Influence of Short-Term Octreotide Administration on Chronic Tissue Injury, Transforming Growth Factor β (TGF-β) Overexpression, and Collagen Accumulation in Irradiated Rat Intestine

JUNRU WANG, HUAIEN ZHENG, and MARTIN HAUER-JENSEN

Departments of Surgery and Pathology, University of Arkansas for Medical Sciences and Central Arkansas Veterans Healthcare System, Little Rock, Arkansas

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ABSTRACT

The somatostatin analog octreotide was recently found to ameliorate radiation-induced tissue injury in rat intestine. The present study addressed whether octreotide reduces chronic intestinal radiation fibrosis, whether enteroprotection is conferred by direct or indirect mechanisms, and whether the effects are dose-dependent. Using a rat model designed for fractionated irradiation, a segment of small intestine was sham-irradiated or exposed to 67.2 Gy X-radiation in 16 daily fractions. Octreotide (0, 2, or 10 μg/kg/h) was administered subcutaneously by osmotic minipumps for 4 weeks, from 2 days before to 10 days after irradiation. Tissue injury was assessed at 2 weeks (early phase) and 26 weeks (chronic phase) by quantitative histopathology and morphometry. Epithelial and smooth muscle cell proliferation was assessed by proliferating cell nuclear antigen staining; connective tissue mast cell hyperplasia by metachromatic staining; and TGF-β1 and collagen protein and mRNA by quantitative immunohistochemistry, in situ hybridization, and/or real-time fluorogenic probe reverse transcription-polymerase chain reaction. Octreotide conferred dose-dependent protection against early (p = 0.0003) and chronic (p < 0.0001) tissue injury. Octreotide abrogated radiation-induced increases in extracellular matrix-associated TGF-β (p < 0.0001), collagen I (p = 0.0001), and collagen III (p = 0.0002) immunoreactivity. Octreotide did not affect radiation-induced changes in steady-state TGF-β1 mRNA levels, mast cell hyperplasia, or smooth muscle cell proliferation. Octreotide reduced crypt epithelial cell proliferation (p = 0.01), but did not otherwise affect unirradiated intestine. Octreotide confers dose-dependent protection against delayed small bowel radiation toxicity and ameliorates radiation fibrosis predominantly by reducing acute mucosal injury. These data strengthen the rationale for using somatostatin analogs as enteroprotective agents in clinical radiation therapy.

Intestinal radiation toxicity (radiation enteropathy) is an important dose-limiting factor in radiation therapy of abdominal tumors and adversely affects the quality of life in a large cohort of cancer survivors. Depending on its clinical presentation in relation to radiation therapy, radiation enteropathy is classified as acute or delayed. Acute radiation enteropathy is a result of mitotic cell death in the intestinal crypts, disruption of the epithelial barrier, and mucosal inflammation. Delayed radiation enteropathy, on the other hand, is a chronic condition characterized by vascular sclerosis and progressive intestinal wall fibrosis.

The pathogenesis of chronic intestinal radiation fibrosis is multifactorial and involves direct radiation responses in the stromal compartment (so-called “primary” late effects), as well as indirect mechanisms secondary to acute mucosal inflammation (so-called “consequential” late effects). For example, radiation-induced overexpression of transforming growth factor β1 (TGF-β1) and connective tissue mast cell hyperplasia promote fibrosis through mechanisms that appear to be a result of stromal cell radiation responses and thus independent of mucosal barrier integrity (Zheng et al., 2000a). On the other hand, breakdown of the mucosal barrier during the acute phase of injury exposes intestinal tissue to intraluminal factors that trigger prominent inflammatory responses and endothelial dysfunction, thus causing or exacerbating tissue injury.

We recently demonstrated that octreotide, a synthetic somatostatin analog, preserves epithelial barrier function and

**ABBREVIATIONS:** TGF-β, transforming growth factor β; MMC, mucosal mast cell; PCNA, proliferating cell nuclear antigen; CTMC, connective tissue mast cell; ABC, avidin-biotin complex; RT-PCR, reverse transcription-polymerase chain reaction; SSC standard saline citrate; PAR-2, protease-activated receptor 2; Gy, gray.
reduces tissue injury in a rat model of radiation enteropathy (Wang et al., 1999). The present study examined the effect of octreotide on mucosal and stromal changes involved in the development of chronic radiation fibrosis. The results suggest that octreotide ameliorates chronic fibrosis in an indirect manner, i.e., by reducing the severity of acute radiation mucositis. These data also corroborate the notion that interventions aimed at minimizing acute toxicity effectively reduce the severity of chronic fibrosis.

