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Expression of pro-inflammatory genes in lesions, spleens and blood neutrophils after burn injuries in mice treated with silver sulfodiazine

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ABSTRACT

Objective(s): It is now supposed that cytokines released during the burn injuries have a great impact on the immunological and pathological responses after the burn. The main objective of this study was to investigate the expression of some pro-inflammatory genes in the wound, spleen and blood neutrophils during the healing process of burn wounds in a murine model.

Materials and Methods: The expression of ten pro-inflammatory genes were examined in wounds, spleens and blood neutrophils of mice with burn injuries treated with either silver sulfodiazine or phosphate-buffered saline (PBS) using RT-PCR at the end of the first and second weeks after injuries.

Results: None of the pro-inflammatory genes were expressed in the skin, spleen and blood neutrophils of healthy mice. In the group control, IL-12P35, IL-12P40, CCR5, IL-1β and IFN- γ were expressed in the spleen and blood neutrophils in the first week. Instead, CCL5, CCR5, IL-1β and IFN- γ were expressed in the wound, but in the second week, the expression of the genes became similar. In the test group, in the first week, TNF-α, IL-12P35, IL-12P40 and IL-1β were expressed in the lesions, CCL4, IL-1α, IL-12P35, IL-12P40, CCR5 and IFN-γ were expressed in the spleen and no pro-inflammatory gene expression was detected in blood neutrophils.

Conclusion: IL-1β and IFN- γ are expressed in wound, spleen and neutrophils of untreated mice, but not in silver sulfodiazine treated mice. Hence, treatment with silver sulfodiazine suppressed the expression of pro-inflammatory genes in some stages of healing.

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Introduction

The wound healing process is divided into 3 main steps, where the wound undergoes sequential phases of inflammation, repair, and remodeling in which many cellular events including platelet degranulation, leukocyte migration and accumulation, and epithelial cell migration are involved. The secretion from wounds clearly indicates a continuous set of intercellular communication in the healing process, which mainly involves the production of pro-inflammatory cytokines. It is now supposed that these cytokines are responsible for initiating and regulating the sequential phases of the wound healing (1).

Burn wounds differ from other types of wounds not only in the cause of injury but also in the direction, the cellular mechanisms and the healing process. In the burn wound, the heat-induced tissue coagulation is occurred at the time of injury (2) and, in contrast to other types of wounds, the predominant direction of tissue injury is horizontal, not vertical. These exclusive characteristics of thermal wounds result in more slowly healing in part due to extensive necrosis, edema formation, and relatively hypoxia (3). Furthermore, large burn injuries induce deregulation in immune responses (4-6) either systematically or locally in the site of burning (7-8).

Several attempts have been made to determine the systemic and local immune responses to thermal injuries in terms of the inflammatory cytokine production, the local wound milieu, or the expression profiles of the genes responsible for wound healing. The aim of present study was to characterize and compare sequential changes in the expression of CCL4/MIP-1β, CCL3/MIP-1α, TNF-α, ll-1α, IL-12P35, IL-12P40, CCL-5, CCR5, IL-1β and IFN- γ genes in the wound milieu, spleen and peripheral blood neutrophils during the healing process of full-thickness burn in a murine model in order to understand the gene expression pattern during the healing process of normal burn wounds.

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Materials and Methods

Animals

Eight-weeks-old female BALB/c mice were purchased from Pasteur institute of Iran and housed in BU-Ali Sina university animal house according to the standard of ethical agreement for keeping inbred animals.

Murine full-thickness burn

All procedures were carried out based on the guidelines of the Ethics Committee of the International Association for Studies of Pain (9). All protocols were reviewed and approved by the research council of Bu-Ali Sina University, Hamedan, Iran.

