Clinical Research

High mobility group box 1 protein (HMGB1) as an immune-modulating factor for polarization of human T lymphocytes

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Abstract

Objective This study was performed to investigate the effect of high mobility group box-1 protein (HMGB1) on immune function of human T lymphocytes in vitro and explore its potential role in cell-mediated immune dysfunction.

Methods

Fresh blood was obtained from healthy adult volunteers and peripheral blood mononuclear cells (PBMCs) were isolated, then rhHMGB1 was added to PBMCs. Four-color flow cytometric (FCM) analysis was used for the measurement of intracellular cytokine including interleukin IL-4 and interferon IFN-\(\gamma\). ELISA kits were employed for the determination of IL-2 and soluble IL-2 receptor (sIL-2R) protein levels in cell culture supernatants.

Results

(1) Different stimulating time and dosage of rhHMGB1 did not alter the number of IFN-\(\gamma\)-positive cells (Th1). rhHMGB1 stimulation provoked a dose-dependent and time-dependent increase in Th2 subset and decrease in ratio of Th1 to Th2. (2) Compared with the untreated cells, when the cells were coincubated with rhHMGB1 (10-100ng/ml) for 12 hrs, protein release of IL-2 and sIL-2R were significantly up-regulated. At 48 hrs, in contrast, protein production were relatively lower in cells after exposure to 100-1000 ng/ml rhHMGB1.

Conclusions

These findings demonstrated that HMGB1 had a dual influence on immune functions of human T lymphocytes.

Key Words High mobility group box-1 protein; Immunity; T lymphocytes; Th1/Th2

Introduction

High mobility group box-1 protein (HMGB1), a 215 amino acid residue-containing nuclear protein abundantly present in all mammalian tissues and cells, also known as amphoterin, is highly conserved among various species, and structurally organized into two DNA-binding domains (termed A and B box) and a negatively charged C-terminal tail\(^1\). Recently, HMGB1 has been identified as a proinflammatory cytokine that mediates endotoxin lethality, local inflammation, and macrophage activation in sepsis\(^2,3\). The biology of HMGB1 has been extensively studied as a proinflammatory cytokine of systemic inflammation, while little is known in regard to its potential contribution to the host cell-mediated immunity, particularly for human T lymphocytes.

It has been demonstrated that in the development of sepsis, human organism may have experienced a phase of immune suppression. In this stage, the lymphocytic reproductive activity has remarkably attenuated and the Th2 immune reaction has become predominant. These changes can compromise the ability of the patient to eradicate the primary infection and predispose to secondary nosocomial infection\(^4,5\). Studies have indicated that shift from Th1 to Th2 response was a contributory factor to marked suppression of cell-mediated immunity in sepsis, though the underlying mechanism has not been fully elucidated\(^6,7\). However, the biological activities of extracellular HMGB1 and its effect on the innate immune response have not been well illustrated. In this study, we investigated the effect of HMGB1 on immunity of human T lymphocytes in vitro and explore its potential role in cell-mediated immune dysfunction.

Materials and Methods

Experimental design

The study population comprised of 26 healthy volunteers. All of them with no detectable physical or clinical laboratory abnormality were demanded to refrain from taking any drug in one month. 8 volunteers (5 men and 3 women, mean age 24±9 years old, range 18-42 years old) participated in the experiment of polarization of the T-helper
lymphocyte activity; 8 volunteers (4 men and 4 women, mean age 25±6 years old, range 22-37 years old) participated in the lymphocyte proliferation assay; 10 volunteers (7 men and 3 women, mean age 29±7 years old, range 25-42 years old) were enrolled for the experiment to determine the protein levels of IL-2, sIL-2R. Written informed consents were signed before the beginning of experiments. The experimental protocol was approved by the Ethical Review Committees of Postgraduate Medical College of PLA, Beijing.