Materials and Methods

Experimental Model. One hundred and thirteen male Sprague-Dawley rats (Harlan, Indianapolis, IN), 43 to 49 days of age (175–200 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 12-h 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.

After 1 week acclimatization, a previously validated surgical model for fractionated small bowel irradiation was prepared as described in detail elsewhere (Hauer-Jensen et al., 1988). The rats were fasted overnight, anesthetized, orchietomized, and an in continuity loop of ileum was sutured to the inside of the scrotum. The resulting “scrotal hernia” contained a 4-cm loop of small intestine, accessible for localized irradiation without additional surgery. With this model, the intestine remains technically within the abdominal cavity, and the surgical procedure does not cause appreciable long-term structural, functional, cellular, or molecular alterations. The model allows delivery of fractionated radiation as used in the clinic, minimizes manipulation of the intestine during irradiation, and produces radiation-induced changes similar to those seen in patients.

Octreotide administration and irradiation were started after 3 weeks postoperative recovery. Seventy-four rats scheduled for irradiation received saline vehicle (n = 28), low-dose octreotide (2 μg/kg/h, n = 25), or high-dose octreotide (10 μg/kg/h, n = 21). An additional 39 control rats were scheduled for sham-irradiation and received saline vehicle, low-dose octreotide, or high-dose octreotide.

Osmotic minipumps (models 2002 and 2 ML2; Alza Scientific Products, Palo Alto, CA) were filled with octreotide (Sandоз Pharmaceutical Corp., Earl Hanover, NJ) or 0.9% saline vehicle (for controls) and implanted in a subcutaneous pocket under methoxyflurane anesthesia (Metyfan; Pitman-Moore, Washington Crossing, NJ); 2 days before the scheduled start of irradiation. Model 2002 contained 200 μg of octreotide (1 μg/μl) and delivered 0.5 μl/h, whereas model 2 ML2 contained 1000 μg of octreotide (0.5 μg/μl) and delivered 5.0 μl/h, both over a 2-week period. Two weeks after implantation, the old minipumps were removed and new pumps filled with octreotide or vehicle were placed in a new site, for a total of 4 weeks of administration of octreotide or saline.

Irradiation was performed under methoxyflurane anesthesia as described previously (Hauer-Jensen et al., 1988). The small bowel in the scrotal hernia was sham-irradiated or exposed to a total radiation dose of 67.2 Gy, given in fractions of 4.2 Gy on 16 consecutive days without weekend break. This dose-fractionation schedule produces dose-response curves with slopes appropriate for studies of response modifiers and consistently elicits moderate-to-severe chronic radiation enteropathy. Irradiation was performed with a Seifert Isovolt 320 X-ray machine (Seifert X-Ray Corporation, Fairview Village, PA), operated at 250 kVp and 15 mA with 3-mm Al-added filtration. The resulting half-value layer and dose rate were 0.85 mm copper and 4.49 Gy/min, respectively. Details of the radiation procedure and radiation dosimetry have been reported elsewhere (Hauer-Jensen et al., 1988; Langberg et al., 1992).

Histological and Morphometric Analysis. Rats from each experimental group were killed humanely 2 and 26 weeks after the last irradiation. These observation times correspond to the early and delayed (chronic) phase of injury in our model system. Irradiated and sham-irradiated intestines were procured and snap-frozen in liquid nitrogen for RNA extraction or fixed in methanol-Carnoy’s solution for immunohistochemical and histochemical staining or in 10% formalin for in situ hybridization.

