Interleukin-4- and -13-induced hypercontractility of human intestinal muscle cells—implication for motility changes in Crohn’s disease

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CROHN’S DISEASE IS A CHRONIC inflammatory condition that may involve any part of the gastrointestinal tract. Although the etiology of Crohn’s disease is unknown, it is generally considered to reflect the convergence of environmental and genetic factors, with the loci of susceptibility lying mainly in the regulation of intestinal inflammation (37, 49). Traditionally, studies on the pathogenesis and pathophysiology of Crohn’s disease have focused on the mucosal compartment, despite the fact that serious complications such as the development of strictures and fistulae emphasize the transmural nature of the inflammatory process. Recent evidence suggests that the presence of T cells in the deeper neuromuscular layers of the gut may more accurately predict postoperative recurrence of the disease than mucosal changes (15, 16). Although medical treatment of Crohn’s disease may improve mucosal lesions, it seldom prevents the development of strictures, and this may reflect differences in immune activity between the mucosal and subserosal compartments of the gut.

Cardinal symptoms of Crohn’s disease include abdominal pain and diarrhea, which reflect, in part, changes in gastrointestinal motility. Studies (3, 24, 29) have shown delays in gastric and intestinal transit that cannot be accounted for on the basis of mechanical obstruction and are therefore likely due to inflammation-induced alterations in the motility apparatus. Altered motility in Crohn’s disease involves changes in neural control mechanisms (47) as well as alterations in the contractility of intestinal smooth muscle (53); we have previously shown that muscle from the inflamed intestine of Crohn’s disease patients exhibits increased contractility in vitro following stimulation by carbachol (53). However, the mechanisms underlying this altered contractility are unknown.

Studies using animal models have shown that mucosal inflammation in the gut leads to changes in the growth and contractile properties of smooth muscle (12). In addition, activation of T cells in vivo in normal mice alters the contractility of smooth muscle (39). In a model of nematode infection, mucosal injury is accompanied by the infiltration of the muscularis externa by T lymphocytes that contribute to the hypercontractility of smooth muscle (50). T cells play a critical role in the pathogenesis of Crohn’s disease, with inflammation driven predominantly by IL-12 and IFN-γ producing Th1 cells in the mucosa (36). However, a recent study demonstrated activated lymphocytes in the muscularis externa in Crohn’s disease (18), inviting speculation that lymphocytes and their products mediate the previously described changes in muscle cell contractility (53).

Recently we have shown that the IL-4 receptor (IL-4R) is expressed on murine intestinal smooth muscle cells (ISMC), and exposure of IL-4 or IL-13 enhances muscle contractility induced by carbachol (2). These results demonstrate that IL-4 or IL-13 has a direct effect on smooth muscle cells. IL-4 exerts its biological effects by binding to the IL-4Rα chain, a component of both the type 1 and the type 2 IL-4R (14, 19, 57). In the type 2 IL-4R, IL-4Rα is paired with IL-13Rα1, to which IL-13 also binds (28, 54). Signal transduction may occur by phosphorylation and activation of STAT6 by Janus kinase (JAK)1 and JAK3, with subsequent binding to specific promoter regions to regulate gene transcription (41, 45, 56).

In the present study, we evaluated the presence of lymphocytes and the cytokines IFN-γ and IL-4 in the muscularis externa in Crohn’s disease. Our results indicate that the lymphocytes exhibit a dominant Th2 profile in the muscularis externa, as reflected by increased IL-4 production. We show that in human intestinal muscle cells, IL-4 or IL-13 induces a

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STAT6-mediated hypercontractile state that is similar to that observed in muscle cells isolated from Crohn’s disease. We conclude that in Crohn’s disease, the immune response in the muscularis externa differs from that in the mucosa and that this contributes to the pathophysiology and clinical expression of the disease.

**MATERIALS AND METHODS**

**Materials.** Acrolein, lefunomide, phenylmethylsulfonyl fluoride (PMSF), salmon testes DNA, dL-DTT, N, N', N'-tetramethylethylenediamine (TEMED), aprotinin, pepstatin A, leupeptin, and Mayer’s hematoxylin were from Sigma (St. Louis, MO); HEPES and ammonium persulfate were from BioShop Canada; human IL-4, human IL-13, IL-4, and IFN-γ immunoassay kit were from R & D Systems (Minneapolis, MN); DME, antibiotic-antimycotic, fetal bovine serum, acrylamide, N,N'-methylenebisacrylamide, and Trypan Blue were from GibCO-BRL Life Technologies (Gaithersburg, MD); Poly(dI-dC) was from Pharmacia Biotech (Uppsala Sweden); STAT6 Ab was from Santa Cruz Biotechnology (Santa Cruz, CA); [γ-32P]ATP was from Amersham (Arlington Heights, IL); Faramount aqueous mounting medium was from DAKO Diagnostics (Mississauga, ON, Canada); human ISMC (CRL-1692) were from ATCC (Manassas, VA).

