Cellular Sources of Transforming Growth Factor- β Isoforms in Early and Chronic Radiation Enteropathy

Junru Wang, Huaïen Zheng, Ching-Ching Sung, Konrad K. Richter, and Martin Hauer-Jensen

From the Departments of Surgery and Pathology, University of Arkansas for Medical Sciences, Little Rock, Arkansas

The three mammalian transforming growth factor (TGF)- β isoforms (TGF- β 1, TGF- β 2, and TGF- β 3) differ in their putative roles in radiation-induced fibrosis in intestine and other organs. Furthermore, tissue specificity of TGF- β action may result from temporal or spatial changes in production and/or activation. The present study examined shifts in the cell types expressing TGF- β mRNA relative to TGF- β immunoreactivity and histopathological injury during radiation enteropathy development. A 4-cm loop of rat small intestine was locally exposed to 0, 12, or 21-Gy single doses of x-irradiation. Sham-irradiated and irradiated intestine were procured 2 and 26 weeks after irradiation. Cells expressing the TGF- β 1, TGF- β 2, or TGF- β 3 transcripts were identified by *in situ* hybridization with digoxigenin-labeled riboprobes. Intestinal wall TGF- β immunoreactivity was measured using computerized image analysis, and structural radiation injury was assessed by quantitative histopathology. Normal intestinal epithelium expressed transcripts for all three TGF- β isoforms. Two weeks after irradiation, regenerating crypts, inflammatory cells, smooth muscle cells, and mesothelium exhibited increased TGF- β 1 expression and, to a lesser degree, TGF- β 2 and TGF- β 3 expression. Twenty-six weeks after irradiation, TGF- β 2 and TGF- β 3 expression had returned to normal. In contrast, TGF- β 1 expression remained elevated in smooth muscle, mesothelium, endothelium, and fibroblasts in regions of chronic fibrosis. Extracellular matrix-associated TGF- β 1 immunoreactivity was significantly increased at both observation times, whereas, TGF- β 2 and TGF- β 3 immunoreactivity exhibited minimal postradiation changes. Intestinal radiation injury is associated with overexpression of all three TGF- β isoforms in regenerating epithelium. Radiation enteropathy was also associated with sustained shifts in the cellular sources of TGF- β 1 from epithelial cells to cells involved in the pathogenesis of chronic fibrosis. TGF- β 2 and TGF- β 3 did not exhibit consistent long-term changes. TGF- β 1 appears to be the predominant isoform in radiation enteropathy and may be more important in the mechanisms of chronicity than TGF- β 2 and TGF- β 3. (*Am J Pathol* 1998, 153:1531-1540)

The ubiquitous growth regulatory protein transforming growth factor (TGF)- β is involved in a wide spectrum of biological activities, many of which are characterized by stimulation of mesenchymal cell proliferation and inhibition of epithelial and neuroectodermal cell proliferation. TGF- β stimulates connective tissue deposition through chemotaxis of fibroblasts and inflammatory cells, increased collagen production by fibroblasts, decreased extracellular matrix degradation, and regulation of angiogenesis. Platelets are the most concentrated source of TGF- β , but virtually every nucleated cell is capable of TGF- β production.

TGF- β has been implicated in the pathogenesis of normal tissue radiation injury in several organs.¹⁻⁵ Although the biological activities of the three mammalian TGF- β isoforms are similar, differential expression during development,⁶ in normal adult tissues,⁷ and in disease states⁸⁻¹⁰ suggests different functions *in vivo*.

In the intestine, TGF- β is involved in regulating epithelial regeneration, inflammation, and extracellular matrix deposition.¹¹⁻¹³ Intestinal radiation injury is associated with increased steady-state TGF- β mRNA levels and changes in the distribution of TGF- β protein and receptors.^{1,10,14-18} Furthermore, recent studies from our laboratory indicate that tissue specificity of TGF- β action in radiation enteropathy may be directed by temporal and spatial changes in sites of production¹⁹ and/or sites of activation.²⁰

In the present study, time- and dose-dependent shifts in the cells expressing TGF- β transcripts were examined in an *in vivo* model of radiation enteropathy using *in situ* hybridization. The association among localization of TGF- β mRNA, structural alterations, and TGF- β immunoreactivity was also assessed. The data indicate that all three TGF- β isoforms are overexpressed in regenerating intestinal epithelium during the early postradiation phase. However, more striking and sustained shifts in the cell types expressing TGF- β 1 suggest a dominant role for this isoform in the pathogenesis of radiation enteropathy, particularly in the regulation of extracellular matrix deposition during the chronic phase.

Supported by National Institutes of Health (grant CA-71382) and Central Arkansas Radiation Therapy Institute.

Accepted for publication August 6, 1998.

Address reprint requests to Dr. Martin Hauer-Jensen, Arkansas Cancer Research Center, 4301 West Markham, Slot 725, Little Rock, AR 72205. E-mail: mhjensen@life.uams.edu.

Materials and Methods

Experimental Model, Irradiation, and Tissue Procurement

A total of 32 male Sprague-Dawley rats (Harlan, Indianapolis, IN), 43 to 49 days of age (175 to 200 g) at the time of surgery, were housed in conventional cages with free access to tap drinking water and standard rat chow (Formulab Chow 5008, Purina Mills, St. Louis, MO). A pathogen-free environment with controlled humidity, temperature, and a 12/12-hour light/dark cycle was maintained. The experimental protocol was reviewed and approved by the University of Arkansas for Medical Sciences Animal Care and Use Committee.

