Enhanced production of monocyte chemotactic protein 3 in inflammatory bowel disease mucosa

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Abstract

Background—The β chemokine monocyte chemotactic protein 3 (MCP-3) has chemoattractant and activating capabilities in monocytes, lymphocytes, eosinophils, and basophils.

Aims—To investigate MCP-3 expression in inflammatory conditions of the human intestinal mucosa.

Patients—Forty five colon biopsy specimens from 18 patients with inflammatory bowel disease (IBD; 16 specimens from inflamed and 10 from non-inflamed areas) and 19 control patients were examined.

Methods—Immunohistochemical staining and reverse transcription polymerase chain reaction (RT-PCR) were used for MCP-3 detection in tissue sections. Intestinal epithelial cell lines (HT-29, Caco-2, T-84) were stimulated with interleukin (IL) 1 β , IL-6, and tumour necrosis factor α (TNF- α) and examined for MCP-3 protein and mRNA expression using immunocytochemistry and RT-PCR, respectively.

Results—In tissue sections, MCP-3 protein was detected predominantly in epithelial cells, both in patients with IBD and in controls. MCP-3 staining was particularly pronounced at sites of active mucosal inflammation. The intensity of MCP-3 staining was positively correlated with the extent of epithelial destruction. In intestinal epithelial cell lines, MCP-3 mRNA was expressed, whereas MCP-3 protein was not consistently detected.

Conclusions—Our data show that MCP-3 protein is present in normal and inflamed intestinal tissue. MCP-3 production is substantially enhanced in areas of active inflammation, suggesting an immunoregulatory role of MCP-3 in intestinal inflammation.

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Keywords: ulcerative colitis; Crohn's disease; epithelial cells; monocyte chemotactic protein 3; chemokines

Chemokines are a growing family of small (8-10 kDa) immunoregulatory peptides that are involved in several types of inflammatory reactions and exhibit mainly chemoattractant and activating capabilities.¹ Altered chemokine expression has been shown in different pathological conditions such as rheumatoid arthritis, psoriasis, reperfusion injury, and glomerulonephritis.²⁻⁵ Furthermore, chemokines may regulate viral infection, as it has

recently been shown that the β chemokine receptors CCR-3 and CCR-5 are cofactors (together with the CD4 molecule) for entry of HIV into macrophages, while CXCR-4 permits entry of lymphotropic virus strains.^{6,7}

The family of chemokines consists of two main families defined according to the arrangement of the first two N terminal cysteines, the α chemokines (C-X-C) in which the cysteines are separated by another amino acid residue, and the β chemokines (C-C), in which the first cysteines are adjacent.^{1,8} The C-X-C chemokines (for example, interleukin 8 (IL-8)) are potent chemoattractants and activators of neutrophil granulocytes, while the C-C chemokines (for example, macrophage inflammatory proteins (MIP), monocyte chemotactic proteins (MCP), RANTES) act primarily on monocytes, lymphocytes, basophils, and eosinophils.^{8,9}

In inflammatory bowel disease (IBD), MCP-1 and RANTES are the only β chemokines which have been studied so far. Reinecker and colleagues¹⁰ found MCP-1 mRNA expression in macrophages, endothelial cells, and epithelial cells of human colonic mucosa. They showed that MCP-1 expression was upregulated during inflammatory processes. Grimm and colleagues¹¹ also found a significant increase in MCP-1 expression in inflamed intestinal mucosa. Furthermore, Mazzucchelli and colleagues¹² found increased numbers of lamina propria cells expressing mRNA for MCP-1 and RANTES in biopsy specimens from patients with IBD.

