Expression and possible function of
granulocyte chemotactic protein-2 (GCP-2/CXCL6)
in inflammatory bowel diseases and gastrointestinal tumors

Klara Gijsbers

Promoter: Prof. Dr. Jo Van Damme
Co-promoter: Prof. Dr. Karel Geboes

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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS</td>
<td>acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>5-ASA</td>
<td>5-aminosalicylic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CCL</td>
<td>CC chemokine ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>CC chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn’s disease</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CI</td>
<td>chemotactic index</td>
</tr>
<tr>
<td>Con A</td>
<td>concanavalin A</td>
</tr>
<tr>
<td>CXCL</td>
<td>CXC chemokine ligand</td>
</tr>
<tr>
<td>CXCR</td>
<td>CXC chemokine receptor</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double-stranded RNA</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>EGM-2</td>
<td>endothelial cell growth medium with supplements</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ELR</td>
<td>Glu-Leu-Arg tripeptide</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagle’s minimum essential medium with Earle’s salts</td>
</tr>
<tr>
<td>ENA-78</td>
<td>epithelial cell-derived neutrophil attractant-78</td>
</tr>
<tr>
<td>EpCAM</td>
<td>epithelial cell adhesion molecule (also called KSA)</td>
</tr>
<tr>
<td>FAE</td>
<td>follicle-associated epithelium</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>GALT</td>
<td>gut-associated lymphoid tissue</td>
</tr>
<tr>
<td>GCP-2</td>
<td>granulocyte chemotactic protein-2</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine 5’-diphosphate</td>
</tr>
<tr>
<td>GI</td>
<td>gastrointestinal</td>
</tr>
<tr>
<td>GIST</td>
<td>gastrointestinal stromal tumor</td>
</tr>
<tr>
<td>GRO</td>
<td>growth-related oncogene</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine 5’-triphosphate</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks’ balanced salt solution</td>
</tr>
<tr>
<td>HCC-1</td>
<td>hemofiltrate CC chemokine-1</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HMVEC</td>
<td>human dermal microvascular endothelial cells</td>
</tr>
<tr>
<td>HSA</td>
<td>human serum albumin</td>
</tr>
</tbody>
</table>
HUVEC  human umbilical vein endothelial cells  
IBD  inflammatory bowel diseases  
IEL  intraepithelial lymphocytes  
IFN-γ  interferon-γ  
IL-  interleukin-  
IP-10  interferon-γ-inducible protein-10  
ISH  in situ hybridization  
KDEL  Lys-Asp-Glu-Leu tetrapeptide  
KSA  epithelial cell adhesion molecule (also called EpCAM)  
LPL  lamina propria lymphocytes  
LPS  lipopolysaccharide  
M cell  microfold cell  
MCP-1  monocyte chemotactic protein-1  
MEC  mucosae-associated epithelial chemokine  
MIP-1α  macrophage inflammatory protein-1α  
MMP-9  matrix metalloproteinase-9  
mRNA  messenger RNA  
NBT/BCIP  nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate  
NT-buffer  NaCl/Tris-HCl buffer  
PAMP  pathogen-associated molecular pattern  
PARC  pulmonary and activation-regulated chemokine  
PBMC  peripheral blood mononuclear cells  
PBS  phosphate buffered saline  
PF-4  platelet factor-4  
PIC  synonym for poly rI:rC (polyriboinosinic-polyribocytidylic acid)  
PMA  phorbol myristate acetate  
poly rI:rC  polyriboinosinic-polyribocytidylic acid  
PRR  pattern-recognition receptor  
RT-PCR  real time-polymerase chain reaction  
SDS-PAGE  sodium dodecyl sulfate-polyacrylamide gel electrophoresis  
SEA  Staphylococcus aureus enterotoxin A  
SEM  standard error of the mean  
TAM  tumor-associated macrophage  
Th  helper T lymphocyte  
TLR  Toll-like receptor  
TNF-α  tumor necrosis factor-α  
TNM  tumor-nodes-metastasis classification  
tricine  N-tris-[hydroxymethyl]methylglycine  
Tris  tris-[hydroxymethyl]aminomethane  
UC  ulcerative colitis  
VEGF  vascular endothelial growth factor  
VKC  vernal keratoconjunctivitis  
WHO  world health organization
Literature study
Chemokines in gastrointestinal disorders

1. Introduction

The human body functions remarkably well despite the fact that it is constantly challenged in a variety of situations. Stress conditions include the contact with infectious organisms (viruses, bacteria, fungi, protozoa and multicellular parasites) and potentially noxious foreign agents (plant products, chemical agents,…) as well as physical damage (UV radiation, bruise, knife-cut, burn,…). In the human body, the gastrointestinal tract is a major site of contact with microorganisms and foreign agents. On the one hand, commensal bacteria and nutritional agents are necessary for the proper working of this tissue and need to be tolerated. On the other hand, efficient resistance against pathogens is essential [1]. To avoid tissue damage in case of infection, the human body displays a number of fine-tuned immunological defence mechanisms that rely on the interplay between innate and adaptive immunity.

The innate immune response represents a non-specific defence system that is rapidly activated upon infection. In response to chemoattractants, neutrophils and monocytes infiltrate the inflamed tissue. These phagocytes then engulf foreign material and break it down by the release of their granular content [2]. In addition, phagocytes carry specific pattern-recognition receptors (PRR) that recognize pathogen-associated molecular patterns (PAMP) present on microorganisms but not on host cells. Toll-like receptors (TLR) are an example of these PRR and show great similarity with the Toll receptor, present in Drosophila, which indicates that the innate immune response is highly conserved throughout evolution [3-6]. In the
CHEMOKINES IN GASTROINTESTINAL DISORDERS

gastrointestinal tract, TLRs convert the recognition of PAMPs into signals for anti-
microbial peptide expression, barrier fortification and proliferation of epithelial cells [7]. Microbial TLR agonists such as bacterial endotoxin and viral double-stranded RNA are potent inducers of cytokines, the hormones of the immune system [8,9].

The adaptive immune response is only present in higher animals, is much slower than the innate immune response and consists of a specific reaction against antigens that is dependent upon the action of T and B lymphocytes.

Chemokines (chemotactic cytokines) are small, secreted proteins that are important immune modulators as they assure the directed movement (chemotaxis) of both phagocytes and lymphocytes within the body [10]. In addition, they activate these immune cells and stimulate the expression of adhesion molecules on endothelial cells, which facilitates the migration of inflammatory cells out of the blood stream into the inflamed tissue [11].

The present review focuses on the expression of chemokines in inflammatory bowel diseases as assessed in plasma, tissue sections, tissue homogenates and organ cultures. Data on therapeutic experiments are not included. In gastrointestinal tumors, the effect of induced chemokine expression was evaluated.

2. Chemokines

The first chemokine to be discovered was platelet factor 4 (PF-4), but without any clear reference to leukocyte chemotaxis. Afterwards, the cDNA of structurally similar proteins was identified, but it was only in 1987, with the discovery of interleukin-8 (IL-8), that the chemoattractant property of these structurally related proteins became clear. Now, not even 30 years later, chemokines are accepted as key mediators in immunity and more than 45 different molecules of this family have been identified [12].

Chemokines are classified upon the position of the first two out of four conserved cysteine residues. The two main subgroups are the CC and CXC chemokines, where the two N-terminal conserved cysteine residues are adjacent or separated by an arbitrary amino acid, respectively. CXC chemokines can be further subdivided into ELR+ and ELR–CXC chemokines based on the presence or absence,
respectively, of a Glu-Leu-Arg tripeptide just in front of the CXC-motif. In contrast to CC chemokines, that are mostly active on monocytes and lymphocytes, ELR\(^\text{+}\)CXC chemokines are specific activators of neutrophilic granulocytes [13]. The ELR-motif is indispensable for this chemotactic activity since it allows binding to the CXC chemokine receptor CXCR2, present on neutrophilic granulocytes [14,15]. ELR CXC chemokines mainly attract lymphocytes and natural killer cells [16].

The denomination of a chemokine was originally derived from its functional activity on its target cell e.g. granulocyte chemotactic protein-2 (GCP-2) and monocyte chemotactic protein-1 (MCP-1) are neutrophil and monocyte chemoattractants, respectively. In some cases, the nomenclature also indicated the producer cell e.g. epithelial-cell derived neutrophil attractant-78 (ENA-78) and mucosae-associated epithelial chemokine (MEC). Another, more uniform nomenclature obtruded itself when it became clear that most chemokines are capable of recruiting more than one type of leukocyte and are produced at different sites throughout the body. As a consequence of both this apparent redundancy and ambiguity, a new chemokine nomenclature was introduced designating each chemokine with a name composed of its structurally conserved motif of cysteine residues and followed by a number that indicates its gene number (designated chronologically) (Table 1.1) [17]. For example, the CXC chemokine IL-8 was baptized CXCL8. Chemokine receptors had already received a similar nomenclature where the ‘L’ for ‘ligand’ is replaced by ‘R’, indicating ‘receptor’ e.g. CXCR1 for CXC chemokine receptor 1 [11].

There are three chemokines that do not belong to either the CC or CXC chemokine subfamily. Two chemokines, namely XCL1 and XCL2, have only one conserved N-terminal cysteine residu. CX\(_3\)CL1, formerly known as fractalkine, is the only chemokine that has three amino acids separating the first two conserved cysteine residues. CX\(_3\)CL1 is the only chemokine that exists as a membrane-anchored protein with a long intracellular tail and a mucin stalk on the outside of the plasma membrane [18,19]. Proteolytic cleavage at the base of the mucin-stalk of this membrane-anchored protein releases a soluble form.
Table 1.1  Human chemokines and their main receptors

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Functional name</th>
<th>ELR</th>
<th>Angiogenesis</th>
<th>Receptor</th>
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<tbody>
<tr>
<td>CXCL1</td>
<td>GRO-α/MGSA-α</td>
<td>+</td>
<td>+</td>
<td>CXCR2</td>
</tr>
<tr>
<td>CXCL2</td>
<td>GRO-β/MGSA-β/MIP-2α</td>
<td>+</td>
<td>+</td>
<td>CXCR2</td>
</tr>
<tr>
<td>CXCL3</td>
<td>GRO-γ/MGSA-γ/MIP-2β</td>
<td>+</td>
<td>+</td>
<td>CXCR2</td>
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<tr>
<td>CXCL4</td>
<td>PF-4</td>
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<td>-</td>
<td>unknown</td>
</tr>
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<td>CXCL4L1</td>
<td>PF-4α</td>
<td>-</td>
<td>-</td>
<td>unknown</td>
</tr>
<tr>
<td>CXCL5</td>
<td>ENA-78</td>
<td>+</td>
<td>+</td>
<td>CXCR2</td>
</tr>
<tr>
<td>CXCL6</td>
<td>GCP-2</td>
<td>+</td>
<td>+</td>
<td>CXCR1, CXCR2</td>
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<td>NAP-2/β-TG</td>
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<td>+</td>
<td>CXCR2</td>
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<td>IL-8</td>
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<td>MIG</td>
<td>-</td>
<td>-</td>
<td>CXCR3</td>
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<td>IP-10</td>
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<td>I-TAC</td>
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<td>-</td>
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<td>SDF-1</td>
<td>-</td>
<td>+</td>
<td>CXCR4</td>
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<td>BCA-1</td>
<td>-</td>
<td>-</td>
<td>CXCR5</td>
</tr>
<tr>
<td>CXCL14</td>
<td>BRAK</td>
<td>-</td>
<td>-</td>
<td>unknown</td>
</tr>
<tr>
<td>CXCL16</td>
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<td>-</td>
<td>+</td>
<td>CXCR6</td>
</tr>
<tr>
<td>XCL1</td>
<td>lymphotactin/SCM-1α</td>
<td></td>
<td></td>
<td>XCR1</td>
</tr>
<tr>
<td>XCL2</td>
<td>SCM-1β</td>
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<td></td>
<td>XCR1</td>
</tr>
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<td>CX3CL1</td>
<td>fractalkine</td>
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<td>+</td>
<td>CXCR1</td>
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<td>CCL1</td>
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<td>CCR8</td>
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<td>CCL2</td>
<td>MCP-1</td>
<td>+</td>
<td>+</td>
<td>CCR2</td>
</tr>
<tr>
<td>CCL3</td>
<td>MIP-1α/LD78α</td>
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<td>CCR1, CCR5</td>
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<tr>
<td>CCL3L1</td>
<td>LD78β</td>
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<td>CCL4</td>
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<td>CCL5</td>
<td>RANTES</td>
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<td>CCL7</td>
<td>MCP-3</td>
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<td>CCR1, CCR2, CCR3, CCR5</td>
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<td>CCL8</td>
<td>MCP-2</td>
<td>+</td>
<td>CCR1, CCR2, CCR3, CCR5</td>
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<tr>
<td>CCL11</td>
<td>eotaxin</td>
<td>+</td>
<td>CCR2, CCR3</td>
<td></td>
</tr>
<tr>
<td>CCL12</td>
<td>MCP-4</td>
<td>+</td>
<td>CCR1, CCR2, CCR3</td>
<td></td>
</tr>
<tr>
<td>CCL13</td>
<td>HCC-1</td>
<td>+</td>
<td>CCR1, CCR5</td>
<td></td>
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<tr>
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<td>HCC-2/leukotakin</td>
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<td>CCR1, CCR3</td>
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<tr>
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<td>HCC-4/LEC</td>
<td>+</td>
<td>CCR1</td>
<td></td>
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<td>TARC</td>
<td></td>
<td>CCR4</td>
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<td>CCL18</td>
<td>ELC/MIP-3β/exodus-3</td>
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<td>CCR7</td>
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<td>CCL19</td>
<td>LARC/MIP-3α/exodus-1</td>
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<td>CCR6</td>
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<td>CCL20</td>
<td>SLC/6Ckine/exodus-2</td>
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<td>CCR7</td>
<td></td>
</tr>
<tr>
<td>CCL21</td>
<td>MDC/STCP-1</td>
<td>+</td>
<td>CCR4</td>
<td></td>
</tr>
<tr>
<td>CCL22</td>
<td>MIPF-1</td>
<td>+</td>
<td>CCR1</td>
<td></td>
</tr>
<tr>
<td>CCL23</td>
<td>eotaxin-2/MIPF-2</td>
<td>+</td>
<td>CCR3</td>
<td></td>
</tr>
<tr>
<td>CCL24</td>
<td>TECK</td>
<td></td>
<td>CCR9</td>
<td></td>
</tr>
<tr>
<td>CCL25</td>
<td>eotaxin-3</td>
<td></td>
<td>CCR3, CCR10</td>
<td></td>
</tr>
<tr>
<td>CCL26</td>
<td>CTACK/ILC</td>
<td></td>
<td>CCR10</td>
<td></td>
</tr>
<tr>
<td>CCL27</td>
<td>MEC</td>
<td></td>
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<tr>
<td>CCL28</td>
<td>none</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

a) This table is a compilation of data derived from different recent reviews [11,12,17,20-29]
b) The nomenclature of chemokines is based on the structurally conserved motif of cysteine residues followed by a number that indicates the corresponding gene number of the ligand. Missing ligands are chemokines that have only been described in rodents but for which no human counterpart has been discovered yet.
c) Chemokines were originally denominated with functional names that indicated their functional activity and/or producer cell.
d) The presence (+) or absence (-) of an ELR-motif roughly divides the CXC chemokines into neutrophil and lymphocyte chemoattractants, respectively.
e) Angiogenic (+) and angiostatic (-) properties have been attributed to chemokines. The exact mechanism by which chemokines influence angiogenesis is, however, still unclear.
f) Chemokines exert their function by binding to specific seven-transmembrane G protein-coupled receptors.
To exert biological activity on their target cell, chemokines bind to specific G protein-coupled receptors with seven hydrophobic transmembrane domains (Table 1.2). Upon binding of the chemokine ligand, the receptor becomes activated and the complex of G proteins, associated on the inside of the membrane with the receptor, exchanges guanosine 5’-diphosphate (GDP) for guanosine 5’-triphosphate (GTP). In this activated conformation, the trimeric complex of G\(_{\alpha}\)-, G\(_{\beta}\)- and G\(_{\gamma}\)-subunit dissociates into an \(\alpha\)-subunit and a \(\beta\gamma\)-subunit that each stimulate specific downstream pathways leading to activation of the cell (oxidative burst, release of lysosomal enzymes, chemotaxis,…) [20,30].

Table 1.2  Cell-specific expression of chemokine receptors

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Receptor (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>neutrophils</td>
<td>CXCR1, CXCR2</td>
</tr>
<tr>
<td>eosinophils</td>
<td>CCR1, CCR3</td>
</tr>
<tr>
<td>basophils</td>
<td>CCR2, CCR3</td>
</tr>
<tr>
<td>monocytes/macrophages</td>
<td>CCR1, CCR2, CCR5, CX3CR1, CXCR4</td>
</tr>
<tr>
<td>immature dendritic cells</td>
<td>CCR1, CCR2, CCR5, CXCR4</td>
</tr>
<tr>
<td>mature dendritic cells</td>
<td>CCR7, CCR9, CXCR4</td>
</tr>
<tr>
<td>naïve T lymphocytes (CD4(^+) and CD8(^+))</td>
<td>CCR7, CXCR4</td>
</tr>
<tr>
<td>Th1 lymphocytes (CD8(^+))</td>
<td>CCR2, CCR5, CXCR3</td>
</tr>
<tr>
<td>Th2 lymphocytes (CD4(^+))</td>
<td>CCR4, CCR8</td>
</tr>
<tr>
<td>natural killer cells</td>
<td>CCR2, CCR5, CXCR3</td>
</tr>
<tr>
<td>B cells</td>
<td>CXCR4, CXCR5</td>
</tr>
</tbody>
</table>

\(^a\) The main functional chemokine receptors, expressed by a certain cell type, are represented based upon different reviews [20,31-36].

3. **The intestine**

The human body relies on a continuous exchange of nutrients and waste products to maintain its normal functions. This exchange takes place at different sites. Oxygen uptake occurs via the airway system. The gastrointestinal system on the other hand, fragmentizes the food and breaks it down into smaller molecules by the use of enzymes. These small components that serve as important energy sources are then absorbed by the small intestine and further transported by the blood and lymph circulation to supply all cells of the human body.
Figure 1.1  Schematic representation of the immune system in the small intestinal mucosa

The mucosa, the inner layer of the small intestine, comprises the lamina propria that is lined by a single layer of tightly connected columnar epithelial cells, facing the lumen, and by the muscularis mucosae facing the submucosa and muscularis propria. The total contact surface of the small intestine with the intestinal content is enlarged by folds in the mucosa: the villi and crypts. The epithelium of the mucosa contains various cell types. Nutrient-absorbing enterocytes and mucus-secreting goblet cells are present in the villi. Entero-endocrine cells, Paneth cells and pluripotent stem cells are located in the crypts of Lieberkühn. The lamina propria is composed of connective tissue but also contains several types of inflammatory cells including eosinophils, mast cells and macrophages. In addition, smooth muscle cells are present as well as blood and lymph vessels that take care of the transport of nutrients, delivered by the enterocytes. Lymphocytes are present in between epithelial cells (IEL) or scattered throughout the lamina propria (LPL). The majority of lymphocytes, however, is located in organized lymphoid structures such as Peyer's patches that consist of aggregates of lymphoid follicles. The follicle-associated epithelium (FAE) overlying the lymphoid structures of a Peyer's patch consists of cubical enterocytes and M cells that are specialized for sensing and uptake of antigens. M cells are specialized enterocytes that have microfolds at the apical site where endocytosis of the antigen occurs and an intraepithelial "pocket" at the basolateral site where it binds to the immunoglobulin receptor on lymphocytes that reside there.
The wall of the small intestine is composed of multiple layers and contains many different cell types (Figure 1.1). The inner layer or mucosa comprises the lamina propria that is lined by a single layer of tightly connected columnar epithelial cells, facing the lumen, and by the muscularis mucosae facing the submucosa and muscularis propria [37]. The total contact surface of the human intestine with the intestinal content is about 400 m² and is obtained by multiple folding of the mucosa by the formation of plicae circulares, villi and crypts. Plicae circulares are folds of mucosa and submucosa while villi and crypts are folds restricted to the mucosa.