One week was allowed for acclimatization before surgery. The surgical procedure has been described elsewhere.²¹ Briefly, the rats were anesthetized with 60 mg/kg ketamine hydrochloride (Ketacet, Aveco, Fort Dodge, IA) and 10 mg/kg xylazine (Gemini, Rugby, Rockville Center, NY) given intramuscularly. A 2-cm lower midline abdominal incision was made, bilateral orchiectomy was performed, and a 4-cm loop of distal ileum was sutured to the inside of the scrotum. The procedure creates an artificial scrotal hernia containing a segment of small intestine that can subsequently be exposed to localized irradiation with minimal manipulation, producing clinical complications and histopathological injury similar to those seen in some patients after abdominal radiation therapy. This operative procedure does not affect weight gain, intestinal morphology, epithelial cytokinetics, or intestinal transit.^{21,22}

Animals were randomly assigned to sham irradiation ($n = 8$) or localized single-dose irradiation with 12 Gy ($n = 12$) or 21 Gy ($n = 12$) to the transposed small bowel segment contained within the scrotal hernia. These doses were chosen to elicit minimal and severe chronic radiation enteropathy, respectively.^{23,24} Irradiation was performed under methoxyflurane (Metofane, Pitman-Moore, Washington Crossing, NJ) inhalation anesthesia after a 3-week postoperative recovery period, using a Seifert Isovolt 320 x-ray machine (Seifert X-Ray Corp., Fairview Village, PA) operated at 250 kVp and 15 mA, with 3-mm Al added filtration and a 4-cm tube with internal diameter of 2.5 cm. The resulting half-value layer was 0.85 mm Cu, and dose rate was 4.49 Gy/minute. Dosimetric considerations have been described.²³

The animals were monitored for development of radiation-induced small bowel complications (intestinal obstruction or fistula formation) and euthanized if clinical signs of such complications occurred. Rats from each experimental group underwent scheduled euthanasia 2 and 26 weeks after irradiation. These observation times in this model correspond to the early and chronic phase in the development of radiation enteropathy.^{21,23,25–28} Irradiated and sham-irradiated intestines were procured under anesthesia, and the animals were subsequently killed by bilateral pneumothorax induction. Specimens were fixed in methanol-Carnoy's solution, blocked in paraffin, and cut at 4 to 5 μm for histopathological examination

and immunohistochemistry. Specimens for *in situ* hybridization were fixed and processed as described below.

Assessment of Radiation Injury

The severity of radiation enteropathy was assessed using a histopathological radiation injury scoring system.^{23,25} Seven parameters of radiation injury (mucosal ulcerations, epithelial atypia, thickening of subserosa, vascular sclerosis, intestinal wall fibrosis, ileitis cystica profunda, and lymph congestion) were assessed and graded according to severity. Two researchers evaluated all specimens. The sum of the scores for the individual alterations constitutes the radiation injury score (RIS). The RIS accounts for several alterations that comprise the pleomorphic picture of late radiation enteropathy, whereas discrete histopathological alterations may be assessed across time and dose groups. The RIS has been shown to be a reliable indicator of the severity of intestinal radiation injury, exhibits a consistent dose-response relationship, and correlates well with the incidence of radiation-induced intestinal complications.^{21,23–25}

In Situ Hybridization

A plasmid containing rat TGF- β 1 cDNA (601 to 1585 bp; a gift from Dr. Anita Roberts, National Institutes of Health, Bethesda, MD) was used for TGF- β 1 mRNA *in situ* hybridization. Plasmids containing mouse TGF- β 2 cDNA (831 to 1440 bp) and TGF- β 3 cDNA (1511 to 1953 bp) were provided by Dr. Harold L. Moses (Vanderbilt University, Nashville, TN).^{29,30} To generate antisense and sense cRNA riboprobes, the plasmids were linearized with restriction endonucleases *Xba*I and *Eco*RI for TGF- β 1, *Eco*RI and *Xho*I for TGF- β 2, and *Hind*III and *Xho*I for TGF- β 3, respectively. Digoxigenin-labeled cRNA probes were produced by *in vitro* transcription with either T3 or T7 RNA polymerase, using the DIG RNA labeling kit (Boehringer Mannheim Corp., Indianapolis, IN).

All chemicals and glassware were RNase-free throughout pretreatment and hybridization. Tissue was procured from sham-irradiated and irradiated animals as described, immediately fixed in 10% neutral buffered formalin for 15 hours, automatically processed overnight, embedded in paraffin, and cut at 4 μm . The sections were deparaffinized, rehydrated, permeabilized with 0.1% Triton X-100 in PBS for 10 minutes, and incubated with proteinase K (10 $\mu\text{g}/\text{ml}$) for 15 minutes at 37°C. The sections were then fixed in 4% paraformaldehyde for 10 minutes at 4°C and rinsed in 0.1 mol/L triethanolamine/HCl (pH 8.0) for 1 minute and then 0.25% acetic anhydride in triethanolamine/HCl (pH 8.0) for 10 minutes. Sections were prehybridized with hybridization solution without probe (Boehringer Mannheim) at 50°C for 30 minutes. Hybridization was performed at 50°C in a 50% formamide-saturated humidified chamber overnight. Mouse lung tissue was used as positive control.^{8,29} Sense probes and hybridization buffer without probes were used as negative controls. Sense and antisense

probes were applied to paired serial slides. No-probe slides were prepared as additional controls for every six slides.

After hybridization, the sections were washed in 2X SSC, 1X SSC at 37°C for 15 minutes respectively, digested for 30 minutes at 37°C with 20 μ g/ml RNAse A, and then rinsed in 2X SSC, 1X SSC, and 0.2X SSC at 37°C for 20 minutes, respectively. Detection of the probes was conducted according to the guidelines provided in the DIG nucleic acid detection kit (Boehringer Mannheim). The TGF- β mRNA signals were detected as dark blue or purple precipitates. Sections were counterstained with methyl green or left without counterstaining and observed using a standard light microscope.

Immunohistochemistry and Computerized Image Analysis

Two polyclonal antibodies to TGF- β 1, TGF- β 1 (anti-CC) and TGF- β 1 (anti-LC), were used (gift from Dr. Kathy Flanders, National Institutes of Health). These antibodies were made in rabbits using different preparations of the same synthetic peptide, corresponding to the 1 to 30 amino-terminal amino acids of mature/active TGF- β 1.³¹ Antibodies to pan-TGF- β (AB-100-NA), TGF- β 2 (AB-12-NA), and TGF- β 3 (AB-244-NA) were purchased from R&D Systems (Minneapolis, MN).

