Bacterial cell wall polymers promote intestinal fibrosis by direct stimulation of myofibroblasts

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Van Tol, Eric A. F., Lisa Holt, Feng Ling Li, Feng-Ming Kong, Mitsu Yamauchi, Jolanta Puciloswka, P. Kay Lund, and R. Balfour Sartor. Bacterial cell wall polymers promote intestinal fibrosis by direct stimulation of myofibroblasts. Am. J. Physiol. 277 (Gastrointest. Liver Physiol. 40): G245–G255, 1999.—Normal luminal bacteria and bacterial cell wall polymers are implicated in the pathogenesis of chronic intestinal inflammation. To determine the direct involvement of bacteria and their products on intestinal fibrogenesis, the effects of purified bacterial cell wall polymers on collagen and cytokine synthesis were evaluated in intestinal myofibroblast cultures established from normal fetal and chronically inflamed cecal tissues. In this study, the intestines of Lewis rats were intramurally injected with peptidoglycan-polysaccharide polymers. Collagen and transforming growth factor (TGF)-β1 mRNA levels were measured and correlated with mesenchymal cell accumulation by immunohistochemistry. The direct effects of cell wall polymers on fibrogenic cytokine and collagen α1 (type I) expression were evaluated in intestinal myofibroblast cultures. We found that intramural injections of bacterial cell wall polymers induced chronic granulomatous enterocolitis with markedly increased collagen synthesis and concomitant increased TGF-β1 and interleukin (IL)-6 expression. Intestinal myofibroblast cultures were established, which both phenotypically and functionally resemble the mesenchymal cells that are involved in fibrosis in vivo. Bacterial cell wall polymers directly stimulated collagen α1 (I), TGF-β1, IL-1β, and IL-6 mRNA expression in the intestinal myofibroblasts derived from both normal and inflamed cecum. Neutralization of endogenous TGF-β1 inhibited in vitro collagen gene expression. From our results, we conclude that increased exposure to luminal bacterial products can directly activate intestinal mesenchymal cells, which accumulate in areas of chronic intestinal inflammation, thus stimulating intestinal fibrosis in genetically susceptible hosts.

intestinal myofibroblast; Lewis rats; experimental colitis

THE CHRONIC INFLAMMATORY bowel diseases, ulcerative colitis and Crohn’s disease, are multifactorial processes characterized by abnormally aggressive immune responses in genetically susceptible hosts (39). Recent studies implicate normal intestinal bacteria in the development of chronic enterocolitis (35, 40), possibly through a mechanism involving the breakdown of peripheral tolerance to autologous intestinal flora (9). Intestinal bacteria and their products induce inflammation in different models of experimental enterocolitis, and increased immune reactivity against resident luminal bacteria has been shown in patients with inflammatory bowel disease (3, 9, 19, 23, 25). In the present study, we investigated the role of the bacterial cell wall polymers peptidoglycan-polysaccharide (PG-PS) in immune-mediated intestinal fibrosis and activation of intestinal myofibroblasts. PG-PS are structural components of almost all bacteria and share many immunologic properties with lipopolysaccharides (LPS) (43). Intramural injection of PG-PS from group A streptococci (PG-APS) causes a biphasic, spontaneous relapsing granulomatous enterocolitis and fibrosis in genetically susceptible Lewis rats with immunopathological features resembling Crohn’s disease (41, 60). This development of chronic granulomatous enterocolitis and associated fibrosis depends on the long-term presence of these poorly degradable cell wall polymers (42).

Stricture formation with subsequent obstruction is one of the most common complications of Crohn’s disease. In these patients, abundant collagen deposition is found in stenotic segments (14), accompanied by increased transcription and submucosal concentrations of various types of collagen, including collagen type I (14, 27). Mesenchymal cells isolated from the strictured intestinal segments of Crohn’s disease patients have increased spontaneous and transforming growth factor (TGF)-β1-stimulated collagen synthesis (45). TGF-β1 is one of the most potent fibrogenic cytokines implicated in arthritis, hepatic cirrhosis, glomerulonephritis, pulmonary fibrosis, and pancreatitis (5, 26, 29, 31, 38, 44, 53, 59). TGF-β1 stimulates extracellular matrix formation, is a potent chemoattractant for monocytes and fibroblasts, and stimulates interleukin (IL)-1β mRNA expression (16, 29, 31, 34, 56, 57). Local administration of TGF-β1 stimulates fibrosis associated with chronic inflammation in various organs (7, 38, 44, 53), whereas neutralization of TGF-β1 reduces inflammation and matrix deposition (4, 6, 55). Hence, despite its beneficial role in tissue regeneration and wound healing, chronic TGF-β1 production in inflammatory foci may cause pathogenic fibrosis.

