Response of the Endothelium to the Epicutaneous Application of Leukotriene B₄

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Abstract

Background: Vascular changes, both endothelial and functional, are crucial events in inflammatory responses. **Objectives:** To investigate the dynamics of endothelial cell (EC) and functional changes during acute inflammation in an in vivo model of the skin using leukotriene B₄. **Methods:** EC proliferation, vascular network size, vessel diameter (VD), and hypoxia-inducible factor (HIF)-1 α were studied by immunohistochemical CD31/Ki67 double staining and single staining of HIF-1 α . Cutaneous perfusion (CP) was assessed using the Twente Optical Perfusion Camera. **Results:** The initial phase illustrated an increase in VD, Ki67+ EC, and HIF-1 α expression and late-phase vascular expansion. The HIF-1 α and Ki67+ EC expression was limited. CP and VD were augmented after 24 h. **Conclusion:** The early phase of inflammation is characterized by EC proliferation and HIF-1 α expression. Vascular expansion continues over time. CP and VD are seen in both phases of inflammation. Angiogenesis, vascular network formation, and perfusion are time-dependent processes which are mutually related during inflammation.

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Introduction

Inflammatory diseases including rheumatoid arthritis, atherosclerosis, inflammatory bowel disease, and chronic inflammatory skin diseases are characterized by vascular modifications [1–6].

The well-known vascular changes during inflammation are vascular enlargement, changes in the vessel diameter (VD), and increased vessel permeability [1, 4, 5, 7, 8]. Expansion of the vascular network (microvascular density, MVD, and vascular area ratio, VAR) can be achieved by angiogenesis, a process that is tightly regulated by pro- and anti-angiogenic and -inflammatory factors [9–11]. The moment in which pro-angiogenic factors exceed anti-angiogenic factors can be referred to as the angiogenic switch. The exact factors causing this angiogenic switch and when this occurs is not yet fully elucidated. Apart from factors of the inflammatory defense system, hypoxia plays an important role in the occurrence of this angiogenic switch [12–14].

Hypoxia has been implicated in the pathogenesis of various forms of inflammatory diseases [15–18]. Hypoxia-inducible factor (HIF)-1 α is a key transcription factor involved in the maintenance of oxygen homeostasis and could be a significant trigger of inflammatory chang-

es in the endothelium. Under normoxia, HIF-1 α is subject to a tightly regulated prolyl hydroxylase (PHD 1–3)-mediated degradation. In hypoxia, reduction in PHD activity results in HIF-1 α accumulation. Many HIF-1 α target genes are important in physiology, including genes that encode proteins involved in the proliferation and/or apoptosis and chemotaxis [14]. Moreover, HIF-1 α has a critical role in angiogenesis control via the modulation of several key factors, such as vascular endothelial growth factor (VEGF)-A, fibroblast growth factor-2, and VEGF receptors [14]. Overproduction of HIF-1 α target gene VEGF in turn induces the formation of dilated and leaky vessels – properties that are also observed in blood vessels in an inflamed area. Additionally, HIF-1 α targets inducible nitric oxide (NO) synthase, an enzyme producing NO that induces cutaneous vasodilatation in response to injury, hypoxia, or local heat [14].

In addition, inflammatory mediators, including arachidonic acid metabolites such as leukotriene (LT) B₄, influence endothelial cells (ECs). Various inflammatory diseases, including atopic dermatitis [19, 20], rheumatoid arthritis [12, 21], and psoriasis [19, 22], are associated with increased LTB₄ tissue concentrations, and in some of these diseases LTB₄ reflects disease activity [23, 24]. LTs are metabolites from arachidonic acid from the 5-lipoxygenase pathway [25, 26]. These cytokines are formed by innate immune cells such as neutrophils, macrophages, and mast cells in response to a variety of stimuli [24]. LTB₄ has an important function in the activation and recruitment of inflammatory cells, e.g., neutrophils, eosinophils, monocytes/macrophages, mast cells, dendritic cells, and effector T cells [24]. Moreover, LTB₄ enhances vascular permeability and endothelial adhesion molecule expression and probably affects smooth muscle cell contraction [27].

Topical application of LTB₄ on the skin has been used as an in vivo model for induced local skin inflammation [28]. This application causes a dose-dependent acute inflammatory response: initially, an accumulation of neutrophils followed by T-cell infiltration and epidermal proliferation. The concentration of 100 ng/10 μ L has been selected based on previous results showing well-reproducible substantial inflammatory responses. Accordingly, this is a suitable in vivo model for studying a number of early alterations and consecutive cascades often seen in inflammatory cutaneous diseases. Clinically, mild erythema and edema are seen at the site of LTB₄ application.