Histological and morphometric alterations of the intestinal mucosa and wall structures were assessed in a “blinded” manner as described previously (Hauer-Jensen et al., 1983; Langberg et al., 1992, 1996). In brief, radiation injury score was calculated as the sum of seven histopathological parameters of radiation enteropathy (mucosal ulceration, epithelial atypia, thickening of subserosa, vascular sclerosis, intestinal wall fibrosis, ileitis cystica profunda, and lymph congestion). Mucosal surface area was assessed according to Baddeley et al. (1986) and modified by us (Langberg et al., 1996). Intestinal wall thickness was measured with a microruler as described elsewhere (Zheng et al., 2000a).

Histologically, normal rat intestine exhibits an abundance of mucosal mast cells (MCCs), whereas connective tissue mast cells (CTMCs) are virtually absent. Radiation causes a sharp decrease in MCCs, followed by progressive CTMC hyperplasia in areas of fibrosis. Studies in c-kit mutant ( mast cell-deficient ) rats have confirmed a mechanistic role for mast cells in both early and delayed radiation enteropathy (Zheng et al., 2000a). CTMC hyperplasia was assessed in sections stained for 30 s in 0.5% toluidine blue in 0.5 N HCl and lightly counterstained. This staining protocol is specific for CTMCs (i.e., does not stain MCCs), as verified by Safranin-Astra blue staining of formalin-acetic acid-fixed specimens (Matsson, 1992). The average number of mast cells per unit area in each specimen was determined by light microscopy using a 20× objective and a 250 × 250-μm eyepiece grid, applied according to a predefined pattern in parallel columns that included serosa, muscularis, and submucosa.

Cell Proliferation Assays. Epithelial cell proliferation rate influences intestinal wound healing, radiation-induced mitotic cell death, and postradiation mucosal barrier restitution. The effect of octreotide on crypt cell cytokinetics in unirradiated intestine was examined using proliferation cell nuclear antigen (PCNA) as proliferation marker. Sections were stained immunohistochemically with anti-PCNA monoclonal antibody (NA03, 1:100 dilution; Calbiochem, Cambridge, MA); standard avidin-biotin complex (ABC) technique, diaminobenzidine chromogen; and hematoxylin counterstaining. Specificity was controlled by the omission of primary antibody, as well as by substituting primary immune antibody with nonimmune IgG (DAKO, Carpintera, CA).

The total number of intestinal crypt cells (excluding Paneth cells), number of mitotic figures, and number of PCNA-positive cells were counted in 15 longitudinally sectioned crypts per specimen. Labeling index (PCNA positive cells/total cells) and mitotic index (mitotic cells/total cells) were calculated for each crypt, and the arithmetic mean in each specimen was considered a single value for statistical calculations.

Smooth muscle cells are the predominant producers of intestinal collagen in many situations, including early radiation enteropathy. Radiation increases intestinal smooth muscle cell proliferation, collagen expression, and TGF-β expression (Wang et al., 1998). Intestinal smooth muscle cell proliferation was assessed in irradiated and unirradiated intestine by counting the number of total smooth muscle cells and PCNA-positive smooth muscle cells in 10 square areas of 62,500 μm² each, selected according to a predefined pattern. The total area counted in each specimen contained at least 1000 smooth muscle cells, and the results were normalized to PCNA-positive cells per thousand.

Quantitative Immunohistochemistry. Intestinal radiation fibrosis is characterized by increased expression of TGF-β and collagen accumulation in submucosa and subserosa (Langberg et al., 1996; Wang et al., 1998). Immunoreactivity levels for extracellular...
matrix-associated TGF-β, type I collagen, and type III collagen were assessed in methanol-Carnoy's-fixed sections using standard ABC staining technique and computerized image analysis.

Immunohistochemical staining was performed with polyclonal antibodies against TGF-β (AB-100-NA, 1:300 dilution; R&D Systems, Minneapolis, MN), type I collagen (1310-01, 1:100 dilution; Southern Biotechnology Associates, Birmingham, AL), and type III collagen (1330-01, 1:100 dilution; Southern Biotechnology Associates) and standard ABC technique.

Computer-assisted image analysis was performed with the SAMBA 4000 system (Dynatech Laboratories/Imaging Products International, Chantilly, VA). Extracellular matrix-associated TGF-β immunoreactivity was measured as described previously (Richter et al., 1997). Collagen immunoreactivity was measured according to Raviv et al. (1997). The arithmetic mean for each specimen and parameter was considered a single value for statistical purposes.

