ABSTRACT

PURPOSE: Evaluate the expression profile of genes related to Innate and Adaptive Immune System (IAIS) of human Primary Epidermal keratinocytes (hPEKP) of patients with severe burns.

METHODS: After obtaining viable fragments of skin with and without burning, culture hKEP was initiated by the enzymatic method using Dispase (Sigma-Aldrich). These cells were treated with Trizol® (Life Technologies) for extraction of total RNA. This was quantified and analyzed for purity for obtaining cDNA for the analysis of gene expression using specific IAIS PCR Arrays plates (SA Biosciences).

RESULTS: After the analysis of gene expression we found that 63% of these genes were differentially expressed, of which 77% were repressed and 23% were hyper-regulated. Among these, the following genes (fold increase or decrease): IL8 (41), IL6 (32), TNF (-92), HLA-E (-86), LYS (-74), CCR6 (- 73), CD86 (-41) and HLA-A (-35).

CONCLUSIONS: This study contributes to the understanding of the molecular mechanisms underlying wound infection caused by the burn. Furthermore, it may provide new strategies to restore normal expression of these genes and thereby change the healing process and improve clinical outcome.

Key words: Gene Expression; Burn; Innate and Adaptive Immune System; Human Keratinocytes.
Introduction

The high susceptibility of burned patients to infections is a concern regarding the evolution and success of clinical treatment, being directly related to morbidity and mortality of the group. To prevent the entry and spread of pathogens the body has two types of immune response: innate immunity, or natural and acquired immunity, or specific. The first is responsible for the initial reactions to any infection and triggers the response of lymphocytes and humoral its products, which are components of acquired immunity, providing a specific and durable late response.

Burns produce changes in the pattern of the immune response of the patient, both represented by stimulating the production of genes related to innate immune response and suppression of genes related to adaptive immune responses, especially those related to antigen presentation and activation of T lymphocytes. These genes inhibited relate predominantly to the response of T helper lymphocyte-1 (Th1) and producers of cytokines such as interleukin-2 (IL-2), interferon-gamma (IFN-gamma), and tumor necrosis factor-beta (TNF-beta). Contributing to the polarization of adaptive immunity thus established is increased the response of T helper-2 (Th2) represented by the secretion of interleukin-4 (IL-4) and interleukin-5 (IL-5) by stimulating production of antibodies by B lymphocytes such bias leads the individual to an immunodeficiency and increased incidence of sepsis among critically injured patients as victims of trauma or burn.

The analysis by flow cytometry of blood samples from patients suffering from burns low amounts of Th1 lymphocytes have been found associated with low levels of IL-12 and no IFN-γ during the first month after injury. In the second month significantly high levels of Th2 lymphocytes were found compared to the control group. In burned patients developing hypertrophic scars and tumor growth factor-beta (TGF-beta) was also increased early after injury, returning to normal levels within 6 months.

Regulatory Lymphocytes T (RLT) are able to control the response of CD4+ antigen-specific cells in burned mice. Mice with normal levels of RLT have a reduced proliferation of CD4 + T cells and the production of Th1 type cytokines, whereas mice with RLT depletion show higher levels of CD4 + T cells and cytokines in response to antigenic stimulation (MACCONMARA et al., 2011). Continuous analysis of peripheral blood samples burned showed great a progressive decrease in the percentage of CD8+ T lymphocytes, while the number of CD4 lymphocytes was similar to the control group. The production of IL-4 was over-stimulated, indicating the activity of Th2 cells. The amount of serum IFN-γ, indicative of Th1 activity, showed a minor increase. These studies demonstrate that CD8+ and CD4+ cells are crucial for polarization of post-traumatic immune response related to Th2.

Studies with murine models of burn provide important information about the sequence of events and relationships between effector cells of acquired immunity, cytokines, and proteins produced during the inflammatory response. Therefore, the burn amplifies the function of CD4 + CD25 + regulatory lymphocytes, which contribute to immunosuppression after injury (CHOILEAIN et al., 2006). In another experiment, the expression of toll-like receptor 4 (TLR4), a protein important for antigen recognition and activation of innate immune response is significantly increased in CD4+ and CD8+ T lymphocytes associated with increased lymphocytes in the number populations of memory T lymphocytes and CD4+ CD44 CD44 CD8+ T6.

