Deactivation of endothelium and reduction in angiogenesis in psoriatic skin and synovium by low dose infliximab therapy in combination with stable methotrexate therapy: a prospective single-centre study

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Abstract

Psoriasis and psoriatic arthritis are inflammatory diseases that respond well to anti-tumour necrosis factor-α therapy. To evaluate the effects of anti-tumour necrosis factor-α treatment on expression of adhesion molecules and angiogenesis in psoriatic lesional skin and synovial tissue, we performed a prospective single-centre study with infliximab therapy combined with stable methotrexate therapy. Eleven patients with both active psoriasis and psoriatic arthritis received infusions of infliximab (3 mg/kg) at baseline, and at weeks 2, 6, 14 and 22 in an open-label study. In addition, patients continued to receive stable methotrexate therapy in dosages ranging from 5 to 20 mg/week. Clinical assessments, including Psoriasis Area and Severity Index (PASI) and Disease Activity Score (DAS), were performed at baseline and every 2 weeks afterward. In addition, skin biopsies from a target psoriatic plaque and synovial tissue biopsies from a target joint were taken before treatment and at week 4. Immunohistochemical analysis was performed to detect the number of blood vessels, the expression of adhesion molecules and the presence of vascular growth factors. Stained sections were evaluated by digital image analysis. At week 16, the mean PASI was reduced from 12.3 ± 2.4 at baseline to 1.8 ± 0.4 (P ≤ 0.02). The mean DAS was reduced from 6.0 ± 0.5 to 3.6 ± 0.6 (P ≤ 0.02). We found some fluctuations in DAS response as compared with the change in PASI, with the latter exhibiting a steady decrease over time. After 4 weeks the cell infiltrate was reduced in both skin and synovium. There was a significant reduction in the number of blood vessels in dermis and synovium at week 4. A significant reduction in the expression of αvβ3 integrin, a marker of neovascularization, was also found in both skin and synovium at week 4. In addition, a significant reduction in the expression of adhesion molecules was observed in both skin and synovium at week 4. We also observed a trend toward reduced expression of vascular endothelial growth factor in both skin and synovium. In conclusion, low-dose infliximab treatment leads to decreased neoangiogenesis and deactivation of the endothelium, resulting in decreased cell infiltration and clinical improvement in psoriasis and psoriatic arthritis.

Keywords: Angiogenesis, immunotherapy, inflammation, psoriasis, psoriatic arthritis

Introduction

Tumour necrosis factor (TNF-α) has been recognized as a pivotal proinflammatory cytokine in several inflammatory diseases, including Crohn’s disease and rheumatoid arthritis. Binding of TNF-α by infliximab, a chimeric IgG1 anti-TNF-α antibody, has been shown to reduce clinical signs and symptoms of disease activity in several clinical trials [1-3]. Psoriasis and psoriatic arthritis (PsA) are inflammatory diseases that also respond to anti-TNF-α therapy [4-10]. Psoriasis is a common chronic skin disease that is characterized by hyperproliferation and abnormal differentiation of keratinocytes, as well as by infiltration of activated...
T cells in the epidermis and papillary dermis. PsA develops in 5–25% of patients with psoriasis. This destructive joint disease is characterized by symmetrical, oligoarthritis, axial and/or distal interphalangeal joint involvement without the presence of rheumatoid factor [11]. Histological features of PsA synovial tissue include infiltration by macrophages, T cells, and other inflammatory cells [12-14].

In addition to the inflammatory component described above, more recent studies on the histology of psoriasis and PsA revealed an important role for endothelial cells. In psoriasis, an abundance of blood vessels is present in the papillary dermis, showing microvascular changes such as pronounced dilatation and tortuosity [15]. Expansion of the microvascular dermal plexus is believed to be mediated by angiogenesis, which is an active vasoproliferative process [16,17]. In PsA the synovium appears more vascular than in rheumatoid arthritis. Macroscopic observations of distinct changes in vascularity in PsA suggested possible pathogenetic differences between the two diseases. A typical morphology described as tortuosity and higher intensity of villous vascularization has been reported in PsA [12,18].