The sections were deparaffinized with xylene, rehydrated in graded ethanol solutions, and treated with freshly prepared 1% (v/v) hydrogen peroxide in absolute methanol for 30 minutes at room temperature to remove endogenous peroxidase activity. Nonspecific protein binding was blocked by incubation for 1 hour in 10% normal goat serum for pan-TGF- β , TGF- β 1 (anti-CC), TGF- β 1 (anti-LC), and TGF- β 2 and in 10% normal rabbit serum for TGF- β 3. Sections were incubated for 1 hour with specific polyclonal rabbit pan-TGF- β antibody at a concentration of 20 μ g/ml for 2 hours with antibodies against TGF- β 1 (anti-CC), TGF- β 2, and TGF- β 3 at a concentration of 1 μ g/ml or overnight with the anti-TGF- β 1 (LC) at a concentration of 1 μ g/ml. Biotinylated goat anti-rabbit antibody (for the pan-TGF- β , TGF- β 1 (anti-CC), TGF- β 1 (anti-LC), and TGF- β 2 antibodies) or biotinylated rabbit anti-goat antibody (for the TGF- β 3 antibody) was applied for 30 minutes at a concentration of 1:400. After removing unbound antibody by three 3-minute rinses in TBS (0.05 mol/L Tris buffer containing 0.15 mol/L NaCl, pH 7.6), sections were incubated with avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA) for 30 minutes. Subsequently, the slides were developed in a solution of 3,3'-diaminobenzidine tetrahydrochloride solution (DAB) and hydrogen peroxide and counterstained in Gill's number 2 hematoxylin (Sigma Chemical Co., St. Louis, MO).

Specificity of staining was ascertained by omission of primary antibody as well as by substitution of primary immune antibody with nonimmune IgG (Dako, Carpinteria, CA). Immunohistochemical staining was also assessed in frozen intestinal and positive control tissues and compared with methanol-Carnoy's-fixed samples.

Positive control specimens for pan-TGF- β antibody were prepared by staining human psoriatic skin.³² Methanol-Carnoy's-fixed xenografts of human tumor cells modified by transfection to overexpress latent TGF- β 1 (B9) or constitutively active TGF- β 1 (C19), respectively (gift from Dr. Mary Helen Barcellos-Hoff, University of California, Berkeley, CA), were used as positive controls for TGF- β 1 (anti-CC) and TGF- β 1 (anti-LC).³³

TGF- β immunoreactivity in the intestinal wall was assessed by computerized image analysis (SAMBA 4000, Dynatech Laboratories/Imaging Products International, Chantilly, VA) using a previously validated measurement technique.^{1,14,18} Twenty 40 $\times$ fields (each covering an area of 23,124 μ m²) were measured per slide. The entire intestinal wall except mucosa was measured according to a predefined grid pattern in columns, two field widths apart, until 20 fields had been measured. Pixel units (PUs) were used as the unit of measurement.¹⁴ The reproducibility of this method is excellent, with a correlation coefficient for repeated measurements of 0.96 and a coefficient of concordance for batch-to-batch variability of 0.99.^{1,14}

Statistical Methods

Statistical analysis was performed with StatXact 3 for Windows, a computer program for exact nonparametric inference. Differences in RIS and intestinal wall TGF- β immunoreactivity (normalized by log-transformation) among treatment groups were assessed with the Jonckheere-Terpstra test. This test is similar to the Kruskal-Wallis nonparametric one-way analysis of variance, except that it assumes that populations are ordered by different levels of a quantifiable variable (ie, radiation dose). Associations among immunoreactivity levels were assessed with Pearson's correlation coefficient. Two-sided *P* values <0.05 were considered statistically significant.

Results

Histopathological Injury

One animal from the 21-Gy group developed radiation-induced small bowel obstruction. There were no clinical signs of intestinal complications in the 12-Gy group or among sham-irradiated animals. The histopathological changes after irradiation were in agreement with previous experiments in our laboratory and consisted of mucosal ulceration with associated inflammatory cell infiltrates, thickening of submucosa, vascular sclerosis, and subserosal thickening. Marked thickening of submucosa and subserosa was frequently associated with chronic mucosal ulcerations at 26 weeks. Inflammatory cells were seen surrounding acute or chronic mucosal ulcers, in thickened submucosa and subserosa, and, to a lesser extent, in specimens with transmural fibrosis. Sham-irradiated intestine did not show intestinal wall thickening, epithelial damage, or fibrosis. As shown in Figure 1, RIS was significantly higher in the 21-Gy group than in the 12-Gy

Figure 1. Radiation injury score 26 weeks after sham irradiation or local, single-dose irradiation of intestine with 12 or 21 Gy.

group and sham-irradiated group ($P < 0.001$). The difference between the 12-Gy group and sham-irradiated group at 26 weeks did not reach statistical significance.

In Situ Hybridization

All three antisense probes showed excellent specific hybridization with strong signals localized to the cytoplasm and nucleolus. As described by others, TGF- β 1, TGF- β 2, and TGF- β 3 mRNA were identified in a wide variety of cells in the mouse lung positive control tissue, including bronchiolar epithelium, alveolar macrophages, alveolar type II cells, and mesenchymal and mesothelial cells.^{8,29,34} Sense probes and hybridization buffer without probes did not show any mRNA signal.

The *in situ* hybridization results are summarized in Table 1. Sham-irradiated and unirradiated intestine demonstrated positive signal for TGF- β 1 mRNA in the epithelial cells, mainly localized to the crypt epithelium (except Paneth cells), with a pronounced decrease along the crypt-villus axis (Figure 2, A and B). The neurons in Auerbach's plexus and Meissner's plexus also exhibited dense TGF- β 1 mRNA signal (Figure 2C). Endothelial cells in small vessels were weakly stained. Similar to TGF- β 1, expression of TGF- β 2 and TGF- β 3 mRNA in normal in-

testine was mainly confined to epithelial cells and some nerve plexus cells. However, compared with TGF- β 1, there were less consistent and less prominent differences between crypt cells and villus cells (Figure 2, D and E).

Intestinal tissue procured 2 weeks after irradiation exhibited several striking changes. Epithelial TGF- β 1 mRNA expression was increased in crypt cells and extended further toward the villus tip than in sham-irradiated controls. Regenerating epithelium adjacent to mucosal ulcers exhibited particularly strong signals (Figure 3A). Inflammatory cells in areas of mucosal ulcerations were strongly positive for TGF- β 1 mRNA (Figure 3B). In the same areas, TGF- β 1 mRNA was expressed in fibroblasts in submucosa and subserosa, smooth muscle cells in muscularis propria and muscularis mucosae, and mesothelial cells (Figure 3C). In contrast, these cell types remained largely negative in areas away from the ulcer. There was increased signal in vascular endothelium.

