Tumor and Stem Cell Biology

Genetic Suppression of Inflammation Blocks the Tumor-Promoting Effects of TGF-β in Gastric Tissue

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Abstract

The contributions of TGF-β signaling to cancer are complex but involve the inflammatory microenvironment as well as cancer cells themselves. In mice encoding a TGF-β mutant that precludes its binding to the latent TGF-β binding protein (Tgfb1⁰⁻/⁰⁻), we observed multiorgan inflammation and an elevated incidence of various types of gastrointestinal solid tumors due to impaired conversion of latent to active TGF-β1. By genetically eliminating activators of latent TGF-β1, we further lowered the amount of TGF-β, which enhanced tumor frequency and multiorgan inflammation. This model system was used to further investigate the relative contribution of TGF-β1 to lymphocyte-mediated inflammation in gastrointestinal tumorigenesis. Toward this end, we generated Tgfb1⁰⁻/⁰⁻,Rag2⁻/⁻ mice that lacked adaptive immune function, which eliminated tumor production. Analysis of tissue from Tgfb1⁰⁻/⁰⁻,Rag2⁻/⁻ mice indicated decreased levels of P-Smad3 compared with wild-type animals, whereas tissue from Tgfb1⁰⁻/⁰⁻ mice had normal P-Smad3 levels. Inhibiting the inflammatory response normalized levels of interleukin (IL)-1β and IL-6 and reduced tumor cell proliferation. In addition, Tgfb1⁰⁻/⁰⁻,Rag2⁻/⁻ mice exhibited reduced paracrine signaling in the epithelia, mediated by hepatocyte growth factor produced by gastric stroma. Together, our results indicate that many of the responses of the gastric tissue associated with decreased TGF-β1 may be directly or indirectly affected by inflammatory processes, which accompany loss of TGF-β1, rather than a direct effect of loss of the cytokine. Cancer Res; 74(9); 2642–51. ©2014 AACR.

Introduction

TGF-β1 was described as a stimulator of mesenchymal cell growth (1). However, subsequent studies revealed that this cytokine also has an important function as an inhibitor of epithelial cell proliferation. As such, TGF-β1 acts as a tumor suppressor, a view supported by data from numerous human and animal studies demonstrating that inactivating mutations in the TGF-β signaling pathway result in different types of carcinomas (2). The multiple and sometimes apparently contradictory actions of TGF-β1 are also evident by the fact that although TGF-β1 is a growth suppressor in early-stage tumors, it may have a promoting effect in late-stage tumors (3–6). The reasons for this difference in response to TGF-β are unclear but may relate to the acquisition of mutations by later-stage tumors that block TGF-β’s growth-inhibiting activity but accentuate its ability to initiate an epithelial-to-mesenchymal transition, a process that enhances the invasive and metastatic phenotypes (7).

In addition to its growth-regulatory capacity, a second important property of TGF-β1 is the promotion of T regulatory cell maturation and the consequent suppression of inflammation (8). Thus, TGF-β1 null mutations in mice are associated with severe multiorgan inflammation and rapid death after weaning (9, 10). Because inflammation and inflammatory mediators promote tumor formation, loss of TGF-β or loss of TGF-β signaling might have a dual effect upon tumorigenesis both by relieving the growth-suppressive activity and by initiating the stimulation associated with inflammation. There are three TGF-β genes (Tgfb1, 2, and 3). Mutations of TGF-β2 or TGF-β3 genes are not known to be associated with tumor production (11–13). Loss of TGF-β1 results in severe multiorgan inflammation and early death (9, 10). Tumor generation has also been reported in TGF-β1–null mice but has been difficult to study because of rapid death after weaning due to the strong phenotype.

