

## Keratinocyte growth factor, tumor necrosis factor-alpha and interleukin-1 beta gene expression in cultured fibroblasts and keratinocytes from burned patients<sup>1</sup>

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### ABSTRACT

**PURPOSE:** To evaluate the gene expression of *KGF*, *TNF-alpha* and *IL-1 beta* in skin fibroblasts and keratinocytes cultured from burned patients.

**METHODS:** Three patients with large burns and three patients with small burns, as well as two controls, were included. The cell culture was initiated by the enzymatic method. After extraction and purification of mRNA, qPCR was used to assess the gene expression of *KGF*, *TNF-alpha* and *IL-1 beta*.

**RESULTS:** The expression of *KGF* was increased on average 220-fold in large burns and 33.33-fold in small burns in fibroblasts, and 11.2-fold in large burns and 3.45-fold in small burns in keratinocytes compared to healthy patients ( $p < 0.05$ ). Expression of *TNF-alpha* was not observed. *IL-1 beta* is down-regulated in fibroblasts of burned patients, and much more repressed in small burns (687-fold,  $p < 0.05$ ). In keratinocytes, the repression of *IL-1 beta* expression occurs in patients with small burns (28-fold), while patients with large burns express this gene intensively (15-fold).

**CONCLUSIONS:** The study showed a quantitative pattern in the expression of KGF gene, which is more expressed according to the size of the burn. TNF-alpha was not expressed. A qualitative pattern in the expression of IL-1 beta gene was demonstrated.

**Key words:** Burns. Gene Expression. Fibroblasts. Keratinocytes. Tumor Necrosis Factor-alpha. Fibroblast Growth Factor 7. Interleukin-1.

## Introduction

Burn injuries can influence many body functions and the consequences can be severe for these patients shortly after the accident. An intense inflammatory response is triggered in burns that affect a large total body surface area (TBSA)<sup>1</sup>. The beneficial effects of local inflammation include cleaning debris and protection against microbial agents, as well as cell growth and proliferation<sup>2</sup>.

However, the prolongation of the acute inflammatory process impairs wound healing, due to the fact that the high level of cytokines leads to degradation of collagen, apoptosis of keratinocytes, vascular commitment and production of oxygen-free radicals<sup>2</sup>.

Cytokines are the primary mediators of this inflammatory response to thermal injury. They promote communication between various types of cells via autocrine, paracrine and endocrine signaling. Thermal injury increases the serum level of cytokines<sup>1</sup>. Tumor necrosis factor-alpha (TNF-alpha) is involved in this process. The increased production of TNF-alpha is detectable in body fluids within the first twelve hours after trauma and is primarily released by macrophages in sites where it induces neutrophil recruitment and maturation<sup>3</sup>. Separately, it was shown that TNF-alpha inhibits the reepithelialization of wounds<sup>4</sup>. TNF-alpha is able to induce KGF production, which suggests that it operates indirectly in the reepithelialization process<sup>5</sup>.

The keratinocyte growth factor (KGF) or fibroblast growth factor-7 (FGF-7) is an important member of the family of FGFs involved in wound repair. Recent studies have suggested that the increase of *KGF* expression is a response to injuries in general. The repair of the injured epithelium depends on the mitogenic KGF power, and it has been noted that this growth factor can protect the epithelial cells of the skin against various insults *in vivo* and *in vitro*<sup>6</sup>. This factor is produced by dermal fibroblasts and acts by stimulating keratinocytes proliferation through receptors present on these cells<sup>4,7</sup>. The *KGF* gene expression by dermal fibroblasts is increased after cytokine signaling. And some of these cytokines are part of the Interleukin-1 family<sup>5</sup>.

The Interleukin-1 (IL-1) family cytokines are molecules that help to coordinate the early immune response against exogenous or endogenous danger. IL-1 alpha and IL-1 beta were the first members of this family to be described and now has 11 molecules of type IL-1. Due to its central role in the onset of inflammation, IL-1 has been an important therapeutic target. The IL-1 beta is a highly pyrogenic molecule and there are multiple levels of control of both its production and its effect<sup>8</sup>.

A different gene expression pattern is generated after

thermal injury but no data are available in the literature. These genes are modulated during the inflammatory response in order to initiate subsequent events in wound repair<sup>9</sup>.

The aim of this study was to evaluate *KGF*, *TNF-alpha* and *IL-1 beta* gene expression in primary cultured dermal fibroblasts and keratinocytes from patients with large and small burns and compare it with that of the controls.

