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Full Length Research Paper

Effects on TNF-α and HMGB1 secretion of macrophages induced by burn plasma

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The regulation of burn serum on TNF-α and high mobility group proteins 1 (HMGB1) secretion by macrophages is the main preoccupation of this study. For this experiment Ten SD rats were conducted into a burn model to collect burn serum. Thereafter, RAW264.7 cells were treated with different concentrations of burn serum and control serum (0, 0.5, 1.0, 1.5, 2.0 and 2.5 ml, respectively) for different times (0, 3, 6, 9, 12 and 24 hrs, respectively). After separating the serum, the levels of TNF-α and HMGB1 were measured by using enzyme-linked Immunosorbent assay (ELISA) with the double antibody sandwiched method. To observe the effects of secretion on TNF-α and HMGB1 in RAW264.7 cells, cells were treated for 6 hrs with different levels of burn serum concentration, the results indicated that burn serum stimulation gradually increased the levels of TNF-α and HMGB1 in RAW264.7 cells. The results also showed that the levels of TNF-α and HMGB1 obviously increased when treated with the burn serum by 1.5, 2.0 and 2.5 ml for 6 hrs, and there was a significant difference when compared with the group of 0, 0.5 and 1.0 ml (*P*<0.05). In the group of 2.0 ml, the levels of TNF-α and HMGB1 increased over time. The levels of TNF-α began to increase after 6 hrs, and the secretion after 6, 9, 12 and 24 hrs resulted in a significant difference when compared to 0 and 3 h (*P*<0.05). While the levels of HMGB1 began to increase after 9 hrs, the secretion after 9, 12 and 24 hrs resulted in a significant difference when compared to 0, 3 and 6 hrs. There was no difference in the levels of TNF-a and HMGB1 in the normal group after burn serum administration at different times. Burn serum can reduce the release of TNF-α and HMGB1 in rat's macrophages and show a significant dose-time-effect correlation.

Key words: Severe burn, tumor necrosis factor alpha (TNF-α), high mobility group proteins 1 (HMGB1), macrophages, secretion.

INTRODUCTION

Severe burn leads to a series of acute phase reactions, such as the body's immune function disorder, mononuclear phagocyte activation and significant amount of cytokine secretion when excessively activated.

The tumor necrosis factor alpha (TNF-α) is the most important "early" appearing pro-inflammatory mediator, while high mobility group proteins 1 (HMGB1) is the most important "late" appearing inflammatory mediator (Lin et al., 2002). An imbalance between the pro-inflammatory and the counter-inflammatory cytokine will lead to an inflammatory cascade of pro-inflammatory cytokine. Meanwhile, it is considered to be responsible for inducing secondary multiple organ failure, such as systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndromes (MODS), and can result in death if it's serious (Nichoileain et al., 2006). Following the severe burns, patients would further develop into SIRS and MODS, which cause high mortality.

At present, inhibitory factor theory, inhibitory cell theory and immunologic network theory are the three main mechanisms contributing to immune function disorder after serious burns. Immunosuppressive factor is a protein or a

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peptide that can suppress immune function. Serum is the vital mediator in regulating tissues or cells, its composition change after burns could lead to multiple organ dysfunctions. Burn serum is the product of the steady-state imbalance and it shows more complex biological effects than the normal serum in different organization cells and some of the time-dependent (Drost et al., 1993; Scalfani et al., 2007). Nevertheless, rather than serum immunosuppressive factor, vast immunosuppressive materials still exist in burn serum.

Therefore, we study the secretion disciplines of TNF-α and HMGB1 on a cellular level through investigating the levels released by burn serum-induced RAW264.7.

MATERIALS AND METHODS

Materials

Mice mononuclear macrophages RAW264.7 was supplied by Shanghai Cells Resource Center; RPMI1640 (cells culture medium), FBS, pancreatin-EDTA and PBS were purchased from Gibco; Green streptomycin mixture (100×) was purchased from Beijing Solarbio Science and Technology Co., Ltd; TNF-α and HMGB1 ELISA kits (Wuhan Boster Bio-engineering Limited Company).

