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Changes in the inositol lipid signal system and effects on the secretion of TNF-α by macrophages in severely scalded mice

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ABSTRACT

Aim: In order to study the mechanism of abnormal macrophage (Mϕ) function in pro-inflammatory cytokine changes after burn, the inositol lipid signal system and its role in tumour necrosis factor-alpha (TNF-α) secretion by peritoneal Mϕs was observed in severely scalded mice.

Methods: Eighteen percent total body surface area (TBSA) full-thickness scalded mice were used as animal model in this experiment. Peritoneal Mϕs stimulated by lipopolysaccharide *in vitro* were collected at different time intervals (0, 2, 6, 12, 24 and 48 after burn hour (PBH)), The activities of phosphatidylinositol-phospholipase C (PI-PLC), inositol-1, 4,5, -triphosphate (IP_3), protein kinase C (PKC), diacylglycerol (DAG) and TNF-α and the level of Ca^{2+} concentration in peritoneal Mϕs were measured, and the effects of specific PKC inhibitor H-7 and calmodulin antagonist W-7 on the production of TNF-α were also observed.

Results: After scald, increased activities of TNF-α and PLC of Mϕ were observed and peaked at 12 PBH. The activities of DAG and IP_3 and the concentration of Ca^{2+} were markedly increased and reached their peaks at 24 PBH simultaneously. Membrane PKC activity was up-regulated after scald and showed a positive correlation with the change of DAG ($r = 0.83$, $P < 0.05$). There was also positive correlation between IP_3 and Ca^{2+} activity ($r = 0.946$, $P < 0.01$). When 12 PBH was chosen as the time point for *in vitro* intervention with the pre-treatment by H-7, both membrane PKC and TNF-α activity decreased significantly. There was no obvious change of TNF-α activity with the application of W-7.

Conclusions: These results indicated that the abnormal activity of TNF-α of Mϕs might be regulated by the inositol lipid signal system following severe burn. The DAG–PKC signal pathway showed closer relationship than IP_3-Ca^{2+} in TNF-α production and could be the optimal target in the prevention and treatment of the systemic inflammatory response syndrome.

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1. Introduction

It has been recognised that severe burn injury may cause marked alterations of the immune function, resulting in life-threatening systemic infections, sepsis, multiple organ failure and even death. Extensive and deep burns exert widespread and profound impacts on various cells and molecules of the immune system. The general characteristics of abnormal immune responses following major burns are hyper-inflammatory response and hypo-immune response of innate and adaptive immunity, respectively. These are recognised as immune dysfunction after burn (PID). After severe burn, macrophages (Mϕs) are postulated to play a vital role in this response when activated by various stimuli, such as stress, necrotic tissue components, ischaemia, bacteria and some cytokines derived from other inflammatory cells or Mϕs themselves [1–5]. While several groups have addressed the relationship between immune dysfunction and burn injury, the mechanism of tumour necrosis factor-α (TNF-α) secretion in this response has yet to be elucidated.

With regard to this, Mϕ is a major producer of pro-inflammatory mediators (e.g., TNF-α, interleukin (IL)-1), nitric oxide (NO). Moreover, burn increases the productive capacity of Mϕ for these mediators [6–9]. Dysregulation of Mϕ activity, leading to increased release of pro-inflammatory factors (i.e., Mϕ hyperactivity), appears to be of fundamental importance in the development of immune dysfunction after burn [4,10]. TNF-α is believed to be the initiating cytokine that induces a cascade of secondary cytokines and humoral factors that can lead to local and systemic sequelae [11]. Moreover, TNF-α is a potent mediator of the shock-like state associated with severe burn and sepsis [12].

An epoch-making discovery was made in about the mid-1980s; that is, the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PI (4,5) P_2) by phospholipase C (PLC) generates two second messengers, inositol-1, 4,5, -triphosphate (IP_3) and diacylglycerol (DAG), when cells are stimulated with hormones or neurotransmitters [13,14]. DAG mediates the activation of protein kinase C (PKC), and IP_3 triggers the release of calcium from intracellular stores. In 1989, another impressive discovery was reported by C. Cantley's group [15]. They found a novel phosphoinositide kinase, PI3K, which phosphorylated PI (4,5) P_2 at the 3-position of the inositol ring to generate PI (3,4,5) P_3. The latter transduces important signals that induce cell proliferation, motility, etc., and thus defects in the generation/degradation of PI (3,4,5) P_3 cause cancer, diabetes and inflammation [16].

