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Matrix metalloproteinases in inflammatory bowel disease : expression, regulation and clinical relevance

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Chapter 3

Effect of the Anti-Tumor Necrosis Factor- α Antibody Infliximab on the *ex vivo* Mucosal Matrix Metalloproteinase-Proteolytic Phenotype in Inflammatory Bowel Disease (IBD)

Short title: Infliximab and MMPs in IBD

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Abstract

Background/aims. Previous studies have shown an upregulation of matrix metalloproteinases (MMPs) in intestinal tissue of patients with inflammatory bowel disease (IBD) and significant clinical improvement after administration of the anti-TNF- α antibody infliximab. The aims of our study were to determine expression and secretion of MMP-1, -2, -3, -9, and their inhibitors TIMP-1, -2 by IBD versus control intestinal mucosa *ex vivo* and to assess the regulatory capacity by infliximab of the proteolytic phenotype.

Methods. Intestinal mucosal explants from 20 IBD and 15 control patients were cultured with or without infliximab and/or the T-cell activator pokeweed mitogen (PWM). Explants and culture supernatants were analyzed for MMPs, TIMPs, and TNF- α protein, activity and/or mRNA levels. All patients were genotyped for functional TNF- α , MMP, and TIMP single nucleotide polymorphism (SNP) loci.

Results. Expression of MMP and TIMP protein/activity in basal medium was higher in IBD versus control explants. Dependent on genotype at SNP loci, infliximab downregulated MMP-1, -3, and -9 relative to TIMP-1 and -2 and also decreased MMP-1 and -3 activities, while PWM enhanced these levels, partly counteracted again by infliximab. The expression of MMP-2 relative to TIMP did not change by treatment with infliximab and/or PWM.

Conclusions. The high expression of MMPs in patients with IBD suggests a role for these proteinases in the pathogenesis of this disease. Infliximab seems to induce a genotype-associated matrix protective phenotype, which may contribute to the observed therapeutic efficacy of this drug in IBD, particularly at the mucosal surface.

Introduction

Patients with Crohn's disease (CD) suffer from multifocal transmural inflammation potentially affecting the whole gastrointestinal lining, but most often localized in the ileocecal region, while patients with ulcerative colitis (UC) have a more superficial continuous mucosal inflammation, affecting the colon only. In both forms of inflammatory bowel disease (IBD), the sustained inflammation may cause severe tissue damage, translating into clinical complaints such as diarrhea, cramps, weight loss, blood iron deficiency, etc. When refractory to medical treatment or due to complications this may result in surgical resection of the affected bowel. Early studies have shown an upregulation of several cytokines in IBD and TNF- α seems to be pivotal. The TNF- α mRNA and/or protein expression was shown to be upregulated in blood,¹ intestinal mucosa,^{2,3} stools,⁴ and cultured intestinal biopsies.⁵ Considering its pleiotropic proinflammatory nature, a chimeric monoclonal antihuman TNF- α antibody was engineered⁶ and found to be beneficial to a large proportion of CD and UC patients refractory to standard treatment with steroids.⁷⁻⁹ Matrix metalloproteinases (MMPs) constitute a family of diverse matrix degrading zinc- and calcium-activated neutral endoproteinases, some of which are under cytokine control and are implicated in a wide range of biological processes including angiogenesis, implantation, and cancer metastasis.¹⁰ A marked upregulation of MMP-1, -2, -3, -7, -9, -10, -12, -13, and -14 mRNA and/or (active) protein levels has been demonstrated in the intestinal mucosa of IBD patients, shifting the balance between these proteinases and their natural inhibitors, i.e., tissue inhibitors of metalloproteinases (TIMPs),¹¹ toward a net proteolytic activity.¹²⁻¹⁷ The enhanced expression of MMPs near ulcers in inflamed IBD intestinal tissue reinforces the potential pathogenic role of these proteinases. Also, in the rat trinitrobenzenesulfonic acid and human pokeweed mitogen (PWM)-activated fetal intestinal models of IBD the application of MMP inhibitors was associated with a decrease in inflammation score and/or tissue damage,¹⁸⁻²⁰ while in the murine dextran sodium sulfate model of UC inhibition of macrophage migration inhibitory factor was associated with reduction of inflammation, tissue damage, and diminished MMP-13 expression.²¹ Based on these data, we speculated that infliximab might be of clinical benefit through an

inhibition of TNF- α -mediated increase in net MMP activity. We experimentally manipulated the concentration of bioavailable TNF- α in IBD mucosa *ex vivo* and studied the effects on the expression and/or activities of MMP-1, -2, -3, -9, and TIMP-1, -2. Recently, a number of single nucleotide polymorphisms (SNPs) in the genes coding for TNF- α , MMPs, and TIMPs have been described and several of these SNPs were shown to be functional and/or related to the pathogenesis of various diseases.²²⁻²⁷ We therefore also examined the effects of these SNPs on TNF- α -regulated MMP and TIMP expression.

Patients and methods

Patients and tissue samples

Demographic and clinical data of the patients are depicted in Table 1. Diagnosis of CD or UC was based on standard clinical, endoscopic, radiologic, and histopathologic findings. Judged by the presence of ulcers, erythematous appearance, marked bowel wall thickening, and/or loss of circular folding, surgically resected IBD tissue was considered macroscopically normal or affected. The control group consisted of three patients with diverticular disease and 12 patients with neoplasia; normal tissue was collected at least 10 cm away from affected tissue. Most IBD but none of the control patients received immunosuppressive therapy prior to surgery.

Tissue culture

Resection specimens were obtained at the Pathology Department of the Leiden University Medical Center within 1 hour after surgical removal and transported in L-15 medium supplemented with penicillin and streptomycin to the laboratory of the Gastroenterology Department. After thorough washing in L-15, mucosa and submucosa were carefully separated from muscularis externa and serosa. Mucosal layers were cut in \approx 3x3 mm preweighted explants and cultured per 10 parts in 6-well culture plates containing 3.5 mL CMRL-1066 basic medium, modified according to Autrup *et al.*,²⁸ with the omission of cortisone. Culture plates were

Table 1. Demographic and clinical data of the IBD and control patients included in the explant cultures

	CD	UC	Control
No. of patients	11	9	15
Median age in years (range)	34 (16–54)	42 (24–75)	66 (41–83)
Male/female	4/7	4/5	8/7
Ileum/colon	5/16	1/10	1/15
Normal/affected tissue	6/15	1/10	16/0
No. of patients with medication			
— Mesalazine	8	4	-
— Steroids	9	7	-
— Azathioprine, cyclosporine	6	6	-
— Infliximab	1	0	-

IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis.

positioned in a sealed box (Billups-Rothenberg, Del Mar, CA), which was subsequently filled with 95% O₂/ 5% CO₂ according to the manufacturer's instructions and placed on a rocking platform. Explants were cultured for 72 hours at 37 °C with replenishment of medium at 24 and 48 hours, and supernatants and explants were stored at -70 °C until subsequent analysis. Infliximab (Centocor, The Netherlands, final concentration 0.14 mg/mL corresponding to physiological plasma concentration in CD patients and completely neutralizing TNF- α biological activity²⁹) and T/B cell activator pokeweed mitogen (PWM, Sigma, The Netherlands, final dilution 1:100 according to manufacturer's protocol) were added to parallel incubations.

Immunohistochemistry

Frozen explants were cut into 7- μ m sections and fixed in formaldehyde and Carnoy's modified medium. After blocking of endogenous peroxidase and nonspecific binding by 0.3% hydrogen peroxide in methanol and normal goat serum, respectively, sections were incubated overnight with mouse antihuman cytokeratin 18 antibody (Santa Cruz Bio-technology, Santa Cruz, CA).

