Chapter 4

Infliximab Treatment Influences the Serological Expression of Matrix Metalloproteinase (MMP)-2 and -9 in Crohn’s Disease

Short title: Infliximab and gelatinases in CD

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Abstract

**Background/aims.** Matrix metalloproteinases (MMPs) are actively involved in the pathogenesis of Crohn’s disease (CD). We assessed the effect of the anti-tumor necrosis factor- α (TNF-α) monoclonal antibody infliximab on the *in vitro* and *in vivo* expression of MMP-2 and MMP-9 in CD.

**Methods.** Infliximab-treated fistulizing (*n* =10) or active disease (*n* = 7) CD patients, from an in-house study, and fistulizing CD patients (*n* = 42) and active CD patients (*n* = 24) from 2 placebo controlled studies were evaluated for serum MMP levels and clinical response. Biopsies were evaluated immunohistochemically for the MMPs. Whole blood cultures stimulated with lipopolysaccharide (LPS)/infliximab were evaluated for MMP mRNA and protein levels.

**Results.** Serum MMP-2 levels in CD patients increased during follow-up, similarly in responders and nonresponders, by infliximab. Immunohistochemistry showed no clear MMP-2 change in biopsies. Serum MMP-9 levels, however, showed a consistent pattern of decrease in most CD patients, particularly in those responding, and MMP-9-positive polymorphonuclear leukocytes in biopsies also decreased by infliximab. LPS stimulation of whole blood increased the MMP-9 levels in plasma significantly in CD patients and controls, but infliximab had no effect on the secretion. Long-term LPS stimulation raised leukocyte MMP-9 mRNA levels 16-fold and infliximab inhibited this induction by 80%.

**Conclusions.** Infliximab treatment increases MMP-2 and decreases MMP-9 in serum of patients with CD, the latter also in the intestine, which extends and confirms our previous *ex vivo* explants observations. However, these changes were not strictly associated with the response to treatment. The enhanced leukocyte MMP-9 expression in CD seems to be regulated by TNF-α.
Introduction

Infliximab, a chimeric IgG1 anti-tumor necrosis factor (TNF)-α monoclonal antibody, is a successful immunotherapeutic agent for Crohn’s disease (CD). The treatment with infliximab results in a high clinical efficacy, rapid onset of action, and prolonged effect in patients with moderate to severe active CD, which have not responded to conventional therapy, and in fistulizing CD patients. Simultaneously, the quality of life of these patients is essentially improved.\(^1\)\(^-\)\(^3\) The proposed immunological mechanisms of infliximab include the suppression of TNF-α bioactivity and the lysis of TNF-α-producing cells, such as monocytes and lymphocytes, via complement fixation, antibody-dependent cellular cytotoxicity (ADCC), and Fc portion binding of the IgG1 antibody. Furthermore, infliximab downregulates mucosal Th1 cytokines, reduces the expression of IFN-γ and other inflammatory molecules, such as intercellular adhesion molecule (ICAM)-1 and vascular adhesion molecule (VCAM)-1.\(^4\)\(^-\)\(^6\)