Radiation-induced shifts in cell types expressing the TGF- β 2 and TGF- β 3 transcripts were much less striking. Epithelial cells did not exhibit consistent changes in TGF- β 2 expression, whereas TGF- β 3 expression in villus epithelium was somewhat decreased compared with sham-irradiated controls. Similar to TGF- β 1, but to a lesser degree, areas of mucosal injury exhibited additional positive signals in the cytoplasm of inflammatory cells, fibroblasts, smooth muscle cells, and mesothelial cells.

At 26 weeks, epithelial cell TGF- β 1 expression had returned to normal. However, inflammatory cells, fibroblasts, and smooth muscle cells in areas of chronic injury remained strongly positive for the TGF- β 1 transcript, and vascular endothelial cells in fibrotic areas showed even denser TGF- β 1 mRNA signals than at 2 weeks (Figure 3, D and E). In contrast, TGF- β 2 and TGF- β 3 expression in non-epithelial cells in areas of injury were much less pronounced than at 2 weeks.

Immunohistochemistry and Computerized Image Analysis

The staining patterns for TGF- β 1 (anti-CC) and pan-TGF- β in normal and irradiated intestine were in accordance with previous experiments performed in our laboratory.^{1,14,15} Methanol-Carnoy's-fixed sections exhibited similar immunoreactivity to cryosections but superior preservation of morphological features.

Table 1. Expression of TGF- β mRNA in Sham-Irradiated and Irradiated Intestine, 2 and 26 Weeks after Irradiation

	TGF- β 1			TGF- β 2			TGF- β 3		
	Sham	2 weeks	26 weeks	Sham	2 weeks	26 weeks	Sham	2 weeks	26 weeks
Epithelium	++	+++	++	++	++	++	++	++	++
Neurons	+++	+++	+++	++	++	++	++	++	++
Smooth muscle	0	+++	+	0	++	0	0	++	0
Inflammatory cells	0	+++	+++	0	++	+	0	++	+
Fibroblasts	0	+++	+++	0	++	+	0	++	+
Endothelium	+	++	+++	0	++	+	0	++	+
Mesothelium	0	+++	++	0	++	0	0	++	0

Results are based on the following scale: 0, negative; +, weakly positive; ++, positive; +++, strongly positive.

Figure 2. *In situ* hybridization demonstrating expression of TGF- β 1, TGF- β 2, and TGF- β 3 mRNA in normal intestine. **A:** Normal (sham-irradiated) intestine hybridized with TGF- β 1 sense probe (left, no signal) and antisense probe (right, strong signal in normal intestinal epithelium and enteric plexus neurons). Magnification, $\times 100$; bar, 100 μ m. **B:** Normal intestinal crypt with particularly prominent TGF- β 1 mRNA expression in the proliferative zone (lower two-thirds). Paneth cells express minimal TGF- β 1 mRNA. Magnification, $\times 400$; bar, 50 μ m. **C:** Overview (magnification, $\times 200$; bar, 100 μ m) and detail (magnification, $\times 1000$) of TGF- β 1 expression in Meissner's submucosal plexus neurons. Positive signal clearly corresponds to localization of mRNA (cell cytoplasm and nucleolus). **D:** Normal intestine hybridized with TGF- β 2 antisense probe. Strong epithelial signal in crypt and villus cells with no distinct crypt-villus gradient. Magnification, $\times 100$; bar, 100 μ m. **E:** Normal intestine hybridized with TGF- β 3 antisense probe. Strong signal is seen in epithelium and enteric plexus neurons. Epithelial distribution is as for TGF- β 2. Magnification, $\times 100$; bar, 100 μ m.

Both engineered tissues, B9 and C19, exhibited TGF- β 1 (anti-CC) extracellular matrix- and cell-associated immunoreactivity; TGF- β 1 (anti-LC) showed cell-associated immunoreactivity only in C19 sections. Pan-TGF- β immunoreactivity (extracellular matrix-associated only) was detected in both clones.

Sham-irradiated intestine showed weak extracellular matrix-associated TGF- β 1 (anti-CC) immunoreactivity around submucosal vessels and Auerbach's plexus of the submucosa. Two weeks after irradiation, submucosal and subserosal TGF- β 1 (anti-CC) immunoreactivity was significantly increased in the 21-Gy group, especially in areas of mucosal ulcers and subserosal thickening. Inflammatory cells in ulcerated areas exhibited cell-associated staining. At 26 weeks, TGF- β 1 (anti-CC) immunoreactivity remained significantly increased in areas of intestinal wall fibrosis and subserosal thickening. As shown in Figure 4, there was a significant dose-dependent increase in intestinal wall TGF- β 1 (anti-CC) immunoreactivity at both 2 and 26 weeks ($P = 0.0001$ and $P =$

0.0003, respectively). TGF- β 1 (anti-LC) showed weak immunoreactivity in intestinal epithelial cells, more pronounced in crypt cells than in villus cells. After irradiation, TGF- β 1 (anti-LC) staining was slightly increased in crypt cells. Inflammatory cells and fibroblasts in areas of ulceration, intestinal fibrosis, and subserosal thickening also stained positive.

Sham-irradiated intestine exhibited TGF- β 2 staining of epithelial cells with minimal staining of intestinal wall cells. Although there was a very slight dose-dependent increase in TGF- β 2 staining at 2 weeks ($P = 0.02$), staining had returned to control levels at 26 weeks ($P = 0.52$). Intestinal epithelial cells also stained weakly positive for TGF- β 3 in sham-irradiated intestine. Immunoreactivity of crypt epithelial cells, inflammatory cells, and fibroblasts was present 2 weeks after 21 Gy. TGF- β 3 in irradiated intestine 26 weeks after 21 Gy were similar to sham-irradiated controls. Image analysis did not reveal significant differences among treatment groups at either observation time ($P = 0.23$ and $P = 0.57$, respectively).