Subsequently, biotinylated goat antimouse antibody and streptavidin-HRP conjugate (DAKO, Glostrup, Denmark) were added. Conversion of amino-9-ethylcarbazole in the presence of hydrogen peroxide resulted in a clear red staining. Sections were lightly counterstained in Mayer's hematoxylin and mounted in Aquamount.

Protein determination

Tissue homogenates (25 mg/mL) were prepared in 0.1 M Tris-HCl, 0.1% Tween 80, pH 7.5, using a Potter device (B. Braun, Germany).³⁰ The TNF- α (Biosource, Camarillo, CA), MMP-2, -9 (in-house), TIMP-1,-2 (R&D Systems, Minneapolis, MN) protein levels in tissue homogenates and/or culture supernatants were measured in parallel with appropriate standards by highly specific enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions or as described previously.^{31,32} Values of TNF- α obtained from infliximab containing samples were multiplied 5-fold to correct for 80% inhibition of measurement of TNF- α by this antibody.³³ The MMP ELISAs recognize latent, active, and TIMP-complexed forms with comparable high efficiencies. Levels of MMP-1 and -3 were measured by specific immunocapture bioactivity assays from Amersham Biosciences (Arlington Heights, IL).³⁴ Briefly, MMP was captured from samples by immobilized MMP-specific antibodies and incubated in buffer with/without *p*-aminophenyl mercuric acetate (APMA) to measure the total of APMA activatable and endogenously active MMP (MMPt) or active MMP only (MMPa), respectively. Subsequently, pro-urokinase modified to contain an MMP recognition cleavage site was added and the color of chromogenic substrate converted by MMP-activated urokinase was measured at 405 nm. Values are expressed in arbitrary units and show good linear correlation with sample MMP protein.

Semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR)

Intestinal mucosa in 4 mL guanidinium thiocyanate solution was homogenized using a Turrax device (IKA-Werke, Germany) and RNA was isolated as described previously.³⁵ Four micrograms of DNase (Sigma)-treated RNA were reverse

transcribed with MMLV reverse transcriptase (Invitrogen, The Netherlands) in a final volume of 40 μ L according to the manufacturer's protocol with only small modifications. Subsequently, cDNA was diluted 20–40,000-fold in a final volume of 25 μ L and amplified with Red Taq DNA polymerase (Sigma) and target-specific primers using a thermal cycler from Biometra (Germany). The PCR protocol consisted of a 3' DNA denaturation step at 94 °C, 28–33 amplification cycles (30 sec at 94 °C, 45 sec at 56–60 °C and 1 min at 72 °C each) and a final extension step of 7 minutes at 72 °C. Oligonucleotide primers were published elsewhere³⁶ or designed using the Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA) and are as follows (sense, antisense, 5' to 3'): GGGTCAGAAGGATTCTATG and GGTCTCAAACATGATCTGGG (β -actin); GATATCGGGGCTTTGATGTA and TCCTTGGGGTATCCGTGTAG (MMP-1); AGGATCATTGGCTACACACC and AGCTGTCATAGGATGTGCC (MMP-2); CGATGCAGCCATTTCTGATA and TGTGACAAGGTGCAAGCTAAG (MMP-3); CGCAGACATCGTCATCCAGT and GGATTGGCCTTGAAGATGA (MMP-9); CCCAGGGACCTCTCTCTAA and GGAAGACCCCTCCAGATAG (TNF- α) resulting in 238, 398, 535, 486, 406, and 413 bp products. The amplified DNA was visualized on agarose gel by UV transillumination, scanned using SCION software (www.scioncorp.com), and quantified essentially as described by Murphy *et al.*,³⁷ with β -actin normalized values representing expression in arbitrary units relative to a positive standard run in parallel. Appropriate RNA-MMLV and cDNA controls were run along and always found to be negative.

Single nucleotide polymorphism analysis

Genomic DNA reconstituted in Tris-EDTA buffer was routinely obtained using an adapted blood leukocyte DNA isolation protocol.³⁸ The genotype at (SNP) loci MMP-1 -1607 1G/2G, MMP-3 -1613 5T/6T, MMP-9 -1562 C/T, TIMP-1 +372 T/C, TIMP-2 +303 G/A, and TNF- α -308 G/A were determined by restriction fragment length polymorphism (RFLP) analysis. Primers, restriction enzymes, and gel restriction fragment pattern corresponding to genotype are specified in Table 2. The SNP at MMP-2 -1306 C/T was analyzed by tetra primer amplification refractory mutational system (ARMS) PCR, the principles of which are described elsewhere.³⁹

Table 2. Specification of primers and restriction enzymes used for analysis of SNPs in MMP, TIMP, and TNF- α genes with size of PCR products on agarose gel and inferred genotype.

Gene/SNP	Forward and reverse primers	Restriction Enzyme	Products (bp)/genotype
MMP-1 -1607 1G/2G	GAAATTGTAGTTAAATCCTTAGAAAG TATGGATTCTGTCTTTCTTTCTGC	EcoN I	120;120+99+21;99+21 1G1G;1G2G;2G2G
MMP-2 -1306 C/T	ACCAGACAAGCCTGAACTTGTCTGA TGTGACAACCGTCTCTGAGGAATG ATATTCCCCACCCAGCAGCT GCTGAGACCTGAAGAGCTAAAGAGTTG		542+379;542+379+211; 542+211 CC;CT;TT
MMP-3 -1613 5T/6T	CTTCCTGGAATTCACATCACTGCCACCACT GGTTCTCCATTCTTTGATGGGGGAAAGA	Tth111 I	130;130+97+32; 97+32 6T6T;6T5T;5T5T
MMP-9 -1562 C/T	ATGGCTCATGCCGTAATC TCACCTTCTTCAAAGCCCTATT	SpH I	352;352+208+144; 208+144 CC;CT;TT
TIMP-1 +372 T/C	GCACATCACTACCTGCAGTC GAAACAAGCCCACGATTTAG	BssS I	175;175+155+20; 155+20 TT or T;CT;CC or C
TIMP-2 +303 G/A	CCTCCTCGGCAGTGTGTG TAGGAACAGCCCCACTTCTG	TspR I	12+16;112+97+24+16; 97+24+16 GG;GA;AA
TNF- α -308 G/A	GAGGCAATAGTTTTGAGGGCCAT GGGACACACAAGCATCAAG	Nco I	126+21;147+126+21; 147 GG;GA;AA

SNP, single nucleotide polymorphism; PCR, polymerase chain reaction. Analysis of SNP MMP-2 -1306 was performed by tetra primer ARMS PCR which involves four oligonucleotide primers but no restriction enzyme. Primers and restriction enzymes for the analysis of SNPs at MMP-1, MMP-3, TIMP-1 and TNF- α loci are from references 55, 56, 57, and 58, respectively.

Analysis of SNP MMP-2 -1306 was performed by tetra primer ARMS PCR, which involves four oligonucleotide primers but no restriction enzyme. Primers and restriction enzymes for the analysis of SNPs at MMP-1, MMP-3, TIMP-1, and TNF- α loci are from previous publications,⁵⁵⁻⁵⁸ respectively.

Statistical analysis

At the protein level, data are presented as means \pm SEM and statistical significance of differences between groups was assessed by (paired) Student's *t*-test. At the mRNA level, data are presented as median (range) and statistical analysis was performed by Mann–Whitney *U*- and Wilcoxon signed ranks test. Correlations were calculated by Spearman's ranks test; all *P*-values are two-tailed and tests were conducted using the SPSS statistical software, v. 11.0 (Chicago, IL).

Results

Validation of culture

After 72 hours of culture in basal medium, lamina propria, muscularis mucosae, and submucosa were still clearly identifiable. Mucosa contained considerable numbers of crypts and lamina propria was covered by large stretches of cytokeratin-18-expressing columnar epithelial cells, with interindividual variation depending on starting tissue. Occasional mucosal edema was noted (Fig. 1). Addition of PWM caused significant time-dependent deterioration of tissue and after 72 hours crypts and epithelial coverage were no more present. In the lamina propria, condensed nuclei heavily stained with eosin were frequently observed at 72 hours, most likely representing activated lymphocytes. Addition of infliximab to basal medium or next to PWM did not alter explant morphological appearance. Replenishment of medium at 24 and 48 hours more closely maintained physiological pH as judged by medium color but did not improve explant morphology.