The epithelium of the mucosa contains various cell types. Nutrient-absorbing enterocytes, mucus-secreting goblet cells and intraepithelial lymphocytes (IEL) are present in the villi, while entero-endocrine cells, Paneth cells and pluripotent stem cells are located in the crypts (Figure 1.1) [38,39]. On the apical site of the enterocyte, finger-like projections or microvilli enlarge its contact surface with the luminal content. Paneth cells secrete products (e.g. tumor necrosis factor-α, lysozyme, defensins) that protect the intestine from pathogenic microorganisms [39-41]. In the intestinal epithelium, there is a continuous process of cell renewal originating from pluripotent stem cells in the base of the crypts of Lieberkühn. As newly divided cells move upward, they differentiate into one of the different cell types of the epithelium: enterocytes, goblet cells or enteroendocrine cells. Cells that stay in the base of the crypt differentiate into Paneth cells.

The lamina propria is composed of connective tissue but also contains several types of inflammatory cells including eosinophils, lamina propria lymphocytes (LPL), mast cells and macrophages. In addition, smooth muscle cells are present as well as blood and lymph vessels that take care of the transport of nutrients, delivered by the enterocytes. The colon or large intestine displays an architecture similar to that of the small intestine but there are no villi.

The gastrointestinal tract is constantly challenged by food antigens and pathogens present in its lumen. The intestine is therefore in need of a good working innate and adaptive immune system that gives adequate tolerance or defence depending on the antigen it encounters [42,43]. The cell-mediated branch of the adaptive immune response relies on mucosal T lymphocytes whereas the humoral defence is conferred by B lymphocytes and plasma cells secreting IgA molecules [44]. By transcytosis
CHEMOKINES IN GASTROINTESTINAL DISORDERS

through intestinal epithelial cells, these secretory immunoglobulins reach the intestinal lumen where they prevent the attachment of bacteria to epithelial cells. As mentioned before, lymphocytes are present in between epithelial cells (IEL) or scattered throughout the lamina propria (LPL) (Figure 1.1). The majority of lymphocytes, however, is located in organized lymphoid structures (e.g. Peyer’s patches). Together with isolated lymphocytes (IEL and LPL), these lymphoid structures represent the gut-associated lymphoid tissue (GALT), the major immune surveillance mechanism of the gut [45]. Peyer’s patches are located in the mucosa (and submucosa) of the terminal ileum. They consist of aggregates of lymphoid follicles, what makes them macroscopically visible (Figure 1.1). The follicle-associated epithelium (FAE) overlying the lymphoid structures of a Peyer’s patch consists of cubical enterocytes and M cells that are specialized for sensing and uptake of antigens (vide infra). The dome region separates the lymphoid follicles of the Peyer’s patch from this overlying epithelium and contains B cells whereas the germinal center itself contains B cells, CD4$^+$ T cells and antigen-presenting cells. M cells are specialized enterocytes that have microfolds (hence the name M cells) at the apical site where endocytosis of the antigen occurs. After transcytosis, the antigen is released into the intraepithelial “pocket” at the basolateral site where it binds to the immunoglobulin receptor on B cells that reside there [45]. These B cells then interact with antigen-presenting cells, located in the dome region, that present the processed antigen to follicular dendritic cells and CD4$^+$ T cells to initiate an immune reaction [46]. In addition, antigens can be sensed directly by dendritic cells that project their cytoplasmic processes across epithelial tight junctions into the intestinal lumen [47].

4. Chemokines in intestinal homeostasis

The adaptive immune response is pivotal when an adequate immune response is needed. Indeed, to minimize the negative effect of foreign agents, lymphocytes patrol the human body in search of noxious components. As such, lymphocytes constantly migrate from the bloodstream into the secondary lymphoid tissues and then back to the circulation through the efferent lymph [48]. Three chemokines have been
described to be selectively expressed in the intestine and to act as primary mediators in tissue-specific lymphocyte recirculation: CCL20, CCL25 and CCL28 [49].

 Constitutive expression of CCL20 is observed in the epithelial cells of the appendix and the colon, whereas its receptor, CCR6, is expressed on mucosal T cells and other mononuclear cells of the colon [50]. CCL25 is expressed in the small intestine by the epithelium of crypts and villi, but also by the FAE of Peyer’s patches, as well as on the luminal surface of the vascular endothelium [51]. CCL25 is, however, virtually absent in other segments of the gastrointestinal tract, such as the colon [48]. Its receptor, CCR9, is present on lymphocytes of the small intestine, namely IEL, CD4+ LPL, CD8+ LPL, plasma cells in the lamina propria and T cells activated in the mesenteric lymph nodes [51]. As such, CCL25 can mediate the entry of lymphocytes into the small intestine where these cells are essential in the defence against enteric pathogens [51]. In contrast with CCL25, CCL28 is expressed by epithelium in the colon but almost not in the small intestine [12,48]. This chemokine binds to CCR10 on memory T cells and to CCR3 on eosinophils [12]. In addition to these three chemokines that mediate specific recruitment of lymphocytes into the gastrointestinal tract, CX3CL1, produced by intestinal epithelial cells, seems an important mediator for the uptake of luminal bacteria by processes of dendritic cells [52].

5. Gastrointestinal disorders

The close contact of luminal agents with the intestinal wall induces innate (non-specific) and adaptive (specific) immune responses. Normally, these defence mechanisms are tightly regulated and abrogated when the antigen is cleared from the intestinal tract. Chronic, idiopathic inflammatory bowel diseases (IBD), however, are characterized by an uncontrolled immune response, resulting in chronic inflammation of the intestine [53,54]. Crohn’s disease (CD) and ulcerative colitis (UC), the two major types of idiopathic inflammatory bowel diseases, are characterized by alternating episodes of activity and remission [55,56]. The natural evolution of UC shows cycles of epithelial destruction and subsequent repair. The inflammatory reaction is mainly limited to the mucosa and diffuse in distribution (i.e. without
healthy intermediate zones). In CD, inflammation is transmural, involving all layers of
the intestinal wall, and discontinuous, leaving some areas unaffected. Macroscopic
lesions tend to be more constant and progressive in CD. UC affects the colon and
extends proximally, whereas CD can affect any part of the gastrointestinal tract
[54,57,58].

There is no absolute differentiating marker for IBD. Therefore, a combination of
clinical, radiological, endoscopic and histological criteria is used for diagnosis [59].
Clinical symptoms of UC and CD are very similar. For UC, they include bloody
diarrhoea, urgency and rectal tenesmus, abdominal pain, decreased appetite,
dernutation and weight loss. The severity of symptoms parallels the extent and the
intensity of the inflammation [60]. Clinical symptoms of CD include non-bloody or
bloody diarrhoea, abdominal pain, cramping, fever, malabsorption by the loss of
effective digestive and absorptive surface, vitamin B_{12}-deficiency, weight loss and
malaise and depend on the location and the severity of the disease [61]. In ileocecal
disease, symptoms can mimic acute appendicitis. In addition, fistulous penetration that
can cause pyuria, fecaluria and pneumaturia, intestinal obstruction and perforation are
typical for CD. In children, growth retardation in the absence of explicit
gastrointestinal symptoms can be the initial clinical manifestation of CD [61]. In
addition to the intestinal problems that characterize IBD, extra-intestinal
manifestations of the joints, skin and eyes are common side effects.

Microscopic examination of tissue samples represents a key event in the diagnosis,
as well as for the differential diagnosis between UC and CD. In addition, it is a
technique for scoring the activity of disease, the influence of medication and the early
detection of neoplasm formation. Granulomas are useful for the differentiation
between UC (granuloma negative) and CD [59]. These are lesions that are composed
of lymphocytes, epithelioid cells (these are cells of monocytic lineage with a large
amount of cytoplasm that look like epithelial cells) and giant cells. Normally, the
presence of lymphocytes, plasma cells and monocytes/macrophages in tissue is
indicative for inflammation. In the intestinal mucosa, however, these cells are
constantly present. A change in the number, distribution or composition of the
mucosal cellular infiltrate marks inflammation both for IBD and other types of colitis
[59]. As such, the infiltration of neutrophils in the lamina propria and epithelium is
used as a parameter for active disease. Neutrophils present in the crypt epithelium and lumen (crypt abscess) or solely in the crypt epithelium (cryptitis) are the main source of toxic molecules (oxygen and nitrogen metabolites) and can cause tissue destruction and amplification of inflammation [59,62]. Data suggest that a Th₁-mediated immune response predominates in CD while in UC inflammation, the cytokine response is less clear [63]. In addition, IBD patients have a variety of circulating auto-antibodies although none of these has been shown to cause gut inflammation and seldom a correlation with disease activity is found [56].

IBD is characteristic for developed countries and has a prevalence of about 1 in 1,000 individuals. The disease mostly strikes young adults (between the age of 20 and 30 years) and has a serious impact on the social life and mental well-being of these persons. The disease affects genetically susceptible persons and is thought to result from a defect in the regulatory immune response (i.e. the capacity to induce suppressor T cells) against the resident microbial flora [64]. Indications for a pivotal role of the resident intestinal flora came from experiments with animal models that showed that genetically engineered animals grown in germ-free conditions did not develop IBD. Exposure to normal enteric bacteria, however, rapidly induced development of the disease [65,66]. The fact that IBD mostly occurs in developed countries might indicate that hygienic conditions could make persons more susceptible for developing the disease. This is, however, not confirmed unequivocally. Therefore, other possibilities related to western life must and have been considered, including infections (*Mycobacterium paratuberculosis*, measles virus,...), allergy, microparticles (in toothpaste, for example) and dysbiosis (imbalance between harmful and protective bacteria) [67,68]. Additional experimental data indicate the importance of other environmental factors such as diet and smoking [63]. There is, however, an inverse association between UC and CD for smoking. Smoking would be beneficial in the protection against UC, whereas it has an adverse effect on the progression of CD where it influences disease activity and is a risk factor for recurrence after surgery [63]. There is, however, no explanation for the difference between UC and CD. The beneficial effect of diets in reducing symptoms is not universally accepted although it is possible that diets may affect intestinal permeability [56]. In CD, intestinal permeability might be of importance as it is increased in first-degree relatives. A
possible explanation could be that increased intestinal permeability facilitates antigen absorption [56]. The precise etiology of IBD remains, however, unknown.

Indications for genetic contributions in IBD came from the high familial occurrence of both diseases and the high rate of concordance between monozygotic twins in CD (but lower rate of concordance in UC). Although many susceptibility genes already have been described, enormous heterogeneity exists depending on the study population. An exception is the recently discovered Nod2/CARD15 gene [69,70]. Increased susceptibility to CD is contributed to a defect in this Nod2/CARD15 gene encoding an intracellular receptor for bacterial peptidoglycan [71]. Moreover, a 40-fold increased risk to develop CD was observed in patients homozygous for the mutated Nod2/CARD15 gene. The precise mechanism by which this mutation confers susceptibility to CD is, however, still unclear [71-73]. Taken together, IBD are complex diseases and the absence of a simple Mendelian inheritance pattern indicates that it is impossible to explain IBD by a single cause/ effect relation.

6. Chemokines in IBD

To defend against pathogens or in response to dietary antigens present in the bowel, inflammatory cells constantly migrate into and out of the lamina propria. The low-level movement of immune cells is called ‘physiological inflammation’. This basal defence mechanism can turn into ‘pathological inflammation’ when the immune response persists and increased levels of immune cells enter the intestinal mucosa [74].

Chemokines are believed to be key mediators in this process of aberrant leukocyte recruitment out of the blood stream into the intestinal tissue. Inflammatory bowel diseases are characterized by a massive infiltrate of immune cells in the intestine, which causes tissue damage by the release of inflammatory mediators including proteolytic enzymes.
To better understand this process, many researchers have investigated in detail the \textit{in vitro} induction of chemokine release by microbial products and endogenous cytokines. In addition, much research focused on analyzing the expression of chemokines in human IBD tissue as well as in tissue from rodent models of IBD (Table 1.3 and Table 1.4) \cite{75-78}. \textit{Ex vivo} detection of chemokine expression in healthy as well as in inflamed intestine helps to decipher the underlying mechanisms of cellular infiltration during the intestinal immune response in IBD.

<table>
<thead>
<tr>
<th>TABLE 1.3</th>
<th>Cell types expressing chemokine protein in involved intestinal tissue of patients with inflammatory bowel disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemokine</td>
<td>Expression in IBD intestine</td>
</tr>
<tr>
<td>CXCL2</td>
<td>epithelial cells</td>
</tr>
<tr>
<td>CXCL5</td>
<td>epithelial cells, mainly of crypts</td>
</tr>
<tr>
<td>CXCL8</td>
<td>CD68$^+$ macrophages, neutrophils, CD3$^+$ T lymphocytes, UC: mononuclear cells in lamina propria and occasionally endothelial cells</td>
</tr>
<tr>
<td>CXCL9</td>
<td>eosinophils</td>
</tr>
<tr>
<td>CXCL10</td>
<td>UC: mononuclear cells in lymphocyte-rich areas of the lamina propria</td>
</tr>
<tr>
<td>CXCL13</td>
<td>associated with follicular dendritic cells</td>
</tr>
<tr>
<td>CCL2</td>
<td>cells in the lamina propria: mononuclear cells resembling lymphocytes and macrophages, spindle cells resembling smooth muscle cells and fibroblasts, endothelial cells lining small venules, surface epithelium? \textsuperscript{c)}</td>
</tr>
<tr>
<td>CCL3</td>
<td>CD68$^+$ macrophages, CD3$^+$ T lymphocytes and endothelial cells below ulcerated epithelium</td>
</tr>
<tr>
<td>CCL5</td>
<td>T lymphocytes and endothelial cells at tissue locations enriched with eosinophils</td>
</tr>
<tr>
<td>CCL7</td>
<td>epithelial cells of surface and crypt epithelium? \textsuperscript{c)}</td>
</tr>
<tr>
<td>CCL11</td>
<td>mononuclear inflammatory cells, eosinophils, fibroblasts and endothelial cells in tissue showing eosinophilia</td>
</tr>
<tr>
<td>CCL20</td>
<td>epithelial cells, mainly of crypts</td>
</tr>
</tbody>
</table>

\textsuperscript{a)} In some studies, a chemokine was only investigated in patients with ulcerative colitis (UC) or Crohn’s disease (CD).

\textsuperscript{b)} The expression of CX$_3$CL1 by endothelial cells and dendritic cells is controversial and could be due to the use of a non-specific antibody \cite{118,119}.

\textsuperscript{c)} The expression of CCL2 and CCL7 by epithelial cells is controversial.
Table 1.4  Chemokine expression levels in inflamed intestinal tissue of patients with inflammatory bowel disease a)

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Specimen b)</th>
<th>Technique c)</th>
<th>Crohn’s disease d)</th>
<th>Reference</th>
<th>Ulcerative colitis d)</th>
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<td>[80,81]</td>
</tr>
<tr>
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<td>ELISA</td>
<td>+</td>
<td></td>
<td></td>
<td>[81]</td>
</tr>
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<td>CXCL2</td>
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<td>[86]</td>
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Table 1.4 Continued

<table>
<thead>
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<th>Chemokine</th>
<th>Specimen b)</th>
<th>Technique c)</th>
<th>Crohn’s disease d)</th>
<th>Reference</th>
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<td>homogenate</td>
<td>ELISA +</td>
<td>[113] =</td>
<td>[113]</td>
<td>[80,113,114] =</td>
<td>[114]</td>
</tr>
<tr>
<td></td>
<td>organ culture</td>
<td>ELISA +</td>
<td>[114] +</td>
<td>[80,114]</td>
<td>[114] +</td>
<td>[114]</td>
</tr>
<tr>
<td></td>
<td>tissue section</td>
<td>IHC +</td>
<td>[114] +</td>
<td>[114]</td>
<td>[114] +</td>
<td>[114]</td>
</tr>
<tr>
<td>CCL22</td>
<td>homogenate</td>
<td>RT-PCR +</td>
<td>[114] +</td>
<td>[82,90]</td>
<td>[81,85,91-95] +</td>
<td>[82,90]</td>
</tr>
<tr>
<td>CCL24</td>
<td>serum</td>
<td>ELISA =</td>
<td>[110] =</td>
<td>[110]</td>
<td>[81,85,91-95] =</td>
<td>[81,85,91-95]</td>
</tr>
</tbody>
</table>

a) This table summarizes the literature on the expression level of chemokines in inflamed intestinal tissue specimens of inflammatory bowel disease patients as compared with healthy intestinal tissue specimens.
b) Specimens for the measurement of chemokine expression were obtained from inflamed intestinal tissue or from peripheral blood.
c) ELISA = enzyme-linked immunosorbent assay, IFS = immunofluorescence staining, IHC = immunohistochemistry, ISH = in situ hybridization, RIA = radio-immuno assay, RT-PCR = real time-polymerase chain reaction
d) Data indicate a higher (+) or unchanged (=) expression level of chemokines observed in inflamed intestinal tissue specimens of patients with Crohn’s disease and ulcerative colitis, respectively. ND indicates that the chemokine was not detectable. Where no reference is mentioned, there are no literature data available.

6.1 CXC chemokines in IBD

6.1.1 ELR⁺CXC chemokines

In 1987, ten years after its discovery, elevated levels of CXCL4 were detected in the plasma of patients with CD (± 30-fold augmentation) in comparison with healthy persons (5.6 ± 4.8 ng/ml) by means of a radioimmunoassay. No correlation with disease activity, site of inflammation or treatment was found [86]. In 1992, CXCL8, a potent granulocyte chemoattractant and intensively studied CXC chemokine in IBD, was detected by ELISA or RT-PCR in intestinal tissue homogenates from IBD patients. Tissue homogenates of patients with active UC showed significantly higher expression levels of CXCL8 (mRNA and protein) than those of patients with active CD or control patients [82,90]. Later, most other groups indicated similarly elevated CXCL8 mRNA and protein levels in patients with active UC and CD compared with inactive IBD patients or control subjects [81,85,91-95]. A possible explanation for the discrepancy in these observations lies in the fact that it is not possible to distinguish between the discontinuous inflammatory nature of CD and the diffuse nature of UC with tissue homogenates. In addition, homogenates of mucosal biopsies probably give a better reflection of the disease pattern of UC than of CD since UC only involves the
mucosa whereas CD is transmural. Using tissue homogenates to make statements about differences in chemokine expression and a correlation between chemokine expression and local disease activity therefore remains challenging. By in situ hybridization and immunohistochemical staining of intestinal tissue sections, it was shown that chemokine expression (mRNA and protein) is very often related to the histological grade of intestinal inflammation. The expression of CXCL8 was linked to active lesions and positive cells were evenly distributed in the mucosa of UC patients whereas a more focal pattern was seen in patients with CD [96,100]. CXCL8^+ cells, both in UC and CD, were located in the lamina propria and were predominantly macrophages and infiltrating neutrophils, adjacent to ulcerations and fistulae or present in crypt abscesses, and occasionally endothelial cells [96,98,100]. The co-expression of CD14, a monocyte marker, confirmed that the phenotypically CD68^+ and morphologically macrophage-like cells were recently recruited from the circulation into the inflamed intestine [96]. In tissue sections of IBD-inflamed mucosa without epithelial defects, expression of CXCL8 was negligible [96].

