GROWTH FACTORS, CYTOKINES, AND CELL CYCLE MOLECULES

Involvement of IL-1 and Oncostatin M in Acanthosis Associated With Hypertensive Leg Ulcer


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Hypertensive leg ulcer (HLU) is an inflammatory disease characterized by intense pain, alteration of vascularization, and skin necrosis. The optimal treatment relies on surgical removal of necrotic tissues covered by a split-skin graft. We studied the histomorphology of the lesions and investigated the involvement of inflammatory cells and cytokines to further define the physiopathology of HLU. We report epidemis acanthosis and a preferential occlusion of the precapillary arterioles with infiltration of neutrophils, macrophages, and T lymphocytes in the dermis. OSM, IL-1β, and IL-6 were overexpressed in the ulcer, whereas the Th17-derived cytokines were not. In vitro, the addition of IL-1β and OSM promoted acanthosis and destructuring of reconstructed epidermis. Exogenous IL-1β and OSM synergistically induced epidermal acanthosis in mice. These data show that OSM and IL-1β are not only a biological characteristic signature of HLU, but these cytokines reflect a specific inflammatory state, directly involved in the pathogenesis. We suggest that anti-cytokine biotherapies could be an alternative strategy to surgery to treat HLU. (Am J Pathol. 2013, 182: 806–818; http://dx.doi.org/10.1016/j.ajpath.2012.11.030)
of the local vascular resistance. Histological studies of HLU biopsies reveal a moderate inflammatory infiltrate without detectable tissue infection, a severe thickening of the dermis media arterioles, and a marked acanthosis and parakeratosis.

Proinflammatory cytokines are key factors in inducing an inflammatory phenotype in tissues such as vessels and skin. We previously showed that the proinflammatory Th17 cytokines IL-17 and IL-22, in association with IL-1, tumor necrosis factor-α (TNF-α), and oncostatin M (OSM) directly target keratinocytes for the induction of acanthosis, the synthesis of CXCL8, the secretion of antibacterial peptides (S100A7, human β-defensin 2), and the inhibition of epidermis differentiation. Together, these cytokines have a powerful synergistic activity both in vitro on normal human epidermal keratinocytes and reconstructed human epidermis (RHE), and in vivo to induce a psoriasiform phenotype in mice. The in vitro transcriptomic profile of keratinocytes treated by these cytokines correlated with that of lesional psoriatic skin. Among these cytokines, the STAT3 signaling cytokines, OSM and IL-22, induced epidermis hyperplasia.

Regarding vascular smooth muscle cells (VSMC), inflammatory cytokines and growth factors induce a switch from the contractile to invasive phenotype that is characterized by increased cell proliferation, synthesis of matrix metalloproteinases, expression of cytokines and chemokines (IL-6, CXCL8), and diminution of differentiation. This phenotype is associated with a decrease of contractile markers such as α-smooth muscle actin (α-SMA), myosin heavy chain, and calponin. IL-1β and platelet-derived growth factor (PDGF) have been reported as potent inducers of VSMC proliferation, with a synergistic effect. Under inflammatory stress, VSMC express IL-1α, contributing to the autocrine inflammatory loop. OSM is the only member of the IL-6 cytokine family able to induce VSMC proliferation, and matrix metalloproteinases and cytokine secretion in vitro. Moreover, OSM is expressed in atherosclerotic lesions and is suspected to contribute to atherosclerosis by promoting VSMC proliferation in humans and mice. In addition to epidermis acanthosis, we report herein a preferential occlusion of the precapillary arterioles with infiltration of neutrophils, macrophages, and T lymphocytes in the dermis. Among proinflammatory cytokines, OSM, IL-1β, and IL-6 are overexpressed in the ulcer, reflecting a specific inflammatory signature of HLU. In vitro, IL-1β and OSM induced acanthosis and a detachment between the spinous and granular layer, contributing to a destructuration of the reconstructed epidermis. Exogenous IL-1β and OSM synergistically induced epidermal acanthosis in mice. These data suggest that OSM and IL-1β are directly involved in the pathogenesis of HLU.

Materials and Methods

Prospective Clinical Study

The study included 15 adult patients with a compatible diagnosis of HLU after clinical, biological, and Doppler ultrasound examinations as previously described. All of our studies involving human tissues were approved by the institutional ethics committee on human experimentation “Comité de Protection des Personnes Ouest III” of Region Poitou-Charentes. The study was conducted according to the Declaration of Helsinki principles, and written informed consent was received from participants before being included. Skin biopsies were taken during the surgical treatment of these ulcers, at the border of necrotic skin. Healthy skin of HLU patients was taken from the thigh of the same leg. Skin biopsies of control subjects were obtained from surgical samples of healthy abdominal skin. Biopsies were immediately frozen in liquid nitrogen for RNA quantification and immunofluorescence, stored in formalin for immunohistochemistry (IHC), or cultured for 48 hours in maintenance medium to analyze cytokine release in supernatants.

