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A p55 TNF Receptor Immunoadhesin Prevents T Cell-Mediated Intestinal Injury by Inhibiting Matrix Metalloproteinase Production[1]

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Anti-TNF-α Ab therapy has been shown to be of benefit in the treatment of active Crohn's disease, but the tissue-injuring processes in the gut mediated by TNF-α that might be inhibited by neutralizing Ab are unknown. In this work, we have used a p55 TNF receptor-human IgG fusion protein (TNFR-IgG) to prevent the severe mucosal injury that ensues when lamina propria T cells in explant cultures of human fetal small intestine are directly activated with the lectin PWM. Following T cell activation and associated with mucosal injury, there is a marked elevation of soluble TNF-α in organ culture supernatants and a large increase in TNF-α mRNA transcripts. The addition of TNFR-IgG at the onset of cultures greatly reduced PWM-induced tissue injury, without inhibiting the increase in TNF-α and IFN-γ transcripts seen following T cell activation. Mucosal injury in this model is mediated by endogenously-produced matrix metalloproteinases (MMPs). When TNFR-IgG was added to PWM-stimulated explants, there was a reduction in MMPs in the explant culture supernatants, especially stromelysin-1. Recombinant TNF-α and IL-1β added directly to mucosal mesenchymal cell lines also caused an increase in MMP production, but only the former was inhibited by the TNFR-IgG. These results suggest that one of the ways in which TNF-α causes tissue injury in the gut is by stimulating mucosal mesenchymal cell to secrete matrix-degrading metalloproteinases. Neutralization of this activity should help maintain tissue integrity. *The Journal of Immunology*, **1998, 160: 4098–4103.**

TNF-α is a pleiotropic cytokine produced mainly by monocytes, macrophages, and T cells, which plays a key role in host defense; however, it also contributes to tissue injury in inflammatory and autoimmune diseases, including septic shock and rheumatoid arthritis when produced in excess (1–3). There is increasing evidence that TNF-α is also important in inflammatory bowel disease (IBD).[3] In Crohn's disease, serum TNF-α concentrations are moderately increased (4). TNF-α-positive cells can be detected by immunohistochemistry throughout the mucosa, and elevated numbers of TNF-α-secreting cells are present in single-cell suspensions of mucosal biopsies (5, 6). TNF-α immunoreactive cells are also elevated in the gut in ulcerative colitis, but in this case the increase is restricted to the mucosa (6). High concentrations of TNF-α can be detected in stools of children with active IBD (7), and isolated mononuclear cells from patients with IBD secrete more TNF-α than cells from control patients (8).

Several studies have addressed the potential efficacy of anti-TNF-α treatment in Crohn's disease (reviewed in Ref. 9). Treatment of patients with severe steroid refractory Crohn's colitis with a TNF-α Ab (cA2) resulted in a rapid decrease in Crohn's disease activity index, remarkable healing of mucosal ulcers, complete clinical remission, and few side effects (10). A single infusion of cA2 Ab therapy has been shown to be efficacious in inducing remission in Crohn's disease in a placebo-controlled, double-blind multicenter trial (11). Work that has been reported in abstract form shows that repeated administration of cA2 Ab can also maintain remission (12).

The mechanism by which anti-TNF-α therapy inhibits inflammation in Crohn's disease is unknown. The Abs may be neutralizing soluble TNF-α in the interstitial fluids. Alternatively, the Ab most widely reported, cA2, is of the IgG1 isotype and may bind onto membrane-bound TNF-α and fix complement. Thus, there is a possibility that the therapeutic effect is not due to TNF-α neutralization, but is by the cytotoxic killing of TNF-α-secreting T cells and macrophages, thereby lowering the concentrations of all tissue cytokines. In this regard, another TNF-α Ab of the IgG4 isotype, which is unlikely to fix complement, appears to be less effective in Crohn's disease than the cA2 Ab (13). Underlying all of this is the lack of knowledge of the mechanisms by which increased concentrations of TNF-α cause tissue injury in the gut.

