GENERAL PATHOLOGY AND PATHOLOGICAL PHYSIOLOGY

Stress Response and Apoptosis in Pro- and Antiinflammatory Macrophages

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We showed that stress response and apoptosis in macrophages depend on the phenotype of their secretory activity and specific biological and physical characteristics of the factor inducing stress-response or apoptosis.

Key Words: *macrophages; stress response; HSP70; apoptosis; lipopolysaccharides; S. aureus; heat shock*

The important role of macrophages in immune reactions of the organism is determined by their ability to respond to bacterial products by the release of cytokines and other mediators of inflammation. Cytokines act as messengers in the auto- and paracrine relations between various immunocompetent cells and regulate the inflammatory response, cell differentiation, and immune reactions. Macrophages modulate the immune balance by changing secretory activity and gaining the pro- or antiinflammatory phenotype. The antiinflammatory and proinflammatory phenotype is characterized by primary production of antiinflammatory (interleukin-10, IL-10) and proinflammatory cytokines (IL-1, IL-6, IL-12, and TNF-α) [12].

In the focus of inflammation, macrophages counteract the toxic effect of cytokines and oxidative stress by triggering the self-protecting stress response [4,6]. Inducible heat shock proteins HSP70 play a key role in the stress response [2]. The protective effect of HSP70 is related to chaperon activity [5], ability to disaggregate abnormal aggregated proteins [9], activation of antioxidant protective mechanism [2], involvement in the utilization of irreversibly damaged proteins [3,11], inhibition of NO overproduction [7,10], and antiapoptotic action [1].

Overproduction of inflammatory mediators in macrophages can initiate or promote the development of diseases. The excessive stress response can be limited by triggering apoptosis in macrophages. Cysteine proteases (caspases) play a key role in apoptosis in various cells. These enzymes provide cleavage of cell proteins, fragmentation of DNA, and regulated cell death.

The stress response and apoptosis in macrophages can be initiated by microbial factors, including gram-positive bacteria (*e.g.*, *S. aureus*), their components (lipopolysaccharide, LPS), physical factors, and heat shock (HS). A balance between the stress reaction and apoptosis determines the survival of macrophages in the focus of inflammation. The ratio between pro- and antiinflammatory macrophages plays a role in the pathogenesis of sepsis, atherosclerosis, and ischemic injury of organs. Studies of the stress response and apoptosis in phenotypically different macrophages allow us to develop new approaches to the therapy of diseases associated with inflammation.

Here we studied the stress response and apoptosis in pro- and antiinflammatory macrophages exposed to

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the influence of microbial (LPS and *S. aureus*) and physical factors (HS).

MATERIALS AND METHODS

Macrophages were isolated from 8-12-week old male C3Heb/Fe mice. Thioglycollate broth (4%, 2 ml, Difco Lab.) was injected intraperitoneally 4 days before isolation of macrophages. Peritoneal macrophages were washed with HBSS and resuspended in RPMI 1640 medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum. Macrophages were programmed to the pro- or antiinflammatory response by 6-h exposure with 0.5 or 5 ng/ml LPS, respectively [12]. Macrophages were stimulated with 500 ng/ml LPS (37°C, 72 h), *S. aureus* (200 *S. aureus* per 1 macrophage, 72 h), or HS (42°C, 1 h). The stress response was determined by activity of HSP70 transcription factor (HSF1), HSP70 mRNA, and HSP70. The intensity of apoptosis was estimated by DNA fragmentation.

Oligonucleotide-binding biotinylated DNA were incubated with nuclear extracts of cells to obtain the complex of DNA and transcription factor. DNA was extracted from complexes and hybridized on a Tran-Signal Array membrane (Panomics). The signal that characterized HSF1 activity was detected on a chemiluminescence system.

Cell RNA was extracted with TRIzol reagent (Life Technologies). The reverse transcription reaction was performed with 1.0 μg RNA using GeneAMP RNA PCR Kit (Perkin-Elmer). cDNA was amplified with HSP70 primers (sense, residues 1183-1203, GATGA AGGAGATCGCTGAGG; antisense, residues 1678-1698, GATGCCCTCGAACAGAGAGT). Samples were electrophoretically separated in agarose gel, stained with ethidium bromide, and photographed.

