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Endogenous IGF-I and α V β 3 Integrin Ligands Regulate the Increased Smooth Muscle Hyperplasia of Stricturing Crohn's Disease

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Abstract

Background/Aims—Insulin-like growth factor-I (IGF-I) regulates human intestinal smooth muscle growth by stimulating proliferation and inhibiting apoptosis. IGF-I-stimulated growth is augmented when α V β 3 integrin is occupied by its ligands, fibronectin and vitronectin. Increased IGF-I expression and muscle cell hyperplasia are features of stricturing Crohn's disease, however, the role of IGF-I in stricture formation is unknown. The aim was to identify the functional role of endogenous IGF-I and α V β 3 integrin ligands in regulating muscle cell hyperplasia in stricturing Crohn's disease.

Methods—Smooth muscle cells were isolated from muscularis propria of stricturing Crohn's disease or normal margins. Quantitative PCR, immunoblot analysis and ELISA were used to measure fibronectin, vitronectin, α V β 3 integrin, and IGF-I levels. Activation of the IGF-I receptor, Erk1/2, p70S6 kinase and GSK-3 β was measured by immunoblot. Proliferation was quantified by Ki67 immunostaining and [³H]thymidine incorporation. Apoptosis was measured from caspase-3 cleavage and nucleosome accumulation.

Results—IGF-I, vitronectin and fibronectin RNA and protein levels were increased 1.8 – 3.4 fold in muscle cells from strictures over normal margins. Basal IGF-I receptor phosphorylation was increased 320% in strictured over normal muscle and basal Erk1/2, p70S6 kinase and GSK-3 β phosphorylation was increased 205 - 292% in strictures. In muscle cells from strictures, Ki67 immunoreactivity and [³H]thymidine incorporation were increased and apoptosis was decreased compared to normal margins. Antagonists of the IGF-I receptor or α V β 3 integrin reversed these changes.

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Conclusion—Smooth muscle cell hyperplasia in stricturing Crohn's disease is regulated by increased endogenous IGF-I and $\alpha V\beta 3$ integrin ligands that regulate augmented proliferation and diminished apoptosis.

INTRODUCTION

Crohn's Disease is complicated by stricture formation in ~30% of patients ^{1, 2}. Three features are characteristic of smooth muscle cells in the muscularis propria of stricturing Crohn's disease: increased muscle cell proliferation (hyperplasia), increased muscle cell hypertrophy, and increased net extracellular matrix production ^{3, 4}. Insulin-like growth factor-I (IGF-I) produced in the liver acts in an endocrine fashion, whereas locally produced IGF-I, e.g. by smooth muscle cells acts, in an autocrine fashion to regulate the growth of smooth muscle cells ^{5, 6}. Two lines of evidence demonstrate the importance of endogenous IGF-I in regulating the growth of intestinal smooth muscle cells: (i) in mice with a CreLox/P-mediated hepatic deletion of IGF-I, intestinal muscle develops normally ⁷, and (ii) smooth muscle hyperplasia in the muscularis propria develops in mice over-expressing IGF-I ^{8, 9}.

In human intestinal smooth muscle cells IGF-I and $\alpha V\beta 3$ integrin share a unique relationship. Occupancy of $\alpha V\beta 3$ integrin (vitronectin receptor) by its ligands, vitronectin and fibronectin, augments the intensity and duration of IGF-I-stimulated IGF-I receptor activation, and muscle growth ¹⁰⁻¹². Interplay between IGF-I and $\alpha V\beta 3$ is thought to play a role in pathophysiologic responses of other smooth muscle types: atheroma formation in vascular muscle and fibroid formation in uterine muscle ^{8, 13, 14}. Activation of the IGF-I receptor tyrosine kinase in human intestinal smooth muscle is augmented by $\alpha V\beta 3$ ligands and is coupled to Erk1/2 and p70S6 kinase activation, which jointly mediate IGF-I-stimulated proliferation, and to GSK-3 β activation, which mediates IGF-I-stimulated inhibition of apoptosis ¹⁵⁻¹⁷.