Matrix metalloproteinases (MMPs) compose a family with over 20 members of Zn\(^{2+}\)-containing neutral proteinases.\(^7\) Usually, MMPs are synthesized as preproenzymes and are secreted in a proenzyme form that requires proteolytic cleavage for activation in most cases.\(^8\)\(^,\)\(^9\) The activity of MMPs is precisely regulated within tissues by the balance between zymogen activation and enzyme inhibition. Factors that regulate activity of the MMPs include endogenous inhibitors, α-macroglobulins, and tissue inhibitors of metalloproteinases (TIMPs) and the MMPs themselves.\(^10\)\(^,\)\(^11\) MMPs are implicated in the inflammatory response, wound healing, tissue remodeling, cell growth, migration, apoptosis, cell-cell communication, tumor invasion, and metastasis.\(^8\)\(^,\)\(^12\)\(^-\)\(^15\) MMPs exert their activity by the degradation of a class of biological molecules that include not only the components of extracellular matrix (ECM) but also an increasing family of bioactive modulators, such as cytokines, growth factor receptors, other proteinases, coagulation factors, chemotactic molecules, and adhesion molecules.\(^14\) In the pathogenesis of CD, MMPs are believed to be associated with the injury of gut tissue mediated by TNF-α and Th1 cytokines. One of the mechanisms by which TNF-α causes intestinal tissue injury is believed to be the enhancement of the MMP production at local sites.\(^16\)\(^-\)\(^18\) MMP-2 (72 kDa, gelatinase A) and MMP-9 (92
kDa, gelatinase B) are the two members of the gelatinase subgroup of MMPs. The substrates of MMP-2 and MMP-9 specifically include not only basement membrane (BM) type IV collagen and other components like gelatin, collagen type I, V, VII, X, elastin, laminin, and fibronectin, but also numerous bioactive molecules, such as fibroblast growth factor receptor (FGFR)-1, prointerleukin (IL)-1, and ICAM-1.\textsuperscript{19-22} Previous studies showed that MMP-2 and -9 are actively involved in the pathophysiological processes, including fistula formation, in the intestine of inflammatory bowel disease (IBD) patients.\textsuperscript{23-30} After treatment with infliximab the elevated levels of MMP-1 and -3 in serum of patients with rheumatoid arthritis were reported to be reduced.\textsuperscript{31} The role of MMPs in the treatment of CD patients with infliximab, however, is still poorly understood. Recently, we showed that infliximab induces a matrix protective MMP-phenotype in \textit{ex vivo} cultures of mucosa from patients with inflammatory bowel disease.\textsuperscript{32} In the present study we explored further this relationship between the clinical efficacy of infliximab and the expression levels of MMP-2 and -9 in patients with CD and describe the \textit{in vivo} and \textit{in vitro} regulation of the expression of these 2 gelatinases by infliximab.

**Patients and methods**

**Clinical studies**

In the present study we included patients that participated in an expanded access program for infliximab treatment of CD in our institute, i.e., in-house study,\textsuperscript{3} and a subgroup of patients that participated in an international multicenter, placebo-controlled trial of infliximab either for the treatment of fistulas in patients with CD or for the treatment of active CD.\textsuperscript{1,2} The in-house study was approved by the medical ethics committee and patients were only included after informed consent. The eligibility of patients in these studies was described previously.\textsuperscript{3,33} Briefly, the age of confirmed patients with CD had to be between 18 and 65 years. For inclusion in the fistula treatment groups, patients had to have single or multiple draining abdominal or perianal fistulas of at least 3 months’ duration. Patients who had had CD for at least 6 months, with CD Activity Index (CDAI) scores equal or above 220, were eligible for the treatment with infliximab for active CD. Analyses of efficacy evaluated the number of patients with a reduction of half or more in the number of
Infliximab and gelatinases in CD

Changes in scores of the CDAI and the open fistulas scores were also evaluated. Failure of treatment was defined as changes in medication that were not permitted in the protocol, surgery related to CD, or no return for follow-up visit.

**Protocol 1. Fistulas**

Within 2 weeks of screening, eligible patients \((n = 10)\), one patient also with active disease) for the in-house study received infliximab 5 mg/kg (body weight) and patients from the international trial were randomly assigned to receive 1 of 3 treatments at weeks 0, 2, and 6: placebo \((n = 14)\) or 5 or 10 mg/kg of infliximab (total \(n = 28\)). Study drug was administered intravenously over a 2-hour period. After the first infusion of study medication, patients returned for clinical and laboratory assessments at weeks 2, 6, 10, 14, and/or 18. Serum samples were collected, if possible, at each study visit through week 18.

**Protocol 2. Active disease**

Patients in the in-house study received a single dose of 5 mg of infliximab per kilogram of body weight (total \(n = 7\)) in an intravenous infusion, administered over a 2-hour period. Disease activity according to the CDAI and/or blood serum samples were assessed at days 0, 3, weeks 2, 4, 8, and 12. In the international study patients were randomly assigned to receive a single dose of either placebo \((n = 7)\) or 5, 10, or 20 mg/kg of infliximab (total \(n = 17\)). Disease activity was assessed and serum samples were collected at weeks 0 and 4.

