Transforming Growth Factor- β Mediates Intestinal Healing and Susceptibility to Injury *in Vitro* and *in Vivo* Through Epithelial Cells

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***In vitro* studies suggest that transforming growth factor (TGF)- β has potent effects on gastrointestinal mucosal integrity, wound repair, and neoplasia. However, the multiplicity of actions of this peptide on many different cell types confounds efforts to define the role of TGF- β within the intestinal epithelium *in vivo*. To delineate these effects selective blockade of intestinal epithelial TGF- β activity was undertaken through targeted expression of a dominant-negative (DN) TGF- β RII to intestinal epithelial cells *in vitro* and *in vivo*. Stable intestinal epithelial cell (IEC)-6 lines overexpressing TGF- β RII-DN (nucleotides -7 to 573) were established. Transgenic mice overexpressing TGF- β RII-DN under the regulation of a modified liver fatty acid-binding promoter (LFABP-PTS4) were constructed. *In vitro* healing was assessed by wounding of confluent monolayers. Colitis was induced by the addition of dextran sodium sulfate (2.5 to 7.5% w/v) to their drinking water. Overexpression of TGF- β RII-DN in intestinal epithelial cell-6 cells resulted in a marked reduction in cell migration and TGF- β -stimulated wound healing *in vitro*. TGF- β RII-DN transgenic mice did not exhibit baseline intestinal inflammation or changes in survival, body weight, epithelial cell proliferation, aberrant crypt foci, or tumor formation. TGF- β RII-DN mice were markedly more susceptible to dextran sodium sulfate-induced colitis and exhibited impaired recovery after colonic injury. TGF- β is required for intestinal mucosal healing and TGF- β modulation of the intestinal epithelium plays a central role in determining susceptibility to injury. (Am J Pathol 2003, 162:597-608)**

with single transmembrane domains, which form homo- and heterodimer complexes that bind TGF- β , inducing phosphorylation and signal transduction.^{1,2} Members of this family mediate cell differentiation, cell migration, extracellular matrix formation, immune responsiveness, and wound healing.^{3,4} This diversity of effects reflects the widespread production of TGF- β isoforms by nearly all cell types and the nearly ubiquitous presence of receptors among different cell populations.² Most of the specific functional effects of TGF- β have been defined *in vitro* using reductionist models. Interpretation of the relevance of actions observed *in vitro* can be difficult because of the multiplicity of cell targets *in vivo*. Indeed various functional effects on a given cell type delineated *in vitro* could be counterbalanced *in vivo* by functional responses to TGF- β by other cell populations. Thus, TGF- β can exert both pro- and anti-inflammatory as well as immune-activating and immune-suppressing effects depending on the cell types studied. Global targeted deletion of TGF- β 1 results in a rapidly fatal multifocal inflammatory disease.^{5,6} However, the pathogenesis of these processes is uncertain because of the variety and potentially intersecting functional effects on differing cell populations.⁴

These complexities have made the definition of the role of TGF- β in the intestinal mucosa, and specifically, the intestinal epithelial cell especially challenging. The intestinal epithelium is a highly specialized cell population undergoing continuous rapid turnover. Most importantly, it is vulnerable to injury from many different processes including, drugs, radiation therapy, infectious agents, and inflammation associated with Crohn's disease and ulcerative colitis. When injury occurs, rapid restoration of the continuity of the epithelial barrier and ultimately normal mucosal architecture is essential. Indeed TGF- β has been found, *in vitro*, to exert potent effects on the intestinal epithelium, which appear to modulate these responses.^{7,8} However, some of these effects could be counterbalanced by opposing functional actions.^{4,9} TGF- β is a potent inhibitor of intestinal epithelial cell proliferation. Indeed, inactivation of TGF- β receptor II

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The actions of the transforming growth factor (TGF)- β family are mediated through at least three types of TGF- β receptors, denoted TGF- β RI, RII, and RIII. TGF- β RI and RII are transmembrane serine/threonine kinase receptors

signaling has been described in many colorectal cancers and presumably contributes to lack of regulation of control of cell proliferation.^{10–12} In addition, TGF- β stimulates production of extracellular matrix components including collagen, which may also facilitate repair but can ultimately contribute to fibrosis, a nonphysiological outcome of healing.^{4,13–15} Furthermore, TGF- β has potent effects on the many other cell populations present in the intestinal mucosa that could dominate the overall outcome of mucosal injury including lymphocytes, macrophages, fibroblasts, and when present, neutrophils.

To determine the functional significance of TGF- β in the intestinal epithelial cell compartment, we have selectively expressed a truncated TGF- β RII in this cell compartment. Truncation of the intracytoplasmic tail results in a receptor that binds ligand but is unable to signal. The truncated receptor acts in a dominant-negative (DN) manner and thus renders the cell unable to respond to TGF- β irrespective of the multiple potential cellular sources of production.^{16–22} Selective overexpression of a TGF- β RII-DN in the intestinal epithelium permits assessment of the functional role in the epithelial compartment without the confounding effects of altered function of other cell types, and also ensures intestinal epithelial resistance to TGF- β despite both autocrine and multiple paracrine sources of TGF- β that could act on the intestinal epithelial cell.