Despite strong indications for a pathogenic role of normal intestinal bacteria in clinical and experimental intestinal inflammation, little is known about the role of bacteria and their products in the development of fibrosis associated with chronic enterocolitis. We therefore investigated whether bacterial cell wall polymers used to induce chronic granulomatous enterocolitis directly affect the synthesis of collagen α1 (type I) in cultured intestinal myofibroblasts and the in vivo and in vitro expression of cytokines involved in fibrosis such as TGF-β1, IL-1β, and IL-6. We present evidence for the direct action of normal luminal bacterial products in the activation of intestinal myofibroblasts, which could
contribute to the development of fibrosis during chronic intestinal inflammation.

MATERIALS AND METHODS

Induction of enterocolitis. Sterile PG-APS polymers were prepared from group A, type 3 strain D58 Streptococcus pyogenes (46). The preparation was sonicated immediately before use, and the concentration was calculated based on rhamnose content (8). Experiments were performed with female, inbred, specific-pathogen-free Lewis rats (145–160 g, Charles River Laboratories, Raleigh, NC) in compliance with regulations from our Institutional Animal Care and Use Committee. Animals were anesthetized with 1.3 ml/kg body wt innovar (Pitman-Moore, Washington Crossing, NJ). Intestines were exposed by aseptic laparotomy and subserosally injected with PG-APS (n = 6, 12.5 µg rhamnose/g body wt) or human serum albumin (HSA; n = 5, 37.5 µg/g body wt, Baxter Health Care) into seven sites within the distal ileum and cecum, including the lymphoid aggregate at the cecal tip, the midcecum, the junction of the mesentery and distal ileum, and two distal ileal Peyer’s patches, causing chronic granulomatous enterocolitis as described previously (28, 41). The animals were killed during the chronic phase of inflammation (17 and 26 days after PG-APS injection), and both macroscopic and histological inflammation were evaluated.

Tissue collection and processing. Cecal tissue samples were snap frozen and stored at −80°C for isolation of RNA and protein. Formalin-fixed, paraffin-embedded sections from the cecal tip were used to study collagen deposition using Masson’s trichrome staining and for immunohistochemical localization of TGF-β1, α-smooth muscle actin (α-SMA), and vimentin (as detailed in Immunohistochemistry). Total RNA was isolated from cecal tissue by the guanidine thiocyanate method (28).

Intestinal (myo)fibroblast cultures and Rat-1 cell line. Normal intestinal tissue from neonatal Lewis rats was dissected and washed in DMEM-F12 with 2 mM L-glutamine and 10 mM HEPES with 10 times the usual concentration of antibiotics (penicillin, 1,000 U/ml), streptomycin (1 mg/ml), and amphotericin B (2.5 µg/ml). The tissue was cut into small pieces, washed, and centrifuged in a tabletop centrifuge at 500 rpm for 1 min to select for tissue fragments, and the supernatant was then decanted. This procedure was repeated four times to minimize bacterial contamination, and the remaining small explants were seeded into 75-cm² tissue culture flasks in RPMI 1640 medium supplemented with antibiotics (10×) and 20% FCS in 5% CO₂ at 37°C. After 24 h, the tissue fragments were collected, transferred to a new flask, and cultured for two to three more days, after which adherent and remaining floating tissue fragments were rinsed off and discarded. The outgrowing cells were cultured in DMEM-F12 growth medium with L-glutamine, HEPES, 10% FCS, and antibiotics (1×). After at least four passages, clusters of cells were selected based on a more discoid than spindle-shaped morphology and grown on culture chamber slides (Lab-Tek, Nunc, Naperville, IL) to permit phenotypic characterization. Cells were characterized by immunostaining with specific antibodies to α-SMA, vimentin, and TGF-β1.