Histology and Histomorphometry

Skin biopsy slices (3 μm thick) were stained with H&E and used for routine diagnosis of HLU. They were screened to measure the epidermal thickness and all blood vessel characteristics (diameters and surfaces), using an Eclipse Nikon 80 I microscope (Nikon, Tokyo, Japan), an AVT Pike F-421 C camera (Allied Vision Technologies, Stadtdrofa, Germany), an HP Workstation XW4600 (Hewlett-Packard, Palo Alto, CA), and TRIBVN ICS WF software version 2.8.14 (TRIBVN, Chatillon, France). For each vessel, total surface/lumen ratio was expressed as a percentage of the vessels of the control subjects.

Quantitative Real-Time PCR Analysis

Skin total RNA were isolated and reverse transcribed as previously described. Quantitative real-time PCR (qPCR) was performed using the LightCycler-FastStart DNA MasterPlus SYBR Green I kit on a LightCycler 480 (Roche Diagnostics, Meylan, France). The reaction components were 1 × DNA Master Mix and 0.5 μmol/L high-performance liquid chromatography purified sense and antisense oligonucleotides purchased from Eurogentec (Eurogentec France, Angers, France), designed using Primer3 software version 3.0.0 (http://frodo.wi.mit.edu, last accessed July 29, 2010). Samples were normalized to the independent control housekeeping gene G3PDH and reported according to the ΔΔCT method as RNA fold increase: 2ΔΔCT = 2ΔCT sample – ΔCT reference.

Cytokine Measurement by ELISA

Skin biopsies from the HLU ulcer border (three skin punches of 4 mm diameter) were placed together in 3 mL of medium containing RPMI, 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL gentamicin. Supernatant was collected at 48 hours and centrifuged. The detection of IL-1β and OSM was conducted using enzyme-linked immunosorbent assay (ELISA) Duo-Sets from R&D
Systems (Minneapolis, MN) and IL-6 by a Standard ELISA Development Kit from PeproTech (Rocky Hill, NJ).

IHC on Human Skin Biopsies

IHC was performed on tissue sections from archived formalin-fixed paraffin-embedded tissue blocks from patients. Block serial sections of 3 μm were cut, deparaffinized in xylene, and hydrated in a graded series of alcohol. After antigen retrieval in citrate buffer, staining was performed using the BenchMark automated staining system (Ventana Medical System, Tucson, AZ) for Ki-67 (IgG1, MIB-1; Dako Cytomation, Glostrup, Denmark), α-SMA (IgG2a, clone 1A4; Dako Cytomation), cytokeratin 10 (IgG1, SC-23877; Santa Cruz Biotechnology, Santa Cruz, CA), IL-1β (rabbit polyclonal, sc-7884; Santa Cruz Biotechnology), and OSM (IgG2a, MAB295; R&D Systems). Appropriated irrelevant polyclonal or monoclonal antibodies were used as negative controls (Supplemental Figure S1). Basal keratinocyte Ki-67 expression was measured at three representative areas for each specimen (HLU lesions or healthy skin). Expression of Ki-67 in VSMC was measured at three representative areas for each specimen (HLU). A tissue microarray (TMA) of dermis had been made with four samples per subject. Briefly, tissues were chosen from H&E-stained slices. Biopsies were performed to retrieve paraffin-embedded tissues and then aggregated on blocks in arrays. From each TMA paraffin-embedded block, serial sections of 3 μm were cut, deparaffinized in xylene, and then hydrated in a graded series of alcohol. After antigen retrieval in citrate buffer, immunofixation was performed by antibodies against CD1a (IgG1, clone O10; Dako Cytomation), CD3 (rabbit antihuman; Dako Cytomation), CD8 (IgG1, clone C8/144B; Dako Cytomation), CD20 (IgG2a, clone L26; Dako Cytomation), and CD68 (IgG1, clone KP1; Dako Cytomation) antigen or by appropriate isotype controls, and staining was revealed by peroxidase with diaminobenzidine substrate. The surface stained on every spot was acquired by a tissue arrayer with GoodSpeed version 1.0 and Spot Browser software version 3.0 (ALPHELYS, Plaisir, France), which was divided by the tissue area on the spot measured using ImageJ software version 1.46 (NIH, Bethesda, MD).