Fluorogenic Probe Reverse Transcription-Polymerase Chain Reaction (RT-PCR). TGF-β1 is overexpressed in many fibrotic conditions and is mechanistically involved in radiation enteropathy (Zheng et al., 2000b). Steady-state TGF-β1 mRNA levels in irradiated and unirradiated intestine were measured with real-time fluorogenic probe (TaqMan) quantitative RT-PCR using the ABI Prism 7700 Sequence Detection System (Perkin Elmer/ Applied Biosystems, Foster City, CA). The 26-mer fluorogenic oligonucleotide probe, 6FAM-CC AAG GGC TAC CAT GCC AAC TTT CGT-6FAM (base pairs 1353–1378), forward primer 5′ TAG GAA GGA CCT GGG TTG GAA G 3′, and reverse primer 5′ AGG GCA AGG ACC TTG CTG TAC T 3′ were designed according to the rat TGF-β sequence (Qian et al., 1990) and synthesized by Perkin-Elmer.

A TGF-β1 cDNA plasmid (gift from Dr. Michael Sporn, National Cancer Institute, Bethesda, MD) was linearized with EcoRI and in vitro RNA transcription was performed with the Riboprobe Transcription System (Promega, Madison, WI). A dilution series of the 940-base nucleotide cRNA was used to construct the standard curve.

Total RNA was extracted from intestinal specimens using TRI-Reagent solution (Molecular Research Center, Cincinnati, OH). Single-tube reverse transcription and amplification were carried out according to protocols optimized in our laboratory. Reverse transcription was carried out at 48°C for 30 min, followed by 10 min at 95°C to activate the AmpliTaq DNA polymerase. Amplification (35 cycles) was performed by denaturing at 95°C (15 s) and annealing/ extending at 60°C (1 min). All samples were run in duplicate with appropriate standards and no-template controls.

Syntactic Hybridization of Collagens I and III. Cellular sources of type I collagen and type III collagen were identified by in situ hybridization using digoxigenin-labeled riboprobes on formalinfixed tissue sections. Bluescript SK+ plasmids containing 2-kb rat type I (α1) and III (α1) collagen cDNA (gifts of Dr. Yamada, National Institutes of Health) were linearized using restriction endonucleases BamHI and Xhol for type I collagen, and EcoRI and Xhol for type III collagen. In vitro transcription and labeling to generate antisense and sense cRNA probes were performed using the DIG RNA labeling kit (Boehringer-Mannheim, Indianapolis, IN) according to the manufacturer's instructions.

All chemicals and glassware were RNase-free during pretreatment and hybridization. Tissues for in situ hybridization were fixed in 10% neutral-buffered formalin for 15 h, automatically processed overnight, embedded in paraffin, and sectioned at 4 μm. The sections were deparaffinized in xylene, rehydrated in graded ethanol and water, and digested with proteinase K (20 μg/ml) for 15 min at 37°C. The sections were then fixed in 4% paraformaldehyde for 10 min at 4°C and rinsed in 0.1 M triethanolamine-HCl (pH 8.0) and 0.25% acetic anhydride in triethanolamine-HCl (pH 8.0). Sections were incubated in prehybridization buffer (Boehringer-Mannheim) for 30 min at 37°C. Hybridization was performed overnight at 55°C. Both sense probes and hybridization buffer were used as negative controls.

After hybridization, the sections were washed in 2× SSC and 1× SSC, digested with 20 μg/ml RNase A, and then rinsed in 2× SSC, 1× SSC, and 0.2× SSC. Detection of the probes was conducted according to the instructions supplied in the DIG nucleic acid detection kit (Boehringer-Mannheim). The type I and III collagen mRNA signals were detected as dark blue or purple precipitates.

Statistical Methods. Differences in the various endpoints as a function of treatment (irradiation, sham-irradiation), response modifier (vehicle, low-dose octreotide, high-dose octreotide), and observation time (2 weeks, 26 weeks) were assessed using 3-way analysis of variance (NCSS2000; NCSS, Kaysville, UT). Post hoc testing of the effect of octreotide on the various endpoints was performed with the Jonckheere-Terpstra test (for assessment of dose dependence) or the Mann-Whitney U test (for pairwise comparisons) using exact non-parametric inference (StatXact 4; Cytel Software, Cambridge, MA). Values of p that were less than 0.05 are considered statistically significant.