## Methods

The project was approved by the Ethics Committee of Federal University of Sao Paulo (UNIFESP) (0689/11) and registered in Clinical Trials (NCT01302223).

This study had an experimental design *in vitro*, it was observational, analytical, controlled and it was conducted in a single center.

Samples were taken from three patients with large burns and three with small burns; two healthy patients in the control group were included in this study.

These samples were obtained from patients admitted to the Burns Treatment Unit, Plastic Surgery Division, Federal University of Sao Paulo, University Hospital (Table 1). The control group comprised two healthy, non-smoking female patients with no previous disease or medication use and undergoing aesthetic surgery. The first patient was 38 years old and underwent breast lift surgery; the second patient was 32 years old and underwent abdominoplasty.

**TABLE 1** – Data (n, age, gender, mean % TBSA) of burned patients included in the study.

Group	n	Mean Age	Gender*	Mean % TBSA
Large Burns	3	31.5 years	2 M 1 F	32 %
Small Burns	3	37.75 years	2 M 1 F	4 %

\*M = Male; F = Female

### *Inclusion, non-inclusion and exclusion criteria*

Inclusion criteria for the study were patients of both genders, over 18 years old, who agreed to participate and signed a consent form, being hospitalized in Burns Unit and requiring surgery. A criterion was added to the group with large burns: having deep partial thickness or full thickness burns affecting between 25% and 50% of TBSA or which require partial skin graft in 10% TBSA. For the small burns group, the criterion added was that the TBSA be affected by 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.

Inclusion criteria were also that the patient has not had previous skin diseases (psoriasis and similar), not had illnesses that might interfere directly in the inflammatory process (rheumatic diseases in general) and agreed to participate in the study.

Exclusion criteria were contamination of the culture flasks, low proliferation rate without achieving confluence of 80% of the cells in the flasks, insufficient quantity of extracted RNA that prevents the evaluation of patient data or loss of the extracted material.

### *Surgery*

The debridement of burned tissue with deep partial thickness or full thickness burns occurred three to five days after injury. Normal skin around the lesion is removed because of the surgical procedure itself.

### *Keratinocyte culture*

Keratinocytes derived from a skin fragment of burn patient were isolated and cultured according to the standard method, adapted in laboratory with changes as described below<sup>7</sup>.

In a 60 mm culture plate, the skin fragment was placed and sectioned into small pieces with size of about 0.5 cm<sup>3</sup>. These fragments were placed in a 50 ml sterile tube with 30 ml of dispase (Boeringer Mannheim, Indianapolis, IN; cat. no. 165859) and kept refrigerated at 4°C until the next morning, totaling 15 hours of action of dispase. After this period, each fragment was gently detached epidermis from the dermis with forceps; the epidermis was intended for isolation of keratinocytes, while the dermis underwent enzymatic dissociation with collagenase as described below.

### *Fibroblast culture*

The culture was initiated by the enzymatic method using collagenase<sup>10</sup>. The dermis was placed in sterile Petri dishes. Collagenase solution sterile type-2 (Gibco, Grand Island, NY, USA - 17101-015) and diluted in PBS (3000 units/ml) and 3 ml of the solution for every gram of tissue was placed on the plate. This was made overnight at 37°C. After digestion of the tissue, the suspension was filtered through a 100micron filter and centrifuged at 400g for 10 min. The cells were centrifuged and placed in a flask

with fibroblast culture medium<sup>11</sup>.

### *RNA isolation and purification*

RNA was extracted from the cells using Trizol Reagent (Invitrogen, Foster City, CA) following the manufacturer's instructions. After complete dissociation of nucleoprotein complexes, phase separation was achieved with chloroform and centrifugation. The precipitated RNA from the aqueous phase was washed with 75% ethanol. The RNA was dried and dissolved in RNase-free water. Total RNA was then purified with QiagenRNeasyMiniKit (Qiagen, Valencia, CA) and submitted to DNase treatment. The amount and quality of the extracted RNA were assessed by spectrophotometry using NanoDrop v3.3.0 (NanoDrop Technologies Inc., Rockland, DE).

