

Human beta defensin-4 and keratinocyte growth factor gene expression in cultured keratinocyte and fibroblasts of burned patients¹

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ABSTRACT

PURPOSE: To evaluate KGF and human beta defensin-4 (HBD-4) levels produced by dermic fibroblasts and keratinocytes cultivated from burned patients' skin samples.

METHODS: Keratinocytes and fibroblasts of 10 patients (four major burns, four minor burns and two controls) were primarily cultivated according to standard methods. HBD-4 and KGF genes were analyzed by quantitative PCR.

RESULTS: In fibroblasts, KGF gene expression was 220 ± 80 and 33.33 ± 6.67 (M \pm SD; N=4), respectively for major and minor burn groups. In keratinocytes, KGF gene expression was 11.2 ± 1.9 and 3.45 ± 0.37 (M \pm SD; N=4), respectively for major and minor burn groups. In fibroblasts, HBD-4 gene expression was 15.0 ± 4.0 and 11.5 ± 0.5 (M \pm SD; N=4), respectively for major and minor burn. In keratinocyte, HBD-4 gene expression was 0.0 ± 0.0 and 13.4 ± 4.8 (M \pm SD; N=4), respectively for major and minor burn.

CONCLUSIONS: KGF expression was increased in burn patient fibroblasts compared to control group. In keratinocytes culture, KGF suppression is inversely proportional to burn extension; it is active and increased in major burn but decreased in minor burn. HBD-4 expression was increased in fibroblasts and decreased in keratinocytes from all burned patients.

Key words: Burns. Gene expression. Fibroblasts. Keratinocytes. Fibroblast growth factor 7. Human beta defensin 4.

Introduction

The high incidence of infection among burn patients is a major concern in its clinical evolution, which is directly related to morbidity and mortality rates of this group. Antimicrobial peptides, components of innate immune response, protect the organism by the activation of inflammatory cells or inactivating pathogen action directly. Therefore, the increased expression in these peptides by the epithelial cells improves hosts resistance against microbial infection^{1,2}. Among the great diversity of antimicrobial peptides involved in the inflammatory response, we highlight here the defensin family. Defensins are able to permeabilize bacterial membranes selectively, as has been proved in the study of peptides Crp-4, HBD-2, HBD-3, RTD-1 e BTD-7³.

HBD-4 presents a high antimicrobial activity against *Pseudomonas aeruginosa* when expressed by genetically modified primary epidermal keratinocytes⁴. HBD-3 shows strong bactericidal activity against *Staphylococcus aureus* and MRSA when its expression is induced in human keratinocytes and fibroblasts⁵.

HBD-2 inhibits in vitro growth of the dermatophytes *Trichophyton rubrum*, *T. mentagrophytes*, *Microsporum canis* e *Epidermophyton floccosum*, despite not as much as psoriasis, RNAse 7 and fluconazol⁶.

Studies concerning keratinocyte growth factor (KGF) actions in skin lead to quantitative relations between KGF levels and immune response antimicrobial peptides. It was demonstrated that RNAm codifying HBD-2 and cathelicidin-37 quantity is increased proportionally to the KGF gene expression in cultured cells^{7,8}.

Keratinocytes cultures treated with interleukin-1-alpha presents high level of the antimicrobial peptides human beta-defensin 1 and 2, IL-37 and antileukoprotease. When infected with *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, its antibacterial activity is significantly increased⁹.

In direct contact with bacteria as *Pseudomonas aeruginosa*, KGF alone is unable to prevent their proliferation. However, cultured human keratinocytes that produced KGF or other culture supplemented with this growth factor are able to exuberantly inhibit bacterial proliferation. Therefore, it is suggested that KGF is a skin immune response modulator, possibly through inducing defensins production by keratinocytes¹⁰.

We infer that KGF acts indirectly, as an immune response modulator, as the substance itself does not present any effect in the microorganisms. The present study hypothesis is that KGF might be the trigger to some antimicrobial peptides

production by keratinocytes, such as human beta defensins. Therefore, our objective is to perform a quantitative analysis of keratinocyte growth factor (KGF) gene expression and human beta-defensin-4 (HBD-4) produced by fibroblasts and keratinocytes, respectively, from cultured burned patients skin samples.

Methods

The project was approved by the Ethics Committee of Federal University of Sao Paulo (UNIFESP) in October 14th, 2011 (1454/11). The present study is experimental, in vitro, using discarded skin from burned patients. It is observational, analytic, controlled and conducted in a single center. Patients included in this study have read and signed the Free and Clarified Contentment Term.

The patients recruited to this study were burn victims admitted in the Burns Treatment Unit, Plastic Surgery Division, University Hospital (UNIFESP), and the control group with healthy patients, non-smoking, submitted to mammoplasty in the Plastic Surgery Division. The culture of cells was realized, and we have created a cell bank with the characteristics of these patients, which have been previously published¹¹.