We have recently demonstrated that following activation of lamina propria T cells in explant cultures of human fetal small intestine with the lectin PWM there is complete destruction of the mucosa (14, 15). This is a relatively acute model of tissue injury compared with the chronic response seen in Crohn's disease in patients, and obviously lacks the complexity seen in vivo. Nevertheless, in both Crohn's disease and in the fetal gut system, Th1 cells appear to be of major importance (15–17). The final mediators of tissue injury in the fetal gut explant model appear to be matrix metalloproteinases (MMPs), especially stromelysin-1. When stromal cells from fetal intestine are stimulated in vitro with TNF-α they produce extremely large amounts of MMPs (15). Therefore a possible therapeutic effect of anti-TNF-α therapy is to

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[3] Abbreviations used in this paper: IBD, inflammatory bowel disease; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinase; IL-1RA, IL-1 receptor antagonist; H&E, hematoxylin and eosin.

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interrupt this pathway of tissue injury and prevent mucosal matrix degradation. In this work, we have therefore used a soluble TNF-α receptor-human IgG fusion protein to attempt to inhibit injury in the fetal gut model. We show that the fusion protein is highly effective in preventing injury and that this is related to a down-regulation of MMP production.

Materials and Methods[4]

TNFR fusion protein

TNFR-IgG fusion protein consists of the extracellular portion of human p55 TNFR linked to the hinge, C_H2 and C_H3 domains of human IgG1 heavy chain (18). The TNF-α-binding affinity of TNFR-IgG is 75 to 100 pM (18, 19). It has been shown that this TNFR-IgG is more potent than anti-TNF-α mAb in protecting against rat endotoxic shock in vivo (18, 20) and that its ability to neutralize the activity of endogenous TNF-α in murine listeriosis is 10-fold that of anti-TNF-α mAb TN3-19.12 (21).

Organ culture of human fetal small intestine

Second-trimester human fetal small intestine was obtained within 2 h of surgical termination from the Medical Research Council Tissue Bank, The Hammersmith Hospital, London, U.K. All the specimens used in this study were aged between 16 and 18 wk gestation.

Culture of human fetal small intestine explants in serum-free medium was performed as previously described (22, 23). Mucosal T cells were activated by the addition of PWM (10 μg/ml, Sigma Chemical, Dorset, U.K.) to the explant culture. TNFR-IgG fusion protein (10 μg/ml, Genentech, South San Francisco, CA) was added together with PWM at the onset of culture. Human IgG (10 μg/ml, Sigma Chemical) was used as a control.

Fetal explants were cultured up to day 3 unless otherwise stated. Tissue explants were snap-frozen in liquid nitrogen-cooled 2-methylbutane and the culture supernatants were spun at 1200 × *g* for 10 min to remove cell debris and stored at −70°C for further analysis.

TNF-α production in human fetal small intestine explant culture

Aliquots of 100 μl of explant culture supernatant from each 20-explant culture were assayed for TNF-α by an ELISA (R&D Systems Europe, Abingdon, U.K.). All assays were standardized using recombinant protein and were done in duplicate. The lower limit of sensitivity of the assay was 1 pg/ml. The production of TNF-α was normalized to explant tissue protein. Culture explants were homogenized in buffer that contains 1% Triton X-100 in TBS. The protein content was determined by the Bio-Rad Protein Assay.

Quantification of cytokine mRNA

To examine whether the TNFR-IgG fusion protein had any effect on the T cell-mediated immunity of the intestinal explants, the expression of IFN-γ and TNF-α mRNA was examined by competitive reverse transcriptase PCR according to the method of Jung et al. (24). A DNA construct pHCQ1 was used that encodes the primer sites for various cytokines, a kind gift from Dr. Martin Kagnoff (Department of Medicine, University of California at San Diego, La Jolla, CA). This was transcribed in vitro using T7 RNA polymerase (Promega, Madison, WI) according to the manufacturers instructions to yield a synthetic RNA encoding the cytokine primer sites identical to those on cellular target RNA. A competitive PCR was then performed after the standard RNA and test RNA were coreverse transcribed. PCR products were electrophoresed in 1% agarose gels. Bands were visualized and their intensities were quantified by densitometry (Seescan 1D gel analysis package v1.00, Seescan, Cambridge, U.K.). The ratios of the band intensities of the PCR products from the standard RNA and the target RNA were plotted against the starting amount of standard RNA molecules on a semilogarithmic scale. In this way, the point at which the starting number of standard RNA transcripts is equal to the starting amount of cellular target RNA transcripts can be determined. This technique allows us to quantify the number of cytokine transcripts in a tissue sample down to as little as 1,000 transcripts per microgram of total RNA.

