normally bind to LTBP, were changed to serines (25). Animals (Tgfb1C33S/C33S) with this mutation should produce all of their TGF-β1 as SLC. If LTBP is required for proper TGF-β1 sequestration and activation, these mice should display a TGF-β1-null-like phenotype. If LTBP is not required for TGF-β1 generation, the mice should display a normal phenotype. The mutation of the cysteines to serines also allows the SLC to be readily secreted; therefore, mutant phenotypes would not be caused by decreased extracellular latent TGF-β1 (14, 26).

Tgfb1C33S/C33S mice displayed phenotypes, such as shortened life span, lack of epidermal Langerhans cells, and multiorgan inflammation, consistent with decreased active TGF-β1 (25). The mutant mice also developed tumors of the stomach, colon, cecum, rectum, and anus. Measurement of active TGF-β1 in these animals was hypomorphs rather than nulls for TGF-β1 because the inflammation and shortened life span were not as severe as observed in TGF-β1 null mice. We hypothesized that the Tgfb1C33S/C33S animals activated some secreted TGF-β1 SLC, albeit inefficiently.

Here, we report a series of experiments designed to test this contention. We reasoned that if we further lowered active TGF-β1 levels in Tgfb1C33S/C33S mice by eliminating activators of the SLC or by deleting 1 Tgfb1 allele, the abnormal phenotype would be enhanced to resemble more closely the Tgfb1−− phenotype.

**Materials and Methods**

**Mouse lines and reagents**

Tgfb1C33S/C33S, Tgfb1−−, Tsp1−−, and Rgb8−− mice have been described previously (25, 27–30). All mice were housed in a specific pathogen-free facility and routinely checked for infections and parasites. All procedures were conducted according to the regulations of the New York University (NYU) Langone Medical Center Institutional Animal Care and Use Committee.

**TGF-β assays**

The preparation of sera and measurement of TGF-β1 with the Quantite (R&D Systems) Kit have been described previously (25). Tissue extracts were prepared from wet tissue samples. The samples were homogenized at 4°C in Tissue Protein Extraction Reagent and Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). The extracts were centrifuged at 8,000 × g for 10 minutes at 4°C. After the protein concentrations in the supernatants were determined, the levels of total and active TGF-β1 were measured using a mouse TGF-β1 duo-set (DY1679) according to the manufacturer’s instructions (R&D Systems). Total TGF-β1 levels were determined by acid activation (final concentration 0.2 mol/L hydrochloric acid, 10 minutes, room temperature) of the latent TGF-β1 in the homogenate. Untreated and activated samples from the same homogenate were assayed in parallel.

**Immunoblotting**

Western blotting was carried out as described by Yoshinga and colleagues (25).

**Immunohistochemistry**

Preparation of tissues, sectioning, and antibody staining were conducted as described in Yoshinaga and colleagues (25).

**Quantitative real-time PCR analysis**

RNA was extracted from 5 pairs of mice from each genotype using TRIzol (Invitrogen). Reverse transcription was conducted using 1 μg of RNA and the SuperScript III Reverse Transcriptionase (Invitrogen; 50°C, 60 minutes). The resulting cDNA was used for quantitative real-time PCR (qRT-PCR) analysis. qRT-PCR was conducted using specific primers and Quantifast SYBR Green PCR Kit (Qiagen) on an iCycler Thermal Cycler (Bio-Rad). Each target transcript expression was quantified by comparing the threshold cycle (Ct) with that of β-actin by using the comparative Ct method (31). The primers used are described in Supplementary Table S1.

**Statistical analysis**

Descriptive statistics were conducted with StatView J-4.5 program (SAS Institute). The Kaplan–Meier method was used to estimate all survival curves from mouse studies. The log-rank statistic was used to compare the overall survival distributions.

**Pathologic scoring of inflammation**

A scoring system in which 3 parameters were monitored in individual histologic sections was used for quantification of inflammation (25). The 3 parameters were: inflammation, which was measured as cell infiltration of the mucosa by mixed populations of inflammatory cells and edema; hyperplasia, which was monitored by the hyperplasia of the mucosal epithelium including lengthening of crypts, increased density of epithelial cells, and crypts and thickening of mucosa; and necrosis/ulceration, which was monitored my examining for...
necrosis of mucosal epithelial cells with attenuation, erosion, or ulceration of the epithelial barrier. A scoring system for each parameter was used in which 0 = within normal limits, 1 = minimal to mild, 2 = moderate, and 3 = severe. Two individuals monitored slides in a blinded fashion. Within the stomach tissue, we observed no necrosis and both the wild-type and mutants showed hyperplasia. Therefore, only the inflammation parameter was used.