Figure 3. *In situ* hybridization demonstrating TGF- β 1 mRNA expression in irradiated intestine. **A:** TGF- β 1 expression in regenerating crypt at the edge of a radiation-induced mucosal ulcer 2 weeks after 21-Gy single-dose irradiation. Magnification, $\times 200$; bar, 100 μ m. **B:** Inflammatory cells and fibroblast-like cells in the base of a radiation-induced mucosal ulcer expressing TGF- β 1 2 weeks after 21-Gy single-dose irradiation. Magnification, $\times 400$; bar, 50 μ m. **C:** Intestinal wall beneath a radiation-induced mucosal ulcer (right overview; magnification, $\times 100$; bar, 100 μ m). Strong TGF- β 1 expression in smooth muscle cells (left upper panel; magnification, $\times 400$) and peritoneal mesothelium (left lower panel; magnification, $\times 400$) is seen 2 weeks after 21-Gy single-dose irradiation. **D:** TGF- β 1 expression in vascular endothelial cells and perivascular cells 26 weeks after 21-Gy single-dose irradiation. Magnification, $\times 400$; bar, 50 μ m. **E:** Fibroblasts in fibrotic area expressing TGF- β 1 mRNA 26 weeks after 21-Gy single-dose irradiation. Magnification, $\times 400$; bar, 50 μ m.

Immunoreactivity levels for TGF- β 1 (anti-CC), TGF- β 2, and TGF- β 3 are shown in Figure 5.

The extracellular matrix-associated pan-TGF- β staining pattern was similar to that of TGF- β 1 (anti-CC), and the immunoreactivity levels, as determined by image analysis, were highly significantly correlated ($r = 0.90$; $P < 0.0001$; 95% confidence interval, 0.82 to 0.98). In contrast, there was no statistically significant or biologically meaningful correlation between the intestinal wall immunoreactivity levels of pan-TGF- β and TGF- β 2 ($r = 0.24$; $P = 0.25$) or TGF- β 3 ($r = 0.13$; $P = 0.50$).

Discussion

The risk of damage to the intestine is often the main dose-limiting factor during radiotherapy for pelvic and abdominal cancer. Despite efforts to not exceed presumed tolerance doses, chronic radiation enteropathy occurs in some patients and remains a major determinant

of quality of life in a large cohort of cancer survivors. During the early phase of radiation enteropathy, epithelial and mucosal changes predominate. In contrast, progressive fibrosis of the deeper layers of the bowel wall is the hallmark feature during the chronic stage and is responsible for severe long-term morbidity. Hence, pelvic radiotherapy results in permanent impairment of gastrointestinal function in more than one-half of the patients³⁵ and serious chronic toxicity in 10 to 15%.^{36,37}

A central role in radiation-induced fibrosis in many organs has been ascribed to the potent fibrogenic and immunomodulatory cytokine TGF- β .^{1-5,17,38} Studies using exogenous addition of TGF- β and/or antibodies against TGF- β *in vivo* have confirmed that TGF- β affects epithelial cell proliferation and wound healing in the intestine^{12,39} as well as inflammation and fibrosis in other organs.⁴⁰⁻⁴³

The regulation of TGF- β production and activation is complex and involves transcriptional, translational, and

Figure 4. Extracellular matrix-associated TGF- β 1 (anti-CC) immunoreactivity in the wall of sham-irradiated and irradiated intestine. The dose-dependent increase in TGF- β 1 (anti-CC) immunoreactivity was highly statistically significant both 2 weeks ($P = 0.0001$) and 26 weeks ($P = 0.0003$) after irradiation.

post-translational mechanisms. The complexity of these regulatory processes may be responsible for the apparent inconsistencies between *in vitro* and *in vivo* data as well as for species and organ differences. For example, there are conflicting reports regarding radiation effects on TGF- β gene and protein expression. Furthermore, the three mammalian TGF- β isoforms are expressed differentially in normal tissue, exhibit different temporal and spatial shifts during the acute phase after irradiation, and differ to some extent in their putative role in the mechanisms of fibrosis. For example, radiation up-regulates TGF- β 1 mRNA expression but down-regulates TGF- β 3 mRNA expression in rat mesangial cells *in vitro*.⁴⁴ TGF- β 1, - β 2, and - β 3 all stimulate fibroblast procollagen production *in vitro*, but the isoforms are differentially expressed during bleomycin-induced lung fibrosis.⁸ Wound-healing studies have shown that the time course

Figure 5. Intestinal wall immunoreactivity for TGF- β 1 (anti-CC), TGF- β 2, and TGF- β 3 2 weeks (left) and 26 weeks (right) after 21-Gy single-dose irradiation. Very prominent TGF- β 1 immunoreactivity and minimal TGF- β 2 and TGF- β 3 immunoreactivity is seen at both observation times.

of induction and sites of expression of the TGF- β isoforms differ. Scarring in cutaneous rat wounds is reduced by neutralization of TGF- β 1 and TGF- β 2 or exogenous addition of TGF- β 3.⁴⁵

Consistent early changes in the expression of all three TGF- β isoforms were shown in our study. However, TGF- β 1 exhibited the most striking shifts in cellular sources and the most prominent long-term alterations. Our data suggest that all three isoforms play a role in early response to irradiation, ie, in regulating epithelial regeneration and inflammatory response. However, TGF- β 1 appears to be the dominant isoform during the later stages, which are characterized by progressive vascular sclerosis and extracellular matrix deposition. Our data also suggest that, during the transition from the early phase to the chronic phase of intestinal radiation injury, shifts in the cellular sources may be responsible for the tissue specificity and change in pathophysiological role of TGF- β .

The negative crypt-villus gradient of transcript level and immunoreactivity of TGF- β 1 found in the present study is consistent with other studies in rat and human intestine.^{13,46–48} In contrast, mouse intestine exhibits higher mRNA levels and more intense staining on the villi than in the crypts.^{10,49} This apparent inconsistency may reflect species differences and, with regard to immunoreactivity, differences among antibodies or fixation techniques.^{33,50,51} TGF- β 1 also exerts a dichotomous effect on the mucosa by inhibiting proliferation and stimulating migration of intestinal epithelial cells.^{11,52} It remains to be elucidated whether the net effect *in vivo* of TGF- β overexpression after irradiation is stimulation or inhibition of ulcer healing.

The significant increase in TGF- β 1 expression by endothelial cells, particularly during the chronic phase, may relate to the critical role of the vascular compartment in the pathogenesis of chronic radiation injury. TGF- β 1 down-regulates the expression of thrombomodulin, a pivotal endothelial surface anticoagulant.⁵³ Clinical and experimental studies from our laboratory have shown a significant down-regulation of endothelial thrombomodulin in submucosal vessels from irradiated intestine.^{18,54} Decreased thrombomodulin likely leads to local hypercoagulability, platelet aggregation with release of TGF- β , autoinduction of TGF- β synthesis, and additional thrombomodulin down-regulation. Up-regulation of adhesion molecules and inflammatory cell chemotaxis may further autoinduce and sustain TGF- β production.⁵⁵ These self-perpetuating processes may be important in the mechanism of chronicity of radiation toxicity. The apparent increase in TGF- β 1 mRNA in endothelial and perivascular cells from 2 weeks to 26 weeks supports this paradigm.