Morphologic and histologic assessment of fetal explants

The morphology of the living explants was assessed by inverted phase contrast microscopy and the changes in gross morphology were assessed by the criteria described previously (23). In brief, normal explants showed long villi and there was little surface debris. The explants showing adaptive changes had partial villus atrophy and crypt hyperplasia with extensive debris on the surface of the mucosa; the villi were short and of variable length but clearly identifiable. However, no villi were visible in those destroyed explants. The surface of the explants was covered with debris and when the cell debris was shaken off the avillous surface was clearly apparent. Frozen sections of explants were stained with hematoxylin and eosin (H&E) to view the histology.

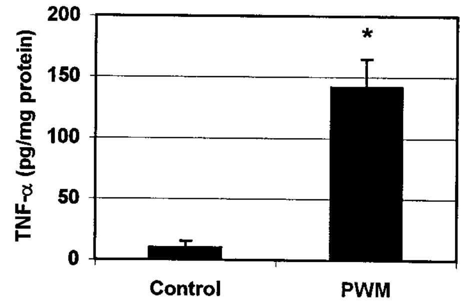

FIGURE 1. The concentration of TNF-α in human fetal small intestine explant cultures. Culture supernatants were collected after 3 days of culture with or without PWM stimulation (20 explants per dish). Bars show the mean ± SEM of three separate experiments using tissue from different fetuses. The increase in TNF-α in PWM-treated explants was statistically significant (**p* < 0.025, Mann-Whitney *U* test).

Mucosal mesenchymal cells

Human fetal mesenchymal cells were isolated and characterized according to the method described previously (15). A total of 5×10^5 cells were seeded into 25-cm^2 area culture flasks and maintained in MEM and 10% FCS until confluence. The cell layer was then washed twice in serum-free MEM and cultured with rTNF-α (1 ng/ml; R&D Systems Europe) or IL-1β (1 ng/ml; R&D Systems Europe) in serial dilutions of TNFR-IgG for 48 h. Culture supernatants were removed and spun at 1200 × *g* for 10 min to remove cell debris.

Detection of MMPs

To determine the effect of TNFR-IgG on MMP and TIMP production by explants and mucosal mesenchymal cells, Western blotting and substrate gel electrophoresis were conducted using the reagents and according to the methods described previously (15). In all cases, equivalent amounts of protein were loaded onto each lane of the gel. Computer-assisted scanning densitometry (SeeScan) was used to analyze the intensity of the immunoreactive bands.

Statistical analysis

Differences between groups were compared using either the Mann-Whitney *U* test, if the data were not normally distributed, or Student's *t* test, if the observations were consistent with a sample from a normally distributed population.

Results

Production of TNF-α protein and mRNA in PWM-stimulated human intestinal explants

Following activation of lamina propria T cells with PWM, there was a 10-fold increase in TNF-α protein secreted in the day 3 PWM-stimulated explant culture supernatants compared with those of unstimulated controls (Fig. 1). Using competitive quantitative PCR, it is also clear that T cell activation induces a significant increase in TNF-α transcripts in the fetal gut explants (a 1000-fold increase in the number of TNF-α transcripts in PWM-stimulated explants compared with controls), as well as a large increase in IFN-γ transcripts (Fig. 2).

TNFR-IgG fusion protein prevents tissue injury

In two separate experiments, PWM produced severe tissue injury with complete mucosal destruction in 100% of the explants. If

[4] This study received ethical approval from the Hackney and District Health Authority, London, U.K.