To circumvent this problem, we generated TGF-β1 hypomorphic mice by permuting the mechanism used to generate active TGF-β1 (14). Although all TGF-βs are initially produced as homodimeric proproteins and the bond between the

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prosequence and the mature molecule is cleaved within the cell, the propeptide, named the latency-associated protein (LAP), remains associated with the signaling molecule after secretion (15). This complex of TGF-β and LAP is called the small latent complex (SLC). The continued interaction of cytokine with its propeptide renders TGF-β latent, because in this form TGF-β cannot interact with its receptor. A variety of mechanisms for the activation, i.e., the liberation of TGF-β from the SLC, of latent TGF-β have been described, and all involve the perturbation of the binding between TGF-β and LAP by integrins, proteases, thrombospondin 1, or reactive oxygen (15). The TGF-β–LAP complex is usually linked to a second gene product, a latent TGF-β binding protein (LTBP), through cysteine residues in LAP forming the large latent complex (LLC). Four LTBP genes exist, three of which, LTBP-1, -3, and -4, bind to TGF-β–LAP isoforms (16). LTBPβs target the latent TGF-β complex to the matrix and may participate indirectly in activation of the latent complex by the integrin αvβ6 (15). The αvβ6-mediated activation process depends upon the integrin binding to an RGD sequence in the TGF-β1 propeptide and the latent complex binding to the matrix via the LTBP. When the cell applies force to the complex by cytoskeleton contraction mediated by the integrin, tethering to the matrix via the LTBP results in distortion of the TGF-β1–LAP complex and release of active TGF-β1 (17, 18). Mutation of either the RGD to a non-integrin binding sequence (RGE) or deletion of the matrix-binding regions in LTBP blocks latent TGF-β activation (19, 20).

To perturb latent TGF-β activation, we mutated the TGF-β1 LAP cysteine (residue 33), which binds LTBP, to serine (14). Thus, TGF-β1 produced by these mutant Tgfb1/C33S/C33S mice is unable to form a complex with any LTBP. With this approach, we demonstrated that the binding of TGF-β1 LAP to an LTBP is crucial for proper TGF-β1 function, as Tgfb1/C33S/C33S mice resemble Tgfb1+/− mice with respect to shortened lifespan, multiorgan inflammation, and lack of epidermal Langerhan's cells (14). However, the shortened lifespan and multiorgan inflammation of Tgfb1/C33S/C33S mice are not as severe as with Tgfb1−/− mice, suggesting that Tgfb1/C33S/C33S animals are hypomorphic rather than null for TGF-β1. The amount of active TGF-β1 is decreased as measured by both indirect and direct assays. The mutant mice develop tumors of the stomach, colon, and rectum at an overall frequency of 20% within 12 to 16 weeks. Tumor generation is enhanced upon further reduction of the TGF-β1 level by elimination of the αvβ6 integrin, which is an activator of soluble SLC, or by generating Tgfb1−/−C33S/C33S mice, which have one Tgfb1 null allele and one C33S allele (21). With this approach, the tumor incidence in Tgfb1−/−C33S mice is as high as 73%. It was notable that the Tgfb1−/−C33S genotype yielded high numbers of gastric tumors, an effect not observed in other models of TGF-β1 deficiency (14, 22). Concomitant with the increase in tumors, we observed a decrease in TGF-β signaling and a concordant increase in the degree of inflammation (21). Therefore, Tgfb1−/−C33S/C33S mice provide a system in which the level of active TGF-β1 can be manipulated genetically and the properties of selective phenotypes examined over an extended period of time.

We hypothesized that we could use Tgfb1−/−C33S mice to examine the contribution of inflammation to tumorigenesis by constructing mice with Tgfb1−/−C33S alleles combined with Rag2−/− alleles. These animals would produce little active TGF-β1 and no mature T or B cells; therefore, by monitoring tumor frequency, we could discern the contribution of lymphocyte-mediated inflammation to tumor production. In this article, we describe the phenotypes of these compound mutant mice. Because of the paucity of genetic models of gastric cancer, we focused our analysis on gastric tumors in Tgfb1−/−C33S mice, although there were additional rectal–anal tumors in the mutant animals. We show that Tgfb1−/−C33S, Rag2−/− mice display decreased levels of inflammation and a severe reduction in tumorigenesis compared with Tgfb−/−C33S mice. We found that in the compound mutant animals, the loss of gastric tumor production is accompanied by reductions in interleukin (IL)-1β and IL-6 cytokine production as well as decreased signaling through the hepatocyte growth factor (HGF) receptor cMet, and cRon.