Peripheral blood T lymphocyte isolation and counting

Thirty milliliters of heparinised blood was diluted in Hanks’ balanced salt solution, and Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO) was used for isolation and preparation of peripheral blood lymphocytes. The isolated lymphocytes were washed twice with phosphate buffered saline (PBS), centrifuged (500g, 4 minutes), resuspended in basal medium (unstimulated culture) RPMI-1640 (Gibco-BRL, Gaithersburg, MD) containing 10% fetal calf serum (Seromed, Berlin, Germany), 1% penicillin/ streptomycin (Sigma Chemical Co., St. Louis, MO), and 1% glutamine (ICN, Eschwege, Germany). Then cell populations were counted under a light microscope. Based on the different experimental demand, the peripheral blood mononuclear cells (PBMC) suspension was diluted with RPMI-1640 to 2×10⁶ cells/ml.

Determination of intracellular cytokines in lymphocytes

Cells (2×10⁶ cells/ml) were inoculated to 24-well plates at 1ml per well and incubated for 2 hrs at 37°C in 5% CO₂. Phytohemagglutinin (PHA) 1% was added to the culture. At 24, 48, and 60 hrs, rhHMGB1/PBS was added to the PBMC suspensions to with the final concentration of HMGB1 at 0, 1, 10, 100, and 1000 ng/ml per well respectively, with three wells for each concentration. After incubation for 68 hrs, supernatants and cells were suctioned and cultured. A pial of RPMI-1640 (Gibco-BRL, Gaithersburg, MD) containing PMA (Sigma Chemical Co., St. Louis, MO) 100ng/ml, ionomycin (Sigma Chemical Co., St. Louis, MO), and 2 µg/ml GolgiStop (Pharmingen, San Diego, CA) 0.75ìl/ml was added, then incubated for 6 hrs at 37°C in 5% CO₂.

Cell suspension was harvested, washed once with phosphate-buffered saline (PBS), and centrifuged (500g, 4 mins), and the cells were resuspended in basal medium 200µl RPMI-1640. 20 µl CD3-PerCP (Becton-Dickinson, San Jose, CA) and 5ìl CD8-APC (Becton-Dickinson, San Jose, CA) were added. Cells were stored at room temperature in darkness. Then washed once with 2ml PBS, centrifuged (500g, 4 mins), resuspended in 300µl RPMI-1640. Two samples of supernatant (100µl) were added in two flow cytometry (FCM) test tubes. Two samples were labeled respectively as the following methods: (1) Intracellular cytokine staining: cells were resuspended in 0.5ml perforation fluid containing 10% AB serum (impacting medium; Hyclone, USA). They were incubated for 10 mins at room temperature in darkness with the following directly conjugated anti-cytokine/surface marker antibodies: 20µl mAb-IL-4-PE/IFN-α-FITC (homeotype negative comparison was done with 20µl β2aFITC/ κPE). All mAbs were acquired from Becton Dickinson (San Jose, CA). Then, cells were stained for 30 mins at room temperature in darkness, washed with 2ml PBS, and centrifuged (500g, 4 mins), fixed with 0.5ml 4% paraformaldehyde (RiedeldeHaen AG, Seelze, Germany); (2) Negative comparison staining: the method was the same with that of intracellular cytokine staining.

Before the FCM analysis, PBMCs were cultured for 6 hrs with 50ng/ml PMA, 11g/ml ionomycin, and 2µM GolgiStop. The Th1/Th2 ratio was evaluated by calculating intracellular IFN-κ/IL-4 ratio. 1×10⁶ cells were analyzed in each experiment using the CellQuest program (Becton-Dickinson, San Jose, CA) after setting the quadrants using isotype controls.

Determination of IL-2, sIL-2R protein levels

The IL-2 and soluble IL-2 (sIL-2R) protein levels in cell supernatants were analyzed by the enzyme-linked immunosorbent assay (ELISA) according to the procedures provided by the manufacturers. The kits for ELISA were provided by Genzyme Corporation, USA. The determinations were carried out according to the instruction which was included in the kit. Absorbance of each well was read at 450nm and the values were derived from the standard curve. A SLT 400 SFC Lab instrument Austrian computer was used to measure absorption.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). One-way ANOVA was used for the data as obtained at same stimulating time and different dosages, or same dosage and different stimulating time. The Mann-Whitney U test was performed for the data of heterogeneity of variance or nonnormal distribution in groups. Statistical analysis for the data of two-sets comparisons was performed using the Student’s t-test. The statistical packages Stata8. 0 was used for all statistical calculations. The significance of differences was set at P<0.05 and the highly significance of differences was set at P<0.01.

