The effect of adalimumab on the vasculature in psoriatic skin lesions

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Introduction

Psoriasis, a chronic inflammatory skin disease, is characterized by raised, sharply demarcated, erythematous-squamous plaques ranging from a few millimeters to several centimeters in diameter. Histological features of psoriasis include epidermal hyperplasia, impaired epidermal differentiation, accumulation of inflammatory cells and excessive angiogenesis with enlarged, tortuous and hyperpermeable dermal blood vessels (1).

In psoriatic plaque development, one of the earliest events is a vascular network expansion, which particularly occurs in the papillary part of the dermis and appears to take place early, before epidermal changes and persists after epithelial and inflammatory alternations (2). In addition to an expanded vasculature, an increased blood perfusion is observed in psoriatic lesions (3).

Vascular network expansion occurs by the process of neovascularization, new blood vessel development, by two mechanisms: angiogenesis and vasculogenesis. Angiogenesis occurs when endothelial cells (ECs) sprout from preexisting blood vessels, migrate, and proliferate to form new blood vessels. Vasculogenesis is de novo formation of new vessels from the differentiation of endothelial progenitor cells.

Neovascularization plays a crucial role in psoriasis; the exact triggers that initiate the capillary morphological remodeling and the timeframe in which they occur in psoriasis development are still indistinct. This is an important niche in our understanding of the pathogenesis of psoriasis because vascular remodeling and its timeframe may be of major significance to the genesis and maintenance of psoriatic lesions. More insight on this matter could increase the possibility of related targets for therapy. In addition, more knowledge about the role and reaction of the vasculature might be relevant in an early assessment of therapy effectiveness; perhaps using noninvasive methods like cutaneous Doppler ultrasound.

In psoriatic skin, pro-angiogenic mediators, such as vascular endothelial growth factor (VEGF), tumor necrosis factor-α (TNF-α) and hypoxia inducible factor (HIF) are increased (4–8). TNF-α, a naturally occurring cytokine, performs a crucial role in various physiological processes including the immune defense and regulation of vascular properties. TNF-α stimulates angiogenesis and increases vessel permeability (9–14). Numerous clinical trials demonstrated an impressive clinical efficacy (15–18) of TNF-α inhibitors in the treatment of psoriasis.

Adalimumab, a TNF-α inhibitor, often prescribed in psoriasis, is a recombinant human Immunoglobulin (IgG) monoclonal antibody. Adalimumab binds soluble TNF-α with high affinity and specificity, resulting in neutralization of the biological function of this cytokine by blocking its interaction with the cell surface TNF-α-receptors.

Adalimumab modulates biological processes induced or regulated by TNF-α, including changes of expression of adhesion molecules. Adhesion molecules, proteins located on the cell surface,
are involved in binding with other cells or with the extracellular matrix. Adhesion molecules play a key role in the recruitment and migration of immune cells such as leukocytes; it is presumed that adhesion molecules also play a crucial role in the regulation of neovascularization and formation of atherosclerosis (4). Various authors investigated the mechanism of action of TNF-α inhibitors in psoriatic patients, focusing on different features of the pathogenesis of psoriasis, such as immune responses and keratinocyte differentiation. Although, TNF-α also has effects on the endothelium, these effects of anti-TNF-α treatment on the (micro) vasculature in psoriasis have not been studied before.

The aim of this study was to investigate the endothelial changes (vascular network size, vessel diameter and endothelial cell proliferation rate) in psoriatic skin during a 16 week treatment with adalimumab. In this assessment, the vascular changes are analyzed in different parts of the dermis; the papillary and reticular dermis. In this way, the impact of treatment can be assessed in different regions of the (epidermal) psoriatic skin.

**Methods**

**Subjects**

Specimens of human psoriatic skin were obtained from a previous study performed at our department (19). Biopsies were attained from 10 psoriasis patients (five women and five men; age 42–73 years) with moderate-to-severe chronic plaque psoriasis (PASI score >10 or a PASI score ≥8 in combination with a Skindex total score ≥35) treated with adalimumab for 16 weeks. All experiments were approved by the local medical ethics committee prior to the study and were conducted according to the principle of the Declaration of Helsinki and in accordance with the Medical Research Involving Human Subjects Act (WMO). In all patients a written informed consent was acquired before inclusion.