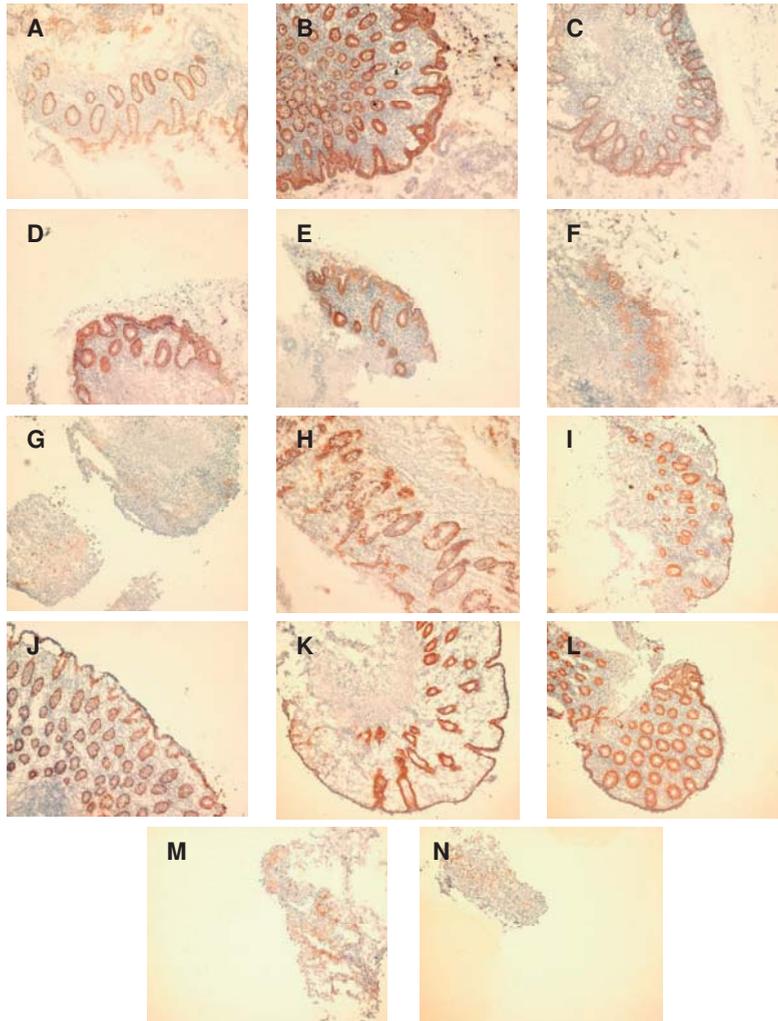
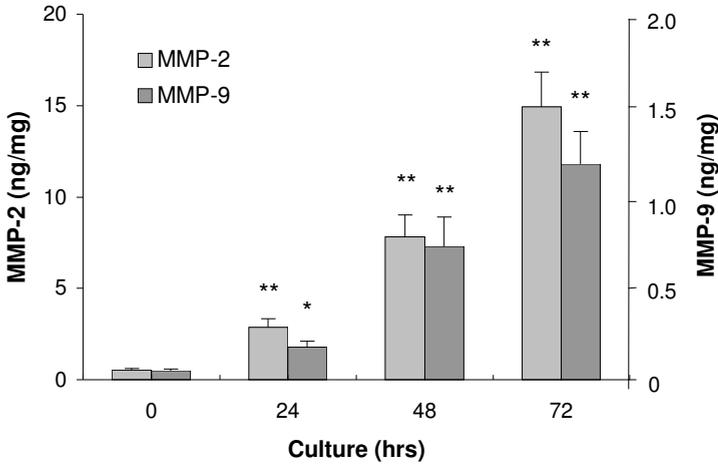


Figure 1. Immunohistochemical staining of cultured intestinal explants. Preculture tissue of a control patient (A) and after 24, 48, and 72 hours in basal medium (B–D, respectively). Note the good preservation of morphology and presence of red-colored cytokeratin-18-expressing epithelial cells. After incubation with PWM for 24 hours, tissue was still well preserved (E). However, prolonged incubation resulted in a gradual deterioration at 48 and 72 hours (F versus G). Fibrotic (H,I) and normal-appearing tissue (J–N) from a CD patient. After 72 hours culture in basal medium, morphology is well preserved (I, K) compared with preculture tissue (H, J). Incubation with PWM resulted in severe tissue destruction at 72 hours (M) and infliximab did not improve morphology, either next to PWM treatment (N) or added to basal medium (L). Similar results were obtained for (affected) CD, UC, and controls.

A)



B)

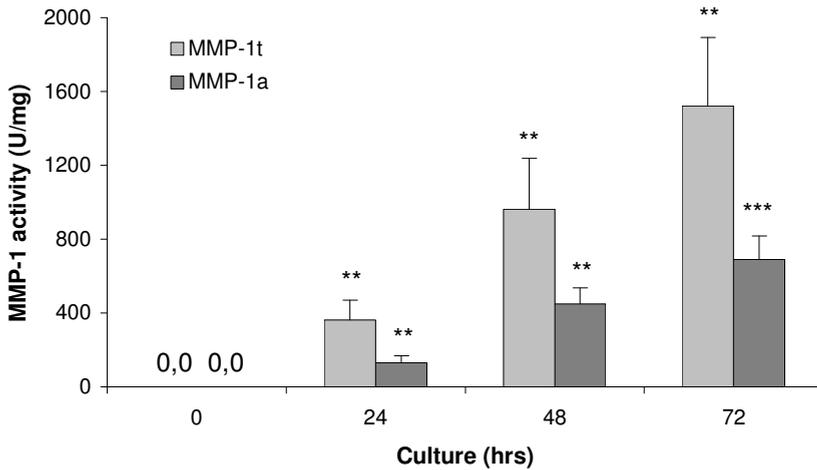
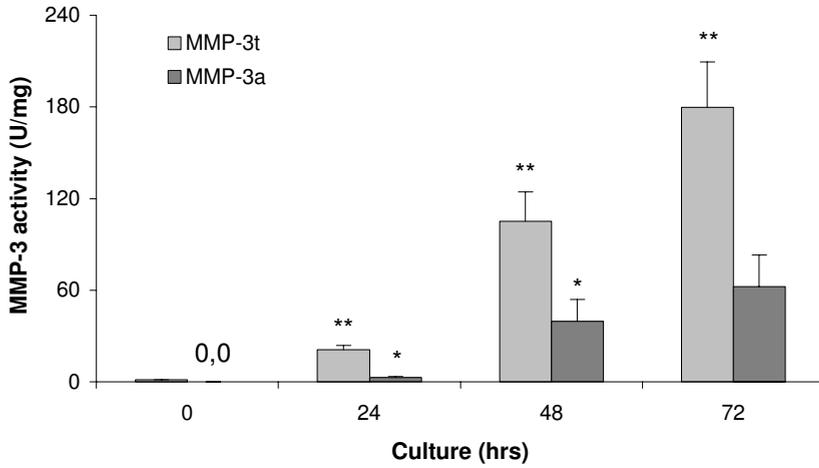


Figure 2. A–E: Basal protein expression/secretion by control intestinal explants. Preculture tissue homogenates (0) and supernatants at indicated times were measured. A: MMP protein in ng MMP/mg preincubation explant weight (mean \pm SEM), $n = 10 - 15$. ** $P < 0.01$, * $P < 0.05$ versus level 24 hours earlier. B: MMP-1 activity in U MMP/mg preincubation explant weight (mean \pm SEM) $n = 9 - 10$. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ versus level 24 hours earlier.