**In vitro study**

**Patients, volunteers, and blood samples**

Patients with CD \((n = 7)\) were treated with infliximab for fistulizing and/or active disease in the in-house study. Blood from healthy volunteers \((n = 5)\) was obtained, with their permission, from the central blood transfusion laboratory. Heparinized blood samples were obtained from patients before and 2 hours after a single infusion of infliximab of 5 mg/kg over a 2-hour period. For the blood samples from
the healthy volunteers a concentration of 75 μg infliximab per mL blood was added. At 37 °C and 5% CO₂, whole blood samples with/without infliximab were stimulated with/without lipopolysaccharide (LPS) (Sigma, St. Louis, MO) at 0.1 μg/ml blood for 1.5–24 hours for MMPs and TNF-α mRNA and protein determinations.³⁴ The plasma was subsequently separated from the blood sample by centrifugation at 4 °C, and stored at -70 °C until further analysis. Leukocytes isolation was performed by adding lysis buffer containing 0.16 M NH₄Cl, 10 mM KHCO₃, and 0.01 mM K₂-EDTA (pH 7.4 at 0°C) to the samples. After erythrocytes were degraded the sample was centrifuged at 4 °C and lysis was repeated to obtain pure leukocytes. The leukocytes were immediately used to isolate RNA.

**Determination of MMPs by ELISA**

MMP-2 and MMP-9 levels in the samples were measured by our highly specific enzyme-linked immunosorbent assays (ELISA), which measure the total of proenzyme, active-, and inhibitor-complexed forms of the respective MMP, as described previously.³⁰,³²,³⁵ In brief, a polyclonal anti-MMP-2 antibody or monoclonal anti-MMP-9 antibody was used as catching antibody and appropriately diluted samples were incubated overnight at 4 °C. Immunodetection of MMP-2 was performed using polyclonal anti-MMP-2 followed by biotin-labeled goat antirabbit-IgG and of MMP-9 with biotin-labeled polyclonal anti-MMP-9 antibody. After incubation with avidin-peroxidase the chromogenic substrate 3,3,5,5-tetramethyl benzidine in the presence of hydrogenperoxide was added and the reaction was stopped with H₂SO₄ and the absorption was measured at 450 nm. The amount of MMP was calculated from the parallel standard curve and expressed in ng MMP per mL serum or plasma.

**Immunohistochemical staining for MMPs**

Standardized colonic biopsies of 6 patients from the in-house study (4 with fistulizing and 2 with active disease) were obtained at the start of the study, as well as at day 3 and week 2 of follow-up. To assess the localization of MMP-2 and MMP-9 within the intestinal tissues, indirect immunohistochemical staining of the MMPs was performed as described previously.³⁰ In brief, paraffin tissue sections,
treated with proteinase K for MMP-2 antigen retrieval, were incubated with rabbit polyclonal antihuman MMP antibodies, similar to those used in the ELISAs. Subsequently, the sections were incubated with biotinylated goat antirabbit Ig, peroxidase-labeled streptavidin, and stained with 3-amino-9-ethylcarbazole and hematoxylin. Control sections incubated with preimmune serum or buffer instead of the primary antibodies showed no staining. The immunohistochemical staining was semiquantitatively assessed using the following scoring system: 0 = no staining, 1 = a few positive cells/areas of tissue or a low staining intensity in all cells, 2 = a minority of the cells/areas of tissue positive or a moderate staining intensity in all cells, 3 = a majority of the cells/areas of tissue positive and/or a moderate staining intensity in all cells, 4 = all cells or areas of tissue strongly positive.