Cell proteins were extracted after lysis of cells in a buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 25% glycerol, and 0.01% Bromophenol Blue and separated in 7.5% polyacrylamide gel. The gels were transferred to PVDF membrane in a buffer containing 48 mM Tris, 380 mM glycine, 0.1% sodium dodecyl sulfate, and 20% methanol (pH 8.3). Nonspecific binding to the membrane was blocked with 5% delipidated dry milk. The blots were incubated with primary anti-HSP70 antibodies in a 1:1000 ratio (Stressgen, SPA-810). Anti-mouse peroxidase-conjugated antibodies (1:3000, Santa Cruz, sc-2005) served as secondary antibodies. Blots were detected with a Santa Cruz developing system (sc-2048).

DNA content was measured as described elsewhere [8]. Macrophages were stained with 0.1% hypotonic sodium citrate, 0.1% triton X-100, 20 μg/ml RNase, and 50 μg/ml propidium iodide. Stained nuclei were assayed by means of flow cytometry on a Coulter EPICS XL-MCL flow cytometer (Couler). Macrophages with fragmented DNA formed a specific subdiploid peak in fluorescence histograms.

Experiments were performed in 7 repetitions. The results were analyzed by ANOVA and Student's *t* test.

RESULTS

LPS in a dose of 500 ng/ml significantly activated the stress response in proinflammatory macrophages, but not in antiinflammatory cells (Fig. 1). The contents of active HSF1, HSP70 mRNA, and HSP70 in LPS-stimulated proinflammatory macrophages were much higher than in antiinflammatory cells. Therefore, LPS-induced activation of the stress response in macrophages is determined by their phenotype. It should be emphasized that the LPS-induced stress response in

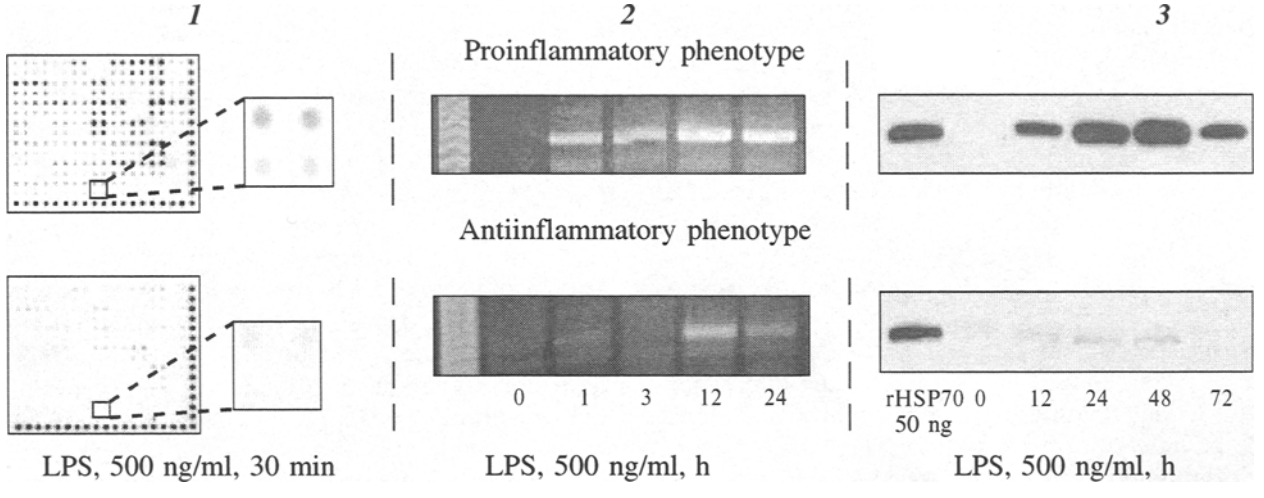

Fig. 1. Activation of transcription factors for heat shock proteins HSF1 (*1*) and accumulation of HSP70 mRNA (*2*) and HSP70 (*3*) in proinflammatory and antiinflammatory macrophages stimulated with LPS.

