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Expression of natural antimicrobial peptide β-defensin-2 and Langerhans cell accumulation in epidermis from human non-healing leg ulcers

Hanna Galkowska[1] , Waldemar L. Olszewski[1,3] and Urszula Wojewodzka[2]

[1]Department of Surgery and Transplantation and [2]Laboratory of Cell Ultrastructure, Medical Research Center, Polish Academy of Sciences;
[3]Central Clinical Hospital, Ministry of Internal Affairs, Warsaw, Poland

Abstract: Chronic wounds like venous calf and diabetic foot ulcers are frequently contaminated and colonized by bacteria and it remains unclear whether there is sufficient expression of defensins and recruitment of epidermal Langerhans cells in the margin of ulcer compared to normal skin. The aim of this study was to examine immunohistochemically the expression of β-defensin-2 (hBD2), GM-CSF, VEGF growth factors and accumulation of CD1a+ Langerhans cells (LC) in epidermis from chronic skin ulcers and to compare it to normal skin from the corresponding areas. Studies were carried out in 10 patients with diabetic foot, 10 patients with varicous ulcers of the calf and 10 patients undergoing orthopedic surgery (normal skin for control). Biopsy specimens were immunostained using specific primary antibodies, LSAB+ kit based on biotin-avidin-peroxidase complex technique and DAB chromogen. Results were expressed as a mean staining intensity. Statistical analysis of staining showed significantly higher staining of hBD2 in both normal and ulcerated epidermis from foot sole skin compared to calf skin (normal and ulcerated, $p<0.05$). Chronic ulcers showed the same expression of hBD2 as normal skin. There was significantly lower accumulation of CD1a+ LC in normal epidermis from foot sole skin compared to normal calf skin ($p<0.05$). Accumulation of CD1a+ LC and GM-CSF upregulation at the border area of diabetic foot ulcer and reduction of LC concentration at the margin of venous calf ulcer compared to normal skin were observed. It seems that normal calf and sole epidermis is, unlike in the mechanisms of innate immunity, influenced by the different keratinocyte turnover and bacterial flora colonizing these regions. Insufficient upregulation of hBD2 in both diabetic foot and venous calf ulcers may suggest the pathological role of this protein in the chronicity of ulcers.

Key words: Innate immunity - β-defensin-2 - Langerhans cells - Leg ulcers

Introduction

Skin is an active immune organ and its injury and microbial invasion trigger the activity of two important components of its innate immunity, namely epidermal dendritic cells (Langerhans cells) and antimicrobial peptides (AMPs). The latter natural antibiotics with broad activity against bacteria, fungi and enveloped viruses participate in the epithelial innate immune processes and are used as the first line of immune defence. AMPs are divided into several categories on the basis of their structure. In humans two major families of AMPs have been characterized: defensins (α and β) and cathelicidins. They were isolated from granules of human peripheral blood neutrophils as well as from macrophages, epithelial cells of the alimentary, respiratory and urogenital systems, and skin epidermis [8, 15]. Skin keratinocytes express three β-defensins: human β-defensin-1, -2, -3 (hBD1, 2, 3). In normal human skin, hBD1 is produced constitutively. *In vitro*, hBD2 synthesis is upregulated in keratinocytes by contact with microorganisms such as *E.coli*, *Staphylococcus epidermidis* and *aureus* and *Candida albicans* [1, 3, 6, 9, 12]. IL1 and IL6 enhance the epidermal antimicrobial response [5]. The hBD2 may also participate in other aspects of innate antimicrobial immunity since it chemoattracts monocytes and immature dendritic cells [4, 14]. Other factors, such as granulocyte-macrophage colony stimulating factor (GM-CSF) and vascular endothelial growth factor (VEGF) are also critical for dendritic cell recruitment *in vivo* and differentiation *in vitro* [7, 11]. Chronic wounds

Correspondence: H. Galkowska, Dept. Surgery and Transplantation, Medical Research Center, Polish Academy of Sciences, Pawinskiego 5, 02-106 Warsaw, Poland; e-mail: hgalk@cmdik.pan.pl