Freshly isolated intestinal epithelial cells from involved tissue (of IBD patients as well as of inflammatory controls) showed a marked upregulation in the expression of CXCL8 mRNA compared to cells isolated from non-involved intestinal tissue [95]. Therefore, Izutani et al. proposed that (especially in UC) these cells might be of importance for the release of CXCL8 and the subsequent recruitment of neutrophils into the intestine. Mazzucchelli et al. confirmed low-level expression of CXCL8 mRNA in intestinal epithelial cells of tissue sections from IBD patients and inflammatory controls (acute appendicitis) by in situ hybridization [100]. There are, however, conflicting reports on the expression of CXCL8 by epithelial cells. Indeed, others could not observe any CXCL8 mRNA or protein expression by intestinal epithelial cells, nor in inflamed mucosa (with or without intact epithelium), nor in healthy colon [92,96]. The finding that epithelial cells would express CXCL8 might otherwise be an artefact due to contamination of the investigated epithelial cell preparations with other cell types. Izutani and colleagues stated, however, that their preparations had a purity of 98-99% and that only occasional lymphocytes were present, but no macrophages [95].
To evaluate whether chemokine concentrations in blood samples could be useful to distinguish between UC and CD, CXCL8 was tested in plasma and serum samples from IBD patients but no measurable concentrations of CXCL8 were detected (<7 pg/ml) [79,89-91]. Polymorphonuclear cells, purified from peripheral blood of patients with CD, were more responsive to CXCL8 in chemotaxis assays than cells from healthy subjects indicating that in CD these cells are in a continuous state of activation [79]. In contrast to CXCL8 (vide supra), CXCL1 was detectable in plasma of control subjects (±100 pg/ml) and was significantly upregulated (±230 pg/ml) in CD patients (but there was no correlation with disease severity) [79]. Homogenates of inflamed colonic mucosa derived from IBD patients contained significantly higher concentrations of CXCL1, CXCL2 and CXCL3 mRNA in comparison with homogenates of non-involved tissue from IBD patients or healthy tissue from control subjects [80,81,83,85]. Isaacs et al. detected a marked elevation of CXCL2 mRNA in tissue samples of active UC and inflammatory controls compared to inactive UC or non-inflammatory controls [82]. However, the results they obtained in tissue samples from patients with CD were less uniform and they could not observe a significant difference between inactive and active CD [82]. Homogenates of affected UC colon mucosa expressed significantly higher levels of CXCL5 mRNA and protein than unaffected biopsy samples [81,87]. Also in tissue homogenates of active CD enhanced levels of CXCL5 (mRNA and protein) were observed although they were not significantly higher [87]. In contrast, even in inactive CD tissue homogenates significantly higher levels of CXCL5 mRNA were measured by Autschbach and colleagues [84]. Positivity for CXCL5 (mRNA and protein) in inflamed intestinal tissue sections of IBD patients was predominantly situated in crypt epithelial cells and inflammatory cells in the lamina propria and was significantly elevated compared with controls where staining was virtually absent [87,88]. In contrast to CXCL8 mRNA, the expression of CXCL5 mRNA was observed to be more predominant in tissue with mild and moderate disease activity than in tissue with severe disease activity [88,99]. It seems thus that CXCL5 and CXCL8, although both neutrophil chemoattractants, are differentially expressed in IBD. Indeed, the in vitro expression of CXCL5 by intestinal epithelial cell lines (Caco-2 and T-84) was delayed and prolonged compared with that of CXCL8 [87]. CXCL5 was released 8-24 h after stimulation of these cells with
IL-1β or tumor necrosis factor-α (TNF-α) whereas CXCL8 release occurred 4-12 h after stimulation [87]. This strengthens the idea that these chemokines can act complementary for the recruitment of leukocytes into inflamed tissue [87]. Comparison of CXCL6 and CXCL8 showed that both chemokines are expressed by leukocytes in the lamina propria of intestinal tissue sections of IBD patients and that this expression correlated with disease activity [89]. In addition, specific expression of CXCL6 in IBD tissue sections was attributed to a subset of endothelial cells at places of epithelial damage, e.g. in an ulcer base. Expression of CXCL8 by endothelial cells was, however, not detected [89]. These data indicate that CXCL6 might have a specific role at places where angiogenesis is essential for the new-formation of tissue and that the expression of CXCL6 by endothelial cells might be limited to newly formed blood vessels. A distinct role for CXCL6 and CXCL8 in IBD was further indicated by the use of mononuclear cells that were isolated from peripheral blood [89]. When these mononuclear cells were stimulated in vitro with a distinct set of inducers (bacterial, viral and plant products) CXCL8 production was significantly lower in CD patients compared with UC patients or healthy subjects. CXCL6 production by stimulated mononuclear cells remained below the detection limit in healthy controls as well as in IBD patients (< 0.2 ng/ml) [89]. The fact that this lowering in chemokine production was specific for CD and CXCL8, but independent of the inducers used, might strengthen the idea of a more important role of genetic predisposition for CD compared with UC.

Taken together, the local expression of different ELR+CXC chemokines seems to represent a key event in the destruction of intestinal tissue by the recruitment of neutrophils, loaded with proteases and toxic molecules, from the blood circulation.

6.1.2 ELR+CXC chemokines

In similarity with ELR+CXC chemokines, ELR+CXC chemokines were found to be upregulated in inflamed intestinal tissue. By immunohistochemical staining of colonic tissue sections of UC patients, CXCL10 was observed in mononuclear cells of the lamina propria [98]. Its expression level was significantly upregulated in comparison with control tissue where there were barely positive cells [98]. Similar results were obtained for CXCL10 mRNA in involved tissue from CD and UC patients with in situ
hybridization [102]. Expression of CXCL10 mRNA was observed in CD3+ T lymphocytes, endothelial cells and CD68+ macrophages in a perivascular distribution [102]. CXCL10 expression was not observed in human epithelial cells of healthy or IBD-involved colon by in situ hybridization or immunohistochemistry [98,102]. In normal colon of mice, however, CXCL10 was described to be constitutively expressed by epithelial cells in the crypt base, the zone from which the proliferation of intestinal epithelial cells is regulated [120]. In a mouse model of colitis, neutralization of CXCL10 protected the mice from epithelial ulceration, whereas recombinant CXCL10 inhibited epithelial cell proliferation [120]. This finding indicates that, at least in mice, CXCL10 is an important mediator of crypt cell proliferation, a process that becomes detrimental in ulcerations. mRNA for the CXCR3 ligands CXCL9, CXCL10 and CXCL11 was significantly upregulated in inflamed colonic tissue homogenates of IBD patients and other inflammatory disease patients in comparison with healthy tissue from control subjects [81,84,85]. In CD lesions, CXCL9 protein was expressed by eosinophils [116]. The B cell chemoattractant CXCL13, binding to CXCR5, was immunohistochemically visualized in relation to peripheral dendritic cells of lymphoid follicles in Peyer’s patches and normal colon [117]. CXCL13 expression was associated with follicular dendritic cells but also with extracellular fibrils in GALT structures. In UC lesions, CXCL13 was also expressed in lymphoid aggregates [117]. As such, the expression of ELR CXC chemokines is of importance in mediating the recruitment of CXCR3+ T lymphocytes and CXCR5+ B lymphocytes into the intestinal tissue.

6.2 CC chemokines in IBD

Data on the expression of CCL2 (mRNA and protein) in normal colon are not really consistent. Some groups barely detected CCL2+ cells [98,105], while one group stained surface epithelium and less crypt epithelium and cells of the lamina propria [104]. Another group observed CCL2+ staining of endothelial cells, smooth muscle cells and intravascular mononuclear cells in the lamina propria of normal colon [106]. In patients with IBD, CCL2 expression (mRNA and protein) was mainly observed at places of epithelial damage and correlated with disease activity [97,98,104-106].
Positive cells, present in the lamina propria and the submucosa, were CD14$^+$ CD68$^+$ macrophages (mainly in a perivascular distribution), as well as smooth muscle cells and endothelial cells [104-106]. Although an initial publication, using immunohistochemical staining, showed CCL2 expression by surface epithelium, other groups were unable to confirm any staining of epithelial cells for CCL2 (mRNA or protein) [98,104-106]. In line with the results obtained with tissue sections, an increase in CCL2 mRNA and protein expression was observed in homogenates of inflamed colon biopsies of UC and CD patients in comparison with uninflamed tissue or homogenates of control biopsies of normal colon [81,83,85,93].

In situ hybridization showed CCL3 and CCL4 mRNA expression by CD68$^+$ macrophages, CD3$^+$ T lymphocytes and endothelial cells in actively inflamed colon tissue from IBD patients but only minimal expression in normal or non-inflamed tissue [102]. The expression of CCL3 and CCL4 mRNA correlated with the grade of activity and was mainly observed in the submucosa and the muscularis propria below ulcerated epithelium as well as in granulomas of CD patients [102]. The expression of CCL3 protein as observed with immunohistochemistry is controversial in that one group saw a significant upregulation in IBD tissue versus controls although another group could not observe positive staining for CCL3 protein [98,102]. Tissue homogenates of active IBD colon showed an increase of CCL3 and CCL4 mRNA compared to homogenates of healthy colon [81,84].

In normal colon, in situ hybridization showed occasional CCL5 mRNA expression in IEL and CD3$^+$ T lymphocytes in the lamina propria [102,106,108]. Constitutive expression of CCL5 mRNA and protein was confirmed with RT-PCR and ELISA on tissue homogenates of normal colon [81,93]. In inflamed colon of IBD patients, Mazzucchelli et al. predominantly observed CCL5-positive staining in mucosa with signs of chronic inflammation but rarely in areas with florid inflammation [106]. By contrast, according to Grimm et al., the expression of CCL5 mRNA correlated with the grade of inflammation and, besides CD3$^+$ T lymphocytes, positivity was also observed in perivascular CD68$^+$ macrophages and endothelial cells below ulcerated epithelium [102]. Although the positive staining of endothelial cells for CCL5 mRNA was not confirmed by Berrebi et al., immunohistochemistry for CCL5 protein also stained endothelial cells as well as T lymphocytes at places with increased eosinophil
numbers [107,108]. A significantly increased level of CCL5 expression (mRNA and protein) in inflamed tissue of IBD patients versus controls was confirmed with RT-PCR and ELISA on colon homogenates [81,93]. CCL5 attracts CD4+/CD45RO+ ‘memory’ T lymphocytes releasing soluble mediators that activate other cell types such as monocyte/macrophages, mast cells, basophils and eosinophils. The recruitment of peripheral CD4+ T cells may therefore upregulate mucosal immune responses and intensify chronic inflammation.

Some groups observed CCL7 expression by epithelial cells (especially of surface epithelium) in healthy and inflamed intestinal tissue [97,109]. In healthy tissue, this staining by immunohistochemistry was homogeneous, whereas in tissue sections of active IBD, staining correlated with the extent of epithelial destruction [109]. Another group was not able to confirm the positive staining of epithelial cells and observed CCL7 positivity in inflammatory cells infiltrated into the lamina propria [98]. CCL8 was reported in one study as being expressed by both epithelial cells and inflammatory cells [97]. The positive staining by inflammatory cells was significantly more pronounced in tissue of IBD patients than in control tissue samples and correlated with disease activity [97]. Immunohistochemical staining of CD and UC tissue showing eosinophilia demonstrated CCL11+ mononuclear inflammatory cells, fibroblasts and endothelial cells, whereas CCL11 expression was virtually absent in normal mucosal tissue [107]. Whether the expression of this eosinophil chemoattractant is significantly increased in serum of IBD patients versus controls or whether CCL11 is undetectable remains unclear [89,111]. By RT-PCR, increased levels of CCL17 and CCL19 mRNA were measured in homogenates of inflamed IBD tissue compared with healthy colon tissue [84,112]. Finally, CCL20 mRNA was upregulated in colon samples from active UC and CD compared to uninflamed IBD colon tissue or healthy colon homogenates and the expression level correlated with inflammation [80,113,114]. Immunohistochemistry of control tissue showed barely CCL20+ cells according to Kaser et al., whereas Kwon et al. stained crypt and surface epithelium as well as cells of the lamina propria [113,114]. Staining of intestinal tissue sections of IBD tissue showed CCL20 protein in the follicle-associated epithelium (FAE) covering lymphoid follicles as well as in crypt epithelial cells and some cells of the lamina propria [113,114]. Its receptor, CCR6, was expressed by CD45RO+ T lymphocytes in
associated lymphoid follicles and the subepithelial region [114]. Thus, the elevated expression of CCL20 might play a crucial role in the recruitment of these memory T cells into the inflamed intestine.

6.3 Other chemokines in IBD

Chemokines from all subgroups have been described in IBD. The intestinal expression of fractalkine/CX3CL1, the only chemokine with a CX3C-motif, by epithelial cells was markedly elevated in active CD [118,119]. The most predominant expression of CX3CL1 was observed by epithelial cells of the lamina propria where the chemokine attracts CX3CR1+ T lymphocytes into the lamina propria of the gut [119]. This expression was markedly upregulated in active IBD. The reported expression of CX3CL1 by endothelial cells and immature dendritic cells is controversial and was probably due to the use of a non-specific antibody that crossreacted with CD84 [118,119]. In inflamed intestinal tissue of CD patients, activated T cells expressed significantly higher levels of lymphotactin/XCL1 – a chemokine bearing only one conserved N-terminal cysteine residue – than in control tissue [103]. Mast cells and dendritic cells also expressed XCL1 but to a lesser extent compared to T cells. Dexamethasone and cyclosporine A, both used in IBD therapy, reduced the expression of XCL1 by T cells stimulated in vitro with phorbol myristate acetate or concanavalin A [103]. Although Yang and colleagues observed constitutive expression of XCL1 mRNA in normal colonic mucosa homogenates, they did not observe a significantly elevated expression in homogenates of active UC colon [81].

All together, these data on chemokine expression in IBD indicate that many different chemokines are upregulated in inflamed tissue of IBD patients, explaining the massive infiltration of immune cells in the intestine. It is likely that by preventing this upregulation in chemokine expression with anti-cytokine therapy (e.g. anti-TNFα), we can prevent the exaggerated infiltrate of immune cells and the accompanying tissue damage [121]. Since data on chemokine expression in IBD have rather been descriptive until now, further research is needed to evaluate the possible benefit of inhibiting these small, but very potent proteins to prevent chronic inflammation.
7. Chemokines and cancer

A tumor or neoplasm is a clone of cells showing uncontrolled proliferation as a result of multiple genetic alterations. Most of the time, genetic alterations have no perceptible effect but DNA mutations are tumorigenic when they cause defects in signals that control cell proliferation, differentiation and programmed cell death (apoptosis). Damage to the DNA can be introduced spontaneously during replication of the DNA (germline or somatic mutations) or can be caused by different types of carcinogens (chemical agents, radiation, viruses,…). Normally, defects in the DNA can be corrected by the cell, but sometimes tumors arise from errors introduced in genes that are responsible for DNA repair. Genes prone to transform normal cells into tumor cells include proto-oncogenes and tumor suppressor genes that stimulate and inhibit growth, respectively. Genomic instability due to impairment in DNA repair or dominant negative mutations leads to activation of proto-oncogenes into oncogenes and/or inactivation of tumor suppressor genes. After malignant transformation, cells have gained new properties (reduced requirement of serum or growth factors, loss of anchorage dependency, growth independent of cell density, immortality) that make these cells capable to generate tumors. A malignant tumor or cancer differs from a benign tumor in that it has an indefinite growth and invades the neighbouring tissue. In addition, these cancer cells can penetrate the blood or lymph circulation and form cell deposits or metastases in other tissues [122].

7.1 The leukocyte infiltrate in solid tumors

Tumors are not just a compilation of tumor cells but are composed of non-malignant cells, so-called ‘stromal cells’, as well. These stromal cells are mainly macrophages, lymphocytes, endothelial cells and fibroblasts [123]. Local chemokine production by tumor cells and stromal cells determines the composition of the leukocyte infiltrate present in solid tumors [124]. Leukocytes may contribute to the progression of a tumor by releasing matrix metalloproteinases and growth factors, as well as mediators of angiogenesis (vide infra) [125,126]. It is generally accepted that inflammation is important for tumor progression [127]. The exact role of the leukocyte
infiltrate is, however, still debated since it is possible that tumor-associated macrophages (TAM) are rather beneficial by inhibition of tumor growth and destruction of tumor cells [128]. On the other hand, tumor-derived chemokines are able to attract neutrophils loaded with matrix-degrading enzymes and can hence favor tumor cell migration towards the blood circulation to form distant metastases [129-131]. Alternatively, as reviewed by Van Damme et al., processing of chemokines by proteases can also affect tumor development [132]. Indeed, it is well established that proteolytic processing of chemokines by matrix metalloproteinases can generate chemokine isoforms with enhanced or reduced activity, depending on the chemokine itself and the level of truncation [133].

Chemokine expression has been extensively studied in cancer and different chemokines seem to have their own expression pattern in different tumors. As an example, elevated CCL18 levels were observed in serum of children with acute lymphoblastic leukemia and significantly higher levels of CXCL8 and CCL18 were detected in ascitic fluid of patients with ovarian carcinoma [134,135]. While many studies have been devoted to the investigation of specific chemokine production by tumors [136-139], knowledge about chemokine receptor expression is rather recent [140]. It is, however, also of major importance to get a clear picture of the expression of chemokine receptors on tumor cells since there is increasing evidence that chemokine production at distant sites might explain organ-specific metastasis of tumor cells [140]. The inhibition of the interaction between chemokines and their receptors might thus be important to prevent metastasis. On the other hand, it could be possible that local chemokine production in primary tumors can otherwise retain the tumor cells, expressing chemokine receptors, in the primary tumor and prevent invasion and metastasis. There is also increasing evidence that certain chemokines have therapeutic potential in anti-tumor therapy since chemokine expression can be important to attract immunocompetent cells to the tumor to combat cancer [35,141]. For example, the expression of CCL7 by transduced HeLa tumor cells led to secretion of high levels of CCL7 and to significant retardation of tumor growth in recipient mice, as compared with HeLa cells that were either buffer-treated or infected with a CCL7-free vector [142,143].
7.2 Chemokines and angiogenesis

To survive and grow, a tumor is dependent on nutrients and oxygen. In addition, the blood and lymph circulation function as a kind of “highway” for tumor cells to reach distant organs and to metastasize [125,144]. Therefore, angiogenesis, the formation of new blood vessels is essential [145,146]. This process consists of a sequence of events: first, endothelial cells become activated and start degrading the surrounding basement membrane. Endothelial cells then invade the surrounding stroma and proliferate. After migration and proliferation, endothelial cells tightly adhere to each other to form a new vessel lumen [147]. Angiogenesis is the result of an ‘angiogenic switch’ where the balance between angiogenic and angiostatic mediators shifts in favor of formation of new blood vessels [148]. Hypoxia, the limitation of oxygen, is a key regulatory factor because it induces the expression of multiple factors that favor angiogenesis, such as vascular endothelial growth factor (VEGF) and certain chemokines and their receptors [149,150]. The release of chemokines, for example by endothelial cells, at places of inflammation may be another factor inducing angiogenesis [89,131].

It is well established that chemokines are important mediators of angiogenesis [151,152]. The presence or absence of the ELR-sequence just in front of the CXC-motif roughly divides the CXC chemokines into angiogenic and angiostatic mediators, respectively [13,152]. Although the ELR-motif seemed to determine the angiogenic property of a CXC chemokine, angiogenic properties have also been ascribed to the ELR-CXC chemokine CXCL12 and to some CC chemokines as well (Table 1.1) [151,153]. The exact mechanism by which chemokines exert their effect on the angiogenesis process is, however, still unknown.

8. Chemokines and gastrointestinal tumors

Gastrointestinal malignancies are common tumors that predominantly affect the colorectum. Histologically, they are a heterogeneous group of neoplasms [154]. Epithelial malignancies, mainly adenocarcinomas, are the most common type. The prognosis of these malignancies is variable but on the average poor, except when the
lesion is detected at an early stage. Since the etiology of gastrointestinal cancer remains largely unknown, treatment of early lesions is extremely important and clear terminology and definitions are therefore essential [155,156].

The scientific literature on chemokine expression in gastrointestinal tumors is limited and most data focus on cancer gene therapy (Table 1.5). A first way to study the role of chemokines in tumor biology with gene therapy is by evaluating their effect on the ability of established tumors to grow and metastasize. As a model, subcutaneous injection of tumor cells into mice is often used. Mostly, these tumor cells are of murine origin but sometimes, human cancer cell lines are used as well. After establishment of the tumor, the same type of tumor cells, transfected with an expression plasmid bearing the desired chemokine gene, is injected into the tumor and causes local expression of the chemokine. Another approach focuses on the possible inhibitory effect of constitutive chemokine expression on tumorigenesis (initial tumor growth). Therefore, healthy mice are injected with genetically modified tumor cells expressing the chemokine under investigation. For comparison, mice are injected with tumor cells transfected with a control plasmid (i.e. without the chemokine gene).

Legend to Table 1.5:

a) This table summarizes recent literature that evaluated the effect of chemokine expression on tumorigenesis and metastasis. As a model, subcutaneous injection of tumor cells into mice was used and the ability of tumors to grow and metastasize was evaluated. Section A shows the effect of transfected or transduced cells on initial tumor growth (tumorigenesis). Therefore, healthy mice were injected with genetically modified tumor cells expressing the chemokine under investigation. Tumor development was compared with mice injected with tumor cells transfected with a control plasmid (i.e. without the chemokine gene).