Results

**Tumor production in Tgfβ1<sup>C33S/C33S</sup>;thrombospondin1<sup>−/−</sup> mice**

To test our hypothesis that Tgfβ1<sup>C33S/C33S</sup> activate TGF-β1 SLC, we first generated Tgfβ1<sup>C33S/C33S</sup> mice deficient in either of 2 known activators of SLC, the matricellular protein TSP-1 or the integrin β8 (17, 18), and examined these animals for changes in inflammation, tumor number, and markers for TGF-β activity.

Tgfβ1<sup>C33S/C33S</sup>;Tsp1<sup>−/−</sup> mice displayed a phenotype that was essentially unchanged compared with Tgfβ1<sup>C33S/C33S</sup> mice (Supplementary Table S2; Tsp1<sup>−/−</sup> mice, used as controls, show no phenotype). There was a slight increase in the degree of inflammation in the colon and rectum when Tgfβ1<sup>C33S/C33S</sup>; Tsp1<sup>−/−</sup> and Tgfβ1<sup>C33S/C33S</sup>;Tsp1<sup>−/−</sup> mice were compared, but inflammation in all other organs was essentially equivalent among mice with the 2 genotypes [Supplementary Table S2A (12 weeks) and Supplementary Fig. S1]. There was no change in survival of Tgfβ1<sup>C33S/C33S</sup>;Tsp1<sup>−/−</sup> mice compared with Tgfβ1<sup>C33S/C33S</sup> mice (data not shown). When these mice were examined for presence of tumors, a slight but statistically insignificant increase in tumor incidence was observed upon elimination of TSP-1 (Supplementary Table S2B). The distribution of tumor types was approximately equivalent in Tgfβ1<sup>C33S/C33S</sup>;Tsp1<sup>−/−</sup> and Tgfβ1<sup>C33S/C33S</sup>;Tsp1<sup>−/−</sup> mice and similar to results in our previous studies (25). Therefore, Tsp1 seems to make only a minor, if any, contribution to the level of active TGF-β in tumor production in Tgfβ1<sup>C33S/C33S</sup> mice.

**Tumor production in Tgfβ1<sup>C33S/C33S</sup>;Itgb8<sup>−/−</sup> mice**

We next examined the degree of inflammation and tumor production in Tgfβ1<sup>C33S/C33S</sup>;Itgb8<sup>−/−</sup> mice. The integrin αvβ8 is an important modulator of TGF-β1 levels within the intestine (32, 33). Unlike Tgfβ1<sup>C33S/C33S</sup>; Tsp1<sup>−/−</sup> mice, Tgfβ1<sup>C33S/C33S</sup>;Itgb8<sup>−/−</sup> mice presented with significant changes compared with Tgfβ1<sup>C33S/C33S</sup> animals. The compound mutant mice had a shortened survival compared with Tgfβ1<sup>C33S/C33S</sup> mice but similar to Itgb8<sup>−/−</sup> mice (Fig. 1A). The level of inflammation was increased in the stomach, cecum, colon, and rectum (Table 1). The increase in inflammation was clearly apparent when tissues from the different

<table>
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<th>Genotype</th>
<th>N</th>
<th>Lung</th>
<th>Heart</th>
<th>Stomach</th>
<th>Cecum</th>
<th>Colon</th>
<th>Rectum</th>
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<tr>
<td>WT</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
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<td>~++</td>
<td>~++</td>
<td>~+++</td>
<td>~++</td>
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<td>++</td>
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**Table 1. Inflammation and tumor production in Tgfβ1 mutant mice. Inflammation in Tgfβ1<sup>C33S/C33S</sup> and Tgfβ1<sup>C33S/C33S</sup>;Itgb8<sup>−/−</sup> mice**

NOTE: ~, no inflammation; +, mild inflammation; ++, moderate inflammation, WT, Control Tgfβ1<sup>+/+</sup> or Tgfβ1<sup>−/−</sup> animals; ~, indicates an intermediate value.