Reconstructed Human Epidermis

Tissues were obtained from the Department of Plastic Surgery (University-Hospital of Poitiers) with approval of the ethics committee. Normal human epidermal keratinocytes were isolated from surgical samples of adult healthy breast skin.

Table 1  Clinical data summary of HLU patients

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>Cell type</th>
<th>Reference</th>
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<tr>
<td>Sex Ratio (M:F)</td>
<td>Leukocytes</td>
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</tr>
<tr>
<td>Age</td>
<td>Neutrophils</td>
<td></td>
</tr>
<tr>
<td>Skin necrosis (cm²)</td>
<td>Eosinophils</td>
<td></td>
</tr>
<tr>
<td>Both legs affected</td>
<td>Basophils</td>
<td></td>
</tr>
<tr>
<td>High blood pressure</td>
<td>Lymphocytes</td>
<td></td>
</tr>
<tr>
<td>Diabetes mellitus type 2</td>
<td>Monocytes</td>
<td></td>
</tr>
<tr>
<td>Diabetes mellitus type 1</td>
<td>Immature neutrophils</td>
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<table>
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<tr>
<th>Cell type</th>
<th>Blood count (10⁶/mm³)</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Leukocytes</td>
<td>11.0 ± 3.5</td>
<td>4.0–10.0</td>
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<tr>
<td>Neutrophils</td>
<td>8.1 ± 3.2</td>
<td>1.5–7.0</td>
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<tr>
<td>Eosinophils</td>
<td>0.38 ± 0.13</td>
<td>&lt;0.8</td>
</tr>
<tr>
<td>Basophils</td>
<td>0.12 ± 0.15</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>2.0 ± 0.61</td>
<td>1.0–4.0</td>
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<tr>
<td>Monocytes</td>
<td>1.02 ± 0.39</td>
<td>&lt;1.0</td>
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<tr>
<td>Immature neutrophils</td>
<td>0.46 ± 0.5</td>
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Inflammation Patients

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<tr>
<td>CRP (mg/L)</td>
<td>92 ± 58.9 (28–258)</td>
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</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>6.9 ± 1.4 (5.1–8.7)</td>
<td>2.0–4.0</td>
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Treatments

<table>
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<th>Cell type</th>
<th>Blood count (10⁶/mm³)</th>
<th>Reference</th>
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<td>Major morphin agonist</td>
<td>53%</td>
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<tr>
<td>Antihypertensive drugs</td>
<td>2.3 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>Statin</td>
<td>46%</td>
<td></td>
</tr>
<tr>
<td>Oral anticoagulant</td>
<td>40%</td>
<td></td>
</tr>
<tr>
<td>Antiangregant</td>
<td>40%</td>
<td></td>
</tr>
<tr>
<td>Corticotherapy</td>
<td>26%</td>
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Data are expressed as × 10⁶/mm³.
Briefly, the skin was rinsed in phosphate-buffered saline (PBS; Invitrogen, Cergy Pontoise, France), and subcutaneous fat was removed with scissors; the remaining tissue was cut and then exposed to dispase in Keratinocyte-SFM medium (K-SFM; Invitrogen). The epidermis was lifted off the dermis with pliers and transferred into trypsin for 15 minutes. After centrifugation, living cells were counted using a standard trypan blue (Sigma-Aldrich, St. Louis, MO) exclusion assay. Cells were seeded in K-SFM/gentamicin supplemented with 25 μg/mL pituitary extract and 0.25 ng/mL epithelial growth factor (Invitrogen). Once keratinocytes reach 80% confluency, cells were detached using trypsin and used for

Figure 1  Histological analysis of HLU lesions. Skin biopsy from the wound edge of patient HLU lesions (P-HLU) (A, E, and H) and control healthy skin (C-HS) (D and G) were stained by H&E (representative of eight subjects). A: The focus is on the edge of the necrosis, Dotted line indicates the limits of necrosis; arrowheads indicate blood vessels. I, inflamed skin; N, necrosis. B: The focus is on parakeratosis. C: The focus is on dermal inflammatory infiltrate. D and E: The focus is on the epidermis and (G and H) on dermis blood vessels. F: The epidermal thickness is expressed as the mean of three measures per subject (n = 6). I: Dermal vascular lumen and wall surfaces of P-HLU were normalized and expressed as the percentage of C-HS (n = 8 in each group, five measures per subject). The relation of the lumen to the total surface of dermal skin arterioles, and their nonlinear regression, in C-HS (J, n = 83) or P-HLU (K, n = 167) are shown. L: Graphic analysis of the tangent to the nonlinear regression of the P-HLU curve at 9000 μm², and the tangents of the P-HLU curve and the C-HS curve at 22,000 μm² is shown. Thin solid lines indicate tangents; thick line indicates P-HLU; dashed line indicates C-HS. Scale bar = 50 μm.
RHE, prepared as previously described. Suspensions of cultured keratinocytes were further cultivated on poly-carbonate culture inserts (Millipore, Billerica, MA) in Epilife medium supplemented with 1.5 mmol/L calcium chloride and 50 μg/mL ascorbic acid, and then transferred at the air—liquid interface for 5 or 12 days (D5 and D12, respectively).