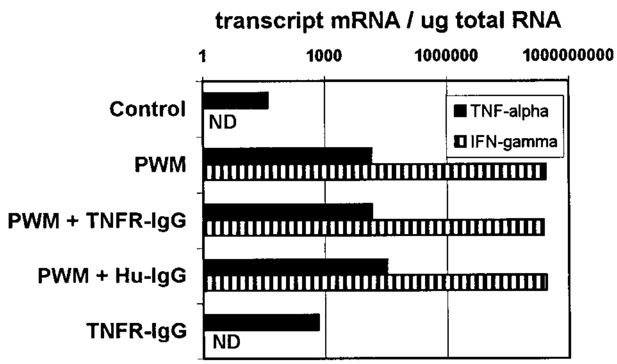

FIGURE 2. Effects of the TNFR-IgG fusion protein on the number of IFN-γ and TNF-α transcripts using quantitative competitive reverse transcriptase-PCR. The fusion protein was added at the onset of culture, and the transcripts were measured on day 1. ND = not detectable.

TNFR-IgG was added at the onset of the cultures along with the PWM, about 93% (37/40 explants) were morphologically normal (Table I). Control normal human IgG had no effect on tissue injury. Histology showed that control explants had normal villi, short crypts, and columnar epithelium, while in day 3 explants cultured with PWM, there was severe tissue injury with epithelial cell shedding and only shreds of the mucosa remaining (Fig. 3). The addition of TNFR-IgG at 10 μg/ml dramatically inhibited tissue injury, and villus morphology was retained even though there is some crypt hypertrophy (Fig. 3).

To ensure that these effects were not due to some unexpected inhibitory effects of the fusion protein on T cell activation, quantitative PCR was conducted. It is clear that there was no difference in the numbers of TNF-α transcripts and IFN-γ transcripts in the PWM-treated explants cultured with TNFR-IgG compared with PWM alone, and that they were markedly elevated above the values detected in control explants and explants cultured with the fusion protein alone (Fig. 2).

TNFR-IgG fusion protein inhibits the production of MMPs

As we have shown that activation of lamina propria T cells results in increased concentrations of MMPs in the organ culture supernatants and that inhibition of their enzymatic activity ameliorates injury (14), Western blotting was used to investigate the effect of TNFR-IgG on PWM-induced MMP production. In control supernatants, collagenase, stromelysin-1, gelatinase A and B, and TIMP-1 were detectable. T cell activation with PWM resulted in increased amounts of both the inactive and active forms of MMP-1, and MMP-3, increased amounts of the inactive forms of gelatinase A and B, and increased TIMP-1. Normal human IgG did not have any inhibitory effect on PWM-induced MMP production (Fig. 4); however, TNFR-IgG produced a slight decrease in collagenase and gelatinase B, had little effect on gelatinase A and TIMP-1 immunoreactivity, but markedly reduced stromelysin-1. When MMP-specific bands (both latent form and active form) were analyzed by quantitative scanning densitometry, the total

Table I. *Morphological appearance of human fetal explants on day 3*

	Normal	Adaptive Changes (%)	Destruction
Control	100	0	0
PWM	0	0	100
PWM + TNFR-IgG	93	7	0
PWM + human IgG	0	5	95
TNFR-IgG	100	0	0

