Basic and clinical aspects of mucosal inflammation and healing in Crohn's disease

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Chapter 8

General Discussion and Summary

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In recent years, great advancements have been achieved into the insight of the function of immune mediators involved in intestinal inflammation of IBD. In this thesis, clinical and experimental evaluations of several immunological mediators, relevant to the destruction and healing of intestinal tissue of IBD, are described. In *in vitro* and/or *in vivo* studies the regulatory mechanisms of TNF-α, MMPs, bFGF and its receptors, at the protein and mRNA level, were assessed in intestinal mucosa of IBD patients and in leucocytes from patients with CD. In addition, the effect of anti-TNF-α therapy in CD, on the expression of bFGF, MMP-2 and MMP-9 were evaluated.

bFGF is a pivotal angiogenic and mitogenic polypeptide for endothelial cells, fibroblasts, smooth muscle cells, and epithelial cells. The pleiotropic biological activities of bFGF are mediated by a dual receptor system, which consists of high-affinity tyrosine kinase receptors and low-affinity heparan sulphate proteoglycans. The bFGF, FGFR-1 and syndecan-1 complex was found to play an active role in IBD, and TNF-α to be involved in the regulatory mechanisms of bFGF expression in leucocytes of CD patients. In inflammatory cells and granulation tissue a high level of bFGF protein and mRNA indicates that bFGF not only participates in tissue repair and wound healing, but also joins the acute reaction of inflammation in the intestinal tissue. The enhanced bFGF in inflamed tissue regions is probably the result of the stimulation by pro-inflammatory mediators and the demand by tissue healing. bFGF is believed to facilitate PMNL emigration during acute inflammation, and has anti-inflammatory actions during chronic inflammation and angiogenesis by inhibition of endothelial activation for leucocyte recruitment [1]. The results of our *in vivo* and *in vitro* experiments suggest that TNF-α, a key player in IBD immunopathogenesis, especially in CD, is one of the factors which induces the expression of bFGF. Previous studies already showed that TNF-α increases the expression of bFGF in endothelial cells and other stromal cells [2;3]. Our studies showed that TNF-α is a regulator in the expression of bFGF in leucocytes of CD, particularly at the mRNA transcription level. LPS was found to induce the expression of bFGF at both the transcription and secretion level, and the induction of mRNA is inhibited by the neutralization of TNF-α with infliximab. The mechanism of bFGF release from producing cells is uncertain. After synthesis, bFGF is believed to be released from cells by a mechanism of exocytosis via an endoplamic reticulum (ER)-Golgi-independent pathway,

since 18 KDa bFGF lacks a conventional signal peptide [4]. Our study indicates that TNF-α is not involved in this release process.

The signal of bFGF is transduced via the complex of bFGF-FGFR-syndecan. Similar to bFGF, the expression of FGFR-1 and syndecan-1 in intestinal IBD tissue was affected in the presence of inflammation. However, the influence of TNF-α neutralization on the expression of FGFR-1 and syndecan-1 in intestinal tissue was not evaluated and therefore remains unclear. *In vitro* studies do indicate, however, that pro-inflammatory cytokines, including TNF-α, are capable of down-regulating syndecan-1 expression in colonic epithelial cell lines [5], which endorses our impression of a shift of the bFGF complex from epithelium to the lamina propria in inflamed IBD tissue.

The treatment of fistulizing CD patients with infliximab significantly decreases the serum bFGF level in responding patients, i.e., closure of fistulas is accompanied by a reduction of serum bFGF. This phenomenon supports the notice that bFGF might serve as a tissue damage marker [6] and is an important participator in wound healing in IBD. Strikingly, we did not find this downregulation of bFGF in serum and intestinal tissue after the clinical improvement by infliximab of active CD patients. A reason for this discrepancy might be that the decrease in CDAI in active patients does not strictly parallel the histopathological improvement at local intestinal sites [7;8]. Although clinical remission has been achieved, the repair and healing process might still actively proceed in the intestinal tissue. It can not be excluded that in the regulation of bFGF expression at inflammatory sites, particularly in the two main bFGF producing cells fibroblasts and endothelial cells, there are different mechanisms involved in patients with different disease phenotypes, i.e., fistulizing versus active disease. These results and suggestions merit further exploration of the regulation of bFGF expression in IBD.