Materials and Methods

Mouse lines

The generation and phenotypes of Tgfb1−/−C33S mice have been described in ref. 21. Tgfb1−/−C33S;Rag2−/− mice were generated by breeding Rag2−/− (TACONIC; ref. 23) on a C57BL/6 background with Tgfb1+/−C33S or Tgfb1−/−C33S mice on a predominantly C57BL/6 background. The offspring, Tgfb1−/−C33S;Rag2−/− and Tgfb1−/−;Rag2−/− mice, were crossed to generate Tgfb1−/−C33S;Rag2−/− mice. Tissues were collected at 3 months of age unless otherwise noted. All procedures were conducted according to the regulations of the New York University Langone Medical Center Institutional Animal Care and Use Committee.

TGF-β assays

The levels of total and active TGF-β1 were measured in sera using a mouse TGF-β1 duo-set (DY1679) according to the manufacturer’s instructions (R&D Systems).

Histology

Stomach and rectal tissues were dissected, opened, and the contents flushed with ice-cold saline. The tissue was immersed and fixed in 10% formalin and embedded in paraffin. Each tissue was serial sectioned and a minimum of five hematoxylin and eosin (H&E)–stained sections were examined that were at least 100-μm apart.

Pathologic scoring of inflammation

A scoring system in which three parameters were monitored in individual histologic sections was used for quantification of inflammation (14, 21). The three parameters were: (i) inflammation, measured as cell infiltration of the mucosa by mixed populations of inflammatory cells and edema; (ii) hyperplasia, monitored by the degree of hyperplasia of the mucosal epithelium including lengthening of crypts, increased density of epithelial cells and crypts and thickening of mucosa; and (iii) necrosis/ulceration, monitored by 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.

**Immunohistochemistry**

Preparation of tissues, sectioning, and antibody staining were performed as described in ref. 14. Antibodies used were purchased from the following sources: calponin (EP798Y, ab46794), CD3 (ab5690-100), F4/80 (CL:A3-1, ab6640), and phospho-SMAD3 (phospho S423+S425, ab51451) were from Abcam; HGF (N-terminal, LSB4012) was from LifeSpan Biosciences; Ki67 (NCL-Ki67p) was from Leica Biosystems; phospho-cMet (pYpYpY1230/1234/1235, 44888G) was from Invitrogen; phospho-ERK (THR202/TYR204, 4370p), and phospho-STAT3 (9145), were from Cell Signaling Technology; phospho-histone H3 (Ser10, 06-570), was from Millipore; and P-MSPR/RON (AF1947) was from R&D Systems.

**Ki67 and P histone H3 analysis**

Staining and analysis were performed as described in ref. 21.

**Image analysis**

Stained slides were scanned using a Leica SCN400F. Slides were analyzed using Ariol image analysis system. The settings were adjusted in a process called "training of classifiers" to analyze objects of specific color, size, and shape, and to distinguish positive from negative cells. Only areas of glandular epithelium were included. Images of all sections were analyzed using the settings established during the training. To quantify the degree of inflammation, positive areas for F4/80 and CD3 staining in the submucosal layer and above were measured using ImageJ software 1.47v (24).