Apparatus

Healforee Bechtop (Shanghai Shenli Science Instruments Company); SHEL-LAB2300 CO_2 incubator (Sanyo Chemical Industries, Ltd); Low temperature desktop centrifuge (Eppendorf); Bio-RAD550 automatic enzyme standard instrument readings (Bio-Rad, USA); Inverted microscope (Olympus, Japan).

Burn serum collection

Ten adult healthy Sprague–Dawley rats (SPF, supplied by the Experiment Animal Center of Sun Yat-sen University), weighing 220–260 g, were kept in wire-bottomed cages under a 12 hr light/dark cycle and controlled temperature (25 ± 1°C) and relative humidity of 40–60%, and had free access to standard lab chow and tap water. Rats were anesthetized with 10% chloral hydrate (1 ml/kg) by intraperitoneal injection, and body hair on their backs where closely clipped. The surface of the skin exposed through the aperture in the template was immersed in 96°C water for 13 s on the back and accomplished third degree full-thickness dermal burns in over 35% of the total body surface area (TBSA). Immediately after i.p injection of saline (30 ml/kg) postburn, rats were anesthetized with 10% chloral hydrate (1 ml/kg) 6 hrs later, blood was collected by heart puncture, and then centrifuged at 3000 r/min for 10 min. Serum was removed and stored at -80°C for later analysis after the complements destructed in 50°C water for 30 min (Wang, 2002).

Cell culture

Mice macrophage cell line RAW264.7 was cultured in RPMI1640 medium containing 10% fetal bovine serum and 1% antibiotics at 37°C in humidified air containing 5% CO_2 at the ratio of 1:10. Cells were harvested by 0.25% trypsin when cells reached about 80 to 90% confluence.

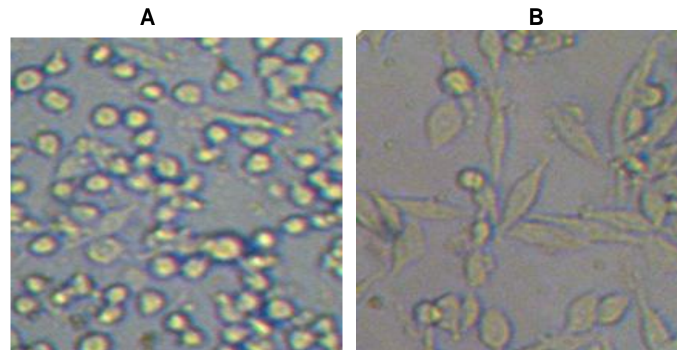

Figure 1. RAW264.7 cells' morphology after treated with normal and burn serum for 6 h under an inverted microscope. A: RAW264.7 cells after treated with normal serum for 6 h (×100); B: RAW264.7 cells after treated with 1.5 ml burn serum for 6 h (×100).

Burn serum processing dose-effect group

Cells in logarithmic growth phase were obtained to co-incubate with 0, 0.5, 1.0, 1.5, 2.0 and 2.5 ml burn and normal serum for 6 h, respectively. Then the cultures were obtained and centrifuged at 5000 r/min for 30 min, and the supernatant were collected to examine TNF-α and HMGB1 secretion with ELISA.

Burn serum processing time-effect group

Cells in logarithmic growth phase were obtained to co-incubate with 2.0 ml burn and normal serum for 0, 3, 6, 9, 12 and 24 h, respectively. Then the cultures were obtained and centrifuged at 5000 r/min for 30 min, and the supernatant were collected to examine TNF-α and HMGB1 secretion by ELISA.

Statistic analysis

All values are expressed as mean ± SE of mean. Student's *t*-test was used to assess an overall difference among the groups for each of the variables. Probability values less than 0.05 were considered statistically significant (analysis was performed using SPSS for Windows, Version 11.0).

RESULTS

Culture results of RAW264.7 cells

In the normal control group, RAW264.7 cells were polygonal shape, narrow intercellular gap, uniform size, and showed oval or irregular in shape under high magnification. But the cells' morphology changed obviously after they were treated with different concentrations of burn serum for 6 h and we could see that the cells became chain-like with intercellular widening, and erasing large areas of cells off (Figure 1).