Materials and Methods

The cDNA with human TGF- β RII (H23FF) was the kind gift of Dr. R. Weinberg¹⁶ from the Whitehead Institute, Cambridge, MA. A DN TGF- β RII was generated by polymerase chain reaction (PCR) with primers coding for a segment corresponding to nucleotides –7 through 573 and adding compatible restriction enzyme sites. A similar fragment has been previously used successfully as a TGF- β RII DN receptor in other transgenic model systems.^{17–19} This fragment was then cloned into an expression vector downstream of a 5' *c-myc* tag (EQKLISEEDL). For cell culture studies the pcDNA 3.1 (in vitro) vector was used and for transgenic studies the CDM 7 vector was used (gift from Dr. Brian Seed, Massachusetts General Hospital, Boston, MA). A cytomegalovirus (CMV) promoter was used in cell culture studies. To direct expression to the epithelial cells of the colon and small intestine a modification of the liver fatty acid-binding protein promoter that improved colonic expression (LFABP-PTS4) was used (obtained from J. Gordon, Washington University, St. Louis, MO).^{20,21} All constructs were subjected to restriction analysis and sequences were confirmed before use in cell culture and animal studies.

Establishment of Stable Cell Lines Expressing TGF- β RII DN

Stable cell lines were established by transfecting the TGF- β RII-DN construct into a rat nontransformed intestinal epithelial cell line (IEC)-6 using Lipofectamine Plus

(Life Technologies, Gibco BRL, Boston, MA) per the manufacturer's directions. Clones were grown in G418 selection media and were screened with primers from 5' location in the *c-myc* tag and 3' in the TGF- β RII-DN and Western blotting.

Assessment of Stable Cell Lines Expressing TGF- β RII DN

Cell Proliferation

Stable cell lines expressing either the TGF- β RII-DN construct or empty vector were studied. To assess baseline cell proliferation, cells were plated at the same cell density on 100-mm plates in regular media (Dulbecco's Modified Eagle Medium (DMEM) 10%, fetal calf serum, G418). The number of days to reach confluence was determined. These initial studies were correlated with thymidine incorporation assessed by seeding 12-well plates to 50% confluence followed by the addition of 0.4 or 40 pmol/L of TGF- β for 24 hours. [³H]-Thymidine (NEN Life Science Products Inc., Boston, MA) was added to each well and after 4 hours the cells were rinsed in cold phosphate-buffered saline (PBS) two times, rinsed with trichloroacetic acid (TCA) (10%), and then 1 ml of 10% cold TCA was added to each well. TCA was removed and 1 ml of 0.1 mol/L NaOH was added to the well and incubated on ice for 10 minutes. After pipetting up and down, the suspension was added to vials containing 5 ml of scintillation fluid and counted. All experiments used a minimum of five plates/well/group and were repeated a minimum of three times.

Basal cell proliferation was also assessed by plating equal numbers of either the TGF- β RII-DN or empty vector cell lines and determination of the duration of time it took to reach confluency. Eight 10-cm plates per group were used in each study and the study was repeated three times. All plates were assessed in a blinded manner every 12 hours from cell plating.

Migration and Wounding Assay

An intestinal epithelial cell wound-healing model was used.⁸ In brief, IEC-6 were plated and allowed to reach confluency in normal growth media. A wound was then made in the epithelial cell monolayer with a razor blade and the wound edge marked. Migration of cells from the wound edge at various times after wound induction and the distance of cell migration from the wound edge were measured using a standard cell culture microscope equipped with an ocular micrometer. Stable cell lines expressing the TGF- β RII-DN under regulation by a CMV promoter, or an empty vector control were studied. Cells were plated at ~80% confluency and wounding performed after confluent. TGF- β was added on wounding at a dose of 40 pmol/L and cell migration from the wound edge was determined on coded plates in a blinded manner at 24 hours. Each study consisted of a minimum of 12 plates per group and was repeated three times.

TGF- β RII-DN-Expressing Transgenic Mice

The construct expressing TGF- β RII-DN under the control of the LFABP-PTS4 promoter construct was used in the transgenic studies. The construct was prepared by cloning the LFABP into the Spe and *Hind*III sites and downstream of a *c-myc* tag (EQKLISEEDL) the TGF- β RII-DN was cloned, in-frame, into *Nco*I and *Not*I sites of the CDM 7 vector that contained a SV40 polyadenylation sequence. The linearized construct was purified by agarose gel electrophoresis followed by extraction using QIAquick gel extraction kits (Qiagen Inc., Santa Clarita, CA). After dialysis against injection buffer (5 mmol/L Tris, pH 7.4, 5 mmol/L NaCl, and 0.1 mmol/L ethylenediaminetetraacetic acid, pH 8.0), the DNA was used for pronuclear injection of 129/SvJ mouse oocytes that were transferred to pseudopregnant mice using standard techniques. Mice were screened by a PCR-based approach as well as by Southern blotting. Three lines of TGF- β RII-DN mice were established on a DBA background and negative littermates bred as controls.