Our finding of sustained TGF- β 1 mRNA expression in fibroblasts in regions of fibrosis is consistent with *in vivo* studies of radiation-induced dermal fibrosis.^{2,38} TGF- β 1 is a potent fibroblast chemoattractant and mitogen and induces terminal differentiation of progenitor fibroblasts into postmitotic, collagen-producing fibrocytes *in vitro*.⁵⁶ However, our finding of intense TGF- β 1 mRNA expression in smooth muscle cells in areas of histopathological

injury is especially pertinent to the development of intestinal fibrosis. In the intestine, smooth muscle cells produce more collagen than fibroblasts, and TGF- β 1 selectively augments myocyte collagen synthesis.^{57,58} The finding of increased TGF- β 1 expression in smooth muscle cells is also consistent with recent data (unpublished) from our laboratory showing highly significant increases in collagen types I and III in muscularis mucosa and muscularis propria in irradiated rat small bowel.

Fibrotic thickening of subserosa, especially in areas of subacute and chronic ulceration, is a hallmark feature of radiation enteropathy. The current *in vivo* study showed that the peritoneal mesothelium did not express TGF- β 1 transcript during the normal condition but became strongly positive after irradiation. This was particularly apparent in areas of mucosal ulceration, corresponding to the localization of histopathological fibrosis and TGF- β 1 immunoreactivity. These observations are analogous to *in vitro* studies showing that pleural mesothelial cells produce TGF- β 1 and fibronectin, thus attracting fibroblasts and stimulating extracellular matrix deposition.^{59,60} TGF- β is the strongest chemotactic factor for mast cells.⁶¹ Hence, chemotaxis of mast cells from the peritoneal cavity mediated by mesothelial expression of TGF- β 1 may be responsible for the subserosal accumulation of connective tissue mast cells observed in radiation enteropathy.¹

The physiological significance of the prominent TGF- β 1 mRNA expression in submucosal and myenteric nerve plexus neurons remains to be elucidated. The enteric nervous system regulates intestinal secretion, absorption, motility, and immune functions, all of which are affected in radiation enteropathy. Enteric nerve plexus cells express TGF- β as well as other cytokines, including platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and interleukin (IL)-1 α ¹⁵ (and unpublished data). Changes in gut neuroendocrine products have been implicated in the pathogenesis of postradiation motility disorders.⁶² The close spatial association of enteric nerve cells and mucosal mast cells in normal bowel also supports a role for the enteric nerve system in the mechanisms of radiation enteropathy. Hence, nerve degeneration is observed in proximity of degranulating mucosal mast cells during intestinal inflammation.⁶³

The present study suggests an important role for TGF- β in the pathogenesis of radiation-induced intestinal fibrosis. However, it is not known whether persistent production of TGF- β is a direct effect of radiation or occurs secondary to, for example, mucosal ulceration or ischemia due to progressive vascular sclerosis. Additional *in vivo* studies should be performed to address this issue as well as the significance of interactions of TGF- β with other cytokines, growth factors, and cell types believed to be involved in the mechanisms of fibrosis.

Conclusion

All three TGF- β isoforms are expressed in normal enterocytes and overexpressed in regenerating intestinal epi-

thelium during the early postradiation phase. TGF- β 2 and TGF- β 3 immunoreactivity and mRNA expression do not exhibit consistent long-term changes, suggesting a role for these isoforms mainly during the initial mucosal injury. In contrast, there were prominent shifts in the cell types expressing the TGF- β 1 transcript during the early phase as well as during the chronic phase of radiation enteropathy. The shift in TGF- β 1 overexpression from epithelial cells to other cell types involved in the pathogenesis of fibrosis suggests a dominant role for TGF- β 1 in radiation enteropathy, particularly in the regulation of extracellular matrix deposition during the chronic phase.

References

1. Richter KK, Langberg CW, Sung CC, Hauer-Jensen M: Increased transforming growth factor β (TGF- β) immunoreactivity is independently associated with chronic injury in both consequential and primary radiation enteropathy. *Int J Radiat Oncol Biol Phys* 1997, 39:187-195
2. Martin M, Lefaix JL, Pinton P, Crechet F, Daburon F: Temporal modulation of TGF- β 1 and β -actin gene expression in pig skin and muscular fibrosis after ionizing radiation. *Radiat Res* 1993, 134:63-70
3. Finkelstein JN, Johnston CJ, Baggs R, Rubin P: Early alterations in extracellular matrix and transforming growth factor β gene expression in mouse lung indicative of late radiation fibrosis. *Int J Radiat Oncol Biol Phys* 1994, 28:621-631
4. Barcellos-Hoff MH: Radiation-induced transforming growth factor β and subsequent extracellular matrix reorganization in murine mammary gland. *Cancer Res* 1993, 53:3880-3886
5. Anscher MS, Crocker IR, Jirtle RL: Transforming growth factor- β 1 expression in irradiated liver. *Radiat Res* 1990, 122:77-85
6. Schmid P, Cox D, Bilbe G, Maier R, McMaster GK: Differential expression of TGF- β 1, - β 2, and - β 3 genes during mouse embryogenesis. *Development* 1991, 111:117-130
7. Unsicker K, Flanders KC, Cissel DS, Lafyatis R, Sporn MB: Transforming growth factor β isoforms in the adult rat central and peripheral nervous system. *Neuroscience* 1991, 44:613-625
8. Coker RK, Laurent GJ, Shahzeidi S, Lympny PA, du Bois RM, Jeffery PK, McAnulty RJ: Transforming growth factors- β 1, - β 2, and - β 3 stimulate fibroblast procollagen production *in vitro* but are differentially expressed during bleomycin-induced lung fibrosis. *Am J Pathol* 1997, 150:981-991
9. Robson H, Spence K, Anderson E, Potten CS, Hendry JH: Differential influence of TGF β 1 and TGF β 3 isoforms on cell cycle kinetics and postirradiation recovery of normal and malignant colorectal epithelial cells. *Int J Radiat Oncol Biol Phys* 1997, 38:183-190
10. Ruifrok AC, Mason KA, Lozano G, Thames HD: Spatial and temporal patterns of expression of epidermal growth factor, transforming growth factor α and transforming growth factor β 1-3 and their receptors in mouse jejunum after radiation treatment. *Radiat Res* 1997, 147:1-12
11. Barnard JA, Beauchamp RD, Coffey RJ, Moses HL: Regulation of intestinal epithelial cell growth by transforming growth factor type β . *Proc Natl Acad Sci USA* 1989, 86:1578-1582
12. Potten CS, Owen G, Hewitt D, Chadwick CA, Hendry H, Lord BI, Woolford LB: Stimulation and inhibition of proliferation in the small intestinal crypts of the mouse after *in vivo* administration of growth factors. *Gut* 1995, 36:864-873
13. Babyatsky MW, Rossiter G, Podolsky DK: Expression of transforming growth factors α and β in colonic mucosa in inflammatory bowel disease. *Gastroenterology* 1996, 110:975-984
14. Richter KK, Langberg CW, Sung CC, Hauer-Jensen M: Association of transforming growth factor β immunoreactivity with specific histopathologic lesions in subacute and chronic experimental radiation enteropathy. *Radiother Oncol* 1996, 39:243-251
15. Langberg CW, Hauer-Jensen M, Sung CC, Kane CJ: Expression of fibrogenic cytokines in rat small intestine after fractionated irradiation. *Radiother Oncol* 1994, 32:29-36