Section B shows the effect of transduced cells (continuously expressing the chemokine under investigation) on tumors that were grown with parental tumor cells.

b) CT-26 is an undifferentiated murine colon adenocarcinoma cell line [171]. CMT93 is a murine rectal polyploid carcinoma cell line [172]. HCT116 is a human colorectal carcinoma cell line [173].

c) NK, natural killer cells; DC, dendritic cells.
Table 1.5 Gene therapy with chemokines to evaluate their effect on tumor growth and/or metastasis

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Cell line b)</th>
<th>Effect c)</th>
<th>Result</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Tumors grown in mice with genetically engineered tumor cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>huCXCL8 transfection</td>
<td>colon 26 (clone 20)</td>
<td>infiltration of neutrophils and macrophages</td>
<td>no effect</td>
<td>[157]</td>
</tr>
<tr>
<td>muCXCL9 + intravenous huIL-2 transfection</td>
<td>CT-26</td>
<td>chemotaxis and activation of CD4+ and CD8+ T cells</td>
<td>lifespan ↑, tumor burden ↓, dissemination to lung ↓, angiostatic effect on tumor vasculature ↑</td>
<td>[158]</td>
</tr>
<tr>
<td>muCXCL12-KDEL retroviral vector</td>
<td>CT-26</td>
<td>cell-surface expression of CXCR4 blocked</td>
<td>metastasis to lung and liver ↓ due to impaired outgrowth of micrometastases (but no impaired invasion)</td>
<td>[159]</td>
</tr>
<tr>
<td>muXCL1, CX,CL1 adenoviral vector</td>
<td>CT-26</td>
<td>XCL1: no effect on tumor CX,CL1: slight delay in tumor growth</td>
<td></td>
<td>[160]</td>
</tr>
<tr>
<td>huCCL2 transfection</td>
<td>CT-26</td>
<td>CCL2 and bacterial endotoxins synergize to activate macrophages to become tumoricidal</td>
<td>tumorigenicity ↓ and significantly less lung metastases</td>
<td>[161]</td>
</tr>
<tr>
<td>muCCL2 adenoviral vector</td>
<td>CT-26</td>
<td>no effect</td>
<td></td>
<td>[162]</td>
</tr>
<tr>
<td>hu, muCCL3 transfection</td>
<td>colon 26 (clone 20)</td>
<td>infiltration of neutrophils and macrophages</td>
<td>necrosis of tumor cells, tumorigenicity ↓ and immunity to subsequent challenge (memory because of cytolysis)</td>
<td>[157]</td>
</tr>
<tr>
<td>muCCL3 transfection</td>
<td>CMT93</td>
<td>number of CD8+ T cells, NK, DC ↑</td>
<td>tumorigenesis ↓</td>
<td>[163]</td>
</tr>
<tr>
<td>muCCL7 transfection</td>
<td>CMT93</td>
<td>number of infiltrating leukocytes ↑</td>
<td>tumors grow more slowly, no metastasis</td>
<td>[164]</td>
</tr>
<tr>
<td>muCCL17,19 adenoviral vector</td>
<td>CT-26</td>
<td></td>
<td>tumor growth slightly delayed</td>
<td>[160]</td>
</tr>
<tr>
<td>muCCL20 retroviral vector</td>
<td>CT-26</td>
<td>large infiltrate of DC, CD8+ and CD4+ T cells (not NK cells)</td>
<td>complete tumor regression</td>
<td>[165]</td>
</tr>
<tr>
<td>muCCL20 transfection</td>
<td>CMT93</td>
<td>number of CD8+ T cells, NK, DC ↑</td>
<td>tumorigenesis ↓</td>
<td>[163]</td>
</tr>
<tr>
<td>muCCL20-22,27 adenoviral vector</td>
<td>CT-26</td>
<td>tumor growth unaffected (CCL20,22) or slightly delayed (CCL21,27)</td>
<td></td>
<td>[160]</td>
</tr>
<tr>
<td><strong>B. Tumors grown in mice with parental tumor cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>huCXCL4 protein injected in tumor</td>
<td>HCT116</td>
<td>suppression of tumor-induced neovascularization</td>
<td>inhibition of tumor growth</td>
<td>[166]</td>
</tr>
<tr>
<td>muCXCL10 + IL-12 adenoviral vector</td>
<td>CT-26</td>
<td>infiltration of CD4+ and CD8+ T cells, NK cells</td>
<td>full tumor eradication of injected and distant tumor nodules</td>
<td>[167]</td>
</tr>
<tr>
<td>muXCL1 adenoviral vector</td>
<td>CT-26</td>
<td>infiltration of CD4+ and CD8+ T cells and NK cells</td>
<td>suppression of tumor growth</td>
<td>[168]</td>
</tr>
<tr>
<td>muCCL4 transfection with viral liposomes</td>
<td>CT-26</td>
<td>accumulation of CD4+ T cells</td>
<td>increased survival period</td>
<td>[169]</td>
</tr>
<tr>
<td>muCCL21 vaccinia virus vector</td>
<td>CT-26</td>
<td>infiltration of CD4+ T cells ↑</td>
<td>inhibition of tumor growth</td>
<td>[170]</td>
</tr>
</tbody>
</table>
8.1 Effect of chemokine expression on tumorigenesis

CT-26 is an undifferentiated murine colon adenocarcinoma cell line that was derived by intrarectal injection of N-nitroso-N-methylurethane in a female BALB/c mouse [171]. A colon carcinoma model is often established by subcutaneous injection of CT-26 cells in mice. In 1996, Nakashima and colleagues injected the footpad of BALB/c mice with colon 26 adenocarcinoma cells (clone 20) that were transfected with a huCXCL8, huCCL3 or muCCL3 expression plasmid. In all three conditions, a marked infiltration of neutrophils and macrophages was observed at the site of inoculation. However, only the site injected with huCCL3 or muCCL3 transfected cells showed reduced tumorigenesis and necrotic destruction of tumor cells [157]. In addition, mice that had rejected CCL3-transfected tumor cells were protected against a subsequent challenge with parental cells (i.e. non-transfected tumor cells). BALB/c mice that were subcutaneously injected with CT-26 cells, infected with recombinant adenovirus encoding muCCL2, developed rapidly growing tumors indicating that CCL2 was unable to reduce tumorigenesis [162]. Crittenden et al. showed that transfection of murine CMT93 colorectal tumor cells with a muCCL3 or muCCL20 expression plasmid was able to decrease tumorigenesis upon subcutaneous injection of these cells into the flank of syngeneic C57BL/6 mice [163]. The reduction of tumorigenesis was ascribed to an increase in CD8$^+$ T cells and natural killer cells, as demonstrated with immunodeficient mice. In contrast with murine B16 melanoma cells, transfection of these colorectal tumor cells with the chemokine-encoding plasmid alone was sufficient to reduce tumorigenesis [163]. A similar technique was used to evaluate the effect of muCCL7 on tumor formation [164]. Transfection of CMT93 cells with a muCCL7 expression plasmid did not alter their tumorigenicity upon subcutaneous injection into the back of C57BL/6 mice. The tumor, however, grew more slowly and was marked by infiltrated leukocytes. In addition, tumor metastases were absent [164]. The anti-tumor effect of a series of murine chemokines (CCL17, CCL19, CCL20, CCL21, CCL22, CCL27, XCL1 and CX$^+_3$CL1) on CT-26 cells was analysed using an adenoviral vector [160]. Tumor growth was not affected (XCL1, CCL20, CCL22) or only slightly delayed (CX$^+_3$CL1, CCL17, CCL19, CCL21, CCL27) [160].
8.2 Effect of chemokine expression on growth of established tumors

There are even indications that chemokines, besides preventing the new-formation of a tumor can help in clearing an established tumor. To analyze the anti-tumoral effect of murine CCL21, tumors were established by subcutaneous injection of murine CT-26 cells into BALB/c mice. Recombinant vaccinia virus, expressing muCCL21, was locally injected into the established tumor and resulted in an enhanced infiltration of CD4+ T cells, which correlated with inhibition of tumor growth [170]. Also the intratumoral injection of an adenovirus expressing XCL1 significantly suppressed the growth of an established CT-26 tumor [168]. Treatment of the mice with a XCL1-expressing adenovirus led to the expression of IL-2 and IFN-γ mRNA and increased the activities of natural killer cells and cytotoxic T lymphocytes [168].

Since angiogenesis is most important for tumor growth, Maione et al. investigated the effect of the angiostatic chemokine CXCL4 on tumor growth. A tumor was established in nude mice (C57BL6/J strain) by subcutaneous injection of human HCT-116 colon carcinoma cells and tumor growth was inhibited by injection of recombinant huCXCL4 into the established tumor. Since nude mice lack a thymus and are therefore unable to modulate a T cell response, this inhibition was ascribed to suppression of tumor-induced neovascularization [166]. It seems thus that, as mentioned earlier, angiogenesis plays an important role in tumor formation. Therefore, the combination of a therapy that induces a good immune response together with the prevention of angiogenesis will be most efficient in combatting tumor growth and metastasis formation.

Furumoto et al. observed complete tumor regression upon expression of muCCL20 at the tumor site, either by CCL20 gene transduction of CT-26 tumor cells or by intratumoral injections of CCL20 recombinant protein [165]. In both cases, CCL20 attracted dendritic cells into the tumor core. This might be of importance since in most studied cancer patients dendritic cells are mainly found at the periphery of tumors, which may limit their interaction with the tumor cells. Intratumoral expression of CCL20 induced a specific CD4+ and CD8+ T cell response against the parental CT-26 tumor cells, suggesting that dendritic cells present in the tumor were not inhibited by the tumor milieu. The low number of tumoral dendritic cells is probably the main
limiting factor in dendritic cell-mediated anti-tumor immunity against CT-26 tumors. The anti-tumor effect mediated by CCL20 was dependent mainly on the induction of a specific anti-tumor CD8\(^+\) T cell response since depletion of CD8\(^+\) T cells abrogated the therapeutic effect induced by CCL20. The observation that CCL20 was also able to induce a clinical response against distal parental CT-26 tumors suggests the induction of a systemic immune response that may be able to induce the regression of metastatic tumors [165]. In contrast to CT-26 cells, CCL20 expression in B16 melanoma cells was not sufficient to induce an anti-tumor response despite a clear increase of tumor-infiltrating dendritic cells [165].

### 8.3 Effect of chemokine expression on tumor metastasis

Miyata and colleagues injected murine CT-26 cells into the peritoneum of BALB/c mice and allowed the tumor cells to disseminate [169]. After 24 h, liposomes containing a muCCL4-expressing plasmid were injected into the peritoneal cavity of the mice to examine the effect of CCL4 on disseminated colon cancer [169]. CCL4 caused accumulation of CD4\(^+\) T lymphocytes and increased the survival period of the diseased mice [169]. Upon transfection with a huCCL2-encoding plasmid, murine CT-26 cells showed decreased tumorigenicity as compared to parental CT-26 cells or control-transfected cells when injected subcutaneously [161]. In addition, a significant reduction in the formation of lung metastases was observed although this was not due to the inability of the transfected cells to arrest and survive in the lung microvasculature. The reduced ability to form lung metastases could not be ascribed to a T cell response, since injection of CCL2-transfected CT-26 cells in athymic nude mice showed a similar inability to form lung metastases. However, histological examination of lung nodules, created by intravenous injection of CCL2-transfected CT-26 cells, showed a large number of infiltrating macrophages that might be tumoricidal [161]. It was indeed further demonstrated that CCL2-transfected cells are more susceptible to lysis by activated macrophages than parental CT-26 cells or control-transfected cells [161].

CXCR4 expression is a general characteristic of malignant epithelial cells whereas the expression of this receptor is low or absent on epithelia of normal breast, ovaria
CHEMOKINES IN GASTROINTESTINAL DISORDERS

and prostate. In contrast, CXCR4 is present on normal colonic epithelium [123]. VEGF can induce the expression of CXCR4 on breast cancer cells and in ovarian cancer and synergizes with CXCL12 in angiogenesis [174]. An essential role for CXCR4 in the outgrowth of colon carcinoma micrometastases was outpointed by the use of the “intrakine” or intracellular chemokine approach [159]. This technique was originally developed for AIDS gene therapy and prevents the expression of a specific chemokine receptor on the cell surface. Therefore, CXCL12, the natural chemokine ligand for CXCR4, is extended with a KDEL-amino acid sequence and will bind to the KDEL receptor. This intracellular receptor has the function to retain resident proteins in the endoplasmatic reticulum [175]. As a consequence, when CXCL12-KDEL binds CXCR4, this newly synthesized receptor is prevented from being expressed on the cell surface. Zeelenberg et al. used this technique to block CXCR4 function in murine CT-26 cell, transfected with a plasmid expressing muCXCL12-KDEL [159]. When the spleen of a first group of BALB/c control mice was injected with non-transfected murine CT-26 cells, part of the cells formed a local tumor, whereas another part left the spleen via the portal circulation to form liver metastases. Upon injection of non-transfected CT-26 cells into the tail vein of another group of control mice, metastases were formed in the lungs. When transfected CT-26 cells, expressing muCXCL12-KDEL, were injected into the spleen or tail, local tumor formation was similar to the control mice. However, the blocked CXCR4 function in these cells caused the inhibition of metastases in the liver and lung. Although the cells were still capable to invade the liver and lung tissue, the outgrowth of these micrometastases (small, almost undetectable amounts of tumor cells) to clearly detectable (macro)metastases was severely impaired. CXCR4-deficient cells were thus able to invade and survive but did hardly proliferate [159].

8.4 Effect of chemokine expression, combined with immunotherapy, on tumorigenesis and metastasis

Ruehlmann and colleagues transfected CT-26 cells with a plasmid expressing EpCAM/KSA, an epithelial cell adhesion molecule present on human carcinoma cells [158]. As such, a docking site was created for a fusion protein between IL-2 and an
antibody against huKSA, which enabled the local expression of IL-2 within the tumor. The resulting CT26-KSA cells were additionally transfected with a plasmid encoding muCXCL9 and subcutaneously injected into BALB/c mice. The expression of CXCL9 markedly reduced the tumor volume and intravenous application of the fusion protein indicated that local activation of the recruited immune cells by IL-2 amplified this anti-tumoral response [158]. The combination of CXCL9 gene therapy and IL-2 immunotherapy indeed generated chemoattraction as well as activation of CD4⁺ and CD8⁺ T cells. As a result, the volume of subcutaneously growing tumors was reduced and survival was prolonged by abrogation of disseminated pulmonary metastases, combined with an angiostatic effect of CXCL9 decreasing neovascularization of the tumor cells [158]. Narvaiza et al. obtained total eradication of subcutaneously established CT-26 tumors by co-injection of a recombinant adenovirus encoding muCXCL10 and one encoding IL-12 into the tumor [167]. The anti-tumoral effect, obtained with IL-12, was indeed markedly enhanced by muCXCL10 and characterized by the infiltration of CD4⁺ and CD8⁺ T cells. Intratumoral injection of only recombinant adenovirus bearing the muCXCL10 gene was, however, not curative [167].

Taken together, these data on gene therapy indicate that in most cases, the expression of chemokines causes the recruitment of T lymphocytes, dendritic cells and natural killer cells into the tumor. If a co-stimulatory molecule is present to activate the recruited cells, the resulting cell-mediated immune response will reduce or even prevent tumorigenesis. In established tumors, this specific immune response can cause regression or even total clearance of the tumor and prevent metastasis. The possible tumor-suppressive activity obtained with chemokine gene therapy seems, however, greatly influenced by the kind of tumor and the activation state of the host’s immune system.
General aims

As mentioned before, a new nomenclature for chemokines was recently introduced (see Chapter 1; Table 1.1). For clarity and ease of reading, however, the old nomenclature is used henceforth.

From the beginning of the chemokine era, the laboratory of Molecular Immunology has focused on the purification and characterization of proteins with chemoattractant properties. In 1988, a protein with chemotactic activity selective for neutrophilic granulocytes was identified and therefore denominated “granulocyte chemotactic protein” (GCP) [176]. Other groups identified the protein independently and it was decided to designate it “interleukin-8” (IL-8), the name that became widely used for this chemokine. Interleukins are a subgroup of cytokines that are produced by leukocytes and act on other leukocytes. Cytokines are small molecular weight messengers of the immune system, secreted by one cell to alter the behaviour of itself or another cell. Cytokines are thus the “hormones” of the immune system. At the time IL-8 was discovered, no one could realize that this protein belonged to a real subfamily of cytokines with chemoattractant properties - later called chemokines (chemoattractant cytokines) - with more than 40 members to be identified. Additional research unveiled that, unlike interleukins, chemokines exert their functions via specific G protein-coupled receptors with seven hydrophobic transmembrane domains and are produced by a wide variety of cells including epithelial, mesenchymal as well as hematopoietic cells. The denomination of the chemokine IL-8 was therefore somewhat inappropriate.
In 1993, another neutrophil chemoattractant was identified from conditioned medium of human MG-63 osteosarcoma cells and was named GCP-2 [138]. Like IL-8, GCP-2 is a CXC chemokine that possesses a Glu-Leu-Arg-motif. The presence of this ELR-motif is essential for functional binding to one of its functional receptors CXCR1 and CXCR2. These receptors are abundantly expressed on neutrophilic granulocytes.

All ELR\textsuperscript{+}CXC chemokines selectively bind to the chemokine receptor CXCR2, whereas only IL-8 and GCP-2 functionally bind to CXCR1 as well [177,178]. Although GCP-2 is structurally most related to ENA-78 (77\% identical amino acids but does not chemoattract through CXCR1), it is very likely that the biological functions of GCP-2 and IL-8 (30\% identical amino acids) more closely resemble each other because of their identical receptor usage. It is therefore speculated that these related chemokines are selectively produced to play a distinct or complementary role in specific diseases. However, IL-8 does not exist in the mouse system and one could rather argue for redundancy. Alternatively, chemokines play an important role in other processes than leukocyte migration such as angiogenesis and hematopoiesis for which their receptor and signaling system are less well understood.

At the start of this thesis, already a number of characteristics of GCP-2 were analyzed \textit{in vitro} as well as \textit{in vivo} using experimental animals. There was, however, little or nothing known about \textit{in vivo} expression of GCP-2 under physiological or pathological conditions in humans. In collaboration with the Department of Morphology and Molecular Pathology, the idea evolved at determining the \textit{in vivo} expression and potential role of GCP-2 in human intestinal tissue samples in relation to gastrointestinal disorders (both inflammation and cancer) and to compare it with IL-8 and other chemokines.

In inflammatory bowel diseases, neutrophils are abundantly present in zones of active inflammation. Therefore, our first aim was to determine whether the structurally and functionally related neutrophil chemoattractants GCP-2 and IL-8 were differentially expressed in inflammatory bowel diseases and to investigate whether it would be possible to use one of these chemokines to distinguish between Crohn’s disease and ulcerative colitis.
GCP-2 and IL-8 are not only neutrophil chemoattractants but can additionally act as angiogenic mediators [14,179]. In addition, chemokines can attract tumor cells, expressing chemokine receptors, and can thereby facilitate metastasis [140]. In a second part of this thesis research focused on the expression and possible role of GCP-2 in angiogenic processes involved in tumor progression and metastasis.
Materials and methods
1. GENERALLY APPLIED REAGENTS AND TECHNIQUES

1.1 Inducers

To measure chemokine production by different cell types, cells were stimulated with a diverse set of inducers: recombinant TNF-α, IL-4, IL-10, interferon-γ (IFN-γ) (all from Peprotech, Rocky Hill, NJ, USA), pure natural IL-1β [180] or IFN-β [181], the viral double-stranded RNA (dsRNA) polyriboinosinic-polyribocytidylic acid (poly rI:rC or PIC), phorbol myristate acetate (PMA) (both from Sigma, St. Louis, MO, USA), the bacterial endotoxin lipopolysaccharide (LPS; *E.coli* 0111:B4; Difco Laboratories, Detroit, MI, USA), *Staphylococcus aureus* enterotoxin A (SEA; Toxin Technology, Sarasota, FL, USA) or the plant lectin concanavalin A (Con A; Calbiochem, La Jolla, CA, USA) (Table 3.1).

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>Peprotech</td>
</tr>
<tr>
<td>IL-4</td>
<td>Peprotech</td>
</tr>
<tr>
<td>IL-10</td>
<td>Peprotech</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Peprotech</td>
</tr>
<tr>
<td>IFN-β</td>
<td>Rega Institute</td>
</tr>
<tr>
<td>PIC or poly rI:rC</td>
<td>Sigma</td>
</tr>
<tr>
<td>PMA</td>
<td>Sigma</td>
</tr>
<tr>
<td>LPS</td>
<td>Difco Laboratories</td>
</tr>
<tr>
<td>SEA</td>
<td>Toxin Technology</td>
</tr>
<tr>
<td>Con A</td>
<td>Calbiochem</td>
</tr>
</tbody>
</table>

1.2 Induction of endothelial cells

Macrovascular human umbilical vein endothelial cells (HUVEC) and human dermal microvascular endothelial cells (HMVEC) were purchased from Cambrex Bio Science (Walkersville, MD, USA) and cultured following the manufacturer’s instructions in endothelial cell growth medium with supplements (in EGM-2 BulletKit and EGM-2 MV BulletKit, respectively). Confluent monolayers were left untreated (control) or were stimulated with different inducers in endothelial cell growth medium with supplements. Conditioned media were collected and stored at −20°C until assay.
1.3 Isolation and induction of peripheral blood mononuclear cells (PBMC)

For chemokine induction experiments, blood samples were collected on EDTA (Vacutainer K$_2$E; BD Biosciences, Franklin Lanes, NJ, USA). Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation (430 g; 30 minutes; 15°C) on Ficoll-sodium diatrizoate (Lymphoprep; Invitrogen, Paisley, Scotland) and seeded at $1\times10^6$ cells/ml in Eagle’s minimum essential medium with Earle’s salts (EMEM; Invitrogen) with 2% fetal bovine serum (FBS; Cambrex Bio Science). PBMC were induced for 20 hours.