In the literature, various methods have been proposed in order to investigate vascular modifications. (Patho)-physiological changes, e.g., altered perfusion, can be investigated using diagnostic imaging methods like echo-

Doppler perfusion or scintigraphic imaging techniques [29, 30]. Perfusion can be described as the volume of blood in a certain amount of tissue per unit of time. Anatomical changes, such as the size of the vascular network, e.g., VAR, MVD, and VD can better be investigated using a static method such as immunohistochemistry.

The aim of the present study is to elucidate the dynamics of cellular and functional changes in the endothelium during acute inflammation using cellular parameters (EC proliferation, vascular network size, and HIF-1 α) and functional parameters (vasodilatation and cutaneous perfusion, CP).

Materials and Methods

Subjects

Sequential biopsies were performed in 10 healthy volunteers (7 females and 3 males; 19–56 years old) after epicutaneous application of LTB₄, which was applied on 5 areas of the lower back. Eight, 24, 32, 48, and 72 h after LTB₄ application, 3-mm punch biopsies were obtained under local anesthesia. One additional biopsy taken at baseline from unchallenged skin served as internal control. Biopsies were used from a previous study performed at our center [28].

Three healthy female volunteers (aged 21–30 years) participated in the perfusion study. None of the volunteers had signs or a history of skin disorders. The local ethics committee approved the study, and written informed consent was obtained from all volunteers before study entry.

LTB₄ Application

All volunteers received an aliquot of 100 ng LTB₄ dissolved in 10 μ L ethanol which was applied on the skin of the lower back through a plastic cylinder (8 mm in diameter). The ethanol was evaporated using a stream of nitrogen gas, leaving the LTB₄ on the skin. The test site was covered with an impermeable dressing for 8 h, which was held in place by tape to prevent LTB₄ from being displaced; the dressing was removed by the investigators. Eight, 24, 32, 48, and 72 h after LTB₄ application, CP was assessed using the Twente Optical Perfusion Camera (TOPCam).

TOPCam

TOPCam [31] is a noninvasive laser Doppler perfusion imaging device, which can be used to visualize superficial microcirculation. The mathematical product of concentration and velocity of moving erythrocytes is measured in the microcirculation of the skin in a whole field of up to 15 $\times$ 15 cm. The whole-field LPDI device uses an expanded rectangular laser beam at a wavelength of 671 nm. Additional information on the TOPCam, including its signal processing, has been described previously [31].

In all volunteers, multiple perfusion images and color photographs were performed 8, 24, 32, 48, and 72 h after LTB₄ application. Before perfusion images were constructed, target regions were marked with a marker for orientation. Patients were instructed to avoid local pressure, scratching, and heavy physical exercise for at least 30 min before measurements were performed, as these

activities can influence the local microcirculation. All measurements were performed by the same investigator (A.G.M.H.).

TOPCam was used at a distance of 40 cm from the skin, corresponding to a measured tissue area of approximately 7×7 cm. The incorporated CMOS camera provides black-and-white photographs from the same location as the perfusion image; these photographs were used for orientation purposes afterwards. The diaphragm was adjusted manually to have a maximum intensity of approximately 70–95% of the saturation level. All measurements were performed in the same air-conditioned room, the illumination was turned off, and the curtains were closed to minimize the influence of ambient light. The perfusion intensity per pixel was estimated as M_0/DC ; this is the first-order moment M_0 of the power spectrum of photocurrent fluctuations, normalized with the average photocurrent value DC. Background correction was conducted to minimize variations in the microcirculation by environmental influences such as temperature and internal influences from the heart cycle. During 7 s, consecutive images at 24.4 frames/s were made to visualize the perfusion cycle due to heart-beat. Eight representative images measured during diastole were used to calculate the average perfusion image by averaging each individual pixel. Within this average diastole image, an internal control was selected from an area of unchallenged skin by drawing 3 rectangles, which were kept the same during the successive measurements. The mean of these 3 rectangles was calculated in all evaluations, and this average was defined as “normal.” Subsequently, all consecutive images were rescaled to obtain this normal perfusion value within these rectangles.