*Eight to 12 weeks of age.*
In the canonical TGF-β signaling pathway, P-Smad2 is an intracellular mediator of TGF-β signaling. Increases in the level of P-Smad2 were observed in the rectal tumors of Tgfb1\(^{C33S/C33S}\);Itgb8\(^{-/-}\) mice, as compared with wild-type (WT) samples. The level of C-Myc expression, which is normally suppressed by TGF-β signaling, was enhanced in the Tgfb1\(^{C33S/C33S}\);Itgb8\(^{-/-}\) rectal tissue compared with wild-type tissue (Fig. 3A).

Table 2. Tumors in Tgfb1\(^{C33S/C33S}\) and Tgfb1\(^{C33S/C33S}\);Itgb8\(^{-/-}\) mice.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N</th>
<th>Stomach</th>
<th>Cecum</th>
<th>Colon</th>
<th>Rectum</th>
<th>Total</th>
<th>(%)</th>
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<tbody>
<tr>
<td>WT</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Itgb8(^{-/-})</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tgfb1(^{C33S/C33S})</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>19</td>
</tr>
<tr>
<td>Tgfb1(^{C33S/C33S});Itgb8(^{-/-})</td>
<td>15</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>7</td>
<td>8</td>
<td>53</td>
</tr>
</tbody>
</table>

NOTE: Total refers to total number of animals with tumors and includes animals with multiple tumors.

*Twelve weeks of age.

*P < 0.05 vs. Tgfb1\(^{C33S/C33S}\).

Five mice had multiple tumors.

Groups were compared by a scoring method that quantified inflammation, hyperplasia, and necrosis/ulceration (Supplementary Fig. S2A). Tumor incidence also was substantially increased from 19% to 53% when Tgfb1\(^{C33S/C33S}\) animals were compared with Tgfb1\(^{C33S/C33S}\);Itgb8\(^{-/-}\) animals (Table 2). Although only rectal tumors were observed in Tgfb1\(^{C33S/C33S}\) mice, more than half of the tumor-bearing Tgfb1\(^{C33S/C33S}\);Itgb8\(^{-/-}\) mice had multiple tumors and these occurred in several different organs. In Fig. 2, images of a rectal adenocarcinoma (Fig. 2A, a) and rectal adenocarcinoma (Fig. 2A, c) are illustrated. All of the tumors were invasive, as tumor cells were observed in the submucosal muscle layer.

To determine if the increased tumor incidence in Tgfb1\(^{C33S/C33S}\);Itgb8\(^{-/-}\) mice correlated with changes in TGF-β levels and/or TGF-β signaling, we analyzed stomach and rectal tissue from wild-type, Tgfb1\(^{C33S/C33S}\), and Tgfb1\(^{C33S/C33S}\);Itgb8\(^{-/-}\) animals for markers of TGF-β1 activity. Because TGF-β1 is an inhibitor of epithelial cell growth, decreases in TGF-β1 signaling should result in enhanced epithelial proliferation. Consistent with potentially diminished levels of active TGF-β1, Tgfb1\(^{C33S/C33S}\);Itgb8\(^{-/-}\) mice displayed increased [more than twice that observed in wild-type (WT) tissues] cell proliferation of the epithelial cells of the mucosal layer in the rectal epithelium, as monitored by immunostaining for the proliferation marker KI-67 (Fig. 3A). We observed a progressive increase in the number of proliferating cells when WT samples were compared with Tgfb1\(^{C33S/C33S}\) and Tgfb1\(^{C33S/C33S}\);Itgb8\(^{-/-}\) samples (Fig. 3A).