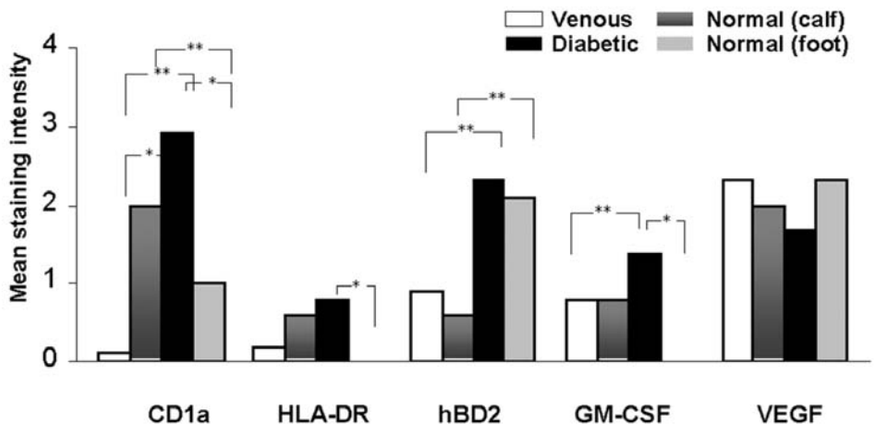

Fig. 1. The mean staining intensity of hBD2, VEGF, GM-CSF in keratinocytes and of CD1a, HLA-DR in Langerhans cells in regenerating epidermis of venous and diabetic foot ulcers and topographically corresponding control skin (*p<0.05 venous or diabetic *vs.* normal; **p<0.05 calf *vs.* foot).

like venous leg and diabetic foot ulcers are frequently contaminated and colonized by bacteria and one may ask the question as to whether there is sufficient expression of defensins and accumulation of epidermal Langerhans cells (LC) in the margin of ulcer compared to normal skin.

The aim of the present study was to examine immunohistochemically the expression of hBD2, GM-CSF and VEGF as well as LC accumulation in epidermis of chronic skin ulcers, compared to normal skin from the corresponding areas.

Materials and methods

Patients. The biopsy material investigated in this study originated from (1) ten patients, mean age 62 years, with type 2 diabetes complicated by foot ulcers grade 2-4 according to Wagner's classification of 3 to 6 months duration, (2) ten patients, mean age 67 years, with varicous ulcers of the calf of more than one year duration, and (3) eight nondiabetic patients, mean age 62 years, undergoing elective orthopedic surgery, providing control skin. All patients were treated in a uniform fashion with hydrocolloid dressings. At the time the biopsies were taken, no signs of inflammation were seen around the ulcers. Also no significant numbers of isolates were cultured from the bottom of ulcers. Occasionally, simple colonies of *Proteus* and *Staphylococcus aureus* were identified. This study was approved by the Ethical Committee of the Medical Research Center, Warsaw, Poland.

Skin biopsies. Skin biopsies were obtained under local anesthesia with 2% lignocaine from the border area of leg ulcers and from the topographically corresponding sites of normal subjects undergoing surgery for fracture correction. Two rectangles of tissue approximately 10 mm in length and 3 mm wide, comprising the ulcer edge and surrounding skin were excised and one specimen was snap frozen in a dry ice-cold acetone and stored at -70˚C, whereas the other one was fixed in 4% phosphate buffered formalin and embedded in paraffin. The paraffin-embedded specimens were cut into 4 μm sections and placed on silane-coated glass slides (Dako, Gloustrup, Denmark). Frozen serial sections 4 μm thick mounted on poly-L-lysine (Sigma, Pool, UK) coated glass slides were dried at room temperature (RT) overnight and stored at -20˚C.