C)



D)

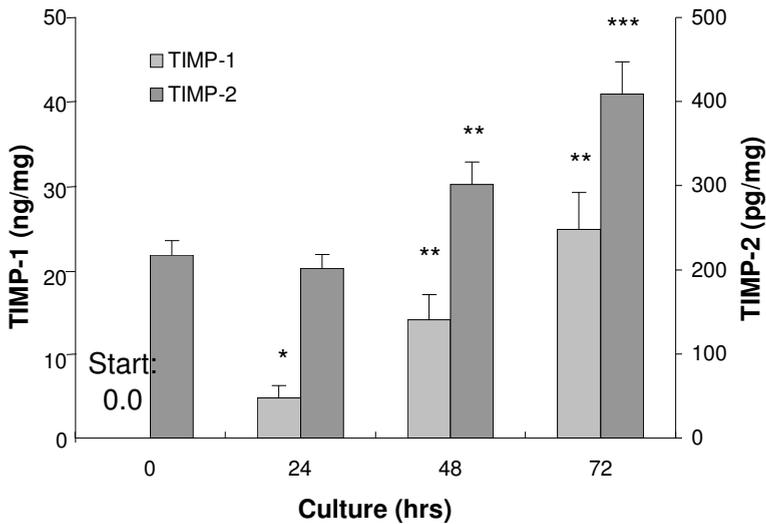


Figure 2 continued. C: MMP-3 activity in U MMP/mg preincubation explant weight (mean \pm SEM) $n = 6$. ** $P < 0.01$, * $P < 0.05$ versus level 24 hours earlier. D: TIMP protein in ng or pg TIMP/mg preincubation explant weight (mean \pm SEM), $n = 6$. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ versus level 24 hours earlier.

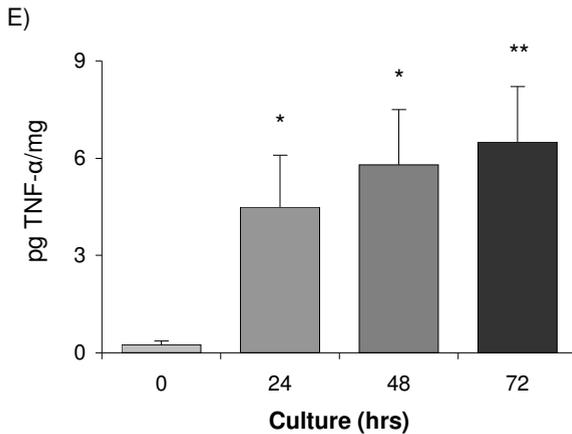


Figure 2 continued. E: TNF- α protein in pg TNF- α /mg pre-incubation explant weight (mean \pm SEM), $n = 9 - 15$. ** $P < 0.01$, * $P < 0.05$ versus level 24 hours earlier.

Production of MMPs

During culture significant amounts of MMPs were continuously secreted into the medium (Fig. 2A,B), with a substantial fraction of MMP-1, -3 (30–50%) and the gelatinases (MMP-2: 15–55%, MMP-9: > 75%, data not shown) in the active form, as determined by BIA and gelatin zymography, respectively. Before culture, IBD explants already contained more MMP-2 (2-fold, $P = 0.005$), MMP-3t (3-fold, NS), and MMP-9 (10-fold, $P = 0.002$) compared to control tissue, while preculture MMP-1 and MMP-3a levels were below the detection limit of our BIA assay. After 72 hours MMP secretion in culture medium with/without infliximab and/or PWM by IBD explants was also significantly higher (Figs. 3, 4). Within the IBD group, UC compared to CD explants expressed higher MMP levels, i.e., basal MMP-1t: 44.6 ± 15.1 versus 27.6 ± 7.4 (10^3) U/mg, MMP-2: 27.3 ± 3.1 versus 24.4 ± 2.4 ng/mg, MMP-3t: 7.2 ± 2.4 versus 4.3 ± 1.0 (10^2) U/mg, and MMP-9: 3.1 ± 0.7 versus 2.2 ± 0.5 ng/mg at 72 hours, although not statistically significant. Addition of PWM resulted in opposite MMP-responses: MMP-2 level was downregulated about 50% compared to basal supernatants, whereas MMP-1, -3, and -9 levels were upregulated up to 40%. Incubation with infliximab also revealed divergent regulation pathways: basal and PWM immunostimulated MMP-2 secretion were not affected, but secretion of MMP-1, -3, and -9 was significantly downregulated by up to 40%, irrespective of tissue group. Remarkably, macroscopic disease had no major effect on MMP-2 and -9 production (e.g., basal MMP-9 secretion by affected

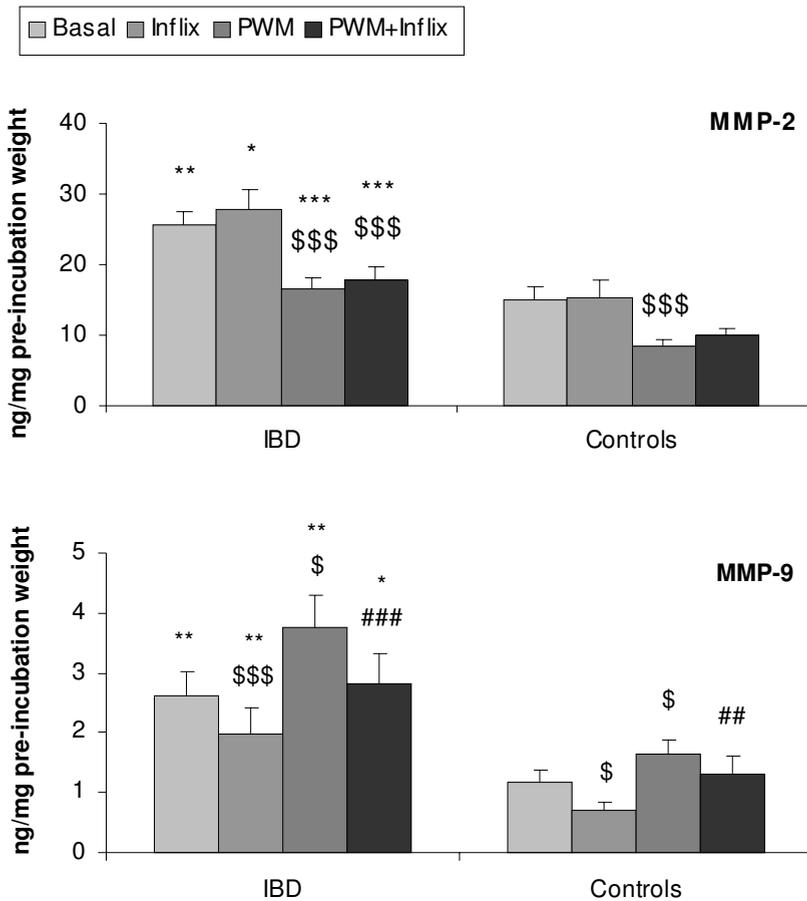


Figure 3. Cumulative MMP protein secretion by cultured intestinal explants at 72 hours (in ng MMP/mg preincubation explant weight, mean \pm SEM). Explants from 15-20 IBD and 7-15 control patients were cultured w/w/o infliximab and/or PWM. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ versus corresponding explants from controls. \$\$\$ $P < 0.001$, \$\$ $P < 0.01$, \$ $P < 0.05$ versus basal incubation within same tissue group; ### $P < 0.001$, ## $P < 0.01$ versus PWM incubation within same tissue group.