1.4 Measurement of chemokines by ELISA

For the measurement of human GCP-2 and IL-8 in serum or conditioned medium of cell cultures, a classic sandwich ELISA was used (Table 3.2 and 3.3).

Table 3.2 Antisera used to perform sandwich ELISA a)

<table>
<thead>
<tr>
<th>Coating Ab</th>
<th>Secondary Ab b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type</td>
<td>Origin</td>
</tr>
<tr>
<td>GCP-2</td>
<td>Poly</td>
</tr>
<tr>
<td>HCC-1</td>
<td>Poly</td>
</tr>
<tr>
<td>IL-8</td>
<td>Poly</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Poly</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>Poly</td>
</tr>
<tr>
<td>PARC</td>
<td>Poly</td>
</tr>
</tbody>
</table>

a) Bios: Biosource; Mono: monoclonal; Poly: polyclonal; PT: PeproTech; R&D: R&D Systems; Rega: antibody generated and purified in the Rega Institute.

b) Peroxidase-labeled goat anti-mouse IgG or peroxidase-labeled donkey anti-rabbit IgG (Jackson ImmunoResearch) were used as detection antibody.

Plates were coated with polyclonal rabbit anti-human GCP-2 (1/500 dilution; PeproTech) or polyclonal goat anti-human IL-8 (1/800) [182] antibody in phosphate buffered saline (PBS; Cambrex Bio Science) (Table 3.2). Purified synthetic GCP-2 [177] and natural IL-8 [182], served as a standard (range of 0.002-2 ng/ml and 0.025-25 ng/ml, respectively) (Table 3.3).
Table 3.3 Chemokines used as standard to perform sandwich ELISA a)

<table>
<thead>
<tr>
<th>Standard</th>
<th>Type</th>
<th>Source</th>
<th>Detection limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCP-2</td>
<td>Synth</td>
<td>Rega</td>
<td>0.002</td>
</tr>
<tr>
<td>HCC-1</td>
<td>Rec</td>
<td>PT</td>
<td>0.05</td>
</tr>
<tr>
<td>IL-8</td>
<td>Nat</td>
<td>Rega</td>
<td>0.025</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Rec</td>
<td>Rega</td>
<td>0.025</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>Rec</td>
<td>R&amp;D</td>
<td>0.025</td>
</tr>
<tr>
<td>PARC</td>
<td>Rec</td>
<td>R&amp;D</td>
<td>0.01</td>
</tr>
</tbody>
</table>

a) Nat: natural; PT: PeproTech; R&D: R&D Systems; Rec: recombinant; Rega: antibody generated and purified in the Rega Institute; Synth: synthetic.

c) The detection limit (ng/ml), representing the lowest amount of chemokine measurable in the ELISA assay, is shown.

Murine monoclonal anti-human GCP-2 (1/2000) or anti-human IL-8 (1/10^4) (both from R&D Systems, Abingdon, UK) were added as secondary antibody. Detection was performed with a specific peroxidase-labeled antibody (1/5000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and visualized with 3,3’,5,5’-tetramethylbenzidine dihydrochloride hydrate (Aldrich Chemicals, Milwaukee, WI, USA). Human MCP-1, hemofiltrate CC chemokine-1 (HCC-1), pulmonary and activation-regulated chemokine (PARC) and macrophage inflammatory protein-1α (MIP-1α) were measured according to the same procedure. Plates were coated with polyclonal rabbit anti-human MCP-1 (1/400; Rega Institute), polyclonal goat anti-human HCC-1 (1/600; R&D Systems), polyclonal goat anti-human PARC (1/500; R&D Systems) or polyclonal goat anti-human MIP-1α (1/500; R&D Systems) antibody. As secondary antibody, monoclonal mouse anti-human MCP-1 (1/5000; R&D Systems), polyclonal rabbit anti-human HCC-1 (1/2500; PeproTech), polyclonal rabbit anti-human PARC (1/500; PeproTech) or monoclonal mouse anti-human MIP-1α (1/20000; Biosource, Camarillo, CA, USA) respectively, was used. Recombinant human MCP-1 (Rega Institute), HCC-1 (PeproTech), PARC or MIP-1α (R&D Systems) served as a standard (range of 0.025-25 ng/ml; 0.05-50 ng/ml; 0.01-10 ng/ml and 0.025-25 ng/ml, respectively). Eotaxin levels were measured with a commercial cytoset of antibodies (Biosource). All ELISAs were specific in that other chemokines or chemokine inducers were not detectable.
1.5 Western blot

Equal amounts (300 ng/lane) of natural human IL-8 [176], recombinant ENA-78 (R&D Systems), recombinant GCP-2(1-77) and GCP-2(9-77) protein [183] were loaded onto a Tris/tricine gel and subjected to SDS-PAGE under non-reducing conditions [184]. The separating, spacer and stacking gels contained 13% T (total % concentration of acrylamide and bisacrylamide monomers) and 5% C (% concentration of bisacrylamide cross-linker relative to T), 10% T and 3.3% C and 5% T and 5% C, respectively. Prestained molecular weight markers (Bio-Rad Laboratories, Hercules, CA, USA) were soybean trypsin inhibitor, lysozyme and aprotinin (Mr 28,000; 20,000 and 6,400; respectively). Proteins were transferred to a polyvinylidene difluoride membrane (Hybond-P; Amersham Pharmacia Biotech, Piscataway, NJ, USA) by electroblotting. To block non-specific binding sites, the blot was immersed in NT-buffer [100 mM NaCl, 10 mM Tris-HCl, pH 7.4] with 3% (w/v) bovine serum albumin (BSA), before incubation with murine monoclonal anti-human GCP-2 antibody (R&D Systems; 1/300 dilution in NT-buffer). After three washes in wash-buffer (NT-buffer with 0.01% Tween-20 and 0.3% (w/v) BSA), the blot was incubated with alkaline-phosphatase conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories; 1/2000 dilution in wash-buffer). The blot was again washed and protein was visualized with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP; Roche Diagnostics, Mannheim, Germany).

1.6 Immunohistochemistry

For immunohistochemical staining, freshly isolated tissue samples were snap frozen in isopentane (cooled with liquid nitrogen) and stored at -80°C until use. Cryostat sections (6 µm) were air dried and fixed in absolute acetone during 10 minutes. To block endogenous peroxidase activity, the slides were treated with 2% hydrogen peroxide in methanol for 5 minutes. Then, slides were incubated at room temperature for 3 hours with primary antibody diluted in PBS. Antibodies used were murine anti-human GCP-2 (R&D Systems; 1/100), polyclonal rabbit anti-human ENA-78 (PeproTech; 1/100), murine anti-human CD31 and CD45 (both from Dako,
Carpinteria, CA; 1/50), rabbit anti-human VEGF (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1/100), murine anti-human gelatinase B (REGA-2D9; [185]; 1/200) and murine anti-human IL-8 (R&D Systems; 1/100). After incubation with peroxidase-labeled antiserum (EnVision+, Dako) for 30 minutes, the reaction product was visualized by 3-amino-9-ethylcarbazole, resulting in bright-red immunoreactive staining. To visualize tissue morphology, slides were faintly counterstained with Mayer’s hematoxylin. As a negative control, the primary antibody was omitted or replaced by IgG, purified from serum of non-immunized mice or rabbits.

1.7 Granulocytosis

Male New Zealand White rabbits were intravenously injected with 10 or 30 µg/ml GCP-2(9-77) [183] diluted in 0.9% NaCl. As a negative control, pyrogen-free saline was injected. Blood samples (1 ml) were drawn from the ear vein at different time points before and after injection of the sample and were collected in EDTA tubes. For each time point, a blood smear was made to determine the number of granulocytes and mononuclear cells per 100 leukocytes. Fixation and staining of cells was performed with Hemacolor solutions (Merck, Darmstadt, Germany). To determine the total leukocyte count of every blood sample, cells were stained with Türk's solution (VWR International, Leuven, Belgium) and counted microscopically in a haemocytometer.

1.8 Thymidine incorporation for the measurement of endothelial cell proliferation

To measure endothelial cell proliferation, HMVEC were grown in glass vials (Milli-6 glass LSC vials, Lumac 5105, PerkinElmer Life Sciences, Boston, MA, USA). After 4 days, the growth medium was replaced by assay medium (EBM-2 without growth factors, but enriched with 3% FBS, 10 µg/ml gentamycin, 0.1 mg/ml L-ascorbic acid, 50 nM hydrocortisone and 10 mM HEPES). Cells were stimulated with GCP-2(1-77) [183], assay medium (negative control) or basic fibroblast growth factor (b-FGF; PeproTech) (positive control). After 48 hours, the cells were incubated
with \[^{3}H\]-thymidine (Amersham Biosciences, Buckinghamshire, UK) (0.6 µCi/vial) for 5 hours at 37°C. Then, the cells were washed twice with ice-cold trichloroacetic acid 5% and once with ice-cold ethanol and allowed to dry for 30 minutes. To determine the incorporation of \[^{3}H\]-thymidine, 100 µl of a liquid scintillation counting cocktail (OptiPhase HiSafe 2; PerkinElmer Life Sciences) was added to the cells and the amount of radioactivity, incorporated into the DNA, was measured by liquid scintillation counting. The obtained results are the mean of 3 to 9 replicates in two independent experiments. As a correction for inter-assay variability, \[^{3}H\]-thymidine incorporation was expressed as the percentage of radioactivity in comparison to the negative control. Endothelial cell proliferation was considered statistically significant (Mann-Whitney U-test) if p-values were < 0.05. Data are represented as mean ± standard error of the mean (SEM).

### 1.9 Purification of neutrophils

For chemotaxis experiments, human neutrophils were purified from fresh blood samples, obtained from healthy volunteers (Blood Transfusion Center of Antwerp, Belgium and Laboratory of Experimental Immunology, University of Leuven, Belgium). Blood was collected upon heparin and mononuclear cells were separated from the granulocytes and erythrocytes by density gradient centrifugation (430 g; 30 minutes; 15°C) on Ficoll-sodium diatrizoate (Lymphoprep; Invitrogen). To remove erythrocytes, the granulocyte pellet was mixed with one volume of PBS and one volume of hydroxyethylstarch solution (Plasmasteril; Fresenius, Bad Homburg, Germany) and erythrocytes were allowed to sediment during 30 minutes at 37°C. The supernatant was centrifuged during 10 minutes at 218 g. The remaining erythrocytes in the granulocyte pellet were lysed by hypotonic shock in distilled water for 30 seconds. Finally, granulocytes were washed in PBS and suspended in chemotaxis buffer i.e. Hank’s Balanced Salt Solution (HBSS; Invitrogen) supplemented with 1 mg/ml human serum albumin (HSA; Belgian Red Cross).
1.10 Chemotaxis

To measure chemotactic activity, a diluted chemokine sample was added to the lower well of a Boyden microchamber (Neuro Probe, Gaithersburg, MD, USA) in triplicate and covered with a polyvinyl pyrrolidone-free polycarbonate filter with 5.0 µm pores (Nuclepore; Whatman, Clifton, NJ, USA) (Figure 3.1).

Figure 3.1. Boyden microchamber
A Boyden microchamber is used for the measurement of the chemotactic activity of a chemokine. Therefore, a dilution of the chemokine is added in triplicate to the lower wells of the microchamber. The upper wells are filled with cells and separated from the lower wells by a polycarbonate filter. A silicone gasket is used to prevent leakage. This setting is incubated for a certain period (dependent on the cell type used) in a CO₂-incubator. Afterwards, the chamber is dismounted and the migrated cells are fixed on the filter. To express the chemotactic activity of the chemokine, a chemotactic index is used by expressing the number of cells that migrated towards the chemokine dilution divided by the number of cells that migrated spontaneously (towards the dilution buffer).

To perform chemotaxis, recombinant GCP-2 [183], natural IL-8 [176] and natural MCP-1 [186] were used, while chemotaxis buffer was used as a negative control. The upper wells of the chamber were filled with a neutrophil suspension (10⁶ cells/ml) and separated from the lower wells by a silicone gasket. Cells were allowed to migrate for
45 minutes at 37°C. A chemotactic index (CI) was used to express chemotactic activity and was measured by calculating the number of cells migrated to the chemokine dilution, divided by the number of cells that migrated spontaneously (i.e. to the chemotaxis buffer). Synergy experiments were performed by adding two different chemokines together to the lower wells of the chamber. To see whether the chemotactic activity of the combination of chemokines statistically differed from the sum of that of the chemokines alone (= synergy), statistical analysis was performed using the Mann-Whitney U-test. Differences between the CI were considered statistically significant if p-values were < 0.05. Data are represented as mean ± SEM.

Inhibition of chemotaxis was performed by the addition of chemokine receptor antagonists to the cells just before transfer to the upper well of the microchamber. SB225002 (Calbiochem) and RS102895 (Sigma) were used as CXCR2- and CCR2-antagonists, respectively. At the concentrations used (10 µM), cytotoxic side effects of the antagonists in neutrophil chemotaxis were excluded since C5a-induced neutrophil migration remained unaffected.

2. PATIENT SAMPLES USED FOR THE STUDY OF IBD

2.1 Patients

In total, blood samples were obtained from 51 CD patients, 46 UC patients and 29 healthy volunteers. The sera from 30 CD and 30 UC patients and from 12 healthy subjects were used to determine chemokine protein levels in the blood circulation. The blood samples from the remaining patients (21 CD, 16 UC, 17 controls) were used to isolate PBMC for chemokine induction. Of the latter patients, 8/21 CD and 13/16 UC were treated with 5-aminosalicylic acid (5-ASA). Of the UC patients treated with 5-ASA, 6 persons received steroids in addition. Some CD patients received azathioprine (n=3), methotrexate (n=1) or a combination of drugs (n=5). The diagnosis of CD and UC was based upon classical clinical, endoscopic, radiological and histological criteria. All CD and UC patients had active disease as assessed with clinical, microscopic and serological (C reactive protein) parameters.
Biopsy samples of macroscopically involved and non-involved intestinal areas were obtained from surgical specimens from another group of patients (29 CD and 31 UC) and were used for immunohistochemistry. Of the CD biopsy samples, 19/29 were obtained from colon tissue, the other 10 samples were from ileal origin. Biopsies of macroscopically normal colon (n=11) and small intestine (n=3), obtained from patients operated for colorectal cancer, as well as samples of normal conjunctiva (n=6) from patients operated for strabism were used as non-inflammatory controls. Samples of inflamed appendices (n=2), obtained from patients operated for appendicitis, and samples of conjunctiva from patients with vernal keratoconjunctivitis (VKC; n=13) were used as intestinal and extra-intestinal inflammatory controls, respectively. VKC was chosen because, like CD and UC, it is a chronic, relapsing inflammatory disorder.

To evaluate the immunoreactivity in the intestine and conjunctiva, the number of positive leukocytes in the mucosa was counted per high power field (400x magnification) in five fields of the most affected zone of the biopsy. All sections were coded and scored blindly. Disease activity was assessed using established CD and UC scoring systems [187,188]. In addition, GCP-2 immunoreactivity was analyzed according to the following scoring system: 0 = no positive cells, + = 1 to 5 positive cells, ++ = 6 to 10 positive cells and +++ = more than 10 positive cells per high power field (400x magnification).

2.2 Statistics

Statistical analysis was performed using the Mann-Whitney U-test. As a correction for multiple testing, Bonferroni corrections were applied. Differences between measured chemokine concentrations were considered statistically significant if p-values were < 0.05.
Biopsy samples were obtained from 22 patients operated for gastrointestinal malignancy (12 female; 10 male; mean age: 63 years; range 24-83 years). Malignancies included 3 adenocarcinomas of the distal oesophagus, 3 adenocarcinomas and 1 gastrointestinal stromal tumor (GIST) of the stomach, 6 adenocarcinomas of the colon, 1 GIST and 1 B cell lymphoma of the small intestine and 7 ductal adenocarcinomas of the pancreas (Table 3.4).

Table 3.4  Origin of biopsy samples for immunohistochemistry a)

<table>
<thead>
<tr>
<th>Location of tumor</th>
<th>Type of tumor</th>
<th>Staging of tumor b)</th>
<th>Number of biopsies</th>
</tr>
</thead>
<tbody>
<tr>
<td>oesophagus</td>
<td>adenocarcinoma</td>
<td>T3N1V0M0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T3N2V0M0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>mucinous adenocarcinoma</td>
<td>T3N1V0M0</td>
<td>1</td>
</tr>
<tr>
<td>stomach</td>
<td>adenocarcinoma</td>
<td>T3N0V0M0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T3N2V1M1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>GIST c)</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>colon</td>
<td>adenocarcinoma</td>
<td>T3N0V0M0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T3N2V1M1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T3N2V0M0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>mucinous adenocarcinoma</td>
<td>T3N0V0M0</td>
<td>2</td>
</tr>
<tr>
<td>small intestine</td>
<td>GIST c)</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>B cell lymphoma</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>pancreas</td>
<td>ductal carcinoma</td>
<td>T3N0V0M0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T3N0V0M0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T3N1V0M0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T3N1V0M1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T3N1V0M1</td>
<td>1</td>
</tr>
</tbody>
</table>

a) For immunohistochemistry, biopsy samples were obtained from patients operated for gastrointestinal malignancy

b) Tumors were classified and staged according to the WHO histological classification [189] and the TNM classification of malignant tumors [190]. T = tumor, N = node, V = vessel, M = metastasis.

c) GIST = gastrointestinal stromal tumor.

Surgical resection specimens containing the tumor and normal tissue, distant from the lesion, were received immediately after operation and routinely processed. For diagnosis and staging of the tumors, sections were made from formalin-fixed and
paraffin-embedded tissue blocks and stained routinely with haematoxylin and eosin. Extra stainings for diagnostic purposes were performed if needed and included stains for B cells (CD20) and T cells (CD3 and CD5) as markers for lymphomas and for mucin secretions and CD117 as markers for adenocarcinomas and GIST, respectively. The tumors were classified and staged according to the WHO histological classification [189] and the TNM classification of malignant tumors [190] (Table 3.4). For immunohistochemistry, cryostat sections were made from tumoral tissue. Normal, non-involved tissue sections of the same patients were used as controls.

Positive controls for immunohistochemistry included colon samples from patients with Crohn’s disease (GCP-2 and gelatinase B), normal skin (CD31), lymph nodes (CD45) and samples from patients operated for breast cancer (VEGF).
Results
GCP-2 in inflammatory bowel diseases

The results presented in this chapter are modified from

Introduction

In vitro, the ELR+CXC chemokine GCP-2 induces a transient increase of the intracellular calcium level in neutrophils, induces the release of the matrix metalloproteinase MMP-9/gelatinase B [138] and is chemotactic for neutrophilic granulocytes [138] and endothelial cells [14]. In vivo, GCP-2 causes local neutrophil accumulation and plasma extravasation [177]. Although GCP-2 is structurally most related to ENA-78, it is very likely that the biological functions of GCP-2 and IL-8 more closely resemble each other because of their identical receptor usage [177,178]. It is therefore speculated that these related chemokines are selectively produced to play a distinct or complementary role in specific diseases.

IBD are characterized by an uncontrolled immune response, resulting in chronic inflammation of the intestine [53,54]. Pro-inflammatory cytokines and chemokines exacerbate intestinal inflammation by mediating a constant influx of leukocytes out of the blood stream into the mucosa [76]. CD and ulcerative colitis (UC) are chronic, idiopathic IBD, characterized by alternating episodes of activity and remission. Mucosal expression of several chemokines such as MCP-1, MIP-1α and IL-8 has been described to be upregulated in both UC and CD, explaining the massive infiltrate of leukocytes in active disease [84,97]. These data are in accordance with other studies showing increased mRNA expression levels for a large number of chemokines in active UC and CD [84].