Inclusion, exclusion and non-inclusion criteria

Inclusion criteria for the study were patients of both genders, over 18 years old, for the group with major burns: having deep partial thickness or full thickness burns affecting between 25% and 50% of total body area surface (TBSA) or which require partial skin graft in 10% TBSA. For the minor burns group, the criterion added was that the affected TBSA should be 5% or less, for deep partial thickness or full thickness burns and the need of partial skin graft. To the control group was included the criterion of not having previous diseases, not smoking, and performing aesthetic surgery.

Patients who had previous skin diseases or conditions that should interfere directly in the inflammatory process were not included. Exclusion criteria were the lost of the extracted material. The surgical procedure, fibroblasts and keratinocytes cultures, and total RNA extraction were reported earlier¹¹.

Commercially available TaqMan Gene Expression Assay kits (Applied Biosystems, Foster City, CA) were used to assess HBD-4 (Hs00823638_m1) and FGF7 (KGF) (Hs00384281_m1). B-actin gene (Hs99999903_m1) was used as housekeeping gene^{11,12}. All qPCR reactions were performed

on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using the fluorescent Taqman methodology (TaqMan One Step qPCR Master Mix Reagentes, Applied Biosystems, Foster City, CA). 100 ng of total RNA) was used for each qPCR reaction in a total volume of 50µL according to the manufacturer's protocol. Thermal cycling conditions were as follow: 30s at 48°C, 10 min at 95°C, 40 cycles of 15s, denaturation at 95°C, and 60 s annealing at 60°C. Quantification Cycle (Cq) values were used as endpoint defined as the PCR cycle number in which the fluorescence generated by the amplification crosses the threshold. Each gene was submitted to an absolute quantification assay by means of a standard curve, produced by dilutions to calculate the amount of a target sequence in a solution. The results were expressed in the same measure units as the standard curve. Three replicate reactions per sample were run to ensure statistical significance.

The assays were made in triplicate and the experimental values obtained in the study were presented by mean (M) and standard deviation (SD), because it is a cultured cells assay, where there is the homogenization of the cells lineage, with any stimulation differing from the culture medium. The calculation of the Cq were obtained as described in previous study¹¹. The comparative Cq method (also known as the 2-DDqC method)¹³ was used for data analysis in order to calculate relative quantities (RQs) of gene expression among the samples. DataAssist™, software developed for quick analysis of TaqMan real-time PCR (Applied Biosystems, Foster City, CA), was used to confirm the data calculations performed in Microsoft Office Excel. Whenever receptor quantification exceeded 36 amplification rounds (qC), quantities were considered to be undetectable or very low, and measurements were taken as invalid. Statistical significance was assessed using the Student's t-test. Statistical significance was defined at $p < 0.05$.

Results

Keratinocyte growth factor (KGF) expression in fibroblast culture

The gene responsible for KGF synthesis is over expressed in burn patients' fibroblasts compared to the control group (Figure 1). Apparently there is no difference between expression levels among the minor and major burn groups and there is only a tendency to significance with $p = 0.081$. The mean of the minor and major burn groups were (M ± SD): 33.33 ± 6.67 (n=4) and 220 ± 80 (n=4), respectively.

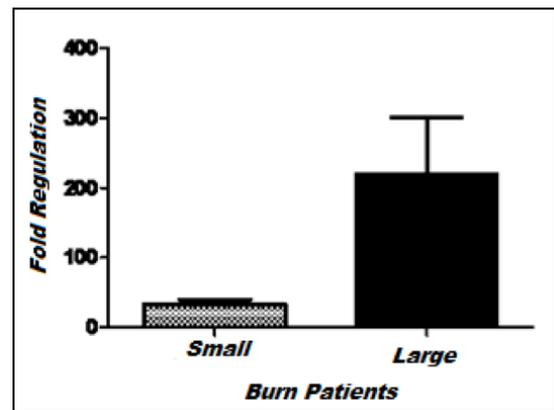


Figure 1. KGF (FGF-7) gene expression in burned patients' fibroblasts.

Keratinocyte growth factor (KGF) expression in keratinocyte culture

In keratinocyte culture, the expression of the KGF gene seems to be directly proportional to burn injury extension. It is active and over expressed in the major burn group and under expressed in the minor burn group (Figure 2). There was a difference ($p = 0.019$) between both groups. The mean obtained for minor and major burn groups were (M ± SD): 3.45 ± 0.37 (n=4) and 11.2 ± 1.9 (n=4), respectively.

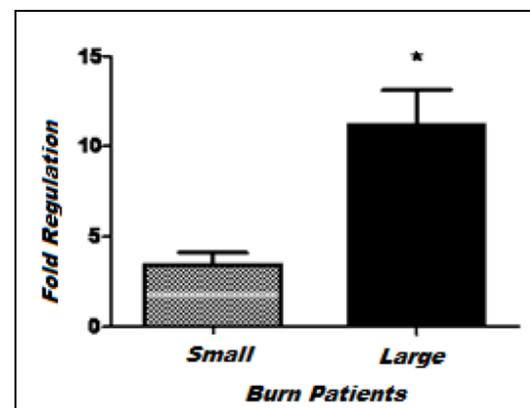


Figure 2. KGF gene expression in burned patients' keratinocyte.