IBD tissue was 1.9 ± 0.7 compared to 1.6 ± 0.5 ng/mg for normal IBD tissue, $n = 5$, paired t -test, $P = 0.4$; in case of MMP-1 and -3 only inflamed tissue was analyzed). Also, tissue location, i.e., ileum versus colon, had no effect (data not shown). Subsets of inflamed IBD and control tissues were also analyzed for mRNA expression. Preculture MMP mRNA levels were very low but higher in CD and UC

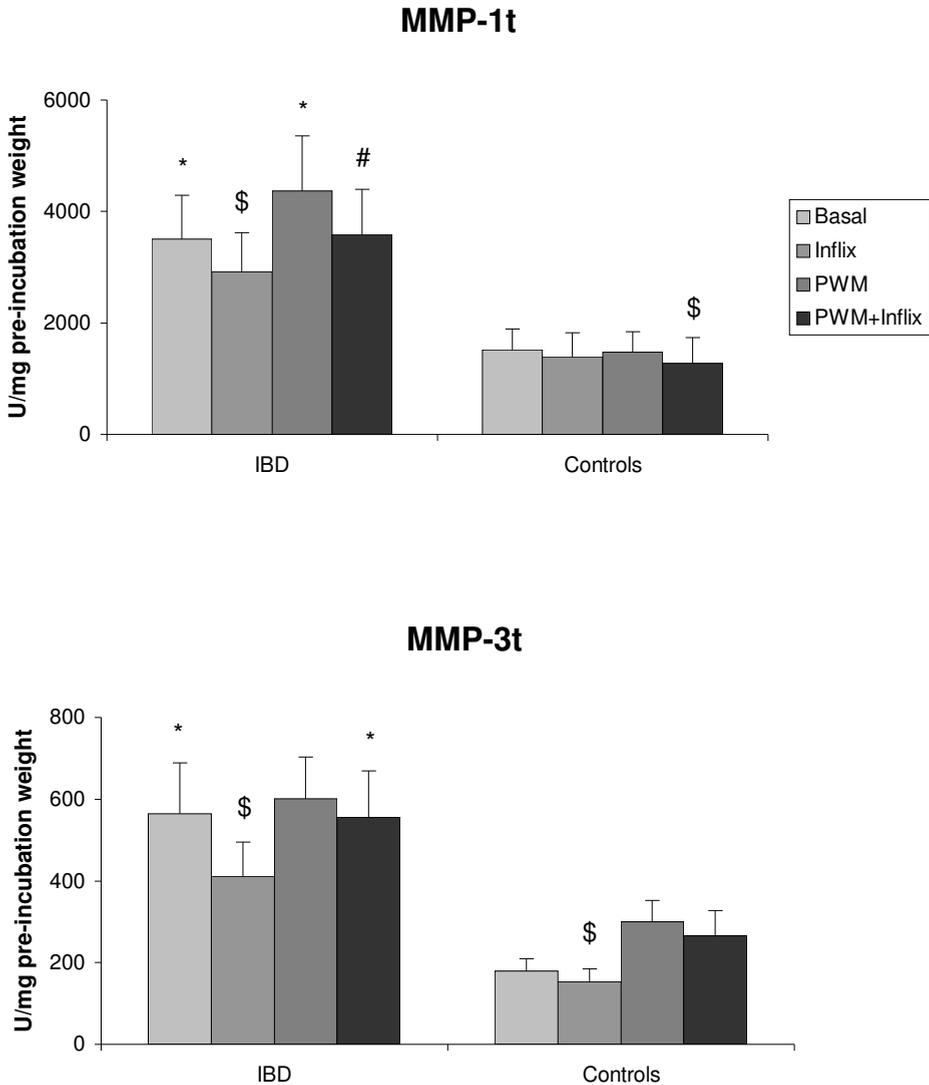


Figure 4-A. Cumulative MMP-t activity secretion (in U MMP/mg preincubation explant weight, mean \pm SEM) by cultured inflamed IBD and control explants (MMP-1: $n = 14-16$ and $6-9$; MMP-3: $n = 13$ versus 6 , respectively) after 72 hours. $**P < 0.01$, $*P < 0.05$ compared to corresponding control incubation, $$$P < 0.01$, $$P < 0.05$ versus basal incubation within same group, $##P < 0.01$, $#P < 0.05$ versus PWM incubation within same group. MMP activity was measured after preincubation with APMA.

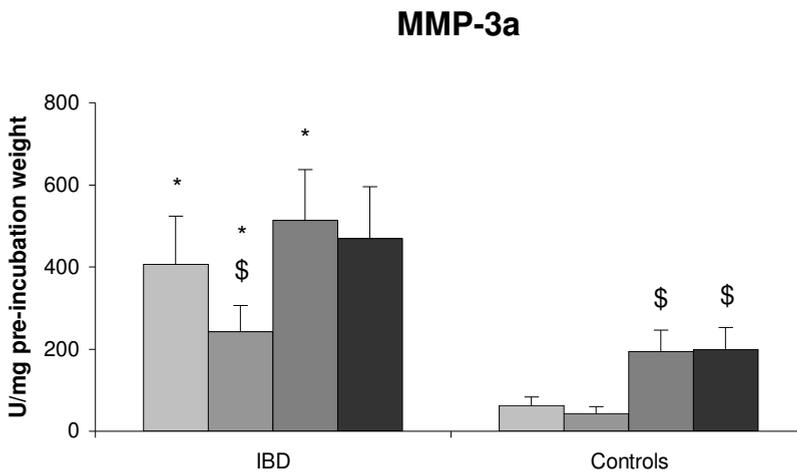
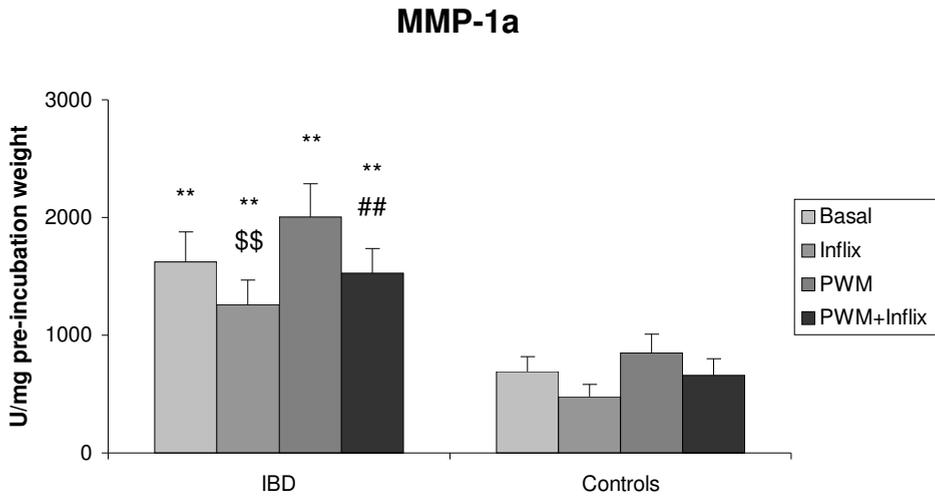


Figure 4-B. Cumulative MMP-a activity secretion (in U MMP/mg preincubation explant weight, mean \pm SEM) by cultured inflamed IBD and control explants (MMP-1: $n = 14-16$ and $6-9$; MMP-3: $n = 13$ versus 6 , respectively) after 72 hours. $**P < 0.01$, $*P < 0.05$ compared to corresponding control incubation, $$$$P < 0.01$, $$P < 0.05$ versus basal incubation within same group, $##P < 0.01$, $#P < 0.05$ versus PWM incubation within same group. MMP activity was measured after preincubation without APMA.

compared to controls, and significant induction was observed during culture (MMP-2, -9: 4–16-fold and MMP-1, -3: 100–500-fold, see also Table X online supplementary material). Opposite to the protein, CD, UC, and control MMP mRNA levels at 72 hours appeared similar in corresponding incubations, although some differences were observed. Infliximab downregulated basal MMP mRNA expression, particularly MMP-1 and -3 in CD and controls but not UC, and when the results from CD and control tissue were combined, this was highly statistically significant (median percent change (range): - 40.9 (- 74.1 to 89.9, $P < 0.05$) and - 51.0 (- 78.8 to 37.4, $P < 0.01$), MMP-1 and -3, respectively). Incubation with PWM upregulated MMP mRNA expression 1.5–12-fold but concurrent incubation with infliximab could not prevent this increase.

