Chapter 4

Influence of infliximab on the expression of basic Fibroblast Growth Factor (bFGF) in patients with Crohn's disease

Short title: bFGF affected by infliximab in CD

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Abstract

Background and aim: Basic fibroblast growth factor (bFGF) is a pleiotropic regulatory peptide, involved in inflammation/tissue repair. We assessed the effect of immunoneutralization of TNF-α by infliximab on the expression of bFGF mRNA and protein in Crohn's disease (CD).

Methods: Patients with fistulizing CD (n=10) were administered infliximab three times and evaluated for bFGF and clinical response up to 14 weeks. Patients with active CD patients (n=7) received infliximab once and their assessment was until week 8. Biopsies from these patients stained for bFGF. Blood from 7 CD patients and 4 volunteers were stimulated with LPS with/without infliximab for bFGF mRNA and protein determination by RT-PCR and ELISA, respectively.

Results: The median serum bFGF levels in responding patients with fistulas decreased from 12.0 pg/ml at inclusion to 10.3 pg/ml at week 14 (P=0.08). Median open/draining fistulas scores (OFS) decreased from 3 to 1 (P=0.03). The bFGF levels and OFS remained stable in the nonresponders. The significant clinical improvement of active CD patients was, however, not accompanied by a decrease of serum bFGF levels. The immunoreaction to bFGF in biopsies showed no effect of treatment. Blood leucocytes from CD patients and controls secreted significantly more bFGF by 24-hours LPS stimulation compared to control incubation (12.3 vs 8.2 pg/ml, P=0.003), with no effect of infliximab, whereas leucocyte bFGF mRNA increased 24-fold, which was inhibited for 70% by infliximab.

Conclusion: TNF-α-regulated bFGF seems to play a role in the inflammation/tissue repair process of CD, particularly in fistulizing disease.
Basic fibroblast growth factor (bFGF or FGF-2) is a member of the heparin-binding regulatory peptide family, that comprises over 20 members with multiple functions [1;2]. FGFs are, for example, involved in the regulation of cell proliferation and differentiation, cell survival and apoptosis, as well as in the interaction of cells with matrix and epithelium with mesenchyme. bFGF is also a potent angiogenic factor in (patho)physiological processes, like wound healing, tissue repair and muscle cell growth [3-6].

Idiopathic inflammatory bowel disease (IBD), i.e., ulcerative colitis (UC) and Crohn's disease (CD), is a chronic and recurrent inflammation of the alimentary tract, causing longterm impairment of intestinal structure and functions. Fistulization is one of the major complications in CD, which commonly occurs during severe intestinal ulceration, often resulting in the interference by surgery. Cumulating data suggest that microbial agents initiate an aberrant immunologic reaction in genetically susceptible subjects, leading to intestinal inflammation and tissue damage in IBD [7-9]. The proinflammatory cytokine tumour necrosis factor (TNF)-α is a key player in the development of CD, mainly produced by activated monocytes/macrophages and lymphocytes [10-12]. TNF-α induces or upregulates the production of other inflammatory mediators, such as interleukin (IL)-1, IL-6, IL-8 and interferon (IFN)-γ, promotes the release of procoagulating substances and proteases, and is also known to cause the activation of macrophages, priming of neutrophils, and the enhancement of epithelial permeability [9;13]. The microbial product lipopolysaccharide (LPS or endotoxin) is the most potential stimulatory factor for monocytes/macrophages to express TNF-α [14]. It has been reported that systemic endotoxaemia was correlated with the activity and extent of IBD [15;16]. Infliximab, a human-murine chimeric IgG1 anti-TNF-α antibody, is one of the most effective biological therapeutic agents up to date in the treatment of patients with active, treatment refractory CD and of CD patients with fistulas [17-19].

Several studies showed that bFGF levels in serum and faeces were elevated in paediatric and adult patients with IBD [20-23]. bFGF is known to promote proliferation of endothelial cells and enhances the number and activity of (myo)fibroblasts [24;25]. The effect of bFGF on the secretion of collagen from fibroblasts is believed to be relevant to the healing of ulceration in IBD [26], and the increase of bFGF was proposed to be related to the activity of inflammation and tissue healing. In addition, TNF-α was shown to increase bFGF in endothelial cells and other stromal cells [27;28]. However, the influence of infliximab therapy in CD on the expression of bFGF and the regulatory activity of TNF-α on the transcription of its mRNA and the synthesis and secretion of its protein are not elucidated yet.