Blood vessels in both psoriatic skin and synovial tissue express a variety of adhesion molecules, including intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and E-selectin [13,19]. In addition, over-expression of vascular endothelial growth factor (VEGF), which is involved in neoangiogenesis, and of its endothelial cell receptors has been reported in psoriatic skin [20] and synovium [21]. The prominent role played by neovascularization in the evolution of psoriatic plaques is underscored by the reported dose-dependent effect of neovastat, an inhibitor of angiogenesis, which resulted in improvement in psoriasis [22]. Since TNF-α is known to promote angiogenesis [23,24], TNF-α blockade might be capable of inhibiting angiogenesis. Of interest, previous studies conducted in patients with rheumatoid arthritis have shown that infliximab is able to deactivate the synovial endothelium [25,26]. There are only limited data for PsA, but examination of serial synovial biopsies in four patients suggested an inhibitory effect on synovial vascularity 12 weeks after initiation of therapy with 5 mg/kg infliximab [27].

The aim of the present study was to evaluate the early effects of low-dose anti-TNF-α therapy on vascularity, in both psoriatic lesional skin and PsA synovial tissue, in relationship to the clinical effects. In short, we found that low-dose infliximab treatment in combination with stable methotrexate therapy leads to decreased neoangiogenesis and deactivation of the endothelium, resulting in decreased cell infiltration and clinical improvement in psoriasis and PsA.

Materials and methods
Study design
The study was a 24-week, single-centre, prospective, open-label trial. Adult patients with a diagnosis of active PsA despite concomitant methotrexate therapy were recruited at the Academic Medical Center/University of Amsterdam. Active psoriasis was defined as at least two psoriatic plaques; active arthritis was defined as at least three tender and swollen joints, and a physician’s joint assessment of moderate or worse.

A washout period of 28 days before study entry was applied in those patients who were receiving topical high-potency corticosteroids, phototherapy (including artificial tanning beds), and disease-modifying antirheumatic drugs other than methotrexate. A washout period of 14 days was applied in those patients who were receiving low and moderate potency topical corticosteroids, topical vitamin D analogues, topical retinoids, keratolytics, or coal tar, other than on the scalp, palms, groin and/or soles of the feet. No topical treatment was allowed during the study except for emollients. The dosage of methotrexate was kept stable at least 28 days before inclusion. After inclusion, patients received infusions of 3 mg/kg infliximab at baseline, and at weeks 2, 6, 14 and 22.

The protocol was reviewed and approved by the medical ethics committee, and all patients gave their written informed consent before enrollment. The study was conducted according to the principles set out by the Declaration of Helsinki.

Assessments
Clinical evaluation
Clinical assessments were performed at baseline and at weeks 2, 4, 6, 8, 12, 14, 16, 20, 22 and 24. The clinical response of psoriatic skin lesions was measured using the Psoriasis Area and Severity Index (PASI), body surface area and the Physician’s Global Assessment on a 7-point scale (ranging from 0 [clear] to 6 [very marked plaque elevation, scaling, or erythema]). The percentages of patients achieving a 50%, 75%, or 90% reduction in PASI from baseline (PASI-50, PASI-75, and PASI-90, respectively) were calculated. The clinical response of arthritis was measured using a modified Disease Activity Score (DAS; 28 joints and ankles [DAS 30]) [28] and using the Health Assessment Questionnaire [29].

Skin biopsies
At baseline and 4 weeks after initiation of treatment, 4-mm punch biopsies were taken from the inside border of a target psoriatic plaque, preferentially from a non-sun-exposed area. Biopsies from each individual patient were obtained from the same target lesion, separated by at least 1 cm. The biopsy samples were randomly coded, snap frozen in Tis-
sue-Tek OCT (Miles, Elkhart, IN, USA), and stored at -70°C until further processing. Cryostat sections (5 µm thick) were cut and mounted on glass slides (Star Frost Adhesive Slides, Knittelgläser, Germany) and stored at -70°C until immunohistochemical staining. All skin biopsies were analyzed in triplicate to minimize random variation.