Effects on secretion of TNF-α and HMGB1 after treated with different dosage burn serum

To observe the effects of secretion on TNF-α and

Table 1. Effects on secretion of TNF-α and HMGB1 after treated with different concentration of serum ($\bar{x} \pm s$).

Concentration of serum (ml)	TNF-α (pg/ml)	HMGB1 (μg/L)
0	1441.6±548.2	259.2±98.6
0.5	1565.2±595.2	281.4±107.0
1.0	1939.3±737.5	348.6±132.6
1.5	3608.4±1372.2	648.7±246.7
2.0	7340.5±2791.5	1319.6±501.8
2.5	12415.5±4721.4	2232.0±848.8

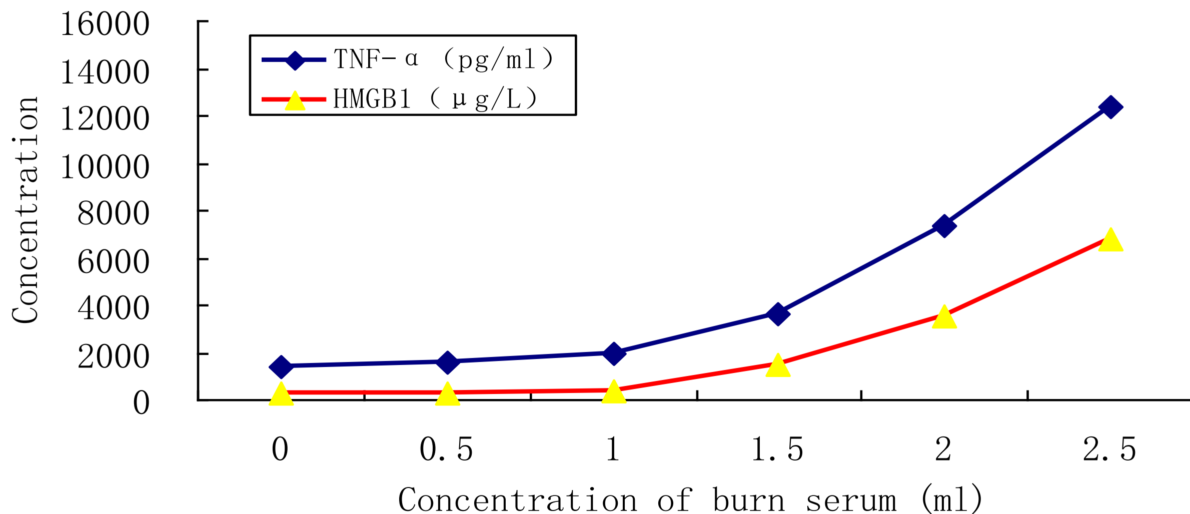

Figure 2. Effects on secretion of TNF-α and HMGB1 after treated with different concentration of serum.

Table 2. Effects on secretion of TNF-α, IFN-γ and IL-8and HMGB1 after treated with normal serum ($\bar{x} \pm s$).

Concentration of serum (ml)	TNF-α (pg/ml)	HMGB1 (μg/L)
0	448.9±170.7	118.3±45.0
0.5	579.7±220.4	147.7±56.2
1.0	568.8±216.3	147.1±55.9
1.5	585.7±222.7	202.2±76.9
2.0	585.7±222.7	160.7 ±61.1
2.5	503.4±191.4	186.3±70.8

HMGB1 after treatment with different concentration of burn serum and normal serum for 6 h in RAW264.7 cells, the results indicated that burn serum stimulation gradually increased the levels of TNF-α and HMGB1. The levels of TNF-α and HMGB1 obviously increased when treated with the burn serum by 1.5, 2.0 and 2.5 ml after 6 hrs, resulting in significant differences when compared with group of 0, 0.5 and 1.0 ml (P<0.05). Table 1 and Figure 2 show effects of secretion on TNF-α and HMGB1 after treatment with normal serum. The results showed that the secretion of TNF-α and HMGB1 after treatment with normal serum at 6 h did not change significantly (Table 2 and Figure 3).