**Quantitative real-time PCR analysis**

Half of the tissue was immersed in RNAlater RNA Stabilization Reagent (Qiagen) overnight at 4°C. The tissue was
removed, frozen in liquid nitrogen, and stored at \(-80^\circ\) C. RNA was extracted using RNeasy Mini Kit (Qiagen). Reverse transcription was performed using the SuperScript III Reverse Transcriptase (Invitrogen; 50\(^\circ\)C, 60 minutes) according to the instructions. The resulting cDNA was used for quantitative real-time PCR (qRT-PCR) analysis using specific primers and the Quantifast SYBR Green PCR Kit (Qiagen) on an iCycler Thermal Cycler (Bio-Rad). Expression of each target transcript was quantified by use of calibration curves and normalized to the expression of \(\beta\)-actin. The primers are described in Supplementary Table S1.

**Statistical analysis**

Descriptive statistics were performed with StatView J-5.0 program (SAS Institute). The Kaplan–Meier method was used to estimate survival curves. The log-rank statistic was used to compare the overall survival distributions. Errors were computed in Fig. 1 as SE and in all other figures as SD.

**Results and Discussion**

**TGF-\(\beta\) production in \(\text{Tgfb1}^{-/-}\text{Rag2}^{+/+}\) mice**

When \(\text{Tgfb1}^{+/+}\text{Rag2}^{+/+}\) and \(\text{Tgfb1}^{-/-}\text{Rag2}^{+/+}\) mice were crossed, \(\text{Tgfb1}^{-/-}\text{Rag2}^{-/-}\) mice were born at the expected frequency (Supplementary Table S2). Although only 55% of the \(\text{Tgfb1}^{-/-}\text{Rag2}^{+/+}\) mice survived at 12 weeks, survival of \(\text{Tgfb1}^{-/-}\text{Rag2}^{+/+}\) mice was considerably better, as by 12 weeks of age 72% of the animals were alive (Supplementary Fig. S1A). By 20 weeks of age, only 33% of the \(\text{Tgfb1}^{-/-}\text{Rag2}^{+/+}\) mice survived, whereas approximately 68% of the \(\text{Tgfb1}^{-/-}\text{Rag2}^{-/-}\) mice survived. Interestingly, the death of \(\text{Tgfb1}^{-/-}\text{Rag2}^{-/-}\) mice seemed to occur in two phases. The first phase, between weeks 2–4, displayed a steep decline with 30% of the animals dying during this period. The second phase, after week 4 and continuing for at least 19 weeks, displayed a more gradual decline. During the first 4 weeks, \(\text{Tgfb1}^{-/-}\text{Rag2}^{-/-}\) mice displayed survival kinetics similar to those of \(\text{Tgfb1}^{-/-}\) animals, but were protected against the second phase of decline. Thus, two processes seem to be responsible for the death of \(\text{Tgfb1}^{-/-}\) mice, an early process, which occurs even in the absence of mature T and B cells, and a second process related to inflammation, which is almost completely negated upon elimination of RAG2 activity. The actual causes of death at these two times remain unknown.

\(\text{Tgfb1}^{-/-}\) and \(\text{Tgfb1}^{-/-}\text{Rag2}^{-/-}\) animals weighed less than wild-type (WT) or \(\text{Rag2}^{-/-}\) animals (Supplementary Fig. S1B). The weights of the spleen and thymus standardized to total body weight in \(\text{Rag2}^{-/-}\) and \(\text{Tgfb1}^{-/-}\text{Rag2}^{-/-}\) mice were decreased compared with those of WT mice, consistent with a lack of T and B cells, whereas the weight and size of the spleen in \(\text{Tgfb1}^{-/-}\text{Rag2}^{-/-}\) mice were increased compared with WT, presumably because of inflammation (Supplementary Fig. S1C and S1D).