### *qPCR*

Commercially available TaqMan<sup>®</sup> Gene Expression Assay kits (Applied Biosystems, Foster City, CA) were used to assess TNF-alpha (Hs00174128\_m1), FGF7 (KGF) (Hs00384281\_m1), IL-1 beta (Hs01555410\_m1) and B-actin (Hs99999903\_m1) gene expressions on all samples. B-actin was used as housekeeping gene<sup>12</sup>. 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). Total RNA (50 ng, 100 ng or 300 ng per well) was used for each qPCR reaction in a total volume of 25µL according to the manufacturer's protocol. Thermal cycling conditions were as follow: 30 s at 48°C, 10 min at 95°C, 40 cycles of 15 s, 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. Three replicate reactions per sample were run to ensure statistical significance.

### *Statistical analysis*

Comparative Cq method (also known as the 2<sup>-ΔΔqC</sup> method)<sup>13</sup> was used for data analysis in order to calculate relative quantities (RQs) of gene expression among the samples. DataAssist<sup>™</sup>, software developed for quick analysis of TaqMan<sup>®</sup> real-time PCR (Applied Biosystems, Foster City, CA), was used to confirm the data calculations performed in Microsoft<sup>®</sup> 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**

*Extraction, purification and measurement of RNA*

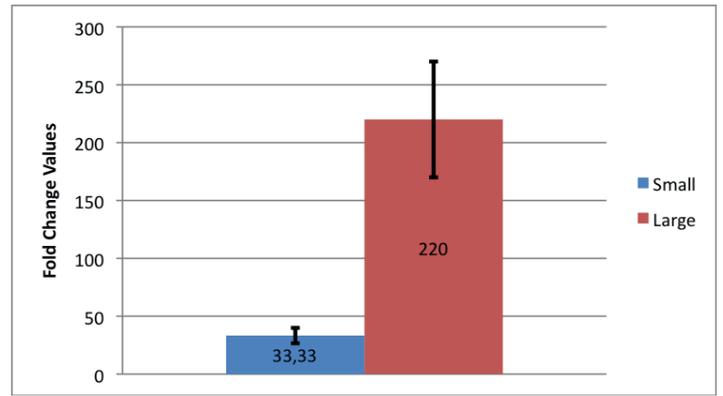
Samples of fibroblasts and keratinocytes were subjected to RNA extraction. Both the data from dosing solutions as well as their purity are presented in Table 2. The solutions presented RNA 260nm/280nm ratio values between 1.9 and 2.1, i.e., a purity range considered suitable for gene expression analysis.

**TABLE 2** - Concentration of RNA solutions obtained by means of spectrophotometry and purity range.

Cell	/	Group	Concentration (ng/ul)	260/280 Ratio
Fibroblasts		Large Group Patient 1	699	2.1
		Large Group Patient 2	667	2.1
		Large Group Patient 3	868	2.0
		Small Group Patient 4	946	2.0
		Small Group Patient 5	144	2.0
		Small Group Patient 6	112	2.0
	Control Group	634	2.0	
Keratinocytes		Large Group Patient 1	593	2.1
		Large Group Patient 2	658	2.1
		Large Group Patient 3	918	2.0
		Small Group Patient 4	919	2.0
		Small Group Patient 5	108	2.1
		Small Group Patient 6	90	1.9
	Control Group	398	2.0	

*KGF gene expression in fibroblasts*

The KGF gene was significantly upregulated in fibroblasts from burned patients when compared to the control group. The small burns group presented a fold change value of 33.33, while in the large burns group this value was 220. There is no statistical difference between small and large burns groups (Figure 1).

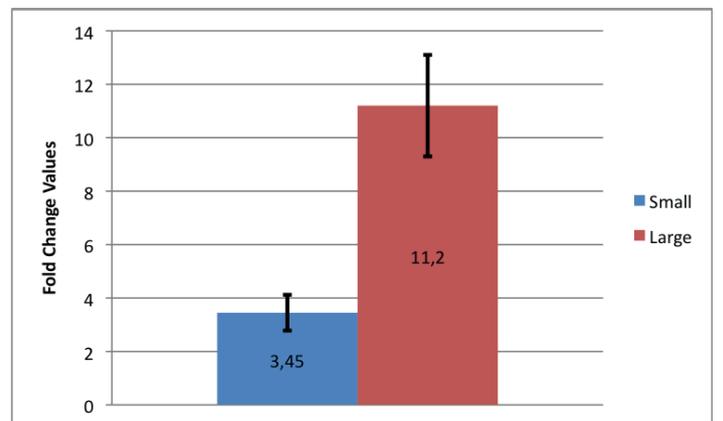


Unpaired t test: p value = 0.081 (significant trend)  
 Are means significant different? ( $p < 0.05$ ) = No

**FIGURE 1** - KGF gene expression in fibroblasts from small and large burned patients compared to a non-burned control patient. Mean  $\pm$  SEM of Small group =  $33.33 \pm 6.67$  (n=3). Mean  $\pm$  SEM of Large group =  $220 \pm 80$  (n=3).

