Functional Modulation of Crohn's Disease Myofibroblasts by Anti-Tumor Necrosis Factor Antibodies

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Background & Aims: **Infliximab induces immune cell apoptosis by outside-to-inside signaling through transmembrane tumor necrosis factor-α (mTNF). However, in inflamed gut, myofibroblasts also produce TNF-α, and the affects of anti-TNF antibodies on these structural cells are unknown. We investigated the action of infliximab on apoptosis, the production of matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMP)-1, and migration of Crohn's disease (CD) myofibroblasts.** ***Methods:*** **Colonic myofibroblasts were isolated from patients with active CD and controls. mTNF was evaluated by Western blotting and flow cytometry. Infliximab-treated myofibroblasts were analyzed for apoptosis by Annexin V staining and caspase-3. TIMP-1 and MMPs were measured by Western blotting, and fibroblast migration was assessed by using an in vitro wound-healing scratch assay.** ***Results:*** **CD myofibroblasts showed higher mTNF expression than control myofibroblasts. Infliximab had no effect on CD myofibroblast apoptosis, caspase-3 activation, and production of MMP-3 and MMP-12. However, infliximab induced a significant dose-dependent increase in TIMP-1 production, which was inhibited by the p38 mitogen-activated protein kinase inhibitor SB 203580. The anti-TNF agents adalimumab, etanercept, and p55 TNF-receptor–human IgG fusion protein also increased TIMP-1 production. The migration of CD myofibroblasts was enhanced significantly by infliximab and recombinant human TIMP-1, and infliximab-induced migration was inhibited by anti–TIMP-1 neutralizing antibody. Infliximab also decreased CD myofibroblast collagen production.** ***Conclusions:*** **Our findings show a novel therapeutic pathway for anti-TNF therapies in enhancing TIMP-1 production and myofibroblast migration, which may reduce MMP activity and facilitate the wound healing.**

Infliximab promotes rapid closure of fistulas and sustained mucosal healing in active Crohn's disease (CD).[1–3] The effectiveness of infliximab is linked not only to the neutralization of soluble tumor necrosis factor (TNF)-α and transmembrane TNF-α (mTNF), but to the induction of apoptosis by reverse signaling through mTNF.[4,5] Monocytes and T lymphocytes, which express high amounts of mTNF, are particularly susceptible to infliximab-induced caspase-dependent apoptosis.[6–8]

Myofibroblasts are key cells in the process of tissue injury and wound healing in the gut.[9] They cause gut damage by secreting matrix metalloproteinases (MMPs),[10] which are calcium ion–dependent and zinc ion–containing neutral endopeptidases involved in extracellular matrix (ECM) degradation.[11] MMP activity is under tight physiologic control by tissue inhibitors of metalloproteinases (TIMPs).[12] Tissue-degrading MMPs act as end-stage effectors of several disorders in which there is an excess of TNF-α,[11,13,14] and their increase in the inflamed gut has been associated with mucosal degradation, ulcerations, and fistulas.[15–20] TNF-α blockade prevents ECM degradation concomitant with inhibition of MMP production.[21]

Myofibroblast migration is an important component of intestinal wound healing.[22] Myofibroblasts become activated and proliferate in the early stage of wounding. They respond to proinflammatory cytokines with elaboration of ECM proteins and additional growth factors.[9,11,22] Recently, persistent mucosal wounding and ulcerations have been associated with a reduced migratory potential of intestinal myofibroblasts in CD, and TNF-α appears to have a role in inhibiting this migration.[23]

Although most attention in inflammatory bowel disease (IBD) has focused on TNF production by T cells and

Abbreviations used in this paper: ECM, extracellular matrix; FITC, fluorescein isothiocyanate; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; mTNF, transmembrane tumor necrosis factor-α; p55-TNFR-IgG, p55 tumor necrosis factor receptor–human IgG fusion protein; TIMP, tissue inhibitor of metalloproteinases; TNF, tumor necrosis factor; TGF, transforming growth factor.

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macrophages, TNF also is made by other cell types, including myofibroblasts.[24] However, there is no information on the effect of infliximab on myofibroblasts. Thus, in this study we have determined whether CD myofibroblasts express mTNF, and whether infliximab and other anti-TNF reagents alter myofibroblast function.

Materials and Methods

Patients

Endoscopic biopsies or surgical specimens were taken from macroscopically and microscopically inflamed and unaffected colonic mucosa of 15 patients affected by active CD (mean age, 35.6 y; range, 20–59 y). The diagnosis of CD was ascertained according to the usual clinical criteria,[25] and the site and extent of the disease were confirmed by endoscopy, histology, and enteroclysis in all patients. Disease activity was assessed by the Crohn's Disease Activity Index. Patients with scores of less than 150 were classified as being in remission, whereas patients with scores higher than 450 had severe disease.[25] In 9 patients the primary site of involvement was ileocolonic, and colonic in the remaining 6 patients. Four were untreated at the time of biopsy, being at the first disease presentation; 5 were treated with mesalazine, steroids, or antibiotics, and 6 were treated with only mesalazine at the time of biopsy and had suspended the steroid treatment at least 3 months earlier. None of them had ever been treated with cyclosporine, methotrexate, or infliximab. Mucosal samples also were collected from the colon of 7 subjects who turned out to have functional diarrhea at the end of their diagnostic work-up, from macroscopically and microscopically unaffected colonic areas of 7 patients undergoing colectomy for colon cancer (mean age, 37.8 y; range, 22–65 y), and from macroscopically and microscopically inflamed and unaffected colonic areas of 7 patients affected by active ulcerative colitis (UC) (mean age, 31.4 y; range, 19–53 y), used as disease control group. Two UC patients had pancolitis, the remaining 5 had left-sided colitis. Three of them were untreated at the time of biopsy, being at the first disease presentation; 2 were treated with mesalazine and topical steroids; and 2 were treated with only mesalazine at the time of biopsy, and had suspended the steroid treatment at least 3 months earlier. Some of the mucosal samples were used to isolate myofibroblasts, some others for organ culture experiments. Each patient who took part in the study was recruited after appropriate local ethics committee approval (both in London and Southampton) and informed consent was obtained in all cases.

Cell Isolation and Culture

Mucosal myofibroblasts were isolated as previously described.[26] Briefly, the epithelial layer was removed by 1 mmol/L ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich, Poole, UK) for two 30-minute periods at 37°C. After EDTA treatment, mucosal samples were denuded of epithelial cells, and subsequently were cultured at 37°C in a humidified CO_2 incubator in Dulbecco's modified Eagle medium (Sigma-Aldrich) supplemented with 20% fetal calf serum, 1% nonessential amino acids (Invitrogen, Paisley, UK), 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μg/mL gentamycin, and 1 μg/mL amphotericin (Sigma-Aldrich). During culture, numerous cells appeared both in suspension and adherent to the culture dish. The cells in suspension were removed after every 24- to 72-hour culture period, and the denuded mucosal tissue was maintained in culture for up to 6 weeks. Established colonies of myofibroblasts were seeded into 25-cm^2 culture flasks and cultured in Dulbecco's modified Eagle medium supplemented with 20% fetal calf serum and antibiotics. At confluence, the cells were passaged using trypsin-EDTA in a 1:2 to 1:3 split ratio. Cells were grown to at least passage 4 before they were used in stimulation experiments, and were characterized by immunocytochemical staining as previously described.[27] The following antibodies were used for the myofibroblast characterization: anti–α-smooth muscle cell actin (clone 1A4; DAKO, High Wycombe, UK), antivimentin (clone V9; Santa Cruz Biotechnology, Wiltshire, UK), anti-PR2D3 (a kind gift from Dr P. Richman, Imperial Cancer Research Fund, London, UK), antidesmin (clone D33; DAKO), anti–cytokeratin-18 (clone CY-90; AbCam, Cambridge, UK), anti-CD3 (clone UCHT1; DAKO), anti-CD68 (clone PG-M1; DAKO), and appropriate isotype-matched controls (Sigma-Aldrich).