Antibodies and immunohistochemistry. The specific primary antibodies against the following antigens were applied (code number and working solution in parentheses): human β-defensin-2 (sc-10854, 1/200) from Santa Cruz Biotech (Santa Cruz, Ca); CD1a (M721, 1/50), HLA-DR (M746, 1/30) from Dako (Glostrup, Denmark); GM-CSF (AB-215-NA, 1/50) and VEGF (AB-293-NA, 1/50) from R&D Systems (Abingdon, United Kingdom). The paraffin-embedded sections used for staining with anti-VEGF antibody were deparaffinized at 57˚C for 15 min and then dewaxed in xylene, taken through graded ethanol and rehydrated in TBS (TRIS-buffered saline). They were boiled two times for 5 min in 0.01 M sodium citrate in microwave oven and after washing blocked with 50% normal swine serum for 20 min. The frozen sections were allowed to thaw at RT before being unwrapped and then they were fixed in cold acetone for 10 min at RT. To remove endogenous peroxidase activity, all slides were incubated in 3% H_2O_2 for 5 min and washed in TBS. Tissue sections were incubated with primary antibodies diluted in TBS for 30 min (CD1a, HLA-DR, GM-CSF) or 60 min at RT (anti-defensin) or overnight at 4˚C (VEGF). Control sections were processed parallelly without the primary antibody. A standard labelling technique using avidin-biotin-immunoperoxidase complex (Dako) was employed (LSAB2 kit for mouse and rabbit immunoglobulins and LSAB+ kit for goat globulins and paraffin-embedded sections). Sections were processed simultaneously for all antigens according to the procedure outlined by the manufacturer. Each incubation step was 18 min with 5 min washes inbetween. Color reaction was performed with DAB (Sigma Chemical Co, St Louis, USA) substrate for 5 min. Finally, the sections were counterstained with 0.2% Mayers hematoxylin (Sigma) for 1 min. Frozen sections were mounted in glycergel (Dako) and paraffin embedded ones in Canada balsam (Fluka Chemie AG, Buchs, Switzerland).

The staining assessment. The mean staining intensity of hBD2, HLA-DR, GM-CSF, VEGF and CD1a was assessed semi-quantitatively using a score system: 0 = no staining; 1 = weak staining; 2 = moderate staining; 3 = strong staining. All samples were evaluated microscopically by two study-blinded investigators. The two scores of three serial tissue sections were averaged for each specimen and these averaged values were used for comparisons.

Data analysis. The Monte Carlo exact test was used to investigate the significance of differences in the mean intensities of staining between studied groups. P-value <0.05 was considered statistically significant.

Results and discussion

The immunostaining demonstrated a variable expression of hBD2 and accumulation of CD1a+ LC in epidermis depending on the area (Fig. 1). There was a