MCP-3 shares a 71% amino acid homology with MCP-1, but is only about 30% homologous to RANTES.^{13,14} MCP-3 combines receptor binding properties of RANTES and MCP-1.¹⁵ Monocytes, T lymphocytes, eosinophils, and basophils can be attracted and activated by MCP-3.^{16,17} Therefore, MCP-3 is thought to be important for the regulation of immunocompetent and inflammatory cells. Recent studies indicate that MCP-3 is upregulated in inflamed human bronchial mucosa.^{18,19}

MCP-3 may be of relevance in IBD and other gastrointestinal inflammatory disorders, characterised by infiltration of the intestinal mucosa by multiple inflammatory cells including macrophages, lymphocytes, eosinophils, and neutrophils. As most of these cell types can be attracted by MCP-3,¹⁴⁻¹⁶ we examined the

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Abbreviations used in this paper: CD, Crohn's disease; IBD, inflammatory bowel disease; IL, interleukin; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; TNF, tumour necrosis factor; UC, ulcerative colitis.

expression of this potent chemokine in human colonic mucosa from patients with IBD and healthy controls.

Methods

PATIENTS

Forty five colonic tissue specimens from 37 patients were examined. Endoscopy was performed for clinical reasons in the Department of Gastroenterology and Hepatology, Medical School Hannover, with an Endoflex coloscope (Olympus, Hamburg, Germany) after patients gave written consent. The patients were divided into three groups according to clinical diagnosis: ulcerative colitis (10 patients), Crohn's disease (eight patients), and controls (19 patients). Diagnosis of IBD was based on clinical, radiological, endoscopic, and histological findings. In patients with IBD, biopsy specimens from both actively inflamed and normal tissue areas were taken, if possible. From patients with ulcerative colitis, eight specimens from macroscopically inflamed and six specimens from macroscopically normal mucosa were obtained. From patients with Crohn's disease, eight specimens from macroscopically inflamed and four specimens from macroscopically normal mucosa were obtained. The control group consisted of patients with carcinoma (n=9), polyps (n=5), angiodysplasia (n=1), irritable bowel syndrome (n=3), and diverticulosis (n=1). In control patients, biopsy specimens were taken from areas of macroscopically normal mucosa. The mean age of IBD patients was 37 years (range 19–54); that of the control group was 52 years (range 27–73).

IMMUNOHISTOCHEMISTRY

Immunohistochemistry was performed as described previously.^{20,21} Briefly, biopsy specimens were fixed in paraformaldehyde 4%, embedded in paraffin wax, and sectioned at 3 µm. Endogenous peroxidase activity was blocked by hydrogen peroxide 3%. Non-specific staining was reduced by preincubation of sections with 10% non-immune rabbit serum (Zymed, San Francisco, California, USA) for 10 minutes at room temperature. Subsequently, sections were incubated overnight at 4°C with the primary antibody monoclonal mouse antihuman MCP-3, isotype IgG1, tested negative for cross reactions with MCP-1, MCP-2, or other structurally related human chemokines (PeproTech, Rocky Hill, New Jersey, USA), at a final concentration of 50 µg/ml. In some experiments, epithelial cell lines were stained additionally with another monoclonal mouse antihuman MCP-3, isotype IgG1, which also showed no cross reactions with other chemokines such as MCP-1, MCP-2, and eotaxin (LeukoSite Inc., Cambridge, Massachusetts, USA). Non-immune mouse monoclonal IgG1 (Immunotech, Marseille, France) diluted at 1/200 in phosphate buffered saline was used as negative control antibody. In addition, the specificity of MCP-3 staining was verified by preincubation of the monoclonal mouse antihuman MCP-3 IgG1

antibody with a 20-fold excess of human MCP-3 (PeproTech) on a molar basis.

After washing, sections were incubated with a biotinylated secondary antibody for 10 minutes, followed by exposure to streptavidin conjugated horseradish peroxidase for 10 minutes. The substrate chromogen mixture (hydrogen peroxide/AEC (3% 3-amino-9-ethylcarbazole in N,N-dimethylformamide)/peroxidase) was added for seven minutes, which coloured immunoreactive cells red (Histostain-SP Kit, Zymed, San Francisco, California, USA). Slides were counterstained with Mayer's haemalaun (E. Merck, Darmstadt, Germany) and mounted in Kaiser's glycerol gelatine (E. Merck). Serial sections of six tissue samples from inflamed and non-inflamed areas showing strong MCP-3 protein expression were also stained with monoclonal mouse antihuman CD3, CD4, or CD8 antibody, respectively (Dako Diagnostics, Hamburg, Germany). These slides were treated for 10 minutes in target unmasking fluid (Kreatech Diagnostics, Amsterdam, the Netherlands) at 90°C after deparaffinisation and rehydration. Slides were then processed as described above.