**Inflammation**

As expected, \(\text{Tgfb1}^{-/-}\text{Rag2}^{-/-}\) mice displayed less inflammation than \(\text{Tgfb1}^{-/-}\) animals by several measures. \(\text{Tgfb1}^{-/-}\text{Rag2}^{-/-}\) stomachs and colorectums weighed significantly less than corresponding \(\text{Tgfb1}^{-/-}\) tissues when normalized to total body weight (Fig. 1A). The degree of inflammation, as measured by a system that scored inflammatory cell infiltration and edema, hyperplasia, and necrosis, was elevated in \(\text{Tgfb1}^{-/-}\) mice but was reduced to normal in \(\text{Tgfb1}^{-/-}\text{Rag2}^{-/-}\) animals (Fig. 1B and C). There was little hyperplasia or necrosis, but this may be a feature of gastric

![Figure 2. TGF-\(\beta\) signaling in \(\text{Tgfb1}^{-/-}\text{Rag2}^{-/-}\) gastric tissue. To monitor TGF-\(\beta\) signaling, P-Smad3-positive cells and expression levels of five TGF-\(\beta\) responsive genes were quantified. A-C, P-Smad3 levels decreased in \(\text{Tgfb1}^{-/-}\text{Rag2}^{-/-}\) tissue and this effect was reversed upon introduction of the Rag2\(^{+/+}\) gene. D, quantification of positive cells indicated that there was a 3-fold difference among mice of the different genotypes. N = 3 per group. E, the expression of three genes, C-Myc, Id1, and IkB, whose expression is negatively regulated by TGF-\(\beta\), displayed enhanced expression in \(\text{Tgfb1}^{-/-}\) animals that was reversed in \(\text{Tgfb1}^{-/-}\text{Rag2}^{-/-}\) mice. Two other genes, Pax1 and Ctgf, whose expression is positively regulated by TGF-\(\beta\), showed no significant changes dependent upon the \(\text{Tgfb1}^{-/-}\) mutation. N = 6 per group.](www.aacrjournals.org)
results were observed for the rectal–anal tissue (data not shown). The degree of inflammation was quantified by measuring the positively stained areas in slides of tissue samples from animals of the three genotypes. The ratios of the positively stained areas for the macrophage marker F4/80 for WT, Tgfb1−/−C33S, and Tgfb1−/−C33S;Rag2−/− mice were 1:10.0:1.4, whereas the ratios for the T-cell marker CD3 were 1:24.3:2.1.

**TGF-β levels**

The amount of total TGF-β1 was decreased in Tgfb1−/−C33S and Tgfb1−/−C33S;Rag2−/− sera compared with WT (Supplementary Fig. S1E), as expected. However, total TGF-β1 was significantly lower than the 50% expected for these two mutant genotypes. The reason for this is not clear but was also observed in our earlier studies (21). It is possible that the SLC is less stable in serum than the LLC. The amount of active TGF-β1 in Tgfb1−/−C33S and Tgfb1−/−C33S;Rag2−/− sera was significantly less than that found in WT or Rag2−/− mice (Supplementary Fig. S1F), as reported in ref. 21.

### Table 1. Tumor incidence (16–19 weeks)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>N</th>
<th>Stomach</th>
<th>Rectum</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Rag2−/−</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Tgfb1−/−C33S</td>
<td>15</td>
<td>5</td>
<td>2</td>
<td>6a (40)b</td>
</tr>
<tr>
<td>Tgfb1−/−C33S;Rag2−/−</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

aIncluding one double tumor-bearing mouse.
bP < 0.05 versus Tgfb1−/−C33S;Rag2−/−.