The clinical results achieved by infliximab treatment confirmed that TNF-α is a pivotal player in the pathogenesis of CD. The application of this anti-TNF-α antibody to active or fistulizing CD patients results in not only clinical remission in most of the patients but also causes substantial changes of the immunological circumstance in these patients. The efficacy mechanisms of infliximab for CD range from neutralization of biologically active TNF-α to the induction of death of TNF-α-producing cells, acting as a toxin for immune cells [9;10]. For instance, a reduction of activated macrophages and T lymphocytes has been found in the intestinal mucosa of CD patients after infliximab therapy [10;11], and down regulation of pro-inflammatory Th1 cytokine production in CD and RA, in serum or tissue, has been observed [12]. The above described influence on the expression of bFGF can now be added to the mechanisms of this anti-pro-inflammatory cytokine therapy. Unfortunately, in a considerable number of CD patients (30-50%) infliximab treatment is not successful, and attempts have been made to elucidate the mechanisms of failure. Increased TNF-α secretion by whole blood LPS-stimulation was thought to be a good indicator, where genetically determined reactivation of the immune system was supposed to be the cause of infliximab failure [13]. However, we found that the determination of TNF-α in whole blood plasma by an ELISA method is severely interfered by infliximab. Furthermore, our results indicate that infliximab does not directly affect the capability of peripheral blood mononuclear cells to produce TNF-α, and corrects the conclusion that infusion of infliximab causes inhibition of TNF-α synthesis by killing the TNF-α-producing cells in the circulation [13]. Apparently, the efficacy of infliximab is mainly based on its effect on the recruitment and activation of immune cells in inflammatory regions [14].

A timely breakdown and reconstitution of the ECM is essential for tissue remodelling and an aberrant remodelling is related to the formation of fistulas and strictures in IBD. Increasing data suggest that MMPs are actively involved in the pathogenesis of IBD. MMP activity is not only relevant to acute tissue injury but also related to matrix modelling and angiogenesis. Previous studies and studies in this thesis showed that neutrophil derived-MMP-9 contributes to the acute intestinal tissue inflammation in IBD, where MMP-9

facilitates the migration of neutrophils from vessels into the matrix [15;16]. Overexpression of MMP-9 is believed to prevent wound healing in chronic inflammation [19]. Our *in vitro* study demonstrated that TNF-α is a regulator in the MMP-9 expression at the mRNA and protein level in leucocytes from CD patients, although the secretion of MMP-9 was found to be TNF-α independent. In intestinal IBD tissue the enhanced MMP-9 mRNA and protein in inflamed regions was observed. In follow-up studies on CD patients treated with infliximab we found a decrease in MMP-9 levels, both in serum and intestinal tissue, which was not strictly related to the clinical improvement. These results further demonstrate that MMP-9 is an active player in the pathogenesis of CD. On the other hand, in tissue repair studies an elevated MMP-9 level has also been considered a factor facilitating re-epithelialization and degradation of denatured collagen [20]. These observations illustrate the complexity and continuum of tissue damage and tissue repair.

With its unique regulatory pathway MMP-2 is constitutively expressed and not directly affected by proinflammatory cytokines, such as TNF-α, because of the lack of a transcriptional factor AP-1 binding site, which exists in other MMP genes. However, it is reported that TNF-α mediates the release of MMP-2 from endothelial cells induced by Angiotensin II (ANG II) [22]. MMP-2 can also be regulated at the level of pro-enzyme activation, as a result from the coordination of MMP-14 and tissue inhibitor of the metalloproteinase (TIMP), and TNF-α has been found to stimulate the activation of MMP-2 through NF-κB mediated induction of MMP-14 [23].

In the chronic inflammation of IBD, MMP-2 is relevant to the tissue remodelling rather then to the acute tissue injury. MMP-2 plays a surveillance role in maintenance of collagen homeostasis in tissues. Regarding the immunohistochemical localization of MMP-2, we found an elevated expression in inflamed areas of the gut. In addition, after the treatment with infliximab an increase of serum MMP-2 levels was observed. These observations further illustrate the importance of MMP-2 in the process of matrix remodelling during wound healing. MMPs, particularly MMP-2 and MMP-14, are also believed to be contributors to (neo)angiogenesis in numerous other diseases conditions [24].