Fertility, growth, and survival were determined for all lines of animals. Mice were sacrificed at 2, 4, 12, 24, and 52 weeks of age. All organs were examined including the intestine, liver, and kidneys. Tissue was processed for histology in a routine manner after hematoxylin and eosin (H&E) staining and examined by light microscopy using coded slides in a blinded manner.

Characterization of the TGF- β RII-DN Transgenic Mice

Myeloperoxidase (MPO) Assay

MPO was determined to assess colonic granulocyte infiltration in all animals sacrificed at the above time points. Tissue samples from the proximal and distal colon were removed, rinsed in saline, and then immediately snap-frozen on dry ice and processed as described previously.²² An enzyme-linked immunosorbent assay plate reader was used to assess absorbance and was set at 460 nm and absorbance determined for three separate 30-second intervals. One unit of MPO activity was defined as 1 μ mol of H₂O₂ broken down to H₂O and O (resulting in a change in absorbance of 1.13×10^2).

Aberrant Crypt Foci and Cell Proliferation

Wild-type and TGF- β RII-DN mice were sacrificed at 3 and 12 months of age for assessment of crypt foci and epithelial cell proliferation (a minimum of five animals per group). For assessment of aberrant crypt foci the entire small bowel and colon was removed and rinsed with ice-cold saline. The bowel was fixed by filling it with 10% phosphate-buffered formalin (pH 7.4) and ligating both ends. After 1 hour the bowel was incised and fixed for a further 24 hours after by staining with 0.2% methylene blue in 0.9% saline. The number of aberrant crypt foci in the small intestine and colon was determined by an observer blinded to the animal group and age using a

dissecting microscope at $\times 40$ magnification as previously described.²³

Cell proliferation was determined by bromo-2-deoxyuridine (BrdU) (Sigma Chemical Co., St. Louis, MO) staining. Mice, 3 and 12 months of age, were assessed with a minimum of five animals per group (wild type *versus* TGF- β RII-DN). BrdU (50 mg/kg) was administered intraperitoneally 1 hour before sacrifice and tissue was processed and stained with anti-BrdU antibodies as described previously.²⁴ One unit in a BrdU-labeling index was defined as the number of positive staining cells $\times$ 100/total number of cells.²⁴ A total of 10 complete colonic crypts were read per animal, 5 from the proximal colon and 5 from the distal colon. Similarly, five crypts from the proximal small intestine (jejunum) and five from the distal small intestine (ileum) were assessed for the small intestine.

Western Blotting for *c-myc*-Tagged TGF- β RII-DN

The colon and small bowel were removed, rinsed in ice-cold $1 \times$ PBS, and suspended in ice-cold lysis buffer (600 μ l/100 mg tissue) containing $1 \times$ PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.7 mmol/L ethylenediaminetetraacetic acid, and Roche Molecular Biochemicals complete Mini protease inhibitor cocktail tablets (Roche Molecular Biochemicals, Indianapolis, IN). Colonic and small intestinal mucosal isolates were prepared using the edge of a clean microscope slide to scrape the epithelium to submucosa from the muscularis in the presence of lysis buffer. These isolates were homogenized for 15 seconds using a Polytron tissue homogenizer (Brinkmann Instruments Inc., Westbury, NY), and centrifuged at $16,000 \times g$ for 20 minutes at 4°C. The samples were heated in loading buffer for 2 minutes at 85°C, and loaded into a 10 to 20% Tricine gel (Novex, San Diego, CA) and transferred onto polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). Blots were blocked for 1 hour at 23°C in a blocking solution containing 5% dry milk, 0.1% bovine serum albumin, and 0.05% Tween-20 in $1 \times$ PBS, and then incubated in mouse monoclonal anti-*c-myc* antibody 10 μ g/ml (clone 9E10, Ab-1; Calbiochem, Cambridge, MA) in the above blocking solution. Blots were then washed in $1 \times$ PBS and 0.05% Tween-20 (three washes of 20 minutes each) and incubated with horseradish peroxidase-linked goat anti-mouse antibody (Amersham Life Sciences, Arlington Heights, IL) at 1:10,000 in the same blocking solution for 60 minutes at 23°C. Antibody detection was performed using Renaissance chemiluminescent reagents (NEN Life Science Products Inc., Boston, MA), according to the manufacturer's instructions.

Immunohistochemical Localization of TGF- β RII-DN

Serial 6- to 10- μ m frozen sections were rehydrated and treated for 10 minutes at 90°C in TUF reagent (Cedarlane Laboratories Ltd.). The sections were rinsed in PBS,

blocked for 2 hours at room temperature with 15% normal goat serum in PBST (PBS containing 0.1% Triton X-100), washed in PBST, and incubated with 1:500 anti *c-myc* antibody (clone 9E10, Ab-1; Calbiochem) in 15% normal goat serum/PBST, overnight at 4°C. Sections were washed and incubated with the secondary antibody (goat anti-mouse IgG conjugated to CY3; Jackson ImmunoResearch Laboratories, West Grove, PA). After washing, slides were mounted in Fluorsave mounting media (Calbiochem). Anti-nuclear staining was done with a DNA-specific dye Hoechst 33258 (Sigma Chemical Co.) as per the manufacturer's guidelines.