Effects on secretion of TNF-α and HMGB1 after treated with 2 ml burn serum for different time

The levels of TNF-α and HMGB1 in RAW264.7 cells that were treated with 2 ml burn serum up-regulated over time. The secretions of TNF-α began to rise at 6 h and the secretions at 6, 9, 12 and 24 h was significantly

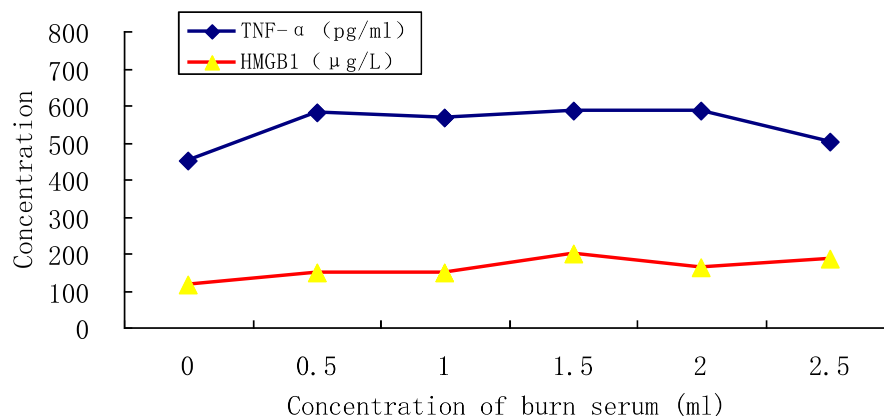

Figure 3. Effects on secretion of TNF-α and HMGB1 after treated with normal serum.

Table 3. Effects on secretion of TNF-α and HMGB1 after treated with 2 ml burn serum for different time ($\bar{x} \pm s$).

Time (h)	TNF-α (pg/ml)	HMGB1 (μg/L)
0	1430.1±543.8	257.1±97.8
3	2073.3±788.4	930.1±353.7
6	7340.5±2791.5	1280.0±486.8
9	8294.7±3154.3	5470.3±2080.3
12	8869.5±3372.9	6738.8±2562.6
24	9335.2±3550.0	7026.2±2671.9

different compared to 0 and 3 h (*P*<0.05). While the levels of HMGB1 began to increase at 9h and the releases at 9, 12 and 24 h were significantly different when compared to 0, 3 and 6 h (Table 3 and Figure 4).

Effects on secretion of TNF-α and HMGB1 after treated with 2 ml normal serum for different time

The levels of TNF-α and HMGB1 had no significant difference in treatment for 0, 3, 6, 9, 12 and 24 h respectively in normal serum group (Table 4 and Figure 5).

DISCUSSION AND CONCLUSION

Burn injury is a kind of severe local injury that can lead to imbalance of the whole internal environment, and finally affect every system of the body. Compared to normal serum, burn serum shows different and complicated biological effects in different tissues. And cytokines (CK) can regulate cell immune response. Generally, when burns are serious, its abnormal regulation or generation and release possibly contribute to organ failure. Releasing large amount of CK will cause influences on the local and systemic pathophysiology, such as immune defense, change hemorheology, inflammation and metabolic disorder (Kuang et al., 2002).

TNF-α is an endogenous regulatory factor without species-specificity that generates by activated mononuclear phagocyte system. Thus, it participates not only in the immune defense as one of the cytokines released by immune response, but also in certain pathophysiological processes, like mediating shock, inflammation, tissues and organ injury. Usually, the concentration of TNF-α is relatively low and reasonable, therefore, concentration is necessary for the protective response.

However, over-expression of TNF-α will induce injury to the body (Zhang et al., 2003). Severe burn generally accompany massive endotoxin that can up-regulate the levels of TNF-α and endotoxin also relates to the severity and prognosis of burn injury (Niederbichler et al., 2009).

Not as a primary pre-inflammatory factor, TNF-α but as a chemokine that motivates secondary inflammatory factor. And the level of TNF-α grows with the burn area and increases the possibility of infection.

Yin et al. (2007) found that burn serums will significantly up regulate the concentration of TNF-α in mice mononuclear macro-phages, and its release increases dose-dependently with burn serum

Table 4. Effects on secretion of TNF-α and HMGB1 after treated with 2 ml normal serum for different time ($\bar{x} \pm s$).