Similar results were found in stomach tissues from the 3 genotypes (data not shown). TGF-β signaling is usually marked by increases in the level of P-Smad2, an intracellular mediator of the canonical TGF-β signaling pathway. P-Smad2 immunostaining in the rectum was heterogeneous in Tgfb1\(^{C33S/C33S}\);Itgb8\(^{-/-}\) mice with some areas showing increased intensity, whereas other areas seemed to have normal levels (Fig. 4A, a–d). In general, there seemed to be less P-Smad2 in the rectal tissue of each of the 2 mutant genotypes and significantly less staining in the double mutant. However, to survey the entire rectum for P-Smad levels, we conducted immunoblotting and scanning after SDS-PAGE on the soluble proteins from rectal tissue (Fig. 4A, e). The results show a clear loss of P-Smad2 reactivity in the double-mutant tissue, whereas tissue from wild-type with only the Tgfb1\(^{C33S/C33S}\) or Itgb8\(^{-/-}\) mutations seemed to contain amounts of P-Smad2 close to or slightly more than that of the wild-type sample. The level of C-Myc expression, which normally is suppressed by TGF-β, was enhanced in the Tgfb1\(^{C33S/C33S}\);Itgb8\(^{-/-}\) rectal tissue compared with wild-type tissue (Fig. 3A).
5A). We also measured the transcript levels for the cell-cycle regulators p15, p21, and p27, which are known to be regulated by TGF-β, in the 3 genotypes (Fig. 5A). There was only a slight decrease, which was not statistically significant, in p21 expression in Tgfb1<sup>C33S/C33S;Itgb8<sup>−/−</sup> mice compared with either wild-type or Tgfb1<sup>C33S/C33S</sup> mice. However, there were pronounced decreases in the expression of the negative regulators p15 and p27 compared with wild-type in both Tgfb1<sup>C33S/C33S</sup> and Tgfb1<sup>C33S/C33S;Itgb8<sup>−/−</sup></sup> rectal tissues consistent with a decrease in active TGF-β1.

Next, we measured TGF-β1 in sera from WT, Itgb8<sup>−/−</sup>, Tgfb1<sup>C33S/C33S</sup>, and Tgfb1<sup>C33S/C33S;Itgb8<sup>−/−</sup></sup> mice (Fig. 6A). When total TGF-β1 was quantified after acid activation of mouse sera, the Tgfb1<sup>C33S/C33S</sup>, Itgb8<sup>−/−</sup>, and Tgfb1<sup>C33S/C33S;Itgb8<sup>−/−</sup></sup> samples were equivalent and slightly lower than the control WT animal samples (Fig. 6A). When active TGF-β1 in serum was quantified, the samples from Tgfb1<sup>C33S/C33S</sup> and Tgfb1<sup>C33S/C33S;Itgb8<sup>−/−</sup></sup> mice were approximately half of the control values (Fig. 6B). However, we did not detect a difference between those 2 samples. This may indicate that the contribution of integrin αvβ8 to the activation of latent TGF-β1 in serum is negligible. We also evaluated the amount of total and active TGF-β1 in rectal tissue extracts (Supplementary Fig. S3A and S3B). Although there were small differences with respect to total TGF-β1 among the 4 experimental groups, there was no statistical difference in the amount of active TGF-β1 among the samples. The reasons for this are not known.