Myofibroblasts from chronically inflamed tissues were isolated from cecal granulomas of Lewis rats 24 days after PG-APS injection. Granulomas were carefully dissected from tissues and washed in medium as described above. Explants were seeded on collagen IV-coated filters in hormonally defined medium (36) supplemented with 5% FCS and antibiotics (1×). Outgrowing cells were cultured in DMEM-F12 with L-glutamine, HEPES, 2% FCS, and antibiotics (1×) as described above. A control Rat-1 embryonic fibroblast cell line was maintained in DMEM-F12 culture medium with 5% FCS, L-glutamine, HEPES, and antibiotics.

Immunohistochemistry. Immunostaining was performed on paraformaldehyde-fixed, paraffin-embedded sections of the cecal tip from PG-PS- or HSA-injected Lewis rats or on intestinal myofibroblasts that were cultured in chamber slides and postfixed in 4% paraformaldehyde. Primary antibodies used were as follows: anti-TGF-β1 rabbit polyclonal antibody for immunohistochemistry (21) and immunofluorescence using confocal imaging (SC146; Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:50–1:100; anti-α-SMA and anti-vimentin mouse monoclonal antibodies (Dako, Carpenteria, CA) at dilutions of 1:25–1:100. Normal rabbit serum and mouse IgG were used as negative controls for antibody specificity. In addition, detailed characterization of the specificity of the TGF-β antibody has been reported previously, including selective blockade of immunostaining with TGF-β1 and not other TGF-β family members as well as specific recognition of TGF-β1 on Western blots (20). Indirect immunostaining was performed using an avidin-biotin peroxidase complex (ABC) kit (Vector, Lab, Burlingame, CA) or by indirect immunofluorescence and confocal microscopy. In each case, sections were preincubated with 2% blocking serum (normal goat serum) before incubation with primary antibodies overnight at 4°C. Sections were then washed in PBS. For ABC immunostaining, sections were incubated with biotinylated goat anti-rabbit IgG or biotinylated rat adsorbed horse anti-mouse IgG (1:400 dilution; Vector Lab) for 45 min, washed in PBS, and then incubated with the avidin and biotin components of the Vectastain kit for 45 min. Red precipitation product was then developed using an aminoethylcarbazole substrate kit (Zymed Labs, San Francisco, CA). Sections were counterstained with Gill’s hematoxylin (Fisher Scientific) and coverslipped with Crystal/Mount (Biomeda, Foster City, CA). For indirect immunofluorescence and colocalization of TGF-β1 and α-SMA, sections were incubated simultaneously with both primary antibodies and then primary antibodies were localized using Cy2-conjugated AffiniPure goat anti-rabbit IgG and Cy5-conjugated AffiniPure goat anti-mouse IgG, both from Jackson Immunoresearch (West Grove, PA). Digital images of immunofluorescent staining were obtained via a Leica TCS 4D confocal microscope.

In vitro stimulation of myofibroblasts with PG-APS and TGF-β1. Cultures of subconfluent intestinal myofibroblasts were switched from growth medium to DMEM-F12 medium supplemented with 0.5% serum, L-glutamine, HEPES, and antibiotics for 24 h. The cultures were incubated simultaneously with both primary antibodies and then primary antibodies were localized using Cy2-conjugated AffiniPure goat anti-rabbit IgG and Cy5-conjugated AffiniPure goat anti-mouse IgG, both from Jackson Immunoresearch (West Grove, PA). Digital images of immunofluorescent staining were obtained via a Leica TCS 4D confocal microscope.

Fig. 1. Bacterial cell wall injection induces in vivo fibrosis associated with granulomatous enterocolitis in genetically susceptible rats. Autoradiogram of a Northern blot hybridization experiment showing markedly increased collagen α1(I) mRNA in inflamed cecal tissues of Lewis rats 26 days after subcutaneous injection of peptidoglycan-polysaccharide polymers obtained from group A streptococci (PG-APS) compared with the noninflamed human serum albumin (HSA)-injected control animals. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control.
collagen and cytokine gene expression, and total RNA was isolated using the TRIzol method (GIBCO).

In vitro blockade of endogenous TGF-β1 in PG-APS-stimulated myofibroblasts. Subconfluent intestinal myofibroblasts from neonatal Lewis rats were cultured in DMEM-F12 medium supplemented with 10% serum, antibiotics, L-glutamine, and HEPES. Cells were exposed to rabbit anti-TGF-β1 polyclonal antibody (0–100 ng/ml, R&D Systems) for 1 h and then stimulated with or without PG-APS (40 µg/ml) for 8 h. Total RNA was isolated from the cells by
the TRIzol method and used to evaluate collagen gene expression.