Results

Structural Changes and Cell Proliferation. The effects of octreotide on histological features, mucosal surface area, intestinal wall thickness, CTMCs, and smooth muscle cell proliferation are shown in Fig. 1 and Table 1.

Irradiated intestine was histologically normal, exhibited mucosal surface area and intestinal wall thickness within normal limits compared with previous data using unperturbed rat small bowel, very few CTMCs in the bowel wall, and very low smooth muscle cell labeling index. Octreotide did not affect histology, mucosal surface area, intestinal wall thickness, intestinal CTMC numbers, or smooth muscle cell proliferation in sham-irradiated rats. Crypt epithelial labeling index in sham-irradiated intestine from rats treated with high-dose octreotide was significantly lower (40.5 ± 3.0) than in intestine from rats treated with vehicle.
TABLE 1

<table>
<thead>
<tr>
<th>Observation Time</th>
<th>XRT + veh</th>
<th>XRT + octreotide</th>
<th>Shams</th>
<th>XRT + octreotide</th>
</tr>
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<tbody>
<tr>
<td>2 Weeks</td>
<td>51.7 ± 10.1</td>
<td>58.9 ± 9.5</td>
<td>9.5</td>
<td>3.9</td>
</tr>
<tr>
<td>26 Weeks</td>
<td>31.8 ± 0.9</td>
<td>34.8 ± 0.4</td>
<td>0.9</td>
<td>0.7</td>
</tr>
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a Shams; XRT = veh, XRT = octreotide 50.9 g/kg/h, or octreotide 10 μg/kg/h, respectively.

b mm², 20 fields per slide).

c Proliferating cell nuclear antigen-positive smooth muscle cells (number of positive cells per 1000).

d Extracellular matrix-associated TGF-β immunoreactivity levels (pixel units, field size 23.124 mm², 20 fields per slide).

e Steady-state TGF-β1 mRNA levels (copies per 50 ng of RNA).

(50.2 ± 0.8, p = 0.01) or low-dose octreotide (50.9 ± 1.0, p = 0.01). The difference in crypt cell mitotic index did not reach statistical significance (data not shown).

Consistent with previous studies, histological analysis 2 weeks after irradiation revealed epithelial atypia, mucosal ulcerations, inflammation, and increased smooth muscle cell labeling index. Vascular sclerosis, intestinal wall fibrosis, and chronic ulcers were characteristic features at 26 weeks. Compared with sham-irradiated intestine and consistent with previous studies, analysis of variance showed that irradiated intestine exhibited increased radiation injury score \((p < 0.0001)\), increased bowel wall thickness \((p < 0.0001)\), reduced mucosal surface area \((p < 0.0001)\), and CTMC hyperplasia \((p < 0.0001)\) at both observation times, as well as increased smooth muscle cell labeling index at 2 weeks \((p < 0.005)\).

Post hoc testing showed that octreotide conferred highly significant, dose-dependent protection against overall intestinal tissue injury \((p = 0.003\) and \(p < 0.0001\) at 2 weeks and 26 weeks, respectively), intestinal wall thickening \((p = 0.05\) and \(p < 0.0001)\), and reduction in mucosal surface area \((p < 0.0001\) and \(p < 0.0001)\) in irradiated rats. Octreotide did not significantly affect postradiation mast cell hyperplasia or smooth muscle cell labeling index.

**TGF-β1.** The immunohistochemical staining pattern for TGF-β was similar to previous studies (Wang et al., 1998). Unirradiated intestine exhibited minimal TGF-β immunoreactivity, mainly around blood vessels and Auerbach’s nerve plexus. Irradiated intestine exhibited increased TGF-β immunoreactivity at both 2 and 26 weeks, most prominent around mid-sized vessels and newly formed capillaries, as well as in areas of intestinal wall fibrosis. Octreotide did not significantly influence TGF-β immunoreactivity at 2 weeks, mainly due to large variability in immunoreactivity levels in intestine from rats treated with vehicle or low-dose octreotide (Table 1). However, octreotide caused a highly significant, dose-dependent reduction in TGF-β immunoreactivity in irradiated intestine procured 26 weeks after irradiation \((p < 0.0001)\).