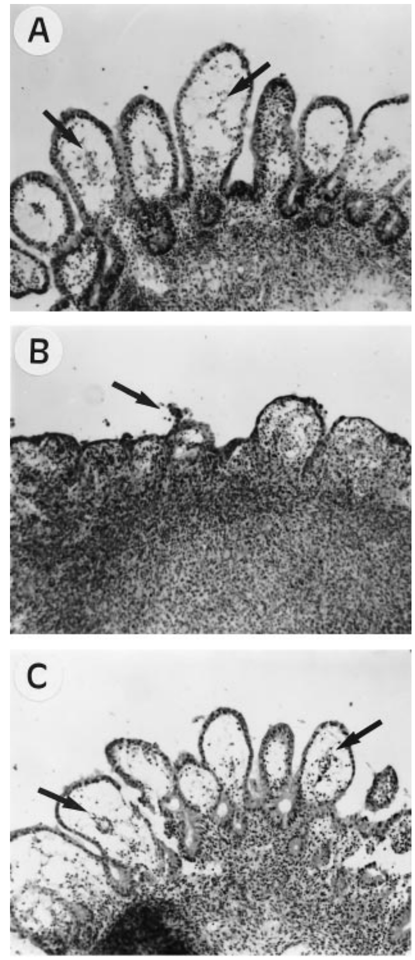

FIGURE 3. Effects of the TNFR-IgG on T cell-mediated tissue injury. Sections were stained with H&E. *A* shows day 3 control explants; villi were present, and the crypts were short. After 3 days' culture, villi in fetal explants were swollen due to edema, but in the villus core, stromal cells were still clearly identifiable (arrows). *B*, the morphology of an explant cultured with PWM for 3 days is shown; only the stumps of the villi remain, and shed epithelial cells (arrow) can be seen on the explant. *C*, a day 3 explant in which 10 μg/ml TNFR-IgG fusion protein was added at the onset of culture along with PWM; there was some crypt hypertrophy, but villus morphology and cells in the villus core were retained (arrows). H&E stain; original magnification, ×100.

amount of interstitial collagenase was 1.7 times lower, stromelysin-1 was 16.8 times lower, and gelatinase-B was 7.8 times lower in explants cocultured with PWM and TNFR-IgG fusion protein than in those cultured with PWM and normal human IgG. When TIMP-1 bands were scanned, the density was three times higher in PWM-stimulated cultures than in control explant supernatants alone but was only slightly decreased in explants cocultured with PWM and TNFR-IgG fusion protein.

TNFR-IgG inhibits TNF-α but not IL-1β-induced production of MMPs and TIMP-1 by mucosal mesenchymal cells

TNF-α (1 ng/ml) causes an increase in MMP (especially collagenase and stromelysin-1) and TIMP-1 production by mucosal mesenchymal cells (Fig. 5*A*). When graded doses of TNFR-IgG were added along with the TNF-α, significant inhibition of collagenase, stromelysin-1, gelatinase B, and TIMP-1 was seen, even at 10 ng/ml TNFR-IgG. Gelatinase A was unaffected. This was confirmed by zymography (Fig. 5*B*). As a specificity control, IL-1β