EVALUATION OF MUCOSAL BIOPSY SPECIMENS

Stained tissue sections were examined with a Labophot-2 microscope (Nikon, Tokyo, Japan) at a 400× magnification. Immunohistochemical staining was graded semiquantitatively on a scale 0–4 (0 = no staining, 1 = sporadic weak positive cells, 2 = sporadic strong positive cells, 3 = many weak positive cells, 4 = many strong positive cells). In parallel, histopathological grading was performed by a pathologist not involved in the further immunohistochemical investigations. Histological examination included grading of epithelial destruction (1 = no destruction, 2 = mild destruction, 3 = notable destruction) and cellular infiltration of the lamina propria with inflammatory cells (1 = no, 2 = mild, 3 = notable).

EPITHELIAL CELL LINES AND CELL CULTURE

Human intestinal epithelial cell lines (Caco-2, HT-29, and T-84) were cultured to confluent layers as previously described.^{22–24} For RNA extraction, cells were cultured in 10 cm dishes. For immunohistochemical studies, cells were grown in chamber slides (Nunc, Roskilde, Denmark) (HT-29 and Caco-2) or in 5 cm dishes (T-84). Prior to cytokine stimulation, cells were serum starved for 24 hours with medium containing 0.1% foetal bovine serum. Cells were then stimulated for 24 hours with human recombinant IL-1β (Biogen-Glaxo SA, Geneva, Switzerland), IL-6 (Amgen Inc., Thousand Oaks, California, USA), and TNF-α (Genentech, Vienna, Austria), all at final concentrations of 10 ng/ml. After removal of medium, chamber slides or culture dishes, respectively, were dried at room temperature and fixed in acetone/methanol (vol/vol: 1:1) for 10 minutes followed by 70% ethanol for 30 minutes, both at 22°C. Slides were stored at –20°C. For immunocytochemical staining,

slides were thawed, rehydrated, and stained as described above.

RNA PREPARATION, RT-PCR, DNA SEQUENCING, AND RNASE PROTECTION ASSAY

Total RNA was prepared from cultured intestinal epithelial cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany). For RT-PCR, 1 µg of total RNA was treated for 15 minutes at 37°C with 10 U RNase free DNase (Promega, Madison, Wisconsin, USA) to remove genomic DNA. After denaturation for 10 minutes at 70°C, cDNA was synthesised for one hour at 37°C by adding Superscript reverse trans-

criptase (Life Technologies, Eggenstein, Germany) and 20 pmol oligo dT primers (Pharmacia, Uppsala, Sweden). A 1/10 volume of the cDNA was used for one PCR reaction. PCR was performed with 2.5 U Taq DNA polymerase (Life Technologies) and 20 pmol of the primers for MCP-3 (sense: 5'-GCCTCTG-CAGCACTTCTGTG-3'; antisense: 5'-CATTCTGTGTGGGGTCAGC-3') in a reaction volume of 50 µl. Positive controls were performed with primers for GAPDH (sense: 5'-ACCACAGTCCATGCCATCAC-3'; antisense: 5'-TCCACCACCCTGTTGCTGTA-3'). cDNA was amplified using a Peltier

Figure 1 Immunohistochemical staining of colonic mucosal sections with mouse antihuman MCP-3 monoclonal antibody. (A–C): macroscopically normal tissue derived from control patients; (D): negative control section derived from the same tissue sample as H; (E, F and H): macroscopically inflamed tissues derived from patients with ulcerative colitis or (G) Crohn's disease. Original magnification $\times 400$ (A, E) or $\times 1000$ (B–D, F–H).