16. Hauer-Jensen M, Richter KK, Wang J, Abe E, Sung CC, Hardin JW: Changes in transforming growth factor β 1 (TGF- β 1) gene expression and immunoreactivity levels during development of chronic radiation enteropathy. *Radiat Res* (in press)
17. Canney PA, Dean S: Transforming growth factor β : a promotor of late connective tissue injury following radiotherapy? *Br J Radiol* 1990, 63:620-623
18. Richter KK, Fink L, Hughes BM, Sung CC, Hauer-Jensen M: Is the loss of endothelial thrombomodulin involved in the mechanism of chronicity in late radiation enteropathy. *Radiother Oncol* 1997, 44: 65-71
19. Sung CC, Wang J, Richter KK, Abe E, Langberg CW, Hauer-Jensen M: Differential expression of transforming growth factor- β (TGF- β) isoforms in experimental radiation enteropathy. *Radiat Res Soc* 1997, 45:194
20. Wang J, Richter KK, Sung CC, Hauer-Jensen M: Potential role of the mannose 6-phosphate/insulin-like growth factor II receptor in regulating transforming growth factor β 1 tissue specificity in chronic radiation enteropathy. *Radiother Oncol* (in press)
21. Hauer-Jensen M, Poulakos L, Osborne JW: Effects of accelerated fractionation on radiation injury of the small intestine: a new rat model. *Int J Radiat Oncol Biol Phys* 1988, 14:1205-1212
22. Hauer-Jensen M, Theus JW, Bradley JD, Baker ML, Langberg CW: Localized subclinical radiation enteropathy prolongs intestinal transit time: an experimental study in a rat model. *Radiat Oncol Invest* 1995, 3:157-166
23. Langberg CW, Sauer T, Reitan JB, Hauer-Jensen M: Tolerance of rat small intestine to localized single dose and fractionated irradiation. *Acta Oncol* 1992, 31:781-787
24. Scott BR, Langberg CW, Hauer-Jensen M: Models for estimating the risk of ulcers in the small intestine after localized single or fractionated irradiation. *Br J Radiol* 1995, 68:49-57
25. Hauer-Jensen M, Sauer T, Devik F, Nygaard K: Late changes following single dose roentgen irradiation of rat small intestine. *Acta Radiol Oncol* 1983, 22:299-303
26. Langberg CW, Waldron JA, Baker ML, Hauer-Jensen M: Significance of overall treatment time for the development of radiation-induced intestinal complications: an experimental study in the rat. *Cancer* 1994, 73:2663-2668
27. Hauer-Jensen M, Poulakos L, Osborne JW: Intestinal complications following accelerated fractionated x-irradiation: an experimental study in the rat. *Acta Oncol* 1990, 29:229-234
28. Langberg CW, Sauer T, Reitan JB, Hauer-Jensen M: Relationship between intestinal fibrosis and histopathologic and morphometric changes in consequential and late radiation enteropathy. *Acta Oncol* 1996, 35:81-87
29. Miller DA, Lee A, Pelton RW, Chen EY, Moses HL, Derynck R: Murine transforming growth factor- β 2 cDNA sequence and expression in adult tissues and embryos. *Mol Endocrinol* 1989, 3:1108-1114
30. Miller DA, Lee A, Matsui Y, Chen EY, Moses HL, Derynck R: Complementary DNA cloning of the murine transforming growth factor- β 3 (TGF- β 3) precursor and the comparative expression of TGF- β 3 and TGF- β 1 messenger RNA in murine embryos and adult tissues. *Mol Endocrinol* 1989, 3:1926-1934
31. Flanders KC, Thompson NL, Cissel DS, Van Obberghen-Schilling E, Baker CC, Kass ME, Ellingsworth LR, Roberts AB, Sporn MB: Transforming growth factor- β 1: histochemical localization with antibodies to different epitopes. *J Cell Biol* 1989, 108:653-660
32. Kane CJM, Knapp AM, Mansbridge JN, Hanawalt PC: Transforming growth factor β 1 localization in normal and psoriatic epidermal keratinocytes in situ. *J Cell Physiol* 1990, 144:144-150
33. Barcellos-Hoff MH, Ehrhart EJ, Kalia M, Jirtle R, Flanders K, Tsang MLS: Immunohistochemical detection of active transforming growth factor- β in situ using engineered tissue. *Am J Pathol* 1995, 147:1228-1237
34. Khalil N, O'Connor RN, Flanders KC, Unruh H: TGF- β 1, but not TGF- β 2 or TGF- β 3, is differentially present in epithelial cells of advanced pulmonary fibrosis: an immunohistochemical study. *Am J Respir Cell Mol Biol* 1996, 14:131-138
35. Yeoh E, Horowitz M, Russo A, Muecke T, Ahmad A, Robb T, Chatterton B: A retrospective study of the effects of pelvic irradiation for carcinoma of the cervix on gastrointestinal function. *Int J Radiat Oncol Biol Phys* 1993, 229-237
36. Letschert JGJ, Lebesque JV, Aleman BMP, Bosset JF, Horiot JC, Bartelink H, Cionini L, Hamers JP, Leer JWH, Van Glabbeke M: The volume effect in radiation-related late small bowel complications: results of a clinical study of the EORTC Radiotherapy Cooperative Group in patients treated for rectal carcinoma. *Radiother Oncol* 1994, 32:116-123
37. Eifel PJ, Levenback C, Wharton JT, Oswald MJ: Time course and incidence of late complications in patients treated with radiation therapy for FIGO stage IB carcinoma of the uterine cervix. *Int J Radiat Oncol Biol Phys* 1995, 32:1289-1300