Real-time fluorogenic probe RT-PCR demonstrated increased steady-state TGF-β1 mRNA levels in irradiated intestine compared with sham-irradiated intestine at 2 weeks \((p < 0.0001)\) and, consistent with previous data (Hauer-Jensen et al., 1998), to a lesser extent at 26 weeks \((p = 0.05)\). Although TGF-β1 mRNA levels in sham-irradiated intestine were 2 to 3 times higher in octreotide-treated than in vehicletreated animals, the differences did not reach statistical significance. Octreotide administration did not significantly affect radiation-induced TGF-β1 mRNA expression at either time point (Table 1).

**Collagens.** Normal (sham-irradiated) intestine exhibited type III collagen immunoreactivity in the epithelial basement membrane, submucosa, and serosa, as well as between intestinal smooth muscle cells in the circular and longitudinal muscle layers. Type I collagen immunoreactivity was restricted to submucosa and serosa, with only slight staining of the epithelial basement membrane and smooth muscle. Octreotide treatment did not affect collagen immunoreactivity in sham-irradiated intestine.

Irradiated intestine exhibited increased type III collagen immunoreactivity at both observation times, mainly in lamina propria adjacent to the muscular mucosae, submucosa,
subserosa, and within the circular and longitudinal smooth muscle cell layers (Fig. 2). Type I collagen immunoreactivity increased in the submucosa and subserosa in fibrotic areas, but to a much lesser extent in bowel wall smooth muscle.

The results of in situ hybridization of type I and type III procollagens reflected their immunohistochemical staining patterns, demonstrating increased type I and type III hybridization signals in fibrotic areas in irradiated intestine and, in addition, prominent type III transcripts in intestinal smooth muscle cells (Fig. 3).

Computerized image analysis of immunoreactivity levels confirmed the radiation-induced increase in both types of collagen at both observation times (Fig. 4). Octreotide did not affect early postradiation collagen immunoreactivity (at 2 weeks). In contrast, octreotide caused a highly significant dose-dependent reduction in both type I and type III collagen at 26 weeks after irradiation \((p = 0.0001\) and \(p = 0.0002,\) respectively).

**Discussion**

Radiation enteropathy is a significant obstacle to uncomplicated cures in cancer therapy. Therefore, the development of effective and safe methods to protect the intestine from radiation toxicity has been a long-standing focus, both clinically and experimentally. Strategies that have shown enteroprotective effects include trophic peptide hormones, growth factors, or amino acids; cytokines or cytokine antagonists; sucralfate; prostaglandins; elemental diets, sulphydryl compounds; antioxidants; and immunomodulators. However, most of these response modifiers are associated with concerns related to efficacy, drug toxicity, and/or the possibility that the compound may also protect the tumor. There is currently no effective and safe strategy for protecting the intestine during clinical radiation therapy.

We recently demonstrated that a moderate dose of octreotide ameliorates tissue injury in irradiated rat intestine (Wang et al., 1999). Somatostatin analogs have significant potential as response modifiers in clinical cancer therapy, mainly because of their exceptional safety profile, beneficial effect on symptoms of gastrointestinal toxicity, and intrinsic antitumor and antiangiogenic properties (Weckbecker et al., 1992; Patel et al., 1994). The present study was performed to address whether the enteroprotective effect of octreotide is dose-dependent, and to examine cellular and molecular alterations involved in primary and consequential radiation fibrosis to obtain information about likely mechanisms of action.

The higher of the two doses of octreotide used in the present study \((10 \mu g/kg/h)\) conferred an impressive degree of protection, both against acute radiation enteropathy, as well as against chronic structural changes. In rats, continuous subcutaneous infusion of octreotide with osmotic minipumps at \(10 \mu g/kg/h\) produces steady-state plasma octreotide levels of 10 to 20 ng/ml. In humans, continuous subcutaneous infusion of 1.6 mg/day results in steady-state levels of 10 ng/ml. Therefore, achieving plasma levels in patients similar to the present rat study would require a dose of approximately 2 mg/day (Peter Marbach, Novartis, Basel Switzerland, personal communication, November 2000).