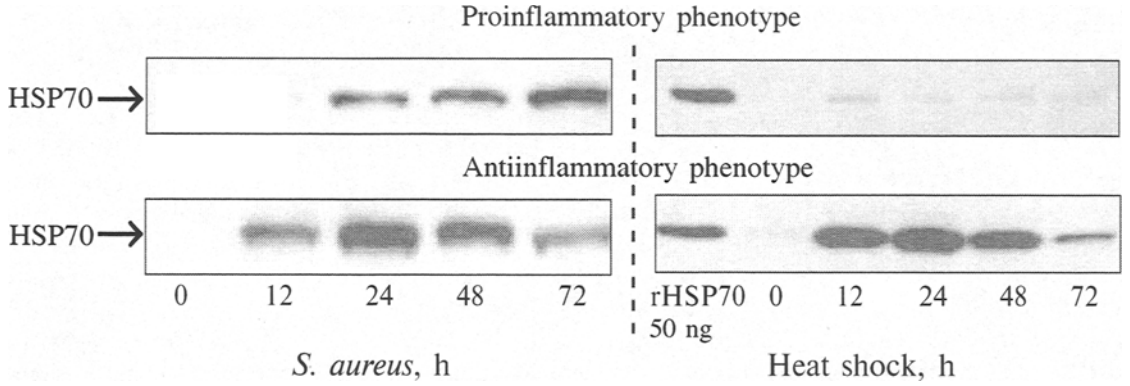

Fig. 2. Accumulation of HSP70 in proinflammatory and antiinflammatory macrophages in response to stimulation with *S. aureus* and heat shock.

antiinflammatory macrophages is suppressed at the level of transcription.

LPS is the major membrane component of gram-negative bacteria. LPS activates macrophages by interacting with surface receptors on CD14 macrophages. The reaction triggers signal pathways that stimulate production of cytokines and activate the stress response (synthesis of HSP70). *S. aureus* belongs to gram-positive bacteria. Macrophages interact with *S. aureus* and phagocytize them. Differences in the ability of macrophages to interact with pathogenic products (receptor-mediated activation of signal pathways or phagocytosis) determine variations in the stress response of macrophages. As differentiated from LPS, *S. aureus* more significantly activated the stress response in antiinflammatory macrophages than in proinflammatory cells (Fig. 2).

At the early stage of infection *in vivo*, macrophages can be rendered to pro- or antiinflammatory phenotype by microbial products in substimulatory concentrations (*e. g.*, LPS). Generalization of infection and fewer result in the development of HS, which serves as a stress factor for macrophages. In our experiments phenotypically different macrophages were *in vitro* exposed to HS. As distinct from the LPS-induced stress response, HS was followed by accumulation of HSP70 in antiinflammatory macrophages, but not in proinflammatory cells (Fig. 2). The data indicate that bacterial and physical factors have different ability to activate the stress response in macrophages with various phenotypes.

Our results show that differentiation of macrophages into pro- and antiinflammatory cells is accompanied by specific changes in the stress-sensitive mechanism. Macrophages are characterized by high plasticity in modulating the stress response, which depends on the phenotype of cells and nature of stress factors.

Fragmented DNA was revealed in 15±3% proinflammatory macrophages stimulated with 500 ng/ml LPS for 72 h. However, fragmentation of DNA did not occur in antiinflammatory cells. The LPS-mediated induction of HSP70 in proinflammatory macrophages was more pronounced than in antiinflammatory cells (Fig. 1). At first glance it would seem that our results contradict published data on antiapoptotic activity of HSP70. The DNA synthesis inhibitor quercetin (50 μM) suppresses LPS-induced accumulation of HSP70, but potentiates DNA fragmentation in macrophages. These data confirm the hypothesis that HSP70 play an important role in the resistance of macrophages to apoptosis. *S. aureus* and HS caused apoptosis in antiinflammatory macrophages (17±3 and 31±4 cells, respectively), but not in proinflammatory cells. Thus, antiinflammatory macrophages exhibit considerable antiapoptotic activity under the influence of LPS in high doses. However, proinflammatory cells were resistant to apoptosis produced by *S. aureus* or HS.

Apoptosis in macrophages depends on the phenotype of secretory activity and specific biological and physical characteristics of the inducing factor (gram-positive or gram-negative bacteria, bacterial components, and physical factors).

It could be suggested that variations in apoptosis in phenotypically different macrophages induced by biological and physical factors are related to differences in caspase activity. However, our experiments with caspase inhibitor Z-VAD-FMK (25 μM) showed that induction of apoptosis in phenotypically different macrophages exposed to the action of microbial and physical factors is a caspase-independent process. The dependence of apoptosis in macrophages on the phenotype of secretory activity remains to be estimated.

Various apoptotic factors can selectively eliminate macrophages with various phenotypes. This phenomenon determines the course of pro- or antiinflammatory immune reactions to bacterial and physical factors.

The first stage in differentiation of the immune response (pro- or antiinflammatory) includes programming of macrophages by subthreshold concentrations

of bacterial products during the early period of infection. Under the influence of bacterial products in increasing concentrations some macrophages can be programmed to the proinflammatory phenotype, while others gain the antiinflammatory phenotype. At the second stage of differentiation the inducing factor selectively eliminates macrophages with the "unnecessary" phenotype and determines the type of the immune response.

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