thermal cycler (PTC200, MJ Research, Watertown, Massachusetts, USA). Thirty five cycles (60 seconds at 94°C, 80 seconds at 60°C, 70 seconds at 72°C) were performed followed by a five minute extension at 72°C after the last cycle. The PCR products contained transcripts of 239 bp (MCP-3) and 452 bp (GAPDH), respectively. A 10 µl aliquot of the PCR product was separated on 1% agarose gel containing ethidium bromide (500 ng/ml) and photographed. To observe relative changes in mRNA expression of MCP-3 the "primer dropping" method established by Wong *et al* was used.²⁵ Duplex PCRs were started with the primer pair for MCP-3. After 12 cycles the primer set of GAPDH was added. In each case 10 µl of the reaction was taken in the course of exponential DNA increase after 31 cycles, 33 cycles, and 35 cycles, separated on 1% agarose gels containing ethidium bromide and analysed using an automated bioimaging analyser (Fuji BAS-1000, Raytest, Germany). To be certain to obtain MCP-3 derived cDNA, a 239 bp fragment of cDNA was subcloned into a plasmid (pCR-Script SK from Stratagene, La Jolla, California, USA) and sequenced by the dideoxy method using the T7 SequencingTM kit (Pharmacia).²⁶ MCP-3 mRNA was also analysed using the RNase protection assay performed according to the manufacturer's instructions (Promega).

STATISTICS

Data were generally expressed as mean (SE). Data from multiple groups were compared by analysis of variance (ANOVA) and the Bonferroni test. A value of $p < 0.05$ was considered to be statistically significant. Correlations were assessed by the Spearman rank correlation coefficient (r_s). For statistical calculations, the software package SPSS for Windows, release 6.0, was used.

Results

IMMUNOHISTOCHEMISTRY FOR MCP-3

MCP-3 protein was detected in biopsy specimens both from patients with IBD and controls by immunohistochemistry using the antihuman MCP-3 monoclonal antibody from PeproTech. In control specimens, MCP-3 was found almost exclusively in intestinal epithelial cells with strong staining in the surface epithelium and little or no immunoreactivity in the crypts (fig 1A–C). Epithelial cells contained low amounts of MCP-3 and staining showed a homogeneous, seam like distribution. In contrast, the distribution of MCP-3 staining in inflamed tissue derived from patients with Crohn's disease (fig 1G) or ulcerative colitis (fig 1E, F, H) was notably inhomogeneous compared with non-inflamed areas. MCP-3 positive cells were found in both the surface and crypt epithelium. Some intestinal epithelial cells within inflamed areas stored large amounts of MCP-3 (fig 1E–H), which was not seen in normal tissue sections. No substantial differences in MCP-3 staining pattern were found between active ulcerative colitis and Crohn's disease.

Figure 2 Semiquantitative distribution of MCP-3 in human colonic epithelial cells. UC, ulcerative colitis; CD, Crohn's disease; CP, control patients; +, biopsy specimens from macroscopically inflamed areas; -, biopsy specimens from macroscopically normal mucosa.

Outside the epithelium, MCP-3 positive cells were found only sporadically. Occasionally, the lamina propria contained MCP-3 positive cells independent of the degree of mucosal inflammation. These findings were confirmed by experiments with freshly isolated human lamina propria cells, unstimulated or stimulated *in vitro* by ionomycin and phorbol myristate acetate (PMA), which did not express MCP-3 as revealed by immunocytochemical staining of cytopins (data not shown; for methods, see Bischoff and colleagues^{20, 27}). To exclude the possibility that the fixation procedure affected the staining properties, we repeated the experiments using cryostat sections (5 µm), and obtained analogous results.

Figure 2 summarises the immunohistochemical results and shows that MCP-3 staining was enhanced in macroscopic inflamed tissue (2.9 (0.3) of a semiquantitative grading using a scale from 0 to 4, $n=16$) compared with macroscopic normal mucosa from patients with IBD (1.4 (0.2), $n=10$, $p=0.002$) or with control biopsy specimens (1.8 (0.2), $n=19$, $p=0.006$). In patients with ulcerative colitis, a significant difference of MCP-3 staining intensity between inflamed and macroscopically normal tissue was found ($p=0.005$). However, this difference was less pronounced in patients with Crohn's disease and was not statistically significant.