The IGF-I gene is alternatively spliced with the main isoform of IGF-I encoded by the IGF-IEa isoform. IGF-IEa expression is increased in the muscularis propria of active and stricturing Crohn's disease over that in normal intestinal margin at the time of resection ¹⁸. Expression was increased in muscle cells, and fibroblasts but IGF-IEa expression was not observed in the inflammatory cells infiltrating the muscular layer ¹⁸. While endogenous IGF-I has been shown to regulate growth of normal intestinal smooth muscle cells, neither the functional significance of increased IGF-I expression in Crohn's disease nor the mechanisms that regulate increased muscle cell hyperplasia of stricturing Crohn's disease have been identified.

This paper shows that the expression of IGF-I, and the $\alpha V\beta 3$ integrin ligands, fibronectin and vitronectin, are increased in smooth muscle cells isolated from the muscularis propria of stricturing Crohn's disease over that in normal muscle. Basal IGF-I receptor activity and that of its signaling intermediates coupled to stimulation of proliferation and inhibition of apoptosis are also increased in muscle cells of stricturing Crohn's disease. The results indicate that the increased proliferation and decreased apoptosis in intestinal smooth muscle cells in stricturing Crohn's disease, compared to normal intestine, are regulated by endogenous IGF-I and $\alpha V\beta 3$ integrin ligands. The results also suggest that the long term sequelae of these two complementary processes that regulate growth may be smooth muscle cell hyperplasia of the muscularis propria, one characteristic of stricturing Crohn's disease.

MATERIALS AND METHODS

Isolation of Intestinal Muscle Cells from Human Intestine

Segments of intestine were obtained from patients undergoing ileal or ileo-cecal resection for stricturing Crohn's Disease according to a protocol approved by the VCU Institutional Review Board. Muscle cells were isolated from the ileal circular muscle layer using previously reported

techniques from regions of stricturing Crohn's Disease and from the normal proximal ileal resection margin^{6, 10, 19, 20}. Demographic data on patients consenting to provide tissue for this study are presented in Table 1. Muscle cells isolated by enzymatic digestion were used to prepare RNA, and whole cell lysates or placed into cell culture. Epithelial cells, endothelial cells, neurons and interstitial cells of Cajal are not detected in cells isolated in this fashion²¹. These cells possess a smooth muscle phenotype: immunostaining for smooth muscle markers but not fibroblast markers, expression of γ -enteric actin, and the physiologic characteristics of contractile intestinal smooth muscle. Each characteristic is retained by the muscle cells in culture²¹.

Preparation of RNA and quantitative real-time PCR (qRT-PCR)

The expression of fibronectin (Hs.203717), vitronectin (Hs.2257), α V (Hs.436873) and β 3 (Hs.218040) integrin subunits, and IGF-IEa (Hs.160562) were measured by qRT-PCR. Total RNA is prepared using RNAqueous™ kits (Ambion, Austin, TX). First stand cDNA synthesis is performed from two μ g of total RNA using qScript™ kits (Quanta, Gaithersburg, MD). Quantitative RT-PCR reactions are performed in 25 μ l volume containing TAQman master mix, 2.5 pmol of forward and reverse primers (Applied Biosystems, Foster City, CA), and 2 μ l of cDNA using a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). Standard curves for amplicons are generated from a dilution series of cDNA from normal cells and results quantified using the $2^{-\Delta C_t}$ method based on β -actin amplification²².

Preparation of whole cell lysates

Cell lysates were prepared as described previously in immunoprecipitation buffer (in mM): 50 Tris-HCl (pH 7.5), 150 NaCl, 50 NaF, 1 Na orthovanadate, 1 dithiothreitol, 1 phenylmethylsulfonyl fluoride and 0.5% NP-40 to which was added 1 μ g/ml leupeptin, 1 μ g/ml pepstatin A, and 1 μ g/ml aprotinin^{10, 20, 23}. Lysates were centrifuged at 14,000g for 10 min at 4°C prior to use for immunoprecipitation or immunoblot analysis.

Immunoprecipitation of IGF-I receptor

IGF-I receptors were immunoprecipitated as previously described^{10, 20, 23}. Lysates containing 1 mg protein were incubated with 2 μ g of rabbit anti-IGF-IR β -subunit overnight at 4°C with Protein A-agarose beads, washed three times, resuspended in 25 μ l sample buffer and boiled for 5 min prior to immunoblotting for phosphorylated IGF-IR identified in phosphotyrosine immunoblots as described below.