Northern blotting. Samples of 2.5–10 µg of total RNA extracted from cecal tissues and myofibroblast and Rat-1 cultures were electrophoresed in a 14% agarose-formaldehyde gel. The size-fractioned RNA was transferred to a nylon membrane (Hybond-N, Amersham Life Sciences) and hybridized with a [32P]dCTP-labeled (3,000 Ci/mmol; ICN, Costa Mesa, CA) 1300-bp cDNA probe encoding rat collagen α1 (I). Northern hybridization of rat mRNA reveals two different molecular weight transcripts of rat α1 (I) collagen of 4.7 and 5.7 kb, which is characteristic for rat collagen α1 (I) as described by Genovese et al. (12). Hybridizations were performed in Rapid-Hyb buffer (Amersham) for 2–3 h at 65°C followed by washing under high-stringency conditions to reduce background using variable (2–0.1×) concentrations of sodium chloride-sodium phosphate-EDTA buffer with 0.1% SDS. The membranes were exposed to Kodak X-OMAT film (Kodak, Rochester, NY) at −80°C using an intensifier screen. Blots were reprobed with a 32P-labeled commercially available rat pTri-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (Ambion, Austin, TX).

RT-PCR. For semiquantitative analysis of TGF-β1, IL-1α, IL-6, and actin mRNAs, 1 µg of total RNA from cecal tissues and cell cultures was reverse transcribed in a volume of 50 µl containing 200 units of Moloney murine leukemia virus RT and transcription buffer (GIBCO BRL, Gaithersburg, MD), 10 mM dithiothreitol (GIBCO), 1 mM dNTP (Perkin-Elmer/ Applied Biosystems, Foster City, CA), 1 mM random hexamers (Perkin-Elmer), and 0.5 U/µl RNase inhibitor (Perkin-Elmer). PCR amplifications were performed using a GeneAmp PCR system 9600 (Perkin-Elmer) in a 50-µl reaction volume containing 1 µl of first-strand cDNA, 0.1–0.25 mM of each antisense and sense primer, 0.1–0.25 mM dNTP (Perkin-Elmer), and 0.5 U/µl RNase inhibitor (Perkin-Elmer). PCR amplifications were performed using a GeneAmp PCR system 9600 (Perkin-Elmer) in a 50-µl reaction volume containing 1 µl of first-strand cDNA, 0.1–0.25 mM of each antisense and sense primer, 0.1–0.25 mM dNTP (Perkin-Elmer), 1.5 units of Taq polymerase (Perkin-Elmer), and buffer (Perkin-Elmer). For each PCR reaction, the optimal amplification conditions for primer and dNTP concentrations were worked out within the indicated ranges. Primers for TGF-β1 (product size 405 bp) were sense 5′-CGTCGAGTGGACCTGGGAC and antisense 5′-CTCCACCTGGGGCTTGGGC, for IL-1α (size 543 bp) sense 5′-GCTACCTATGTCCTTGCCGT and antisense 5′-GACCATGGCTGTTCCTAGG, for IL-6 (size 504 bp) sense 5′-CTCCAGCGAGTGGCTCTT and antisense 5′-GAGGACCTGGGAGTGTTGG, and for β-actin (size 281 bp) sense 5′-ACCACAGCTGAGGAAATCG and antisense 5′-AGAGGCTTTACGGATGTCACAG. Conditions for amplification were 94°C for 45 s and 58°C for 1 min, followed by 72°C for 1.5 min, and samples were evaluated and compared in the linear range of amplification. Optimal cycle number for each cytokine is listed in Figs. 6 and 7. PCR products were analyzed in agarose gels. β-Actin mRNA served as a control to ensure that any observed changes in abundance of TGF-β1, IL-1α, or IL-6 mRNAs were specific. Specificity was confirmed by size and restriction enzyme digests and by including a water control instead of the first-strand cDNA.

Statistics. Data are presented as means ± SE, and differences between the groups were analyzed using the two-tailed Student’s t-test.