Immunohistochemistry

All biopsies were formalin fixed, embedded in paraffin and sectioned at 4 μ m. The paraffin-imbedded sections were deparaffinized, and rehydrated. In sections stained for HIF-1 α antigen, retrieval was achieved using citrate buffer (pH 6.0, 20 min at 96°C). Endogenous peroxidase activity was blocked by 2% H₂O₂ in phosphate-buffered saline. The sections were incubated overnight at 4°C with HIF-1 α antibody (1:20; clone: MOPI; BD Transduction Laboratories) dissolved in 1% bovine serum albumin (BSA). Visualization of the antibody was achieved with a peroxidase-based EnVision kit (DAKO, UK), 3,3'-diaminobenzidine (DAB) metal-enhanced substrate kit (Thermo Scientific, USA). Counterstaining was performed with Mayer's hematoxylin (Sigma-Aldrich).

Double staining of Ki67/CD31 was used to identify proliferating ECs; these ECs stain both CD31 (EC marker) and Ki67 (proliferation marker). In the sections stained for Ki67/CD31, antigen retrieval was achieved by EDTA buffer (pH 9.0, 10 min at 96°C), and endogenous peroxidase activity was blocked with 2% H₂O₂ in PBS. Sections were incubated for 1 h at room temperature with Ki67 antibody (1:100; clone: MIB-1; DAKO Ltd) dissolved in 1% BSA. Visualization of the antibody was achieved with a peroxidase-based EnVision kit (DAKO) and a metal-enhanced DAB substrate kit (Thermo Scientific). Subsequently, sections were incubated for 1 h at room temperature with CD31 antibody (1:20; clone: JC/70A; DAKO) dissolved in 1% BSA. Visualization of the antibody was achieved with BrightVision poly-AP IgG (ImmunoLogic, The Netherlands), stained with permanent red (DAKO), and counterstained with Mayer's hematoxylin (Sigma-Aldrich). Substitution with 1% PBS served as negative control.

Table 1. Endothelial cell (EC) proliferation in unchallenged healthy skin (0 h) and biopsies of skin challenged with epicutaneous leukotriene B₄ application (8–72 h)

Time point	Proliferating EC/ mm ² endothelial surface	<i>p</i> value vs. 0 h
0 h	0	–
8 h	56.08±7.36	0.005
24 h	48.33±8.36	0.008
32 h	42.92±7.70	0.005
48 h	28.53±7.36	0.012
72 h	21.25±6.44	0.028

Means ± SEM. Wilcoxon matched-pair signed-rank test.

Quantification of Cells and Surface Area

Image capture of the immunohistochemistry samples was performed using a Mirax Midi scan slide scanner (3DHitech Ltd, Budapest, Hungary). Slides were scanned in batch mode with fully automated focusing and tissue finding, using a $\times 20$ objective lens (Plan-Apochromat; Carl Zeiss, Jena, Germany; NA = 0.8). Images were stored in a proprietary image format (3DHitech), based on jpg compression (set to a compression ratio of 80%). The specimen level pixel size for this setup is 0.23 μ m [32].

All image analyses were performed using Fiji 1.47. Regarding the epidermis, an area across the whole section was measured, excluding the stratum corneum. Regarding the dermis, the region was defined as the surface from the basement down to 350 μ m. In the tissue sections, the size of the vascular network was investigated using VAR and MVD. VAR is defined as area occupied by (micro)vessel within a selected area, i.e., CD31 staining in the area to the total selected area. MVD represents the number of (micro)vessels in a selected area. VD can be determined as the smallest part of a vessel lumen (in μ m) surrounded by ECs stained for CD31. Proliferating ECs can be defined as ECs that stain both CD31 (endothelial marker) and Ki67 (proliferation marker). These proliferating ECs were counted and expressed as the number of Ki67+ ECs relative to the total surface of the endothelium. Keratinocytes positive for Ki67 were determined and expressed as the number of positive cells per millimeter length of the basement membrane and HIF-1 α -positive keratinocyte nuclei as percentage of the total keratinocyte count.

Statistical Analysis

Data from image analyses were expressed as means ± SEM. The Wilcoxon matched-pair signed-rank test was used to determine statistically significant differences. Values of $p < 0.05$ were regarded as significantly significant.

Results

Histologically, LTB₄ application resulted in acanthosis, parakeratosis, and inflammatory infiltrates in all healthy volunteers.

Fig. 1. Overview of immunohistological results. Microvascular density (**a**), vessel diameter (**b**), vascular area ratio (VAR), i.e., the fraction of the area occupied by (micro)vessels within a selected area (**c**), endothelial cell (EC) proliferation (**d**), and HIF-1α+ (**e**) and Ki67+ keratinocytes per millimeter basement membrane (**f**) at baseline (0 h) and 8, 24, 32, 48, and 72 h after LTB₄ application. Means ± SEM. * *p* < 0.05 vs. baseline.