In a previous immunohistochemical study we showed that an elevated bFGF protein level was present in intestinal inflammatory cells, with a concomitant decreased expression of bFGF in epithelial cells of inflamed mucosa, indicating the involvement of bFGF in the inflammatory process of IBD [29]. In the present study we describe the effect of immunoneutralization of TNF-α by infliximab on the in vitro and in vivo expression of bFGF in Crohn's disease.

Materials and Methods

Patients and volunteers

All patients participated in the expanded access program for anti-TNF-α treatment of CD, which was approved by the Medical Ethics Committee of our institution [30]. For eligibility the patients had to be between 18 and 65 years of age, and Crohn's disease had to be confirmed by radiography or endoscopy. For inclusion in the fistula study, patients had to have a single or multiple open/drainaging enterocutaneous fistulas resistant to conventional treatment for at least three months. Patients who had had moderately to severely active
Crohn's disease for at least six months, with activity index (CDAI) scores equal or above 220, refractory/dependent on corticosteroid therapy, and no response to or intolerance of other immunosuppressive treatment, were eligible for the study of infliximab for active Crohn's disease.

Four healthy volunteer controls were between 28 and 50 years of age.

Clinical study

Protocol 1. Fistulas

Within two weeks of screening, eligible patients received 5 mg of infliximab per kilogram of body weight (total n=10, one patient also had active disease), all to be given at weeks 0, 2, and 6. The intravenous infusion of infliximab lasted over a two-hour period. Patients returned for clinical and laboratory assessments at day 3, week 2, 6, 10 and 14.

Efficacy was evaluated by the numbers of patients with a reduction of half or more of the number of open/draining fistulas from baseline at two consecutive visits, defined as responders. Changes in scores of the CDAI and the open/draining fistulas scores (OFS), i.e., the number of open/draining fistulas per patient, were also evaluated. Failure of treatment was defined as changes to medication that were not permitted in the protocol, surgery related to Crohn's disease, or no return for follow-up visit.

Protocol 2. Active disease

Patients received a single dose of 5 mg of infliximab per kilogram of body weight (total n=7) intravenously over a two-hour period. Disease activity scores and/or serum blood samples were obtained at day 0, day 3, week 2, 4, and 8.

Immunohistochemistry

Intestinal biopsies from macroscopically non-inflamed areas of patients in the fistula study (n=10) and from inflamed areas in the active disease patients (n=6) were obtained at the start of the study, as well as at day 3 and from some at week 2 of follow-up. Paraffin embedded tissue examples were cut into 4 μm sections. Immunohistochemistry for bFGF was performed with a polyclonal anti-bFGF antibody (code no. sc-79-G, Santa Cruz Biotechnology Inc., U.S.A.) for detection. This rabbit affinity-purified antibody to bFGF was raised with a peptide mapping within the amino-terminal domain of bFGF of human origin. Secondary biotinylated antibodies, i.e., goat anti-rabbit IgG were purchased from Dako, Denmark. The colour reaction was developed by 3-aminio-9-ethylcarbazole in acetate buffer containing H₂O₂. Specificity of the immunohistochemical staining was assessed by neutralization incubation of the primary antibodies with a five to ten-fold (by weight) excess of blocking peptide (sc-79-P, Santa Cruz Biotechnology Inc., USA).