The reconstructed epidermis was fixed in 10% formalin in PBS, dehydrated, and embedded in paraffin. Sections (5 μm) were cut, deparaffinized in xylene, and hydrated in a graded series of alcohol. Sections were stained with H&E and with an anti-cytokeratin 10 antibody (SC-23877; Santa Cruz Biotechnology).

**In Vivo Murine Skin Inflammation**

Outbred C57B6 mice were purchased from Charles River Laboratories (Chatillon, France). Ear intradermal injections were performed under brief isoflurane (Forene; Abbott France, Rungis, France) gas anesthesia. The 250-ng carrier-free OSM (R&D Systems Europe), IL-1β (PeproTech), IL-6 (PeproTech), or PBS were injected in a total volume of 20 μL. After either 3 or 7 days, ears were collected and frozen immediately in liquid nitrogen for H&E staining or IHC analysis.

**Immunofluorescence of Mouse Skin**

The 6-μm sections of mouse ear were taken from frozen tissues and fixed in 10% formalin in PBS. Ear thickness was evaluated after H&E coloration. Ear thickness was enhanced by using monoclonal rabbit anti-mouse PCNA (EPR3821; Epitomics, Burlingame, CA) and for granulocytes by using rat IgG2b anti-mouse Ly-6G monoclonal antibody (mAb) (Gr-1; BD Biosciences, San Jose, CA) or with isotype control (IgG2b, Caltag; Invitrogen Life Technologies, Carlsbad, CA) associated with a donkey anti-rat IgG Alexa Fluor 488—conjugated secondary antibody (Invitrogen Life Technologies). Confocal microscopy was carried out on an Olympus FV1000 confocal microscope (Olympus, Tokyo, Japan). Pictures are representative of three experiments.

**Statistical Analysis**

Contingency tests were performed using Fisher’s exact test. Statistical analysis of significance was calculated using either the U-test for comparison of two groups or Kruskal-Wallis one-way analysis of variance with a Dunn’s post hoc test, when applicable, for three groups. P values lower than 0.05
were considered as significant. Data are either represented as box plots or mean and SEM. Nonlinear regression was calculated using third-degree polynomial formulas.

Results

Characteristics of HLU Patients

Fifteen patients presenting typical lesions of HLU, as reported previously, were included in the study (Table 1). All patients but two presented long-standing HBP, 53% associated with diabetes. HLU ulcers were shallow lower-limb ulcers, painful, with a central necrosis surrounded by an inflammatory and purpuric border. HLU diagnosis was retained after exclusion of arterial and vein occlusion by ultrasound Doppler and by common differential diagnosis. The sex ratio did not differ significantly from our previous study (Fischer’s exact test \( P = 0.09 \)). HBP was treated by a mean of 2.3 ± 1.1 classes of antihypertensive drugs (60% angiotensin-converting enzyme inhibitors or sartans, 73% diuretic agents, 40% calcium channel blockers, or 60% beta-blockers). Forty-six percent of patients were treated by statins, 40% by oral anticoagulants, and 40% by antiaggregants. One patient took oral corticotherapy, and 20% of patients were treated by topical corticotherapy before surgery. Ulcers were extremely painful, requiring partial (20%) or pure (60%) morphine agonists. Non-opioid drugs, such as antidepressants (40%), anxiolytic agents (60%), pregabalin (13%), or gabapentin (37%), were also associated with insufficient benefit. Surgical excisions were performed by removing the ulcer including the inflammatory border, and loss of skin tissue was reconstructed by meshed split skin graft. Preoperative blood tests showed systemic inflammation. C-reactive protein serum levels were elevated in all patients, and white blood cell counts showed hyperleucocytosis (mean: 11,000/mm³) with elevated neutrophils, immature neutrophils, and monocytes, whereas circulating lymphocytes, basophils, and eosinophils remained unchanged. Standard laboratory tests showed normal electrolyte concentrations (Table 1). Five of 15 patients underwent several surgical procedures due to recurrence of skin necrosis at the border of previously excised skin, reflecting an active and inflammatory state of the disease.