Induction and Assessment of Colitis

All mice were of a DBA background and matched by age, sex, and body weight. Animals were ear-tagged in a blinded manner so investigators assessing mice daily were also blinded to group. Animal experiments were performed in accordance with National Institutes of Health guidelines and protocols approved by the Subcommittee on Research Animal Care at the Massachusetts General Hospital and Harvard Medical School.

Colitis was induced by addition of dextran sodium sulfate (DSS) (molecular weight, 40,000, lot no. 3073B; ICN Biomedical, Aurora, OH) to drinking water (2.5% or 7.5% w/v in distilled water) as described previously.²⁵ Animals were assessed daily and mean DDS/water consumption and body weights were recorded. The severity of diarrhea was assessed daily using a 0 to 3 scale; 0 = normal, 1 = soft, 2 = very soft but formed, 3 = liquid stool. Fecal blood was assessed by resuspending a fecal pellet in 400 μ l of H₂O. After brief centrifugation 40 μ l of supernatant was added to a 0.5 $\times$ 0.5-cm piece of SENSAs paper (SmithKline Diagnostics Inc., San Jose, CA), allowed to air-dry and developed with one drop of SENSAs developer solution. The presence of blood results in a white to blue change that is proportional to the amount of blood presence in the sample. The intensity of the SENSAs color change was scored by observers blinded to animal group and treatment on a 0 to 4 scale; 0 = nil, 1 = faintly blue, 2 = moderately blue, 3 = dark blue, and fecal blood visible to the eye was scored as a 4. The reproducibility of both the diarrhea and fecal blood scoring systems has been reported previously.²⁵ Adhesion score was defined as: 0 = no adhesions to the colon, 1 = one area of adhesion to the colon, 2 = two areas of adhesion to the colon, 3 = three or more areas of adhesion to the colon. Macroscopic bowel thickening was assessed and measured and presented as the percentage of colon length thickened. All assessments were done in a blinded manner on coded animals.

On sacrifice the colon was removed, opened along the mesenteric border, and fecal material removed. Tissue was removed, fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with H&E in standard manner. MPO assay was also performed at various time points as an index of colonic inflammation as noted above.

To assess recovery after induction of colitis, DSS-colitis was induced in TGF- β RII-DN and age- and sex-

Figure 1. a: Expression of TGF- β RII-DN blocks TGF- β inhibition of IEC-6 proliferation. Proliferation was assessed by determination of thymidine incorporation in IEC-6 cell lines stably transfected with either the TGF- β RII-DN or empty vector under regulation of the CMV promoter. Cells were exposed to a final concentration of 0, 4, or 40 pmol of TGF- β for 24 hours followed by incubation in tritium-labeled thymidine. All experiments contained a minimum of five plates/well per group and repeated a minimum of three times (**, $P < 0.01$). **b:** Confirmation of TGF- β RII-DN in stably transfected IEC-6 cells. *c-myc* Western blot showing expression of the TGF- β RII-DN (21 kd) in stably transfected IEC-6 cell lines.

matched control mice by adding low-dose DSS (2.5% w/v) to their drinking water. Mice were followed as above for a period of 15 days at which time DSS was discontinued. Mice from both groups were assessed as above and the remainder of the animals in both groups was followed for a further 10 days after discontinuation of DSS exposure. Mice were then sacrificed and assessed as above.

Statistical Analysis

Data are presented as the mean $\pm$ SEM. Parametric data were analyzed using a one-way analysis of variance followed by a Dunnett multiple comparisons posttest. Nonparametric data (scoring) were analyzed using a Kruskal-Wallis test (nonparametric analysis of variance) followed by a Dunn's multiple comparisons posttest. An associated probability (P value) of <0.05 was considered significant. Survival curves were created using the Kaplan-Meier method and survival comparisons were performed using the log-rank or Mantel-Haenszel test that generate a two-tailed P value. All statistical analysis was performed with Graph Pad InStat and Prism 3.0 programs (San Diego, CA).

Results

To define the role of TGF- β in the intestinal epithelium, a construct encoding a mutant TGF- β receptor (TGF- β RII-DN) that blockades responses to the ligand was prepared. Before expression *in vivo*, this construct was studied in an intestinal epithelial cell line *in vitro*. IEC-6 cell lines stably expressing TGF- β RII-DN or empty vector were established and expression confirmed by reverse transcriptase-PCR (not shown) and Western blotting (Figure 1a). Cell lines expressing TGF- β RII-DN were markedly resistant to TGF- β -induced inhibition of cell proliferation at both 4 and 40 pmol of TGF- β (Figure 1b)

Figure 2. a and b: Blockade of TGF- β signaling reduces intestinal epithelial cell migration and *in vitro* wound repair. Stable IEC-6 cell lines transfected with either the TGF- β RII-DN or empty vector under regulation of the CMV promoter were plated and allowed to grow to confluency and then wounded with a razor blade. The CMV-TGF- β RII-DN lines had reduced spontaneous and TGF- β -induced (40 pmol/L) migration across the wound edge at the 24 hour time point. Cells were assessed by direct inspection (**a**) and quantified by cell counting (**b**) as described in Materials and Methods. Each study consisted of minimum of 12 plates/well per group and the study was repeated three times on two different stable cell lines per group (***, $P < 0.001$).