**Adalimumab treatment**

Patients were treated with an initial dose of 80 mg adalimumab given subcutaneously, followed by 40 mg adalimumab subcutaneously every other week starting 1 week after the initial dose. At the baseline visit, one representative psoriasis lesion was defined as the ‘target lesion’. At baseline, after 10 days and 16 weeks of treatment, SUM scores (scoring the erythema, infiltration, and desquamation) were assessed from the target lesion and a 4 mm punch biopsy was taken from this lesion under local anesthesia.

**Immunohistochemistry**

The skin biopsies were formalin-fixed, embedded in paraffin and sectioned at 4 μm; previous analysis revealed that 4 μm was the most optimal slice thickness for both immuno histochemical markers (CD31 and Ki67) used in this study (results of this previous analysis are not included in this paper).

The paraffin-embedded sections were dewaxed, rehydrated and antigen retrieval for the Ki67/CD31 double staining was attained using a tris-EDTA buffer (pH 9.0, 10 min at 96 °C). After which these were incubated primarily with Ki67 (proliferation marker) and visualization of the Ki67 antibody was achieved with a peroxidase-based EnVision-kit (DAKO, Glostrup, Denmark) 3–3′-diaminobenzidine metal enhanced (Metal Enhanced DAB substrate-kit, Thermo Scientific, US). Subsequently, the sections were incubated with CD31 (endothelial cell marker) in 1% bovine serum albumin, followed by the visualization of CD31 which was achieved with BrightVision (BrightVision Poly-AP anti-Ms IgG from ImmunoLogic, the Netherlands). All tissue samples were counterstained with Mayer’s hematoxylin (Sigma-Aldrich). Substitution with 1% PBS served as negative controls.

**Quantification of cells and surface area**

The immunohistochemical tissue samples were captured using the Mirax Midi Scanner (3DHISTECH, Budapest, Hungary) and scanned in batch mode with tissue finding and fully automated focusing, using an ×20 objective lens [Plan-Apochromat (Carl Zeiss, Jenna, Germany), NA = 0.8]. Tissue images were set to a compression ratio of 80% and stored in jpg-compression based format (3DHistech). The specimen level pixel size for this setup is 0.23 μm (20).

The open source imaging processing package Fiji 1.47 was used for all image analyses. The regions of interest of the dermal compartment were divided in papillary dermis (PD) and de reticular dermis (RD). The PD is defined as the dermis area within the papillae up to the base of the basement membrane. The RD can be described as the surface from the basement down to 350 μm.

The vascular network size was examined using the vascular area ratio (VAR) and the microvascular density (MVD). The VAR represents the area occupied by (micro) vessels; CD31 positive area in a selected area. The MVD refers to the number of vessels in a selected area. The vessel diameter (VD) can be defined as the smallest part of vessel-lumen (in μm) surrounded by CD31 stained Endothelial cells (ECs).

Proliferating endothelial cells (pECs) can be described as ECs that double stain the Ki67 (proliferation marker) and CD31 (endothelial marker). The pECs were numerated and expressed as Ki67-positive ECs relative to the total surface of the endothelium.

With respect to the epidermis an area was measured across the whole section, without stratum corneum. Keratinocyte Ki67+ nuclei were expressed as the sum of positive cells per millimeter length of the basement membrane.

**Statistics**

Statistical analysis was performed using SPSS 20.0 statistical software (SPSS, Chicago, USA). Data were expressed as mean ± standard error of mean (SEM). Time-related changes were assessed using a one-way ANOVA repeated measures test, followed by a Bonferroni post hoc test. Throughout the analyzes, p values < .05 were considered statistically significant.