The immunohistochemical staining was semiquantitatively assessed using the following scoring system: - = no staining, + = a few positive cells / areas of tissue, ++ = a minority of the cells / areas of tissue positive, +++ = a majority of the cells / areas of tissue positive, ++++ = all cells or areas of tissue strongly positive.
In vitro study

Heparinized blood samples (10 ml/each) were obtained from a group of randomly selected CD patients (n=7) before and two hours after infusion of infliximab. For the blood samples from the volunteers (n=4) a concentration of 75 μg infliximab per ml blood was used. Whole blood LPS stimulation: at 37°C, 5% CO₂, the blood samples with/without infliximab were stimulated with/without LPS (Sigma, St. Louis, U.S.A) 0.1 μg/ml blood for 1.5-24 hours for bFGF mRNA or/and protein determination, as described previously for TNF-α [31]. The plasma was subsequently separated from the blood sample by centrifugation at 2800 rpm for 5 min. at 4°C, and stored at -70°C until further analysis. Leucocytes were isolated by the addition of erythrocyte lysis buffer, containing 0.16 M NH₄Cl, 10 mM KHCO₃, and 0.01 mM K₂-EDTA (pH 7.4 at 0°C), to the cell pellets. After lysis of the erythrocytes the samples were centrifuged at 1500 rpm, 4°C for 5 min., and the procedure was repeated to obtain pure leucocytes which were immediately used to isolate RNA.

Enzyme-Linked Immunosorbent Assay (ELISA) for bFGF

The amount of bFGF in serum was determined with the high sensitivity Quantikine ELISA kit (HSFB75, R&D System, Inc. Minneapolis, MN, USA), performed according to the instructions of the manufacturer. At appropriate serum/plasma dilutions the sensitivity of this ELISA was <0.23 pg/ml. The intra- and inter-assay coefficients of variation were 3.9% and 11.6%, respectively.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Reagents used in the RT-PCR were purchased from GIBCO BRL, USA, unless otherwise specified. Oligonucleotide primers (Table 1) were derived from DNA sequence database of NCBI (Bethesda, MD, USA). Primer sets were designed using the Primers3 Output computer program (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). The bFGF sense primer crossed an intron to prevent interference of contamination by genomic DNA. Specificity of the primers was checked with the NCBI BLAST program. β2-microglobulin (M) was used as a control to normalize PCR signals from different groups.

Total RNA was isolated from tissue samples by the method of Chomczynski and Sacchi [32]. The integrity and quality of the purified RNA were analysed by agarose gel/ethidium-bromide staining and the 260/280 nm absorbance ratio. After cDNA synthesis with Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase. The cDNA was amplified in a PCR mixture. The reaction of PCR was performed by 35 cycles for bFGF and 28 cycles for β2-M in Whatman T Gradient cycler (Biometra, Goettingen, Germany). The amplified products were electrophorized on a 1.5% agarose gel containing ethidium-bromide and visualized under ultraviolet light. The Scion imaging program (Frederick, Maryland, USA. www.scioncorp.com) was used to quantify the band density in the gels.

Table 1. Oligonucleotide primers for RT-PCR

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Gene</th>
<th>Sense primer</th>
<th>Antisense primer</th>
<th>Product size</th>
</tr>
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<tr>
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<td>GACCCCTCACATCAAGCTACA</td>
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<td>NM-004048</td>
<td>β2-M</td>
<td>CCAGCAGAATGGAAGATC</td>
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</table>
Statistical analysis

The results are given as median with inter quartile range (IQR). The Wilcoxon’s signed-rank test was used to evaluate differences between paired data and the Mann-Whitey U-test for comparison between groups. Differences were considered significant when \( P \leq 0.05 \).

Results

Clinical study

The serum bFGF levels in responding patients with fistulas (n=6) decreased from 12.0 (10.0-16.7) pg/ml at the beginning of the study to 10.3 pg/ml (8.8-10.7) at week 14 (P=0.08). Correspondingly, open/draining fistulas scores in these patients were changed at the end of follow-up from 3 (2-6) to 1 (0-1) (P=0.03), most of these responders already had an improvement at week 2. The levels of bFGF in the nonresponders of the patients with fistulas (n=4) remained relatively stable with 12.0 (8.7-14.0) pg/ml at the beginning and 12.9 (8.7-23.3) pg/ml at the end of follow-up. The open fistulas scores in this group remained at 1 (Figure 1), and all these nonresponding patients had genitourinary fistulas.