Hypertensive Leg Ulcer Lesions Display Acanthosis and Microvascular Stenosis

H&E staining of skin biopsies performed in the margins of ulcers showed typical HLU lesions as reported. The dermis was infiltrated by inflammatory cells (Figure 1, A, C, E, and H). When compared to normal skin, HLU epidermis showed acanthosis and parakeratosis surrounding the necrotic area (Figure 1, A, B, E, and F) \( (P < 0.001) \). Skin

Figure 3  Expression of \( \alpha \)-SMA and cytokeratin 10 (CK10) in HLU. IHC of patient healthy skin (P-HS) and patient HLU (P-HLU) biopsies stained for \( \alpha \)-SMA (A and B) or CK10 (C and D) antibodies, images are representative of four different patients. B: qPCR analysis of skin biopsies from control healthy skin (C-HS), P-HS, and P-HLU was carried out for \( \alpha \)-SMA and CK10, normalized using a housekeeping gene \( (n = 8 \text{ per group}) \). Scale bar = 50 \( \mu \text{m} \). \( * P < 0.05, ** P < 0.01, \) and *** \( P < 0.001 \).
arteriole wall thickness was increased when compared to controls, leading to substantial obstruction of some vessels (Figure 1, A, H, and I) \( (P < 0.001) \). Extracellular deposits in the arterial wall were not detected (Figure 1H). The reduction of the lumen in HLU was quantified by measuring whole-vessel and lumen sectional surfaces in control and HLU sections. A correlation between total and lumen surfaces was observed for healthy skin vessels (Figure 1J) \( (R^2 = 0.95) \). In HLU lesions, vessels with a total surface between 2 and \( 15 \times 10^4 \mu m^2 \) had reduced lumen surface when compared to controls (Figure 1K) \( (R^2 = 0.62) \). The vascular lumen was decreased in HLU patients compared to controls (Figure 1, H and I) \( (P < 0.001) \). We further analyzed the derivatives of the regression curves of the lumen surface compared to the total vessel surface. The HLU vessel primary derivative showed a second polynomial shape with an inflection point at 9700 \( \mu m^2 \). At this inflection point, we observed a weaker tangential slope to the curve for the smallest vessels (Figure 1L), whereas the slope for the largest vessels paralleled that of healthy skin.

**KI-67 Expression Indicates Proliferation of Keratinocytes and VSMCs in HLU**

Epidermal acanthosis and the increase of the vascular wall thickness led us to investigate the proliferative state of keratinocytes and VSMCs using KI-67 immunostaining. Fifty-six percent of basal keratinocytes from HLU lesions expressed KI-67 antigen compared to 7% in normal skin \( (P < 0.001) \) (Figure 2, A, B, and C). The expression level of KI-67 in HLU dermal vessels was enhanced when compared to normal dermal vessels (respectively, \( 0.5 \times 10^{-3}/\mu m^2 \) versus \( 1.5 \times 10^{-3}/\mu m^2 \), \( P < 0.0001 \)) (Figure 2, D, E, and F). The percentage of arteriolar VSMC expressing KI-67 is enhanced in HLU when compared to control (60% versus 37% positive staining, respectively) (Figure 2, G and H). The expression of KI-67 antigen was particularly higher than in healthy skin, mostly in the smallest dermal blood vessels (Figure 2, G and H). To a lesser extent, this phenomenon was observed for dermal blood vessels with a diameter ranging from 40 to 70 \( \mu m \) (Figure 2, G and H).

**Hypertensive Leg Ulcers Display a Modified Differentiation State of Keratinocytes and VSMCs**

IHC staining of \( \alpha \)-SMA revealed an increase in the number of capillaries in HLU, reflecting angiogenesis (Figure 3A), whereas the decreased \( \alpha \)-SMA mRNA in HLU (Figure 3B) reflected the dedifferentiated and proliferative state of VSMC. Cytokeratin 10 (CK10) immunostaining and transcriptomic analysis showed a decreased expression in HLU.

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**Figure 4** Leukocyte immunolabeling of paraffin-embedded tissues from P-HS and P-HLU that constitute the TMA \( (n = 8, 4 \) samples per tissue). A: Representative slide staining for anti-CD1a, anti-CD3, anti-CD20, or anti-CD68 antibodies. B: Quantification of the stained surface. ***\( P < 0.001 \).
lesion compared to normal skin, indicating a less differentia-
ted state of epidermis (Figure 3, C and D).