Somatostatin receptors are G protein-coupled receptors that are widely distributed throughout the gastrointestinal tract, including intestinal smooth muscle (Corleto et al., 1999). The cyclic octapeptide octreotide predominantly activates the type 2 receptor. Furthermore, somatostatin analogs

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**Fig. 2.** Collagen III immunohistochemistry and image analysis. Unprocessed (top) and digitally processed (bottom) images of intestinal smooth muscle from sham-irradiated (left) and irradiated (right) intestine, stained immunohistochemically for collagen III. The images demonstrate a clear increase in collagen III immunoreactivity in the smooth muscle cell layer of irradiated intestine. Original magnification, 400×.
are considered “universal gastrointestinal inhibitors” with prominent effects on intestinal secretion, motility, cell proliferation, blood flow, immune function, and bilo-pancreatic secretions. Hence, somatostatin and its analogs regulate a large number of biological processes that may affect the intestinal radiation response and/or clinical symptoms of radiation toxicity.

The present study and previous data from our and other laboratories suggest that the most likely mechanism by which octreotide ameliorates mucosal injury and subsequent fibrosis is by reducing the intraluminal content of pancreatic secretions. Quastler suggested already in the 1950s that proteolytic enzymes secreted by the exocrine pancreas exacerbate intestinal radiation toxicity (Quastler, 1956). Subsequent studies in our and other laboratories corroborated this hypothesis (Morgenstern and Hiatt, 1967; Morgenstern et al., 1970; Mulholland et al., 1984; Hauer-Jensen et al., 1985) and provided the initial rationale for a “pharmacological, reversible pancreatectomy” using somatostatin analogs. Nevertheless, it is not clear how reduced intraluminal proteolytic activity confers enteroprotection or, conversely, how pancreatic enzymes exacerbate radiation enteropathy.

Proteolytic enzymes may adversely affect radiation enteropathy in several ways. First, pancreatic enzymes may exert nonspecific proteolytic effects on cellular and extracellular tissue components during the period of postradiation epithelial barrier disruption. This is analogous to clinical and preclinical studies that demonstrate fibrosing enteropathy when pancreatic enzyme therapy is combined with agents that disrupt mucosal barrier integrity (Smyth et al., 1994; Lloyd-Still et al., 1998). Second, one may speculate that pancreatic enzymes increase crypt cell death by increasing cell proliferation rate, accelerate postradiation epithelial desquamation (analogous to trypsinizing cell cultures), or delay epithelial reconstitution by affecting the extracellular matrix “scaffold”. Third, trypsin, a serine protease and major component of the exocrine pancreatic secretions, may enhance the formation of reactive oxygen species (Bounous, 1986). This may be particularly pertinent during the latter part of a fractionated radiation schedule, when large amounts of free radicals are generated in a situation with pre-existing epithelial barrier compromise. Finally, trypsin is a major activator of the G-protein coupled receptor, protease-activated receptor 2 (PAR-2). PAR-2 is strongly expressed in normal intestine and although its exact roles in physiological and pathological processes are still incompletely understood, it appears to be involved in the regulation of epithelial and stromal cell proliferation, intestinal inflammation, and postinflammatory fi-
brosis. Hence, studies in our laboratory have shown spatial and temporal changes in the expression of PAR-2 mRNA and protein strongly suggesting involvement in radiation enteropathy (Wang et al., 2000), and that these changes can be modulated by octreotide administration (J. Wang, H. Zheng, M. D. Hollenberg, S. J. Wijesuriya, and M. Hauer-Jensen, unpublished data). Further preclinical and clinical studies are needed to determine the extent to which each of these mechanisms contributes to the enteroprotective effects of octreotide.