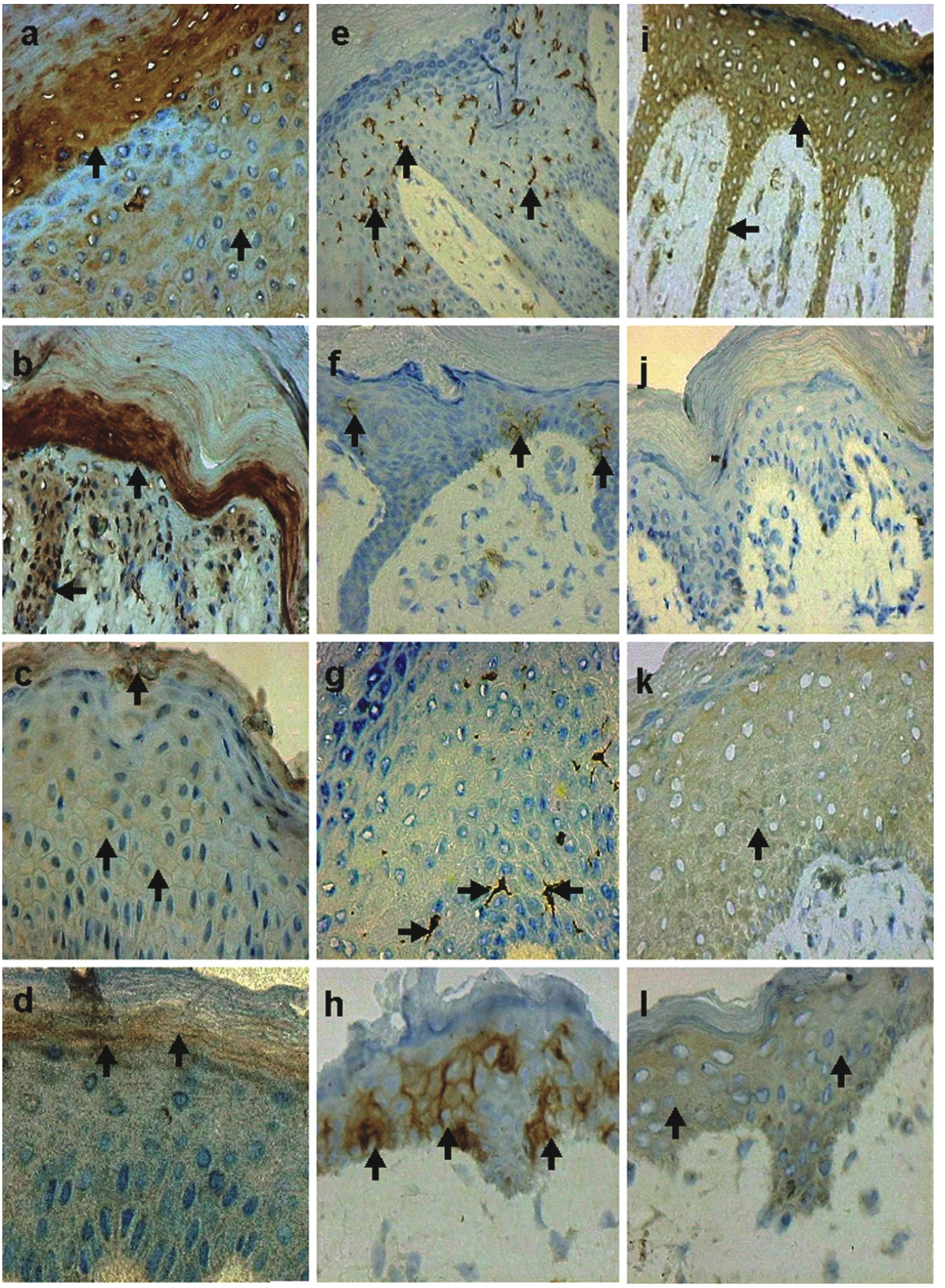

Fig. 2. Immunostaining of hBD2 (**a-d**), CD1a+ Langerhans cells (**e-h**) and GM-CSF (**i-l**) in epidermis at the border area of diabetic foot (**a, e, i**) and venous calf (**c, g, k**) ulcers and in normal foot (**b, f, j**) and calf (**d, h, l**) skin. b,e,f,i,j: × 100, a,c,d,g,h,k,l: × 200.

significantly enhanced staining intensity of hBD2 in keratinocytes of both normal and ulcerated epidermis from foot sole skin compared to the weak staining of calf skin (normal and ulcerated, $p<0.05$), (Fig. 2 a-d). Staining was localized mainly in stratum corneum and in the interkeratinocyte space of the Malpighian layer. In contrast, there was a significantly reduced concentration of CD1a+ LC in normal epidermis from foot sole skin compared to normal skin of calf ($p<0.05$), (Fig. 2 e-h). We observed the reduced expression of CD1a marker at the edge of venous calf ulcers ($p<0.05$) compared to control skin and significantly increased expression of both CD1a and HLA-DR markers of LC on the margin of diabetic foot ulcer compared to normal sole skin ($p<0.05$). In contrast to lack of GM-CSF staining in normal sole epidermis, keratinocytes at the border of diabetic foot ulcer expressed GM-CSF ($p<0.05$), (Fig. 2 i-l). The intensity of this staining was significantly higher than that observed in calf epidermis. All studied epidermal samples revealed a comparable, moderate to strong, expression of VEGF (Fig.1).

Our results are consistent with previous reports that hBD2 is detectable in normal human skin and that sole skin demonstrates intense immunostaining [1]. Low expression of hBD2 in calf epidermis in contrast to sole skin may reflect differences in keratinocyte turnover [10] or bacterial colonization. Production of hBD2 can be activated in keratinocytes by IL1β [13] *in vitro* and by wounding of normal skin [2]. However, no upregulation of hBD2 immunostaining compared to normal skin was observed by us in the chronic ulceration, both in venous calf and diabetic foot ulcers. This can suggest that insufficient expression of hBD2 may play a pathogenic role in chronic wounds.

Moreover, we demonstrated a variable accumulation of epidermal CD1a+ LC depending on the leg area, low in sole and enhanced in calf. The observed high level of LC in diabetic foot ulcer could be associated with simultaneously enhanced expression of GM-CSF, a growth factor critical for dendritic cell survival and differentiation [11]. Other factors, hBD2 and VEGF, supposed to influence dendritic cell accumulation [14] or reduction in skin [7] appeared not to be correlated with expression of epidermal CD1a marker in diabetes.

We suggest that normal calf and sole epidermis are, unlike as in the mechanisms of innate immunity, influenced by the different keratinocyte turnover and bacterial flora colonizing these regions. Chronic wounds, both diabetic foot and varicous calf ulcers, showed insufficient expression of hBD2 as compared to normal skin suggesting the pathological role of this protein in chronicity of ulcers.

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