**Synovial biopsies**

At baseline and 4 weeks after initiation of treatment, a small-bore arthroscopy was performed under local anesthesia of the same knee or wrist joint, which had been clinically active at the time the first biopsy was performed. An average of at least 12 synovial tissue samples was obtained from the entire joint using a 2.5-mm grasping forceps (Storz, Tuttingen, Germany) on each occasion, as described previously [30]. Six samples were fixed in formaldehyde and embedded in paraffin, and six samples were snap-frozen en bloc in Tissue-Tek OCT (Miles) and stored in liquid nitrogen until sectioning. Sections (5 µm thick) were cut in a cryostat and mounted on glass slides (Star Frost Adhesive Slides), which were stored at -70°C until immunohistochemical analysis could be performed.

**Immunohistochemistry**

Skin and synovial tissue sections were stained with anti-CD3 mAb (Becton Dickinson, San Jose, CA, USA) to detect T cells. In addition, synovial tissue sections were stained with anti-CD68 mAb (clone EBM11; Dako, Glostrup, Denmark) to detect macrophages. Epidermal hyperproliferation was evaluated by keratin-16 expression (Sigma, St Louis, MI, USA). To analyze the expression of adhesion molecules, sections were stained with anti-VCAM-1 (CD106, 51-10C9; Becton Dickinson), anti-ICAM-1 (CD54, BBIG-L1; R&D Systems Inc., Minneapolis, MN, USA), and anti-E-selectin (CD62E, 68-5H11; Becton Dickinson) mAbs. To study (factors involved in) vascularity, sections were stained with anti-VEGF (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-αvβ3 integrin (CD51/CD61; Santa Cruz Biotechnology Inc.), and anti-von Willebrand Factor (anti-vWF; Dako) mAbs. The staining procedure was performed as described previously [31]. After a primary step of incubation with mAb, bound antibody was detected according to a three-step immunoperoxidase method. Horseradish peroxidase activity was detected using a hydrogen peroxide as substrate and amino-ethylcarbazole as dye, producing a reddish colour.

**Digital image analysis**

All sections were randomly coded and analyzed by computer-assisted image analysis as described previously [32]. In short, images were acquired and analyzed using a Syntom algorithm on a Qwin-based analysis system (Leica, Cambridge, UK). In skin biopsies, 20 high-power fields/section were analyzed. In synovial biopsies, 18 high-power fields from different parts of the section were analyzed. Positive staining of cellular markers was expressed as positive cells/mm² (dermis and synovium) or as positive cells/mm (epidermis). Positive staining of adhesion molecules, angiogenesis markers and growth factors was expressed as integrated optical density/mm². In skin sections, epidermal thickness was measured and expressed in millimeters.

**Statistical analysis**

SPSS 10.1.4 for Windows (SPSS, Chigago, IL, USA) was used for statistical analysis. The Wilcoxon signed rank test for matched pairs was used to compare baseline data with week 4 data. Results are expressed as mean ± standard error of the mean.

**Results**

**Clinical improvements in skin disease and arthritis activity after infliximab treatment**

Eleven patients with active PsA were included in the study and received infusions of low-dose infliximab (3 mg/kg). Baseline characteristics are summarized in Table 1. Patients had active disease despite methotrexate treatment. Two patients experienced adverse events during the study. One patient suffered from a bursitis of the elbow and from a cold, and another patient experienced headache, dry eyes, and restless feet. These adverse events were listed as mild events and were all of short duration. No serious adverse events were observed during the course of this study.