RESULTS

Collagen α1 (I) expression during chronic granulomatous enterocolitis. Collagen α1 (I) mRNA expression was examined in cecal tissues by Northern blot hybridization (Fig. 1). Marked increases of tissue collagen α1 (I) expression occurred in all animals with PG-APS (n = 6)-induced chronic intestinal inflammation compared with low constitutive expression levels in the cecum of noninflamed HSA (n = 4)-injected control animals (Fig. 1). Quantitation of the collagen α1 (I) mRNA expression in the Northern blot experiments revealed a sixfold increase (3.28 ± 0.52 inflated vs. 0.53 ± 0.1 control, P = 0.0026) in chronically inflamed cecal tissues when normalized for GAPDH expression. This increase in active collagen synthesis during PG-APS-induced chronic granulomatous enterocolitis in Lewis rats is in agreement with our previous findings of significantly increased fibrillar collagen in chronically inflamed cecal tissue homogenates (54).

Localization of collagen, TGF-β1, and mesenchymal cells in chronically inflamed cecal tissues of PG-APS-treated rats. Masson’s trichrome stain showed collagen deposition to be abundant in the submucosal layer of chronically inflamed cecum with distinct accumulation of collagen in the mesenchymal rim of granulomas (Fig. 2A). Immunohistochemistry showed TGF-β1 staining to be localized adjacent to areas of collagen deposition surrounding the granulomas in areas of mesenchymal cell accumulation (Fig. 2B). In addition, aggregates of intense TGF-β1-positive mononuclear cells with a macrophage-like morphology were found scattered throughout the mucosa and submucosa (Fig. 2C) as well as in areas of subserosal granulomatous inflammation (data not shown). Control incubations using normal rabbit serum or rabbit IgG were uniformly negative.

Antibodies against cytoskeletal filaments were used on adjacent sections of cecum from PG-APS-injected...
Fig. 4. Phenotypic characterization of cultured intestinal mesenchymal cells isolated from the cecum of a neonatal Lewis rat after 4 passages shows the abundant expression of both α-SMA-positive (A, ×200) and vimentin-positive (B, ×200) filaments. All cells were found to express these markers, although intensity of the staining varied between cells as shown in this representative picture. Isotype control antibody staining (C, ×100) was uniformly negative.
rats to determine the phenotype of the mesenchymal cells in the capsule surrounding the mature granulomas in areas of dense collagen deposition. These mesenchymal cells stained positive for \( \alpha \)-SMA (Fig. 2D) and vimentin (Fig. 2E), which is typical of myofibroblasts, whereas the control staining with mouse IgG isotype was negative (Fig. 2F). Vascular smooth muscle cells (arrowheads in Fig. 2D) stain only for \( \alpha \)-SMA.

Because initial immunohistochemistry indicated that sites of TGF-\( \beta \) localization corresponded to areas of mesenchymal cell accumulation and overlapped with sites of \( \alpha \)-SMA staining, confocal microscopy was performed to localize these antigens on the same section incubated simultaneously with \( \alpha \)-SMA and TGF-\( \beta \). As shown in Fig. 2, G-I, this staining revealed colocalization of TGF-\( \beta \) and \( \alpha \)-SMA in mesenchymal cells at the periphery of granulomas.

RT-PCR analysis of TGF-\( \beta \) and IL-6 mRNA expression. RT-PCR analysis of TGF-\( \beta \) and IL-6 mRNA expression in the cecum of HSA- or PG-APS injected animals is shown in Fig. 3. Cecal tissue samples taken from animals with PG-APS-induced chronic granulomatous enterocolitis had 2.5-fold higher TGF-\( \beta \) mRNA levels when normalized for actin mRNA compared with HSA-injected control animals \([\text{in densitometry units:} 23 \pm 4 \,(n = 6) \text{ vs.} 9 \pm 2 \,(n = 4); P = 0.02]\). The increased TGF-\( \beta \) mRNA expression corresponded with the increased abundance of TGF-\( \beta \)-positive cells in the chronically inflamed cecum as determined by immunohistochemical staining. The PG-APS-induced chronic granulomatous inflammation was also characterized by dramatic induction or upregulation of IL-6 mRNA levels in HSA-injected control animals that had no macroscopic or histological signs of intestinal inflammation. Interestingly, relatively low levels of IL-6 mRNA in two of the PG-APS-injected rats (Fig. 3, lanes 5 and 6) corresponded with low cecal granuloma scores.