Three groups of 7 animals weighing 20–25 g were used in the study. In two groups, appropriate analgesia induced using 2% Leidocaine, 10 mg/kg SC at the dorsum of animals. To obtain a third-degree burn injury, the dorsum of the mice was first cleansed and shaved, and a 1×1 cm scald wound was created by placing an electrical hat plate (200° C) for 4- 5 sec on the skin (10). The third degree of the burn was confirmed by either blister or the presence of edema and underlying tissue damage. The mice were then housed to get better health condition in a warm and dry cage with water and food, and monitored regularly for signs of distress. Wounds were daily monitored and treated either with commercial silver sulfadiazine cream in the test group or phosphate-buffered saline (PBS) in the control group for three weeks. The complete healing was confirmed with the presence of scarring and wound contraction. The third group including 7 healthy mice was used as the second control group. In this group, the mice were euthanized and samples from the same place as burnt groups were taken for pathological analysis.

Size assessment and sampling

Size of the wounds were assessed every two days using the equation used by Cribbs (10).

$P=(D + d)/2$

(D=the large diameter, d=the small diameter, P=the average size of the lesion)

Samples were collected from the spleens, wounds and blood neutrophils of mice with burning injuries on day 0 (before starting the treatment), 7 and 14 of the experiment.

Primers

All samples were examined for expression of CCL4, CCL3, TNF-α, IL-1α, IL-12P35, IL-12P40, CCL5, CCR5, IL-1β and IFN-γ pro-inflammatory genes using specific primers. Sequences of the primers are shown in Table 1.

Isolation of neutrophils

Neutrophils isolation was performed according to the protocol explained by Rezapour (11). In brief, heparinized blood samples taken from the heart of the mice were diluted with equal volumes of NaCl 0.85%. Meglumine compound (meglumine diatrizoate 66%, and sodium diatrizoate 10%) was diluted in 3 times of NaCl 0.85% volume and used for isolation of neutrophils. Five milliliter of the diluted blood samples were slowly added to 4 ml of the diluted meglumine compound being careful not mixing the two phases of the blood and the meglumine compound. The samples were centrifuged for 15 min at 250 g and the supernatant was replaced with 0.5 ml of PBS. The pellet was homogenized by slow pipetting and then two 25 sec hypotonic lyses were applied with distilled water and NaCl 2.55% followed by centrifugation at 200 for 5 min and replacing the supernatant with NaCl 0.85%.

Table1. Sequences of primers designed for pro-inflammatory genes

Gene	Accession	Sequence
CCL4/MIP-1β	NM_013652.2	F: CAG CCC TGA TGC TTC TCA CT R: GGGAGA CAC GCG TCC TAT AAC
CCL3/MIP-1α	NM_011337.2	F: TCT GCG CTG ACT CCA AAG AG R: GTG GCT ATC TGG CAG CAA AC
TNF-α	M38296.1	F: TAT AAA GCG GCC GTC TGC AC R: TCT TCT GCC AGT TCC ACG TC
IL-1α	CCDS16725	F: CAG TTC TGC CAT TGA CCA TC R: TCT CAC TGA AAC TCA GCC GT
IL-12P35	M86672.1	F: ATG ATG ACC CTG TGC CTT GG R: CAC CCT GTT GAT GGT CAC GA
IL-12P40	M86671.1	F: CTG CTG CTC CAC AAG AAG GA R: ACG CCA TTC CAC ATG TCA CT
CCL-5	NM_013653.3	F: GTG CTC CAA TCT TGC AGT CG R: AGA GCA AGC AAT GAC AGG GA
CCR5	NM_009917.5	F: ATT CTC CAC ACC CTG TTT CG R: GAA TTC CTG GAA GGT GGT CA
IL-1β	CCDS16726.1	F: TTG ACG GAC CCC AAA AGA TG R: AGA AGG TGC TCA TGT CCT CA
IFN- γ	MN-008337	F: GCT CTG AGA CAA TGA ACG CT R: AAA GAG ATA ATC TGG CTC TGC