Expression of TIMP-1 and TIMP-2

Affected IBD and normal control tissues were also analyzed for TIMP secretion. Both TIMPs were continuously produced and secreted throughout culture (Fig. 2C), with part of the TIMP-2 secretion reflecting release from the large protein pool already present in starting tissue. Before culture, IBD and control explants ($n = 13$ versus 6) contained similar amounts of TIMP (60 ± 50 versus 0 ± 0 pg/mg TIMP-1 and 267.2 ± 20.4 versus 217.5 ± 17.5 pg/mg TIMP-2, both NS) but 72 hours basal secretion was higher by IBD explants, especially in the case of TIMP-2 (Fig. 5). Within IBD, UC compared to CD explants expressed somewhat more TIMP, i.e., basal TIMP-1, -2 at 72 hours 48.0 ± 9.2 versus 28.9 ± 5.6 ng/mg and 7.5 ± 0.7 versus 6.2 ± 0.3 (10^2) pg/mg, $P = \text{NS}$, respectively. Addition of infliximab and/or PWM decreased TIMP protein secretion in all groups, but did not affect relative protein levels between corresponding incubations of IBD versus control tissue.

Production of MMP relative to TIMP

The effect of treatment on the weight ratio between MMP and TIMP is shown in Table 3. The MMP-2/TIMP ratios remained relatively stable. However, addition of infliximab to basal medium decreased the weight ratio between MMP-1t, MMP-3t, and MMP-9 versus TIMP-1 and/or TIMP-2 in CD, UC, and controls and when all groups were combined this effect was highly statistically significant. Apparently,

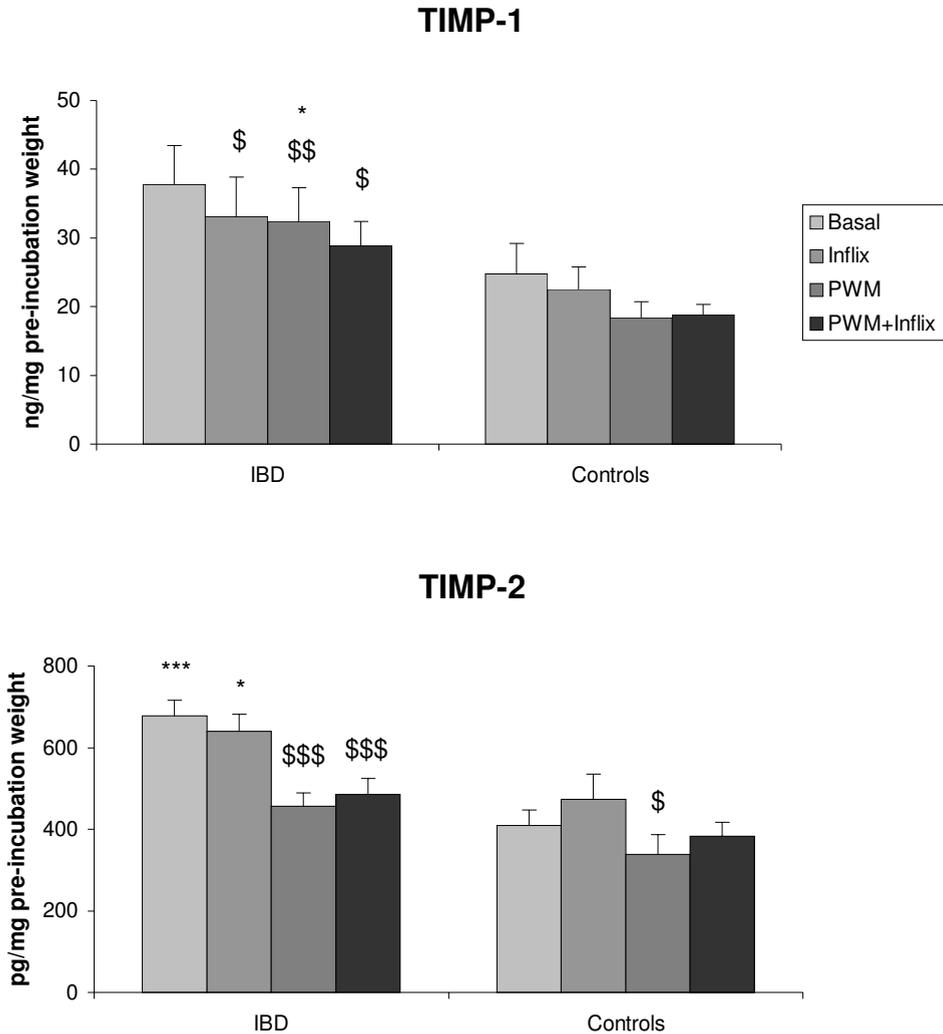


Figure 5. Cumulative TIMP protein secretion (in ng or pg TIMP/mg preincubation explant weight, mean \pm SEM) by cultured intestinal inflamed IBD and control explants ($n = 13$ and 6 , respectively) after 72 hours. *** $P < 0.001$, * $P < 0.05$ compared to corresponding control explant cultures, \$\$\$ $P < 0.001$, \$\$ $P < 0.01$, \$ $P < 0.05$ versus basal incubation within same group.

Table 3. Effect of incubation with infliximab and PWM on secretion of MMP relative to TIMP protein (mean \pm SEM) by combined cultured inflamed CD, UC, and control intestinal explants ($n = 13$ and 6 , respectively)

Ratio	Basal	Infliximab	PWM	PWM + infliximab
MMP-2/TIMP-1	0.8 \pm 0.1	1.0 \pm 0.1	0.7 \pm 0.1	0.7 \pm 0.1
MMP-2/TIMP-2	37.1 \pm 1.9	38.6 \pm 2.7	36.5 \pm 3.1	36.2 \pm 2.7
MMP-9/TIMP-1 ($\times 10^3$)	55.5 \pm 8.9	42.0 \pm 8.0**	121.9 \pm 15.0**	80.2 \pm 11.2* ^{##}
MMP-9/TIMP-2	3.4 \pm 0.7	2.4 \pm 0.6**	7.7 \pm 1.0***	4.6 \pm 0.7* ^{###}
MMP-1t/TIMP-1	93.2 \pm 15.3	87.8 \pm 14.0	127.8 \pm 22.1**	110.0 \pm 18.4*
MMP-1t/TIMP-2	4.9 \pm 0.9	4.1 \pm 0.8**	7.5 \pm 1.6**	5.8 \pm 1.1* [#]
MMP-3t/TIMP-1	12.4 \pm 1.4	11.2 \pm 1.3	19.7 \pm 2.1**	17.7 \pm 2.1**
MMP-3t/TIMP-2	0.7 \pm 0.1	0.5 \pm 0.1*	1.2 \pm 0.2***	1.0 \pm 0.2 [#]

CD, Crohn's disease; UC, ulcerative colitis; PWM, pokeweed mitogen. MMP-2 and MMP-9 versus TIMP reflect actual weight ratios, MMP-1 and MMP-3 versus TIMP values are in arbitrary units. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ versus basal incubation, ^{###} $P < 0.001$, ^{##} $P < 0.01$, [#] $P < 0.05$ versus PWM.

levels of these MMPs are more dramatically reduced than either TIMP-1 or TIMP-2 when infliximab is added. Conversely, incubation with PWM increased these MMP/TIMP ratios toward a more proteolytic phenotype, and infliximab was able to partially inhibit these increases, although not fully back to control levels. The MMP/TIMP ratios in corresponding incubations from CD and UC compared to controls were higher, but statistical significance was not reached.