Cell Stimulation

After 24-hour culture in serum-free Dulbecco's modified Eagle medium, subconfluent monolayers of myofibroblasts seeded in 12-well plates at 3×10^5 cells per well were incubated for 24 hours with infliximab (Remicade; Schering-Plough, Milan, Italy) added to the culture medium at different concentrations (10 and 100 μg/mL) or its isotype-matched control (human IgG1, Sigma-Aldrich). In parallel experiments, cells treated with infliximab or IgG1 were incubated with 10 μmol/L mitogen-activated protein kinase (MAPK) p38 inhibitor SB 203580 (SB 203580 hydrochloride; Calbiochem, La Jolla, CA), or 1 ng/mL recombinant human interleukin-1β (R&D Systems, Abingdon, UK). Additional experiments were performed by incubating cells for 24 hours with recombinant human transforming growth factor (TGF)-β1 (10 ng/mL; R&D Systems), etanercept (10 μg/mL; Enbrel; Wyeth Europa, Maidenhead, UK), p55 TNF-receptor–human IgG fusion protein (10 μg/mL, p55-TNFR-IgG; Genentech, San Francisco, CA), and adalimumab (10 μg/mL, Humira; Abbott Laboratories, Chicago, IL).

The human Jurkat T-cell line was stimulated in anti-CD3–coated 96-well plates (BD Biosciences, Oxford, UK) with anti-CD28 antibody (0.5 μg/mL; eBioscience, San Diego, CA), and then incubated for 24 hours with 10

μg/mL infliximab or human IgG1. Cell lysates were used in Western blotting as control for caspase-3 detection.

Wound-Healing Scratch Assay

Myofibroblast migration was assessed according to the method of Rodriguez et al[28] and modified by us. Briefly, cells (2×10^5) were seeded into Nunc cell culture dishes (Nalge Nunc International, Rochester, NY) with 2-mm grids, size 35 × 10 mm, in 2 mL of Dulbecco's modified Eagle medium supplemented with 20% fetal calf serum and antibiotics. The cells were maintained at 37°C and 5% CO_2 until confluent. Once confluent, each dish of monolayer cells was given a mechanical wound by scoring with a 200-μL pipette tip, parallel to the grid bars along the central grid line. This permits easy viewing of the cells growing back together, and ensures that the 2-mm grid may be used as a reference so that the wound areas can be measured and compared. Wound placement was checked with an Olympus inverted CK2 microscope (Olympus UK Ltd., London, UK). The medium then was removed, and the cells were washed 5 times with HL-1 serum-free medium (Cambrex Bio Science, Nottingham, UK) supplemented with antibiotics, and then replaced with 1.5 mL HL-1 medium with the following treatments: human IgG1 (100 μg/mL), infliximab (10 and 100 μg/mL), adalimumab (100 μg/mL), etanercept (100 μg/mL), p55-TNFR-IgG (100 μg/mL), neutralizing anti–TIMP-1 antibody (5 μg/mL, Calbiochem, Nottingham, UK), control mouse IgG (100 μg/mL, Sigma-Aldrich), and recombinant human TIMP-1 (10^{-8} mol/L; a kind gift from Dr A. Docherty, Celltech Pharmaceuticals, Slough, UK). Some of the wells were pretreated with TNF-α (5 ng/mL, National Institute for Biological Standards and Controls, Hertfordshire, UK) for 24 hours, then washed 5 times with HL-1 serum-free medium to remove TNF-α. Cells then were treated with infliximab (100 μg/mL) or human IgG1 (100 μg/mL). Photographs of the cells in each grid along the induced wound were taken at 0, 2, 4, 8, 16, and 24 hours using a digital camera (Olympus Camedia 34-40 zoom, 20× magnification) attached to a light microscope. The computer program Image J (National Institutes of Health, Bethesda, MD) was used to measure the area of initial damage (images taken at time 0) and of the remaining damage at subsequent time points. Each grid image was observed separately; 2 points per grid at the same position at every time point were measured using imaging software at the same magnification. The percentage of wound repair then was calculated.

Flow Cytometry

Myofibroblasts were washed twice in phosphate-buffered saline containing 2% fetal calf serum and incubated at 4°C for 30 minutes with infliximab at a concentration of 1 μg/mL. A fluorescein isothiocyanate (FITC)-labeled anti-human IgG1 antibody (DAKO) was used as secondary antibody. Appropriate isotype-matched control antibodies (BD Biosciences) were included in all experiments. After washing twice with 250 μL fluorescence-activated cell sorter buffer (phosphate-buffered saline containing 1 mmol/L EDTA and 0.02% sodium azide), cells were fixed with 2% paraformaldehyde, and analyzed by flow cytometry using a FACSCalibur Flow Cytometer (BD Biosciences). The level of mTNF on cell populations was determined by geometric mean fluorescence intensity with subtraction of values for isotype-matched controls. Apoptosis was quantified using FITC–Annexin V (Zymed Laboratories, San Francisco, CA). Cells were stained with 5 μL of FITC–Annexin V diluted 1:10 in buffer. After incubation for 15 minutes, the cells were analyzed by flow cytometry using a FACSCalibur Flow Cytometer (BD Biosciences).

Organ Culture

Colonic biopsy specimens were placed on iron grids in the central well of an organ culture dish and the dishes were placed in a tight chamber with 95% O_2/5% CO_2 at 37°C, at 1 bar. Infliximab (10 and 100 μg/mL) or human IgG1 were added and, after 24 hours, proteins were extracted from the tissue and determined by Western blotting.

Western Blotting

Western blotting was performed according to a modified method described previously.[27] In brief, cells or tissue samples were lysed in ice-cold lysate buffer (10 mmol/L EDTA, 50 mmol/L pH 7.4 Tris-HCl, 150 mmol/L sodium chloride, 1% Triton X-100, 2 mmol/L phenylmethylsulfonyl fluoride, 2 mmol/L sodium orthovanadate, 10 mg/mL leupeptin, and 2 mg/mL aprotinin) and the amount of protein was determined by the Bio-Rad Protein assay (Bio-Rad Laboratories, Hemel Hempstead, UK). A total of 10 μg of protein or 15 μL of cell culture supernatants were loaded in each lane and were run on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions. After electrophoresis, protein was transferred to nitrocellulose (Bio-Rad); a sheep anti-human MMP-3 polyclonal antibody (1:500 dilution; The Binding Site, Birmingham, UK), a mouse anti-human MMP-12 monoclonal hemopexin-like domain antibody (1:200 dilution; R&D Systems), a mouse anti-human TIMP-1 monoclonal antibody (1 μg/mL; Calbiochem), a rabbit anti-human caspase-3 monoclonal antibody (1:200 dilution; Upstate, Lake Placid, NY), and infliximab (1 μg/mL) were used as primary antibodies. Rabbit anti-sheep, rabbit anti-mouse, goat anti-rabbit, or rabbit anti-human antibodies conjugated to horseradish peroxidase (DAKO) were used as secondary antibodies, and the reaction was developed with the ECL plus kit (Amersham Biosciences, Little Chalfont, UK). When required, blots were stripped and analyzed for β-actin, as an internal loading control, using a polyclonal rabbit anti-human β-actin (1:5000 dilution,

Table 1. Immunocytochemical Characterization of Colonic Myofibroblasts Isolated From 8 CD Patients and 8 Control Subjects

	Percentage positive cells (mean ± SD)						
	Anti-α-SMA	Vimentin	PR2D3	Desmin	Cytokeratin	CD3	CD68
CD	86.0 ± 3.2	94.9 ± 1.2	24.2 ± 8.4	34.6 ± 10.7	7.0 ± 2.2	0	0
Controls	85.7 ± 2.2	95.5 ± 1.8	28.8 ± 9.9	38.7 ± 6.1	6.1 ± 2.3	0	0

Anti–α-SMA, α-smooth muscle actin marker; antivimentin, cytoplasmic intermediate filament marker; anti-PR2D3, pericryptal mesenchymal cell marker; antidesmin, smooth muscle cell marker; anti-cytokeratin, cytokeratin-18 marker; anti-CD3, T-cell marker; anti-CD68, macrophage marker.