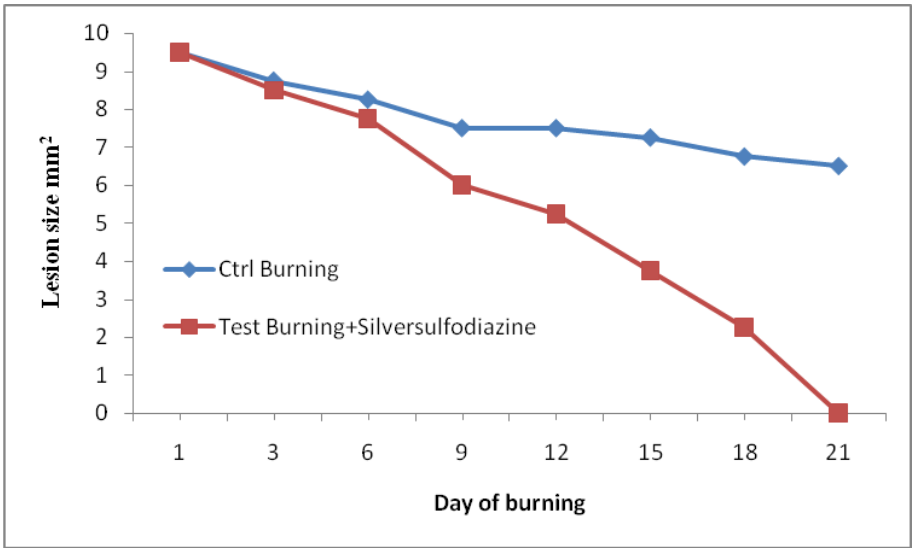

Figure 1. Induction of burning lesions in BALB/c mice. Burn wounds were induced in two groups of BALB/c mice with a hot metal instrument at 200 °C for 5 sec. The first group was treated with commercial silver sulfadiazine cream and the second group was taken as the control and treated with phosphate-buffered saline (PBS). The lesion sizes were measured every three days

RNA extraction

Total mRNA was extracted from samples according to the protocol explained by Rezvan using DENAzist commercial kit (DENAzist Asia, Iran) (12). One hundred milligram of tissue or cell pellets was sonicated for 20 min. Then, 1 ml of the lysing buffer included in the kit was added to the samples vortexing for 15 sec and incubating at room temperature for 5 min. The samples were centrifuged at 12,000 g for 10 min at 4 ºC and the supernatant was transferred into a new tube. Afterwards, 200 µl of chloroform was added to the tubes and vortexed for 15 sec followed by incubation at room temperature for 3 min. The samples were then spun at 12,000 g for 15 min at 4 ºC and the top phase was transferred into a new tube mixing with an equal volume of isopropanol. An equal volume of the G2 buffer included in the kit was added and mixed followed by incubation at room temperature for 10 min and centrifuged at 10000 g for 10 min at 4 ºC. After discarding the supernatant and adding 1 ml of 70% ethanol, the tubes were quickly vortexed and centrifuged at 10000 g for 5 min at 4 ºC. The supernatant was then discarded and 30-100 µl of nuclease-free water was added.

RT-PCR

RT-PCR was performed according to the protocol explained by rezvan using Denazist commercial kit (DENAzist Asia, Iran) (12). Briefly, a mixture of 10 µg total RNA, 1 µl oligo dT primer, 1 µl dNTP and nuclease-free water up to 10 µl was prepared. The mixture was incubated at 65°C for 5 min and chilled on ice for 2 min. A mixture of cDNA synthesis (10X Buffer M-MuLV 2 µl, M-MuLV reverse transcriptase 100 units, nuclease-free water top up to 10 µl) was prepared and 10 µl of the cDNA synthesis mixture was added to each RNA-primer incubating at 42 °C for 60 min followed by an extra incubation at 85 °C for 5 min and chilling on ice. The PCR was programmed as 40 cycles of denaturation at 95 °C for 30 sec, annealing at 4 °C less than the melting temperature of the primers for 30 sec and an extension step at 72 °C for 20 sec. An initial denaturation step of 95 °C for 10 min before the cycles and a final extension step at 72 °C for 10 min after the cycles were also applied.

Results

Monitoring of healing in burn wounds

The burning wounds in the mice were monitored in two days interval for three weeks. The size of the wounds were measured and compared in the test group treated with silver sulfadiazine and the control group treated with PBS. The size of wounds is shown in Figure 1.