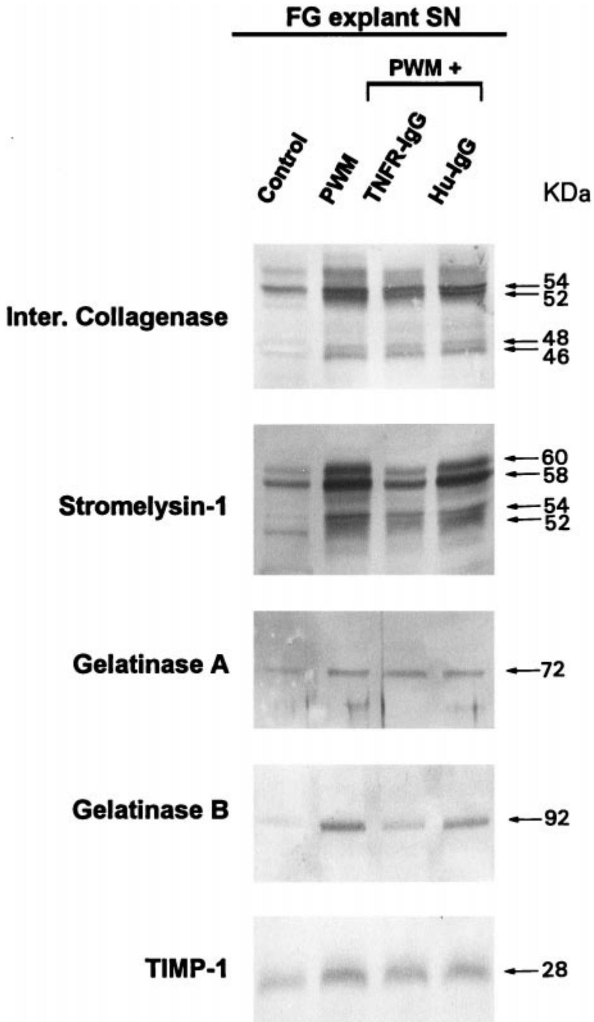

FIGURE 4. Effect of TNFR-IgG fusion protein on MMP and TIMP-1 production in PWM-stimulated human fetal small intestine explants. TNFR-IgG fusion protein and normal human IgG (10 μg/ml) were added at the onset of culture. Supernatants were collected after 3 days of culture, concentrated 6- to 7-fold, and run on 10% SDS-PAGE under reducing conditions. Arrows indicate MMP-specific m.w. detected by polyclonal anti-MMPs and mAb TIMP-1. The example shown is representative of four separate experiments.

was also investigated (Fig. 5, *C* and *D*). It also produced an increase in MMPs, but had less effect on TIMP-1. However, in the presence of graded doses of TNFR-IgG, IL-1β-induced MMP production was unaltered (Fig. 5, *C* and *D*).

Discussion

In this work we show clearly that a TNFR-IgG can prevent the severe tissue injury that occurs following lamina propria T cell activation with the lectin PWM in explant cultures of human fetal small intestine. The fetal gut system differs from clinical intestinal inflammation in many ways. Although T cell-mediated (22, 23), it is a relatively speedy response in comparison with the chronic immune activation seen in Crohn's disease. Tissue injury can manifest as villous atrophy and crypt cell hyperplasia or as severe injury with complete mucosal destruction similar to that seen in severe graft-vs-host and late stage intestinal allograft rejection (22, 23). PWM-induced tissue injury is almost completely inhibited by cyclosporin A and FK506 (23), indicating that injury is T cell mediated and not due to the nonspecific effect of the lectin. In addition, there is no contribution by blood-borne inflammatory cells such as neutrophils and eosinophils. Nonetheless, we believe that it gives important insights into the pathways by which activated T cells, macrophages, and stromal cells interact during mucosal cell-mediated immune responses.

There was no evidence that the TNFR-IgG had any unexpected immunosuppressive effects since neither TNF-α or IFN-γ transcripts were reduced, and therefore we can attribute its effects to neutralization of the TNF-α within the tissue. Whether this occurs by binding soluble TNF-α trimers or membrane-bound molecules is unknown; however, we can exclude complement-mediated destruction of TNF-secreting cells since the explants were cultured in serum-free medium.

Elevated concentrations of TNF-α were present in organ culture supernatants following activation of lamina propria T cells with PWM. This TNF-α likely originates from lamina propria T cells (25), or tissue macrophages activated by T cell products. In patients with IBD, the majority of the TNF-α-secreting cells are monocytes recently extravasated from the blood (26); there is no a priori reason why resident macrophages should not make TNF-α, especially in the fetus where there are marked differences between the class II$^+$ MHC cells compared with postnatal intestine (27). In any regard, the cellular source of the TNF-α is probably less important than its functional consequences, since in IBD, TNF-α has been detected in epithelial cells, fibroblasts, neutrophils, mast cells, eosinophils, Paneth cells, and macrophages (28), but how many of these cells produce meaningful amounts is unknown.

The effects of TNF-α in the gut have been studied in some detail. In a series of experiments, Hsueh and colleagues (29, 30) showed that TNF-α, synergizing with endotoxin, caused elevated mucosal phospholipase A2 and increased local concentrations of platelet-activating factor, resulting in bowel necrosis. TNF-α has also been reported to kill epithelial cells (31) and impair epithelial barrier function (32). It can also up-regulate adhesion molecule expression on human mucosal vascular endothelial cells in culture (33). All of these pathways probably play a role in tissue injury in the gut. However, since the lamina propria is a soft tissue consisting of structural collagens, proteoglycans, and glycoproteins such as fibronectin, glycosaminoglycans, and ground substance, we have been interested in the role of matrix-degrading enzymes in tissue injury in the gut.