Reverse transcription–polymerase chain reaction (RT-PCR)
Oligonucleotide primers (Table 1) for RT-PCR were derived from the DNA sequence database of the NCBI (Bethesda, MD). Primer sets were designed using the Primers3 Output computer program (Whitehead Institute for Biomedical Research, Cambridge, MA). The MMP-2 and MMP-9 PCR products span 3 introns to prevent interference of contamination by genomic DNA. Specificity of the primers was checked with the NCBI BLAST program. β2-microglobulin (β2-M) was used as a control to normalize PCR signals from the different samples. Total RNA was isolated from blood samples by the method of Chomczynski and Sacchi. The integrity and quality of the purified RNA were analyzed by 1.5% agarose gel/ethidium-bromide staining and the 260/280 nm absorbance ratio. Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase was used for cDNA synthesis from DNAse-treated RNA. The PCR was started at 94 °C for 3 minutes, followed by 30 cycles for MMP-2 and MMP-9, and 28 cycles for β2-M. Each cycle consisted of a denaturation step (at 94 °C for 30 sec), an annealing step for 45 sec (at 56 °C for MMP-2 and β2-M, 59 °C for MMP-9), and extension step (at 72 °C for 1 min), followed by a final elongation step (at 72 °C for 7 min), as described previously. The reaction was performed in a Whatman T Gradient cycler (Biometra, Goettingen, Germany) and the amplified products were electrophorized on 1.5% agarose gels containing ethidium-bromide (0.5 g/mL) and visualized under
Table 1. Oligonucleotide primers for RT-PCR.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Gene</th>
<th>Sense and Antisense Primer</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM-004530</td>
<td>MMP-2</td>
<td>AGGATCATGGCTACACACC AGCTGTCATAGGATGTCAGCA</td>
<td>535</td>
</tr>
<tr>
<td>NM-004994</td>
<td>MMP-9</td>
<td>CGCAGACATCGTCATCCAGT GGATTGGCTTGGAAGATGA</td>
<td>406</td>
</tr>
<tr>
<td>NM-000594</td>
<td>TNF-α</td>
<td>CCCCAGGGACCTCTCTCTAA GGAAGACCCCTCCAGATAG</td>
<td>413</td>
</tr>
<tr>
<td>NM-004048</td>
<td>β2-M</td>
<td>CCAGCAGAGAATGGAAAGTC GATGCTGCTTACATGTCAG</td>
<td>269</td>
</tr>
</tbody>
</table>

RT-PCR, reverse transcription–polymerase chain reaction; MMP, matrix metalloproteinase.

ultraviolet light. RT-PCR, which contained RNA but not M-MLV reverse transcriptase, was used to check contamination with genomic DNA. The Scion imaging program (Frederick, MD, www.scioncorp.com) was used to semiquantify the band density in the gels, expressed in arbitrary units (AU).

Statistical analysis
The results of the MMP ELISAs are given as mean ± SEM, the clinical, immunohistochemical, and mRNA data are presented as median with interquartile range. The Wilcoxon signed-rank test or the paired Student t-test was used to evaluate difference between paired data and the Mann–Whitney U-test or the Student t-test for unpaired data, where applicable. Differences were considered significant when $P \leq 0.05$.

Results
Clinical studies

**MMP-2**

The serum MMP-2 level in patients with fistulas from the in-house study ($n = 6$) responding to treatment with infliximab showed a steady increase from $605 \pm 78$ (ng/mL) at day 0 up to $834 \pm 46$ at week 14 ($P = 0.08$). Correspondingly, the open/draining fistulas score in these patients was decreased at the end of follow-
up from 3 (2–6) to 1 (0–1) ($P = 0.03$); most of these responders already had signs of improvement at week 2. Except for the first 3 days, where the level of MMP-2 in the nonresponders of the patients with fistulas ($n = 4$) decreased due to 1 outlier, also the nonresponding patients showed a gradual increase up to $649 \pm 107$ at the end of follow-up. The open fistulas scores in this group remained at 1, and all these nonresponding patients had genitourinary fistulas. Similarly, from day 0 to week 10 in the international study the serum MMP-2 level in fistulizing CD patients increased in both placebo (n.s.) and infliximab ($P = 0.003$) treated patients, and in both responders/healers and nonresponders/nonhealers ($0.02 < P < 0.08$), with a general decrease after week 10. Baseline MMP-2 levels at inclusion were comparable between all subgroups (Fig. 1). Also in CD patients with active disease ($n = 7$) from the in-house study who responded to the treatment with infliximab, the improvement of disease activity was accompanied by a significant increase of the serum MMP-2 level from $364 \pm 54$ (ng/mL) at day 0 to $656 \pm 53$ at week 8 ($P = 0.03$). The CDAI decreased significantly from 365 (264–461) down to 50 (10 –189)

![Figure 1.](image)

Figure 1. Serum MMP-2 levels in all subgroups of patients with fistulas from the international study showed a similar pattern, i.e., slightly increasing during the follow-up, with a slight decrease at the end. MMP: matrix metalloproteinase. Placebo: $n = 14$; all infliximab: $n = 28$; responders: $n = 22$; nonresponders: $n = 19$; healers: $n = 18$; nonhealers: $n = 23$. 


at the corresponding timepoints ($P = 0.04$). At the end of follow-up, i.e., week 12, the MMP-2 level started to decrease and the CDAI started to increase again. One patient showed no improvement with the infliximab treatment and the serum MMP-2 level in this patient remained stable over time. The serum MMP-2 level in patients with active disease from the international study did not show consistent and significant changes at the end of 4 weeks follow-up compared with day 0 in both placebo and infliximab-treated groups (Table 2), although at higher dosages a tendency to increase was clearly discernable.