Wound healing is a complicated process that requires interaction between extracellular matrix proteins, including proteases, cytokines, growth factors and collagens. It has been reported that bFGF participates in the regulation of the expression of MMP-1, -3 and TIMP-1, but not of MMP-2, in intestinal myofibroblasts [25]. Although many cytokines and growth factors are identified as regulators in the expression of MMPs, bFGF has been reported not to be involved in the expression of MMP-2 and -9 during wound healing [18;26]. However, there are contradictory reports on the role of bFGF on the regulation of MMP-2 and -9 in various cell types [27-29]. *Vice versa*, the cleavage activity of MMPs is not only limited to the components of the ECM but expand to an increasing class of extracellular proteins, including cell surface receptors. MMP-2 is, for instance, also able to cleave FGFR-1 extracellular domain from the plasma membrane and this FGFR-1 ectodomain retained its ability to bind FGF [30]. The lytic activity of MMP-2 on FGFR-1 apparently modulates the biological activity of FGF. Interestingly, as prominent inhibitors of MMPs, TIMP -1, -2 and -3 also block angiogenesis by bFGF in various tissues in animal and human experiments [31]. Although in this thesis only a partial relationship between bFGF and MMPs is described, the interaction between bFGF and MMPs might be an important element in the orchestration of inflammatory tissue destruction and repair (Figure 1).

The results obtained on the mechanisms of anti-TNF-α treatment, the regulatory mechanisms of bFGF and MMP expression by leucocytes of CD patients and the role of these factors in the intestinal tissue or circulation of patients with IBD are helpful to our understanding of the activity of immunoregulatory peptides in these diseases. The observations add to our knowledge about the activity of inflammatory mediators, like cytokines, growth factors and proteases, in the pathogenesis of IBD and provide leads to novel therapeutic approaches for IBD.