**Human tissue.** Tissues were obtained from surgically resected ileal specimens from 11 patients with active Crohn’s disease (5 males and 6 females, median age 34.1 yr; range 22–56 yr). Studies were performed on the macroscopically inflamed segment as well as on the macroscopically normal proximal margin of each specimen. Specimens were also obtained from 10 patients (7 males and 3 females, median age 61.6 yr; range 56–65 yr) undergoing surgery for carcinoma of the colon, and these were used as noninflamed control tissues. Specimens were placed into saline after initial examination and dissection by the hospital pathologist. The protocol was approved by the Human Ethics Board of Chedoke McMaster Hospital, McMaster University.

**Counting of CD3-positive cells.** Full-thickness specimens were fixed in 10% formalin and embedded in paraffin for histological analysis. Sections were cut and stained with hematoxylin-eosin and examined in a blinded manner using light microscopy using a standardized ocular grid. The number of CD3+ cells was counted in the muscularis externa and the mucosa. We did not include CD3+ cells within lymphoid follicles or granuloma, because we were primarily interested in cells infiltrating the muscle tissue. A total of 10 areas was examined for each compartment in each specimen, and the results were expressed as a mean value for the number of CD3+ cells per 100 mm².

**Measurement of IL-4 and IFN-γ levels.** Total protein was extracted from mucosa and muscle layers separately. The tissues were placed in 1.5 ml of cold lysis buffer (PBS containing 40 μM aprotinin), homogenized, and the supernatants were stored at −70°C until use for enzyme-linked immunoassay according to the manufacturer’s directions.

**Primary cultures of ISMC.** Cells were obtained from explants of the muscularis externa taken from the macroscopically normal end of the small bowel specimens from Crohn’s disease patients. Strips of the longitudinal muscle layer were dissected and explanted in 10-ml tissue culture dishes and cultured in DMEM with 10% fetal bovine serum and 1% antibiotic-antimycotic solution. The explants were maintained at 37°C in a humidified atmosphere of 5% CO₂, and media were changed every 3 days. On reaching confluence, the ISMC monolayer was passaged, the dishes were rinsed in PBS, and the ISMCs were detached by incubation in 0.25% trypsin calcium- and magnesium-free PBS with EDTA (pH 7.4) at 37°C for 3–4 min. Jejunal smooth muscle cells from cell line CRL-1692 (normal 35-yr-old female) were used as controls. Cells from Crohn’s disease patients or the cell line adhered to the bottom of the dish within 24–48 h, began dividing within 72 h, and attained 80% confluence at ~14 days. For the contraction study, cells were grown on a cover glass, which was placed in 2-ml culture dishes up to 20–30% confluence. For the EMSA, cells were maintained in 10-ml culture dishes up to 80% confluence.

Cells from cell line were preincubated with or without cytokines (10 ng/ml IL-4, 10 ng/ml IL-13) for 16 h in the 5% CO₂ incubator. In other experiments, cells were incubated with STAT6 inhibitor lefunomide (100 μM) (44) for 6 h after incubation with IL-4 or IL-13.

**Measurement of contraction in cultured cells.** Cultured cells from the cell line or from Crohn’s disease patients were incubated with carbachol concentrations at room temperature for 30 s before stopping the reaction by adding 1% acrolein as described previously from this laboratory (11). The median cell length of 50 cells on each slide was measured with a microscope using image-splitting micrometry, and the percent decrease from control in the mean cell length was determined.