AbCam). Bands were quantified by scanning densitometry using an LKB Ultrascan XL Laser Densitometer (Kodak Ltd., Hemel Hempstead, UK).

Reverse Gelatin Zymography

Reverse gelatin zymography was performed to detect TIMPs in the myofibroblast culture supernatants. Twenty-five microliters of each sample was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 12% gels containing 1 mg/mL gelatin and 0.2 μg/mL recombinant human Pro-MMP-2 (R&D Systems). Samples were electrophoresed at 120 V for 70 minutes. The gels were washed in a washing buffer (50 mmol/L Tris, 0.2 mol/L NaCl, and 2.5% Triton X-100) for 15 minutes 2 times, and then incubated in a digestion buffer (50 mmol/L Tris, 0.2 mol/L NaCl, 10 mmol/L $CaCl_2$, 0.02% Brij-35) at 37°C for 20 hours. The gels were stained with 0.5% Coomassie blue R-250 in 50% methanol and 10% acetic acid solution for 10 minutes, and destained in 30% methanol and 10% acetic acid solution until the background of the gel became clear. Recombinant human TIMP-1 (Celltech Pharmaceuticals) was used as control protein for reverse zymography at 3 different concentrations (0.025 mol/L, 0.05 mol/L, and 0.01 mol/L).

Collagen Assay

Total soluble forms of collagen were measured in myofibroblast supernatants using the Sircol Collagen Assay kit (Biocolor Ltd, Belfast, UK) according to the manufacturer's instructions. The Sircol dye reagent has been formulated to bind specifically to the [Gly-X-Y]n helical structure found in collagen types I–XIV. The cell supernatant (200 μL) was mixed with 1 mL of Sircol dye reagent, and the tubes were shaken for 30 minutes at room temperature to allow collagen-dye binding to complete. After centrifugation, the supernatant of unbound dye was removed, and the collagen-bound dye pellet was dissolved in the alkali reagent. The recovered dye concentration was measured by a spectrometer with the absorbance of 540 nm. The calibration curve for the spectrometer had been drawn previously using the supplied collagen standard. The collagen content in each sample of cell supernatant was obtained as an average of 3 readings.

Statistical Analysis

Data were analyzed in the GraphPad Prism statistical PC program (GraphPad Software, San Diego, CA) using the paired *t* test and the Mann–Whitney *U* test. A P value of less than .05 was considered statistically significant.

Results

mTNF Expression by Myofibroblasts

First, phenotypic characterization of CD and control myofibroblasts was performed by immunocytochemistry (Table 1).

Second, to determine if intestinal myofibroblasts might be a target of infliximab, mTNF expression was analyzed by Western blotting and flow cytometry using myofibroblasts isolated from both inflamed and uninvolved areas of CD and UC patients, and from control subjects. mTNF expression detected by Western blotting on myofibroblasts was increased significantly ($P < .0001$) in both CD and UC inflamed areas in comparison with uninvolved areas and controls. No significant difference was found between myofibroblasts from IBD uninvolved areas and normal myofibroblasts (Figure 1*A*). These results were confirmed by flow cytometric analysis showing a significantly ($P < .0001$) higher mean fluorescence intensity of mTNF expression in myofibroblasts from both CD and UC lesions in comparison with myofibroblasts from uninvolved areas or control myofibroblasts. Moreover, a significantly ($P < .001$) higher number of mTNF-positive myofibroblasts was found in CD lesions (mean, 24.4% ± 4.8%) and UC lesions (mean, 20.1% ± 4.3%) in comparison with both uninvolved CD areas (mean, 5.9% ± 1.8%) and UC areas (mean, 4.2% ± 1.4%), and controls (mean, 2.8% ± 0.4%) (Figure 1*B*). No significant difference was observed in the expression of mTNF between UC and CD myofibroblasts, both in involved and uninvolved sites.

In Vitro Effect of Infliximab on Apoptosis of Myofibroblasts

To investigate the influence of infliximab on apoptosis, myofibroblasts were cultured with increasing concentrations of infliximab (10 and 100 μg/mL) or IgG1, and apoptosis was analyzed by flow cytometry with FITC–Annexin V staining. Representative results of CD patients show no effect of both concentrations of infliximab on myofibroblast apoptosis (Figure 2*A*). No significant difference was found in CD in the percentage of apoptotic myofibroblasts when cultured with IgG1

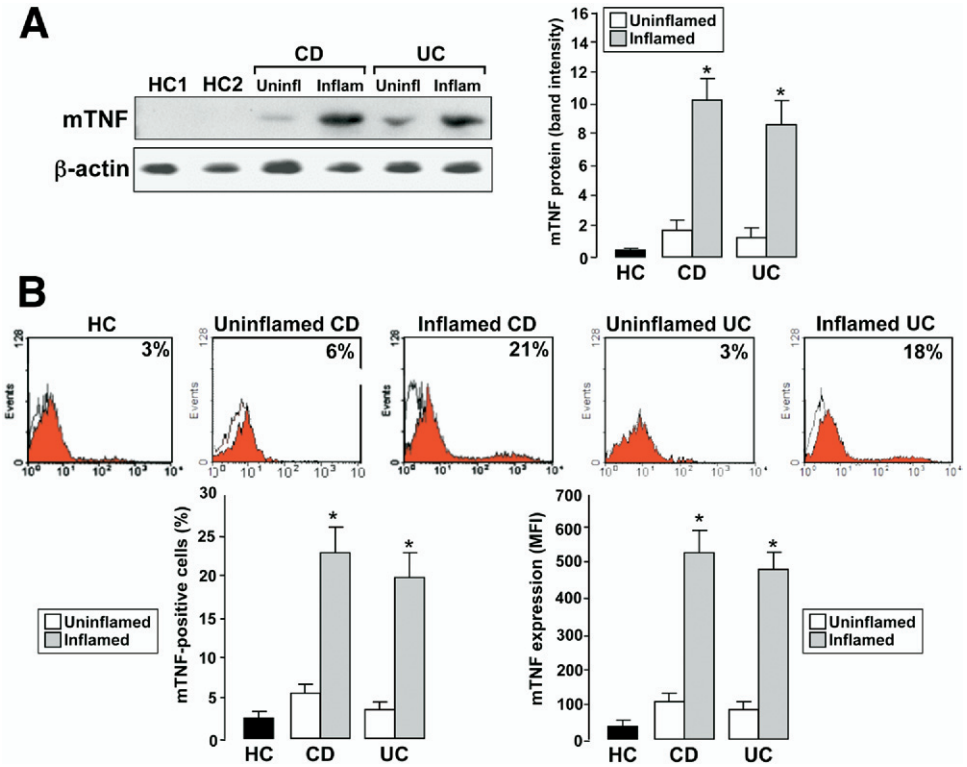

Figure 1. mTNF expression by myofibroblasts. (*A*) Expression of mTNF and β-actin in myofibroblasts isolated from 2 healthy controls (HC1 and HC2), from inflamed and uninflamed areas of a CD patient, and from inflamed and uninflamed areas of a UC patient, detected by Western blotting. The example is representative of experiments performed in 7 subjects for each group. The *right panel* shows the densitometry of Western blots. Results are mean (±SD). (*$P < .0001$ vs unaffected areas and control subjects). (*B*) Flow cytometric analysis of mTNF expression on myofibroblasts isolated from a control subject (HC), from inflamed and uninflamed areas of a CD patient, and from inflamed and uninflamed areas of a UC patient. *Solid histograms* represent staining with infliximab used as the primary antibody and *open histograms* show staining with the isotype control. Numbers within the dot plots represent the percentage of mTNF-positive cells determined with subtraction of values for the isotype-matched control antibody. The example is representative of experiments performed in 7 subjects for each group. *Lower panels* show the percentage of mTNF-positive myofibroblasts and the mean fluorescence intensity of mTNF expression. Results are mean (±SD). (*$P < .001$ vs uninflamed areas and control subjects).