Hypertensive Leg Ulcers Are Infiltrated by Monocytes/ Macrophages, Neutrophils, and T Cells

Hypertensive leg ulcers displayed a mixed leukocyte infiltrate. Surface staining of CD1a, expressed by dendritic cells, was lower in patient HLU (P-HLU) lesions versus patient healthy skin (P-HS) (0.6-fold decrease, 0.0024 versus 0.0014, \( P < 0.001 \)). Surface staining of the CD3 T-cell antigen was significantly increased in P-HLU (0.0016 versus 0.025, \( P < 0.001 \)), whereas the CD20 B-cell antigen was not significantly different. Surface staining of CD68, characteristic of the monocyte/macrophage lineage, was increased by 52-fold (0.0059 versus 0.315, \( P < 0.001 \)). The most represented infiltrating cells were CD68\(^+\) cells (Figure 4, A and B, and Supplemental Table S1).

IL-1\( \beta \), IL-6, and OSM Are Overexpressed in HLU

To identify the cytokine pattern expressed during the inflammatory phase of HLU, we quantified the expression of Th1, Th2, Th17, and proinflammatory cytokine mRNA in lesions and control skin, as previously described for other skin inflammatory diseases.\(^{14,30}\) Healthy skin from HLU patients and controls displayed a similar pattern of cytokine mRNA expression, except for transforming growth factor-\( \alpha \) (TGF-\( \alpha \)), which was decreased in P-HS when compared to control healthy skin (C-HS). As indicated in Figure 5, the expression of IL-1\( \beta \), IL-6, and OSM was significantly increased in HLU lesions compared to P-HS and C-HS. By contrast, we did not observe modifications of the expression of T-cell cytokines, ie, Th1 [interferon-\( \gamma \) (IFN-\( \gamma \))], Th2 (IL-4, IL-5, IL-13), or Th17 (IL-17A, IL-22). In addition, the expression of the chemokine CXCL8 was strongly upregulated in HLU lesions. The expression of growth factors involved in hVSMC proliferation, ie, PDGF-A, PDGF-B, epidermal growth factor (EGF), TGF-\( \alpha \), and TGF-\( \beta \)1, did not differ significantly between P-HS and P-HLU (Figure 5A).

We further examined the production of IL-1\( \beta \), IL-6, and OSM proteins by ELISA in culture supernatants of skin biopsies. As shown in Figure 5B, IL-1\( \beta \) and OSM were significantly increased (respectively, 45 versus 1747 pg/mL, \( P < 0.05 \), and 10.5 versus 175 pg/mL, \( P < 0.05 \)) in HLU

![Figure 5](image-url)  
**Figure 5** Inflammatory cytokine signature of HLU.  
A: Total RNA was extracted from skin biopsies from C-HS, P-HS, and P-HLU and reversed transcribed (\( n = 10 \)). Cytokine mRNA relative expression was quantified by qPCR using GAPDH as a housekeeping gene to normalize gene expression. B: Skin biopsies were harvested in the inflammatory border of P-HLU and in normal skin from C-HS and cultured in medium for 48 hours. Supernatants were harvested to measure cytokine secretion by ELISA (\( n = 5 \)). *\( P < 0.05 \), **\( P < 0.01 \), and ***\( P < 0.001 \).
biopsy supernatants compared to patient healthy skin. Secretion of IL-1β and OSM was similar between C-HS and P-HS. IL-6 production was not different between P-HLU, P-HS, and C-HS (228 versus 168 and 175 ng/mL, respectively). Nevertheless, IL-6 levels associated with HLU remains difficult to appreciate because a surgical procedure is able to rapidly and dramatically induce tissue IL-6 release.31 IL-1β and OSM immunolabeling of HLU slides allowed us to determine the source of the cytokines. We detected IL-1β and OSM labeling in dermis macrophages and, to a lesser extent, OSM labeling in polynuclear cells, lymphocytes, and myofibroblasts (Supplemental Figure S3).

We further performed in vitro and in vivo experiments to assess IL-1β and OSM involvement in the disease.

IL-1β and OSM Induce Thickening and Destructuration of RHE

To further characterize the effects of IL-1β and OSM on keratinocytes, in vitro RHE was exposed to OSM and/or IL-1β at day 8 of culture and collected at day 12 for staining. When compared to control, OSM-exposed RHE showed a global thickening of the stratum spinosum as previously described,12 whereas IL-1 induced a slight thinning. Interestingly, the combination of both cytokines altered significantly the tissue structure, inducing a cleft between the stratum germinativum and the stratum spinosum, whereas overall, the epidermis was thickened. To further evaluate the proportion of detachment, we analyzed three fields on a slide using the cellSens software version 1.3 (Olympus). The proportion of deconstructed RHE was 68 ± 6% in IL-1β- and OSM-treated samples, whereas less than 5% of detachment was observed in other conditions. CK10 expression was slightly decreased by OSM and abolished in the presence of IL-1β, whereas the combination of OSM and IL-1β displayed an intermediate CK10 expression (Figure 6).