**Table 2.** Serum MMP-2 and -9 levels in patients with active disease included in the international study.

<table>
<thead>
<tr>
<th></th>
<th>Placebo $n = 7$</th>
<th>Infliximab 5 mg/kg $n = 4$</th>
<th>Infliximab 10 mg/kg $n = 6$</th>
<th>Infliximab 20 mg/kg $n = 7$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MMP-2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>780 ± 170</td>
<td>935 ± 411</td>
<td>658 ± 126</td>
<td>780 ± 80</td>
</tr>
<tr>
<td>Week 4</td>
<td>715 ± 225</td>
<td>879 ± 160</td>
<td>998 ± 118</td>
<td>898 ± 172</td>
</tr>
<tr>
<td><strong>MMP-9</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>344 ± 79</td>
<td>180 ± 62</td>
<td>286 ± 111</td>
<td>255 ± 48</td>
</tr>
<tr>
<td>Week 4</td>
<td>207 ± 46</td>
<td>194 ± 65</td>
<td>232 ± 86</td>
<td>135 ± 29</td>
</tr>
</tbody>
</table>

MMP, matrix metalloproteinase. MMPs presented in ng/mL (mean ± SEM).

**MMP-9**

The MMP-9 serum level of the in-house patients with fistulizing disease in both responders and nonresponders was hardly affected by the infliximab therapy, although at the end of follow-up a decrease was noticed from 297 ± 41 to 267 ± 76 and from 441 ± 48 to 240 ± 61 ng/mL, respectively. In the international study there seemed to be a general trend to a decreased MMP-9 level where the infliximab-treated patients and responders/healers seemed to have a slightly lower level at week 6 to 18 than the placebo treated and nonresponding/nonhealing patients, although no statistical significance was reached (Fig. 2). Active disease patients from the in-house study who responded to infliximab had a decreased MMP-9
serum level from day 3 after treatment onwards. The MMP-9 serum level fell from 419 ± 88 at the beginning of the study down to 236 ± 26 at week 4 (P = 0.05), and remained at this lower level to the end of the follow-up, 230 ± 52 (P = 0.05), accompanying the decrease in CDAI. The MMP-9 level in the 1 patient who did not respond to the treatment also showed a reduction of MMP-9 serum levels during the follow-up. In the international study the levels of MMP-9 in the active CD patients were also decreased in both the placebo and infliximab-treated groups at the end of the follow-up, except for the 5 mg/kg infliximab group, but no statistical significance was reached (Table 2).

**Immunohistochemical results**

A patchy and relatively strong positive immunoreaction to MMP-2 was present in the ECM of submucosa in noninflamed tissues (Fig. 3B). In inflamed tissues a positive staining of MMP-2 was observed in endothelial cells and the ECM of the lamina propria (Fig. 3B,C). There were no major differences between patients with fistulas or with active disease. Overall, the immunohistochemical expression pattern of MMP-2 did not seem to change by the treatment with infliximab therapy (data not shown). The immunoreactivity for MMP-9 was predominantly present in the polymorphonuclear leukocytes (PMNL). A relatively high PMNL positive staining for MMP-9 was observed in the tissues before treatment [median score 2 (IQR 1–2.5)]. Follow-up biopsies after treatment with infliximab revealed a decreased intensity of MMP-9 staining already at day 3 [1 (1–1.8), n.s.], which was even lower at week 2 [0.5 (0–1.3), P < 0.05] in 5 out of 6 patients, 4 of them with a good clinical response (Fig. 4B,C). Interestingly, in most of the tissue sections we found enteroendocrine cells to be positive for MMP-9, independent of treatment with infliximab (Fig. 4D).

**In vitro study**

Whole blood cultures revealed that the levels of MMP-2 in plasma of both CD patients and controls were not affected by stimulation with LPS or with LPS in the presence of infliximab for 1.5 or 24 hours. However, the levels of MMP-2 in the plasma of CD patients were in general lower than that in healthy controls (Table 3).
Figure 2. In contrast to the serum levels of MMP-2, MMP-9 levels in patients with fistulas from the international study showed a trend to decrease in all groups during the follow-up, with a slight increase at the end. MMP: matrix metalloproteinase. Placebo: $n = 14$; all infliximab: $n = 28$; responders: $n = 22$; nonresponders: $n = 19$; healers: $n = 18$; nonhealers: $n = 23$.