Immunohistochemical results were correlated with histopathological findings. A positive correlation between MCP-3 staining intensity and grade of epithelial destruction (grade 1–3) was found ($r_s=0.52$; $p<0.001$; $n=45$). In contrast, the extent of cellular infiltration (grade 1–3) of the mucosa and MCP-3 expression was only weakly correlated ($r_s=0.35$; $p<0.05$; $n=45$). The analysis of serial intestinal tissue sections stained immunohistochemically with monoclonal antibodies against MCP-3, CD3, CD4, and CD8 did not reveal any association between number and localisation of T cell subsets and local production of MCP-3 in normal and in inflamed tissue (data not shown).

In addition to the immunohistochemical analysis of MCP-3 protein expression, MCP-3 mRNA was detected in human colonic biopsy

Figure 3 MCP-3 mRNA expression in human epithelial cells. (A): Cells were challenged with a buffer control (lanes A) or with TNF- α 10 ng/ml (lanes B) for 24 h. A 10 μ l aliquot of the PCR tube content was taken out in the course of exponential DNA increase after 31 cycles (lanes 1), 33 cycles (lanes 2), and 35 cycles (lanes 3). (B): Time course analysis of MCP-3 mRNA expression by HT-29 cells without stimulation (0 h) and following stimulation with TNF- α for 2, 8, and 24 h. DNA fragments obtained after 35 cycles are shown.

specimens. MCP-3 mRNA expression was found in inflamed as well as in healthy tissue ($n=3$). However, no significant enhancement of MCP-3 mRNA expression was detected in inflamed tissues compared with controls by semiquantitative RT-PCR.²⁵

INTESTINAL EPITHELIAL CELL LINES EXPRESS MCP-3 mRNA

MCP-3 expression was also studied *in vitro* in unstimulated and stimulated epithelial cells using three human intestinal epithelial cell lines (HT-29, Caco-2, T-84). Cells were stimulated with the proinflammatory cytokines TNF- α , IL-1 β , and IL-6, respectively, or challenged with a buffer control. By immunocytochemical means using two different antihuman MCP-3 monoclonal antibodies, MCP-3 protein was found in only three of 12 experiments (three cell lines, four different cell preparations each), if the cells were stimulated with TNF- α , IL-1 β , or IL-6. MCP-3 immunoreactivity was most pronounced in HT-29 cells stimulated with TNF- α . In all unstimulated cell preparations ($n=12$) and in most stimulated cells (nine of 12 experiments) no MCP-3 protein could be detected by immunocytochemistry. MCP-3 immunostaining was slightly weaker using the mAb of PeproTech compared with the antibody of LeukoSite. MCP-3 mRNA expression was detectable in all experiments by RT-PCR but not by RNase protection assay. Following stimulation with TNF- α for 24 hours, MCP-3 mRNA was 2.5-fold increased compared with GAPDH mRNA in HT-29 cells (fig 3A) whereas no clear MCP-3 mRNA increase in response to activation was seen in Caco-2 and T-84 cells. Time course analysis revealed maximal MCP-3 mRNA expression in TNF- α stimulated HT-29 cells after two hours of stimulation with no further increase after eight or 24 hours (fig 3B).

Discussion

The role of chemokines at the gastrointestinal barrier is largely unknown. Among the large family of chemokines, only a few have been

examined for their expression in human gastrointestinal tissue so far. Two of the C-X-C chemokines, IL-8 and ENA-78, and two of the C-C chemokines, MCP-1 and RANTES, were found to be expressed in gastrointestinal tissue.^{10-12, 28-30} All these chemokines were enhanced in inflammatory conditions of the gastrointestinal tract such as IBD. In the meantime, new C-C chemokines such as MCP-3 have been discovered.^{13, 14} The function of MCP-3 in inflammatory diseases is unclear. MCP-3 is upregulated in the course of bronchial asthma,^{18, 19} but data are lacking regarding inflammatory disorders of the gastrointestinal tract. Our data show for the first time that MCP-3 is expressed in human intestinal mucosa. MCP-3 mRNA and protein was detectable in biopsy specimens from patients with IBD and from healthy controls. The major cellular source of MCP-3 was intestinal epithelial cells. In contrast to MCP-1, which is produced by epithelial and lamina propria cells,¹⁰⁻¹² MCP-3 protein expression was low within the lamina propria. These results suggest that in the human intestine, MCP-3 is almost exclusively derived from epithelial cells.