Immunoblot Analysis

Analysis of integrin proteins and phosphorylated signaling proteins was performed as previously described^{10, 16}. Cell lysates containing equal amounts of total protein or immunoprecipitated from samples containing equal amounts of protein (BioRad, Hercules, CA) were separated with SDS-PAGE under denaturing conditions and electrotransferred to nitrocellulose. Membranes were incubated overnight with antibodies: α V, β 3, fibronectin, vitronectin, phosphotyrosine, Erk1/2, phospho-Erk1/2(Thr202/Tyr204), p70S6 kinase or phospho-p70S6 kinase(Ser389), GSK-3 β , GSK-3 β (Ser9), caspase-3, cleaved caspase-3 or β -actin (Cell Signaling Technologies, Beverly, MA; Upstate Biotechnology, Lake Placid, NY; Biosource International, Camarillo, CA; Becton-Dickinson, Franklin Lakes, NJ). Membranes were reblotted to measure total protein or β -actin. Bands were visualized with ECL using a FluoChem 8800; digital images were quantified using AlphaEaseFC version 3.1.2 software (Alpha Innotech, San Leandro, CA). Densitometric values were reported in arbitrary units after normalization to total protein levels or β -actin.

Immunohistochemical staining of Ki67

Proliferating muscle cells was measured by counting Ki67 immunoreactive cells in histologic sections by two blinded investigators in ten successive high power fields. Sections of formalin fixed, paraffin embedded tissue specimens from strictures or normal margins were mounted on charged slides, deparaffinized and dehydrated. Heat-citrate pretreatment was used for antigen retrieval. Sections were incubated with a 1:50 dilution of anti-human mouse monoclonal Ki67 antigen (DAKO, Carpinteria, CA), a secondary biotinylated goat anti-mouse antibody, and a biotin-streptavidin detection kit was used with DAB as substrate.

[³H]Thymidine Incorporation Assay

Proliferation of smooth muscle cells in culture was measured by the incorporation of [³H] thymidine as described previously^{6, 10, 20}. Quiescent cells were incubated for 24 h in serum-free DMEM in the presence or absence of echistatin or AG1024 (Sigma Chemical, St Louis, MO). During the final 4 h of incubation, 1 µCi/ml [³H]thymidine was added to the medium. [³H]Thymidine incorporation into the perchloric acid extractable pool was used as a measure of DNA synthesis.

Nucleosome Apoptosis Assay

Apoptosis in cultured muscle cells was quantified by measurement of cytoplasmic histone-associated DNA fragments (nucleosomes) using a quantitative sandwich-enzyme-immunoassay ELISA (Roche Applied Science, Indianapolis, IN)¹⁹. Quiescent muscle cells were incubated for 24 h in serum-free DMEM in the presence and absence of test agents and lysed. 20 µl of lysate containing cytoplasmic DNA fragment were incubated on streptavidin-coated ELISA plates an antibody to histone-biotin (clone H11-4) and antibody to anti-DNA-POD (clone MCA-33). ABTS solution was added for visualization using a Victor2 1420 multichannel counter (Perkin Elmer, Boston, MA) and reported as ΔA₄₀₅

Statistical analysis

Values given represent the mean ± SE of n experiments where n represents the number of experiments on specimens derived from separate intestinal specimens or primary cultures. Statistical significance was tested by Student's t-test for either paired or unpaired data as was appropriate with P < 0.05.

RESULTS

IGF-I expression is increased in intestinal smooth muscle cells of stricturing Crohn's disease

The IGF-I gene is alternatively spliced. The main circulating isoform of IGF-I is encoded by the IGF-IEa splice variant. Quantitative-PCR analysis confirmed that the IGF-IEa splice variant is expressed in smooth muscle. Levels of IGF-IEa mRNA expression are increased 3.4 ± 0.3 fold in muscle cells isolated from strictured intestine compared to muscle cells from the normal resection margin (Fig. 1A).

IGF-I is produced by smooth muscle cells of human intestine⁶. Levels of IGF-I protein in muscle cells isolated from normal margins (2.1 ± 0.3 ng/mg protein) are increased 62 ± 9% in muscle cells isolated from regions of stricturing Crohn's disease (Fig. 1B).