**Preparation of nuclear extracts and EMSA.** Cultured cells were washed in cold PBS and scraped from the plate. Nuclear extracts were prepared by treating the cell pellet with buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT, 10 μM aprotinin, 2 μg/ml pepstatin A, 2 μg/ml leupeptin, 0.5 mM PMSF) for 10 min on ice. Cells were vortexed and centrifuged at 12,000 g for 10 s, and the supernatant was discarded. The nuclear pellet was resuspended in cold buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, pH 8, 0.5 mM DTT, 10 μM aprotinin, 2 μg/ml pepstatin A, 2 μg/ml leupeptin, 0.5 mM PMSF) for 20 min on ice. The suspension was centrifuged, 12,000 g for 2 min, and supernatants were frozen at −70°C. STAT6 double-stranded consensus oligonucleotide (5'-gatacGCTTCTCTTCCAGGAACTCAATG-3', 3'-CGAGAAGAAGGGTCCTTGAGTTACagct-5') (35) was end-labeled with [γ-32P]ATP (2 mCi/ml). The nuclear extract (15 μg) was incubated with buffer (250 mM Tris-HCl, pH 7.5, 40 mM NaCl, 10 mM EDTA, pH 8, 2.5 mM DTT, 10 mM spermidine, 25% glycerol), 1 μg poly (dI-dC), and 10 μg sperm DNA for 10 min on ice. Two nanograms of anti-STAT6 Ab were added to the sample; 20 pM ds oligonucleotide was used as cold competitor, and the EMSA was performed by incubating 0.1 pmol 32P-end-labeled oligonucleotide with the sample for 30 min at room temperature. Reaction products were separated in a 4.3% polyacrylamide gel in 0.25 × TBE for 4 h at room temperature. The gel was dried and exposed to X-ray film for 72 h, and band density was quantified by Scion Image software.

**Statistics.** Each experiment was performed at least three times, and the results are presented as the means ± SE. Statistical analyses were performed using the Student’s t-test for comparison of two means or a one-way ANOVA for the comparison of more than two means. A P value <0.05 was considered to be statistically significant.

**RESULTS**

**Distribution and density of CD3+ cells in gut specimens of patients with Crohn’s disease and control subjects.** Only occasional CD3+ cells were seen in the muscularis mucosa of segments from control patients. There was a 26-fold increase (P < 0.01) in CD3+ cell number in the muscularis externa from inflamed segments from Crohn’s disease patients compared with controls, and there was a fivefold increase in CD3+ cells in noninflamed segments (Fig. 1).

**Expression of IFN-γ and IL-4 in the muscularis externa in Crohn’s disease.** IL-4 protein expression was increased in the muscle as well as mucosa from Crohn’s disease patients compared with control segments (P < 0.01). This increase was significant in macroscopically involved segments (Fig. 2 A). In
contrast, as shown in Fig. 2B, IFN-γ protein was not significantly increased in either the muscle or the mucosa from Crohn’s disease patients compared with control.

**Contraction of cultured muscle cells.** Carbachol induced contraction of cells from all groups in a concentration-dependent manner. In control muscle cells, the mean cell length was $351.8 \pm 6.2 \mu m$ compared with $395.7 \pm 1.4 \mu m$ in cells from Crohn’s disease ($P < 0.01$). The reduction in mean cell length by 10 pM carbachol was $21.3 \pm 1.5\%$ in control cells compared with $27.8 \pm 1.6\%$ in cells from Crohn’s disease, reflecting a 31% increase in contractility ($P < 0.01$). In addition, as shown in Fig. 3, the ED$_{50}$ for carbachol-induced contraction was 67-fold less in cells from Crohn’s disease (0.03 vs. 2 pM in cells from Crohn’s disease and control, respectively).

We next examined the effects of exposing cells to IL-4 or IL-13 for 16 h before carbachol stimulation. Contraction of cells exposed to IL-4 was $45.6 \pm 1.3\%$, reflecting a 23% increase in contractility compared with control cells ($P < 0.001$). Similarly, as shown in Fig. 4A, contraction of cells exposed to IL-13 was $42.5 \pm 1.0\%$, reflecting a 15% increase above that of control cells ($P < 0.05$). Exposure of cells to either IL-4 or IL-13 alone did not alter the smooth muscle cell length (data not shown).

We next examined the effect of STAT6 inhibitor leflunomide against this cytokine-induced muscle hypercontractility. Leflunomide alone did not alter the effect of carbachol-induced contraction (data not shown), but 100 μM leflunomide inhibited the carbachol-induced contraction following exposure to either IL-4 or IL-13 ($P < 0.0001$; Fig. 4B).