When activated, T-helper lymphocytes 17 (Th-17) secrete IL-6, IL-17, IL-22, IL-23, IL-27 and TGF-beta cytokines. Skin samples extracted from murine models of burns and analyzed by ELISA show a significant increase in IL-17 and IL-22 in the wound area compared to the control group about 3 hours after injury. Thus, there is an early-type response induced by Th-17 Burn7. Analyzing the skin surrounding the wound in a similar model, IL-6 levels were significantly elevated show after 1 hour and after 24 hours decreased. In another study to analyze the activity of dendritic cells in a murine model also, no significant reductions were observed in the expression of costimulatory molecules on the surface of this cell type as CD40, CD80, CD86. There was increased expression of CD86 one day after injury, suggesting that sunburn causes an early activation of dendritic cells. CD4 + T cells stimulated by antigen presentation of dendritic cells showed such levels of IL-2, IFN-gamma, IL-10 and IL-13 similar to the control group.

Immunohistochemical analysis of skin samples from patients with second degree burn shows an increased expression of ICAM-1, adhesion molecule associated with leukocyte transmigration through the vascular endothelium in the burning zone and surrounding basal cells. Keratinocytes in a polar distribution of ICAM-1 predominantly in the newly formed epithelium was observed.

The analysis by in situ hybridization and immunohistochemistry of skin samples from burn patients showed increased expression of MCP-1, monocyte chemotactic protein in keratinocytes of the basal layer of the wound as well as in regions where there granulation tissue.
The analysis of keratinocyte culture medium obtained by debridement of the burned patients showed elevated levels of IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IFN-γ, tumor necrosis factor-alpha (TNF-α) and colony-stimulating macrophages and granulocytes (GM-CSF) compared to the control. These changes in the pattern of immune response of burned patients are directly related to immunodeficiency, which increases their susceptibility to infection and sepsis, we believe it is of paramount importance to study and identify an expression profile of genes related to innate and acquired immune response of patients with severe burns. With the identification of a specific profile we can infer or suggest new treatments or clinical interventions applied to such cases, reducing the rates of morbidity and mortality in the group.

Therefore the aim of this study is to evaluate the expression of 84 genes mediating innate and adaptive immune response by PCR Array of cultured primary human keratinocytes of patients with severe burns.

Methods

Experimental design

The present study design is experimental, in vitro, using donated burn patients tissues. It is observational, analytic, controlled and conducted in a single center. All the patients included in this study have read and signed the Free and Clarified Consent Term.

The project was submitted by the Ethics Committee of Federal University of Sao Paulo (UNIFESP) and approved in November 9th, 2012 (146.468).

Patients

The patients recruited to this study were burn victims (Table 1) admitted in the Burns Treatment Unit, Plastic Surgery Division, Federal University of São Paulo, University Hospital.

Table 1 - Data (n, age, gender, mean % TBSA) of burned patients included in the study.

<table>
<thead>
<tr>
<th>Group</th>
<th>Age Medium (years)</th>
<th>Gender</th>
<th>SCQ Medium (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe Burn</td>
<td>31.5</td>
<td>3 M</td>
<td>32</td>
</tr>
</tbody>
</table>

F, female; M, male.

The control group comprised healthy, non-smoking donors submitted to aesthetic plastic surgery. The first is a 38 years old female who underwent breast lift surgery; the second patient was a 32 years old female who underwent abdominoplasty. All of them fit in the inclusion criteria.

Inclusion, exclusion and non-inclusion 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 severe or large 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. 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, such as psoriasis and similar, superficial skin lesions or illnesses that might interfere directly in the inflammatory process, as rheumatologic diseases in general were not included.

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

Surgical procedure

The skin samples used in this study were obtained by the usual surgical procedure for burn patient care in our Burns Unit. Debridement of dead tissue from a patient with full or partial thickness burn normally was realized 3 or 4 days after the burn; the healthy skin around the burn lesion, which is normally collected and discarded, was destined to skin culture in our laboratory.

Keratinocyte culture

Keratinocytes derived from a skin fragment of a burn patient were isolated and cultured according to the standard method, adapted in our laboratory with changes as described below. In a 60 mm culture plate, the skin fragment was placed and sectioned into small pieces with sizes of approximately 0.5 cm³. Those fragments were placed in a 50 ml sterile tube with 30 ml of dispase (Boeringer Mannheim, Indianapolis, IN) and kept refrigerated at 4°C until the next day, totaling 15 hours of action of dispase. After
this period, each epidermal fragment was gently detached from the dermis with forceps; the epidermis was intended for isolation of keratinocytes, while the dermis underwent enzymatic dissociation with collagenase as described in the next section.