Time (h)	TNF-α (pg/ml)	HMGB1 (μg/L)
0	488.6±185.8	140.8±53.5
3	526.2±200.1	172.4±65.6
6	585. 7±222.7	160.7±61.1
9	586.8±223.1	209.3±79.6
12	567.7±215.9	214.4±81.5
24	535.5±203.6	228.5±86.9

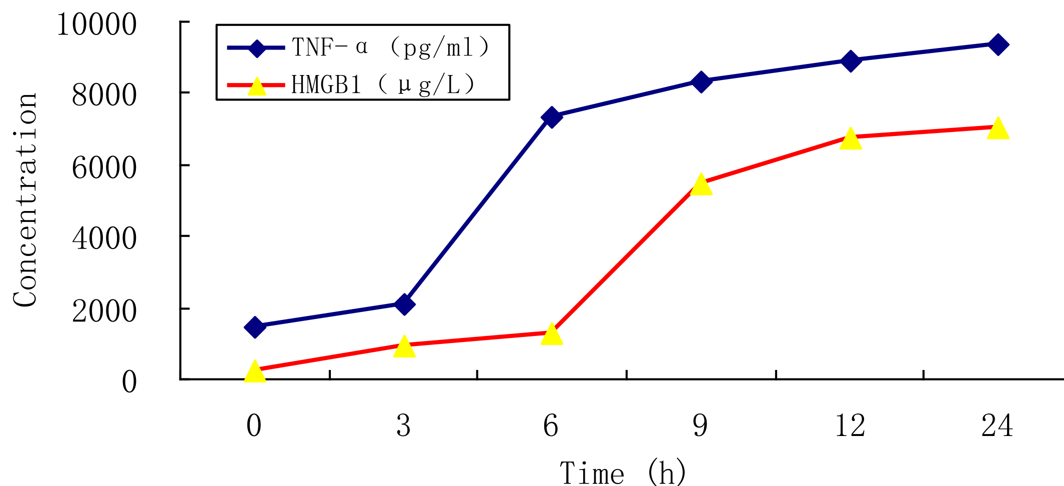

Figure 4. Effects on secretion of TNF-α and HMGB1 after treated with 2 ml burn serum for different time.

concentration Li et al. (2009) revealed that the expression of TNF-α and oxygen radical in the serious burn group was higher than that in the control group, while early enteral nutrition attenuated the overall levels of TNF-α and oxygen radical. It was discovered that the levels of TNF-α in burn patients' serum increased obviously with the levels related to the severity of burns (Barber et al., 2004). Normally, three mechanisms may be responsible for TNF-α increase, these are; (a) Severe burn generally accompany massive endotoxin release that can up-regulate the levels of TNF-α; (b) Severe burn can motivate the neneutrophile granulocyte and promote TNF-α to release (Zhou et al., 2005); (c) Severe burn that can also activate transcription factor NFKB and motivate cytokine cascade, and finally lead to the release of TNF-α, PAF and leukotriene and so on (Man et al., 2006); (d) TNF-α increase not only stimulates the release of neutrophils, but promotes the differentiation of mononuclear macrophage, coagulation and complement system, which induce acute phase proteins synthetization.

It has been confirmed that HMGB1 plays an important role in the inflammatory response since it was found to facilitate inflammation. HMGB1 is a highly conserved protein present in the nucleus of almost all eukaryotic cells. Physiological state of HMGB1 functions as a nuclear binding protein, and once released into the extracellular milieu, it then functions as a late inflammatory factor. Intranuclear HMGB1 can move into extracellular milieu through active secretion of inflammatory cells and passive release of necrotic cells. HMGB1 possess the characteristics of cytokine and can be secreted actively by a set of immunocytes called activated mononuclear cells, macrophages, natural killer cells and dendritic cells (DC). Extracellular HMGB1 acts on immunocytes and endotheliocytes by combining the surface receptors through paracrine and autocrine pathway, and then mobilizes target cells and releases a variety of chemokines, cytokines and adhesion molecules, stimulates inflammatory cells, destructs the epithelial barrier, and thus exaggerate the inflammatory response.