(mean, 5.2% ± 1.2%) or with infliximab at 10 μg/mL (mean, 4.7% ± 1.7%) or 100 μg/mL (mean, 5.5% ± 1.5%). The same trend was observed in control myofibroblasts (mean, 5.4% ± 1.6%, 5.9% ± 1.9%, and 4.8% ± 1.4%, respectively). Western blotting showed that infliximab did not activate caspase-3 in CD myofibroblasts, as indicated by the absence of the active forms of caspase-3, the p12 and p17 subunits that instead were evident in anti–CD3/CD28-stimulated Jurkat T cells treated with infliximab (Figure 2*B*).

In Vitro Effects of Infliximab on Myofibroblast Production of MMP-3, MMP-12, and TIMP-1

As expected, interleukin-1β increased MMP-3 production by CD myofibroblasts. Infliximab did not change MMP-3 levels in the supernatants of both unstimulated and interleukin-1β–stimulated CD myofibroblasts (Figure 3*A*). However, MMP-3 production was decreased significantly ($P < .05$) by TGF-β1 (Figure 3*B*). Infliximab also did not decrease MMP-12 production, although a dramatic effect was seen with TGF-β1 (Figure 3*B*).[29] In marked contrast, there was a significant dose-dependent increase of TIMP-1 in the supernatants of CD myofibroblasts stimulated with increasing concentrations of infliximab (Figure 4*A*; $P < .005$ and $< .0001$ at 10 and 100 μg/mL, respectively). p38 MAPK inhibition by SB 203580 significantly ($P < .01$) decreased infliximab-induced TIMP-1 production by CD myofibroblasts (Figure 4*A*; $P < .01$ and $<.0001$ at 10 and 100 μg/mL, respectively). Infliximab did not show any effect on normal myofibroblasts, both in terms of MMP and TIMP-1 production (data not shown).

To confirm the infliximab-induced up-regulation of TIMP-1 production, we performed reverse gelatin zymography to assess TIMP activity in the culture supernatants of CD myofibroblasts treated with increasing concentrations of infliximab. As shown in Figure 4*B*, infliximab induced a dose-dependent increase of TIMP-1 activity in the supernatants of CD myofibroblasts. We next investigated if other anti-TNF reagents increased CD myofibroblast production of TIMP-1. As

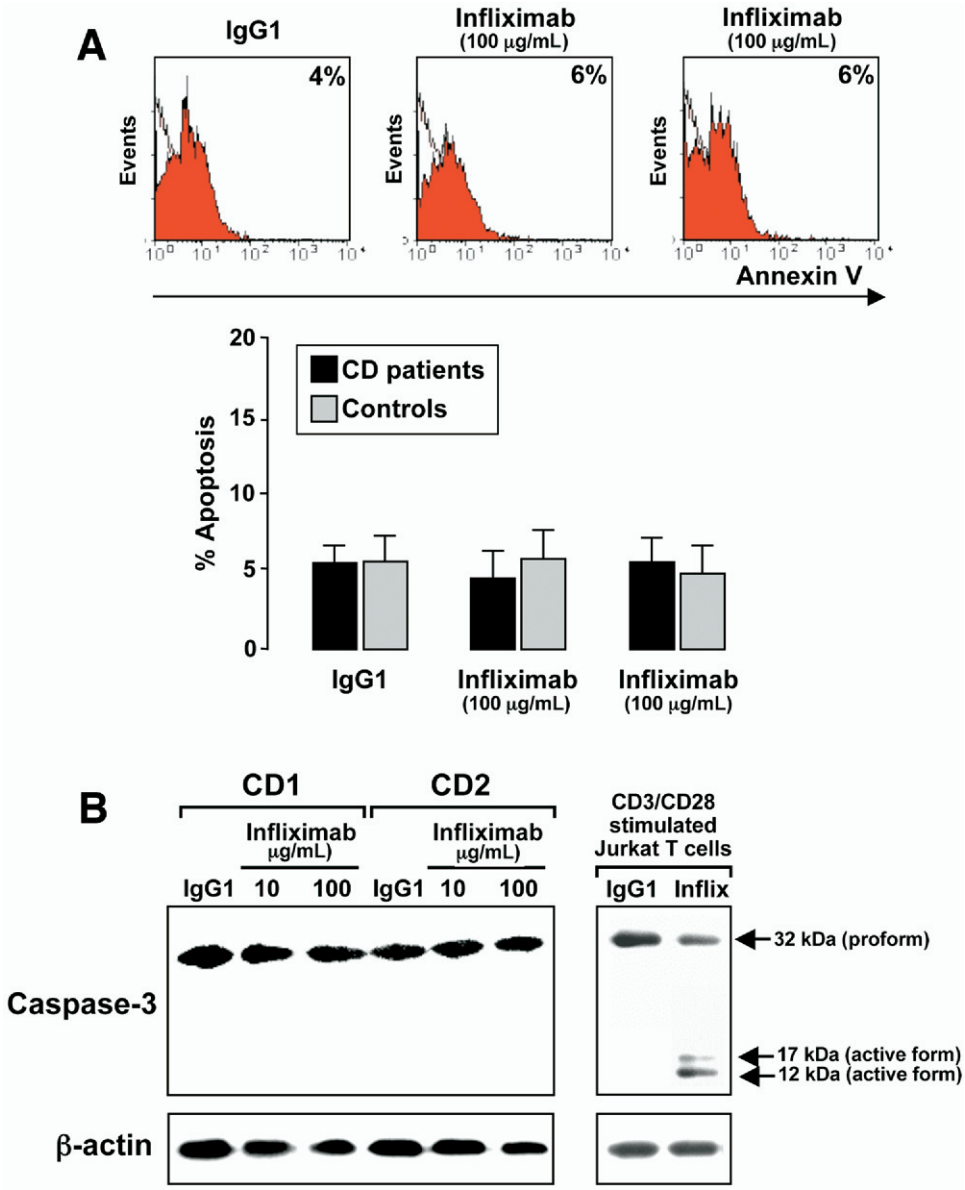

Figure 2. Effect of infliximab on myofibroblast apoptosis. (*A*) CD myofibroblasts were cultured with infliximab added to the culture medium at different concentrations (10 and 100 μg/mL) or its isotype-matched control (human IgG1). The percentage of apoptotic cells was determined by flow cytometric analysis of FITC–Annexin V staining. Numbers within the dot plots represent the percentage of apoptotic cells. The example is representative of experiments performed in 7 subjects for each group. *Lower panel* shows the percentage of apoptotic myofibroblasts cultured with increasing concentrations of infliximab (10 and 100 μg/mL) or IgG1. Results are mean (±SD). (*B*) Myofibroblasts isolated from 2 CD patients (CD1 and CD2) were treated with infliximab at different concentrations (10 and 100 μg/mL) or IgG1. Caspase-3 and β-actin were determined by Western blotting. The *upper blot* shows a band of 32 kilodaltons corresponding to the proform of caspase-3 in infliximab- or IgG1-stimulated CD myofibroblasts. However, no band was evident at the molecular weight of 12 and 17 kilodaltons corresponding to caspase-3 active forms, which instead are evident in anti-CD3/anti-CD28–stimulated Jurkat T cells treated with infliximab. Data are representative of experiments performed in 7 patients with CD.

positive control we used TGF-β1.[30] Adalimumab, etanercept, and p55-TNFR-IgG all significantly ($P < .001$) increased TIMP-1 production by CD myofibroblasts (Figure 4*C*).