Proliferating ECs

Eight hours after LTB₄ application, the number of Ki67+ nuclei in CD31-positive ECs peaked (56.08 ± 7.36 cells/mm²). The level of proliferating ECs declined gradually thereafter. In contrast, proliferating ECs were not found in unchallenged skin biopsies taken at baseline. The values of proliferating ECs and significant differences versus the unchallenged skin biopsies are summarized in Table 1.

HIF-1 α Levels

Results demonstrate that HIF-1 α expression is very limited in normal skin ($0.64 \pm 0.1\%$ of the total number of keratinocytes), but 8 h after LTB₄ application, a significant increase in HIF-1 α expression was seen ($4.67 \pm 0.4\%$, $p < 0.01$). HIF-1 α expression decreased gradually over time.

Size of the Vascular Network

The size of the vascular network was determined on the basis of 2 parameters: VAR and MVD. VAR, the area fraction of ECs in a selected tissue area, was $1.9 \pm 0.1\%$ in unchallenged skin; 24 h after LTB₄ application, VAR was significantly increased ($2.3 \pm 0.1\%$; $p = 0.02$); during the rest of the experiment, a trend to increased levels was noted.

MVD, the number of blood vessels in a selected area, was 28.1 ± 0.8 vessels/mm² in healthy skin. A significant increase in MVD was demonstrated 24 h after skin challenge (31.3 ± 1.2 vessels/mm², $p = 0.03$). The MVD increase corresponds with VAR expansion.

VD Measurements

The mean VD (diameter of the vessel lumen) was 7.4 ± 0.1 μ m in unchallenged skin; 8 h after LTB₄ application, VD was already significantly increased (8.4 ± 0.1 μ m), plateaued at 24–48 h, and then started to decrease (Fig. 1).

CP Measurements

Eight hours after LTB₄ application, a slight increase in CP was noticeable compared to the distant skin in all volunteers. Perfusion intensity evidently rose at 24 h in 1 volunteer, while the other volunteers revealed this evident increase after 32 h. Maximum perfusion intensity was seen at 48 h in all volunteers, followed by a decline after 72 h. The imaging of the superficial microcirculation revealed a fairly homogenous aspect during all the measurements.

Ki67+ Keratinocyte Nuclei

The number of Ki67+ keratinocytes was relatively stable during the first 48 h after LTB₄ application (Fig. 2); there were ± 20 Ki67+ keratinocyte nuclei/mm basement length at all these time points. A marked increase in proliferating keratinocytes, both basal and suprabasal, was observed 72 h after LTB₄ application (72 Ki67+ nuclei/mm basement length, $p < 0.01$) (Fig. 3).

Discussion

In this study, the dynamics of several cellular and functional changes in the endothelium after LTB₄ application were investigated in vivo. The responses to LTB₄ were well reproducible with a differentiation in time. In all subjects, substantial inflammatory responses were observed with respect to the parameters studied in this report. Whereas in normal unchallenged skin, no proliferating ECs were found, the endothelium was activated within 8 h after LTB₄ application. Eight hours after stimulation, the number of proliferating ECs peaked, followed by a gradual decrease during the rest of the experiment.

It is important to note that the HIF-1 α expression curve and the curve expressing EC proliferation show an almost identical course. Various (mainly oncological) studies have demonstrated that there is a strong correlation between HIF-1 α expression and angiogenesis [14, 33]. The present study, which shows a very similar time course, suggests that HIF-1 α probably plays an important role in regulating EC proliferation. The correlation between HIF-1 α expression, EC proliferation, and the increase in the vascular network emphasizes that HIF-1 α is a key factor in angiogenesis control through the modulation of several pro-angiogenic factors (such as VEGF-A and fibroblast growth factor-2) [14].

The size of the vascular network (VAR and MVD) found in our study in healthy skin is consistent with values found in previous studies [34, 35]. VAR and MVD start to expand significantly 24 h after LTB₄ challenge and continue to expand during the remaining experiment. Therefore, vascular changes in the chronic phase seem to be mainly determined by an increase in the vascular network. The growth of the vascular bed is probably the result of the high degree of EC proliferation observed earlier.

Eight hours after LTB₄ application, VD starts to increase and remains relatively stable during the entire experiment. The VD curve appears to be clearly in line with CP measurements at the corresponding time points. It

Color version available online

Fig. 2. Representative photographs of the CD31/Ki67 double and single staining of hypoxia-inducible factor (HIF)-1α of the skin at baseline and 8, 24, 32, 48, and 72 h after leukotriene B₄ application.