Vitronectin and Fibronectin Production is Increased in Intestinal Smooth Muscle Cells in Stricturing Crohn's Disease

Vitronectin and fibronectin, two ligands of αVβ3 integrin (vitronectin receptor), are expressed in intestinal smooth muscle cells^{10, 24-26}. The levels of vitronectin mRNA were increased 1.8 ± 0.3 fold (P < 0.05 vs normal margin) in muscle cells isolated from stricturing Crohn's

disease, and levels of fibronectin mRNA increased 2.3 ± 0.4 fold ($P < 0.05$ vs normal margin) (Fig. 2A).

The levels of vitronectin protein in smooth muscle cells isolated from stricturing Crohn's disease were similarly increased by $170 \pm 25\%$ over the levels in muscle cells from normal margins. Levels of fibronectin protein were increased $160 \pm 20\%$ in muscle cells from stricturing regions compared to normal margins (Fig. 2).

In contrast to $\alpha V\beta 3$ integrin ligand expression, no significant change in either αV or $\beta 3$ integrin subunit protein levels were seen in regions of stricturing Crohn's disease ($36 \pm 25\%$ and $7 \pm 19\%$ above normal margins, respectively).

Basal IGF-I Receptor Phosphorylation is Increased in Muscle Cells of Stricturing Crohn's Disease

Endogenous IGF-I regulates the growth of human intestinal smooth muscle cells by activation of the cognate IGF-I receptor¹⁷. Occupation of $\alpha V\beta 3$ integrin by its ligands, i.e. vitronectin and fibronectin, augments the intensity and duration of IGF-I-stimulated, IGF-I receptor activity¹⁰. We hypothesized that the increased levels of vitronectin and fibronectin in regions of active Crohn's disease in conjunction with the increased expression of IGF-I would increase basal levels of IGF-I receptor tyrosine kinase activity in the intestinal muscle of stricturing Crohn's disease.

Phosphorylation of the IGF-I receptor was observed even under basal conditions in muscle cells isolated from normal resection margins as expected for an autocrine regulator of intestinal smooth muscle growth. Basal IGF-I receptor phosphorylation was an additional $320 \pm 35\%$ higher in muscle cells isolated from strictures (Fig 3).

Basal Activity of IGF-I-stimulated Signaling Intermediates are Increased in Muscle Cells of Stricturing Crohn's Disease

Endogenous IGF-I regulates intestinal smooth muscle cell growth by jointly activating the Erk1/2 and the p70S6 kinase (via PI 3-kinase) signaling pathways, and inhibiting apoptosis via GSK-3 β ^{16, 17}. We hypothesized that activity of these signaling intermediates would be increased in muscle cells isolated from strictures compared to normal margins. Phosphorylation of Erk1/2(Thr202/Tyr204) parallels Erk1/2 activity, GSK-3 β (Ser9) parallels GSK-3 β activity, and phosphorylation of p70S6 kinase(Ser389) most closely parallels p70S6 kinase activity. These phosphoproteins were therefore used in immunoblot analysis as measures of enzyme activity in lysates prepared from isolated muscle cells^{16, 17, 19}.

Erk1/2 was phosphorylated under basal conditions in muscle cells isolated from normal resection margins and increased an additional $251 \pm 34\%$ in the muscle cells isolated from strictures (Fig. 4A). Similarly, p70S6 kinase(Ser389) phosphorylation was observed with basal p70S6 kinase(Ser389) phosphorylation was present in the muscle cells isolated from normal margins and increased an additional $292 \pm 37\%$ in muscle cells isolated from strictures (Figure 4B).

GSK-3 β (Ser9), coupled to IGF-I-stimulated inhibition of apoptosis, was phosphorylated under basal conditions in muscle cells isolated from normal resection margins. Phosphorylation was increased an additional $206 \pm 16\%$ in muscle cells isolated from strictures (Fig. 4C).