After the first infusion of infliximab there was already a significant decrease in PASI, which was maintained throughout the study (Fig. 1). At week 16 the mean PASI was 1.8

### Table 1

| Demographic and clinical data of study patients at baseline |
|-------------------------------------------------------------|
| **Parameter** | **Data** |
| Age (years) | 49 (26–70) |
| Male:female ratio | 6:5 |
| Duration of joint disease (years) | 9 (1–22) |
| Duration of skin disease (years) | 21 (2–41) |
| Disease Activity Score | 6.2 (4.8–8.2) |
| Tender joint count | 14 (2–26) |
| Swollen joint count | 11 (9–21) |
| Visual analogue scale for pain | 69 (36–90) |
| C-reactive protein (mg/ml) | 26 (7–36) |
| Psoriasis Area and Severity Index | 12.2 (1.0–29.8) |
| Methotrexate dosage (mg/week) | 10 (6–20) |

Except for male:female ratio, data are expressed as mean (range) for the 11 patients evaluated. Visual analogue scale values were scored by the patient on a range of 0–100 mm.
± 0.4, as compared with 12.3 ± 2.4 at baseline ($P \leq 0.02$). PASI-50 was achieved by 91% (10/11) of the patients at week 10. At the same time point, PASI-75 was achieved by 82% (9/11) and PASI 90 was achieved by 18% (2/11). The body surface area was reduced from 16.3 ± 4% at baseline to 4 ± 1% at week 16 ($P \leq 0.02$). Clinical pictures of a representative patient are shown in Fig. 2.

Amelioration of skin disease was associated with improvements in arthritis. Two weeks after the first infusion of infliximab a significant and clinically relevant decrease in DAS was observed. At week 16 the mean DAS was 3.6 ± 0.6, as compared with 6.0 ± 0.5 at baseline ($P \leq 0.02$). Ten out of 11 patients (91%) exhibited a DAS response, defined as a decrease by at least 1.2 points. However, there was some fluctuation in the DAS response over time (Fig. 1). Approximately 6 weeks after the last infusion of the loading period (infusions at weeks 0, 2, and 6) DAS tended to be increased, and thereafter it decreased after each subsequent infusion. In contrast, for skin psoriasis we observed steady improvement in erythema and scaling of psoriatic plaques (Fig. 1). The mean Health Assessment Questionnaire score exhibited a rapid and sustained decrease from 3.2 ± 0.5 at baseline to 0.9 ± 0.3 at week 16 ($P \leq 0.02$).

**Immunohistochemical changes in skin and synovium after infliximab treatment**

Skin biopsies from 11 patients were obtained at baseline and week 4. At the same time points, synovial biopsies were obtained from nine patients of the knee joint ($n = 7$) or wrist ($n = 2$). Baseline synovial biopsies from the other two patients were not suitable for immunohistochemical evaluation.

**Decreased cellularity**

The cellular staining findings are shown in Table 2. At week 4 a significant decrease in the mean number of CD3+ T cells was observed in both lesional dermis and epidermis. Similarly, the number of CD3+ T cells and CD68+ macrophages in the synovium tended to be decreased, although the difference did not reach statistical significance, possibly because of the relatively small number of patients.

The mean epidermal thickness was reduced from 0.43 ± 0.04 mm to 0.16 ± 0.02 mm ($P \leq 0.02$). Normalization of keratinocyte hyperproliferation, measured using epidermal keratin-16 expression, occurred in all biopsies obtained at week 4 ($P \leq 0.02$).

**Deactivation of endothelium**

Results of the immunohistochemical analysis of the expression of all adhesion molecules are demonstrated in Table 3. A significant reduction in the expression of all adhesion molecules studied in lesional skin was observed 4 weeks after baseline. Mean E-selectin expression was reduced by 95% at week 4 compared with baseline ($P \leq 0.02$). Mean ICAM-1 expression was reduced by 79% ($P \leq 0.02$) and mean VCAM-1 expression was reduced by 44% ($P \leq 0.05$).