**STAT6-DNA binding in cultured muscle cells.** Nuclear extracts from human smooth muscle cells were incubated with the STAT6 inhibitor leflunomide for 6 h after incubation with IL-4 or IL-13 and subsequently analyzed by EMSA. As shown in Fig. 5A, IL-4 enhanced the STAT6-DNA binding in a dose-dependent manner, and competition with 100-fold molar excess of unlabeled STAT6 oligonucleotide completely abolished DNA binding to the labeled STAT6 oligonucleotide ($P < 0.001$). Addition of anti-STAT6 Ab also inhibited the STAT6 band induced by IL-4 ($P < 0.05$). As shown in Fig. 5C, leflunomide inhibited the STAT6-DNA binding induced by IL-4 ($P < 0.05$). Similarly, IL-13 enhanced the STAT6-DNA binding (Fig. 5D), which was inhibited by 100-fold molar excess of unlabeled STAT6 oligonucleotide ($P < 0.05$).

**DISCUSSION**

We have previously shown that the altered motility in Crohn’s disease is due, at least in part, to a change in the contractility of smooth muscle (53). Although mechanisms underlying this altered contractility are poorly understood, studies in animals confirm that inflammation in the intestinal mucosa is accompanied by increased muscle contractility and that these changes are mediated by lymphocytes present in the muscularis externa (50, 52). Recent studies in a nematode infection model indicate that these changes reflect the actions of the Th2 cytokines IL-4 and IL-13 on smooth muscle (2). The present study builds on those findings in animals by demonstrating increased numbers of T cells and the increased expres-
sion of IL-4 protein in the intestinal muscularis externa of Crohn’s disease patients and by demonstrating the ability of IL-4 or IL-13 to induce hypercontractility of human intestinal muscle cells, similar to that seen in cells isolated from Crohn’s disease patients. Together, these results suggest that T cells in the muscularis externa play a hitherto unidentified role in the pathophysiology of Crohn’s disease by producing changes in intestinal motility that form a basis for symptom generation.

We acknowledge that the use of noninflamed colonic segments as controls in our study may be construed as a potential weakness. This reflects the generally acknowledged difficulty in obtaining normal small intestinal tissue for use as controls, because resection of small intestine for noninflammatory conditions occurs infrequently. We know of no data demonstrating that lymphocyte counts in the muscularis externa differ substantially between the colon and distal small intestine. Furthermore, it is very unlikely that any variation in cell counts between these regions would significantly compromise the observed 26-fold difference between lymphocyte numbers in the muscularis externa of the inflamed small intestine and those of the noninflamed colon. In addition, it should be pointed out that a similar strategy of using noninflamed colonic segments as controls has been used in several published studies identifying important changes in the small intestine of Crohn’s disease patients (33, 46, 53).

Our results also confirm previous work showing the presence of lymphocytes in the muscularis externa in patients with Crohn’s disease (18, 22). Significant numbers of lymphocytes were identified in the overtly inflamed segments as well as in the macroscopically normal margins of the specimens from Crohn’s disease patients. Although we did not determine the precise localization of these cells within the neuromuscular layers, it is likely that lymphocytes are in close proximity to enteric nerves as well as muscle, because this tissue is richly innervated. The presence of lymphocytes in these tissues may have several implications beyond the generation of muscle hypercontractility shown in the present study. Geboes et al. (22) suggested that T cells might mediate neuroimmune communications in Crohn’s disease via MHC II expressed on neural support cells such as enteroglia. In addition to altering the neural modulation of motility in Crohn’s disease, immune cells may influence the natural history of the disease. Recent preliminary reports by D’Haens et al. (15, 16) showed a positive correlation between the perineural infiltration by immunocytes in the myenteric plexus of surgically resected Crohn’s disease specimens and the subsequent development of disease recurrence postoperatively. Taken together, these observations indicate that while the focus of inflammation in Crohn’s disease may be in the mucosa, the presence of T cells

Fig. 3. Dose-response curves for carbachol-induced contraction of human smooth muscle cells from Crohn’s disease (CD) patients and controls. Values are the means ± SE of 4–7 experiments. *Significant difference from control at the same concentration. (P < 0.01).

Fig. 4. A: carbachol (10 nM)-induced contraction of human smooth muscle cells with or without preincubation of IL-4 or IL-13. Incubation with 10 ng/ml IL-4 enhanced the carbachol-induced smooth muscle contraction (+P < 0.001). Similarly, incubation with 10 ng/ml IL-13 enhanced the carbachol-induced smooth muscle contraction (+P < 0.05). Values are the means ± SE of 5–8 experiments. B: the effect of the Stat6 inhibitor leflunomide (L) on carbachol-induced contraction with or without prior exposure to IL-4 or IL-13. Leflunomide inhibited muscle hypercontractility induced by IL-4 (*P < 0.0001) or IL-13 (+P < 0.0001). Values are the means ± SE of 5–8 experiments.
in the muscularis externa may be important not only in symptom generation by altering gut physiology, but also in determining the clinical course of the disease.