Smooth Muscle Cell Proliferation is Increased in Stricturing Crohn's Disease and Dependent on Endogenous IGF-I and α V β 3 Integrin Activation

We hypothesized that the concomitant increase in IGF-I, vitronectin and fibronectin expression; and increased activation of the IGF-I receptor and IGF-I-stimulated signaling intermediates coupled to proliferation would result in increased muscle cell proliferation in regions of stricturing Crohn's disease compared to normal muscle. This was examined in two ways: i) muscle cell proliferation was measured from the number of Ki67 positive cells in histologic sections of stricture and normal margin. Smooth muscle cells were identified morphologically by their characteristic spindle shaped appearance. ii) Increased proliferation specifically in muscle cells was confirmed by measurement [3 H]thymidine incorporation in homogeneous cell cultures.

The number of proliferating muscle cells in histologic sections from normal margins showed a mean of 1.2 ± 0.3 Ki67 positive smooth muscle cells per HPF (Fig. 5A). The number of Ki67 positive muscle cells increased to 4.3 ± 0.2 per HPF in regions of stricturing Crohn's Disease (Fig. 5B).

Basal [3 H]thymidine incorporation in muscle cells isolated and cultured from normal margins was 675 ± 72 cpm/mg protein. Proliferation was inhibited $51 \pm 4\%$ by the IGF-I receptor tyrosine kinase inhibitor, AG1024 (1 μ M) and $61 \pm 3\%$ by the α V β 3 integrin antagonist, echistatin (10 nM) (Fig. 6A)^{27, 28}. Basal [3 H]thymidine incorporation in muscle cells from stricturing Crohn's disease increased to 1750 ± 80 cpm/mg protein, $260 \pm 30\%$ above that in cells from normal margins (Fig. 6A) and was inhibited $54 \pm 4\%$ by AG1024 and $67 \pm 8\%$ by echistatin (Fig. 6A). Similarly, incubation of muscle cells isolated from strictures with a neutralizing antibody to α V β 3 integrin caused concentration-dependent inhibition of proliferation (Fig. 6B). These results indicate that endogenous IGF-I and α V β 3 integrin ligands regulate intestinal smooth muscle proliferation and mediate muscle cell hyperplasia in regions of stricturing Crohn's disease.

Smooth Muscle Cell Apoptosis is Decreased in Stricturing Crohn's disease and Dependent on Endogenous IGF-I and α V β 3 Integrin Activation

Endogenous IGF-I also regulates intestinal muscle cell growth by inhibiting apoptosis¹⁹. We hypothesized that the events that stimulate proliferation would also decrease apoptosis. This notion was tested in two ways: i) by direct measurement of the relative levels of cleaved and total caspase-3 in freshly isolated smooth muscle cells, and ii) by measurement of nucleosome accumulation in cultured smooth muscle cells.

In lysates derived from muscle cells isolated from normal margins, the fraction of cleaved caspase-3/total caspase-3 was 1.36 ± 0.29 . This fraction decreased to 0.53 ± 0.07 in muscle cells isolated from stricturing Crohn's disease reflecting a decrease in the level of apoptosis in these cells (Fig 7A).

Apoptosis was also measured from the level of nucleosomes in muscle cells isolated and cultured from normal resection margins and from strictures. Basal levels of nucleosomes in normal cells increased $66 \pm 15\%$ in the presence of AG1024, and increased $119 \pm 18\%$ in the presence of echistatin (Fig. 8B). In smooth muscle cells isolated from regions of stricturing Crohn's Disease, nucleosome levels were only $90 \pm 4\%$ of that measured in muscle cells from normal margin indicating a lower basal level of apoptosis. In the presence of echistatin, nucleosome levels in cells isolated from strictures increased $63 \pm 8\%$, and in the presence of AG1024 increased by $107 \pm 16\%$ (Fig 8B).

DISCUSSION

Three characteristic features are hallmarks of the muscularis propria in stricturing Crohn's disease: smooth muscle cell hyperplasia, smooth muscle cell hypertrophy and excess net extracellular matrix production. This paper shows that in patients with stricturing Crohn's disease, growth of intestinal smooth muscle in strictures is increased compared to regions of normal muscle. Increased IGF-IEa mRNA and IGF-I protein production by the smooth muscle cells of the muscularis propria of stricturing Crohn's disease is associated with increased smooth muscle cell growth¹⁸. This is in contrast to the ~46% decrease in serum levels of hepatic-derived IGF-I in patients with active Crohn's disease that is thought to arise from the effects of the disease on nutrition and inflammation²⁹. We have previously shown that endogenous IGF-I regulates smooth muscle cell hyperplasia *in vitro* by concomitantly stimulating proliferation and inhibiting apoptosis^{6, 19}. However, the functional role of IGF-I in mediating the increased growth of smooth muscle of the muscularis propria in stricturing Crohn's disease and the mechanism involved have not previously been examined. This paper shows that basal rates of muscle cell proliferation are increased in smooth muscle of strictures, and are accompanied by decreased rates of apoptosis; both are regulated by the increased levels of endogenous IGF-I.