Primary intestinal myofibroblast cultures from neonatal Lewis rats. To address the direct effects of the bacterial cell wall polymers on the function of intestinal mesenchymal cells, we established intestinal myofibroblast culture systems from both normal and chronically inflamed cecum from Lewis rats. Immunohistochemical studies with these mesenchymal cells were done after at least four passages in culture to ensure uniformity of the phenotype of the cells tested. The functional experiments were all done between passages 6 and 12 and only after phenotyping the cultures for cytoskeletal filament expression. The cells were found to have a typical stellate to discoid appearance when cultured at low density and showed a stratified growth pattern on reaching confluency. There were vast bundles of cytoskeletal filaments.
plasmic microfilaments and abundant expression of α-SMA (Fig. 4A) and vimentin (Fig. 4B) as shown by immunohistochemical staining. This confirms a myofibroblast phenotype similar to that described by Valentich et al. (51) for differentiated human intestinal myofibroblasts. There was no staining when sections were incubated with control mouse IgG (Fig. 4C) or rabbit IgG (not shown).

In vitro effects of PG-APS and TGF-β1 on collagen and fibrogenic cytokine synthesis. Functional studies tested the ability of PG-APS and TGF-β1 to activate intestinal myofibroblasts. Rat-1 fibroblasts were first used as positive controls to confirm the capacity of TGF-β1 to stimulate collagen gene expression (Fig. 5A). PG-APS stimulated collagen α1(I) mRNA levels in the Rat-1 embryonic fibroblasts, whereas IL-1β and corticotropin-releasing hormone, representing a cytokine and neuropeptide that are upregulated in the inflamed cecal tissue (28, 54), had no effect (Fig. 5A). The abilities of PG-APS and TGF-β1 to stimulate collagen and cytokine mRNA expression in intestinal myofibroblast cultures were then evaluated. Myofibroblasts from a normal cecum showed high constitutive levels of collagen α1(I) mRNA expression, but PG-APS and TGF-β1 both stimulated an increase in collagen α1(I) expression in a time-dependent fashion (Fig. 5, B and C). Myofibroblasts cultured from PG-APS-induced cecal granulomas were also examined for effects of PG-APS and TGF-β1 on expression of collagen mRNA. PG-APS and TGF-β1 stimulated collagen α1(I) mRNA expression in these myofibroblasts, showing similar kinetics of upregulation as the fetal cecal myofibroblasts (Fig. 5D). Peak collagen stimulation at 8 h was approximately twofold the unstimulated values for PG-APS and threefold for TGF-β1.

The effects of PG-APS on the expression of fibrogenic cytokines by cultured myofibroblasts were further investigated by RT-PCR. The stimulatory effect of TGF-β1 was also addressed, since this cytokine was found to be abundantly present in areas of myofibroblast accumulation during chronic granulomatous inflammation. PG-APS increased the expression of TGF-β1, IL-1β, and IL-6 mRNAs in intestinal myofibroblast cultures from fetal tissues, and TGF-β1 induced expression of TGF-β1 and IL-1β mRNA (Fig. 6). The kinetics and degree of cytokine responses were similar in the fetal and inflamed myofibroblast cells in that PG-APS also rapidly induced expression of IL-1β, TGF-β1, and IL-6 mRNA in myofibroblasts isolated from granulomas (Fig. 7). TGF-β1 upregulated IL-1β and IL-6 mRNA in these cells (Fig. 7) as observed in myofibroblast cultures from normal cecum (Fig. 6). Peak induction at 8 h after TGF-β1 stimulation was 14.8 ± 6.7-fold (IL-1β) and 13.6 ± 6.6-fold (IL-6) higher than unstimulated values in three separate experiments in myofibroblasts isolated from granulomas. TGF-β1 autinduction of TGF-β1 mRNA in these cells was, however, modest (2.7 ± 1.0-fold increased relative to unstimulated).

Because PG-APS upregulated both TGF-β1 and collagen mRNA expression and TGF-β1 independently stimulated collagen expression in intestinal myofibroblasts, we next determined whether induction of TGF-β1 was required for PG-APS-stimulated collagen gene expression in vitro. Neutralization of endogenous TGF-β1 inhibited both constitutive and PG-APS-stimulated collagen α1(I) mRNA expression in a dose-responsive fashion (Fig. 8). PG-APS-stimulated collagen expression was inhibited to a greater extent than constitutive collagen synthesis. Blockade of endogenous TGF-β1 inhibited PG-APS-stimulated collagen synthesis by 70% to below constitutive levels.