38. Randall K, Coggie JE: Long-term expression of transforming growth factor TGF β 1 in mouse skin after localized β -irradiation. *Int J Radiat Biol* 1996, 70:351-360
39. Slavin J, Nash JR, Kingsnorth AN: Effect of transforming growth factor β and basic fibroblast growth factor on steroid-impaired healing intestinal wounds. *Br J Surg* 1992, 79:69-72
40. Border WA, Okuda S, Languino LR, Sporn MB, Ruoslahti E: Suppression of experimental glomerulonephritis by antiserum against transforming growth factor β 1. *Nature* 1990, 346:371-374
41. Giri SN, Hyde DM, Hollinger MA: Effect of antibody to transforming growth factor β on bleomycin induced accumulation of lung collagen in mice. *Thorax* 1993, 48:959-966
42. Wahl SM, Allen JB, Costa GL, Wong HL, Dasch JR: Reversal of acute and chronic synovial inflammation by anti-transforming growth factor β . *J Exp Med* 1993, 177:225-230
43. Wolf YG, Rasmussen LM, Ruoslahti E: Antibodies against transforming growth factor- β 1 suppress intimal hyperplasia in a rat model. *J Clin Invest* 1994, 93:1172-1178
44. Wang J, Robbins ME: Radiation-induced alteration of rat mesangial cell transforming growth factor- β and expression of the genes associated with the extracellular matrix. *Radiat Res* 1996, 146:561-568
45. Shah M, Foreman DM, Ferguson MW: Neutralisation of TGF- β 1 and TGF- β 2 or exogenous addition of TGF- β 3 to cutaneous rat wounds reduces scarring. *J Cell Sci* 1995, 108:985-1002
46. Koyama SY, Podolsky DK: Differential expression of transforming growth factors α and β in rat intestinal epithelial cells. *J Clin Invest* 1989, 83:1768-1773
47. Campbell AP, Smithson J, Lewis C, Kettlewell MG, Mortensen N, Jewell DP, McGee JO: Altered expression of TGF α and TGF β 1 in the mucosa of the functioning pelvic ileal pouch. *J Pathol* 1996, 180:407-414
48. Suemori S, Ciacchi C, Podolsky DK: Regulation of transforming growth factor expression in rat intestinal epithelial cell lines. *J Clin Invest* 1991, 87:2216-2221
49. Barnard JA, Warwick GJ, Gold LI: Localization of transforming growth factor β isoforms in the normal murine small intestine and colon. *Gastroenterology* 1993, 105:67-73
50. James JD, Hauer-Jensen M: Significance of fixation time for quantitative computerized immunohistochemistry. *Natl Soc Histotechnol* 1998, 23:P06
51. Shetye JD, Scheynius A, Mellstedt HT, Biberfeld P: Retrieval of leukocyte antigens in paraffin-embedded rat tissues. *J Histochem Cytochem* 1996, 44:767-776
52. Kurokawa M, Lynch K, Podolsky DK: Effects of growth factors on an intestinal epithelial cell line: transforming growth factor β inhibits proliferation and stimulates differentiation. *Biochem Biophys Res Commun* 1987, 142:775-782
53. Ohji T, Urano H, Shirahata A, Yamagishi M, Higashi K, Gotoh S, Karasaki Y: Transforming growth factor β 1 and β 2 induce down-modulation of thrombomodulin in human umbilical vein endothelial cells. *Thromb Haemost* 1995, 73:812-818
54. Wang J, Richter KK, Sung CC, Hauer-Jensen M: Association of chronic endothelial dysfunction with sustained transforming growth factor- β (TGF- β) overexpression and progression of experimental radiation enteropathy. *Radiat Res Soc* 1997, 45:194
55. Wahl SM, Costa GL, Mizel DE, Allen JB, Skaleric U, Mangan DF: Role of transforming growth factor β in the pathophysiology of chronic inflammation. *J Periodontol* 1993, 64:450-455
56. Rodemann HP, Bamberg M: Cellular basis of radiation-induced fibrosis. *Radiother Oncol* 1995, 35:83-90
57. Graham MF, Drucker DE, Diegelmann RF, Elson CO: Collagen synthesis by human intestinal smooth muscle cells in culture. *Gastroenterology* 1987, 92:400-405
58. Graham MF, Bryson GR, Diegelmann RF: Transforming growth factor

- $\beta 1$ selectively augments collagen synthesis by human intestinal smooth muscle cells. *Gastroenterology* 1990, 99:447–453
59. Bermudez E, Everitt J, Walker C: Expression of growth factor and growth factor receptor RNA in rat pleural mesothelial cells in culture. *Exp Cell Res* 1990, 190:91–98
60. Kuwahara M, Bijwaard KE, Gersten DM, Diglio CA, Kagan E: Mesothelial cells produce a chemoattractant for lung fibroblasts: role of fibronectin. *Am J Respir Cell Mol Biol* 1991, 5:256–264
61. Gruber BL, Marchese MJ, Kew RR: Transforming growth factor- $\beta 1$ mediates mast cell chemotaxis. *J Immunol* 1994, 152:5860–5867
62. Otterson MF, Koch TR, Zhang Z, Leming SC, Moulder JE: Fractionated irradiation alters enteric neuroendocrine products. *Dig Dis Sci* 1995, 40:1691–1702
63. Williams RM, Bienenstock J, Stead RH: Mast cells: the neuroimmune connection. *Chem Immunol* 1995, 61:208–235