The results showed that in the first week, the size of burn wounds in the control group was not significantly different from the test group; however, the test group produced less fibrin deposition. In the second week, healing of the wounds ameliorated in the test group compared to those of the control group. More healing and re-epithelialization was observed in the intervention group by the end of the third week.

Expression of pro-inflammatory genes in lesions, spleens and neutrophils of burnt mice

The expression of ten pro-inflammatory genes including CCL4/MIP-1β, CCL3/MIP-1α, TNF-α, IL-1α, IL-12p35, IL-12p40, CCL-5, CCR5, IL-1β, and IFN-γ was evaluated and semi-quantitatively appraised in the lesions, spleens and peripheral blood neutrophils. In healthy mice, none of the pro-inflammatory genes were expressed in all samples of skin, spleen and neutrophils.

Table 2. Expression of pro-inflammatory genes in wounds, spleens and peripheral blood neutrophils of mice with burning injuries

	Sampling time	Sampling site	CCL4	CCL3	TNF-α	IL-1α	IL-12P35	IL-12P40	CCL5	CCR5	IL-1β	IFN- γ
Healthy tissue	All weeks	wound	-	-	-	-	-	-	-	-	-	-
		neutrophils	-	-	-	-	-	-	-	-	-	-
		spleens	-	-	-	-	-	-	-	-	-	-
Control group (treated with PBS)	Week 1	wound	-	-	-	-	++	+	-	++	++	+++
		neutrophils	-	-	-	-	-	-	+	+	+	+++
		spleens	-	-	+	-	-	-	++	++	+	++
	Week 2	wound	-	-	++	+	+	+	-	-	++	+
		neutrophils	-	-	++	++	+	+	-	-	+	+
		spleens	-	+	++	+	+	+	-	-	+	+
Test group (treated with silver sulfadiazine)	Week 1	wound	-	-	++	-	+	+	-	-	+	-
		neutrophils	-	-	-	-	-	-	-	-	-	-
		spleens	+	-	-	+	+	+	-	+	-	+
	Week 2	wound	-	-	-	-	+	+	-	-	-	++
		neutrophils	-	-	-	-	-	-	-	-	-	-
		spleens	-	-	-	-	+	+	-	-	-	++

The thickness of bands shown in the gel electrophoresis was marked from 1 to 3 plus (low colour bands +, medium colour bands ++ and dark colour bands +++)

In the group without receiving medical treatment (control), in the first week, the expression of the examined pro-inflammatory genes in neutrophils were quite similar to that of the spleen, but the expression of the genes in the lesions was different. In this group, IL-12P35, IL-12P40, CCR5, IL-1β and IFN- γ were expressed in the spleen and blood neutrophils in the first week. Instead, CCL5, CCR5, IL-1β and IFN- γ were expressed in the wounds. In the second week, TNF-α, IL-1α, IL-12P35, IL-12P40, IL-1β and IFN- γ were expressed in all lesions, spleens and neutrophils; moreover, CCL3 was expressed in the spleen.

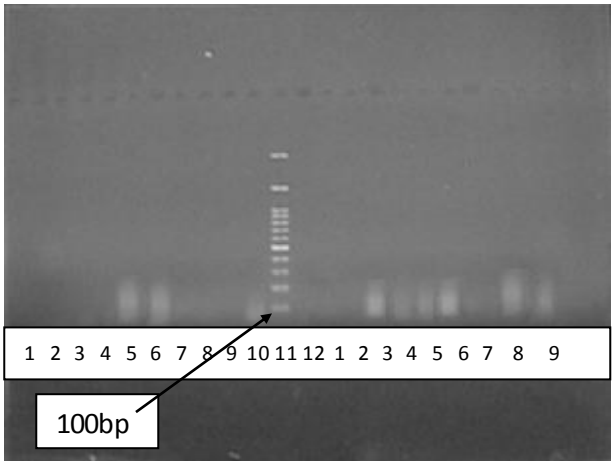

Figure 2. Expression of pro-inflammatory genes in burn wounds. Two groups of mice with burn injuries were treated with either silver sulfodiazine or phosphate-buffered saline (PBS). The expression of ten pro-inflammatory genes was evaluated in the wounds, spleens and blood neutrophils. Bands from 1 to 10 on the left side is for CCL4, CCL3, TNF-α, IL-1α, IL-12P35, IL-12P40, CCL5, CCR5, IL-1β and IFN- γ in wounds treated with silver commercial sulfodiazine cream and on the right side is for the same genes in wounds treated with PBS in last stages of healing (end of the second week). Bands 11 stands for the standard DNA and 12 is for the negative control