**Total RNA extraction**

Each cell type was separately homogenized 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 Qiagen RNeasy MiniKit (Qiagen, Valencia, CA) and submitted to DNase treatment using the RNase-free DNase Set (Qiagen Inc., Valencia, CA, USA). The amount and quality of the extracted RNA were assessed by spectrophotometry using NanoDrop v3.3.0 (NanoDrop Technologies Inc., Rockland, DE) and capillary electrophoresis using Bioanalyzer 2100 (Agilent Technologies Inc., Palo Alto, CA, USA).

**qPCR Array**

The total RNA (1.0 μg) per plate/array from each pool of patient was used for synthesis of cDNA. Samples were treated with buffer coming from the kit and reverse transcription reactions were performed using the RT2 First Strand Kit from SABiosciences (Qiagen Company), according to the manufacturer’s protocol. qPCR Array was performed using the RT2 Profiler™ PCR array of SABiosciences (http://www.sabiosciences.com/ArrayList.php).

For each patient 84 genes were examined for relevant human genes involved in the pathway of Innate and Adaptive Immunity (PAHS-052C). Amplification, data acquisition, analysis curves were performed on an ABI Prism 7500FAST Sequence Detection System (Applied Biosystems, Foster City, CA). In each turn, each gene was checked for efficiency, and minimum and maximum threshold curve pattern. To ensure accurate comparisons between curves the same threshold were established for all genes and races. Three genes were used as internal control and the average Ct value were used to standardize gene expression (2-CT change table) and determine the difference between groups. Gene expression was considered upregulated or downregulated when the difference was more than two times.

**Statistical analysis**

The statistical parameters and the data analysis were performed by the RT2 Profiler PCR Array data analysis version 3.5 (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). For the analysis it was considered significant the fold change/fold regulation that presented a p value less than 0.05.

**Results**

After the expression analysis of the 84 studied genes for each pathway, we observed, for Innate and Adaptive Immune System (IAAS) pathway, that 63% of these genes were differentially expressed, among these 77% were down-regulated and 23% were up-regulated (Figures 1 and 2).

**Figure 1** - Heat Map. In red, up-regulated genes; in green, down regulated genes; and in black, genes not regulated.

**Figure 2** - Scatter Plot. In red, up-regulated genes; in green, down regulated genes; and in black, genes not regulated.
Among differentially expressed genes, we highlight the ten most hypo expressed genes, that were TNF (92 times), HLA-E (86 times), Lyz (74 times), CCR6 (72 times), CD86 (41 times), HLA-A (35 times), IRF3 (25 times), and STAT3 (22 times), TLR2 (21 times), and IL18 (18 times) of decrease (Figure 3).

Tumor necrosis factor (TNF) gene encodes a multifunctional proinflammatory cytokine that belongs to the superfamily. This cytokine is mainly secreted by macrophages. It can bind to, and thus functions through its receptors TNFRSF1A/TNFR1 and TNFRSF1B/TNFBR. This cytokine is involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation. In a recent study, our group evaluates the gene expression of TNF in skin fibroblasts and keratinocytes cultured from burned patients that was not expressed\(^1\). This result obtained by analysis of gene expression of individual gene corroborates our current results using another system for gene analysis by PCR Arrays in keratinocyte cells.

Major histocompatibility complex, class I, E (HLA-E) and A (HLA-A) belongs to the HLA class I heavy chain paralogues. This class I molecule is a heterodimer consisting of a heavy chain and a light chain (beta-2 microglobulin). The heavy chain is anchored in the membrane. HLA-E is a non-classical MHC class that acts as the ligand for the NKG2A inhibitory receptor present on natural killer (NK) and CD8\(^+\) cells. Peptide binding and stabilization of HLA-E is often considered to signal infection or cell stress. Non-inflammatory chronic lesions express significantly less HLA-E protein, comparable to levels found in white matter from controls. Increased HLA-E protein levels were associated with higher scores of inflammation\(^1\). Pustulosis acuta generalisata (PAG) is a rare poststreptococcal disease of the skin, which has been reported after streptococcal throat infection. The patient was found to be HLA positive\(^1\).