The purpose of this study was to compare the expression of the two CXCR1 agonists, IL-8 and GCP-2 in relation to IBD.
Results

1. Immunohistochemical staining of ELR⁺ CXC chemokines in IBD

Because neutrophils are abundantly present in zones of active inflammation in IBD, we investigated the expression of the three structurally and functionally related ELR⁺ CXC chemokines GCP-2, ENA-78 and IL-8 in intestinal tissue sections of IBD patients. Microscopic analysis of intestinal tissue sections of non-inflammatory controls and of non-involved samples from CD and UC patients showed only a small number of leukocytes in the mucosa staining positive for GCP-2, ENA-78 or IL-8. Positive chemokine staining appeared mainly as a diffuse, finely granular, cytoplasmic staining. The number of GCP-2 positive leukocytes in the lamina propria was increased in sections from involved areas of CD and UC patients and correlated with the disease activity observed in the biopsy samples (Table 4.1).

Table 4.1  GCP-2 immunoreactivity of leukocytes in intestinal tissue samples of IBD

<table>
<thead>
<tr>
<th>Grade of immunoreactivity</th>
<th>Crohn’s disease b)</th>
<th>Ulcerative colitis b)</th>
<th>Inflammatory control c)</th>
<th>Non-inflammatory control b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>50</td>
<td>30</td>
<td>30</td>
<td>90</td>
</tr>
<tr>
<td>+</td>
<td>30</td>
<td>50</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>++</td>
<td>20</td>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>+++</td>
<td>0</td>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

a) GCP-2 immunoreactivity of leukocytes was scored as follows: 0 = no positive cells; + = 1 to 5 positive cells; ++ = 6 to 10 positive cells and +++ = more than 10 positive cells.
b) Biopsy samples were obtained from intestinal tissue of IBD patients (CD and UC).
c) Tissue samples from inflamed appendices and conjunctiva were used as intestinal and extra-intestinal inflammatory controls, respectively.
d) Macroscopically normal samples of colon, small intestine and conjunctiva were used as non-inflammatory controls.
e) Data represent the percentage of tissue samples (per patient group) with a certain grade of GCP-2 immunoreactivity of leukocytes.
In addition, pronounced GCP-2 positive staining was observed in endothelial cells (Factor VIII⁺, CD34⁺) of blood vessels located in the ulcer bed in ulcerations and erosions in involved tissue sections of IBD patients (Figure 4.1). Surprisingly, however, no GCP-2 positive endothelial cells were observed in inflamed areas without epithelial or mucosal defects.

Figure 4.1 Ulceration-related expression of GCP-2 by endothelial cells
Panel A: GCP-2 immunoreactivity (red) in endothelial cells from blood vessels located at the base of an ulceration (arrow) in the intestine of a patient with UC. No GCP-2 positive endothelial cells are observed in inflamed areas without epithelial defects (arrowhead). Panel B: Magnification of GCP-2 positive endothelial cells from panel A.

GCP-2 positive staining of leukocytes was also observed in intestinal (appendix) and extra-intestinal (conjunctiva) biopsies from other acute and chronic inflammatory conditions without epithelial defects that were evaluated as inflammatory control samples. As in the non-ulcerated samples, no GCP-2 positive staining of endothelial cells was observed in these samples. In contrast with GCP-2, no ulceration-related endothelial staining was observed for IL-8 or ENA-78, although positively stained leukocytes were present in the mucosa. As for GCP-2, the number of leukocytes staining positive for IL-8 or ENA-78 correlated with the disease activity observed in the biopsy (data not shown and Figure 4.2).
RESULTS: GCP-2 IN INFLAMMATORY BOWEL DISEASES

Figure 4.2 Immunohistochemical staining of inflammatory cells for IL-8, ENA-78 and GCP-2
Intestinal resections of patients with CD show immunoreactivity for IL-8, ENA-78 and GCP-2. Positive cells (red) appear to be inflammatory cells in the mucosa.

Specific determination of GCP-2 in tissue sections may suffer from cross-reactivity of the detecting antibodies with the structurally related ENA-78 and IL-8 (77% and 30% identical amino acids, respectively). Therefore, we verified the specificity of the antibody by Western blotting experiments. Figure 4.3 illustrates that the monoclonal antibody, used for immunohistochemical staining allowed to selectively recognize isoforms of GCP-2, i.e. intact GCP-2(1-77) and N-terminally truncated GCP-2(9-77), whereas equal amounts of ENA-78 and IL-8 were not detected.
RESULTS: GCP-2 IN INFLAMMATORY BOWEL DISEASES

Figure 4.3 Western blot of the GCP-2 antibody used for immunohistochemistry
Equal amounts (300 ng/lane) of GCP-2(1-77), GCP-2(9-77), ENA-78 and IL-8 were loaded onto a Tris/tricine gel, subjected to SDS-PAGE under non-reducing conditions and transferred to a polyvinylidene difluoride membrane by electroblotting. The blot was first incubated with a murine monoclonal GCP-2 antibody (1/300) that was further detected with alkaline-phosphatase conjugated goat anti-mouse antibody. Protein was visualized by NBT/BCIP-staining. Prestained molecular weight markers represent soybean trypsin inhibitor (28 kDa), lysozyme (20 kDa) and aprotinin (6.4 kDa).

2. GCP-2 promotes granulocyte mobilization in vivo

In order to evaluate whether GCP-2 produced in the inflamed intestine may have biologically relevant effects, we investigated whether GCP-2 is capable of inducing leukocyte-blood vessel interaction in vivo. Systemic administration of recombinant GCP-2(9-77) in rabbit (30 µg/kg) revealed an immediate (5 minutes) and severe neutropenia (5-fold reduction in number of circulating granulocytes) followed by a profound granulocytosis (4-fold increase above the starting granulocyte number) within two hours post-injection, and restoration of normal cell counts after 24 hours (Figure 4.4). Mononuclear cell counts in the circulation were not affected by the GCP-2(9-77) treatment and rabbits treated with buffer alone did not show significant changes in neutrophil counts (Figure 4.4). Intravenous injection of a lower dose of GCP-2(9-77) (10 µg/kg) still provoked a significant, but weak neutropenia after 5 minutes (2- to 3-fold reduction) and also the subsequent granulocytosis was less pronounced (2- to 3-fold increase) compared to 30 µg/kg of GCP-2 (Figure 4.4). The presence of bacterial endotoxin in the injected chemokine samples was excluded by the Limulus amebocyte lysate assay (Cambrex Bio Science) indicating an LPS contamination of less than 3.75 pg per injected dose (data not shown). The obtained
results demonstrate that GCP-2 is capable to induce leukocyte blood vessel interactions and to promote recruitment of neutrophils upon \textit{in vivo} expression.

\begin{center}
\begin{figure}
\includegraphics[width=\textwidth]{figure4.4.png}
\caption{Effect of human GCP-2 on circulating granulocyte counts}
\end{figure}
\end{center}

Rabbits were injected intravenously with GCP-2(9-77) at 30 µg/kg or 10 µg/kg or with endotoxin-free saline (negative control). Blood was drawn from an ear vein at different time points before and after injection to determine the number of granulocytes (\textcolor{red}{\text{▲}}) and mononuclear cells (monocytes: \textcolor{blue}{○} and lymphocytes: \textcolor{green}{□}). For each condition, a representative experiment is depicted.
3. The production of GCP-2 and IL-8 by various endothelial cell types is differently regulated

Since neovascularization is an important mechanism in the healing process of intestinal inflammation, we further compared the expression of GCP-2 and IL-8 by endothelial cells in vitro. Therefore, we determined the kinetics of GCP-2 and IL-8 protein production by HUVEC after induction with optimal [180,182] doses of endogenous (the cytokine IL-1β) or exogenous (viral dsRNA, bacterial endotoxin) stimuli (Figure 4.5).

Figure 4.5  Kinetics of IL-8 and GCP-2 production in endothelial cells
Confluent monolayers of HUVEC or HMVEC were stimulated with 50 µg/ml LPS (▲), 100 µg/ml poly rI:rC (♦) or 30 U/ml IL-1β (■) or were left untreated (○). Samples were taken at different time intervals and their IL-8 and GCP-2 protein concentrations were analyzed by ELISA. Results are the mean of three independent experiments. For clarity, no error bars are represented.
IL-1β (30 U/ml) induced moderate quantities (5 ng/ml) of GCP-2 that reached a plateau after 48 hours. For IL-8, a higher production level (200 ng/ml) was obtained, which continued to rise until 120 hours after induction. The viral dsRNA poly rI:rC (100 µg/ml) and LPS (50 µg/ml), were weaker inducers of GCP-2 (1 to 2 ng/ml) and IL-8 (100 ng/ml) than IL-1β. The production of these ELR⁺CXC chemokines by unstimulated cells was 100-fold lower (3 ng/ml for IL-8) than after optimal induction (by IL-1β) or remained below the detection limit (< 0.2 ng/ml GCP-2).

Similar experiments were carried out on HMVEC that are more representative for angiogenesis in the intestine. Figure 4.5 shows that, in contrast to HUVEC, in HMVEC both IL-1β and LPS were better inducers of IL-8 and GCP-2 compared to dsRNA, indicating that bacterial rather than viral products affect IL-8 and GCP-2 expression in these cells. The maximal production levels obtained for IL-8 (150 ng/ml) were again much higher than those of GCP-2 (2 ng/ml) and were reached at 96 hours after induction for both chemokines. Quantitatively, these in vitro data do not reflect the more pronounced expression of GCP-2 versus IL-8 in IBD biopsies, indicating that other, yet undefined, inducing substances are responsible for chemokine expression in vivo.

4. Unchanged chemokine concentrations in serum of IBD patients

To determine whether chemokine concentrations in the blood of IBD patients could be used to differentiate between CD and UC, we measured the serum levels of different CC and CXC chemokines (MIP-1α, HCC-1, PARC, eotaxin, GCP-2 and IL-8) (data not shown). In addition, we investigated the possible impact of different types of IBD medication. For all chemokines tested, the serum levels remained below the detection limit in IBD patients and control subjects, except for eotaxin and the plasma chemokines PARC and HCC-1. However, the mean (± SEM) serum levels of eotaxin, PARC and HCC-1 for both CD patients (n=30; 0.22 ± 0.02, 14 ± 1 and 210 ± 14 ng/ml, respectively) and UC patients (n=30; 0.20 ± 0.02, 16 ± 1 and 193 ± 11 ng/ml, respectively) were not statistically different from those of healthy subjects (n=12; 0.18 ± 0.02, 13 ± 1 and 195 ± 20 ng/ml, respectively). Medication had no clear influence on serum chemokine concentrations.
5. Impaired IL-8 production in PBMC of CD patients

Finally, the participation of leukocytes in the production of ELR\(^+\)CXC chemokines was evaluated in patients with CD and UC. For that purpose, PBMC from healthy control subjects and IBD patients were isolated and stimulated for 20 hours with LPS (5 \(\mu\)g/ml), Con A (10 \(\mu\)g/ml) and poly rI:rC (30 \(\mu\)g/ml). For IBD patients, the possible impact of different types of medication was also evaluated.

In PBMC from healthy volunteers, on average (\(\pm\) SEM) 264 \(\pm\) 28, 252 \(\pm\) 36 and 96 \(\pm\) 14 ng/ml IL-8 protein was induced by LPS, Con A and poly rI:rC, respectively (Figure 4.6). In contrast, no detectable amounts of GCP-2 (< 0.2 ng/ml) were produced by stimulated PBMC (data not shown). When control subjects were compared with UC patients, it was observed that the IL-8 production by PBMC was not significantly different for any of these three exogenous inducers. In contrast, IL-8 production was significantly lower in PBMC of CD patients than in PBMC of healthy subjects in response to LPS (p < 0.001), Con A (p < 0.05) and poly rI:rC (p < 0.05). When compared with UC patients, the IL-8 production in CD patients was again significantly impaired when PBMC were stimulated with LPS (p < 0.001) and Con A (p < 0.001). If statistical analysis was performed on the 7/21 CD and 11/16 UC patients receiving treatment with 5-ASA solely or in combination with steroids, similar statistically significant differences (p < 0.05) were obtained for LPS, Con A and poly rI:rC, indicating that the treatment is not responsible for the observed differences. A statistical analysis on all patients blood formula demonstrated that there was no significant difference between UC and CD patients in monocyte or lymphocyte counts (data not shown). Therefore, the lowered IL-8 production could not be ascribed to a difference in leukocyte formula.

To verify whether the impaired chemokine production in CD patients was specific for IL-8, induction of the CC chemokine MIP-1\(\alpha\) was evaluated as well (Figure 4.6). LPS, Con A and poly rI:rC significantly induced MIP-1\(\alpha\) in PBMC of healthy subjects (mean \(\pm\) SEM were 24 \(\pm\) 2, 20 \(\pm\) 3 and 14 \(\pm\) 2 ng/ml, respectively), but the production in CD or UC patients versus healthy controls was not statistically different (Figure 4.6). Similar results as for MIP-1\(\alpha\) were observed for MCP-1 production by
stimulated PBMC (data not shown), indicating that the reduced chemokine production in CD patients could be restricted to IL-8 and/or other ELR\(^+\)CXC chemokines.

**Figure 4.6** Chemokine production by stimulated PBMC from IBD patients

PBMC from IBD patients (CD; \(n=21\) and UC; \(n=16\)) and healthy persons (Control; \(n=17\)) were isolated and stimulated with 5 \(\mu\)g/ml LPS, 10 \(\mu\)g/ml Con A, 30 \(\mu\)g/ml poly rI:rC (PIC) or were left untreated (Co). After 20 hours of induction, IL-8 and MIP-1\(\alpha\) protein were measured in the conditioned media by ELISA. Measured chemokine concentrations are depicted in box plots. Medians are represented as horizontal lines in the box plots. The box is outlined by the first quartile at the bottom and the third quartile on top and encloses the middle 50% of the data (=interquartile range, IQR). The whiskers enclose values deviating maximally 1.5 times the IQR (from the first and the third quartile, respectively). Outliers (○) and extremes (◊) represent values > 1.5 IQR and > 3 IQR, respectively.
Discussion

By attracting and activating immune cells, chemokines play a crucial role in the onset and perpetuation of inflammatory reactions. In many diseases, such as IBD, overexpression of chemokines causes an uncontrolled immune response that leads to chronic inflammation. Chemokine production in IBD patients has been reported by independent groups and was reviewed recently [76,77]. The results of most groups indicate elevated chemokine levels in IBD patients compared to control subjects [93,100,106,191]. Elevated levels of all chemokine subgroups (CC, CXC, C and CX3C) are believed to explain the massive infiltration of leukocytes into the mucosa, that is crucial in the development and maintenance of these intestinal diseases [103,119]. Increased IL-8 expression has been reported in homogenates of colon biopsies and resected bowel segments of IBD patients versus non-inflammatory controls [92,93]. Until now, the only chemokine with significantly increased serum levels in active CD and UC seems to be eotaxin, as demonstrated by Chen et al. [110].

We have compared the in vitro and in vivo expression of IL-8 and GCP-2, two related ELR+CXC chemokines that functionally bind both CXCR1 and CXCR2 and that chemoattract neutrophils [192]. The choice to investigate these chemokines in intestinal tissue sections of IBD patients by immunohistochemical staining was based upon the fact that neutrophils are abundantly present in zones of active inflammation in IBD [193]. Our immunohistochemical data show that, in biopsies of IBD patients, cells staining positive for the ELR+CXC chemokines GCP-2, IL-8 and ENA-78 were leukocytes in the lamina propria. The number of positive cells correlated with the disease activity observed in the samples (Table 4.1), confirming earlier reports [92,115]. In addition, we observed a prominent expression of GCP-2, but not IL-8 or ENA-78, by endothelial cells in areas of mucosal defects. The selective expression of GCP-2 in endothelial cells at sites of an ulceration probably plays a role in the attraction of neutrophilic granulocytes out of the blood stream into the inflamed ulcer bed. The recruitment of neutrophils can be an essential defense mechanism in the gastrointestinal tract that is constantly in close contact with antigens present in the
intestinal lumen. Our findings of a relation between GCP-2 expression in endothelial cells and mucosal defects are supported by literature data. Indeed, in the rat corneal micropocket model of neovascularization, human GCP-2 was shown to be angiogenic [14] and overexpression of murine GCP-2 in human tumor cell allografts resulted in enhanced angiogenesis in vivo [129]. The specific GCP-2 staining suggests that the function of GCP-2 is different from that of IL-8 and ENA-78 despite their structural relationship. Our findings support that the chemokine network shows complementarity, rather than redundancy [194].

The in vivo relevance of GCP-2 was further evidenced by systemic administration in rabbits, as previously demonstrated for human IL-8 [176]. Intravenous injection of pure GCP-2 provoked a dose-dependent systemic response, composed of an immediate granulopenia, followed by a profound granulocytosis, whereas mononuclear cell levels were not affected. This phenomenon can be explained by an initial adhesion of the granulocytes to the vessel wall, followed by attraction of additional cells from the marginating pool resulting in enhanced granulocytosis upon detachment of the cells from the endothelium.

Since angiogenesis is an important mechanism in healing, the expression of GCP-2 and IL-8 by endothelial cells was investigated in vitro. We demonstrated that GCP-2 and IL-8 expression are differentially regulated in different types of endothelial cells. The pro-inflammatory cytokine, IL-1β, and LPS were equally good inducers of IL-8 and GCP-2 in HMVEC, whereas in HUVEC, IL-1β was clearly the best inducer. The double-stranded RNA, poly rI:rC, was a weak inducer of both GCP-2 and IL-8 in these endothelial cell types. This indicates that bacterial, rather than viral products are responsible for ELR⁺CX₃C chemokine expression in endothelial cells, especially in HMVEC. In vitro, GCP-2 production by various cell types in response to inflammatory stimuli has earlier been demonstrated [195]. The fact that the in vitro expression of GCP-2 and IL-8 is differently regulated, although these CXC chemokines functionally bind to the same receptors (CXCR1 and CXCR2), further supports the idea that these molecules play a different role in inflammation.

We further examined whether chemokine concentrations in the serum of IBD patients could be used to differentiate between CD and UC by measuring the concentrations of different CC and CXC chemokines (MIP-1α, HCC-1, PARC,
eotaxin, GCP-2 and IL-8). In the sera of IBD patients and healthy subjects, we could not measure any detectable level of the inflammatory/inducible chemokines tested (IL-8, GCP-2, MIP-1α). These results are not totally unexpected since IBD is a chronic disease and chemokines are quickly cleared from the circulation. On the contrary, measurable levels of eotaxin and the plasma/constitutive chemokines HCC-1 and PARC were found, but again no differences were observed between healthy controls and IBD patients. This is in contrast to other autoimmune or infectious diseases such as rheumatoid and septic arthritis or to cancer in which enhanced levels of PARC could be detected in body fluids or plasma [135,196].

Finally, we determined whether the differences in IL-8 and GCP-2 expression, as observed in tissue sections, were also detected in isolated leukocytes. Nor in IBD patients, nor in healthy subjects, stimulated PBMC did produce detectable levels of GCP-2. In contrast, PBMC from CD patients, when stimulated with exogenous inflammatory mediators (LPS, Con A or poly rI:rC), produced significantly less IL-8 protein than PBMC from UC patients or healthy subjects. Since this was noticed for several unrelated chemokine inducers, recognizing different cellular receptors, this indicates that a general rather than a specific refractory state is observed in CD patients. The lowered IL-8 protein levels in PBMC of CD patients were not the consequence of the patients’ treatment with anti-inflammatory drugs or of a difference in leukocyte formula. Indeed, when we compared data from CD and UC patients, treated with the same drugs, IL-8 production was still different (p < 0.05). Since blood collection of IBD patients was not directly coinciding with drug intake, our findings do not necessarily contradict the findings of Grimm et al. who found a generalized reduction in chemokine release (both IL-8 and MCP-1) after in vitro stimulation of PBMC by LPS in the presence of anti-inflammatory drugs [96,105]. Since the IL-8 production by stimulated blood leukocytes of CD patients is lowered, high local production of IL-8 by involved intestinal tissue might facilitate the emigration of neutrophils out of the blood stream into the inflamed tissue. However, the question remains whether the lowered IL-8 production by PBMC in CD patients is a tissue specific phenomenon or rather due to a genetic defect. It is indeed tempting to explain the lowered IL-8 production with the defective intracellular receptor for peptidoglycan, Nod2/CARD15, that has been linked with CD [69,70,197]. However,
since the decrease in IL-8 production was noticed for several unrelated inducers (LPS, Con A or poly rI:rC) binding different receptors, a defective Nod2 receptor does not provide a full explanation for the observations in CD patients. In addition, we observed that the production of MIP-1α, a CC chemokine that is well induced by LPS [198], was not affected in PBMC from CD patients. The reduced IL-8 production thus seems to be both chemokine- and disease specific, but not inducer specific and could be useful as a parameter to distinguish between CD and UC.
GCP-2 in gastrointestinal tumors

Introduction

During the host response against an acute infection, the subtle interplay between chemokines and adhesion molecules is needed to selectively attract specific leukocyte subsets, expressing chemokine receptors, to the site of inflammation [199]. In this context, chemokines can synergize with each other or with other inflammatory mediators to enhance leukocyte infiltration in an early phase [200]. At a later stage, chemokines can dampen the inflammatory response when they are processed into receptor antagonists by proteases [201]. In addition to their indispensable role in healing processes, chemokines can become detrimental in pathological situations such as cancer [202], rheumatoid arthritis, multiple sclerosis and many other diseases [203].