Effect of Infliximab on MMP-3, MMP-12, and TIMP-1 Protein Expression in Mucosal Biopsy Specimens Grown Ex Vivo

Mucosal biopsy specimens taken from active lesions of CD patients were cultured with infliximab or IgG1, and MMP-3, MMP-12, and TIMP-1 were determined by Western blotting both in the culture supernatant and tissue (Figure 5). Infliximab showed no effect on MMP-3 and MMP-12 protein expression in both the supernatant and tissue. However, TIMP-1 was increased by infliximab in a dose-dependent manner.

Myofibroblast Wound Healing Response to Anti-TNF Antibodies and Recombinant Human TIMP-1

To evaluate the role of anti-TNF agents on myofibroblast migration, a wound-healing assay was performed using subconfluent monolayers of CD myofibroblasts. Cell migration was measured as the percentage of wound repair (see Materials and Methods section). First, we found that 10 and 100 μg/mL infliximab increased in a dose-dependent manner the migration of CD myofibroblasts in comparison with control IgG1 after 24 hours of culture. However, the statistical significance ($P < .05$) was reached only at the highest concentration of 100 μg/mL (Figure 6*A*). As expected, TNF-α significantly reduced ($P < .05$) CD myofibroblast migration (mean percentage of wound repair, 15.5% in TNF-treated cells vs

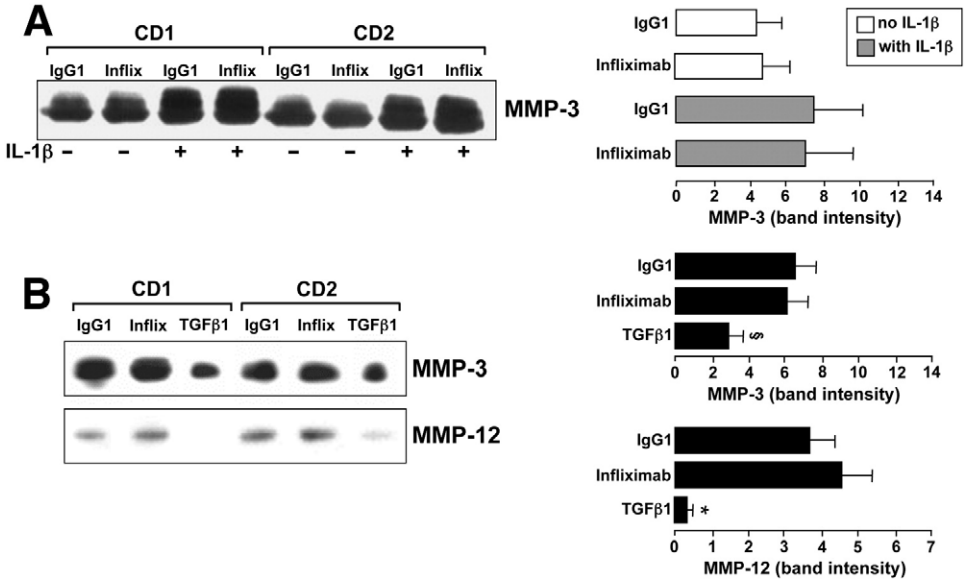

Figure 3. Effect of infliximab on MMP-3 and MMP-12 production by CD myofibroblasts. (*A*) Western blotting detection of MMP-3 protein in the supernatants of interleukin-1β–stimulated myofibroblasts isolated from 2 CD patients (CD1 and CD2) and incubated for 24 hours with 10 μg/mL infliximab (inflix) or its isotype matched control (human IgG1). The example is representative of experiments performed in 8 patients. *Right panel* shows the densitometry of Western blots. Results are mean (±SD). (*B*) Western blotting detection of MMP-3 and MMP-12 in the supernatants of myofibroblasts isolated from 2 CD patients (CD1 and CD2) and incubated for 24 hours with 10 μg/mL infliximab (INFLIX) or IgG1, or with 10 ng/mL recombinant human TGF-β1. The example is representative of experiments performed in 8 patients. *Right panel* shows the densitometry of Western blots. Results are mean (±SD). ($^{\S}P < .05$ vs cells stimulated with IgG1 and infliximab; $^{*}P < .0001$ vs cells stimulated with IgG1 and infliximab).

29.3% in untreated cells), and when infliximab was added to TNF-α–pretreated CD myofibroblasts, it partially restored cell migration (mean, 20.6% in infliximab-cultured cells vs 15.5% in IgG1-cultured cells).

Second, we compared the effect of infliximab with that of other anti-TNF agents (etanercept, adalimumab, and p55-TNFR-IgG) on myofibroblast migration in CD (Figure 6*B*). At 24 hours of culture, both infliximab and etanercept significantly ($P < .05$) increased the wound repair of CD myofibroblasts in comparison with control IgG1. p55-TNFR-IgG enhanced migration, but not significantly, whereas adalimumab slightly induced cells to move faster. All the anti-TNF agents had no effect on migration of normal myofibroblasts (data not shown).

To verify whether TIMP-1 influenced myofibroblast migration in CD, myofibroblast monolayers also were cultured in the absence or presence of recombinant human TIMP-1. TIMP-1 significantly ($P < .05$) increased the wound repair of CD myofibroblasts in comparison with unstimulated conditions at 24 hours of culture (Figure 6*B*). To further support the involvement of TIMP-1 in infliximab-induced myofibroblast migration, an anti–TIMP-1 neutralizing antibody was added to CD myofibroblasts cultured with infliximab. As shown in Table 2, the promigratory action of both infliximab and etanercept was inhibited significantly by the addition of anti–TIMP-1 antibody to the cell cultures.

Effect of Infliximab on Collagen Production by Myofibroblasts

Infliximab significantly ($P < .05$) reduced the production of collagen by myofibroblasts isolated from active lesions of CD patients (Figure 7). No change was seen in collagen production when infliximab-treated myofibroblasts were incubated with SB 203580.

Discussion

In the present study we have shown a novel therapeutic pathway for anti–TNF-α therapies: first, CD myofibroblasts show high expression of mTNF that is required for the outside-to-inside signal transduction of infliximab; second, although infliximab has no effect on CD myofibroblast production of MMP-3 and MMP-12, it may reduce the activity of these potent tissue-degrading enzymes by increasing TIMP-1 production through a p38 MAPK-dependent pathway; third, the resistance of CD myofibroblasts to infliximab-induced apoptosis may ensure the presence of a substantial number of TIMP-1–producing cells in the inflamed gut; fourth, infliximab reverts the T-helper cell type 1 cytokine-induced migration of CD myofibroblasts and reduces collagen production, thus facilitating the wound-healing process.