In the group treated with silver sulfodiazine in the first week, TNF-α, IL-12P35, IL-12P40 and IL-1β were expressed in the lesions, CCL4, IL-1α, IL-12P35, IL-12P40, CCR5 and IFN- γ in the spleen and non of the pro-inflammatory genes were expressed in blood neutrophils (Figure 2). In the second week, IL-12P35, IL-12P40 and IFN- γ were expressed in the lesions and spleen and still none of the genes were expressed in the neutrophils (Table 2).

Discussion

Although there are studies emphasizing on the prominent role of cytokines in the outcome of burn injuries (14), few studies have been carried out to evaluate the effect of burning on the expression of pro-inflammatory genes particularly in neutrophils and the spleen. This study was designed to investigate the expression profile of the pro-inflammatory genes during and after burn injuries in the wounds, spleens and blood neutrophils.

Although no sign of expression for pro- inflammatory genes in the skin, blood neutrophils and the spleen was observed in healthy mice, some of pro-inflammatory genes were expressed in the wounds, neutrophils and the spleen in mice with burn injuries and without medical treatment. This indicates that the skin burn creates a systemic reaction in whole body and induces pro- inflammatory gene expression not only in the site of burn but also in other organs or cells such as the spleen and neutrophils. Surprisingly, in burnt cases without medical treatment, in the first week, the expression profile of pro-inflammatory genes in the spleen was similar to that in blood neutrophils but very different than that of the burn wounds. In this group, the expression of pro-inflammatory genes in wounds, blood neutro-

phils and spleens became very similar in the second week.

In mice treated with silver sulfadiazine, during the first week, unlike the controls (mice without treatment), IFN- γ and CCR5 were not expressed in the burn wounds, but TNF-α was expressed. In this group, TNF-α was expressed in the burn wounds but not spleen, while in the controls it was expressed in the spleen but not in the burn wounds.

It has been postulated that burn injuries induce changes in immunological cytokines, which potentially lead to shock and death in animals, and the size of burn injuries has a direct effect on the figure of the cytokine profile after burning (15).

In patients with large burn injuries, chemokines of the CXC family are up-regulated, where the C, CC and CX3C chemokine family in all burn patients especially in those with small injuries were down-regulated (16). Moreover, by increase of the burn size, the expression of MHC class II antigens are down-regulated causing delay in rejection of allografts after burn injury. The expression of IFN-γ in wounds and the skin is also augmented particularly after minor thermal injury (17).

Changes in expression pattern of pro- inflammatory genes induced by burning have also been reported previously. In a study, an up-regulation in transcript-tion of pro-inflammatory cytokines such as IL-6, TNF-α and keratinocyte-derived chemokine was observed in burn injuries. TNF-α in turn induced systemic inflammatory reactions, which potentially resulted in multiple organ failure (18). Burn injuries also enhanced the expression of IL-1α, IL-2, IL-4, IL-10, IFN-γ and TNF-α in keratinocytes and induced changes in expression pattern of cytokines in these cells (19).

To the best of our knowledge, this is the first study on the changes in the expression profile of pro-inflammatory genes after medical treatment in burn injuries. The results of this study showed that after medical treatment with silver sulfodiazine, a significant suppression was induced on the expression pattern of TNF-α, IL-1α, IL-12P35, IL-12P40, CCL5, CCR5, IL-1β and IFN- γ. An inverse relation has been previously shown between IL-1β and the burn size (20). In patients with burn injuries, the expression of IL-1β was highly suppressed in fibroblasts of patients with small injuries and keratinocytes of those with large injuries (21). In another study on humans, the concentration of IL-1β and TNF-α were not increased in patients with thermal injuries in comparison with those of the controls (22).