**Results**

**Clinical results**

At baseline, the mean PASI (Psoriasis Area and Severity Index) was 16.93 (SEM 2.64). Ten days after treatment initiation, a clinical improvement in PASI score was seen in half of the patients. During adalimumab therapy the PASI declined to 14.94 (SEM 2.63) after 10 days and to 1.07 (SEM 0.42) after 16 weeks. The mean SUM score (score of erythema, infiltration and desquamation) of the target lesion declined from 6.90 (SEM 0.35) at baseline to 6.00 (SEM 0.69) after 10 days an to 0.40 (SEM 0.27) after 16 weeks (19).

**Ki67+ keratinocytes**

At baseline, a number of 173.8 ± 4.8 Ki67+ keratinocytes per mm basement membrane were demonstrated. An obvious decline was already found after 10 days (119.7 ± 6.3; p < .05) and continued to
decline 16 weeks after starting treatment (41.9 ± 2.2; p < .05) (Figures 1 and 2).

Proliferation of endothelial cells
At baseline, a number of 23.0 ± 5.7 Ki67+/ nuclei in the ECs were found in the total dermis; papillary dermis (PD) + reticular dermis (RD). A decrease in proliferating ECs (pECs) in the total dermis is observed 10 days after adalimumab initiation, although this is not significant (p = .28); after 16 weeks of therapy the decline becomes substantial (p < .01). The amount of pECs in the PD is obviously higher than in the RD (Table 1).

Vascular network size
The size of the vascular bed was determined on the basis of 2 parameters, the VAR and the MVD. The VAR, the area fraction covered with vessels, in the total dermis (PD + RD), was 9.1 ± 0.3% at baseline. Only after 10 days, a clear decrease (7.2 ± 0.2%; p < .05) was observed in the total dermis. This decline was even greater at the final measurement, 16 weeks after treatment was started (4.6 ± 0.2%; p < .05).

The surface covered with vessels compared to the total tissue area (VAR) in the PD is considerably higher than in the RD during the entire experiment; the difference in VAR ratio’s among the PD and RD is most pronounced at baseline (before treatment initiation).

The VAR in the PD decreases considerably after the first 10 days of treatment, although this decrease is not yet significant (13.3 ± 0.4 versus 12.9 ± 0.4; p = .12). The decrease is substantial 16 weeks (8.7 ± 0.3%; p < .05) after starting the adalimumab therapy.

The VAR of the RD shows a less marked decrease after 10 days of treatment; it appears to remain more or less the same during the first 10 days of treatment with a VAR of 5.9 ± 0.2%. Sixteen weeks after treatment inception the VAR of the RD declines evidently (4.1 ± 0.2%; p < .05).

The number of (micro) vessels per unit area (MVD) of psoriatic skin (total dermis) was 71.6 ± 1.8 mm² at baseline. Sixteen weeks after initiation of the adalimumab treatment, the MVD was obviously reduced (p < .05).

Vessel diameter (VD)
The mean vessel width of the vessels in involved psoriatic skin was 13.7 ± 0.1 µm before treatment. This value was significantly reduced (12.8 ± 0.1 µm; p < .05) after 10 days of treatment with adalimumab and the decline endured during continuation of the treatment; sixteen weeks after initiation of the Adalimumab treatment the VD was 8.6 ± 0.1 µm (p < .05).

Discussion
Psoriasis is characterized by a high ECs proliferation value, a strong enlargement of vascular bed and aberrant tortuous dilated capillary loops (1). This study revealed that adalimumab treatment of psoriatic skin causes a considerable reduction in ECs proliferation, vascular bed size (vascular area ratio, (micro) vascular density) and vessel diameter. Improvement of the (micro) vascular changes was associated with an evident decrease in keratinocyte proliferation and clinical improvement (SUM score 6.9 at baseline vs. 4.4 after 16 weeks) (19).

The vascular changes (ECs proliferation, expansion of the vascular network size and increase in the vessel diameter) are most pronounced in (the capillary loops in) the papillary dermis of affected psoriatic skin (as opposed to the reticular dermis). This observation was in agreement with previous studies that suggested that EC proliferation appears to arise mostly in the papillary dermis (21,22). This suggests that vascular expansion mainly derives through proliferating ECs in the papillary dermis, and to a lesser extent in deeper layers of the dermis. In the reticular dermis, some sporadic Ki67+/ ECs were observed; in previous studies no proliferating ECs were found in the reticular dermis (21,22).