**Tumor production in Tgfb1<sup>C33S</sup> mice**

We reasoned that if enhanced tumorigenesis in Tgfb1<sup>C33S/C33S;Itgb8<sup>−/−</sup></sup> versus Tgfb1<sup>C33S/C33S</sup> mice was the result of lower TGF-β1 levels, a second approach to decrease active TGF-β1 levels would be to generate Tgfb1<sup>C33S/C33S</sup> mice. As these animals would have only 1 functional TGF-β1 allele and that would be the C33S allele, they should produce approximately half as much TGF-β1 as Tgfb1<sup>C33S/C33S</sup> mice. Therefore, we generated Tgfb1<sup>C33S/C33S</sup> mice by crossing Tgfb1<sup>C33S/C33S</sup> and Tgfb1<sup>−/−</sup> animals. Tgfb1<sup>C33S/C33S</sup> mice were produced at the expected Mendelian frequency (data not shown) and had a shortened life span compared with wild-type or Tgfb1<sup>C33S/C33S</sup> mice (Fig. 1B). Tgfb1<sup>C33S/C33S</sup> mice displayed more severe inflammation in their lungs, heart, stomach, liver, and colon than did Tgfb1<sup>C33S/C33S</sup> mice (Table 3 and Supplementary Fig. S2B). Tumor incidence in Tgfb1<sup>C33S/C33S</sup> mice was also significantly enhanced from 20% to 73% when Tgfb1<sup>C33S/C33S</sup> mice were compared with Tgfb1<sup>C33S/C33S</sup> mice (Table 4). Interestingly, there were only 2 Tgfb1<sup>C33S/C33S</sup> mice with multiple tumors. The reason for this is unclear. The distribution of tumor types in Tgfb1<sup>C33S/C33S</sup> mice was also different compared with Tgfb1<sup>C33S/C33S</sup> mice. By 12 weeks of age, 53% of the Tgfb1<sup>C33S/C33S</sup> animals had gastric adenocarcinomas compared with 9% in Tgfb1<sup>C33S/C33S</sup> mice. [The incidence of gastric adenocarcinomas in Tgfb1<sup>C33S/C33S;Itgb8<sup>−/−</sup></sup> mice was 20% (Table 2).] Two gastric tumors are shown in Fig. 2B. One tumor is an adenocarcinoma (Fig. 2B, a and b) and 1 is a squamous cell carcinoma (Fig. 2B, c and d). Both tumors were invasive, as tumor cells were found in the submucosal muscular layer. The rectal tumors in Tgfb1<sup>C33S/C33S</sup> mice were similar to those observed in animals with the Tgfb1<sup>C33S/C33S;Itgb8<sup>−/−</sup></sup> genotype (data not shown).
We next measured a number of parameters to characterize signaling and TGF-β1 levels in Tgfb1−/−C33S mice, as we did with the Tgfb1−/−C33S/Igfb8−/− mice. We focused on the gastric tumors because of their high incidence and relative rarity in other mouse models. As we observed in Tgfb1−/−C33S/Igfb8−/− mice, stomach tissue from Tgfb1−/−C33S mice had higher levels of KI-67-positive cells than did wild-type or Tgfb1−/−C33S tissues (Fig. 3B). Quantification of the number of KI-67-positive cells revealed that there was a 4-fold increase in Tgfb1−/−C33S tissue compared with controls and a 2-fold increase compared with Tgfb1−/−C33S tissue (Fig. 3B). P-Smad2 staining in the stomachs of wild-type, Tgfb1−/−C33S, and Tgfb1−/−C0 mice revealed that there was progressively less staining, as the expected level of total TGF-β1 decreased (Fig. 4B). We observed that C-Myc expression in the stomach tissue of Tgfb1−/−C33S mice was elevated compared with wild-type mice but was not increased compared with Tgfb1−/−C0 animals (Fig. 5B). We also measured tumor suppressor gene expression. As observed with the Tgfb1−/−C33S and Tgfb1−/−C0 tissues, there was little difference in expression of p21 when WT, Tgfb1−/−C33S, and Tgfb1−/−C0 stomach tissues were compared by qRT-PCR (Fig. 5B). Similar to the samples in Fig. 5A, there was a decrease in the expression levels of p15 among WT, Tgfb1−/−C33S, and Tgfb1−/−C0 tissue (Fig. 5B).

Finally, we measured the level of total and active TGF-β1 in sera from wild-type, Tgfb1−/−C33S, and Tgfb1−/−C0 mice (Fig. 6A). The total TGF-β1 in sera from Tgfb1−/−C33S mice, as measured after acid activation, was equivalent to wild-type as described earlier (25). The Tgfb1−/−C0 mouse sera had less than half of the total amount of TGF-β1 consistent with the loss of 1 allele. Both the mutant sera (Tgfb1−/−C33S and Tgfb1−/−C0) had less than 30% of the amount of active TGF-β1 than the wild-type sample (Fig. 6D). The amount of active TGF-β1 in the Tgfb1−/−C33S mouse sera was slightly more than half of that found in the Tgfb1−/−C33S mouse sera. When the amount of total and active TGF-β1 was measured in tissue, there were small differences in the total and active concentrations of cytokine between the genotypes and the amount of active was approximately 10% of the total (Supplementary Fig. S3C and S3D). There were no statistically significant differences between the Tgfb1−/−C33S and Tgfb1−/−C0 samples, although both of these samples were lower than the wild-type control sample.

Discussion

The association of TGF-β with cancers of the gastrointestinal tract is well established. Extensive work has shown that elimination of the growth factor or interference with its signaling either by TGF-β receptor mutations or mutations in the intracellular signaling pathway yield carcinomas (34). Our previous work showing that blocking the formation of the disulfide bond between the TGF-β1 propeptide and its matrix localizing proteins, the LTBP5s, resulted in the production of gastrointestinal cancers, was consistent with the hypothesis that binding to LTBP was required for proper TGF-β1 generation from its latent complex (25). Interference with active TGF-β1 production yields results similar to ablation of TGF-β1 signaling. Because the inflammatory response observed in

Figure 4. P-Smad levels are decreased in tumor tissue compared with WT. A, P-Smad2 staining in rectal tissue. Staining of WT and Igfb8−/− tissues (a and b) indicated higher levels of P-Smad2 in the epithelium than observed in tissues from Tgfb1−/−C33S and Tgfb1−/−C33S/Igfb8−/− mice (c and d). e, data from scans of immunoblots are presented under each lane as the ratio of P-Smad2 and Smad2. The results indicate no difference between WT and Tgfb1−/−C33S/C33S samples, but there is almost a complete loss of signaling in the Tgfb1−/−C33S/Igfb8−/− tissue. Bars, 100 μm.