Recent research (Yang et al., 2010) indicates that lipopolysaccharide (LPS)-stimulated murine RAW264.7 cells can time-dependently secrete lots of HMGB1. Administration of a high dose of purified restructure HMGB 1 after 2 h can cause sepsis, and a higher dose can cause death in mice (Andersson et al., 2011).

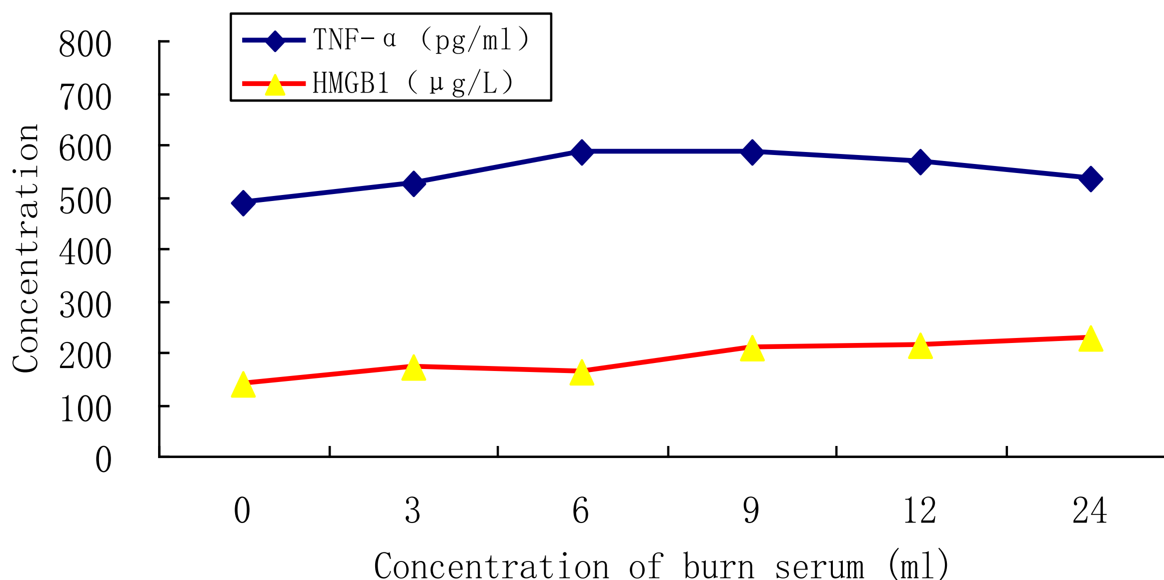

Figure 5. Effects on secretion of TNF-α and HMGB1 after treated with 2 ml normal serum for different time.

Moreover, activated macrophages-released HMGB1 that can also stimulate other inflammatory mediums to release lethality results when over-expressed (Kosai et al., 2008). HMGB1 may be one of the key alarm molecules induced inflammatory responses during cellular damage and cell necrosis. Studies have demonstrated that LPS can time-dose-dependently up-regulate macrophages HMGB1 release (Tsung et al., 2007). Treatment with LPS for 20 h, HMGB1 obviously transfers from the nucleus to cytoplasm and can be detected at extracellular (Reismann et al., 2009). Compared with TNF-α and other inflammatory factors, the late expression kinetics characteristic of HMGB1 makes the HMGB1 antagonize the actual clinical value (Chen et al., 2011).

In this study, we analyzed the expressions of TNF-α and HMGB1 in burn serum-treated mononuclear phagocyte line RAW264.7. The results suggested that burn serum stimulation gradually increased the levels of TNF-α and HMGB1 in RAW264.7 cells. And the levels of TNF-α and HMGB1 obviously increased when treated with the burn serum of 1.5, 2.0 and 2.5 ml by dose-independent. When treated with equal volumes of burn serum for different time, the levels of TNF-α and HMGB1 increased over time, and the increase of TNF-α was relatively different in HMGB1. But they were both time-dependent. While the levels of TNF-α and HMGB1 had no significant difference in treatment at different time inburn serum administration in normal group.

In summary, burn serum can time-dose-independently induce the release of TNF-α and HMGB1. And this provides a new approach to the diagnosis and treatment of severe burns and also provides a new therapeutic target for prevention of SIRS.

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