**DISCUSSION**

The present observations in PG-APS-induced chronic granulomatous enterocolitis in Lewis rats and in cultured intestinal myofibroblasts prompt us to propose a role for bacterial cell wall products in inflammation-induced intestinal fibrosis. We suggest that activated cells with a myofibroblast phenotype typified by expression of α-SMA and vimentin are directly responsive to bacterial stimulation and are important mediators of...
increased collagen synthesis and deposition in the chronically inflamed rat intestine. In vitro cultures were set up with myofibroblasts isolated from both normal fetal cecum and PG-APS-induced cecal granulomas. These intestinal myofibroblasts can be stimulated to synthesize collagen \( \alpha_1 \) (I), TGF-\( \beta_1 \), IL-1\( \beta \), and IL-6 by direct actions of bacterial products such as PG-APS as well as in response to TGF-\( \beta \). Stimulation of collagen expression by PG-APS in these myofibroblasts was dependent on autocrine production of TGF-\( \beta_1 \).

Our results are consistent with other studies that demonstrate that LPS, IL-1\( \beta \), or tumor necrosis factor-\( \alpha \) can induce mesenchymal cells to secrete proinflammatory cytokines. However, these previous studies did not relate to intestinal fibrogenesis. For example, LPS stimulates granulocyte monocyte-colony stimulating factor, IL-1\( \alpha \), IL-1\( \beta \), IL-6, IL-8, and intercellular adhesion molecule 1 expression in human duodenal fibroblasts (32) and stimulates IL-6 and platelet-activating factor secretion in hepatic myofibroblasts (33, 49). Similarly, LPS from Bacteroides stimulates IL-1\( \alpha \) and IL-6 secretion by human gingival fibroblasts (48). Mourelle et al. (30) found that colonic anaerobic bacteria, including Bacteroides species, injected into the bowel wall, stimulate TGF-\( \beta_1 \) secretion and collagen deposition. Our studies demonstrate that bacterial products directly stimulate expression of TGF-\( \beta \) and collagen in intestinal mesenchymal cells in vitro. Although group A streptococci are not found in the intestine, PG-APS is used as a prototype of poorly degradable PG-PS polymers. PG-PS from group D streptococci (Enterococci) and Eubacterial species found in the intestine are also capable of inducing chronic granulomatous inflammation (43, 46).

The effects of TGF-\( \beta_1 \) stimulation on collagen mRNA expression in the intestinal myofibroblasts are consistent with other cell culture systems in which TGF-\( \beta_1 \) upregulates collagen synthesis (2, 13, 38). Moreover, we found that TGF-\( \beta_1 \) increases its own expression in intestinal myofibroblasts, probably through the previously documented autoregulatory stimulation of distinct TGF-\( \beta_1 \) promoter sites (18).

Constitutive expression of IL-1\( \beta \) mRNA in the cultured intestinal myofibroblasts is low; however, the kinetics of IL-1\( \beta \) mRNA upregulation by PG-APS and TGF-\( \beta_1 \) resemble IL-1\( \beta \) expression by these stimuli in monocytes and macrophages (28, 56). In addition, there was a marked stimulation of IL-6 mRNA by PG-APS in cultured myofibroblasts from normal or chronically inflamed cecal tissues. These observations suggest that activated myofibroblasts in areas of intestinal inflammation may attract other mesenchymal cells due to increased secretion of chemotactic IL-6 on bacterial stimulation. Further studies will be of interest to assess whether TGF-\( \beta_1 \) or IL-1\( \beta \) plays an intermediate role in PG-APS stimulation of IL-6 mRNA expression. Increased IL-6 synthesis may not only aggravate inflammation but may also enhance fibrosis through its inhibitory effect on collagen-degrading enzymes (50).

The PG-APS-induced stimulation of TGF-\( \beta_1 \) mRNA in cultured myofibroblasts and protein synthesis in monocytes (26), together with the in vivo production of TGF-\( \beta_1 \) by mononuclear cells and myofibroblasts in areas of collagen deposition, point to a regulatory role of TGF-\( \beta_1 \) in intestinal fibrosis. Indeed, our data show that the increased collagen \( \alpha_1 \) (I) gene expression during PG-APS-induced chronic enterocolitis was accompanied by increased cecal TGF-\( \beta_1 \) and IL-6 mRNA expression. Moreover, abundant TGF-\( \beta_1 \) immunoreactivity was detected in clusters of macrophage-like cells
and in inflammatory cells associated with the granulomas, which is consistent with studies in hepatic granulomas by Manthey et al. (26). Locally increased TGF-β1 expression has also been described in other models of inflammation-induced fibrosis, with mesenchymal cells and monocytes/macrophages being the most abundant sources of endogenous TGF-β1 (1, 17, 26, 53, 59).