Results

Changes in Th1/Th2 subsets of T lymphocytes

Th1 proportion (IFN-κ+ expression): With same duration of exposure but different dosage, or same dosage but different exposure duration there was no significant difference in Th1 proportion. But the proportion of Th1 showed a tendency of elevation after being stimulated with 1~100ng/ml rhHMGB1 for 12 hrs and 24 hrs (Figure 1A).

Th2 proportion (IL-4+ expression): There were sig-
significant differences in Th2 proportion at 24 hrs under different dosages ($F=3.3973, P=0.0189$), and the Th2 proportion at 1000ng/ml was higher than those at 0, 1, 10, 100ng/ml ($P<0.05$), respectively. No significant difference was found at 48 hrs, but Th2 proportions under the dosages of 10, 100ng/ml were obviously higher than that at 0ng/ml ($P<0.01$). At 10ng/ml there was no proved significant differences, while Th2 proportion was shown to be high at 48 hrs than those at 0, 12, 24 hrs ($P<0.05$). There was highly significant difference in Th2 proportion at 100ng/ml with different duration of exposure ($F=9.923, P=0.0192$). The results showed that Th2 proportion was higher at 48 hrs than that at 24 hrs ($P<0.05$) and obviously higher than those at 0, 12 hrs ($P<0.01$). There were obvious differences in Th2 proportion at 100ng/ml with different duration ($F=4.6219, P=0.0095$), and Th2 proportion at 24 hrs was higher than that at 0 hrs ($P<0.05$), and that at 48 hrs was obviously higher than those at 0, 12 hrs ($P<0.01$) (Figure 1B).

**Fig 1** Changes in Th1 and Th2 subsets of T lymphocyte stimulated with rhHMGB1

Th1/Th2 subsets ratio: At 12 hrs there was no significant difference with different concentrations. At 24 hrs highly significant differences in Th1/Th2 ratio in different groups were found ($H=13.489, P=0.0091$), and Th1/Th2 ratio at 1000ng/ml was markedly lower than those at 0, 1, 10, and 100 ng/ml ($P<0.05$ or 0.01). At 48 hrs, the results showed that Th1/Th2 ratios at 120, 1000 ng/ml were lower than that at 0ng/ml ($P<0.05$). There was significant difference in Th1/Th2 ratio at 100ng/ml with different time ($H=13.324, P=0.0040$), and Th1/Th2 ratio at 24 hrs was higher than those at 0, 24 hrs ($P<0.05$). Th1/Th2 ratio at 48 hrs was markedly lower than those at 0, 12, 24 hrs ($P<0.01$). Similarly, there was obvious differences in Th1/Th2 ratio at 1000ng/ml with different duration of exposure ($H=13.324, P=0.0040$), and Th1/Th2 ratio at 24 hrs as well as 48 hrs were lower than those at 0, 12 hrs, respectively ($P<0.05$ or 0.01) (Figure 2).

**Fig 2** Changes in Th1/Th2 subsets of T lymphocyte with Different concentrations of rhHMGB1 and different co-stimulating time

HMGB1 is thought to have the capability of mediating the shift of Th1/Th2 immune functions as shown by above experimental results. HMGB1 at relative low dosages could induce differentiation of lymphocytes to Th1 dominance, and the major effect of HMGB1 was to raise Th1 proportion. However, HMGB1 in high dosages could induce Th2 dominance during long time, through enhancement of the Th2 expression.

Changes in IL-2, sIL-2R protein excretion

When lymphocytes stimulated with PHA for 72 hrs, the sIL-2R protein concentration in supernatants showed a arising tendency. The IL-2 protein concentration increased at 24 hrs, then entered the platform stage (Figure 3).

At 12 hrs, there were significant differences in IL-2 protein levels under the influence of different dosages ($F=2.9119, P=0.0496$). IL-2 protein levels were increased with stimulation with 10, 100, and 1000ng/ml of rhHMGB1 ($P<0.05$), but there was no marked differences among three dosage groups. At various dosages there was no significant difference in sIL-2R protein levels, while the difference in IL-2/sIL-2R ratio at different dosages were obviously different ($F=4.9675, P=0.0061$). The IL-2/sIL-2R ratio increased with 10ng/ml rhHMGB1 ($P<0.05$), and it was elevated obviously with 100, 1000ng/ml rhHMGB1 stimulation ($P<0.01$).