In this study, carbachol induced the contraction of cultured human muscle cells in a concentration-dependent manner similar to that seen in other groups (26). We demonstrate that intestinal muscle cells isolated from Crohn’s disease patients exhibit a substantially greater degree of shortening on exposure to carbachol than control cells. This finding indicates that inflammation-induced hypercontractility is a property of the muscle cells, rather than the syncytium. Exposure of human muscle cells to Th2 cytokines IL-4 and IL-13 induced a hypercontractile state similar to that seen in Crohn’s disease, and this was abolished by 100-fold molar excess of cold STAT6 oligonucleotide (E, lane 2) or by anti-STAT6 Ab (E, lane 3). Leflunomide also inhibited the STAT6 band induced by IL-13 (P < 0.05) (E, lane 4). This figure represents 1 of 4 separate experiments that gave similar results.

The activation of the STAT6 pathway by IL-4 and IL-13 usually occurs via IL-4Rα (19, 28, 41, 45, 54, 56), which is present on several cell types (34). We recently demonstrated IL-4 receptor mRNA expression in the murine muscle (2). Thus, taken together, these findings support our conclusion that IL-4 and IL-13, acting directly on muscle cells via STAT6 pathways, are putative mediators of muscle hypercontractility seen in Crohn’s disease. This is supported by a similar role for IL-4 in animal models (30) and in keeping with the hypercontractile state induced in murine muscle following exposure to IL-4, but not IFN-γ (9), or following the overexpression of IL-4 in muscle using an adenovirus vector (51). Goldhill et al. (23) reported that IL-4 administration in vivo enhanced the response to cholinergic nerve stimulation in murine small...
intestinal longitudinal muscle, and this appeared to be dependent on mast cell release of leukotriene D₄. This does not exclude a direct effect of Th2 cytokines on smooth muscle, because we found that preincubation of longitudinal muscle-myoenteric plexus preparations with IL-4 and IL-13 directly enhanced the carbachol-induced contractile response in dispersed murine smooth muscle cells (2).

Our results reveal a novel finding of a dominant Th2 profile in the muscularis externa of Crohn’s disease tissue. This finding is unexpected in light of studies indicating that Crohn’s disease is associated with a Th1 profile (10, 20, 33, 36, 40, 53). These studies based their conclusions on the cytokine profile of the mucosa, and none examined the muscularis externa. However, it should be pointed out that elevations of IL-4 mRNA in Crohn’s disease have been reported (1, 13). Early ileal lesions in patients with Crohn’s disease after surgical resection were accompanied by a significant increase of IL-4 mRNA in the mucosal biopsy, suggesting a role for IL-4 in the recurrence of the disease (13). Our results clearly demonstrate the dominant expression of the Th2 cytokine IL-4, with little change in the Th1 cytokine IFN-γ in the muscularis externa of segments from Crohn’s disease patients. These results raise the possibility that the nature of the immune response in the mucosa may not necessarily reflect that in the neuromuscular layers. Thus immunomodulatory therapies (4), based exclusively on information derived from studies on the mucosal compartment of the gut in Crohn’s disease, may not necessarily be effective in treating those components of the disease involving deeper tissues. This may be particularly relevant in Crohn’s disease, in which aggressive therapy improves mucosal inflammation and ulceration but may not prevent stricture formation.

Studies in animal models have shown that mucosal inflammation is associated with hypertrophy and hyperplasia of smooth muscle (7) and that this is, in part, T cell mediated (8). The presence of T cells in the muscularis externa in Crohn’s disease may also influence stricture formation, because it is known that muscle cell growth and proliferation, as well as collagen deposition, contribute to strictures (27), likely via the disease may also influence stricture formation, because it is known to be upregulated by IL-4 (17). Thus it is possible that the thickening of the smooth muscle layers in Crohn’s disease is due in part to trophic changes in smooth muscle and that this is mediated by IL-4 and perhaps TGF-β. IL-4 has also been shown to promote collagen synthesis and fibrosis in different tissues (38, 42, 43). Thus IL-4 in the muscularis externa may contribute to fibrotic stricture formation that characterizes Crohn’s disease via effects on smooth muscle growth and collagen synthesis.

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