confirming the functional effect of the construct expression in blocking response to TGF- β . Cell lines expressing TGF- β RII-DN also had more rapid basal proliferation as noted by a shorter time to grow to a confluent monolayer after a 5:1 split (an equal number of cells plated per group) with the TGF- β RII-DN cells reaching confluency by 3.50 ± 0.27 days and control cells by 5.13 ± 0.23 days (eight 10-cm plates assessed per group, $P = 0.0004$). These effects are consistent with concurrent blockade of endogenous TGF- β known to be produced by these cells.

Effect of TGF- β Blockade on Migration and Wound Repair *in Vitro*

Wounds were established in cultured intestinal epithelial cell monolayers to determine and reconfirm the importance of TGF- β in repair processes *in vitro* suggested by earlier studies. After wound induction, the baseline migration on average was 45% slower in the stable cell lines expressing the TGF- β RII-DN compared to cells with

Figure 3. Expression of TGF- β RII-DN in transgenic mice. Tissue expression of the mutant receptor construct was assessed by reverse transcriptase-PCR after DNase treatment was restricted to the colon, ileum, and jejunum. No expression was seen in duodenum, stomach, heart, kidney, liver, or skeletal muscle. Primers: coding, GAG CAG AAG CTG ATC TCT GAG; noncoding, GCC CGG ATC CAA GCG GCC GCT AAC GCG GTA GCA GTA GAA GAT; 38 cycles of 94°C for 1 minute, 61°C for 1 minute, 72°C for 1 minute.

empty vector (mean migration at 24 hours, 9.49 ± 2.19 mm versus 17.20 ± 2.36 mm, respectively; $P < 0.001$), as shown in Figure 2. When TGF- β -induced wound healing was assessed, 40 pmol/L of TGF- β failed to enhance wound healing in the cell lines with TGF- β RII-DN, whereas there was a marked increased rate of migration in the empty vector control cell lines ($P < 0.001$) [data presented as TGF- β -induced change in migration as a percentage of baseline (no TGF- β) migration; Figure 2, a and b].

Effect of Blockade of Intestinal Epithelial Cell Response to TGF- β *in Vivo*

After confirmation of effectiveness of the TGF- β RII-DN as a tool to blockade TGF- β response by intestinal epithelial cells *in vitro*, transgenic mice expressing TGF- β RII-DN restricted to intestinal epithelial cells via the PTS4 modification of the LFABP promoter (LFABP-PTS4) were established. Expression of the TGF- β RII-DN was assessed by reverse transcriptase-PCR after DNase treatment and was found to be restricted to the colon, ileum, and jejunum as anticipated (Figure 3). No significant expression was detected in the stomach, duodenum, heart, kidney, liver, and skeletal muscle. Colonic expression of the encoded protein was confirmed by Western blot analysis and immunohistochemistry (Figures 4 and 5, a and b). Immunohistochemistry demonstrated greater expression in epithelial cells that were closer to the luminal surface compared to epithelial cells located near the crypts (Figure 5, a and b). Two of the three lines established had

Figure 4. Localization of TGF- β RII-DN protein expression in transgenic mice. The transgene-encoded protein was assessed by Western blot with anti-sera to the *c-myc* tag in protein extracts from the colonic epithelium of TGF- β RII-DN transgenic mice *versus* wild-type mice showing PTS4-LFAB directed expression of the TGF- β RII-DN (21 kd).

variable levels of expression of the RII-DN via Western blotting, Northern blotting, and reverse transcriptase-PCR. Immunohistochemical localization studies showed patchy expression of the *c-myc*-tagged RII-DN in these two lines, thus these lines likely represented mosaics that have been commonly described using this promoter.^{26,27} Thus, for all subsequent studies only the line with uniform intestinal epithelial cell expression of the RII-DN was used.

No differences in fertility, body weight, or survival were observed in the TGF- β RII-DN mice when compared to littermate control lines. Of interest, intestinal morphology in TGF- β RII-DN mice, as assessed by light microscopy, was indistinguishable from wild-type controls and no animals developed intestinal inflammation, diarrhea, or fecal blood loss. The latter was confirmed by the lack of any differences in the low baseline colonic content of MPO activity between groups (TGF- β RII-DN, 9.96 ± 0.87 *versus* weight, 10.74 ± 1.97 ; see Figure 10e). Furthermore, no tumors or aberrant crypt foci were noted in any of the animals examined up to 52 weeks of age and there were no differences in intestinal epithelial cell proliferation assessed by BrdU labeling at 12- and 52-week time points (Figure 6). The lack of alteration in cell proliferation is somewhat surprising given that TGF- β is thought to act as a brake on cell proliferation. The finding that cell proliferation was seemingly unaffected suggests that *in vivo* other factors play either a dominant or compensatory role. In addition, no differences in epithelial apoptosis in the small and large intestine as assessed by Apoptag staining were present in the TGF- β RII-DN compared to wild-type mice.