Gastrointestinal (GI) malignancies are common tumors. Histologically, they are a heterogeneous group of neoplasms. Epithelial malignancies, mainly adenocarcinomas are the most common type. The prognosis of these malignancies is variable but on the average poor, except when the lesion is detected at an early stage. For the growth and survival of a tumor, angiogenesis is essential for the supply of oxygen and nutrients [204]. Therefore, inhibition of angiogenesis has become an important target in tumor therapy.

Mesenchymal cells, including fibroblasts and macrovascular endothelial cells, have earlier been shown to produce GCP-2 and to display angiogenic properties in the rat corneal micropocket model of neovascularization [14,195]. Overexpression of the murine GCP-2 analog in human tumor cell allografts resulted in enhanced angiogenesis [129]. Although GCP-2 has thus been shown to be important for angiogenesis in these animal models, the presence and role of GCP-2 in tumor development has not been investigated yet in humans.
Results

1. Immunohistochemical staining of GCP-2 in human gastrointestinal tumors

To investigate the role of GCP-2 in angiogenic processes involved in tumor progression and metastasis, we determined whether GCP-2 was specifically expressed in surgical specimens of patients who were operated for different types of GI malignancies (3 oesophagus, 4 stomach, 6 colon, 2 small intestine and 7 pancreas). GCP-2 was expressed by endothelial cells in the stroma of 15/22 tumor biopsies, but was not expressed by endothelial cells in normal tissue from non-involved areas (Table 4.2). Endothelial cells stained positive for GCP-2 in all the oesophagus (3) and colon (6) tumors (Figure 4.7). Only 2/4 and 3/7 of the stomach and pancreas biopsies, respectively, showed positive endothelial cells in tumoral areas. No staining was observed in the two non-epithelial GIST malignancies, while endothelial cells in the lymphoma stained positive for GCP-2.

Table 4.2  GCP-2 immunoreactivity by endothelial cells per tumor type a)

<table>
<thead>
<tr>
<th>Location of tumor</th>
<th>Type of tumor</th>
<th>Number of cases</th>
<th>GCP-2 a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>oesophagus</td>
<td>adenocarcinoma</td>
<td>3</td>
<td>3/3</td>
</tr>
<tr>
<td>stomach</td>
<td>adenocarcinoma</td>
<td>3</td>
<td>2/3</td>
</tr>
<tr>
<td></td>
<td>GIST c)</td>
<td>1</td>
<td>0/1</td>
</tr>
<tr>
<td>colon</td>
<td>adenocarcinoma</td>
<td>6</td>
<td>6/6</td>
</tr>
<tr>
<td>small intestine</td>
<td>GIST c)</td>
<td>1</td>
<td>0/1</td>
</tr>
<tr>
<td></td>
<td>B cell lymphoma</td>
<td>1</td>
<td>1/1</td>
</tr>
<tr>
<td>pancreas</td>
<td>ductal carcinoma</td>
<td>7</td>
<td>3/7</td>
</tr>
</tbody>
</table>

a) For immunohistochemistry, biopsy samples were obtained from patients operated for gastrointestinal malignancy
b) Tumors were classified and staged according to the WHO histological classification [189] and the TNM classification of malignant tumors [190]. T = tumor, N = node, V = vessel, M = metastasis.
c) GIST = gastrointestinal stromal tumor.
d) The ratio of cases with GCP-2 expressing endothelial cells is represented per tumor type.
To determine whether all endothelial cells in the tumor or only a subset of them expressed GCP-2, endothelial cells were immunohistochemically identified by CD31 staining (Figure 4.7). Of all the GCP-2 positive cases (15/22), only in one oesophagus, one colon, 2 pancreas biopsies and the B cell lymphoma, the distribution of the staining for CD31 was similar to that seen for GCP-2 (i.e. homogeneous throughout the malignant tissue). This indicates that, in most biopsies, not all vascular structures of the tumors stained positive for GCP-2. In three cases (2 colon and 1 stomach), GCP-2 staining was mainly situated at the periphery of the tumor, namely at the junction of tumoral and non-tumoral tissue.

Figure 4.7  Inflammation-related expression of GCP-2 by endothelial cells in cancer
Microphotograph of a moderately well differentiated adenocarcinoma of the colon. Immunohistochemical staining with antibodies directed against GCP-2 shows positively stained endothelial cells (bright-red) of blood vessels in the tumor (arrowhead). The nature of the cells is confirmed by staining serial sections with antibodies against CD31 (arrowhead). CD45⁺ leukocytes (arrowhead) are adjacent to GCP-2⁺ vessels.

VEGF is an important regulator of angiogenesis and has been studied extensively in tumors. Since not all endothelial cells in the investigated tumor biopsies stained positive for GCP-2, we examined whether there was a correlation between GCP-2 positivity in endothelial cells and the presence of the angiogenic factor VEGF in tumor cells (data not shown). In almost all the biopsies from epithelial tumors (17/19), tumor cells stained positive for VEGF. The staining was weak, except for 4 pancreatic tumor biopsies where a prominent, homogeneous VEGF positivity was observed. Two of these four biopsies were negative for GCP-2. The three non-epithelial tumors were negative for VEGF.
Since GCP-2 is a leukocyte chemoattractant, we further determined whether a correlation existed between the presence of leukocytes (producer cells of cytokines that can induce GCP-2) and GCP-2 expression by endothelial cells. Therefore, the tumor samples were tested with the leukocyte marker CD45 and 20/22 tumor biopsies stained positive for CD45. Seven of the CD45+ biopsies were negative for GCP-2, whereas 13 were positive for GCP-2. As expected, the presence and location of leukocytes in tumoral biopsies correlated with GCP-2 expression (Figure 4.7). Because GCP-2 is known to selectively chemoattract neutrophilic granulocytes, we finally stained the biopsies for the matrix metalloproteinase-9 (gelatinase B) that is released from the granules of neutrophils, activated by GCP-2. Leukocytes stained positive for gelatinase B and the positively stained cells were solitary cells or small clusters of cells diffusely distributed throughout the neoplastic tissue (Figure 4.8). Finally, blood vessel staining for the related prototype CXC chemokine IL-8 was negative with the methodology used. Occasional mononuclear cells stained positive (Figure 4.8).
Figure 4.8 Immunohistochemical staining for GCP-2, gelatinase B and IL-8

The upper and middle panels show microphotographs of a poorly differentiated carcinoma of the pancreas stained with antibodies against GCP-2 and gelatinase B showing the diffuse distribution of gelatinase B expressing leukocytes and a relation with GCP-2 expressing blood vessels (arrowhead indicates the same horizontal Y-shape in serial sections). The lower panel shows normal colon mucosa and indicates that leukocytes, staining positive for IL-8, are solitary cells in the stroma.
2. Production of GCP-2, IL-8 and MCP-1 by HMVEC in response to immunomodulators

In view of the in vivo observed expression of GCP-2 by vascular cells, the production of this angiogenic CXC chemokine by endothelial cells was further investigated in vitro and compared in detail with the related neutrophil-attracting chemokine IL-8 and the CC chemokine MCP-1. Therefore, microvascular endothelial cells (HMVEC), which form an essential entity of the neovascularization process of tumors, were stimulated in vitro with various immunomodulators. GCP-2 protein production was determined by a specific ELISA, which did not cross-react with structurally related chemokines, including IL-8 and ENA-78.

The tumor promoting agent PMA, the mitogen Con A, bacterial SEA, and the cytokines IL-4, IL-10, IFN-β and IFN-γ failed to induce significant amounts of IL-8, GCP-2 or MCP-1 in HMVEC (Figure 4.9). In contrast, a dose-dependent co-induction of these two angiogenic CXC chemokines and MCP-1 was obtained with the Toll-like receptor ligands, bacterial LPS and viral dsRNA (poly rI:rC), as well as with the endogenous pro-inflammatory cytokines TNF-α and IL-1β. In all conditions, the production levels of MCP-1 (± 60 ng/ml) and IL-8 (± 20 ng/ml) were higher than those of GCP-2 (± 2 ng/ml). IL-1β seemed to be a better inducer of GCP-2 than TNF-α, whereas the opposite was found for MCP-1. LPS was superior to dsRNA to induce GCP-2, IL-8 and MCP-1. In contrast to HMVEC, carcinoma cell lines (HeLa, Caco-2) did not produce detectable amounts of GCP-2 in response to any of the inducing substances tested nor was the supernatant of stimulated Caco-2 tumor cells able to induce IL-8 or GCP-2 in HMVEC (data not shown). Taken together, these data demonstrate that the simultaneous induction of these chemokines is co-regulated and that GCP-2 is produced in smaller quantities than IL-8 and MCP-1.
Figure 4.9. IL-8, GCP-2 and MCP-1 production by microvascular endothelial cells

Confluent monolayers of HMVEC were stimulated for 72 hours with different doses of TNF-α, IL-1β, IL-4, IL-10, IFN-β, IFN-γ, poly rI:rC, LPS, SEA, ConA, PMA or were left untreated (control). Conditioned media were used to determine IL-8, GCP-2 and MCP-1 protein concentrations by ELISA. Results are the mean ± SEM of six independent experiments. Significant chemokine induction (above constitutive expression) is indicated by asterisks (* p < 0.05; ** p < 0.01; *** p < 0.001).
3. Effect of GCP-2 on endothelial cell proliferation

Although chemokines that bind to the CXC chemokine receptor CXCR2, like IL-8 and GCP-2, have been reported to induce endothelial cell migration [14], the capacity of GCP-2 to stimulate endothelial cell growth has not been established yet. To that goal, HMVEC were treated with GCP-2 in parallel with FGF, a known stimulator of endothelial cell growth. Figure 4.10 illustrates that at 30 ng/ml GCP-2 weakly enhanced (25%) $[^3]H$-thymidine incorporation in HMVEC compared to basal proliferation (medium alone), whereas FGF caused a doubling of the counts at a concentration as low as 1 ng/ml. These findings indicate that GCP-2 alone is rather a marginal stimulus of HMVEC growth compared to FGF.

![Figure 4.10 Chemokine-induced endothelial cell proliferation](image)

HMVEC were stimulated with different concentrations of GCP-2 (1-77). Assay medium and FGF were used as negative and positive controls, respectively. After 48 hours, the cells were incubated with $[^3]H$-thymidine (0.6 μCi/vial) for 5 hours and incorporation of $[^3]H$-thymidine was determined by measuring the amount of radioactivity with a liquid scintillation counter. The obtained results are the mean ± SEM of 3 to 9 replicates in two independent experiments. As a correction for inter-assay variability, thymidine incorporation is expressed as the percentage of radioactivity in comparison to the negative control. Endothelial cell proliferation was considered statistically significant (Mann-Whitney U-test) if p-values were <0.05 (** p < 0.01; *** p < 0.001).
4. **MCP-1 synergistically enhances the in vitro neutrophil chemotactic activity of GCP-2**

Since microvascular endothelial cells produced only very low amounts of GCP-2 in comparison with MCP-1, we studied the possible interplay of these two chemokines that activate different receptors during inflammatory reactions. Therefore, we investigated the neutrophil chemotactic activity of GCP-2(1-77) and GCP-2(9-77) in the Boyden microchamber assay for chemotaxis in combination with MCP-1. GCP-2(1-77) and GCP-2(9-77) are two naturally occurring GCP-2 isoforms that are equipotent neutrophil chemoattractants [205], yielding a chemotactic index (CI ± SEM) of 4.2 ± 1.6 (n=10) and 4.4 ± 2.9 (n=10), respectively, at 15 ng/ml (Figure 4.11).

![Figure 4.11 Synergy between GCP-2 and MCP-1 in neutrophil chemotaxis](image)

To measure the chemotactic effect of GCP-2(1-77), GCP-2(9-77), IL-8 and MCP-1 on neutrophils (10⁶/ml), diluted chemokine samples were added to the lower well of a Boyden microchamber. As a negative control, chemotaxis buffer was used. Synergy experiments (n=3 to 10) were performed by adding two different chemokines together to the lower wells of the chamber. To see whether the chemotactic activity (CI) of the combination of chemokines statistically differed from the sum of that of the chemokines alone (= synergy), statistical analysis was performed using the Mann-Whitney U-test. Differences between the CI were considered statistically significant if p-values were < 0.05 (*) or < 0.01 (**).
The CC chemokine MCP-1, on the contrary, is a weak stimulator of neutrophil chemotaxis (1.4 ± 0.3, n=10) at 100 ng/ml (Figure 4.11). Simultaneous addition, however, of physiological concentrations of MCP-1 (100 or 300 ng/ml) and GCP-2 (15 ng/ml) resulted in a statistically significant increase in neutrophil migration (Figure 4.11). Indeed, a 2- and 15-fold augmentation of the neutrophil chemotactic activity of both GCP-2(1-77) and GCP-2(9-77) was observed by the addition of 100 and 300 ng/ml of MCP-1, respectively. For GCP-2(1-77), the addition of MCP-1 (100 or 300 ng/ml) resulted in a CI ± SEM of 11.5 ± 8.1 (n=10) and 64.7 ± 22.4 (n=10) respectively. For GCP-2(9-77), a CI ± SEM of 9.4 ± 6.3 (n=10) and 97.4 ± 22.8 (n=10), was obtained by the addition of 100 or 300 ng/ml MCP-1, respectively. This indicates that chemokines produced at low levels (GCP-2) can enhance inflammatory reactions in the presence of other chemokines. GCP-2 and MCP-1 mediate leukocyte chemotaxis through different receptors (CXCR2 and CCR2, respectively). Therefore, we investigated whether both receptors are crucial in evoking synergistic neutrophil chemotaxis, or whether one receptor would be sufficient. The possible implication of the two chemokine receptors in the observed synergy was determined by the use of different chemokine receptor antagonists (Figure 4.12). Synergy in neutrophil chemotaxis (n=5, CI ± SEM of 25.0 ± 1.5) induced by the combination of GCP-2(9-77) (15 ng/ml) and MCP-1 (300 ng/ml) was significantly lowered (77% inhibition, p = 0.009) by the addition of 10 µM of the non-peptide CXCR2 antagonist, SB225002, to a CI ± SEM of 5.6 ± 1.8 (n=5). The addition of 10 µM of the CCR2 antagonist, RS102895, also significantly lowered (31% reduction, p = 0.047) the synergy of GCP-2(9-77) with MCP-1 to a CI ± SEM of 17.1 ± 3.0 (n=5). The combination of SB225002 and RS102895 caused an inhibition (76%) similar to that of SB225002 alone, that is, to a CI ± SEM of 6.0 ± 1.5 (n=4).
RESULTS: GCP-2 IN GASTROINTESTINAL TUMORS

Figure 4.12  Inhibition of chemokine-induced synergy by chemokine receptor antagonists

To measure the chemotactic effect of GCP-2(9-77) (15 ng/ml), MCP-1 (300 ng/ml) or their combination on neutrophils, chemokine samples were added to the lower well of a Boyden microchamber. As a negative control, chemotaxis buffer was used. Inhibition of chemotaxis was performed by the addition of chemokine receptor antagonists (10 µM) to the cells (10⁶/ml) just before transfer to the upper well of the chamber. SB225002 and RS102895 were used as specific CXCR2- and CCR2-antagonists, respectively. To see whether the synergy in chemotactic activity (CI) by the combination of chemokines was significantly lowered in the presence of a single receptor antagonist (n=5) or the two antagonists together (n=4), statistical analysis was performed using the Mann-Whitney U-test. Differences between the CI were considered statistically significant if p-values were < 0.05 (*) or < 0.01 (**).
Discussion

In 1995, Strieter et al. demonstrated that CXC chemokines could be classified into angiogenic and angiostatic subgroups, based on the presence or absence of the ELR-motif. ELR$^+$CXC chemokines, such as IL-8, GCP-2 or growth-related oncogene (GRO/CXCL1), display their angiogenic properties through CXCR2 binding [206-208]. This activity is counteracted by ELR$^-$CXC chemokines which recognize CXCR3, such as interferon-γ-inducible protein-10 (IP-10/CXCL10) or PF-4 [209,210]. From this point of view, chemokines have gained importance in vascular biology since their role in angiogenesis extended their function as chemoattractive inflammatory mediators.

The precise molecular mechanisms of chemokine activities related to neovascularization during inflammation or tumor growth are not yet fully understood [22]. However, from this study it is evident that microvascular endothelial cells, which form an essential entity of the neovascularization process of tumors, are a good source of CXC chemokines. Indeed, GCP-2 and IL-8, but also the CC chemokine MCP-1 are co-induced in HMVEC by pro-inflammatory stimuli, including LPS, dsRNA, IL-1β and TNF-α. These findings confirm previous results on macrovascular endothelial cells [195] and could suggest that GCP-2 contributes to neovascularization in an autocrine way. However, GCP-2 provoked only weak endothelial cell growth compared to FGF, which is a standard growth factor for these cells. This proliferative effect of GCP-2 on HMVEC is in agreement with that of IL-8 [211]. In sharp contrast with its weak proliferative effect on endothelial cells, GCP-2 is a potent chemoattractant for neutrophils, even after limited posttranslational N-terminal processing by proteases, such as gelatinase B that is released from GCP-2-activated neutrophils [133,205]. Moreover, we here describe that, at physiologically relevant concentrations, GCP-2 and MCP-1, both secreted by HMVEC, synergize to yield a ten-fold increase in the neutrophil chemotactic response, compared to the combined effect of the two chemokines separately. Synergy between chemokines at suboptimal concentrations may therefore be a mechanism to provide efficient leukocyte
infiltration in mild inflammatory conditions or in the absence of exogenous microbial immune modulators as is the case during tumor development and metastasis. With the use of specific chemokine receptor antagonists, we showed that the observed synergy between GCP-2 and MCP-1 was significantly inhibited by a CCR2 antagonist (for MCP-1) as well as by a CXCR2 antagonist (for GCP-2). It seems thus that both receptors are implicated in this synergistic effect on neutrophil migration. CXCR2, which is more abundantly expressed on neutrophils than CCR2, might play a more important role. Since MCP-1 and GCP-2 use similar but still different signal transduction pathways, the observed synergy between chemokines might occur at this level.

Finally, the presence of GCP-2 was investigated in vivo in relation to GI malignancies. It was shown by immunohistochemistry that GCP-2 was preferentially expressed by blood vessel cells in zones of tumoral neovascularization. The expression pattern, however, did not fully match with that of the angiogenic mediator, VEGF. In addition, staining with the endothelial cell marker, CD31, indicated that only a specific subset of endothelial cells expressed GCP-2. In contrast, GCP-2 staining did correlate with leukocyte infiltration (CD45+ cells) into the tumor as well as with the expression of the matrix metalloproteinase-9, gelatinase B. Taken together, our data strengthen the idea of an important role for chemokines in tumor growth and metastasis [202]. The production of biologically active GCP-2 by endothelial cells within the tumor can contribute to tumor development through neovascularization due to endothelial cell chemotaxis [14] and to tumor metastasis by attracting and activating neutrophils loaded with proteases, such as gelatinase B, which promote matrix degradation and hence the escape of tumor cells by the so-called countercurrent principle [144].
General discussion and conclusions

An important aspect of the inflammatory response against infection or physical damage is the release of chemokines. Through binding to specific membrane-bound receptors, these chemokines exert a plethora of functions. By forming a chemotactic gradient, inducing the release of proteases and increasing expression of adhesion molecules, chemokines enable transendothelial migration of immune cells to the site of inflammation. Besides these inducible/inflammatory chemokines that recruit leukocytes in response to physiological stress, constitutive/homeostatic chemokines are responsible for basal trafficking of leukocytes as well as proper positioning of these leukocytes within secondary lymphoid organs. The expression of inducible chemokines can be elicited in many different cell types by almost any stimulus that alters cellular homeostasis. Although chemokines are essential to assure normal functioning of the innate and adaptive immune system, they can become harmful and evoke disease when their expression becomes persistent or exuberant.