At 48 hrs, no significant differences in IL-2, sIL-2R protein levels at various dosages was found, but IL-2 release showed a diminishing tendency with increasing rhHMGB1 ($P=0.0642$), and the lowest level of IL-2 appeared at 1000ng/ml ($P=0.0513$). The IL-2/sIL-2R ratio at different
dosages was not obviously different, while it was obvi-
ously lower at 1000ng/ml than that at 10ng/ml (P<0.05).

Discussion

Sepsis is considered as a symptom complex resulted
from an invasion of pathogen, provoking a cascade of harm-
ful physiological changes including fulminating inflamma-
tory responses detrimental to structures and functions of
tissues and organs. It is an indocile challenge to human
health and economical development world wide. Mortality
rate of severe sepsis and septic shock may reach 30~80%8,
and it has become the leading cause of death of non-cardiac
diseases in different countries, mainly because its underly-
ing pathogenetic mechanisms have not been fully eluci-
dated9. The key point in treatment of sepsis is to control
inflammatory response in a reasonable degree. The discov-
ery of HMGB1 as a contributory pathogenetic factor in sep-
sis is of great interest as some investigators found that the
elimination of HMGB1 is important in controlling inflamma-
tory response in sepsis.

At present, the mechanism of signal transductions
through which HMGB1 evokes inflammatory response still
remains unclear. HMGB1 can bind to the receptor for ad-
vanced glycation end products (RAGE) in a dose-depen-
dent manner. The interaction between HMGB1 and RAGE
is competitively inhibited by addition of soluble RAGE
or anti-RAGE IgG10. RAGE is expressed on mononuclear
phagocytes and vascular smooth muscle cells, and engage-
ment of RAGE with ligands (such as advanced glycation
end products [AGE], or HMGB1), activates not only the
NF-κB signaling pathway11, but also the mitogen-activated
protein kinase (MAPK) pathway. This suggests that HMGB1
has the biological property of a proinflammatory mediator.
HMGB1 not only participates in inflammatory regulations
in sepsis caused by cytokine networks, but also presents
the dynamic character in a time-dependent manner as a down-
stream or “late” action. In experiment with endotoxin
challenge, infection of the peritoneal cavity12, and burns13
it has been proved that HMGB1 is released in tissues and
peripheral blood after 8~12 hrs, and significant amount of
HMGB1 was detected after 32~72 hrs. This delayed release
of HMGB1 distinguishes it from early cytokines released
after endotoxin challenge. In view of the late and prolonged
action of HMGB1 in animals13,14, we speculated that it was
possible to detect elevated HMGB1 levels in serum in sep-
tic patients, because clinical signs of sepsis usually lagged
behind the early cytokine response to acute infection.

Furthermore, it has been demonstrated that the anti-HMGB1
treatment could alleviate organs damage in sepsis and re-
duce mortality rate15. However, it has been found futile for
anti-HMGB1 treatment to inhibit the release of the pro-in-
flammatory mediators in earlier period. The delayed emerg-
ence of HMGB1 in serum suggested that a delayed admin-
istration of its antibody is rational in order to suppress this
delayed cytokine.

Our preliminary results had proved that HMGB1 was
essential in the process of T lymphocyte adaptive immune
responses. The studies in murine models showed that low
doses of HMGB1 amplified the cytokine cascade during
systemic inflammation. HMGB1, as a cytokine, significantly
increased the release of TNF-α in a dose-dependent
manner. However, with prolonged (24~72 hrs) and high dos-
age (1000 ng/ml) of stimulation with HMGB1, the functions
of macrophages and T lymphocyte proliferation were
inhibited, and apoptosis of T cells intervened (data not
shown). When T cells were treated with HMGB1 in different
dosages for different duration, they would transform into
different subsets of Th1 and Th2. The fact that high dose of
HMGB1 can transform Th1 to Th2 subset implies that there
is an immune suppression in this event16. Based on this
finding, in order to offer the theoretical evidences to inter-
pret its clinical significance of immunologic competence,
we designed to investigate the effects of HMGB1 on human
T lymphocyte immune functions.