TNF-α markedly up-regulates interstitial collagenase and stromelysin-1 production by mucosal mesenchymal cells (15), as it does with mesenchymal cells from other tissues (34–38). In vitro experiments using mesenchymal cell lines (Fig. 5, *A* and *B*) showed that the addition of very low doses of TNFR-IgG (10 ng/ml) were effective at inhibiting TNF-α-induced interstitial collagenase and stromelysin-1 production. The increase in MMPs was also seen in the culture supernatants of explants treated with PWM in which there was extensive mucosal injury, confirming previous studies (15). When the TNFR-IgG was added, there was a marked reduction in stromelysin-1 and gelatinase B, but much less of a reduction in collagenase and gelatinase A. We consider it highly probable that the reduction in stromelysin-1 is the reason for the maintenance of structural integrity in the PWM-stimulated explants cocultured with TNFR-IgG. In previous studies, we have added recombinant gelatinase A and B, stromelysin-1, and interstitial collagenase to explants and only stromelysin causes tissue injury at low concentrations. In addition, a stromelysin/gelatinase inhibitor is much more effective than a collagenase inhibitor in preventing injury (15). We would therefore suggest that a major role for TNF-α in gut injury is to induce stromelysin-1 production by mucosal mesenchymal cells. It is also worthy of comment that