As the results showed, in the control group (mice treated with PBS), TNF-α was switched off in the wounds until the end stages of healing (second week) where in silver sulfadiazine treated groups, TNF-α was expressed in early days of the lesions until the healing was completed.

It has already been shown that during the healing process, the expression of keratinocyte growth factor (KGF) mRNA is up-regulated by TNF-α, IL-1α and IL-1β, but not affected by transforming growth factor-beta1 (TGF-β1) and IFN- γ in human fibroblasts (23). Therefore, the figure of TNF-α expression conforms its importance in the healing process and supporting this idea that the delayed healing observed in the control group compared with the test group is probably due to the early expression of TNF-α in the wounds. In the control group (mice treated with PBS), in the first week, TNF-α was only expressed in the spleen, but in the second week, it was expressed in all wounds, neutrophils and spleens. It seems that the slow healing in the control group induced a systemic expression of TNF-α, which in turn helped the healing process.

It is known that blood neutrophils arrive to the site of inflammation from the blood and do not return back from the injured tissues. The results showed that in the control group, most of the pro-inflammatory genes were expressed in blood neutrophils, but none of the pro-inflammatory genes in these cells were expressed in the test group; however, some of the pro-inflammatory genes were expressed in the wounds or spleens of this group. Therefore, by acceleration of healing with medical treatments, expression of the pro-inflammatory genes in blood neutrophils stopped, but contribution of blood neutrophils in the healing process and expressing the pro-inflammatory genes after burning still needs more investigation. Besides, in the test group, expression of the pro-inflammatory genes in neutrophils is switched off, while some of the pro-inflammatory genes are expressed in the wounds and spleens indicating that other types of cells are also involved in the expression of pro-inflammatory genes.

It is known that T lymphocytes, natural killer (NK) cells, inflammatory monocytes and natural killer T (NKT) cells are perceived as the main source of IFN-γ production (24). Recent studies demonstrated that neutrophils could be assumed as the crucial source of IFN-γ production through toll like receptor-2 (TLR-2) and Dectine-1 (a specific receptor for β-glucans) contributing towards immunity against intracellular pathogens (24-26).

Notably, neutrophils are also capable to produce IFN-γ through TLR-independent pathway, which is different from the conventional pathway (myeloid-differentiation factor 88) (27) exploited by T and NK cells (25). Additionally, different cytokines such as IL-17a, IL-12, and IL-15 or their combination yield IFN-γ transcription (26, 28). In this context, *in vitro* studies indicated that combination of IL-12 and IL-15 induced neutrophils to produce IFN-γ (26).

Constitution of IL-12 cytokine needs production of both IL-12p35 and IL-12p40. IL12p35 is shown to be produced by a wide array of cells including lymphocytes, whilst IL-12p40 is just produced by activated phagocytes (29).

Expression of IL-12p40 gene, detected in neutrophils as activated phagocytes in the control group, was in-line with other recent studies showing that neutrophils produced IL-12 when they were activated through TLR-2 (30). Co-expression of IL-12 (IL-12P35 and IL-12P40) and IFN- γ indicates the activity of IL-12 and IFN- γ axis.

The results showed that in the control group IL-12 and IFN- γ axis was active in all stages of healing in the wounds, but in neutrophils and the spleen, it was only active in the last stages of healing (second week). In the test group, IL-12 and IFN- γ axis was active in the spleen in all stages of healing, but in the wounds, IL-12 and IFN- γ axis was active in the last stages of healing; it was never active in neutrophils. Therefore, IL-12 and IFN- γ axis was probably suppressed by silver sulfadiazine in the wounds and neutrophils.

Conclusion

Burning can create different figures of expression for pro-inflammatory genes in the wounds, spleens and blood neutrophils indicating a systemic effect of burning on the immune system involving different cells and organs. The pro-inflammatory genes were mostly expressed during the healing process, but expression of the genes was down-regulated or stopped when the lesions cured. Chemical treatment with silver sulfodiazine suppressed the expression of some pro-inflammatory genes particularly in blood neutrophils.

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