By contrast, IL-6 alone or in combination with IL-1β displayed only a very discrete effect on RHE acanthosis and on CK10 expression, and no additional effects when compared to IL-1β and OSM (data not shown).

IL-1β and OSM Reproduce Features of HLU Lesions in Mice

IL-1β and OSM were injected intradermally, alone or in combination, in mouse ears. The combination of OSM and IL-1β induced ear redness and swelling after 3 days (Figure 7). Histological analysis revealed some features of HLU lesions, ie, an important inflammatory cellular infiltrate, an epidermal thickening, and a parakeratosis. These inflammatory signs were weak in the presence of the cytokines alone. PCNA labeling was enhanced with IL-1β and to a lesser extent with OSM, and the combination of both cytokines further increased epidermis staining, reflecting the proliferating rate of keratinocytes (Figure 7). Although no clear-cut modification of dermis vessels was observed after 3 days of treatment, we noticed revascularization after 7 days (Supplemental Figure S4). In addition to skin arterioles expressing high levels of α-SMA, we detected additional small vessels expressing low levels of α-SMA in IL-1β- and OSM-treated mice. As for in vitro experiments, IL-6 in combination with IL-1β was much less potent than OSM to modify these in vivo skin parameters (Supplemental Figure S4).

Immunohistological analysis with anti-Gr1 mAb revealed abundant granulocytes in ear tissue from IL-1β- and OSM-injected mice compared with saline-injected mice; whereas only a few cells were detected in the presence of the cytokines alone. In contrast to human HLU skin, CD68 immunostaining revealed a weak infiltration of the monocyte/macrophage cells in dermis in all tested conditions (Supplemental Figure S4).

Figure 6  In vitro exposition of reconstructed human epidermis (RHE) to OSM and IL-1. RHE was exposed to PBS, OSM, IL-1β, or a combination of both OSM and IL-1β (10 ng/mL each), and then fixed and stained with H&E or for cytokeratin-10. Shown are representative images of three experiments.
Discussion

HLU is characterized by skin inflammation that arises on a background of long-lasting HBP. The alteration of large skin arterioles had been reported in HLU. We further observed a strong alteration of the downstream microvasculature, particularly of the precapillary arterioles. The reduced lumen of these arterioles is linked to an important thickening of the media. The increase of KI-67 associated with the decrease of α-SMA expression in VSMC is characteristic of a phenotypic switch from a contractile to a proliferative state as reported in atherosclerosis. We hypothesized that other than HBP, inflammation contributes to the vascular alteration and drives the spreading of HLU lesions by reducing blood perfusion, inducing skin hypoxia and necrosis.

Our HLU patients presented with long-lasting HBP treated with several classes of antihypertensive drugs. Some patients were also treated by corticotherapy, statins, oral anticoagulants, or anti-aggregants. These patients were treated before the onset of HLU, and none was affected by severe or malignant high blood pressure during the disease.

Any of these drugs had a beneficial effect on either HLU onset or progression, pain level, or skin necrosis area, suggesting that HBP alone is not sufficient to explain the HLU pathogenesis.

Histopathological studies of HLU skin biopsies showed a hyperplastic epidermis with hyperproliferative basal cells. Cytokines were examined in these biopsies as potential therapeutic targets.

Figure 7 Mice ear injection of IL-1β and OSM recapitulates some features of HLU. Ears from outbred C57B6 mice (n = 5 for each group) were injected intradermally with 250 ng of IL-1β, 250 ng of OSM, combination of both, or with PBS as a control. On day 3, ears were collected for staining with H&E, immunodetection of neutrophils using anti-Gr-1 mAb and PCNA mAb staining. Scale bar = 100 μm.