In synovial tissue there was a significant reduction (81%) in the expression of ICAM-1 on synovial capillaries ($P \leq 0.05$). Decreased expression of both E-selectin and VCAM-1 was observed in the synovial tissue as well, although the change did not reach statistical significance.
Reduced vascularity
In both lesional dermis and synovial tissue, vascularity was significantly diminished after infliximab therapy, as shown by examination of haematoxylin stained sections. The mean number of blood vessels/mm² dermis was reduced from 27 ± 3 at baseline to 17 ± 2 at week 4 ($P \leq 0.02$). The number of blood vessels/mm² of synovial tissue was reduced from 18 ± 4 to 4 ± 1 ($P \leq 0.02$).

Consistent with these observations, there was a significant decrease in vWF-positive blood vessels and $\alpha_v\beta_3$-positive newly formed blood vessels in the dermis ($P \leq 0.02$). A similar trend was seen for the expression of VEGF ($P = 0.37$) in lesional dermis. This growth factor is involved in blood vessel development. Evaluation of synovial tissue revealed the same pattern, with significant downregulation of both vWF and $\alpha_v\beta_3$-positive vessels after infliximab treatment ($P \leq 0.05$), and a decrease in the expression of VEGF ($P = 0.07$; Table 4). Representative images of immunohistochemical staining are shown in Fig. 3.

Discussion
The results of the present study confirm that anti-TNF-α treatment with infliximab is effective in reducing clinical signs and symptoms of both psoriasis and PsA. In comparison with previously performed clinical trials in PsA with 5 mg/kg infliximab [33], we demonstrated that a low-dose treatment regimen with 3 mg/kg in combination with stable methotrexate therapy was also efficacious, exhibiting a rapid decrease in both PASI and DAS after the first dose of infliximab. The clinical effects confirm and extend the results of another recently reported trial [34]. However, it should be noted that, although the decrease in PASI was sustained at a steady level throughout the study period, the DAS exhibited some fluctuation over time. After each administration of infliximab, a decrease in DAS was observed that was sustained for approximately 6 weeks, after which the score slowly increased to approximately 75% of the baseline value until the next infusion. These data suggest that optimal infliximab therapy for the treatment of PsA might require a shorter dose interval period or higher dosages. In contrast, low-dose infliximab treatment every 8 weeks appears to be sufficient to treat moderate-to-severe plaque psoriasis, at least in patients on stable concomitant methotrexate therapy.

The immunohistochemical evaluation performed in this study may provide insights into the immunomodulatory effects of infliximab on psoriatic skin and synovium in situ. We chose to conduct the immunohistochemical analysis at week 4 in order to ensure observation of the early effects of infliximab. It is known from clinical experience that after 2 weeks of infliximab therapy a beneficial clinical effect can be observed in both skin lesions and inflamed joints in PsA. In addition, we recently showed in patients with rheumatoid arthritis that marked changes can be detected in the synovial tissue as soon as 48 hours after the first infusion of infliximab [35]. Apart from a reduction in clinical parameters of psoriasis and PsA, a decrease was observed in the number of inflammatory cells in lesional skin and synovial tissue biopsies at week 4. Although the reductions in CD3+ T cells and CD68+ macrophages in synovial tissue did not reach statistical significance, this might be accounted for by the relatively small number of patients from whom synovial biopsies could be obtained ($n = 9$).

| CD3 epidermis | Baseline | Week 4 |
|---------------|----------|--------|
| 28 ± 7        | 3 ± 1**  |
| CD3 dermis    | 132 ± 47 | 58 ± 19*|
| CD3 synovium | 83 ± 46  | 14 ± 6  |
| CD68 intimal lining layer | 67 ± 27 | 47 ± 27 |
| CD68 synovial sublining | 112 ± 46 | 36 ± 18 |

Epidermal counts represent positive cells/mm. Dermal and synovial counts are shown as positive cells/mm². The data are expressed as mean ± standard error of the mean. *$P \leq 0.05$, **$P \leq 0.02$, versus baseline.
The mechanism by which the number of lesional inflammatory cells is decreased by low-dose infliximab in psoriasis and PsA is apparently not induction of apoptosis at the site of inflammation, as we recently demonstrated [36]. Conceivably, infliximab treatment might reduce cell migration as well as retention of inflammatory cells in the skin and synovial tissue. A similar mechanism appears to be operative in rheumatoid arthritis [25,35,37].