Lysosome (Lyz) gene encodes human lysozyme, whose natural substrate is the bacterial cell wall peptidoglycan (cleaving the beta[1-4]glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine). Lysozyme is one of the anti-microbial peptides agents found in human milk, and is also present in spleen, lung, kidney, white blood cells, plasma, saliva, skin, and tears. Likewise the HLE, there are no studies that report the expression of Lyz gene in human skin burn. Lyz is a type of mediator release from injured human skin that acts as an important first line defense mechanism in cutaneous injury\(^1\). Our results showed that this gene is hypo expressed in severe burn skin, so, this patient is highly vulnerable to infections.

**Figure 3** - Hypo-expressed genes related to Innate and Adaptative Immunity in severe burn human skin.

**Figure 4** - Hyper-expressed genes related to Innate and Adaptative Immunity in severe burn human skin.

**Discussion**

In the literature we found no studies that evaluated the expression of genes related to innate and adaptive immunity in severe burn human skin, so this work is the first to address this issue. Overwhelmingly, the articles dealing separately genes involved in this system, in addition, in animal models, and a few times were performed protein instead gene analyzes.
Chemokine (C-C motif) receptor 6 (CCR6) gene encodes a member of the beta chemokine receptor family, which is predicted to be a seven transmembrane protein similar to G protein-coupled receptors. The gene is preferentially expressed by immature dendritic cells and memory T cells. These gene results indicate that an important aspect of the healing response to corneal epithelial abrasion includes influx of CCR6 that contributes to the inflammatory response and promotes epithelial healing. In our study CCR6 is repressed in severe burn skin, probably the epithelial healing in these patients are injured.

Elevated CD86 antigen expression has functional consequences for the magnitude of CD4 T cell responses both in vitro and in vivo. These data pinpoint CD86 upregulation as an additional mechanism by which IL-21 can elicit immunomodulatory effects. Exposure to UV radiation led to concentration-dependent increases in sunburn cell formation and decrease in CD86-positive cell number in the epidermis.

Interferon regulatory factor 3 (IRF3) gene encodes a member of the interferon regulatory transcription factor (IRF) family. The innate immune response constitutes the first line of host defence that limits viral spread and plays an important role in the activation of adaptive immune response. Viral components are recognized by specific host pathogen recognition receptors triggering the activation of IRF3. IRF3, along with NF-κB, is a key regulator of IFN-β expression.

Signal transducer and activator of transcription 3 (STAT3) gene is a member of the STAT protein family. In response to cytokines and growth factors, STAT family members are phosphorylated by the receptor associated kinases, and then form homo- or heterodimers that translocate to the cell nucleus where they act as transcription activators. This protein is activated through phosphorylation in response to various cytokines and growth factors including IFNs, EGF, IL5, IL6, HGF, LIF and BMP2. STAT3 signal transduction pathways was facilitated in the hypertrophic scar (HS) that is a serious fibrotic skin condition and a major clinical problem.

Toll-like receptor 2 (TLR2) gene is a member of the Toll-like receptor (TLR) family which plays a fundamental role in pattern recognition and activation of innate immunity. In sites of inflammation or tissue injury, hyaluronic acid (HA), ubiquitous in the extracellular matrix, is broken down into low m.w. HA (LMW-HA) fragments that have been reported to activate immunocompetent cells. We found that LMW-HA induces activation of keratinocytes, which respond by producing beta-defensin 2. This production is mediated by TLR2 and TLR4 activation and involves a c-Fos-mediated, protein kinase C-dependent signaling pathway. After injury, the release of beta-defensin 2 by keratinocytes, can protects cutaneous tissue at a time when it is particularly vulnerable to infection.

Interleukin 18 (IL18) gene is a proinflammatory cytokine that augments natural killer cell activity in splenic cells, and stimulates interferon gamma production in T-helper type 1 cells. IL-18 therapy markedly activated neutrophil functions, thereby increasing survival from postburn Methicillin-resistant Staphylococcus aureus infection.

We saw that he levels of Interleukins 8 (IL8) and 6 (IL-6) significantly increased compared with that of the controls in the human burn skin, so our results corroborate with others authors that observe similar results in the plasma of mice subjected to burn injuries for IL-8 gene and after photodamage caused by chronic UV exposure for IL-6 gene.