**Figure 3.** Gastric and rectal–anal tumors in Tgfb1−/−C33S mice. Representative gastric tissue sections stained with H&E are shown. A, gastric tumor with abnormal gland structure. The arrow points to immune cells infiltrating the stomach tissue. B, higher magnification of the boxed area in A, C, gastric tumor with submucosal invasion and polyp-like lesion. D and E, rectal–anal tumor. Invasive cells are clearly seen in the submucosa.
To determine whether TGF-β signaling was affected by the Rag2 null mutation, we performed two types of experiments. First, we measured P-Smad3 levels in stomach tissue of WT, Tgfb1/C33S and Tgfb1/C33S;Rag2−/− animals using immunohistochemistry (IHC). P-Smad3 levels are surrogate markers for TGF-β signaling, as phosphorylation of Smad3 is part of the TGF-β signaling cascade (25). The amount of P-Smad staining was clearly decreased in Tgfb1/C33S mice compared with WT (Fig. 2A, B, and D). P-Smad3 levels were restored to the level of WT in Tgfb1/C33S;Rag2−/− tissue (Fig. 2C and D). This result was unexpected, as we assumed TGF-β activation and, therefore, TGF-β signaling would remain low in the Tgfb1/C33S;Rag2−/− mice. It is possible that in the absence of T and B cells, there is compensation by other ligands that might increase P-Smad3 levels.

In a second approach, we examined the expression of TGF-β-sensitive genes in mice of the different genotypes (14, 26). The expression of three genes, C-Myc, Id1, and Id2, normally suppressed by TGF-β1, showed increased expression in Tgfb1/C33S mice compared with either WT or Rag2−/− animals (Fig. 2E). Expression of Id1 and Id2 was significantly reduced in Tgfb1/C33S;Rag2−/− mice compared with Tgfb1−/−C33S, suggesting that the observed enhancement of gene expression was related to the inflammatory state. The precise mechanism for enhancement is not known, but could be the result of enhanced BMP signaling. The differences in C-Myc expression between the Tgfb1/C33S;Rag2−/− and Tgfb1/C33S genotypes were not significant. Two other genes, Puls and Cgfl, whose expression is normally stimulated by TGF-β1, displayed no increase in the Tgfb1−/−C33S mutants, consistent with lack of an increase in active TGF-β1 in the mutant genotypes (Fig. 2E).

Tumor incidence

Tgfb1−/−C33S mice had an overall incidence of stomach and rectal tumors of 40% between 16 and 19 weeks (Table 1). All tumors were well-differentiated adenocarcinomas (Fig. 3) and all but one of the stomach tumors were invasive. No metastases were observed. The tumor incidence was slightly less than observed previously (21). This may reflect the genetic background as the animals in this study were in a primarily C57BL/129 background, whereas animals in our earlier studies were outbred. No tumors were found in the stomach or rectum of Tgfb1−/−C33S;Rag2−/− mice (Table 1). We therefore concluded that decreasing the inflammatory response impeded tumor development.

Prevention of tumors in Tgfb1−/−C33S mice that harbor the Rag2−/− mutation contrasts with a report by Engle and colleagues (22), who found that Tgfb1−/−;Rag2−/− mice developed tumors. However, we found mainly gastric and rectal–anal tumors, whereas Engle and colleagues observed colon and cecal tumors. Engle and colleagues also described high levels of granulocytes in early tumor stages, but we failed to observe this. Indeed, we detected very little hyperplasia. There are several technical differences between our model and that of Engle and colleagues that might account for the apparently conflicting results. Perhaps the most significant difference is that our animals are TGF-β1 hypomorphs, whereas the animals that Engel and colleagues studied were TGF-β1 nulls. Thus, tumor type may reflect the absolute level of TGF-β1. In addition, Engle and colleagues used 129 x CF-1 mice, whereas our mice were on a primarily C57BL6 background. Individual mouse strains may exhibit unique responses to TGF-β1 loss due to differences in modifier genes.