*KGF gene expression in keratinocytes*

The KGF gene was upregulated in keratinocytes from burned patients when compared to the control group. The small burns group presented a fold change value of 3.45, while in the large burned group this value was 11.2. There is a significant difference between small and large burns groups ( $p = 0.019$ ) (Figure 2). KGF gene expression was modulated by the burn intensity in keratinocytes.

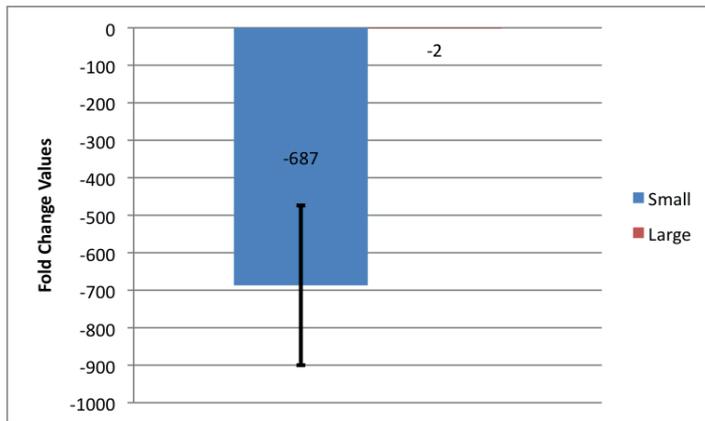


Unpaired t test: p value = 0.019\* (significant different)  
 Are means significant different? ( $p < 0.05$ ) = yes

**FIGURE 2** - KGF gene expression in keratinocytes from small and large burned patients compared to a non-burned control patient. Mean  $\pm$  SEM of Small group =  $3.45 \pm 0.67$  (n=3). Mean  $\pm$  SEM of Large group =  $11.2 \pm 1.9$  (n=3).  $p = 0.019^*$ .

*IL-1 beta gene expression in fibroblasts*

The IL-1 beta gene was down-regulated in fibroblast of the burned patients when compared with the control group, and much more repressed in small burns. The fold change value of the small burned group was -687, while in the large group this value was -2.0 (p=0.033) (Figure 3). Nevertheless, all patients continue to express the gene.

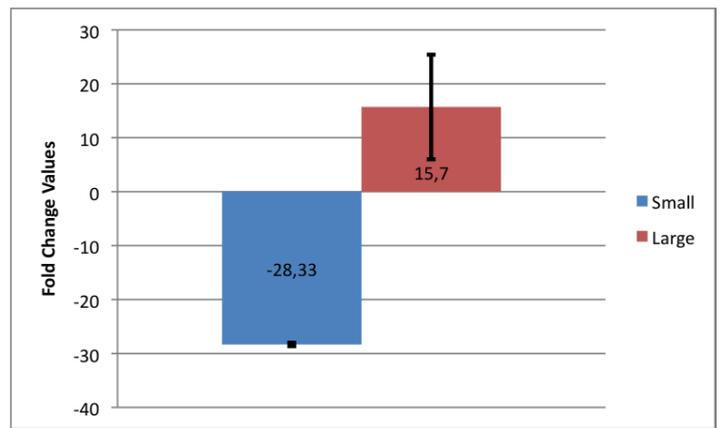


Unpaired t test: p value= 0.033\* (significant different)  
Are means significant different? (p<0.05) = yes

**FIGURE 3** - IL-1 beta gene expression in fibroblasts from small and large burned patients compared to a non-burned control patient. Mean ± SEM of Small group = -687 ± 213 (n=3). Mean ± SEM of Large group = -2.0 ± 0.40 (n=3). p = 0.033\*.

*IL-1 beta gene expression in keratinocytes*

Repression of IL-1 beta gene was high in small burns patients, while large burns patients expressed this gene intensively when compared to control (Figure 4). Burn intensity is controlling which intracellular pathways should be activated and deciding if IL-1B gene should be repressed or activated according to the changes in the microenvironment of the cells.