To study the cause of excessive secretion of TNF-α by Mϕs may be important to explain the functional changes of Mϕs and the mechanism of post-burn immune dysfunction. In our laboratory, the post-burn changes of phagocytic function, antigen presentation and surface receptors by Mϕs were investigated. Furthermore, we explored the mechanism of altered TNF-α expression by peritoneal Mϕs and *Panax notoginseng saponins* (PNS) modulation in light of nuclear factor-kappa B (NF-κB) signal transduction in severely scalded mice [17]. It was reported that lipopolysaccharide (LPS) stimulated normal peritoneal Mϕs to produce TNF-α *in vitro*, primarily mediated by the inositol lipid signal system [18], and inhibition of PLC by their general inhibitors decreased TNF-α expression in Mϕs and keratinocytes during LPS stimulation [19]. However, it remains unclear whether excessive secretion of TNF-α by Mϕs is caused by the disturbance of the inositol lipid signal pathway after severe burn. As thermal injury increases mice peritoneal Mϕ sensitivity to LPS by a mechanism that is both pertussis toxin sensitive and pertussis toxin insensitive [10], in the present study, we investigated peritoneal Mϕ activation stimulated by LPS *in vitro* after severe scald in mice through measuring changes in PLC, IP_3, Ca^{2+}, DAG, PKC and TNF-α, so as to explore the relationship between TNF-α secretion and the inositol lipid signal system.

2. Materials and methods

2.1. Experimental burn and groups

Kunming mice of both genders (6–8 weeks of age, 24 ± 1.5 g) were obtained from the Experimental Animal Center of Third Military Medical University (Chongqing, China). The research was conducted in accordance with the institutional accepted principles for laboratory animal use and care. All the animals were maintained in cages at 20 ± 2 °C with free access to pellet food and water. This study complies with current ethical regulations on animal research of this institute and all animals used in the experiment received human care.

Mice received a full-thickness scald burn as previously described, which was confirmed by pathological slices. Briefly, the entire dorsal surface of anaesthetised mice was shaved and fixed under a thick rubber board with a hole in order to expose 18% total body surface area (TBSA) on the shaved back. Then, the exposed area was subjected to high pressure vapour (1.0–1.2 kg cm^{-2}) at a distance of 4 cm away from its outlet for 10 s to produce 18% TBSA skin-full-thickness scalds; then, mice were resuscitated with 3 ml sterile normal saline intraperitoneally. On waking, the mice were returned to the animal facility in different cages. The normal control group was treated as above, except that the hot vapour was replaced by air and they were not resuscitated.

The mice were randomly assigned into seven groups: a normal control group (control), several post-burn hour (PBH) groups (i.e., 2, 6, 12, 24 and 48 PBH) and an *in vitro* pre-treatment group of 12 PBH. There were eight mice in each group. To further explore the relationship between the inositol lipid signal system and TNF-α, 12 PBH was chosen as the time point for intervention of the peritoneal Mϕs. PKC inhibitor H-7 was used with the final concentration of 10 and 50 μmol l^{-1}, and calmodulin antagonist W-7 was applied with the final concentration of 50 and 100 μg ml^{-1} *in vitro*, respectively.

2.2. Isolation and culture of mouse peritoneal macrophages [4,17]

The injured mice were sacrificed by bleeding at the designated time points after injury (i.e. 0, 2, 6, 12, 24 and 48 PBH) and thoroughly cleansed with 70% ethyl alcohol. A small incision was made into the abdomen, and the peritoneal cavity was rinsed with 5 ml pre-cooled phosphate-buffered saline (PBS) (pH 7.2) twice, aseptically. The rinsed PBS was collected and