Excessive release of anti-inflammatory cytokines is an
important mechanism of immunosuppression in sepsis6.
Recently, studies have been shown that there is abnormal
polarization of Th1 and Th2 cells in peripheral blood in pa-
tients with critical sepsis17. The expressions of both IL-10
and HMGB1 mRNA have notably increased in septic pa-
tients with fatal outcome. Because cytokines might play an
important role in inducing CD4+ T ancillary cells
differentiation, we speculate that HMGB1 as a late cytokine
might influence immune function shift of T lymphocytes from Th1 subset to Th2 subset. Our results showed that Th1/Th2 ratios significantly decreased after 24 hrs of 100ng/ml HMGB1 stimulation, or with 1000ng/ml HMGB1 stimulation for 12 hrs. The results indicate that a prolonged exposure to high dose of HMGB1 can lead to a shift of Th1 to Th2. The abnormal polarization of T cells brings about changes in immune state from continual autoimmune responses to immunological inhibition. In the present study, we adopted the method of intracellular cytokine staining to distinguish Th1 from Th2, the results revealed that the changes between the important proinflammatory cytokine IFN-β and the anti-inflammatory cytokine IL-4 after HMGB1 stimulation. This is similar to variation tendency of cytokines observed in vivo in trauma and septic patients. Our preliminary experiments showed that the number of Th1 in mice splenic lymphocytes with different doses of HMGB1 challenge had obviously decreased. However, the change was not seen in human T lymphocytes stimulated with HMGB1. Such difference might be due to different sensitivities to HMGB1 stimulation of lymphocytes coming from different sources of origin provenance.

Some clinical observations reported that the levels of IL-2 and other proinflammatory cytokines began to decrease after 24 hrs in septic patients, however, the level of sIL-2R increased at the same time in serious trauma, infection and septic patient. Our data showed that the levels of IL-2 protein excretion was up-regulated after 12 hrs of 10~1000ng/ml HMGB1 stimulation in a dose-dependent manner. HMGB1 could also increase the sIL-2R expression. There was a close relation between increased IL-2R expressions and increased HMGB1 after 48 hrs. Up to now, receptors which can crosslink with HMGB1 and transduct the immunoregulation signals have not been identified on surfaces of lymphocyte cells. Whether there are other molecules on the lymphocyte surfaces which possesses characteristics of the receptors of cytokines to activate the transductions of signals and genetic transcriptions is an unknown subject calling for further investigation.

Our results have also proved that IL-2 protein release could be influenced by HMGB1 stimulation. In nuclei of T cells, IL-2 genetic transcription promoter-AP-1 is co-regulated by both nuclear factor NF-AT and gene fos, jun. It is our speculation that the transduction in cells which can influence T cellular immune functions might be mediated by NF-AT and phosphokinase pathways in the upper stream. Our previous studies have shown that Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways were involved in the HMGB1 expression and activation of HMGB1 in macrophages. JAK/STAT is also known as an important pathway which can transmit the IL-2R activating signals. We still do not know how HMGB1 affects the pathology mechanism of T cells. In the future, we will study HMGB1 and NF-κB, AP-1 and so forth, and how to adjust the balance of T cell activation, proliferation and apoptosis in septic patients. From these further studies, we will try to find some new evidence for sepsis cure, meanwhile, and we would discuss immunopathogenesis of other immunologic derangement diseases by the way of HMGB1.

In summary, these findings demonstrated that HMGB1 had a dual influence on immune functions of T lymphocytes. As a ubiquitous novel cytokine with proinflammatory activity, HMGB1 initiated inflammatory response by inducing cytokines with inflammatory properties from T lymphocytes (Th1). On the other hand, with the increased concentration and prolonged stimulation time, HMGB1 might suppress T-cell-mediated immune function with shifting of Th1 to Th2, so that HMGB1, as a late cytokine in sepsis, might contribute to the anti-inflammatory immunosuppressive state. Further studies are needed to elucidate the potential role of HMGB1 in regulation of cell-mediated immunity in sepsis in vivo.

References