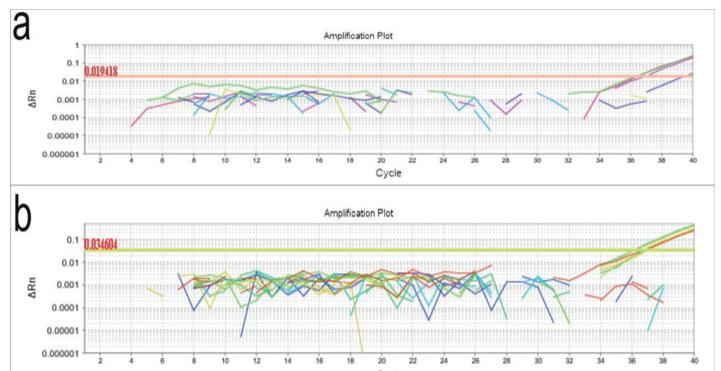


Unpaired t test: p value= 0.011\* (significant different)  
Are means significant different? (p <0.05)= yes

**FIGURE 4** - IL-1 beta gene expression in keratinocytes from small and large burned patients compared to a non-burned control patient. Mean ± SEM of Small group = -28.33 ± 0.33 (n=3) Mean ± SEM of Large group = 15.7 ± 9.7 (n=3). p = 0.011\*.

*TNF-alpha gene expression in fibroblasts and keratinocytes*

TNF-alfa gene expression was either not detected in fibroblasts and keratinocytes or occurred at very low levels (Figure 5).



**FIGURE 5** - TNF-alpha amplification plot in fibroblasts (a) and in keratinocytes (b).

**Discussion**

Studies that evaluate gene expression of cytokines and growth factors by epidermal and dermal cells of the skin from burned patients do not exist in the literature. The few studies published on gene expression in burns were realized in blood cells and offered a vision of systemic inflammation in the body. Nedelec *et al.*<sup>14</sup> demonstrated that a differential effect of burn injury occurs on fibroblasts from wounds and normal skin. Systemic mediators markedly alter the proliferation potential of dermal fibroblasts,

but not fibroblasts from burn injuries. The wound environment substantially alters both the proliferation rate and the responsiveness of fibroblasts to cytokines. Thus, the data support the value of using burn wound fibroblasts *in vitro* experiments to investigate the change of wound healing by cytokine manipulation<sup>14</sup>.

The present study is the first to evaluate the gene expression of KGF, TNF-alpha and IL-1 beta, mediators with a central role in the inflammation process in human skin cells from burned patients.

*KGF* is an important growth factor involved in the reepithelialization of lesions. This factor is produced by dermal fibroblasts, and for this reason *KGF* gene expression is increased in these cells during the acute phase of burns in order to accelerate cleaning and repair of damaged tissues. Our results showed that this gene is upregulated, on average, 125-fold in skin fibroblasts and 7.5-fold in keratinocytes from burned patients than in control patients. This suggests that thermal injury is an event with very strong signal to stimulate the expression, and perhaps the production of *KGF*. This also indicated a quantitative pattern of expression. The greater the extension of burned area higher the *KGF* expression by skin cells of the patient.

Chedid *et al.*<sup>15</sup> studied cytokines and growth factors that influenced the production of *KGF* by fibroblasts. An embryonic fibroblast culture (M426), a postectomy skin fragment of a newborn and an adult dermis fragment were used in this study. It demonstrated that IL-1 induced a marked increase in *KGF* RNA levels. This effect was dose dependent, blocked in the presence of anti-IL-1 and accompanied by an increase of active *KGF* protein. The results suggested that other growth factors such as PDGF-beta, IL-6 and TGF-alpha moderately stimulate *KGF* RNA. These data suggested that a variety of growth factors and cytokines can influence the production of *KGF* *in vivo*<sup>15</sup>.

The increase in *KGF* gene expression for injured tissue has already been demonstrated by Brauchle *et al.*<sup>16</sup>, who used Western blot analysis to show an increased *KGF* expression on surgical specimens of patients suffering from Crohn's disease and ulcerative colitis.

Li *et al.*<sup>17</sup>, through analysis by qPCR, demonstrated that *KGF* is expressed in healthy periodontal tissue, and also found that the expression levels of *KGF* increased significantly in the injured periodontal tissues.