Blockade of Intestinal Epithelial Cell Responses to TGF- β Increases Susceptibility to Mucosal Injury and Delays Repair

Colitis was induced by administration of DSS in drinking water. Transgenic TGF- β RII-DN mice and littermate control animals consumed the same amount of DSS. The TGF- β RII-DN mice were more susceptible to DSS-induced colitis as shown by a greater reduction in basal body weight and more fecal blood loss (Figure 7, a and

b). Although diarrhea scores were equivalent, histological assessment of the surviving animals revealed markedly more severe colitis in the TGF- β RII-DN mice than littermate control mice (Figure 8, a and b). The increased severity of DSS-induced colitis resulted in a marked reduction in survival of the mice expressing the TGF- β RII-DN given the same amount of DSS (Figure 9).

Because TGF- β has been found to be a mediator of wound healing *in vitro*, to determine whether the increased severity of injury in TGF- β RII-DN mice receiving DSS was because of abnormal wound healing the effect on recovery from DSS-induced colitis was assessed. A lower concentration of DSS (2.5%) was administered at which no marked differences between the littermate control lines and the TGF- β RII-DN mice were observed at up to 15 days of DSS exposure. Thus, weight loss, diarrhea score, and fecal blood loss from day 9 to day 15 of DSS exposure at this lower dose of DSS was comparable in the two groups. Similarly, no differences in macroscopic damage ($P = 0.36$), extent of colitis ($P = 0.67$), adhesion score ($P = 0.53$), or tissue levels of MPO activity ($P = 0.75$) were observed.

After 15 days, DSS was removed from the drinking water. During this recovery phase the wild-type animals rapidly improved, returning to their basal body weights, by day 20. In contrast, the basal body weights of TGF- β RII-DN failed to return to baseline even by day 25, 10 days after stopping the DSS (Figure 10a). By day 25 the TGF- β RII-DN mice had significantly more fecal blood loss ($P = 0.002$), diarrhea ($P = 0.045$), and after sacrifice more adhesions ($P = 0.003$) compared to similarly treated wild-type animals (Figure 10b). Macroscopically the extent of thickening of colon was also markedly less in the wild-type animals with only 2.8% ($\pm 1.5\%$) colonic bowel wall appearing thickened *versus* 38% ($\pm 5.2\%$) in the TGF- β RII-DN mice ($P < 0.001$). Comparable differences were also present on histological examination (Figure 10, c and d) and correlated with greater levels of colonic MPO activity in the TGF- β RII-DN mice ($P < 0.001$, Figure 10e). The failure of the TGF- β RII-DN mice to recover from the DSS exposure resulted in increased mortality in these animals compared to wild-type mice (Figure 10f).

Discussion

Intestinal homeostasis is dependent on the ability of the intestinal mucosa to withstand injury from luminal factors and to repair and remodel after damage occurs. Many cytokines, chemokines, growth factors, and other mediators have been implicated in regulating and maintaining intestinal homeostasis *in vitro*.²⁸ TGF- β has been proposed to play a central role in these processes because of the multiple actions on both epithelial and immune cells.^{4,7,9} The potential importance of the role TGF- β plays in these processes is suggested by alterations in TGF- β levels or responsiveness described in both human inflammatory bowel disease and animal models of inflammatory bowel disease, as well as in inflammatory bowel disease-associated malignancies.^{8-12,28-30} Furthermore,

Figure 5. Immunohistochemical localization of the c-myc-tagged TGF- β RII-DN. Specific c-myc staining was localized to epithelial cells. Some specific staining was noted in epithelial cells at the base of the crypts but the highest degree of staining was present in epithelial cells closest to the luminal surface (**a**). No significant specific staining was noted in wild-type mice or in the TGF- β RII-DN mice when the primary antibody (anti-c-myc) was excluded, or an unrelated antibody was substituted for the primary antibody. The secondary antibody was labeled with CY3. Staining was localized to the cytoplasm and cell surface without specific localization to the nucleus as determined by the use of the DNA-specific dye Hoechst 33258 (**b**).

targeted disruption of TGF- β in mice results in a multisystem chronic inflammatory disease leading to premature death.^{5,6} However, because most cells both produce and respond to this growth factor it has limited the ability to determine the role TGF- β plays in regulating intestinal epithelial cell function *in vivo*. To address this, we developed *in vitro* and *in vivo* models that selectively block intestinal epithelial cell responsiveness in the absence of alterations in TGF- β responses in other cell populations

by overexpressing a DN TGF- β receptor RII (TGF- β RII-DN).

TGF- β has been proposed to play a key role in regulating intestinal epithelial cell migration and wound repair. Previous studies using an *in vitro* wounding assay demonstrated that stimulation of IEC-6 migration by interleukin-1 β , interferon- γ , epidermal growth factor, and TGF- α is TGF- β -dependent.^{7,8} Consistent with these findings in the present study stable cell lines expressing TGF- β

Figure 6. Blockade of TGF- β response does not alter proliferation of intestinal epithelial cells. Cell proliferation determined by BrdU staining. BrdU was given to wild-type and transgenic TGF- β RII-DN mice 1 hour before sacrifice at 3 and 12 months of age. Tissue was removed, processed, and stained with anti-BrdU antibodies. There was no difference in cell proliferation as determined by BrdU staining in the TGF- β RII-DN compared to wild-type animals. Expressed as BrdU-labeling index; BrdU-labeling index is defined as the number of positive staining cells $\times$ 100/total number of cells.