Production of TNF- α and relation to MMP/TIMP Expression

Explants also continuously secreted TNF- α into the medium, although peak production was observed during the first 24 hours (Fig. 2D). Secretion was paralleled by a concurrent increase of TNF- α in tissue homogenates (data not shown). Before culture, CD and UC explants contained similar high TNF- α levels compared to controls (0.80 \pm 0.18 versus 0.25 \pm 0.11 pg/mg, $P < 0.05$, $n = 17$ IBD versus 15 controls), and they had secreted more TNF- α , although not statistically significant, after PWM stimulation at 72 hours (Fig. 6). Incubation with infliximab only marginally affected basal TNF- α secretion. PWM, however, increased TNF- α

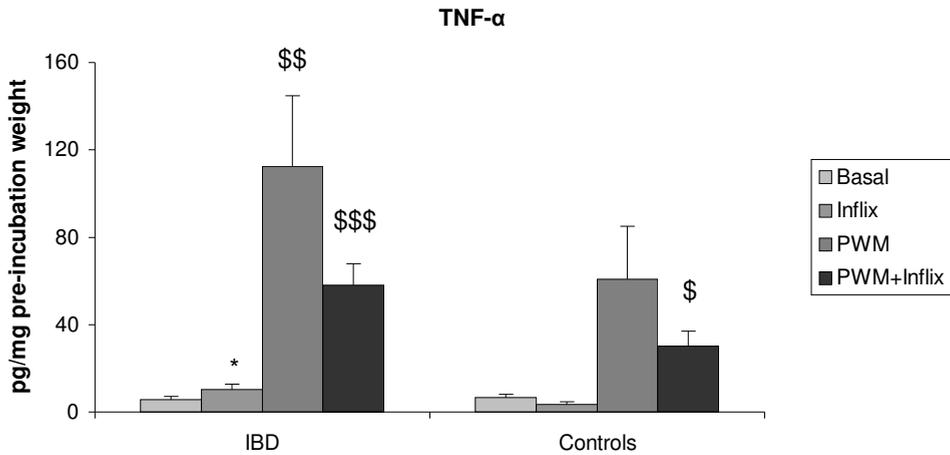


Figure 6. TNF- α protein secretion by intestinal inflamed IBD ($n = 12-13$) and control ($n = 6-9$) explants at 72 hours, cultured w/wo infliximab and/or PWM. Mean \pm SEM in pg TNF- α /mg preincubation weights are shown. The TNF- α levels determined in medium supplemented with infliximab were multiplied 5-fold to correct for 80% inhibition of the TNF- α ELISA results by infliximab. * $P < 0.05$ versus control, \$\$\$ $P < 0.001$, \$\$\$ $P < 0.01$, \$ $P < 0.05$ versus basal incubation within same group.

levels 8–12-fold and infliximab was able to partially prevent this increase, although again not statistically significant. These results were essentially the same at the mRNA level (data not shown). There was a strong positive correlation of 72 hours basal TNF- α levels with MMP-3 total activity, MMP-9 and TIMP-1 protein secretion ($0.58 < r < 0.74$, $P < 0.05$, all groups combined, $n = 18-22$). Importantly, basal TNF- α was also positively correlated with MMP-9/TIMP-1, MMP-9/TIMP-2, and MMP-3t/TIMP-2 ($0.67 < r < 0.85$, $P < 0.01$, $n = 18$), whereas a strong negative correlation was observed versus MMP-2/TIMP-1 ($r = -0.71$, $P < 0.01$). All correlations were lost when explants were treated with PWM. Finally, MMP-3 is known to be a physiological activator of MMP-1. In our experiments, active MMP-3 and active MMP-1 were highly correlated independent of incubation with PWM and/or infliximab (e.g., basal level: $r = 0.74$, $P < 0.001$, $n = 19$).

Genotype at SNP loci and effect on protein and mRNA expression

Using RFLP or tetra primer ARMS PCR, relevant SNP loci in the DNA of IBD patients and controls were genotyped. Explants with at least one 2G, 5T, or A allele at MMP-1 -1607, MMP-3 -1613, or TNF- α -308, respectively, were shown to express more corresponding protein in medium with/without PWM and/or infliximab compared to explants with alternative allelic composition (Table 4), although not statistically significant. Allelic composition often did not affect the response of explants to infliximab or PWM; for instance, PWM significantly decreased MMP-2 protein expression in explants with the CC as well as in explants with the CT or TT genotype (CC basal compared to PWM: 20.1 ± 2.3 versus 12.4 ± 1.5 and CT + TT: 22.4 ± 2.2 versus 13.5 ± 2.0 ng/mg, both $P < 0.001$). However, in explants with the CC but not CT or TT genotype at MMP-9 -1562 infliximab significantly downregulated MMP-9 protein levels, while PWM caused an upregulation. Also, only in explants with at least one 2G or 5T allele at the MMP-1 -1607 versus MMP-3 -1613 locus, infliximab decreased total MMP-1 and MMP-3 secretion, respectively. At the mRNA level, explants with the CC genotype at MMP-2 -1306 expressed more MMP-2 than explants with the CT or TT genotype, although not statistically significant, and only explants with the 6T6T genotype at MMP-3 -1613 responded to incubation with infliximab by decreasing MMP-3 production ($P < 0.05$, see also Table XX online supplementary material).

Discussion

We have shown a generalized increased *ex vivo* expression of MMP-1, -2, -3, -9, and TIMP-1, -2 protein by explants from CD and UC versus control patients. The enhanced production of MMPs and TIMPs by IBD tissue corresponds to results from previous studies concerning expression levels of these markers *in vivo*¹²⁻¹⁷ and could contribute to the tissue damage seen in IBD. In our experiments we observed that UC compared to CD explants expressed more MMP and TIMP, perhaps reflecting the more severe inflammation of the starting tissue of the former as measured by myeloperoxidase content (16.4 ± 1.4 versus 11.4 ± 1.1 U/g, $P < 0.05$). The protein level of MMP-2 dropped nearly 2-fold when the explants were activated by PWM, an inducer of the *in vitro* inflammatory process, while

Table 4. Genotype at selected SNP loci and effect on MMP, TIMP and TNF- α protein expression (mean \pm SEM) by combined IBD and control explants