Figure 8 Hypothetic scheme of the pathogenesis of HLU and potential therapeutic strategies.
keratinocytes and a moderate dermal leukocyte infiltrate consisting of neutrophils, monocyte/macrophage cells, and T cells. Transcriptomic analysis of cytokines and growth factor expression in HLU compared to P-HS or C-HS showed a specific increase of IL-1β, OSM, and IL-6 cytokine expression. If IL-1β and OSM were elevated in P-HLU skin-sample culture supernatants, a weak and insignificant increase of IL-6 production was observed in P-HLU supernatants compared to P-HS or C-HS. This suggests that IL-6 production is more transient than OSM and IL-1β, consistent with our observation, showing a transient production of IL-6 after surgery.21 We showed the presence of IL-1β and OSM in dermis macrophages, and to a lesser extent, OSM labeling in polymuclear cells, lymphocytes, and myofibroblasts, in agreement with the pattern of IL-1β and OSM secretion previously described.33,34 By contrast, expression of the proinflammatory Th17 cytokines (IL-17A, IL-22), as well as TNF-α and IL-1α, was not modified. In addition, we could not detect any variation of expression of the IL-4, IL-5, and IL-13 Th2-derived cytokines or the IFN-γ Th1-derived cytokine (data not shown). Regarding the growth factors, neither PDGF-A, PDGF-B, vascular endothelial growth factor, or TGF-β1 was modified, whereas TGF-α expression was decreased. We detected an increase of CXCL8 expression, in agreement with the granulocyte infiltrate in HLU. Previous studies showed a specific signature in inflammatory skin diseases. Mainly IL-1β, IL-6, IL-17A, IL-22, OSM, and IFN-γ were over-expressed in psoriatic skin; IL-4, IL-13, IL-22, and to a lesser extent OSM in atopic dermatitis; and IL-10, IL-6, IL-17A, and IFN-γ in acne inversa.12,14,30,35–37 Taken together, we described a specific cytokine profile in HLU, which has not been described previously in inflammatory skin pathologies. This profile is distinguished from psoriasis, atopic dermatitis, and acne inversa by the absence of Th1-, Th2-, and Th17-derived cytokines.

We further analyzed the in vitro and in vivo effects of OSM and IL-1β on skin biology to evaluate their involvement in the HLU phenotype. In vitro, OSM alone induced hyperplasia of RHE, whereas the combination of OSM and IL-1β synergistically induced a destructurization of RHE, evoking acantholysis associated with acanthosis and a differentiation arrest of keratinocytes. This phenotype of treated keratinocytes mimicked the feature of skin HLU. By contrast, IL-6 alone or in combination with IL-1β displayed only a very discrete effect on RHE. This is in accordance with previous results demonstrating that IL-6 was clearly less potent than OSM in inducing human β defensin 2 and CXCL8 expression by keratinocytes in vitro, alone, or in association with other proinflammatory cytokines including IL-1.14

Furthermore, injection of IL-1β and OSM in mouse ear skin rapidly and synergistically induced macroscopic inflammation, acanthosis, granulocyte infiltration, keratinocyte proliferation, and revascularization of the dermis. Note that IL-6 in combination with IL-1β has a limited effect on these parameters. In accordance, the phenotypic switch of VSMC toward a proliferative state has been reported in vitro on human and animal cells after exposure to OSM or IL-1.24,38,39 Among the IL-6 cytokine family, only OSM is able to induce VSMC proliferation.39

Skin morphology after OSM and IL-1β treatment resumes some features of the human skin phenotype of HLU. Taken together, those data demonstrate that OSM and IL-1β are not only a phenotypic signature of HLU, but also mimic the feature of the disease by their biological effects on skin and vessels.

We hypothesize that in HLU, a vicious circle spreads out progressively as a consequence of the reduction of the vascular lumen, leading to blood flow reduction and tissue hypoxia (Figure 8). It has been shown that IL-1 and OSM are induced by hypoxia.40–42 Inflammation through cytokine production promotes epidermis hyperplasia and destructuration which, in association with hypoxia, lead to skin ulceration and necrosis. We suggest that factors such as alarmins released during necrosis contribute to the maintenance of the vicious circle by the recruitment of leukocytes. The inflammatory state induces VSMC proliferation and skin media arteriole thickening. In association with vascular lumen alteration, we observe angiogenesis, albeit insufficient to restore blood perfusion and prevent further necrosis.

HLU appears to be a more complicated pathology than expected and cannot be assumed to be only a consequence of long-lasting HBP.5,6 Whatever the class of antihypertensive agent used, we did not observe any difference in disease evolution. To date, surgery remains the gold standard for the treatment of HLU. The removal of the altered tissue can disrupt the vicious circle and allows fast healing. Our results suggest that biotherapies could be alternatives to surgery. Although anti-OSM therapies are not available to date, IL-1—targeting therapies are widely used to treat inflammatory diseases and could be promising to treat HLU by breaking the synergy between OSM and IL-1.43 We are currently investigating the possibilities of treating HLU by this strategy.

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Supplemental Data

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