The relatively modest clinical improvement of psoriasis in the first 10 days after adalimumab therapy (a reduction of the average PASI of 16.93 to 14.94) is reflected in the mild improvement of the endothelium (the relatively small decline of the VD, vascular network size and ECs proliferation value). On the contrary, adalimumab treatment is already accompanied by a significant decrease in the keratinocyte proliferation during the first 10 days. Alternations in the endothelial parameters (decrease in pECs, vascular network size and VD) become distinct after 16 weeks of treatment.

Initially, the relatively mild reduction in vascular network size is probably particularly caused by a declining vessel diameter (and perhaps an associated decline ECs thickness), since the amount of vessels (MVD) appears to remain quite stable during the first 10 days of treatment, while the VD already significantly decreases. This assumption can be supported by Hendriks et al. who observed a marked reduction in the perfusion intensity in psoriatic lesions with obvious clinical improvement on therapy, while the expression of dermal CD31+ remained obviously elevated (23). Hern et al. found similar results using native capillaroscopy

<p>| Table 1. Characteristics of microvasculature in psoriatic plaque skin before and during Adalimumab treatment. |
|---------------------------------------------------------------|--------|--------|--------|--------|</p>
<table>
<thead>
<tr>
<th></th>
<th>Pre-treatment (P1)</th>
<th>15 weeks treatment (P2)</th>
<th>16 weeks treatment (P3)</th>
<th>P1 vs. P2</th>
<th>P1 vs. P3</th>
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<td>Total Dermis</td>
<td></td>
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<tr>
<td>Microvessel density (mm²)</td>
<td>71.6 ± 1.8</td>
<td>70.5 ± 1.6</td>
<td>64.5 ± 1.4</td>
<td>p = .98</td>
<td>p &lt; .05</td>
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<td>Vascular area ratio</td>
<td>0.09 ± 0.00</td>
<td>0.07 ± 0.00</td>
<td>0.05 ± 0.00</td>
<td>p &lt; .05</td>
<td>p &lt; .05</td>
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<tr>
<td>Vessel diameter (µm)</td>
<td>13.7 ± 0.1</td>
<td>12.8 ± 0.1</td>
<td>8.6 ± 0.1</td>
<td>p &lt; .05</td>
<td>p &lt; .05</td>
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<tr>
<td>Endothelial cell proliferation (Ki67+/ nuclei/mm²)</td>
<td>23.0 ± 5.7</td>
<td>11.7 ± 1.6</td>
<td>4.1 ± 2.1</td>
<td>p = .28</td>
<td>p &lt; .05</td>
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<td>Papillary Dermis</td>
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<tr>
<td>Vascular area ratio</td>
<td>0.13 ± 0.00</td>
<td>0.13 ± 0.00</td>
<td>0.09 ± 0.00</td>
<td>p = .92</td>
<td>p &lt; .05</td>
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<td>Endothelial cell proliferation (Ki67+/ nuclei/mm²)</td>
<td>131.9 ± 31.0</td>
<td>75.5 ± 14.4</td>
<td>30.9 ± 16.0</td>
<td>p = .30</td>
<td>p &lt; .05</td>
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<td>Reticular Dermis</td>
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<tr>
<td>Vascular area ratio</td>
<td>0.06 ± 0.00</td>
<td>0.06 ± 0.00</td>
<td>0.04 ± 0.00</td>
<td>p = .94</td>
<td>p &lt; .05</td>
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<tr>
<td>Endothelial cell proliferation (Ki67+/ nuclei/mm²)</td>
<td>6.6 ± 3.4</td>
<td>1.9 ± 1.3</td>
<td>0</td>
<td>p = .85</td>
<td>p = .27</td>
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Results are presented as mean ± standard error of the mean (SEM); n = 10. (One-way ANOVA repeated measures test; Bonferroni post hoc test).
images of plaque microvessels in psoriasis patients treated with a pulsed dye laser (24).

