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