In contrast, in active CD patients (n=7) who responded to the treatment with infliximab, the improvement of disease activity was not accompanied by a decrease of serum bFGF levels at week 4 to 8, in contrast, an opposing trend existed. The bFGF level was found to be 10.6 (5.4-19.7) pg/ml at the beginning and 12.9 (9.4-23.2) pg/ml at the end of follow-up, whereas the CDAI decreased significantly from 365 (264-461) down to 50 (10-189) (P=0.04) (Figure 2). One patient showed no improvement by the infliximab treatment.

![Figure 1. A decrease in the serum bFGF levels in fistula CD responders (R) to infliximab at week 14, comparing with that at the beginning of study (n=6, (+): P=0.08); median open/draining fistulas scores (OFS) in these patients were changed at the end of follow-up from 3 to 1 (x: P=0.03); in the nonresponders both the bFGF levels and OFS remained stable until week 10 (n=4).](image-url)
bFGF affected by infliximab in CD

**Figure 2.** In active CD patients (n=7) who responded to the treatment with infliximab, the improvement of disease activity (+: P=0.04) was not accompanied by a decrease of serum bFGF levels at week 4 to 8.

**Immunohistochemistry**

In colonic biopsies from patients with active CD or with fistulas, immunoreactivity for bFGF was observed in epithelial cells, endothelial cells, and (myo)fibroblasts. Some plasma cells and macrophages were positive as well. In both inflamed and non-inflamed areas, the bFGF reaction was also present in the extracellular matrix (ECM). Overall, at the beginning of study the immunoreaction to bFGF in active disease patients was more intense than that in the tissue from patients with fistulas. At day 3 and week 2, in patients with either fistulas or active disease, the immunostaining of bFGF in intestinal tissue seemed not to be affected by infliximab therapy, either in cells or in ECM (Table 2 and Figure 3).

<table>
<thead>
<tr>
<th>Time point</th>
<th>Fistulas CD</th>
<th>Active CD</th>
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<tbody>
<tr>
<td></td>
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<td>10</td>
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<td>Week 2</td>
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*Table 2. bFGF immunohistochemical staining overall score on intestinal tissues*

**In vitro study**

Leucocytes from CD patients (n=7) and controls (n=4) secreted significantly more bFGF in vitro after 24 hours stimulation with LPS, from 8.2 (7.6-13.1) pg/ml as control incubation to 12.3 (9.4-16.6) pg/ml (P=0.003). The increase of bFGF in plasma by LPS stimulation was not affected by the presence of infliximab, with a level of 12.6 (8.9-16.1) pg/ml. (Figure 4). No differences were observed between patients and controls.

bFGF mRNA expression in leucocytes from CD patients and volunteers was marginally increased after 1.5 hours LPS stimulation, i.e. 1- to 3-fold higher compared to the control incubation. Continuation of the stimulation with LPS for up to 24-hours this increment reached approximately 24-fold. Infliximab inhibited this LPS-induced increase of bFGF mRNA at 24 hours for almost 70% (Figure 5).
This preliminary study revealed that the healing of fistulas in CD by treatment with infliximab is accompanied by a decrease in serum bFGF levels, whereas the improvement of active disease, i.e., a decrease in CDAI, was not related with such a reduction of serum bFGF. Furthermore, LPS stimulation in vitro induced bFGF secretion by leucocytes which was not affected by infliximab, although late-phase bFGF mRNA transcription was largely TNF-α dependent.

Infliximab is known to neutralize the bioactivity of both soluble and transmembrane TNF-α, in addition to the induction of apoptosis of TNF-α-producing cells [33-36]. The reduction of serum bFGF after the healing of fistulas by infliximab therapy seems to be related to the neutralization of TNF-α. TNF-α has been reported to be a stimulator of the expression of bFGF in endothelial cells and stromal cells [27;28], which are essential contributors to tissue repair and wound healing. Furthermore, the activity of stromal fibroblasts in IBD is substantially different to those from normal tissue [26], and a recent study showed that TNF-α reduces the migration of fibroblasts in IBD [37]. As a pleiotropic regulatory peptide, bFGF is involved in the development of embryonic tissues and participates...
in the healing of tissue injury. For example, bFGF is one of the main stimulators for the formation of granulation connective tissue within ulcers [38]. In the reconstitution of tissue in the alimentary tract, bFGF seems to function as promotor of the proliferation of epithelial cells and fibroblasts, the migration of epithelial cell, the metabolism of myofibroblasts, and angiogenesis [38;39]. In our study the demand of fistulizing tissue for bFGF might be decreased when the fistulas were healed by therapy with infliximab via the inhibition of TNF-\(\alpha\), as an additional mechanism of its therapeutic benefit. Recently, Di Sabatino et al. [40] also reported that the elevated serum bFGF decreased, in patients with CD, both fistulizing and active CD, who responded to therapy with infliximab.