Discussion

The association of TGF-β with cancers of the gastrointestinal tract is well established. Extensive work has shown that elimination of the growth factor or interference with its signaling either by TGF-β receptor mutations or mutations in the intracellular signaling pathway yield carcinomas (34). Our previous work showing that blocking the formation of the disulfide bond between the TGF-β1 propeptide and its matrix localizing proteins, the LTBP5s, resulted in the production of gastrointestinal cancers, was consistent with the hypothesis that binding to LTBP was required for proper TGF-β1 generation from its latent complex (25). Interference with active TGF-β1 production yields results similar to ablation of TGF-β1 signaling. Because the inflammatory response observed in
$Tgfb^{C33S/C33S}$ mice was not as severe as that observed in $Tgfb^{1/-}$ mice (28, 29), we presumed that some latent TGF-$\beta_1$ was activated in our mutant animals. Indeed, by further impairing active TGF-$\beta_1$ production either by eliminating an activator of latent TGF-$\beta$ or by simply decreasing the amount of total TGF-$\beta_1$ produced, we enhanced both the tumor and inflammatory phenotypes. Therefore, these mutant animals provide a hypomorphic series in which the relationship of different TGF-$\beta_1$ levels, inflammation, and relatively rapid tumor production can be explored. In addition, the results reinforce our earlier conclusions of the importance of TGF-$\beta$ LLC in TGF-$\beta_1$ biology as well as highlighting the role of the integrin $\alpha_v\beta_8$ in activation of TGF-$\beta_1$ SLC.

Both integrins $\alpha_v\beta_6$ and $\alpha_v\beta_8$ are important for active TGF-$\beta_1$ formation (27, 33). Because $Tgfb^{1/C33S/C33S}$ animals produce only the SLC of TGF-$\beta_1$ and this complex is not activated by $\alpha_v\beta_6$ (14), we focused on the effects of $\alpha_v\beta_8$ loss. Mice that were deficient for integrin $\beta_8$ and that produced $Tgfb^{1/C33S}$ displayed a higher degree of inflammation and considerably more tumors than $Tgfb^{1/C33S/C33S}$ mice. The enhanced inflammation is consistent with published reports that $\alpha_v\beta_8$ is crucial for latent TGF-$\beta_1$ activation by dendritic cells (33) and supports the observed association of inflammation and tumorigenesis (35, 36). Similar results were observed on the degree of inflammation with $Tgfb^{1/C33S/C33S}$ mice that were housed in helicobacter-free conditions (data not shown). Yet, $Igfb8\text{-/-}$ mice do not get tumors, indicating that $\alpha_v\beta_8$ is not the exclusive latent TGF-$\beta_1$ activator in the gastrointestinal tract. The other potential activator of TGF-$\beta_1$ SLC, TSP-1, seemed to make no contribution to the level of TGF-$\beta_1$ in $Tgfb^{1/C33S/C33S}$ mice as determined by our assays. Perhaps analysis of a larger cadre of animals might reveal a minor contribution. Our methods. A, WT, $Tgfb^{1/C33S/C33S}$, and $Tgfb^{1/C33S/C33S}$, $Igfb8\text{-/-}$ mice measured by qRT-PCR as described in Materials and Methods. A, WT, $Tgfb^{1/C33S/C33S}$, and $Tgfb^{1/C33S/C33S}$, $Igfb8\text{-/-}$, $P < 0.01$ versus WT; $t$, $P < 0.05$ versus WT; $\#, P < 0.05$ versus $Tgfb^{1/C33S/C33S}$, $B$, WT, $Tgfb^{1/C33S/C33S}$, and $Tgfb^{1/C33S/C33S}$, $P < 0.01$ versus WT; $t$, $P < 0.05$ versus WT; $\#, P = 0.07$ versus WT.