The present study also found this *KGF* overexpression by fibroblasts and keratinocytes in an injured tissue. In this case, the skin of burned victims was examined, and it was observed that thermal injury significantly increased the gene expression of *KGF* by dermal fibroblasts and keratinocytes that are around the burn.

Analysis of IL-1 beta showed a qualitative pattern on gene expression in the present study. This cytokine expression was substantially suppressed in patients with small burns, both in dermal fibroblasts and in keratinocytes. In keratinocytes of large burns there is an increase in gene expression.

Hu *et al.*<sup>18</sup> investigate the role of IL-1 in incisional wound biology and its effect on wound chemokine production *in vivo* and whether this mechanism could be active in human subjects. A well-characterized murine model of incisional wounding was used to assess the *in vivo* role of IL-1 in wound biology. The amount of seven different cytokines/chemokines produced within an experimentally induced skin incision on a mouse paw and the nociceptive response was analyzed in mice treated with an IL-1 inhibitor. They also investigated whether human IL-1 beta or IL-1 alpha stimulated the production of chemokines by primary human keratinocytes *in vitro*, and whether there was a correlation between IL-1 beta and chemokine levels in two experimental human wound paradigms. Administration of an IL-1 receptor antagonist to mice decreased the nociceptive response to an incisional wound, and reduced the production of multiple inflammatory mediators, including keratinocyte-derived chemokine (KC) and macrophage inhibitory protein (MIP)-1 alpha, within the wounds. IL-1 alpha and IL-1 beta stimulated IL-8 and GRO-alpha (human homologues of murine keratinocyte-derived chemokine) production by primary human keratinocytes *in vitro*. IL-1 beta levels were highly correlated with IL-8 in human surgical wounds, and at cutaneous sites of human ultraviolet B-induced sunburn injury. IL-1 plays a major role in regulating inflammatory mediator production in wounds through a novel mechanism; by stimulating the production of multiple cytokines and chemokines, it impacts clinically important aspects of wound biology. These data suggest that administration of an IL-1 receptor antagonist within the perioperative period could decrease postsurgical wound pain<sup>18</sup>.

With the same approach, in our results, the gene expression of IL-1 beta in human keratinocytes were more expressed in large burns, so if an IL-1 receptor antagonist would be used, we will can control the inflammatory process exaggerated by the stimulus to cytokine production and determine an improved clinical evolution.

This study demonstrated that TNF-alpha was not expressed by dermal fibroblasts and keratinocytes, both in burned patients as the control group, suggesting that during the acute inflammatory process other cells must express this gene and thus secrete the growth factor.

The effects of exogenous TNF-alpha depend on the concentration and duration of exposure, and this emphasizes the

importance of balancing pro-inflammatory signals that control wound healing. At low concentrations, TNF-alpha can stimulate the healing process since it indirectly induces inflammation and releases growth factors<sup>4</sup>.

Cytokines produced during acute inflammation, such as IL-1 and TNF-alpha, stimulate fibroblasts to synthesize other pro-inflammatory cytokines, creating a loop that is responsible for the magnification and even for the perpetuation of the inflammatory process<sup>19</sup>.

Finnerty *et al.*<sup>20</sup>, after realizing that burned adults have higher morbidity and mortality than burned children, compared the temporal profile of cytokine concentrations in plasma of burned adults and children. The temporal profile of cytokines was different between burned children and adults, but no significant differences were detected between TNF-alpha concentrations between the two groups. Other pro-inflammatory and anti-inflammatory cytokines had large differences in concentrations in adults and children with extensive burns. Adults had a hyper-inflammatory pattern compared to children. Plasma concentrations of cytokines were analyzed using a multiplex assay kit array system that evaluated 22 cytokines<sup>20</sup>.

It was demonstrated that these mediators have a central role in inflammation, and they are extremely important to understand the response to thermal injury<sup>6,8,18</sup>. It has also been shown that there is an interaction between IL-1 beta, TNF-alpha and KGF during inflammation and wound repair<sup>21</sup>.

The originality of this article lies in the fact that is the first one to assess the gene expression of TNF-alpha, KGF and IL-1 beta in epidermal and dermal cultured cells of burned patients and compare them with those of healthy people.

## Conclusions

The study showed a quantitative pattern in the expression of KGF gene, which is more expressed accord to the size of the burn. TNF-alpha was not expressed by dermal fibroblasts and keratinocytes, suggesting that during the acute inflammatory process other cells must express this gene. A qualitative pattern in the expression of IL-1 beta gene was demonstrated.

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