The immunohistochemical evaluation of intestinal tissue from patients with active Crohn's disease revealed a relatively high bFGF expression, which indicates that bFGF plays a role in both the inflammatory response stage and in the tissue remodelling period. Several kinds of inflammatory cells, such as activated monocyte/macrophage, express bFGF under certain stimuli, including the bacterial product LPS and TNF-\(\alpha\). After induction of remission in active CD by infliximab, both tissue and serum bFGF remained at a relatively stable level. One reason that there was no decrease of bFGF which paralleled the decrease of the CDAI might be that the correlation between clinical remission and histological improvement of CD is not very strong [41;42]. In the inflamed tissue of active disease patients the expression of bFGF after 3 days and 2 weeks was still at a similar level as before the treatment. Perhaps the fibroblasts and endothelial cells, main producers of bFGF, as well as macrophages were still in an active state during the tissue repair process, even after the suppression of TNF-\(\alpha\) by infliximab. In addition, at all time points the tissue expression of bFGF in the patients with active disease was higher than that of the patients with fistulizing disease. A longer follow-up of the active disease patients at the tissue level might be needed to get a normal bFGF expression. In a model system with experimental ulcers, Vincze et al. [43] showed that the elevated levels of bFGF in local tissues of the upper gastrointestinal tract can return to normal after 1 to 2 weeks as the healing of ulcers occurred.

The immunoregulatory mechanisms involved in the bFGF production was further assessed by our in vitro experiments with blood leucocytes, which showed a significant increase of bFGF mRNA and protein production after stimulation with LPS in both CD patients and healthy subjects, particularly at 24 hours. The results confirmed LPS to be a (co-) factor in the expression of bFGF by leucocytes. Monocytes are probably the major source of bFGF production since Schulze-Osthoff et al. [44] already showed that stimulation by LPS activated monocytes to express bFGF. The role of TNF-\(\alpha\) in this process is rather implicit since the intracellular transduction of the LPS signal results in the activation of the transcription factor nuclear factor kappa B (NF\(\kappa\)B), which is transferred into nucleus to trigger mRNA transcription of TNF-\(\alpha\) and other inflammatory mediators [45].

The elevated transcription of bFGF mRNA by LPS stimulated leucocytes was to a large extent mediated by TNF-\(\alpha\), as infliximab inhibited a major part of this increase. The observation that TNF-\(\alpha\) can enhance the expression of bFGF mRNA was already described for endothelial cells to mediate the angiogenesis [27;28]. In addition, autocrine regulatory mechanism in the bFGF production may be involved. Pohle et al [46], for example, showed that human recombinant bFGF augmented the expression of bFGF mRNA, suggesting a positive feedback loop in the regulation of the bFGF mRNA expression during the healing of gastric ulcers. Regulation of the expression of bFGF is not only at the transcriptional level, however, but also post-transcriptionally [47]. In our study LPS stimulation caused a significant increase in the protein secretion level but infliximab didn't affect this level both in CD patients and healthy volunteers. These results indicated that TNF-\(\alpha\) was not necessary for the translation and secretion of bFGF in LPS-stimulated leucocytes.

In conclusion, bFGF plays a role in the inflammation and tissue repair processes of fistulizing CD. Inflammatory mediators, such as LPS and TNF-\(\alpha\), regulate the expression of bFGF at both the mRNA and protein level.
References


43. Vincze, A.; Sandor, Z.; Nagata, A.; Tarnawski, A.; Szabo, S. The role of endogenous basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) in experimental duodenal ulcer development and healing. Gastroenterology 110, A287. 1996.