Figure 3. In patients with fistulas a patchy MMP-2 expression was immunohistochemically observed in the submucosa (A, negative control, and B, MMP-2 staining, both x200). After infliximab therapy for 2 weeks (C, x200) the immunoreaction to MMP-2 in endothelial cells (arrow) and lamina propria of inflamed tissue was prominent. MMP: matrix metalloproteinase.
MMP-9 immunostaining was predominantly present in polymorphonuclear leukocytes within the lamina propria (A, negative control, and B, MMP-9 staining, both x200). After treatment of this patient with infliximab for 2 weeks MMP-9 staining was found to be decreased (C, x200). The positive immunoreaction of MMP-9 in enteroendocrine cells (arrow) was frequently observed (D, x1000). MMP: matrix metalloproteinase

Figure 4. MMP-9 immunostaining was predominantly present in polymorphonuclear leukocytes within the lamina propria (A, negative control, and B, MMP-9 staining, both x200). After treatment of this patient with infliximab for 2 weeks MMP-9 staining was found to be decreased (C, x200). The positive immunoreaction of MMP-9 in enteroendocrine cells (arrow) was frequently observed (D, x1000). MMP: matrix metalloproteinase

With RT-PCR no detectable MMP-2 mRNA level was found in leukocytes of both CD patients and healthy volunteers. In contrast to the levels of MMP-2, MMP-9 levels in patients’ plasma were higher, compared with those in healthy volunteers. After a 1.5-hour LPS stimulation the levels of MMP-9 were significantly increased in CD patients and in controls, both more than 2-fold higher than the unstimulated cultures, with the increase in the CD patients significantly higher than that in the healthy volunteers ($P = 0.05$). Infliximab was found not to affect MMP-9 protein levels of this short-term LPS stimulation (Table 3). After 24 hours, LPS stimulation
Table 3. Plasma MMP levels from the *in vitro* whole blood cultures of CD patients and healthy volunteers

<table>
<thead>
<tr>
<th></th>
<th>Patients (n = 7)</th>
<th>Volunteers (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.5h</td>
<td>24h</td>
</tr>
<tr>
<td>MMP-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blank</td>
<td>380 ± 108</td>
<td>477 ± 148</td>
</tr>
<tr>
<td>LPS</td>
<td>397 ± 140</td>
<td>639 ± 144</td>
</tr>
<tr>
<td>Infliximab+LPS</td>
<td>462 ± 52</td>
<td>421 ± 98</td>
</tr>
<tr>
<td>MMP-9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blank</td>
<td>541 ± 209</td>
<td>315 ± 55</td>
</tr>
<tr>
<td>LPS</td>
<td>1132 ± 242**</td>
<td>848 ± 204*</td>
</tr>
<tr>
<td>Infliximab+LPS</td>
<td>1296 ± 412*</td>
<td>878 ± 327</td>
</tr>
</tbody>
</table>

MMP, Matrix Metalloproteinase; CD, Crohn’s disease; LPS, lipopolysaccharide. *P <0.05, **P < 0.01 versus blank; ***P < 0.05 versus 1.5h- incubation. MMPs presented in ng/mL (mean ± SEM).

did not further promote leukocytes to synthesize/secrete MMP-9 in CD patients, but in the healthy volunteers LPS stimulated the MMP-9 protein synthesis/secretion about 2-fold, which was significantly higher in comparison with the level after 1.5 hours (*P < 0.05). Infliximab did not affect the MMP-9 protein secretion after 24 hours in either patients or healthy volunteers. Interestingly, the 1.5-hour LPS stimulation hardly changed the respective MMP-9 mRNA level compared to that of the blank samples, whereas TNF-α mRNA was increased 13-fold (Table 4). More MMP-9 mRNA was transcribed in leukocytes after 24 hours stimulation with LPS, raising up to 16-fold. This transcription of MMP-9 mRNA was mediated by TNF-α.