This paper also provides evidence that along with increased IGF-I production in muscle cells of stricturing Crohn's disease, there is increased production of the α V β 3 ligands, fibronectin and vitronectin. Our recent work provides insight on how these events are linked and contribute to increased muscle growth: occupancy of the α V β 3 integrin by its endogenous ligands, vitronectin and fibronectin, in intestinal smooth muscle increases the intensity and duration of IGF-I-stimulated IGF-I receptor tyrosine kinase activity, signaling and growth¹⁰. These results suggest that regulation of muscle growth by IGF-I and augmentation by occupancy of α V β 3 integrin contributes further to the hyperplasia of smooth muscle cells of the muscularis propria in stricturing Crohn's disease.

The evidence supporting the participation of both α V β 3 integrin and IGF-I in the increased smooth muscle growth in strictured intestine of Crohn's disease can be summarized as follows: i. α V β 3 integrin is expressed by intestinal smooth muscle cells and the expression of vitronectin and fibronectin, ligands of α V β 3 integrin, was increased in regions of stricturing; ii. basal IGF-I receptor tyrosine kinase phosphorylation in intestinal smooth muscle is increased in regions of stricturing; iii. basal phosphorylation of Erk1/2 and p70S6 kinase, both coupled to stimulation of proliferation, is increased in muscle cells in regions of stricturing; iv. basal phosphorylation of GSK-3 β , coupled to inhibition of apoptosis, is increased in muscle cells in regions of stricturing; v. basal rates of smooth muscle cell proliferation are increased in regions of stricturing and are dependent on endogenous IGF-I and α V β 3 integrin^{27, 28}; vi. basal levels of smooth muscle cell apoptosis are decreased in regions of stricturing and are dependent on endogenous IGF-I and α V β 3 integrin.

These results corroborate those of Zimmermann et al¹⁸ showing that the levels of IGF-IEa RNA were increased in regions of active Crohn's Disease when compared to uninflamed intestine but further show that IGF-I expression and production is upregulated specifically in intestinal smooth muscles cells. Increased IGF-IEa expression is also observed in animal models of colitis including TNBS-induced, peptidoglycan-polysaccharide-induced, and DSS-induced colitis^{18, 30, 31}. The mechanisms responsible for increased IGF-I expression in stricturing Crohn's disease are unknown. Normally growth hormone (GH) is a main regulator of IGF-I expression. GH-dependent induction of suppressor of cytokine signaling (SOCS)-2 and SOCS-3 inhibits GH-dependent expression of IGF-I through a negative feedback mechanism³². However, a GH-resistant state exists in patients with Crohn's disease and in animal models of colitis where there is normal and stimulated GH secretion yet decreased

circulating (hepatic-derived) IGF-I levels. SOCS-2 and SOCS-3 are also negative modulators of the effects of inflammatory cytokines. While GH did not increase SOCS-3 expression *in vitro* in cultured murine myofibroblasts from full thickness intestine, the Th1 cytokines, TNF- α or IL-6, in combination with GH did³³. Recent (unpublished) data from our lab suggests that increased production of IGF-I by strictured muscle cells is accompanied by altered levels of SOCS-2 and SOCS-3 protein compared to muscle cells from normal margin. This apparent dysregulation of SOCS expression may contribute to the increased IGF-IEa expression in muscularis propria muscle cells of stricturing Crohn's disease.