TNF-α is considered to be centrally involved in the pathogenesis of CD and to play a pivotal role in the inflammatory cascade.[31] It is synthesized as a transmembrane protein (mTNF), which is processed to release the

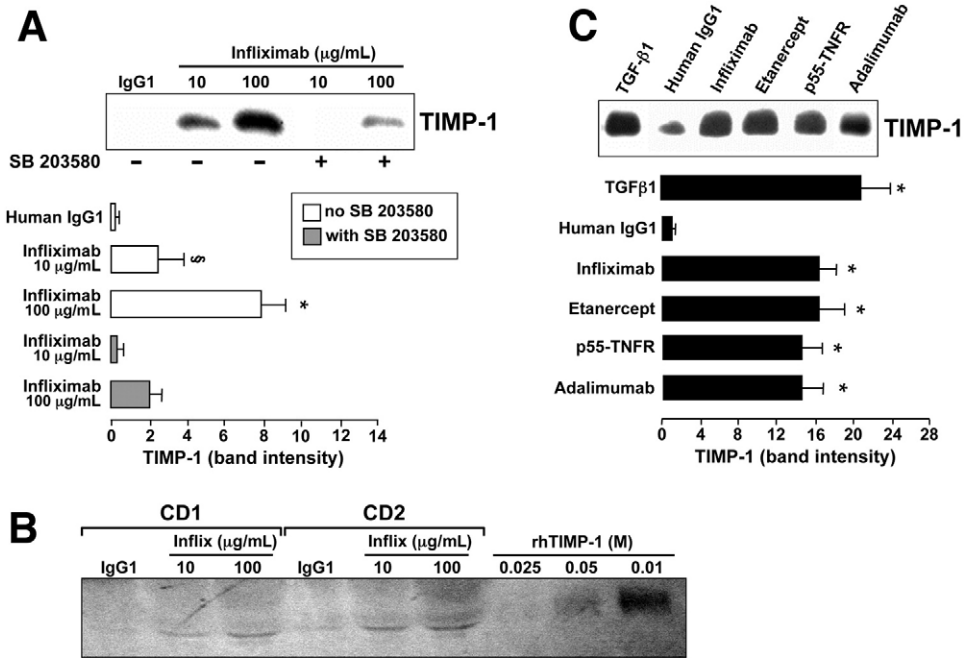

Figure 4. Effect of (*A*) infliximab and other (*B*) anti-TNF reagents on TIMP-1 production by CD myofibroblasts. (*A*) Western blotting detection of TIMP-1 protein in the supernatants of CD myofibroblasts treated with 10 or 100 μg/mL infliximab or its isotype-matched control (human IgG1) in the presence or absence of the p38 mitogen-activated protein kinase inhibitor SB 203580. The example is representative of experiments performed in 7 patients. *Right panel* shows the densitometry of Western blots. Results are mean (±SD). (*$P < .001$ vs IgG1-treated cells cultured in the absence of SB 203580, and vs 100 μg/mL infliximab-treated cells stimulated with SB 203580; §$P < .01$ vs IgG1-treated cells cultured in the absence of SB 203580, and vs 10 μg/mL infliximab-treated cells stimulated with SB 203580). (*B*) Western blotting detection of TIMP-1 protein in the supernatants of myofibroblasts isolated from a CD patient and treated with recombinant human TGF-β1, human IgG1, infliximab, etanercept, p55-TNFR, and adalimumab. The example is representative of experiments performed in 7 patients. *Right panel* shows the densitometry of Western blots. Results are mean (±SD). (*$P < .001$ vs cells treated with IgG1). (*C*) Reverse gelatin zymography performed to assess TIMP activity (see Materials and Methods section) in the culture supernatants of myofibroblasts isolated from 2 CD patients (CD1 and CD2) and treated with 10 and 100 μg/mL infliximab, or control IgG1. Recombinant human TIMP-1 (rhTIMP-1) was used as control protein for reverse zymography at 3 different concentrations (0.025, 0.05, and 0.01 mol/L). The example is representative of experiments performed in 6 patients.

soluble form.[32] Because many biological effects exerted by infliximab, including apoptosis and cell-cycle arrest, are mediated by outside-to-inside signaling through mTNF,[4,5] we first examined the presence of mTNF on CD myofibroblasts. Our data clearly showed that mTNF is expressed constitutively in intestinal myofibroblasts, and that such expression is significantly higher in CD myofibroblasts in comparison with control myofibroblasts, thus suggesting that these cells may be a potential target of infliximab. These results are consistent with a previous study that showed TNF in intestinal myofibroblasts in situ in CD.[24] The lower mTNF expression in CD myofibroblasts from unaffected intestinal areas supports the concept that mTNF expression is influenced by local inflammation rather than being intrinsic to disease. Moreover, the significantly higher expression of mTNF on myofibroblasts from UC lesions provides a further pathophysiologic explanation for the efficacy of anti–TNF-α therapy in UC patients.[33]

In unstimulated conditions CD myofibroblasts showed the same apoptotic rate of control myofibroblasts. This is somewhat different in other immune-mediated disorders, including rheumatoid arthritis,[34] idiopathic pulmonary fibrosis,[35] and systemic sclerosis,[36] where resident mesenchymal cells show resistance to apoptosis. Apoptosis of cultured fibroblasts can be induced by growth factor deprivation, inhibition of protein kinases, or alteration of ECM-fibroblast interactions.[36,37] A role for cytokines as modulators of fibroblast susceptibility to apoptosis also has been proposed. In rheumatoid arthritis, TNF-α–induced synoviolin expression inhibits fibroblast-like synoviocyte apoptosis. Hence, it has been suggested that the therapeutic effect of infliximab may include a reversal of the anti-apoptotic effects of synoviolin in connective tissue diseases.[34]

Our study shows that intestinal myofibroblasts from CD patients do not undergo infliximab-induced apoptosis. Because the proapoptotic action of infliximab strictly depends on the proteolitic activation of caspases,[6–8] we also investigated the expression of the effector caspase-3. Caspase-3 activation was absent when CD myofibroblasts were cultured with increasing concentrations of infliximab. This further strengthens the notion that infliximab does not influence myofibroblast apoptosis in CD. This finding is in keeping with other evidence that the action

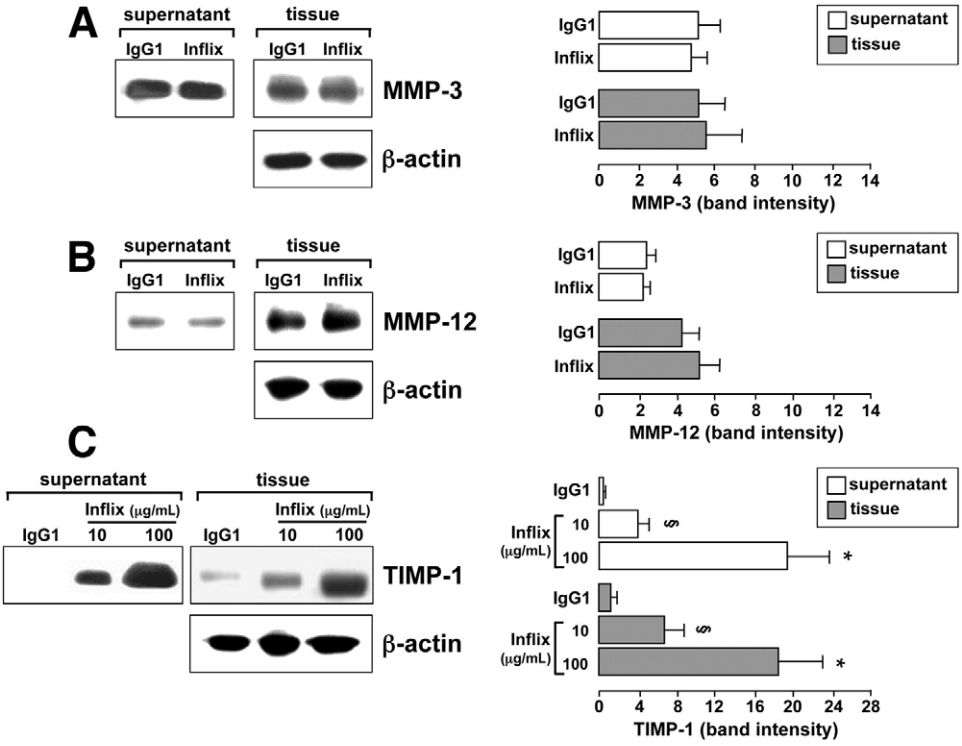

Figure 5. Ex vivo effect of infliximab (INFLIX) on MMP-3, MMP-12, and TIMP-1 expression on CD biopsy specimens. Western blotting detection of (*A*) MMP-3, (*B*) MMP-12, and (*C*) TIMP-1 protein in the supernatant and in the tissue of biopsy specimens taken from CD patients and incubated for 24 hours with 10 and/or 100 μg/mL infliximab (inflix) or its isotype-matched control (human IgG1). Blots of mucosal homogenates were stripped and analyzed for β-actin as an internal loading control. The example is representative of experiments performed in 7 patients. *Right panel* shows the densitometry of Western blots. Results are mean (±SD). (*$P < .001$ vs cells stimulated with IgG1; §$P < .01$ vs cells stimulated with IgG1 and vs cells stimulated with 100 μg/mL infliximab).