Table 4. MMP-9 and TNF-α mRNA levels in cultured leucocytes from healthy volunteers

<table>
<thead>
<tr>
<th>mRNA</th>
<th>1.5h LPS</th>
<th>24h LPS</th>
<th>24h LPS + Infliximab</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-9</td>
<td>0.5 (0.1–1.4)</td>
<td>16 (2–47)</td>
<td>3 (3–7)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>13 (10–121)</td>
<td>4 (3–9)</td>
<td>2 (0.5–4)</td>
</tr>
</tbody>
</table>

MMP, matrix metalloproteinase; LPS, lipopolysaccharide. The results represent 4 experiments, with mRNA levels expressed in median relative densitometry units (inter quartile range) in comparison with blank samples.
as infliximab downregulated the mRNA level by almost 80%. At the same time, the TNF-α mRNA level in the 24-hour samples was enhanced by only 4-fold and even lower, i.e., 2-fold, in the presence of infliximab.

Discussion

Treatment with infliximab is very effective in patients with active or fistulizing CD, although the mechanism(s) of action have not yet been fully elucidated. MMPs have been implicated in both disease phenotypes and infliximab was recently found to be inducing a mucosa protective MMP-phenotype in ex vivo mucosal IBD explants. In the present study, we found that these infliximab-induced alterations in MMPs are to a large extent also reflected in the serum and in intestinal biopsies. We found an increase of serum MMP-2 in both fistulizing and active CD patients by the treatment with infliximab, in comparison to baseline, with a decline at the end of follow-up after cessation of treatment. The cause of the increase in MMP-2 might be related to the turnover of the intestinal tissue in CD, especially the remodeling of the ECM components. CD is a chronic and recurrent inflammation of the alimentary tract, where remission and relapse of the disease alternate and almost inevitably occur. In the inflammatory process the destruction and healing of tissue seems to occur simultaneously. This could partially explain why the increase of serum MMP-2 is not strictly related to the criteria of clinical improvement. During these processes there is formation of granulation tissue, especially at ulcerative and fistulizing sites, where the remodeling of tissue actively takes place. This granulation tissue differs from the normal tissue in composition of cells and matrix components, containing many fibroblasts and endothelial cells. In addition, we previously showed that in the inflamed area MMP-2 is significantly increased. The proliferation, differentiation, and (neo)angiogenesis are highly promoted by stimulation through induced growth factors, such as transforming growth factor (TGF)-β, basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF). The increase of MMP-2 at local sites is probably to meet the demand of active ECM turnover. Ågren also believed that MMP-2 is important during the prolonged remodeling phase in wound healing. Apparently, at
moments of high tissue demand for MMP-2 the serum levels are low and at inclusion of controlled treatment protocols these serum levels start to increase, not strictly related to response. The sequestration of MMP-2 to ECM of intestinal IBD tissue may also partially contribute to the paradoxical phenomenon, i.e., the elevated expression of MMP-2 in inflamed tissue and low level in the circulation.\textsuperscript{18} MMP-2 is constitutively expressed by many cells, especially mesenchymal cells, such as fibroblasts and endothelial cells, and has a ubiquitous tissue distribution.\textsuperscript{20} TNF-\(\alpha\) and other proinflammatory cytokines seem not to be major promoting factors for the expression of MMP-2 because the gene promoter lacks TPA-responsive element (TRE). TGF-\(\beta\), however, is regarded as a stimulator for the expression of MMP-2, which might be relevant to the remodeling processes.\textsuperscript{20} Our \textit{in vitro} results do confirm this since the levels of MMP-2 in plasma were not affected by the incubation of leukocytes with immunomodulators like LPS and/or infliximab. Serum MMP-9 levels, in contrast to MMP-2, in the CD patients were found to be reduced by the treatment, with an increase again at the end of the follow-up. Similar to the duration of the increase of the MMP-2 level, the decrease of MMP-9 also lasted for a time period that coincides with the duration that infliximab is maintained at a detectable level in the circulation.\textsuperscript{6} MMP-9 is thought to be an active participant in the process of inflammation in CD, especially in the acute phase. Unlike other MMPs, MMP-9 is normally stored in secondary and tertiary granules of neutrophils poised for rapid release to participate in the reaction of the host to exogenous and endogenous stimulation. MMP-9 not only lyses components of the ECM, but also influences the generation or activation of c-x-c and other chemokines, which attract neutrophils to migrate across the BM of capillaries to inflammatory sites.