Figure 5. Transcript levels of cell growth regulators are altered in tumor tissue compared with WT. Rectal (A) or gastric (B) tissue from WT and mutant animals was extracted and expression levels of the indicated markers were measured by qRT-PCR as described in Materials and Methods. A, WT, $Tgfb^{1/C33S/C33S}$, and $Tgfb^{1/C33S/C33S}$, $Igfb8\text{-/-}$, $P < 0.01$ versus WT; $t$, $P < 0.05$ versus WT; $\#, P < 0.05$ versus WT; $B$, $P < 0.05$ versus WT; $t$, $P < 0.01$ versus WT; $\#, P = 0.07$ versus WT.

Figure 6. Active TGF-$\beta_1$ levels are decreased in sera from WT mice. TGF-$\beta_1$ levels from WT, $Igfb8\text{-/-}$, $Tgfb^{1/C33S/C33S}$, and $Tgfb^{1/C33S/C33S}$, $Igfb8\text{-/-}$ mouse sera measured by ELISA (A and B). Total TGF-$\beta_1$ was measured after acid treatment of serum samples to activate all of the latent TGF-$\beta_1$ present (A), whereas active TGF-$\beta_1$ was measured in sera without acid treatment (B). There was little difference among the 4 genotypes with respect to total TGF-$\beta_1$. However, $Tgfb^{1/C33S/C33S}$ and $Tgfb^{1/C33S/C33S}$, $Igfb8\text{-/-}$ had significantly less active TGF-$\beta_1$ than did WT or $Igfb8\text{-/-}$ sera. There was no statistical difference in levels of TGF-$\beta_1$ in the $Tgfb^{1/C33S/C33S}$ and $Tgfb^{1/C33S/C33S}$, $Igfb8\text{-/-}$ sera. TGF-$\beta_1$ levels in WT, $Tgfb^{1/C33S/C33S}$, and $Tgfb^{1/-C33S}$ mice were also measured by ELISA (C and D). WT and $Tgfb^{1/C33S/C33S}$ sera had approximately equivalent amounts of total TGF-$\beta_1$, whereas $Tgfb^{1/-C33S}$ sera had less than half the amount of total TGF-$\beta_1$ as did WT sera. When serum-active TGF-$\beta_1$ levels were quantified, $Tgfb^{1/C33S/C33S}$ sera had slightly less than a third the amount found in WT, whereas $Tgfb^{1/-C33S}$ sera had approximately 20% of WT.

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inflammation and tumor frequency. Together, our results with the different mutant animals indicate that tumor production is dependent upon the level of TGF-β1 signaling.

A limitation of our study is that we do not know the precise level of active TGF-β1 in the specific tissues examined. The TGF-β1 levels in serum reflect the expected changes, the secondary markers for TGF-β1 signaling in the tissues reflect decreased TGF-β1, and the level of active TGF-β1 from stomach is decreased when compared with wild-type mice. However, we were unable to detect statistically significant differences in active TGF-β1 when tissue extracts from Tgfb1<sup>C33S/C33S</sup> animals were compared with either Tgfb1<sup>C33S</sup> or Tgfb1<sup>C33S/C33S</sup><sup>+/−</sup> tissue extracts. The reason for this is not apparent but might reflect the fact that the amount of active TGF-β1 in the tissue is close to the limit of sensitivity of the assay. The availability of an assay that directly monitored TGF-β1 activity in the limit of sensitivity of the assay. The availability of an assay that directly monitored TGF-β1 activity would resolve the question of tissue levels of active cytokine.

An interesting question is why the tumor types and distribution seemed to be different between Tgfb1<sup>C33S/C33S</sup><sup>/Itgb8</sup>/<sup>−/−</sup> mice, as each mutation should lower the amount of active TGF-β1. This may reflect the fact that in 1 case, Tgfb1<sup>C33S</sup> mice, the total amount of TGF-β1 was diminished, whereas in the second case, Tgfb1<sup>C33S/C33S</sup><sup>/Itgb8</sup><sup>/−/−</sup>, the total TGF-β1 was not decreased; only one of the potential activators was decreased compared with Tgfb1<sup>C33S</sup>C33S animals. The contribution of integrin β8 to active TGF-β1 levels may vary throughout the gastrointestinal tract, thereby yielding a unique pattern of tumorogenesis. Alternatively, the differences in tumor incidence may reflect the different mouse strain backgrounds of the 2 sets of mutant and control animals generated for our studies. The high8 null mutation is an embryonic lethal when placed in the C57BL genetic background (27). Only when the mutation is placed into mixed background of C57BL plus ICR, do pups with the null mutation survive for up to 8 months; this precludes using inbred animals. Therefore, differences in genetic backgrounds might account for differences in results observed between the 2 sets of animals. Finally, the fact that the microenvironment in the stomach and rectum may be different and may differentially affect tumorogenesis.