Figure 4. Cell proliferation in Tgfb1−/−C33S and Tgfb1−/−C33S;Rag2−/− mice. A–C, sections of gastric tissue stained with an antibody to Ki67, which reveals proliferating cells. (A, WT; B, Tgfb1−/−C33S; C, Tgfb1−/−C33S;Rag2−/−). There is a dramatic increase in the number of positive cells in sample B. This increase is reduced upon introduction of the Rag2−/− mutation (C). When the percentage of positive cells was computed (D), the change in proliferation was evident. N = 3 per group. E–G, cells stained for the proliferation-specific antigen P-HH3. The number of positive cells was greater in the Tgfb1−/−C33S sample (F) than in either WT or Tgfb1−/−C33S;Rag2−/− samples (E and G), indicating that removal of T and B cells decreased the number of dividing cells. When the percentage of dividing cells was computed (H), the changes between the genotypes were obvious. N = 3 per group.
We quantified the numbers of proliferating cells by scoring for Ki67-positive cells (Fig. 4). Gastric tissue samples from WT mice had low numbers of positive cells (Fig. 4A and D), whereas the samples from Tgfb1−/−;C33S mice had a high number of Ki67-reactive cells (Fig. 4B and D). Thus, decreasing TGF-β1 levels results in enhanced proliferation, a situation reversed in the Tgfb1−/−;Rag2−/− sample (Fig. 4C and D). To confirm these results, we also stained stomach epithelium with an antibody against phosphohistone H3 (P-HH3), a proliferation-specific antigen. Again, Tgfb1−/−;C33S samples contained more positive cells than WT, and this number was reduced to normal in Tgfb1−/−;Rag2−/− mice (Fig. 4E–H). Therefore, increased cell proliferation as measured by two markers, correlated with the degree of inflammation. The potential complexity of the contribution of inflammation to tumor production is further highlighted in a recent article that reported that tumor production in Smad3−/− mice was enhanced in Smad3−/−;Rag2−/− animals (27).

Cytokine signaling

To determine the potential inflammatory stimuli contributing to tumor generation, we used qRT-PCR to screen stomach tissue of the different genotypes for a number of cytokines associated with tumor generation (Fig. 5A). The assay revealed increased levels of IL-1β and IL-6 expression in Tgfb1−/−;C33S tissue compared with WT or Rag2−/− samples. This is consistent with the diminution in Th17 cells we reported earlier (28). Both IL-1β and IL-6 have been associated with enhanced tumorigenesis (29, 30). These increases in gene expression were reversed by introduction of the Rag2−/− mutation (Fig. 5A). Heightened levels of IL-10, Mip1α, and Mip1β were also observed in Tgfb1−/−;C33S samples, but these changes were at the limit of statistical significance. We examined IL-1β and IL-6 protein levels in tissues by ELISA but we were unable to detect changes in either gastric tissue or in the sera of Tgfb1−/−;C33S mice compared with WT (data not shown).

Because IL-6 signals through the Stat pathway (31), we monitored tissue from WT, Tgfb1−/−;C33S, and Tgfb1−/−;Rag2−/− stomachs by IHC for the product of Stat activation, P-Stat3. Tgfb1−/−;C33S mice displayed significantly more immunoreactivity than WT mice and the level of reactivity was dramatically reduced in samples from Tgfb1−/−;Rag2−/− mice (Fig. 5B–E). The expression of three genes, Mmp13, Survivin, and Bcl-xl, whose expression is enhanced by P-Stat3,
was assayed (32–34). Mmp13 and Survivin displayed enhanced expression levels in Tgfβ1/−/C33S tissues and normal levels in Tgfβ1/−/C33S;Rag2−/− tissues consistent with Stat3 activation in the compound mutant mice (Fig. 5F). Bcl-xl expression was not significantly enhanced in Tgfβ1/−/C33S gastric tissue. These results suggest that there is enhanced signaling through the