Fig. 3. Perfusion intensity during acute inflammation following cutaneous leukotriene B₄ application.

seems that vasodilatation plays an important role in the acute as well as the late phase of inflammation.

In the acute phase, vasodilatation is probably induced by paracrine agents (such as NO or histamine, for example) and an acutely increased demand for blood, likely as a result of increased oxygen consumption. This can probably be explained by the fact that HIF-1 α targets NO synthase, an enzyme producing NO, which induces cutaneous vasodilatation in response to local injury or hypoxia [14].

In the chronic phase, vasodilatation is probably caused by an increased demand for blood, the effects of inflammatory cells or cytokines, for example, on ECs and the angiogenic switch (characterized by increased VEGF levels), which results in the formation of dilated and hyper-permeable vessels.

Hendriks et al. [28] reported the accumulation of inflammatory cells after LTB₄ challenge. An influx of neutrophils (elastase+ cells) was demonstrated only 8 h after LTB₄ application, coinciding with IL-17 accumulation followed by a decline after 32 h. This increase in inflammatory cells occurs concomitantly with acute endothelial changes. Twenty-four hours after challenging the skin, a statistically significant but modest increase in T-Bet+ cells was demonstrated. In contrast to the course of IL-17+ and elastase+ cells, the number of cells positive for T-Bet remained unchanged till the end of the experiment.

The VD course is very similar to perfusion (physiological counterpart of VD) measured by the TOPCam; perfusion increased simultaneously with VD. Our results suggest that perfusion is more dependent on the degree of vasodilatation than on the size of the vascular bed as the increase in the vascular bed over time is not reflected by increased perfusion. This assumption is supported by

the results of Hendriks et al. [36], who found a spectacular decrease in CP, while the amount of endothelium remained elevated histologically in psoriatic patients treated with betamethasone dipropionate ointment. An enlarged vascular bed, vasodilatation, and increased perfusion have also been observed in several chronic inflammatory diseases, including psoriasis and rheumatoid arthritis [5, 6].

Previously, it has been shown that LTB₄ is metabolized in the skin by Ω -oxidation into 20-OH-LTB₄ and 20-COOH-LTB₄ [37]. It is remotely possible that these metabolites may be responsible for the effects of LTB₄ over time.

In summary, in this in vivo model (LTB₄) of skin inflammation, there seems to be a pathogenic order between HIF-1 α expression, EC proliferation, vascular network size, and functional endothelial changes. The acute phase of inflammation is dominated by HIF-1 α expression and EC proliferation, while the chronic phase is mainly characterized by enlargement of the vascular network. Vasodilatation and increased perfusion (physiological counterpart of VD) seem to be mainly a hallmark in the early inflammatory process.

There was a striking parallel in the time courses of EC proliferation and HIF-1 α , providing support for a causal link between hypoxia and EC proliferation. It is attractive to speculate that EC proliferation de facto represents angiogenesis.

Changes in perfusion and vasodilatation had a similar course over time, whereas perfusion and an enlarged vascular bed were not correlated over time. The present study of cutaneous inflammation suggests that angiogenesis, vascular bed size, and perfusion, all have different dynamics but are interrelated in the context of inflammation.

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Statement of Ethics

The experiments were approved by the local ethics committee and conducted according to the Declaration of Helsinki principles and written informed consent was obtained by all volunteers before inclusion.

Disclosure Statement

A.G.M.H., R.M.C.K., C.J.M.V., and P.E.J.E. have nothing to declare. S.C.A.H. has participated in clinical trials from Eli Lilly and Novartis. M.M.B.S. has received grants from/was involved in clinical trials from Abbvie, Almirall, Astellas, Leo Pharma, and Pfizer. She served as a consultant for Abbvie, Almirall, Boehringer Ingelheim, and Pfizer, gave lectures for Pfizer, and travelled to meetings (Abbvie, Pfizer, and Leo Pharma); fees were paid directly to the institution.

P.C.M.K. has served as a consultant for Schering, Plough, Celgene, Centocor, Allmirall, UCB, Wyeth, Pfizer, Soffinova, Leo Pharma, Actelion, Galderma, Novartis, Janssen-Cilag, and Abbott; and has carried out clinical trials for Allmirall, Celgene, GSK, Eli Lilly, Amgen, Centocor, Wyeth, Schering Plough, Merck Serono, Abbott, and Philips Lighting.

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