Protein	Genotype	No. Patients	Start	Basal	Infliximab	PWM	PWM + Infliximab
MMP-2 (ng/mg)	CC	14-21	0.7 \pm 0.1 [†]	20.1 \pm 2.3	24.1 \pm 3.6	12.4 \pm 1.5 ^{***}	15.1 \pm 2.0 ^{**}
	CT or TT	9-12	1.1 \pm 0.2	22.4 \pm 2.2	22.8 \pm 3.0	13.5 \pm 2.0 ^{***}	15.2 \pm 1.9 [*]
MMP-9 (ng/mg)	CC	18-25	0.3 \pm 0.1	2.0 \pm 0.3	1.5 \pm 0.3 ^{***}	2.9 \pm 0.4 ^{**}	2.2 \pm 0.4 ^{###}
	CT or TT	5-8	0.5 \pm 0.3	2.0 \pm 0.8	1.9 \pm 1.4	2.6 \pm 0.7	2.6 \pm 0.9 [#]
MMP-1t 10 ³ U/mg	1G1G	8-9	ND	2.0 \pm 0.3	1.9 \pm 0.4	2.4 \pm 0.5	2.0 \pm 0.4
	1G2G or 2G2G	12-15	ND	3.4 \pm 0.9	2.9 \pm 0.9 [*]	3.8 \pm 1.1	3.5 \pm 1.0 [*]
MMP-3t (10 ² U/mg)	5T5T or 5T6T	11	0.04 \pm 0.03	4.8 \pm 1.5	3.4 \pm 1.0 [*]	5.5 \pm 1.1	4.9 \pm 1.2
	6T6T	8	0.01 \pm 0.00	3.9 \pm 1.0	3.1 \pm 0.8	4.5 \pm 1.1	4.3 \pm 1.3
TIMP-1 (ng/mg)	TT-♀ or T-♂	7	ND	25.7 \pm 5.6	20.8 \pm 4.0	22.8 \pm 4.3	23.2 \pm 3.6
	CT or CC-♀ or C-♂	12	ND	38.3 \pm 5.7	35.0 \pm 5.8	31.0 \pm 5.3 ^{**}	27.2 \pm 3.7 ^{**}
TIMP-2 (10 ² pg/mg)	GG	12	2.6 \pm 0.2	6.4 \pm 0.6	6.1 \pm 0.5	4.2 \pm 0.3 ^{***}	4.6 \pm 0.4 ^{***}
	GA	7	2.3 \pm 0.3	5.2 \pm 0.5	5.5 \pm 0.7	4.3 \pm 0.6	4.5 \pm 0.5
TNF- α (pg/mg)	GG	12-23	0.5 \pm 0.2	5.5 \pm 1.2	6.7 \pm 1.7	73.3 \pm 17.3 ^{**}	43.8 \pm 7.4 ^{###}
	GA or AA	6-7	0.8 \pm 0.3	5.3 \pm 2.1	10.9 \pm 4.5	135.0 \pm 61.8	58.7 \pm 17.1 [*]

SNP, single nucleotide polymorphism; IBD, inflammatory bowel disease; ND, not determined, below threshold of assays. Distribution of IBD and control tissue over genotypes is similar (Fisher's Exact test, not shown). IBD values in case of MMP-2 and MMP-9 are taken from inflamed and noninflamed tissue, otherwise only inflamed tissue was measured. MMP-9 expression by explants with CT or TT genotype is significantly lower when infliximab is added next to PWM, but is not reflected in depicted figures because PWM value is based on $n = 8$, PWM + infliximab on $n = 5$ and test is based on paired $n = 5$. ^{***} $P < 0.001$, ^{**} $P < 0.01$, ^{*} $P < 0.05$ versus basal incubation, ^{###} $P < 0.001$, [#] $P < 0.05$ versus PWM incubation, [†] $P < 0.05$ versus corresponding incubation of explants with alternative genotype. Statistical significance was not assessed between starting material and medium.

MMP-1, -3, and -9 production was enhanced or remained stable. However, mRNA levels of all four MMPs were similar or increased compared to basal incubation when PWM was added. Apparently, PWM activation may affect MMP posttranscriptional processes, particularly of MMP-2, resulting in the observed divergence of regulation of MMP protein production and secretion. When infliximab was added to basal medium, MMP-2 protein and mRNA levels remained similar, while MMP-1, -3, and -9 protein and/or mRNA were decreased. Clearly, the expression of MMP-2 in this explant culture system is not affected by TNF- α , whereas the basal expression of MMP-1, -3, and -9 protein appears to be substantially dependent on TNF- α , confirming previous observations on the regulation of these MMPs by this cytokine.⁴⁰ The downregulation of MMP-1, -3 protein by infliximab was paralleled by a decrease in corresponding mRNA in CD and control but not UC explants. This might point to aberrant regulation pathways of MMP-1 and -3 in UC compared to CD and controls, possibly related to specific pathogenic mechanisms. It can be argued that both absolute and relative levels of MMPs and TIMPs are physiologically relevant. Infliximab added to basal medium resulted in downregulation of the MMP-9 versus TIMP ratio, while MMP-2 versus TIMP remained constant. Importantly, addition of this drug also decreased the level of total MMP-1 and MMP-3 over TIMP, particularly TIMP-2, together with a downregulation of active MMP-1 and -3. These observations point to an antiproteolytic and matrix protective phenotype induced by infliximab. Conversely, addition of PWM increased the ratio of MMP-1, MMP-3, and MMP-9 to TIMP and also increased net MMP-1 and -3 activity, shifting the balance to a more proteolytic phenotype, possibly resulting in the observed significant epithelial cell targeted tissue degradation. Treatment with infliximab was able to partially prevent this PWM-induced increase, although not fully back to control levels. Therefore, our results indicate a clear relationship between expression of TNF- α and MMP-1, -3, and -9 net activity in CD, UC, and normal control tissue. This is corroborated by the finding of a positive correlation between TNF- α versus MMP-9/TIMP, MMP-3/TIMP-2, and TNF- α versus MMP-3 total activity in basal medium and of similar TNF- α and MMP/TIMP levels in corresponding incubations of IBD compared to control explants. However, despite less impressive differences in

TNF- α secretion, MMP activity levels were much higher in IBD compared to controls, stressing the importance of other proinflammatory pathways in upregulating MMPs. Our results on TNF- α reveal less enhancement compared to previous publications concerning TNF- α production by IBD mucosal biopsies.^{5,41} Obviously, from surgical resection until culture our tissues had been exposed to more severe physical stress, perhaps upregulating TNF- α production processes. The duration of disease in our IBD study population was often several years, and as shown by Dionne *et al*,⁴² this may downregulate TNF- α production. Alternatively, from many of our patients the removed bowel was affected by stricturing processes, perhaps paralleled by decreased capacity for production of TNF- α compared to solely inflamed tissue. Also, a large proportion of our patients were treated with corticosteroids and/or disease-modifying drugs like azathioprine or cyclosporine prior to surgical resection, possibly affecting TNF- α secretion during culture. Several studies have reported on functional SNP loci in the genes coding for MMP, TIMP, and TNF- α . We found increased production of corresponding protein or mRNA by explants with two C or at least one 2G, 5T or A allele at the MMP-2 -1306, MMP-1 -1607, MMP-3 -1613, and TNF- α -308 loci, respectively, confirming results in previous publications.^{24,43-45} We also found an inhibitory effect of infliximab on MMP protein or mRNA production especially in explants with the CC and 1G2G or 2G2G genotype at SNP loci MMP-9 -1562 and MMP-1 -1607, respectively. These results may be relevant for future selection of infliximab responsive patients, but first have to be confirmed in larger studies. Experimental manipulation of cultured (human) intestinal IBD mucosa, as described here, may provide a new valuable tool in this respect. Despite the physical stress exerted on the tissue, the presence of bacteria, and the absence of fetal calf serum, we were able to preserve gross morphology up to 72 hours, with maintenance of crypts and cytokeratin-18-expressing epithelial cells, even when medium was not replenished. Possibly the presence of retinoic acid may play a central role, due to its anabolic effects on epithelial cells⁴⁶ and its attenuation of extracellular matrix degradation by stromal cells.⁴⁷ Given the large availability of surgical resection specimens, culture of IBD intestinal mucosa may offer an inexpensive way of monitoring drug efficacy, cytokine and proteinase expression, etc. In the past there have been several

reports describing potential mechanisms of infliximab action, i.e., downregulation of adhesion molecules on endothelial cells and production of IL-6 by fibroblasts,⁴⁸ antibody and/or complement-dependent (cellular) cytotoxicity,⁴⁹ induction of apoptosis in T lymphocytes,⁵⁰ and restoration of gut barrier function.⁵¹ Our results demonstrate another potential mechanism of action, i.e., downregulation of excess MMP-1, -3, and -9 activity and parallel the observed downregulation of serum levels of various MMP members in rheumatoid arthritis⁵² and CD patients⁵³ treated with infliximab. Our results reinforce the potential destructive capacities of MMPs in IBD pathogenesis, the pivotal role of TNF- α in regulating MMP-1, -3, and -9 activity, and the rationale for developing therapeutic intervention strategies utilizing specific MMP inhibitors for treating patients with CD or UC, as also previously shown in animal models.^{18,54}

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