Human beta-defensin-4 (HBD-4) expression in fibroblast culture

HBD-4 is over expressed in burned patients' fibroblasts of both minor and major groups, compared to the control (Figure 3). No difference was shown between minor and major groups ($p = 0.43$). The mean of minor and major burn were (M ± SD): 11.5 ± 0.5 (n=4) and 15.0 ± 4.0 (n=4), respectively.

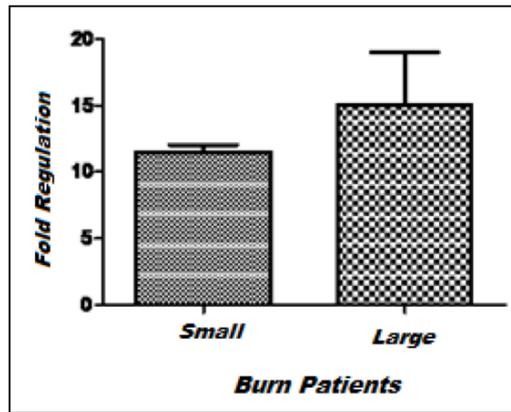


Figure 3. HBD-4 gene expression in burned patients' fibroblasts.

Human beta-defensin-4 (HBD-4) expression in keratinocyte culture

The keratinocytes cultured had the gene HBD-4 repressed in a directly proportional manner to the extension of the burn (Figure 4). In the major burn group, HBD-4 gene is active and over expressed compared to control, while it is no expressed in the minor burn group. The mean of minor and major burn were (M ± SD): 13.4 ± 4.8 (n=4) and 0 ± 0 (non-expressed), respectively.

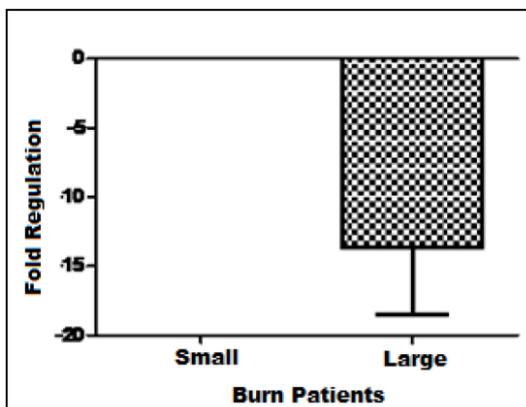


Figure 4. HBD-4 gene expression in burned patients' keratinocytes.

Discussion

A large amount of studies shows KGF's importance as a paracrine mediator of epithelial growth. The induction of increased KGF expression in fibroblasts and keratinocytes grafted in burn models in vivo leads to significant acceleration of the reepithelialization process¹⁴, while KGF ablation leads to decreased proliferation rates, angiogenesis and VEGF mRNA concentration in murine models submitted to burn injury¹⁵.

Corroborating with the results obtained in the present study, assays utilizing RNase has detected KGF expression

in normal skin samples, without any injuries, while in situ hybridization has revealed an important increase in the expression of RNA codifying KGF only 1 day after skin damage. That increase is progressively proportional to the age of the damage infringed⁷.

Murine beta-defensin (mBD) has been detected in normal skin samples taken from burn murine models, while samples taken from areas which were submitted to thermal injury have shown a large decrease in mBD concentration. Gr-1(+)/CD11b(+) cells, capable of suppress antimicrobial peptides production by keratinocytes, have been isolated from the circundating tissue around burned site in murine models. When those cells were inoculated in control rats, without burn injury, antimicrobial peptides production around inoculation site has decreased. Both groups have shown increase of intradermal infection incidence by *Pseudomonas aeruginosa*¹⁶.

Similar to previously reported, our study has shown that occurred a significant increase in HBD-4 expression in area marginal to the burned skin. In detail, our results have shown that in the minor burn group the HBD-4 gene has its activity repressed, while in major burn group it is active, but hypo expressed.

The antibiotic use, although essential to the burned patient treatment, causes selection of multiresistant bacteria. Antibiotic resistant *Acinetobacter baumannii*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* cultures were analyzed. All of them have shown sensibility to HBD-4 antimicrobial activity, without any relation between resistances to the most commonly used antibiotics and the sensibility to the human peptide¹⁷.

These data, with are consistent with results shown in this study, indicates that the hypo expression of HBD-4 in burn patients must be related to the concerning high incidence of infection and sepsis, especially in major burn patients. The advance of knowledge about the altered genes and the possibility of using drugs and substances able to stimulate defensin production by keratinocytes open a possibility to the development of new efficient treatment against antibiotic-resistant pathogens.

Therefore, we have the perspective to ally the knowledge obtained regarding KGF expression and HBD-4 expression to a possible drug therapy to evaluate the control of these genes expression in major burn patients.

Conclusion

KGF gene expression is increased in burn patients fibroblasts, however, in keratinocyte culture, the expression of the KGF gene seems to be directly proportional to burn extension. HBD-4 is overexpressed in burn patients' fibroblasts

and in keratinocyte culture this gene was repressed in a directly proportional manner to the extension of the burn.

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