RII-DN had impaired wound repair in the same *in vitro* wounding assay. Because epithelial cell migration from the lesion edge resulting in re-epithelialization, is one of the initial responses after gastrointestinal injury, disrupting the normal epithelial cell migration conceptually could result in increased susceptibility to injury. This inference was confirmed in the transgenic TGF- β RII-DN mice *in vivo*. Continued exposure to DSS results in epithelial injury and severe disease transmural inflammation. Histological assessment at varying time points during DSS exposure showed both injury and repair with clear re-epithelialization of denuded ulcerated areas. Exposure to high enough concentrations of DSS results in injury that outweighs repair and produces severe extensive ulceration and inflammation.

Intestinal wound repair was indirectly assessed by recovery experiments. In these studies, continued exposure to 2.5% DSS resulted in injury that appeared to be similar in severity in the TGF- β RII-DN and wild-type control mice. When the concentration of DSS was increased (to 7.5%) the TGF- β RII-DN mice clearly were more susceptible to DSS-induced injury. In the recovery study, wild-type animals rapidly recovered from the injury with almost normal appearing mucosa 10 days after cessation of DSS. Conversely, many of the TGF- β RII-DN animals failed to recover and the disease process continued leading to death. Histological assessment showed continued active inflammation in these mice and numerous ulcerated areas failed to become completely re-epithelialized. These *in vivo* phenotypic characteristics closely mirrored the effects seen *in vitro*. Collectively these findings suggest that despite the multiplicity of regulatory peptides found to promote restitution *in vitro*, TGF- β is absolutely required.

The important role of TGF- β signaling in the pathogenesis of inflammatory bowel disease has been further delineated by the studies of Monteleone and colleagues,³¹ which showed that patients with inflammatory bowel disease have unregulated SMAD-7 expression. SMAD-7 inhibits TGF- β signaling by preventing phosphorylation of SMAD 2/3 which in turn prevents the formation of the

Figure 7. Blockade of TGF- β response in intestinal epithelial cells increases susceptibility to injury (a). Changes in baseline body weight during induction of colitis with 7.5% DSS. The TGF- β RII-DN mice were more susceptible to DSS-induced colitis than wild-type mice ($n = 14$ mice per group; *, $P < 0.05$; **, $P < 0.01$). Fecal blood loss during induction of colitis with 7.5% DSS. The TGF- β RII-DN mice were more susceptible to DSS-induced colitis than wild-type mice with significantly greater amounts of fecal blood loss ($n = 14$ mice per group; *, $P < 0.05$; **, $P < 0.01$). See Materials and Methods for scoring system.

SMAD 2/3/4 complex and target gene induction.^{31,32} An anti-sense approach was used to block SMAD7, which restored TGF- β signaling in mononuclear cells isolated from patients with Crohn's disease.³¹ Thus it appears that TGF- β plays a key role in regulating intestinal inflammation and altering normal TGF- β signaling either by targeted disruption of TGF- β , altering the members of the SMAD family of proteins involved in signal transduction or by disrupting signaling via a DN-RII approach, as was done in this study, results in more severe intestinal inflammation.

The role of TGF- β in tumorigenesis has been well documented. The majority of tumors associated with hereditary nonpolyposis colorectal cancer syndrome and ulcerative colitis-associated colonic adenocarcinoma have mutations resulting in down-regulation of TGF- β respon-

Figure 8. The TGF- β RII-DN mice were more susceptible to DSS-induced colitis. **a:** TGF- β RII-DN mice. **b:** Wild-type mice. All of the TGF- β RII-DN mice had extensive areas of ulceration and transmural inflammation; in contrast only superficial ulceration and inflammation was noted in the wild-type mice.

siveness.^{10–12} Remarkably, the specific defects in these tumors in humans result in a similar condition that was induced in the TGF- β RII-DN mice with a mutation resulting in the loss of downstream signaling domain of the TGF- β receptor RII.^{11,12} The exact mechanism by which such TGF- β RII mutations promote tumorigenesis are unclear but TGF- β RII in concert with TGF- β RI normally induce signal transduction to the nucleus via members of the SMAD family of proteins. The importance of TGF- β in tumor suppression was highlighted by a study by Mikhailowski and colleagues³³ that showed that exogenous TGF- β 1 marked reduced colonic tumor formation in a rat model of colon cancer and by the study of MacKay and colleagues that showed transfection of native TGF- β RII

to colon cancer lines resulted in inhibition of cell growth and a reduction in the malignant phenotype of the transfected cells.³⁴ In a study by Zhu and colleagues³⁵ SMAD-3-deficient mice frequently developed metastatic colonic adenocarcinoma at an early age, however, a study by Yang and colleagues³⁶ found that only 1 of 30 mice followed for 6 months developed colon cancer but the mice frequently has intestinal inflammation. The reason for the discrepancy between these studies on the disruption of SMAD-3 is unclear but the targeting vectors were different, as were the murine strains.