of infliximab on apoptosis of mTNF-expressing cells varies according to the type of target cell.[6–8,38,39]

In the intestine, ECM-degrading MMPs play a crucial role both in tissue injury and wound repair after the resolution of inflammation.[11,27] In explants of human fetal gut, where resident T cells have been activated along the T-helper cell type 1 pathway, MMPs have been reported to destroy the mucosa rapidly.[21] In celiac disease, in which a T-helper cell type 1 response to gluten drives tissue remodeling, MMP-3 (stromelysin-1) is overexpressed in subepithelial myofibroblasts,[40] and both transcripts and activity of MMP-12 (macrophage metalloelastase) are increased in the mucosa.[41,42] In IBD, the higher production by activated macrophages of MMP-12 together with the increased release of MMP-3 by T-helper cell type 1-cytokine-activated myofibroblasts is involved in degradation of mucosa, ulceration, and fistulas.[15–20] Interestingly, experimental evidence suggests that TNF-α blockade inhibits MMP production.[21] Because myofibroblasts are a major source of MMPs in the gut,[9–11] we hypothesized that infliximab might influence MMP myofibroblast production. However, we found that infliximab had no effect in down-regulating MMP-3 and MMP-12 production in CD myofibroblasts, and did not reduce MMP-3 and MMP-12 production in CD biopsy specimens cultured with infliximab.

The proteolytic activity of MMPs is controlled tightly by TIMPs via noncovalent binding of the active forms of MMPs at molar equivalence.[43] Because it is the imbalance between MMPs and their inhibitors, rather than MMPs on their own, that play the crucial role in tissue injury in the gut,[11,15,19] we focused our attention on TIMP-1. It is known that TNF-α decreases TIMP-1 production.[44] Infliximab induced a dose-dependent increase in TIMP-1 protein production by CD myofibroblasts, and these results were strengthened further by reverse gelatin zymography showing accordingly an infliximab-induced dose-dependent increase of TIMP-1 activity. Together with unchanged MMP-3 and MMP-12 levels, increased TIMP-1 would be expected to inhibit ECM degradation, thus allowing the healing process. Moreover, infliximab-induced up-regulation of TIMP-1 may compensate for the lack of TIMP-1 increase seen in the inflamed gut of patients with IBD.[20] The resistance of CD myofibroblasts to infliximab-induced apoptosis also assumes another important role because it may ensure the presence in the inflamed gut of TIMP-1–producing cells sufficient to counteract the activity of tissue-degrading MMP-3 and MMP-12.

Organ culture experiments performed with CD biopsy specimens showed the same dose-dependent effect of infliximab on TIMP-1 expression, both in the supernatants and tissue.

We also explored the role of other anti–TNF-α antibodies and fusion proteins that have shown varying degrees of clinical efficacy (or not) in CD.[45] Etanercept (recombinant TNF-RII/IgG1 Fc domain fusion protein), adalimumab (fully human recombinant IgG1), and recombinant human soluble p55-TNFR-IgG also increased TIMP-1 production by CD myofibroblasts, suggesting a class-action effect. We would be hesitant at this stage to overinterpret these data because etanercept clearly in-

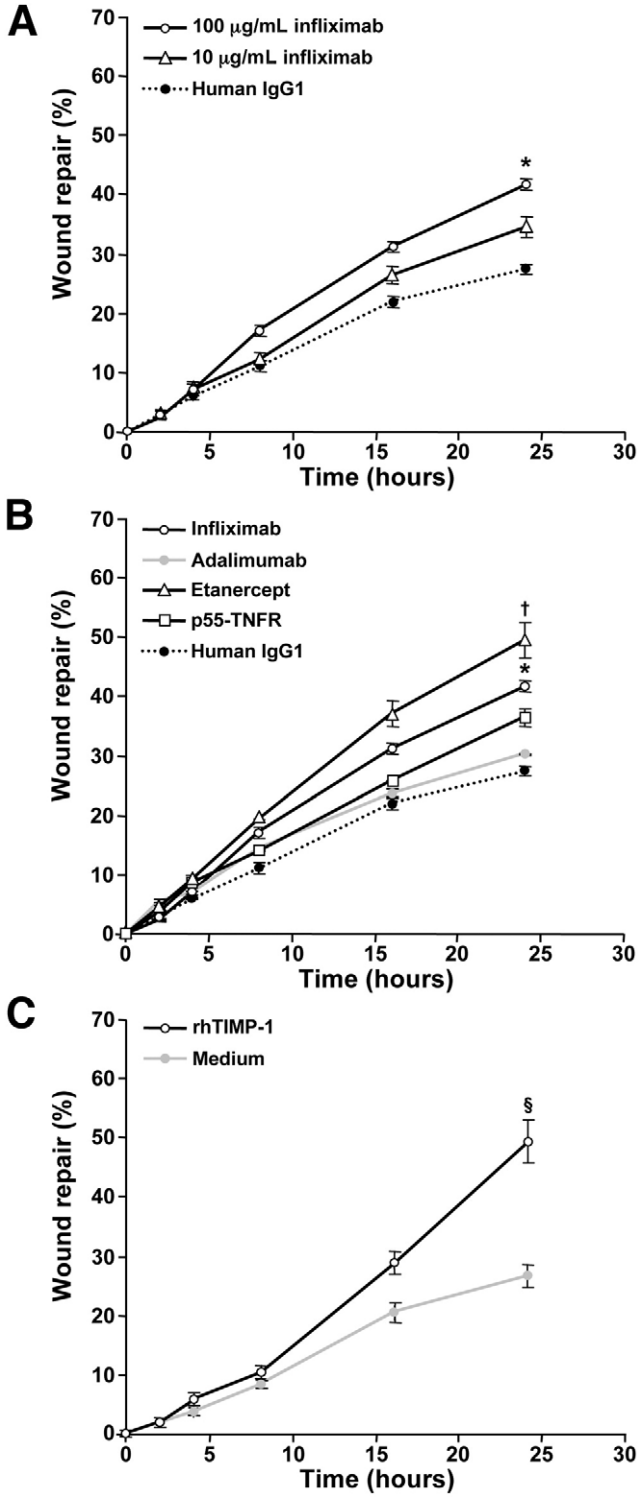

Figure 6. Effect of (*A*) infliximab, other (*B*) anti-TNF reagents, and (*C*) recombinant human TIMP-1 on the migration of CD myofibroblasts assessed by an in vitro wound-healing scratch assay. Cells were cultured with (*A*) increasing concentrations of infliximab (10 and 100 μg/mL) or its isotype-matched control (human IgG1; 100 μg/mL); or (*B*) with adalimumab, etanercept, p55-TNFR, all at the same concentration of infliximab and human IgG1 (100 μg/mL); or (*C*) in the absence or presence of recombinant human TIMP-1 (rhTIMP-1, 10^{-8} mol/L). Results obtained by culturing 8 different cell lines of CD myofibroblasts are mean (±SD). (*$P < .05$, 100 μg/mL infliximab vs IgG1 at 24 h; †$P < .05$, 100 μg/mL etanercept vs IgG1 at 24 h; §$P < .05$, rhTIMP-1 vs medium only).

duced TIMP-1, but was ineffective in CD patients.[46] The effects of anti-TNF agents are complex and of the various pathways shown (apoptosis, neutralization of soluble TNF), it still is not clear which is critical. We would only say in the context of this study that increasing TIMP-1 in disease mucosa is probably a good thing, but how this relates to remission as measured by the Crohn's Disease Activity Index is unclear.