We found that areas of collagen deposition within PG-APS-induced granulomas colocalize with mesenchymal cells expressing α-SMA and vimentin, suggesting that intestinal myofibroblasts mediate in vivo collagen deposition and fibrosis. In situ, these mesenchymal cells have the same myofibroblast characteristics, i.e., expression of α-SMA and vimentin, because the cells were isolated and characterized from normal and inflamed colon. Myofibroblasts are implicated as mediators of fibrosis in various models of inflammation in other organs (7, 21, 44, 59). Consistent with the concept that TGF-β1 causes differentiation of fibroblasts into myofibroblasts (7, 11, 58), we found abundant TGF-β1 immunoreactivity in areas adjacent to or coincident with α-SMA and vimentin staining mesenchymal cells in the capsule of intestinal granulomas. Confocal microscopic analysis colocalized TGF-β1 and α-SMA in a subpopulation of myofibroblasts in the fibrotic rim surrounding granulomas, indicating that myofibroblasts themselves are a source of TGF-β1. This observation supports the concept of paracrine regulatory effects of this cytokine in situ, which is further supported by the inhibition of PG-APS-induced collagen gene expression in vitro by neutralization of endogenous TGF-β1. Thus an intestinal milieu of excessive local TGF-β1 production as we documented may promote the differentiation of mesenchymal cells into a myofibroblast phenotype that then becomes committed to collagen deposition.

The cellular mediators of intestinal fibrosis in vivo are not yet precisely defined. It has been reported that IL-1β stimulates the proliferation of cultured intestinal smooth muscle cells (15, 47), whereas this cytokine inhibits collagen synthesis (15). Similar to our findings, Graham et al. (13) demonstrated TGF-β1 stimulated collagen deposition in human intestinal smooth muscle cells. Fritsch et al. (11) have shown that TGF-β1 causes differentiation of rat intestinal fibroblasts into myofibroblasts and that this growth factor may inhibit proliferation, depending on the cell line used. Furthermore, Stallmach and colleagues (45) found that fibroblasts isolated from strictured, inflamed intestines from patients with Crohn’s disease synthesize more collagen and show increased responsiveness to TGF-β1 stimulation compared with fibroblasts from control intestines. However, myofibroblasts isolated from normal fetal or PG-APS-induced granulomatous cecal tissues displayed similar responses to fibrogenic stimuli. These similar responses probably reflect their identical genetic background (Lewis rat) and the lack of exposure to fibrogenic stimuli such as TGF-β1 during multiple in vitro passages, causing them to revert to their basal unstimulated state. The lack of complete blockade of PG-APS-induced collagen expression in cultured myofibroblasts by neutralization of endogenous TGF-β1 suggests that other growth factors, including other TGF-β isoforms and insulin-like growth factor (60), contribute to fibrogenesis.

In conclusion, we present evidence that myofibroblasts accumulate in areas of fibrosis associated with PG-APS-induced chronic enterocolitis. In vitro studies demonstrated that these myofibroblasts are directly stimulated by poorly degradable PG-APS and soluble products of activated macrophages, thereby increasing the synthesis of collagen and fibrogenic cytokines. PG-APS-stimulated collagen synthesis, and to a lesser extent constitutive collagen gene expression, is dependent on autocrine production of TGF-β1 by intestinal myofibroblasts. Inflammation and fibrogenesis may further be perpetuated by sustained activation of macrophages and mesenchymal cells by TGF-β1, IL-1β, and PG-APS in an autocrine and paracrine fashion and through continued exposure to luminal bacterial products due to enhanced mucosal translocation (23, 40). Indeed, the strategic localization of subepithelial myofibroblasts in the normal human colon, as described by Valentich and Powell (52), may subject these cells to bacterial stimulation during episodes of inflammation. Finally, this persistent activation of myofibroblasts by bacterial products and fibrogenic cytokines during chronic enterocolitis may cause excessive collagen deposition leading to pathogenic fibrosis.

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