In the present study we found that infliximab is capable of reducing the expression of the adhesion molecules ICAM-1, VCAM-1 and E-selectin on endothelium in psoriatic dermis and synovial tissue. ICAM-1 is a member of the immunoglobulin superfamily and is widely distributed in psoriatic skin and synovial tissue [13,19]. Synthesis and expression of ICAM-1 on endothelial cells and keratinocytes can be induced by TNF-α [38,39]. The interaction between leukocyte-function-associated antigen (LFA)-1 and ICAM-1 mediates adherence of leucocytes to endothelial cells, facilitating migration of inflammatory cells to inflamed areas [40]. VCAM-1 is expressed on activated endothelial cells and stimulates transendothelial cell trafficking by binding to its ligand, very late antigen (VLA)-4 on T cells and monocytes [41]. E-selectin mediates T-lymphocyte trafficking to psoriatic lesional skin by binding to cutaneous lymphocyte-associated antigen (CLA) [42,43]. The role of E-selectin mediated cell trafficking in PsA synovium is less clear [44], but studies conducted in rheumatoid arthritis suggest a potential role in the pathogenesis of synovial inflammation [45]. The observed decrease in adhesion molecule expression could be explained in part by the reduction in vascularity discussed below. It should be noted, however, that there was also clearly decreased expression of molecules per blood vessel (Fig. 3).

The mechanism by which the number of lesional inflammatory cells is decreased by low-dose infliximab in psoriasis and PsA is apparently not induction of apoptosis at the site of inflammation, as we recently demonstrated [36]. Conceivably, infliximab treatment might reduce cell migration as well as retention of inflammatory cells in the skin and synovial tissue. A similar mechanism appears to be operative in rheumatoid arthritis [25,35,37].

We found a significant and profound decrease in vascularity and neoangiogenesis in both skin and synovium after treatment. This might be particularly important in psoriasis and PsA because of the prominent role of hypervascularization, and the typical tortuous morphology of the capillaries, in these diseases [12,15,18]. Previous work has shown that serum and tissue levels of VEGF are elevated in
patients with psoriasis and PsA as compared with normal individuals [46-49]. The effect of infliximab on vascularity, as shown in the present study, might be explained in part by reduced VEGF expression at the site of inflammation. Other factors could be involved as well. For instance, recent studies indicate a role for angiogenic peptides such as endothelial-cell stimulating angiogenesis factor (ESAF) and plasminogen activator inhibitor type-1 (PAI-1) in psoriasis [47,50].

The effects reported here could in theory be influenced to some extent by concurrent treatment with methotrexate. This drug has been reported to inhibit neovascularization in vitro and in vivo [51]. Therefore, it might be more difficult to detect an additional reduction in vascularity after adding infliximab to the therapeutic regimen. However, because the dosages of methotrexate were relatively low and were kept stable throughout the study, we do not consider it likely that concurrent methotrexate therapy influenced our results to a great extent.

Conclusion
TNF-α targeted therapy with low-dose infliximab in combination with stable methotrexate therapy confers improvement in clinical signs and symptoms of psoriasis and PsA. Decreased cell infiltration in both skin and synovial tissue associated with clinical improvement might be explained in part by deactivation of vascular endothelium and by inhibition of vascularity, resulting in decreased inflammatory cell migration.

Competing interests
None declared.

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Figure 3
Representative pictures of immunohistochemical stainings. Pictures represent the expression of von Willebrand Factor (vWF), αvβ3 integrin, vascular endothelial growth factor (VEGF) and intercellular adhesion molecule (ICAM)-1 in skin and synovium before (pre) and 4 weeks after (post) initiation of infliximab therapy. Original magnification: 400x.
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