It has been very well established that TNF binding to the TNF receptor activates both nuclear factor κB, and MAPK cascades, which cooperate in the orchestration of inflammatory responses. These molecules include extracellular signal–regulated kinase, c-Jun amino-terminal, and p38 kinases.[47,48] In the context of this article, however, it also is important to stress that p38 MAPK recently has been recognized as one of the signaling pathways that is activated by infliximab binding to mTNF. Indeed, ligation of mTNF by infliximab has been shown to activate p38 MAPK in monocytes and in lamina propria T cells from CD patients.[49–51] To determine if MAPK signaling was required to transduce the infliximab-induced up-regulation of TIMP-1 in mTNF-expressing CD myofibroblasts, we cultured infliximab-treated cells with the p38 inhibitor pyridinyl imidazole derivative SB 203580. Our results showed that SB 203580 inhibits the infliximab-induced dose-dependent increase of TIMP-1 secretion. This finding provides further evidence of the critical role of p38 MAPK cascade in transducing infliximab signaling in mTNF-expressing cells, and suggests a transcriptional control of TIMP-1 production in myofibroblasts.

This study explored the action of infliximab on the migration of intestinal myofibroblasts in CD. It is known that the myofibroblast migration is an important mechanism of intestinal wound healing.[22] In IBD, persistent mucosal ulceration has been associated with insufficient wound repair owing to a reduced migration of intestinal myofibroblasts.[23] Because TNF-α exerts inhibitory effects on the migratory potential of intestinal myofibroblasts,[23] and our data confirmed these findings, we hypothesized a possible role for infliximab in facilitating myofibroblast migration in CD. Migration of myofibroblasts was investigated by using an in vitro wound-healing scratch assay. This assay, which mimics cell migration during wound healing in vivo, is a classic and commonly used method for studying the ability of fibroblasts to recover the wound.[28] We showed that the migratory response of CD myofibroblasts to infliximab was increased dose-dependently. Interestingly, when infliximab was added to TNF-α–pretreated CD myofibroblasts, it partially counteracted TNF-α inhibitory effects by restoring to some extent cell migration.

In addition, to verify whether the stimulatory effects of infliximab on myofibroblast migration was associated with the concomitant increased production of TIMP-1, recombinant human TIMP-1 was used as stimulus in the

Table 2. Effect of TIMP-1 Neutralization on Wound Healing Response to Infliximab and Etanercept by CD Myofibroblasts

Culture conditions	% Wound repair	SEM	*P* value (*t*-test, 2-tailed)
Human IgG1 + mouse IgG	27.3	0.7924	.0150 (vs infliximab + mouse IgG)
Human IgG1 + anti–TIMP-1 Ab	23.2	0.3478	
Infliximab + mouse IgG	41.8	0.6858	
Infliximab + anti–TIMP-1 Ab	21.1	2.4398	.0399 (vs infliximab + mouse IgG)
Etanercept + mouse IgG	49.5	2.9520	.0485 (vs human IgG1 + mouse IgG)
Etanercept + anti–TIMP-1 Ab	21.7	0.0460	.0306 (vs etanercept + mouse IgG)

NOTE. The percentage of wound repair is shown as the mean, and was assessed by using the wound-healing scratch assay after 24 hours of culture (see Materials and Methods section). Human IgG1 was used as control for both infliximab and etanercept, whereas mouse IgG was used as control for the mouse anti-human TIMP-1 neutralizing antibody (anti–TIMP-1 Ab).
SEM, standard error of the mean.

wound-healing scratch assay, and in parallel experiments an anti–TIMP-1 neutralizing antibody was added to CD myofibroblasts cultured with infliximab. TIMP-1 markedly enhanced the migratory potential of CD myofibroblasts, while neutralization of TIMP-1 significantly abolished the promigratory action of infliximab. The same significant inhibitory action of the anti–TIMP-1 antibody was evident on cells treated with etanercept. This latter showed a significant promigratory action of CD myofibroblasts, comparable with that of infliximab and higher in comparison with p55-TNFR-IgG and adalimumab, whose positive influence on migration was limited. This finding is quite surprising if we take into consideration the well-known ineffectiveness of etanercept in clinical trials in CD,[46] and, conversely, the proved efficacy of adalimumab in this condition.[52] However, our in vitro study, focused on a single cell population, does not entirely reflect the in vivo mucosal environment where the actions of anti-TNF agents on survival, cytokine production, activation, proliferation, and migration are exerted on a complex network of a number of immune and nonimmune cells. In ongoing studies, we are investigating whether infliximab-induced myofibroblast migration is associated with an increase in the tyrosine phosphorylation of p125 focal adhesion kinase that is a central regulator of cell migration in health and disease.[23,53]

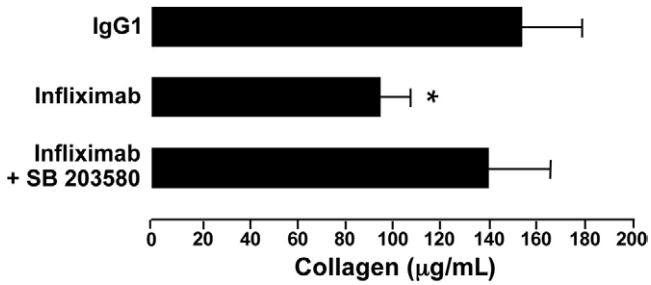

Figure 7. In vitro effect of infliximab on collagen production by CD myofibroblasts. Colonic myofibroblasts isolated from 9 CD patients were incubated with 10 μg/mL infliximab or its isotype-matched control (human IgG1) in the presence or absence of the p38 MAPK inhibitor SB 203580 (10 μmol/L). After 24 hours of culture, soluble collagen was determined in cell supernatants. Results are mean (±SD). (*$P < .05$ vs cells cultured with IgG1 and vs infliximab-stimulated cells cultured with SB 203580).

A number of studies have indicated that changes in the migratory potential of mesenchymal cells often are associated with the development of fibrosis in different diseases.[54,55] Intestinal fibrosis and stricture formation are common features of the natural course of CD, which often require surgical resection.[56] Impaired wound contraction and increased deposition of ECM components as a result of the local increase in the number of CD myofibroblasts with reduced dispersing potential seem to play a role in favoring intestinal fibrogenesis.[23] Hence, it is conceivable that infliximab might prevent the fibrogenic process in CD by restoring myofibroblast migration. Concerns have been raised that rapid healing of narrowed segments induced by infliximab may further narrow the lumen and possibly could provoke obstruction.[57] It therefore has been suggested that patients with possible or documented strictures should not be treated with this agent.[57] We thus measured collagen in the supernatants of CD myofibroblasts treated with infliximab. Our data showed that infliximab down-regulates collagen production. These findings are strengthened further by previous studies showing a role of infliximab in down-regulating the profibrogenic basic fibroblast growth factor in the serum of CD patients.[58,59] The action of infliximab in restoring migration and in reducing collagen production of CD myofibroblasts supports the concept of an antifibrogenic role for this agent. In accordance with this, clinical studies have shown that infliximab treatment does not result in an increased risk of intestinal obstruction, and is effective in preventing postoperative recurrence in CD patients.[60,61] In addition, we found that the action of infliximab on collagen production by CD myofibroblasts is abolished by SB 203580. This result provides further evidence for the role of p38 MAPK in transducing infliximab signaling in CD myofibroblasts.

In conclusion, our study shows a novel therapeutic pathway for anti-TNF therapies in enhancing TIMP-1 production, thereby reducing MMP activity and facilitat-

ing the wound-healing potential of intestinal myofibroblasts.

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