IGF-I also regulates expression of one component of the extracellular matrix, collagen. The upregulation of IGF-I is accompanied by upregulation of IGF binding protein-5 (IGFBP-5) expression and collagens I, III, and V^{18, 34, 35}. Our previous work has shown how these events are linked in human intestinal muscle where IGF-I increases IGFBP-5 expression and production, and both IGF-I and IGFBP-5 increase each others' production in addition to increasing collagen production^{18, 20}. Our recent work has also shown that IGFBP-3 and TGF- β 1 expression is increased in muscle cells isolated from stricturing Crohn's disease; both regulate collagen I expression and production by activating the TGF- β RI/II receptor complex^{36, 37}. While these results suggest that the processes that culminate in increased collagen production play a role in the development of stricture formation in Crohn's disease by increasing collagen production and net extracellular matrix formation, this has not been directly demonstrated.

Production of other extracellular matrix proteins, i.e. vitronectin and fibronectin, are also increased in muscle cells of stricturing Crohn's disease and play a regulatory role in the development of smooth muscle cell hyperplasia by activating α V β 3 integrin and augmenting IGF-I-stimulated smooth muscle cell hyperplasia¹⁰. Recently, α V β 3 integrin-dependent mechanisms mediating angiogenesis in the inflammatory response of the intestinal mucosa and submucosa in Crohn's disease, ulcerative colitis, and murine DSS-induced colitis have been elucidated³⁸⁻⁴⁰. Endothelial α V β 3 integrin expression is increased by inflammation *in vivo* or by experimental application of TNF- α , vascular endothelial growth factor or basic fibroblast growth factor⁴¹. In contrast to the response of intestinal endothelial cells to inflammation, α V β 3 integrin levels in intestinal smooth muscle cells are not altered in stricturing Crohn's disease.

The clinical significance of the findings of this paper is in the setting of active and stricturing Crohn's disease. Levels of the α V β 3 integrin ligands vitronectin and fibronectin are increased and act in concert with increased IGF-I expression to further increase IGF-I-stimulated proliferation and inhibition of apoptosis of intestinal smooth muscle cells. The long term sequelae of these two complementary processes regulating muscle cell growth may be muscle cell hyperplasia in the muscularis propria that is one characteristic of stricturing Crohn's disease.

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Figure 1. IGF-I expression is increased in smooth muscle cells of stricturing Crohn's disease
Panel A: IGF-IEa RNA transcript levels are increased in muscle cells isolated from stricturing Crohn's disease compared to normal margins. Q-PCR results are expressed as fold increase over normal using the $2^{\Delta C_t}$ method with β -actin as control. Panel B: IGF-I protein levels are increased in muscle cells isolated from stricturing Crohn's disease compared to normal margins. Results from ELISA are expressed in pg/mg protein. Values represent the mean $\pm$ SEM of 4-5 separate experiments. * denotes $P < 0.05$ vs normal margin.

Figure 2. $\alpha V\beta 3$ integrin ligands, vitronectin and fibronectin, are increased in smooth muscle cells of stricturing Crohn's disease

Panel A: Vitronectin and fibronectin RNA transcript levels are increased in muscle cells isolated from stricturing Crohn's disease compared to normal margins. Q-PCR results are expressed as fold increase over normal using the $2^{\Delta C_t}$ method with β -actin as control. Panel B: Vitronectin and fibronectin levels are increased in muscle cells isolated from stricturing Crohn's disease compared to normal margins. Inset: Representative immunoblots of vitronectin and fibronectin levels and β -actin. Results are expressed in relative densitometric units normalized to β -actin levels. Values represent the mean $\pm$ SE of 5 separate experiments. * denotes $P < 0.05$ vs normal margin.

Figure 3. Basal IGF-I receptor phosphorylation is increased in smooth muscle cells of stricturing Crohn's disease

Densitometric analysis showing increased basal IGF-I receptor phosphorylation in smooth muscle cells isolated from stricturing Crohn's disease compared to normal margins. Inset: Representative immunoblots of IGF-I receptor phosphorylation (pY) and total IGF-I receptor levels. Results are expressed in relative densitometric units normalized to total IGF-I receptor levels. Values represent the mean $\pm$ SE of 4 separate experiments. * denotes $P < 0.05$ vs normal margin.

p-p70S6 kinase
Total p70S6 kinase

Figure 4. Basal signaling intermediate phosphorylation is increased in smooth muscle cells of stricturing Crohn's disease