It is interesting to speculate on the actual cause of tumors in these mutant mice. Tumor onset is relatively rapid but the frequency is not particularly high. In this respect, our model differs from other models where tumor frequency and rate are quite robust. Usually, these other models involve the alteration of tumor suppressor genes and/or oncogenes. TGF-β1−/−mediated gastric tumor production, therefore, is in some ways closer to that seen in humans. Because all of the epithelial cells have the same genotype, why are there not more tumors or what is unique about the cells comprising the tumors that occur? Thus far, we have not explored what additional genetic changes may have occurred within these tumors because at the time of sacrifice (12 weeks), the tumors are all microscopic. Additional experiments need to be done using macroscopic tumors from older animals and analyzing the involved tissue for genetic alterations.

Tumorogenesis within the gastrointestinal tract due to loss of TGF-β1 is interesting to consider with respect to the

### Table 3. Inflammation in Tgfb1<sup>C33S/C33S</sup> and Tgfb1<sup>+/−C33S</sup> mice<sup>a</sup>

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Lung</th>
<th>Heart</th>
<th>Stomach</th>
<th>Cecum</th>
<th>Colon</th>
<th>Rectum</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>−</td>
<td>~+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Tgfb1&lt;sup&gt;C33S/C33S&lt;/sup&gt;</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Tgfb1&lt;sup&gt;+/−C33S&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

NOTE: −, no inflammation; ~+, mild inflammation; ++, moderate inflammation; ~, indicates an intermediate value; >, indicates greater prevalence.

<sup>a</sup>Twelve weeks of age.

### Table 4. Tumors in Tgfb1<sup>C33S/C33S</sup> and Tgfb1<sup>+/−C33S</sup> mice<sup>ab</sup>

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N</th>
<th>Stomach</th>
<th>Cecum</th>
<th>Colon</th>
<th>Rectum</th>
<th>Total</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tgfb1&lt;sup&gt;C33S/C33S&lt;/sup&gt;</td>
<td>56</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>7</td>
<td>11</td>
<td>20&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tgfb1&lt;sup&gt;+/−C33S&lt;/sup&gt;</td>
<td>15</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>11</td>
<td>73&lt;sup&gt;c,d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

NOTE: Total refers to total number of mice with tumors and includes mice with multiple tumors.

<sup>a</sup>Eight to 12 weeks of age.

<sup>b</sup>Two mice had multiple tumors.

<sup>c</sup>P = 0.0004 vs. Tgfb1<sup>C33S/C33S</sup>.

<sup>d</sup>Two mice had multiple tumors.
cell type responsible for initiation of the lesion. The loss of TGF-β1 signaling within the epithelium removes a potent inhibitor of cell growth and potentially allows for the early growth of initiated cells. In addition, the presence of inflammatory cells of several types in Tgfb1fl/fl mice due to increased inflammation may supply mediators that promote tumorigenesis. However, removal of T and B cells by crossing with Rag2−/− mice seems not to delay colon tumor appearance in Tgfb1fl/fl mice by a significant degree (37). Rather, the absence of TGF-β1 yields disorganized crypt architecture that may predispose the tissue for malignant transformation. Alternatively, 2 other reports describing tumor production using cells deficient in TGF-β signaling describe contributions of either mutant stroma or T cells to gastrointestinal tumor production (38, 39). It is unclear in the mutant mice we have examined if the decreases in TGF-β1 have a direct effect on the epithelium, thereby promoting carcinoma formation or whether the lack of TGF-β1 produced by or signaling through other cell types promotes tumor formation. This question can be further explored with respect to hematopoietic cell contribution to tumor development using bone marrow from Tgfb1−/− mice transferred to WT animals. Such experiments are planned.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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