Notwithstanding these previous observations in colonic tumors, TGF- β does not seem to be essential for the normal development and regulation of intestinal homeostasis because the down-regulation of TGF- β responsiveness in the present study did not result in abnormal intestinal development or baseline intestinal inflammation in a nonsterile environment. No differences in intestinal epithelial cell proliferation or apoptosis could be detected in the TGF- β RII-DN mice suggesting that, at baseline conditions, TGF- β may not be the key regulator of these epithelial cell responses. Furthermore, down-regulation of epithelial cell TGF- β responsiveness did not lead to an increase in dysplasia or neoplasia again suggesting that intestinal epithelial cell proliferation and differentiation are not solely regulated by TGF- β . Alternatively, some residual response may be present in the intestinal epithelial cells of the TGF- β RII-DN mice that was sufficient to regulate basal function but inadequate to respond to a significant insult such as DSS exposure. Another possible explanation for the lack of changes in cell proliferation, apoptosis, and tumorigenesis is that LFABP-PTS4 promoter-directed expression of the TGF- β RII-DN appeared to be less in replicating cells at the base of the crypts but increased dramatically as cells

Figure 9. TGF- β RII-DN mice are more susceptible to colonic injury. Survival curves for TGF- β RII-DN and wild-type (wt) mice during induction of colitis. The marked increased susceptibility of the TGF- β RII-DN to DSS-induced colitis resulted in a significant reduction in survival compared to wild-type mice. Survival curves were created using the Kaplan-Meier method and survival comparisons were performed using the log-rank or Mantel-Haenszel test, which generate a two-tailed *P* value.

Figure 10. Healing is impaired by blockade of intestinal epithelial responses to TGF- β . **a:** To assess effects on repair rather than primary injury, mice were given a low dose of DSS (2.5% w/v). Changes in baseline body weight. There were no significant differences in basal body weights of TGF- β RII-DN mice *versus* wild-type animals during the exposure to 2.5% DSS. Once the DSS was discontinued the wild-type mice rapidly returned to baseline whereas the TGF- β RII-DN mice failed to return to baseline (*, $P < 0.05$; **, $P < 0.01$). **b:** Clinical assessment (day 25). Ten days after discontinuation of DSS the wild-type mice had no fecal blood loss, minimal diarrhea, and on sacrifice had minimal adhesions compared to the TGF- β RII-DN mice. All assessments were done in a blinded manner on coded animals (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). **c** and **d:** Histology (day 25). Ten days after discontinuation of DSS the wild-type animals had no significant signs of ulceration and minimal superficial inflammation, TGF- β RII-DN mice exhibited large areas of ulceration and transmurial inflammation. TGF- β RII-DN mice lack re-epithelialization of the ulcerated area (Figure 10c). **e:** Tissue MPO. Colonic tissue MPO content was determined before induction of colitis (day 0), at the end of DSS exposure (day 15) and on sacrifice on day 25 (***, $P < 0.001$). **f:** Survival curves for TGF- β RII-DN and wild-type (wt) mice during induction of colitis and after discontinuation of DSS (day 15). The impaired recovery of TGF- β RII-DN mice resulted in a significant reduction in survival compared to wild-type mice. Survival curves were created using the Kaplan-Meier method and survival comparisons were performed using the log-rank or Mantel-Haenszel test, which generate a two-tailed P value.

moved up the crypts toward the luminal surface so that the down-regulation TGF- β responsiveness was more marked in the nonreplicating cells closer to the luminal surface.

The role of intestinal goblet cell TGF- β responsiveness was recently assessed by Hahm and colleagues³⁷ using a different DN TGF- β RII approach. In their study the expression of the TGF- β RII-DN was driven by the intestinal trefoil promoter, which should limit expression to intestinal goblet cells. In a nongerm-free environment the intestinal trefoil-TGF- β RII-DN developed spontaneous colitis whereas in a germ-free environment the animals were free of colitis but more susceptible to DSS-induced intestinal injury.³⁷ The role of TGF- β in regulating goblet cell function has not been previously assessed and thus the mechanism by which such a goblet cell-specific construct results in intestinal injury in a nongerm-free setting and increased susceptibility to injury is unclear and was not provided in the study. However, this is consistent with earlier studies from this laboratory demonstrating a role for goblet cells distinct from columnar epithelial cells in determining susceptibility or resistance to colitis.³⁸ The role of TGF- β has been assessed in intestinal epithelial cells *in vitro* and our study presented here clearly shows that overexpression of TGF- β RII-DN in intestinal epithelial cells affects wound healing *in vitro* and down-regulation of epithelial cell TGF- β responsiveness results in increased susceptibility to intestinal injury and delayed wound healing *in vivo*.

In summary, the present studies have allowed the delineation of the functional importance of TGF- β within the epithelial cell compartment without the confounding effects of TGF- β in lamina propria immune cells and other cell populations.

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