The cell proliferation data from the present study are in agreement with other investigators who have shown that octreotide decreases (Thompson et al., 1993; Alper et al., 1997; Turkekul et al., 1998) and growth hormone increases (Silver et al., 1999) intestinal epithelial cell proliferation, anastomotic strength, and wound healing response. Since a decreased crypt cell proliferation rate makes the intestine less susceptible to radiation injury, this observation may partly explain the beneficial effect of octreotide on early radiation mucositis. The inhibitory effect of high-dose octreotide on cell proliferation may, however, also have implications for its use in patients with recent intestinal anastomoses, when a delay in wound healing would be potentially hazardous. The observation that labeling index was affected by octreotide treatment, whereas mitotic index was not, probably reflects the lower absolute numbers and greater relative variability of the latter endpoint, rather than a differential effect on these aspects of cell proliferation. However, the issue of alterations in the length of S phase, relative size of the proliferative pool, and/or cell turnover time could be resolved by combining a mitotic arrest agent with a radioactive proliferation marker, thus allowing simultaneous determination of labeling index, grain-count histograms, and metaphase accumulation (Hauer-Jensen et al., 1992).

Octreotide increases first order jejunal flow (Pofahl et al., 1994), but impairs overall microvascular small bowel perfusion (Heuser et al., 2000). As demonstrated in experiments using degradable starch microsphere injections in rats (Forsberg et al., 1978, 1979) and cats (Lote, 1981), transient intestinal ischemia during irradiation renders the intestinal tissue relatively hypoxic and radioresistant, and ameliorates both acute mucosal injury and chronic fibrosis. It is conceivable that octreotide-induced intestinal hypoperfusion during the period of fractionated irradiation may have contributed to the reduction in injury observed in the present study. Therefore, to further support the use of octreotide in the clinic, studies in tumor-bearing animals should be performed to rule out the possibility that alterations in blood flow will jeopardize tumor control by increasing hypoxia.

Based on the results from the present study, it seems unlikely that direct influence of octreotide on stromal compartments is a major mechanism by which this compound ameliorates radiation fibrosis. The intestine is rather unique in that smooth muscle cells, not fibroblasts, are the main source of TGF-β1 and collagen IV during the early stages of many fibrotic processes, including radiation enteropathy. Somatostatin analogs inhibit expression of early response genes, proliferation, and growth factor production in smooth muscle cells (Bauters et al., 1994; Hayry et al., 1996; Sakamoto et al., 1998), which may influence radiation enteropathy. The modest (and nonsignificant) influence on radiation-induced smooth muscle cell proliferation observed in the present study suggests that, at least for octreotide, these effects may not be major antifibrotic mechanisms in the context of intestinal radiation injury. Since postirradiation increases in TGF-β1 immunoreactivity occur with both primary and consequential chronic injury (Richter et al., 1997), the effect of octreotide on TGF-β1 immunoreactivity levels in the present study is also consistent with this notion. This observation that steady-state TGF-β1 mRNA levels did not correlate with TGF-β1 immunoreactivity levels are in agreement with previous studies from our and other laboratories and may be due to differences in mRNA translation, post-translational processing, TGF-β activation, shifts in cellular sources of TGF-β, and feedback mechanisms (Hauer-Jensen et al., 1998; Wang et al., 1998; Zheng et al., 2000b). Furthermore, although not statistically significant, the 2- to 3-fold increase in TGF-β1 mRNA levels in octreotide-treated compared with vehicle-treated sham-irradiated rats is of similar magnitude as reported by Huynh et al. (2000). Although we do not know whether the increase in TGF-β1 occurs by a direct or indirect mechanism, our data suggest that increased TGF-β1 expression by epithelial cells in octreotide-treated animals may contribute to decreased proliferation. Finally, the lack of a difference in connective tissue mast cell hyperplasia between octreotide-treated and vehicle-treated rats, despite the critical role of these cells in the mechanisms of primary intestinal radiation fibrosis (Zheng et al., 2000a), also supports the notion that octreotide exerts its enteroprotective effects by minimizing mucosal injury, i.e., by ameliorating the consequential (indirect) aspects of chronic fibrosis.

Conclusions

High-dose octreotide confers striking, dose-dependent protection against intestinal radiation fibrosis. The mechanisms by which octreotide ameliorates chronic radiation fibrosis appear to be mainly indirect, by reducing acute mucosal injury, rather than by directly affecting stromal processes. These data strengthen the rationale for the use of somatostatin analogs as enteroprotective agents in clinical radiation therapy.

References