In conformity with the decline in pECs, the size of the vascular network (defined as VAR and MVD) decreases over time. It is noted that ECs proliferation de facto represents angiogenesis and that the values found in our experiment are directly connected to vascular network size.

Adalimumab therapy blocks tumor necrosis factor-α (TNF-α), a cytokine that directly stimulates keratinocyte proliferation as well as angiogenesis and chemotaxis of inflammatory cells. In the context of psoriatic lesion, the effects of adalimumab on the endothelium proved to have a more backward role in the initial phase of the disease, but the vascular parameters correlated well with clinical improvement. In this study it could not be made clear

Figure 1. Overview of Immunohistological results. (a) Microvascular density (MVD): the number of (micro)vessels in a selected area (per mm²) of the total dermis, (b) Vessel diameter (in μm) in the total dermis, (c) Ki67+ keratinocytes per mm length of basement membrane, (d) Endothelial Cell proliferation (Ki67+ EC nuclei/CD31+ mm²) within a selected area of the total dermis, (e) Endothelial Cell proliferation (Ki67+ EC nuclei/CD31+ mm²) within a selected area of the papillary dermis, (f) Endothelial Cell proliferation (Ki67+ EC nuclei/CD31+ mm²) within a selected area of the reticular dermis, (g) Vascular Area Ratio (VAR): fraction of the area occupied by (micro)vessels within a selected area of the total dermis, (h) Vascular area ratio (VAR): fraction of the area occupied by (micro)vessels in a selected area of the papillary dermis, (i) Vascular area ratio (VAR): fraction of the area occupied by (micro)vessels in a selected area of the reticular dermis. The Bar charts represent the results of the analyzed items at baseline and 10 days (1.5 weeks) and 16 weeks after the initiation of Adalimumab treatment. Means ± SEM. *p < 0.05: statistically significant compared to baseline; † statistically significant compared to 2 subsequent biopsies.
whether vascular changes precede changes in keratinocyte-activity.

Results demonstrated that the VD is the first vascular parameter that revealed a significant decline; the VD is inextricably linked to the cutaneous perfusion (25). Therefore monitoring of the endothelium, using the noninvasive approaches (such as cutaneous Doppler ultrasound), may be a valuable marker to assess therapy efficacy and residual disease activity beyond the naked eye.

Ki67/CD31 double-staining proved a straightforward approach to quantify angiogenesis, also providing the opportunity to obtain extra information on other vascular parameters, such as the extent of the vasculature (MVD and VAR) and vessel diameter (VD).

In summary, clinical improvement due to adalimumab in psoriasis during a 16-week study was accompanied by substantial vascular changes in immunohistochemistry: a considerable reduction in endothelial cell proliferation, vascular network size and vessel diameter were observed. The Ki67/CD31 double-staining proved a straightforward approach to quantify angiogenesis, also providing the opportunity to obtain extra information on other vascular parameters, such as the extent of the vasculature (MVD and VAR) and vessel diameter (VD). Especially the endothelial cells in the papillary dermis (as opposed to the reticular dermis) of active psoriatic skin expressed proliferative activity, which was inhibited relatively fast due to adalimumab. In this study it could not be made clear whether vascular changes precede changes in keratinocyte-activity.

The VD (which is inextricably linked to cutaneous perfusion) is the first vascular parameter that declines after starting adalimumab treatment. Monitoring of the cutaneous perfusion (VD), using noninvasive approaches as Doppler ultrasound, may be a valuable marker to assess therapy efficacy. Further studies are needed to find out whether noninvasive skin imaging could contribute to assessment of residual disease activity in psoriasis.

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Disclosure statement

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Dr. Seyger received grants from/was involved in clinical trials from Abbvie, Almirall, Astellas, Janssen, Leo Pharma, Lilly and Pfizer. She served as a consultant for Abbvie, Almirall, Boehringer Ingelheim, Janssen, Lilly and Pfizer, gave lectures for Abbvie, Janssen, Lilly, and Pfizer and traveled with Abbvie, Lilly, Pfizer and Leo Pharma to meetings; fees were paid directly to the institution.

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