\textsuperscript{38} Kirkegaard \textit{et al.}\textsuperscript{29} recently found MMP-9 to be markedly upregulated in and contribute to intestinal fistula formation in CD. During the evolvement of tissue repair, overexpression of MMP-9 has been speculated to prevent the healing process.\textsuperscript{8} In contrast, Salo \textit{et al.}\textsuperscript{39} concluded that MMP-9 plays a prominent role because it participated in every step of the healing process, including detachment of epithelial cells from the basal membrane, rolling of cells to the wound matrix, and remodeling of the granulation tissue. The neutrophil is the most important source of MMP-9 in the acute phase of inflammation.\textsuperscript{40} In the
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In the present study, immunohistochemical evaluation showed that MMP-9 predominately existed in the neutrophils and to a lesser extent in the ECM of severely inflammatory regions. The reduction of the MMP-9 expression in intestinal tissue from the infliximab-treated CD patients is probably related to the decrease in the number of the inflammatory cells, especially neutrophils and monocytes/macrophages.\textsuperscript{41,42} In addition, our previous \textit{ex vivo} mucosal IBD explants study revealed that within 72 hours infliximab is able to markedly reduce the MMP-9 synthesis/secretion also.\textsuperscript{32} MMP-9 was also found to be present in enteroendocrine cells. The significance of this observation needs to be elucidated further. Perhaps there is a similarity with the presence of MMP-7 in intestinal Paneth cells, which is believed to be related to the activation of \(\alpha\)-defensin.\textsuperscript{43,44} Proinflammatory cytokines, such as TNF-\(\alpha\), IL-1\(\beta\), are able to activate the MMP-9 gene through nuclear factor (NF)-\(\kappa\)\(\beta\) to enhance MMP-9 production.\textsuperscript{39,45} LPS stimulates monocytes to express MMP-9, which is partly dependent on TNF-\(\alpha\) because neutralization of TNF-\(\alpha\) significantly downregulated the production of MMP-9.\textsuperscript{46} Our \textit{in vitro} study also showed that MMP-9 is released from leukocytes of both CD patients and healthy volunteers after short-term 1.5 hours LPS incubation. The release of MMP-9 from leukocytes of the CD patients was significantly higher than from healthy volunteers. Most likely, the abundance of MMP-9 in the CD neutrophils occurred during the process of their maturation activation in response to different stimulators, such as TNF-\(\alpha\) and bacterial products.\textsuperscript{47,48} TNF-\(\alpha\) seemed not to be involved in the process of MMP-9 secretion by neutrophils \textit{in vitro}, as infliximab did not affect the level of MMP-9 in plasma. The transcription of MMP-9 mRNA was found to be hardly affected by short-term LPS stimulation, probably because of the increased production of other immediate response mRNAs like that of TNF-\(\alpha\). Further incubation with LPS for 24 hours increased the level of MMP-9 significantly in healthy volunteers rather than in CD patients. The MMP-9 mRNA was also strongly upregulated. Infliximab did suppress this increase of MMP-9 mRNA, therefore we conclude that TNF-\(\alpha\) is involved in the promotion of the transcription of MMP-9 mRNA. In the CD patients the MMP-9-producing blood cells probably lost their potential ability to further induce MMP-9 synthesis. We speculate that monocytes are the main MMP-9-producing blood cell type responding to the
long-term LPS incubation in this study. Pugin et al.\(^4\) previously reported that the maximal level of monocytic MMP-9 synthesis was between 24-48 hours after LPS stimulation. Other studies also showed that LPS could activate monocytes to express the MMP-9 gene and protein, although they did not implicate TNF-α in their studies.\(^49,50\) The MMP-9 changes we observed in the serological follow-up of our patients are, however, most likely a reflection of what is happening at the mucosal level, also based on our explant studies, and less so an immediate effect of infliximab on circulating blood cell MMP-9 production and secretion.

In conclusion, the serum MMP-2 and MMP-9 level in CD patients display an inverse changing pattern, i.e., an increase of MMP-2 and a decrease of MMP-9 during the treatment with infliximab, although not strictly related to the clinical effect of infliximab. The enhanced leukocyte MMP-9 expression in CD seems to be regulated by and responsive to TNF-α mediation. These clinical findings reinforce previous observations in experimental models showing MMP-2 to be protective and MMP-9 to be enhancing dextran sodium sulfate-induced colitis, rendering the latter a potential therapeutic target.\(^51-53\)

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