The protein encoded by interleukin 1, alpha (IL-1A) and interleukin 1, beta (IL-1B) genes are members of the interleukin 1 cytokine family. This cytokines are pleiotropic cytokine involved in various immune responses, inflammatory processes, and hematopoesis. IL-1A cytokine is produced by monocytes and macrophages as a proprotein, which is proteolytically processed and released in response to cell injury, and thus induces apoptosis. IL-1B is produced by activated macrophages as a proprotein, which is proteolytically processed to its active form by caspase 1 (CASP1/ICE). This cytokine is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis. These genes are commonly altered chlorine vapor-induced porcine skin injury. So, these genes are important to defense against infections caused by injured skin like in severe burned skin.

Solute carrier family 11member 1 (SLC11A1) protect from injury CD8 antigen is a important agent in immune response and during the course of a microbial infection, different antigen presenting cells (APCs) are exposed and contribute to the ensuing immune response. CD8α(+) dendritic cells (DCs) are an important coordinator of early immune responses to the intracellular bacteria Listeria monocytogenes (Lm) and are crucial for CD8(+) T cell immunity.

Complement component 3 (C3) gene plays a central role in the activation of complement system. Its activation is required for both classical and alternative complement activation pathways. People with C3 deficiency are susceptible to bacterial infection.

Activation of complement is known to accompany burn injury. C3 fragments is recognized as adhesion molecules by granulocyte receptors, these deposited proteins could promote leukocyte accumulation, thereby contributing to an initiation of an inflammatory cascade at a site of burn injury.
Nucleotide-binding oligomerization domain containing 2 (NOD 2) gene is a member of the Nod1/Apaf-1 family and encodes a protein with two caspase recruitment (CARD) domains and six leucine-rich repeats (LRRs). The protein is primarily expressed in the peripheral blood leukocytes. It plays a role in the immune response to intracellular bacterial lipopolysaccharides (LPS) by recognizing the muramyl dipeptide (MDP) derived from them and activating the NFKB protein. Nod2 has an important role in murine skin wound healing. Cutaneous Nod2 is induced in key wound cell types in response to injury. In the absence of Nod2, mice display a substantial delay in acute wound repair associated with epithelial and inflammatory changes. In recent findings was revealed a novel intrinsic role for Nod2 in cutaneous wound repair in addition to its role in recognizing invading pathogens. We found in our study that this gene was up-regulated in severe burn skin, so that is the importance for stop the infection.

Chemokine (C-C motif) receptor 4 (CCR4) gene belongs to the G-protein-coupled receptor family. It is a receptor for the CC chemokine - MIP-1, RANTES, TARC and MCP-1. Chemokines are a group of small polypeptide, structurally related molecules that regulate cell trafficking of various types of leukocytes. The chemokines also play fundamental roles in the development, homeostasis, and function of the immune system. The loss of a single chemokine perturbs the chemokine network not only in the setting of acute inflammation but even in an isolated inflammatory cell, the macrophage.

Integrin alpha M (ITGAM) gene encodes the integrin alpha M chain. Integrins are heterodimeric integral membrane proteins composed of an alpha chain and a beta chain. This I-domain containing alpha integrin combines with the beta 2 chain (ITGB2) to form a leukocyte-specific integrin referred to as macrophage receptor 1 (‘Mac-1’), or inactivated-C3b (iC3b) receptor 3 (‘CR3’). Using a mouse model of thermal injury, recently it was studied why antimicrobial peptides are not produced at the burn-site tissues and how this defect contributes to the increased susceptibility to Pseudomonas aeruginosa burn-wound infection. The results indicate that sepsis stemming from P. aeruginosa burn-wound infection is accelerated by burn-induced by cells that express ITGAM that has abilities to suppress antimicrobial peptide production by epidermal keratinocytes. Then, the important up-regulation that we saw for this gene in our results can be important to fight the infection in the local of severe burn skin.

**Conclusion**

Cultured primary human Epidermal Keratinocytes can to drive inflammatory responses related to innate and adaptative immunity in these severe burned patients. Therefore, this study contribute to understanding the molecular mechanisms underlying wound infection and provide new strategies that would restore the normal expression of these genes to enhance the healing process and drive these patients to a better outcome.

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**References**