Panel A: Densitometric analysis showing increased basal Erk1/2 phosphorylation in smooth muscle cells isolated from stricturing Crohn's disease compared to normal margins. Inset: Representative immunoblots of phospho-Erk1/2(Thr202/Tyr204) and total Erk1/2 levels. Panel B: Densitometric analysis showing increased basal p70S6 kinase(Thr389) phosphorylation in smooth muscle cells isolated from stricturing Crohn's disease compared to normal margins. Inset: Representative immunoblots of phospho-p70S6 kinase(Thr389) and total p70 S6 kinase. Panel C: Densitometric analysis showing increased basal GSK-3β phosphorylation in smooth muscle cells isolated from stricturing Crohn's disease and normal margins. Inset: Representative immunoblots of phospho-GSK-3β(Ser9) and total GSK-3β. Results are expressed in relative densitometric units normalized to total signaling intermediate levels. Values represent the mean ± SE of 4 separate experiments. * denotes $P < 0.05$ vs normal margin.

Figure 5. Ki67 immunoreactive smooth muscle cells are increased in stricturing Crohn's disease over normal margins

Representative micrographs of muscularis propria of human intestine showing the increased numbers of Ki67 positive smooth muscle cells in stricturing Crohn's disease (Panel B) compared to normal margins (Panel A). Arrows denote Ki67 immunoreactive muscle cells stained with DAB on background hematoxylin/eosin staining identifiable by their characteristic spindle shape appearance. Magnification=200x. Insets: Expanded view of representative Ki67 positive smooth muscle cells.

Figure 6. Smooth muscle cell proliferation is increased in stricturing Crohn's disease and dependent on endogenous IGF-I and α V β 3 integrin ligands

Panel A: Basal [3 H]thymidine incorporation is increased in muscle cells isolated from stricturing Crohn's disease compared to normal margins. Proliferation is inhibited by the α V β 3 integrin antagonist, echistatin (100 nM), or by the IGF-I receptor tyrosine kinase

inhibitor, AG1024 (10 μ M). Panel B: [3 H]thymidine incorporation in muscle cells isolated from stricturing Crohn's disease is inhibited in a concentration-dependent fashion by an α V β 3 integrin neutralizing antibody. Results are expressed in cpm/ μ g total cell protein. Values represent the mean $\pm$ SEM of 5 separate experiments. * denotes $P < 0.05$ vs control muscle cells from normal margins, ** denotes $P < 0.05$ vs control muscle cells from stricturing Crohn's disease, *** denotes $P < 0.05$ vs control muscle cells from normal margins.

Figure 7. Rates of smooth muscle cell apoptosis in stricturing Crohn's disease are decreased and dependent on endogenous IGF-I and α V β 3 integrin ligands

Panel A: Apoptosis, measured as cleaved caspase-3/total caspase-3, is lower in muscle cells isolated from stricturing Crohn's disease compared to normal margin. Inset: Representative immunoblots of total caspase-3, cleaved caspase-3 and β -actin. Results were expressed as cleaved caspase-3/total caspase-3. Panel B: Apoptosis, measured as nucleosome levels, is decreased in muscle cells cultured from stricturing Crohn's disease compared to normal margin. Apoptosis was augmented by the α V β 3 integrin antagonist, echistatin (100 nM), or by the IGF-I receptor tyrosine kinase inhibitor, AG1024 (10 μ M) in both. Results from nucleosome-specific ELISA are expressed as ΔA_{405} . Values represent the mean $\pm$ SEM of 5 separate experiments. * denotes $P < 0.05$ vs control muscle cells from normal margins, ** denotes $P < 0.05$ vs control muscle cells from stricturing Crohn's disease, *** denotes $P < 0.05$ vs control muscle cells from normal margins.

Table 1

Demographics of patients with stricturing Crohn's disease.

	Patient No. (% of total)
<i>Age (years)</i>	
under 20	1 (6.7)
20-29	1 (6.7)
30-39	3 (20.0)
40-49	4 (26.7)
50-59	5 (33.3)
over 60	1 (6.7)
<i>Sex</i>	
Male	3 (20.0)
Female	12 (80.0)
<i>Race</i>	
White	9 (60.0)
Black or African	5 (33.3)
Other/unknown	1 (6.7)