Our data show that, compared with non-inflamed tissue, the immunoreactivity for MCP-3 within the epithelial layer was significantly enhanced in inflamed tissue specimens from patients with IBD. Interestingly, a significant correlation between microscopical epithelial destruction and MCP-3 staining was detectable. Similar findings have been reported previously for IL-8 expression.²⁸ Previous investigations using allergen challenged mice showed inhibition of airway inflammation after treatment with a MCP-3 antibody.³¹ Similar mechanisms could be assumed for the human intestinal mucosa and suggest that MCP-3 may be involved in epithelial destruction during active IBD.

Most intense MCP-3 immunoreactivity was found in inflamed tissue derived from patients with ulcerative colitis. Enhanced MCP-3 immunoreactivity was also observed in active Crohn's disease, although the difference compared with controls was less pronounced compared with active ulcerative colitis. This observation might be due to the fact that ulcerative colitis is primarily a mucosal disease, whereas Crohn's disease affects all tissue layers of the bowel wall. Differences in chemokine expression between Crohn's disease and ulcerative colitis have also been reported for IL-8.²⁸ It remains to be clarified whether the increase in MCP-3 production is specific for active IBD, or an unspecific phenomenon occurring in any kind of intestinal inflammation.

MCP-3 protein expression in non-inflamed tissue was largely restricted to the surface epithelium, while in inflamed tissue MCP-3 positive cells were also found in the lamina propria cells. A possible explanation for these findings may be that MCP-3 production by epithelial cells is induced by luminal and lamina propria derived factors. Luminal antigens such as allergens or bacteria could be a

possible inductor of MCP-3 expression in surface epithelium and therefore contribute to a physiological staining pattern in non-inflamed tissue.^{19 31-34} In IBD, additional triggering events, for example, cytokines derived from lamina propria cells such as TNF- α or IL-1 β known to be released during the inflammatory process, may activate epithelial cells for MCP-3 production.^{35 36}

Confirming previous studies in human epithelial cell lines,³⁷ we found MCP-3 mRNA expression in several human intestinal epithelial cell lines (HT29, Caco-2, T-84). Using semiquantitative RT-PCR, we and Yang *et al*³⁷ found an increase (2.5-fold or fourfold, respectively) of MCP-3 mRNA expression in HT-29 cells on activation with TNF- α . However, MCP-3 mRNA expression was rather moderate, as it was only detectable by RT-PCR but not by RNase protection assay. Accordingly, we could not confirm consistent MCP-3 protein production in human epithelial cell lines, suggesting considerable functional differences between epithelial cells in situ and transformed cell lines. It cannot be ruled out, however, that the most potent inductor molecules for MCP-3 expression in epithelial cells were lacking in our in vitro experiments, for example, bacterial or viral products shown to be capable of inducing chemokine expression in human epithelial cell lines.^{33 34 38}

In summary, our data together with previous data reported by other groups^{10-12 28-30 33 34 37 38} clearly show that epithelial cells are a source of chemokines known to regulate tissue inflammation. Thus, epithelial functions seem not to be restricted to formation of the intestinal barrier, but also include immunoregulatory capacities. This finding may be of particular relevance in the pathogenesis of intestinal inflammatory diseases such as IBD characterised by activation of epithelial and lamina propria cells.³⁹ Indeed, many authors found that chemokine production by epithelial and/or lamina propria cells is enhanced during active IBD.^{10-12 28-30} Our study further confirms this concept by showing that MCP-3 production by human intestinal epithelium is clearly enhanced in active IBD.

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