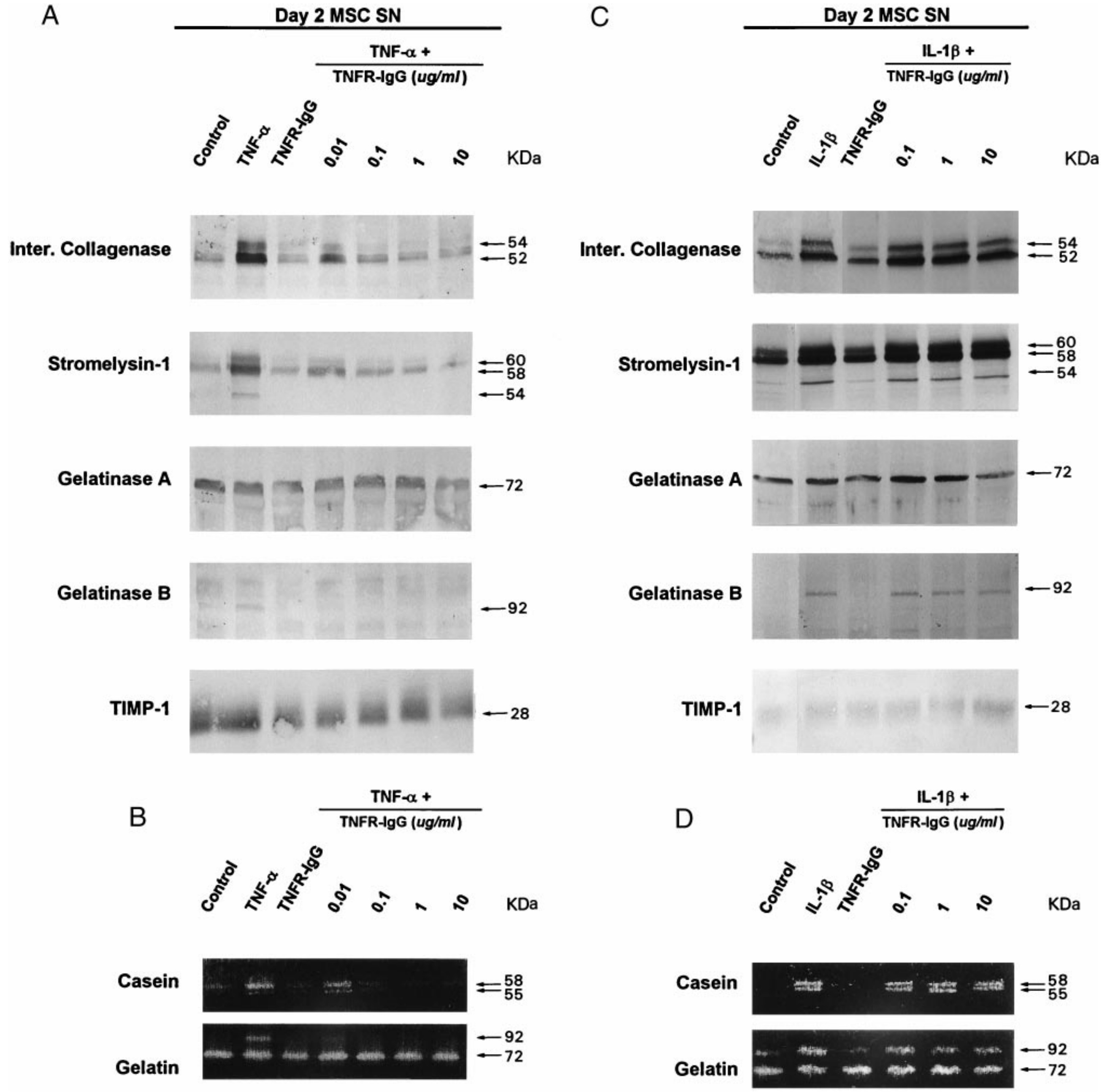

FIGURE 5. Effect of TNFR-IgG fusion protein on MMP production by human fetal mucosal mesenchymal cells stimulated with TNF-α or IL-1β. *A* and *C*, Western blotting of MSC culture supernatants after stimulation with TNF-α or IL-1β for 2 days. Unconcentrated supernatants were loaded onto 10% SDS-PAGE and run under reducing conditions. *B* and *D*, Substrate gel electrophoresis of the culture supernatants described in *A* and *C*. Twenty-fold concentrated culture supernatants were run in casein-embedded gels whereas unconcentrated culture supernatants were run in gelatin-embedded gels. All gels were run under nonreducing conditions. Arrows indicate the MMP-specific m.w. The example shown is representative of two separate experiments. It is clear that, following cytokine activation, stromal cells secrete increased amounts of collagenase and stromelysin, but the increase in gelatinases is modest, and gelatinase B is near the limit of detection.

although interstitial collagenase was markedly increased in PWM-stimulated explants, it was only minimally decreased when TNFR-IgG was added. This also suggests that stromelysin-1 is more important than interstitial collagenase in this model, but it also suggests that, whatever is causing the elevated collagenase, it is a factor other than TNF-α, such as IL-1β. Alternatively, the cell source in the tissue may be different. Perhaps the collagenase is made predominantly by macrophages whereas the stromelysin is made by TNF-α-activated mesenchymal cells.

One of the puzzles in interpreting the therapeutic effects of anti-TNF-α therapy in Crohn's disease as specific inhibition of TNF-α is that the treatment should have no effect on other proinflammatory cytokines such as IL-1β, unless one evokes a complex feedback loop whereby TNF-α promotes the production of other cytokines. IL-1β concentrations are markedly elevated in IBD (39), and in rabbit colitis, IL-1R antagonist (IL-1RA) is highly effective at preventing gut injury (40). Studies in man have revealed an imbalance of IL-1β/IL-1RA ratios (41, 42) in the gut in IBD as well as elevated concentrations of platelet-activating factor, IL-6, IL-8, IL-15, etc. (43). There have, however, been no reported clinical studies on the use of IL-1RA in IBD. Even if one neutralizes TNF-α, however, one would expect that local IL-1β would also act on mucosal mesenchymal cells to increase stromelysin-1 production (Fig. 5) and maintain tissue injury. However, this does not appear to be the case, either in fetal gut explants or in patients, so perhaps in the tissue microenvironment there is sufficient IL-1RA to inhibit IL-1β, despite the altered ratios.

Finally, we also investigated TIMP-1 production following T cell activation in explants and in mesenchymal cells activated with cytokines. In the explants, there was a modest increase in TIMP-1 in the PWM-stimulated explant supernatants, which was only slightly decreased by TNFR-IgG. In vitro, mesenchymal cells secrete marginally more TIMP-1 when stimulated with rTNF-α and this was inhibited by the TNFR-IgG. Nothing is known about the regulation of TIMP-1 production in the gut, but these preliminary results suggest that it may be possible to down-regulate MMPs without such a dramatic effect on TIMP-1, thereby increasing the capacity of the gut's endogenous inhibitors to prevent MMP-mediated matrix degradation.

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