Figure 6. HGF signaling in Tgfβ1/−/C33S mice. Gastric tissue from animals of three genotypes was stained for HGF. Cells in the stroma were positive (A–C). DAPI (4',6-diamidino-2-phenylindole) staining is in blue and HGF in red. The position and shape of these cells were similar to that expected for stromal fibroblasts. When we costained Tgfβ1/−/C33S gastric tissue for HGF and calponin, a marker for fibroblasts (Q–S), all cells positive for HGF were positive for calponin staining. We also measured HGF expression levels (D) and found a significant increase in Tgfβ1/−/C33S tissue, which was normalized in Tgfβ1/−/C33S;Rag2−/− samples. We measured the numbers of cells in the gastric epithelium positive for the activated forms of the HGF receptor c-Met (P-Met) and c-Ron (P-Ron). Both P-Met (E–G) and P-Ron (I–K) levels were increased in Tgfβ1/−/C33S and reduced in Tgfβ1/−/C33S;Rag2−/− samples. The differences in percentage positive cells for both markers are presented in H and L, respectively. We also monitored P-Erk levels in the three genotypes (M–O). P-Erk levels increased in Tgfβ1/−/C33S and decreased in Tgfβ1/−/C33S;Rag2−/− compared with WT. The percentage differences are indicated in P. N = 3 per group for experiments in D, H, L, and P.
Stat3 pathway, perhaps because of increased IL-6, in Tgfb1–/– C3Sl gastric tissue compared with WT and this is reversed in Tgfb1–/– C3Sls Rag2–/– mice.

An earlier report using a conditional deletion of the TGF-β receptor (TβRII) in fibroblasts described gastric tumor formation in TβRII-deficient mice (35). In that system, tumor induction was mediated through a paracrine mechanism in which loss of TGF-β signaling in gastric stroma resulted in enhanced expression of HGF, which stimulated the growth of epithelial cells. Therefore, we examined gastric tissues for HGF levels, as measured by IHC and qRT-PCR. Staining with an antibody for HGF revealed no staining in WT tissue. In samples from Tgfb1–/– C3Sl mice, staining occurred in a pattern corresponding to the morphology of stromal fibroblasts or smooth muscle cells (Fig. 6A–C). To validate that the HGF-positive cells were fibroblasts, we performed colocalization studies using the antibodies to HGF and calponin, a marker for stromal nonepithelial cells (36). We observed coincidence of the two markers (Fig. 6Q–S). We found that HGF expression as measured by qRT-PCR was increased in gastric tissue of Tgfb1–/– C3Sl mice compared with WT animals and was reduced almost to normal levels in Tgfb1–/– C3Sls Rag2–/– mice (Fig. 6D).

Coincident with the increased expression of HGF in Tgfb1–/– C3Sl tissue, there was an increase (7-fold) in cells positive for activated HGF receptor (P-cMet; Fig. 6E–H). This increase was not observed in stomach tissue from Tgfb1–/– C3Sls Rag2–/– mice. We also examined the tissues for P-Ron, as concomitant increases in P-Ron have been reported upon decreased TGF-β signaling (37, 38). As with P-cMet, staining was increased in Tgfb1–/– C3Sl tissue and reversed to WT levels in Tgfb1–/– C3Sls Rag2–/– mice (Fig. 6I–L).

We next examined tissues for the level of the downstream mediator of P-cMet, P-Erk (Fig. 6M–P). There was a large increase (~4-fold) in the percentage of cells that were positive for P-Erk and this increase was reduced by more than 50% when Tgfb1–/– C3Sl and Tgfb1–/– C3Sls Rag2–/– tissues were compared.

These results indicate a potential signaling sequence of decreased active TGF-β1, enhanced levels of inflammation, the production of inflammatory mediators, upregulation of HGF in stromal tissues, and enhanced gastric epithelial proliferation generating adenocarcinomas. The association of inflammation and proliferation in our model is perhaps surprising as in vitro experiments with gastric stromal cell lines have indicated a direct effect of TGF-β1 on HGF expression (37, 38). Our results, however, indicate that inflammatory cells primarily mediated the effect of lowered TGF-β. The difference between our results and previous reports may reflect differences in responses of cell lines versus tissues. Our data suggest that interference with HGF signaling should decrease both gastric epithelial cell proliferation and tumor production. As chemical inhibitors of HGF are available, future experiments are planned to test the dependence of tumor production on HGF. In addition, interference with IL-6 or IL-1β might prevent tumor formation.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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