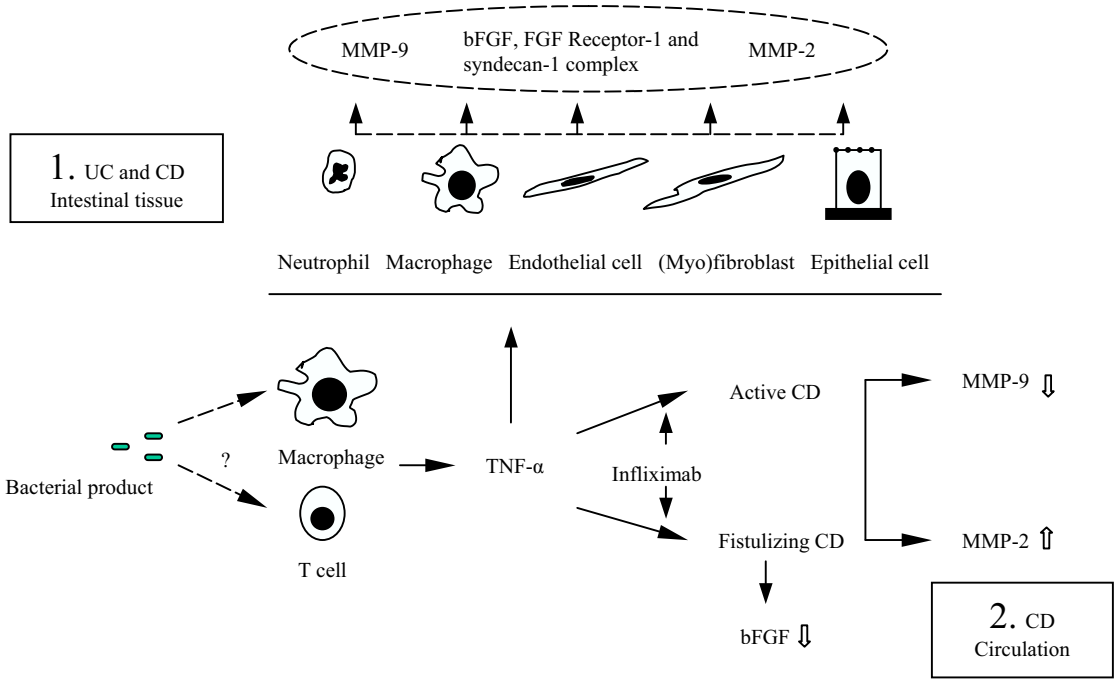

Figure 1. The outline of the results described in this thesis: MMP-2, MMP-9 and the complex of bFGF, FGF Receptor-1 and syndecan-1 were found to actively participate in the inflammatory process, both tissue destruction and healing, of IBD; 1) in intestinal tissues of UC and CD the elevated expression of MMP-2 and -9, and a heterogenic expression of the complex was observed; 2) after treatment with infliximab, serum bFGF was decreased in fistulizing patients who respond to the therapy, whereas serum MMP-2 and -9 in CD patients exhibited an inverse changing partten, i.e., an increase of MMP-2 and a decrease of MMP-9.

Summary

In this thesis studies are described on immunopathogenic aspects of inflammatory bowel disease (IBD), i.e., Crohn's disease (CD) and ulcerative colitis (UC), as well as on efficacy mechanisms of infliximab, an anti-tumor necrosis factor (TNF)-α antibody, for the treatment of CD. We focused on three groups of factors, fibroblast growth factor and its receptors, the gelatinase-type of matrix metalloproteinases (MMPs), and TNF-α, which are all believed to be important mediators in the mucosal processes in IBD (**Chapter 1**). The following observations were made:

1. The ternary complex of bFGF-FGFR-syndecan-1 is actively involved in the inflammatory and healing processes of the intestinal IBD mucosa. A shift in the expression of the bFGF-FGFR-syndecan-1 complex was observed, at both the protein and mRNA level, from mature epithelial cells in normal tissue to cells of the lamina propria in IBD tissue, particularly at sites with increased inflammation (**Chapter 2**). This change in the predominant location of the bFGF-complex is thought to be related to coordinated reparative mechanisms as angiogenesis, tissue reconstitution, cell-cell and cell-matrix interaction in the intestinal mucosa.

2. Healing of fistulizing/perianal CD is reflected by a decrease in high serum bFGF, particularly in relation to treatment with infliximab (anti-TNF-α). In contrast, serum bFGF levels do not relate with response in patients with active CD. These observations confirm that bFGF, in concert with TNF-α, plays a role in the inflammation and tissue repair process in CD patients with a fistulizing disease phenotype (**Chapters 3** and **4**).

3. *In vitro* experiments illustrated further that LPS regulates the expression of bFGF at both the transcriptional (mRNA) and/or post-transcriptional (protein) level in leucocytes from patients with CD and from healthy controls. The transcription regulation of bFGF was found to be mediated to a large extent by TNF-α, as exemplified by interference of infliximab (**Chapter 4**).

4. The determination of TNF-α by immunosorbent assays is strongly interfered by infliximab. The presence of infliximab does not influence the capability of peripheral blood cells, however, to produce TNF-α (**Chapter 5**).

5. Comprehensive tissue analysis showed that MMP-2 and MMP-9 are markedly increased and thus actively involved in the inflammatory and remodelling processes in intestinal IBD mucosa. MMP-2 was found to participate in the stromal processes, whereas MMP-9 was predominantly associated with the leucocyte-mediated inflammatory process (**Chapter 6**).

6. An enhanced MMP-9 secretion by blood leucocytes of CD patients through LPS stimulation was found to be independent of TNF-α inhibition by infliximab. The induction of MMP-9 mRNA transcription in leucocytes after longer LPS stimulation, however, was TNF-α dependent (**Chapter 7**).

7. Treatment of CD patients with infliximab resulted in an inverse changing pattern of serum MMP-2 and MMP-9, i.e., an increase of MMP-2 and a decrease of MMP-9, the latter also in the intestine. However, these changes were not strictly associated with the clinical response, i.e., improvement, to treatment with infliximab (**Chapter 7**).

These observations may be helpful to understand the role of proteolytic enzymes and immunological regulatory peptides in the pathological processes of IBD (Figure 1). In addition, the *in vitro* and *in vivo* studies with infliximab provide further insight into mechanisms of anti-pro-inflammatory cytokine directed immunological therapy for Crohn's disease.

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