The intestine forms the interface of the internal compartment with the environment and needs to ensure proper digestion and uptake of nutritional agents while protecting against nocuous agents. As a consequence, in comparison to other tissues, there are always immune cells present in the intestinal wall (“physiological inflammation”). These immune cells compose the gut-associated lymphoid tissue and confer intestinal homeostasis. The attraction and positioning of immune cells within the intestinal wall is regulated by chemokines. In inflammatory bowel diseases, uncontrolled chemokine expression within the intestine causes a huge infiltration of immune cells that results in damage to the intestine and chronic inflammation. Neutrophils are normally absent.
from the intestine and can therefore be used as a marker for “pathological inflammation” in inflammatory bowel diseases.

In this doctoral thesis, research was performed on the expression and function of the CXC chemokine neutrophil chemoattractant GCP-2 in gastrointestinal diseases, namely inflammatory bowel diseases (IBD) and gastrointestinal malignancies.

In intestinal tissue sections of patients with IBD (ulcerative colitis and Crohn’s disease), both GCP-2 and IL-8 were expressed by leukocytes present in the lamina propria. The number of positive cells correlated with the histological grade of inflammation. The cells staining positive had the morphology of mononuclear leukocytes. In addition to these mononuclear leukocytes, endothelial cells in tissue with epithelial damage expressed GCP-2 but not IL-8. Endothelial cells more remote from the actively inflamed sites, did not express GCP-2 or IL-8. Since sites of epithelial damage are dependent on angiogenesis for healing and new-formation of tissue, we hypothesized that only newly formed endothelial cells expressed GCP-2 (vide infra). On the one hand, the angiogenic properties of GCP-2 could be beneficial for healing of the tissue. On the other hand, neutrophils attracted by the chemokine can worsen tissue damage by releasing proteinases and toxic metabolites.

The expression of human GCP-2 in inflamed intestinal tissue may have biologically relevant effects since this chemokine is a potent neutrophil chemoattractant, as evidenced in rabbits. Within five minutes after administration of GCP-2 into the blood circulation, neutrophil counts dropped 5-fold. This neutropenia was followed by a severe rise in the number of circulating neutrophils (4-fold above starting level) explained by a massive recruitment of neutrophils out of the bone marrow. These results indicate that GCP-2 is a potent neutrophil chemoattractant and that expression by endothelial cells can stimulate the influx of neutrophils into the inflamed tissue.

The presence of neutrophils in tissue at sites of epithelial damage could have a dual effect [62]. On the one hand, neutrophils form the first line of defense against infectious agents or “nonself” substances that can easily enter the intestinal wall in case of epithelial damage. The release of their granules containing reactive oxygen metabolites, hydrolytic enzymes and antimicrobial peptides renders the neutrophil a key player in early host defense [212]. On the other hand, neutrophils can cause severe
tissue damage when they are activated premature or when termination of their activation fails.

IL-8 is the first identified chemokine and hence intensively studied, whereas research on GCP-2 has been less extensive so far. In addition, IL-8 is a most abundantly secreted and potent neutrophil chemoattractant. We propose, however, that structurally related chemokines such as GCP-2, although often co-produced and acting in the same manner, have their own specific role and can act complementary. The differential expression of GCP-2 and IL-8 by endothelial cells at places of epithelial damage strengthens this idea. We therefore investigated the release of these highly similar chemokines more in detail. Hence, we stimulated macrovascular as well as microvascular endothelial cells with exogenous microbial products (viral double-stranded RNA or bacterial endotoxin) as well as the endogenous proinflammatory cytokine IL-1β to stimulate chemokine expression. On both cell types, the optimal condition for induction of GCP-2 and IL-8 were similar. However, the expression levels of IL-8 were always ± 40-fold higher than those of GCP-2, whereas the kinetics of release were similar. Quantitatively, these in vitro data did not reflect the more pronounced expression of GCP-2 versus IL-8 in IBD biopsies, indicating that other, yet undefined substances are responsible for chemokine expression in vivo.

Measuring chemokine levels in the serum of IBD patients would have been an easy way to discriminate between UC and CD. For most chemokines expression was, however, too low to be detectable and the serum levels of those chemokines that were measurable did not differ from those of control subjects. Alternatively, we stimulated peripheral blood mononuclear cells (PBMC) from UC and CD patients with viral double-stranded RNA, bacterial endotoxin or the plant lectin concanavalin A and compared chemokine release with that of PBMC from control subjects. IL-8 expression by PBMC of CD patients was significantly lower than that of UC patients or control subjects. The lowered chemokine production was observed for the different inducers supporting the idea of a general refractory state in CD. A difference in patient treatment or in leukocyte formula between CD, UC or control subjects to explain the lowered chemokine production was excluded. Induction of the CC chemokine MIP-1α was evaluated as well, but the production level was the same for the different patient groups. GCP-2, although functionally similar to IL-8, was not produced at detectable
amounts by stimulated PBMC. Together, these data favour the idea of a genetic basis for the dysfunction observed in CD. In 2001, evidence for a genetic basis of the disease was furnished by the discovery of a susceptibility gene (i.e. NOD2/CARD15), specific for CD but not for UC [69,70]. Homozygous mutations in this gene, encoding an intracellular receptor for bacterial peptidoglycan, are responsible for a 40-fold increased risk to develop CD. Although we observed lowered IL-8 production with inducers, different from peptidoglycan, we believe that other genetic defects could also be responsible for the uncontrolled intestinal immune response in CD. The lowered IL-8 production by PBMC of CD patients do not directly explain the massive infiltration of neutrophils as observed in the intestinal wall. It could be possible, however, that low IL-8 production in the blood circulation, together with high local production of neutrophil-attracting chemokines within the inflamed intestine create a chemotactic gradient that favours the transendothelial migration of neutrophils into the intestinal wall. In addition, other specific neutrophil chemoattractant chemokines such as ENA-78 and GRO could be implicated.

Normal inflammation is self-limiting because the production of anti-inflammatory cytokines closely follows that of proinflammatory cytokines. When inflammation persists, DNA damage can result and give rise to tumor development. As such, the risk for colon cancer is higher for patients that suffer from long and severe colonic inflammation. To grow, a tumor is dependent on growth factors and nutrients delivered by blood vessels. During embryogenesis, endothelial cells are formed from endothelial progenitor cells (vasculogenesis), while in adults new blood vessels originate from the existing vasculature (angiogenesis). Except for the female reproductive cycle, angiogenesis is mostly associated with pathological situations [148]. Therefore, the deprivation of oxygen and nutrients by destroying the vasculature of malignant tissue seems to be an increasingly important target in anti-tumor-therapy. Moreover, tumor blood vessels displaying specific surface markers could be targeted based on recognition by a selective drug [149,213,214]. Since only endothelial cells at places of epithelial damage expressed GCP-2 (vide supra), we proposed the ability of selective GCP-2 expression by newly formed blood vessels. Since tumors are dependent on newly formed blood vessels, we continued with immunohistochemical staining of intestinal tissue sections from gastrointestinal tumors. The expression of
GCP-2 was preferentially observed by endothelial cells at zones of tumoral neovascularization. No tumor cells stained positive for GCP-2 although this chemokine was initially purified from conditioned medium of MG-63 osteosarcoma cells. This probably indicates that the in vivo situation differs from experiments performed in vitro or that the expression of GCP-2 depends on the original tissue from which the tumor develops. Immunohistochemical staining of serial sections for CD31, a marker for endothelial cells, indicated that not all endothelial cells stained positive for GCP-2 reinforcing the idea that GCP-2 expression might be restricted to newly formed endothelial cells. We therefore did additional staining of serial sections for VEGF. This protein is an important mediator of angiogenesis and has recently been correlated with a bad prognosis for tumor progression. Hypoxic conditions favor the production of VEGF and thus of angiogenesis and tumor growth. Since the expression pattern of VEGF and GCP-2 did not fully match, it might be possible that VEGF is associated with angiogenesis due to hypoxia, whereas GCP-2 expression might be rather correlated with angiogenesis due to inflammation. Additional staining for CD45, a marker of leukocytes, confirms the correlation between GCP-2 expression and the presence of inflammatory cells. The relevant effect of GCP-2 as a neutrophil chemoattractant was established by staining of serial sections for gelatinase B, a matrix metalloproteinase released from the granules of neutrophilic granulocytes by GCP-2. Gelatinase B expression in gastrointestinal tumors correlated with the expression of GCP-2. In contrast, blood vessel staining of the intestinal tissue sections was negative for IL-8 although occasional mononuclear cells stained positive. The expression of chemokines within tumors can have a dual effect. On the one hand, chemokine expression is necessary to create an inflammatory infiltrate to clear the pathogen (if there is one) and to create a cell-mediated immune response. On the other hand, inflammatory cells can be harmful by delivering growth factors for the tumor, by releasing proteases and angiogenic chemokines that favor metastasis. In addition, tumor cells bearing chemokine receptors can metastasize to places of chemokine production. Although GCP-2 was formerly shown to attract endothelial cells and to stimulate angiogenesis, endothelial cell proliferation was weak in comparison with FGF.
In chemotactic assays, GCP-2 is a weak chemoattractant in comparison with IL-8. However, in combination with MCP-1, a CC chemokine binding to a different chemokine receptor (CCR2), the neutrophil chemotactic properties of GCP-2 augment significantly (15-fold). A similar synergistic effect in neutrophil chemotaxis can be observed when MCP-1 is combined with IL-8. As such, it seems again that GCP-2, in comparison with IL-8, has little impact. The additive value of these findings, however, lies in the fact that in cases where IL-8 expression is limited or absent, GCP-2 can be an enormously potent neutrophil chemoattractant when MCP-1 is also produced. *In vitro* induction experiments confirm the coexpression of GCP-2 and MCP-1 by microvascular endothelial cells. In contrast to other chemokines, GCP-2 conserves its chemotactic activity after limited proteolytic processing at the NH$_2$-terminus, since GCP-2(9-77) is equipotent to GCP-2(1-77). The chemokine family has often been depicted as redundant (many chemokines can bind more than one receptor and many receptors can bind several chemokines) and as such of little value for therapy. Taken together, however, our findings point to a complementary and even synergistic value of a multiple chemokine system. Rather the type of chemokine, the site and duration of expression as well as the combined effect of multiple chemokines in a network will be of importance than just the individual effect of each chemokine. The fact that GCP-2 and IL-8 seem to have their own specific expression pattern in different diseases (IBD and gastrointestinal tumors) suggests that both chemokines have their own role in inflammatory processes, although they are structurally and functionally related.
During evolution, the immune system of humans has adapted itself to confer protection to a wide range of stress conditions. Besides physical barriers, the human body is equipped with an army of immune cells consisting of different subsets, each fulfilling specific tasks. In case of inflammation due to infection, tumor growth or tissue damage, our body relies on the interplay of the innate immune response, acting fast and non-specific, and the adaptive immune response, acting slower but specific. Neutrophils are present in the blood circulation and belong to the immune cells responding first to microbial agents. Upon stimulation, they leave the blood circulation and enter the inflamed tissue to scavenge harmful microorganisms. When the activation of neutrophils persists or is initiated too early, they can cause severe tissue damage due to the release of toxic metabolites. By attracting and activating immune cells, chemokines play a crucial role in the onset and perpetuation of inflammatory reactions. Chemokines are small proteins that are released by many different cell types upon stimulation with proinflammatory mediators. Besides leukocyte recruitment in response to physiological stress, chemokines are also responsible for basal leukocyte trafficking to secondary lymphoid organs during homeostasis.

In many diseases, overexpression of chemokines causes an uncontrolled immune response that leads to chronic inflammation. Crohn’s disease and ulcerative colitis are inflammatory bowel diseases (IBD) that are characterized by chronic intestinal inflammation and a constant influx of leukocytes, mediated by proinflammatory cytokines and chemokines. Granulocyte chemotactic protein-2 (GCP-2/CXCL6) is an angiogenic chemokine that selectively attracts neutrophilic granulocytes. Functionally,
it shows high homology with interleukin-8 (IL-8/CXCL8) and both chemokines bind the same G protein-coupled receptors (CXCR1 and CXCR2) to exert their activity. In this thesis, the intestinal expression of the chemokines IL-8 and GCP-2 and the participation of immunocompetent cells in IBD were evaluated. IL-8 production by peripheral blood mononuclear cells (PBMC) from IBD patients, stimulated with bacterial endotoxin, viral double-stranded RNA or plant lectin, was significantly lowered in patients with Crohn’s disease, but not in ulcerative colitis patients or healthy subjects. The reduced chemokine production by PBMC from IBD patients was both IL-8 and Crohn’s disease specific, but not inducer dependent. In the serum of IBD patients, chemokine levels were not detectable or remained unaltered compared with control subjects. GCP-2, but not the structurally related chemokine epithelial cell-derived neutrophil attractant-78 (ENA-78/CXCL5), nor IL-8, was expressed by endothelial cells in inflamed intestinal tissue of IBD patients. In contrast, in vitro stimulated macrovascular endothelial cell cultures produced more IL-8 than GCP-2. The selective GCP-2 staining of endothelial cells at sites of epithelial damage suggests that GCP-2, despite its low production level in vitro, plays a role in IBD, different from that of structurally (ENA-78) and functionally (IL-8) related chemokines.

Furthermore, we have shown that GCP-2, IL-8 and monocyte chemotactic protein-1 (MCP-1/CCL2) are co-induced in microvascular endothelial cells after stimulation with proinflammatory stimuli. To mimic endothelial cell-derived GCP-2 in vivo, GCP-2 was intravenously injected in rabbits and shown to provoke a dose-dependent systemic response, composed of an immediate granulopenia, followed by a profound granulocytosis. Moreover, in vitro GCP-2 synergized with MCP-1 in neutrophil chemotaxis which may represent a mechanism for tumor development and metastasis by providing efficient leukocyte infiltration. Immunohistochemical analysis of inflamed intestinal tissue sections from IBD patients suggested that GCP-2 is specifically expressed by newly formed blood vessels. The precise role of chemokines in neovascularization during inflammation or tumor growth is not yet fully understood. By immunohistochemistry, GCP-2 was further shown to be expressed by endothelial cells from human patients with gastrointestinal malignancies. GCP-2 staining correlated with leukocyte infiltration into the tumor and with the expression of the
matrix metalloproteinase gelatinase B that facilitates angiogenesis by degrading the extracellular matrix.

Together, these data suggest that the expression of GCP-2 and IL-8 is differently regulated *in vitro* and *in vivo* and that GCP-2 and IL-8 have their own role in inflammatory processes although they are structurally and functionally related. Thus, the chemokine network shows complementarity, rather than redundancy.
Samenvatting

Doorheen de evolutie heeft het immuunsysteem van de mens zich aangepast aan verschillende stresscondities om zo een geschikte verdediging te verzekeren. Naast fysische barrières beschikt het menselijke lichaam over een waar leger van immuuncellen. Bij ontsteking, als gevolg van een infectie, tumorgroei of weefselschade, doet ons lichaam beroep op de interactie van de aangeboren immuunreactie, die snel en aspecifiek werkt, en de aangepaste immuunreactie, die trager maar specifiek werkt. Neutrofiele granulocyten zijn aanwezig in de bloedsomloop en behoren tot de immuuncellen die als eerste reageren op microbiële infectie. Na stimulatie verlaten ze de bloedsomloop en dringen het ontstoken weefsel binnen om er schadelijke microorganismen op te ruimen. Als de activering van neutrofielen aanhoudt of te vroeg begint, kunnen deze cellen ernstige weefselschade veroorzaken door de vrijzetting van giftige stoffen. Chemokinen spelen een belangrijke rol bij het ontstaan en het in stand houden van een immuunrespons doordat ze immuuncellen aantrekken en activeren. Chemokinen zijn kleine eiwitten die worden vrijgezet door verschillende celtypes na stimulatie met proinflammatoire stoffen. Naast het aantrekken van witte bloedcellen tijdens ontstekingen, zijn ze ook verantwoordelijk voor het gewone verkeer van witte bloedcellen naar de secundaire lymfoïde organen tijdens homeostase.

Overexpressie van chemokinen veroorzaakt bij vele ziektes een ongecontroleerde immuunreactie die aanleiding geeft tot chronische ontsteking. De ziekte van Crohn en colitis ulcerosa zijn inflammatoire darmziekten (IBD) die gekenmerkt worden door
chronische ontsteking van de darmen en een constante infiltratie van witte bloedcellen gemedieerd door cytokinen en chemokinen.

Granulocyt chemotactisch proteïne-2 (GCP-2/CXCL6) is een angiogeen chemokine dat selectief neutrofiele granulocyten aantrekt. Functioneel vertoont het een grote gelijkenis met interleukine-8 (IL-8/CXCL8). Beide chemokinen oefenen hun werking uit door binding aan dezelfde G proteïne-gekoppelde receptoren, nl. CXCR1 en CXCR2. In deze thesis werd de expressie van IL-8 en GCP-2 in de darm van IBD patiënten onderzocht. Daarnaast werd nagegaan hoe immuunmodulatoren de expressie van chemokinen beïnvloeden bij IBD.

Gestimuleerde mononucleaire cellen (geïsoleerd uit perifeer bloed) van patiënten met de ziekte van Crohn zetten significant minder IL-8 vrij dan mononucleaire cellen van patiënten met colitis ulcerosa of van gezonde personen. Verminderde productie van IL-8 werd waargenomen na stimulatie met verschillende ontstekingsmediatoren (bacterieel endotoxine, viraal dubbelstrengs RNA en plantenlectine) maar was specifiek voor patiënten met de ziekte van Crohn. In het serum van IBD patiënten konden geen chemokinen gemeten worden of waren de waargenomen concentraties vergelijkbaar met deze bij gezonde personen. In ontstoken darmweefsel van IBD patiënten werd GCP-2, maar niet het structureel verwante chemokine epitheelcel-afgeleid neutrofiele chemoattractant-78 (ENA-78/CXCL5), noch IL-8, tot expressie gebracht door endothecelcellen. In tegenstelling hiermee, produceerden gestimuleerde in vitro culturen van endothecelcellen meer IL-8 dan GCP-2. De selectieve aankleuring van endothecelcellen op plaatsen waar het epitheel beschadigd is, geeft aan dat GCP-2, ondanks zijn lage concentratie in vitro, een rol speelt in IBD die verschilt van structureel (ENA-78) en functioneel (IL-8) verwante chemokinen. Verder toonden we aan dat GCP-2, IL-8 en monocyt chemotactisch proteïne-1 (MCP-1/CCL2) samen worden vrijgezet door microvasculaire endothecelcellen na stimulatie met proinflammatoire stimuli. Het effect van GCP-2, in vivo tot expressie gebracht door humane endothecelcellen, werd nagebootst door het chemokine intraveneus te injecteren in konijnen. Injectie veroorzaakte een dosis-afhankelijke respons in de bloedbaan bestaande uit een onmiddellijke granulopenie, gevolgd door een sterke granulocytose. In vitro werkte GCP-2 bovendien synergetisch met MCP-1 in het aantrekken van neutrofiele granulocyten.
De precieze functie van chemokinen in nieuwvorming van bloedvaten tijdens ontsteking of tumorgroei is nog niet volledig opgehelderd. Bij het aankleuren van ontstoken darmweefsel van IBD patiënten leek het alsof GCP-2 specifiek door nieuw gevormde endotheelcellen tot expressie werd gebracht. Verder toonde immunohistochimie ook GCP-2 expressie aan in endotheelcellen van personen met gastrointestinale tumoren. Deze aankleuring van GCP-2 kwam overeen met de infiltratie van witte bloedcellen in de tumor en met de expressie van het matrix metalloproteïnase gelatinase B dat angiogenese bevordert door het afbreken van de extracellulaire matrix.

Tot besluit geven deze gegevens aan dat de expressie van GCP-2 en IL-8 verschillend gereguleerd is in vitro en in vivo en dat GCP-2 en IL-8 hun eigen rol hebben in ontstekingsprocessen hoewel ze structureel en functioneel verwant zijn. Het complexe netwerk van chemokinen vertoont dus eerder complementariteit dan overtolligheid.


