Caspase Inhibitor Z-VAD-FMK Potentiates Heat Shock-Induced Apoptosis and HSP70 Synthesis in Macrophages

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Caspase inhibitor Z-VAD-FMK potentiated heat shock-induced apoptosis in macrophages. Z-VAD-FMK did not activate HSP70 synthesis, but significantly increased the intensity of this process during heat shock. It cannot be excluded that caspases abolish HSP70 accumulation under these conditions. The HSP70 synthesis inhibitor quercetin potentiated DNA fragmentation in macrophages cocultured with Z-VAD-FMK after heat shock. HSP70 play an important role in the protection of macrophages from caspase-independent apoptosis.

Key Words: *macrophages; stress response; HSP70; caspase inhibitor; heat shock*

Z-VAD-FMK is a strong inhibitor of caspases [1,4]. It is surprising that Z-VAD-FMK does not prevent and even potentiates LPS-induced apoptosis in macrophages [5]. Published data show that LPS activates macrophageal caspases [3]. Previous studies [5] suggest the existence of Z-VAD-sensitive targets involved in the survival of LPS-stimulated macrophages. Some caspases probably possess antiapoptotic activity. Macrophages play an important role in immune reactions of the organism. LPS-induced death of macrophages can initiate disturbances in the innate and acquired immune response, which underlies the pathogenesis of diseases. Experiments with caspase inhibitors are directed not only to study the role of these enzymes, but also to develop the methods for pharmacological prevention of pathological apoptosis. Previous studies showed that specific characteristics of macrophages should be taken into account during the use of caspase inhibitors to suppress LPS-induced apoptosis [5].

Under conditions of generalized infection and hyperthermia apoptosis in macrophages is induced not only by microbial products, but also by heat shock (HS). The role of caspases in HS-induced apoptosis in macrophages is poorly understood. Moreover, the possibility of preventing apoptosis in these cells by treatment with caspase inhibitors should be evaluated. The development of terminal apoptosis depends on activation of the antiapoptotic mechanism. Activation of HSP70 synthesis during HS is a key antiapoptotic mechanism in various cells [2]. It remains unclear whether caspases regulate the content of HSP70. Further studies are required to estimate whether HSP70 perform similar antiapoptotic functions in macrophages and other cells.

Here we studied the effect of Z-VAD-FMK (caspase inhibitor) on apoptosis and activation of HSP70 synthesis and evaluated the influence of quercetin (HSP70 synthesis inhibitor) on apoptosis in macrophages.

MATERIALS AND METHODS

Macrophages were obtained from male C3Heb/Fe mice aging 8-12 weeks. The animals intraperitoneally received 2 ml 4% thioglycollate broth (Difco Lab.) 4 days before isolation of macrophages. Macrophages

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were isolated from peritoneal lavage with HBSS, resuspended, and cultured in RPMI-1640 medium containing 100 U/ml penicillin, 100 μg/mk streptomycin, and 10% phosphate buffered saline. HS was produced by healing the cell culture to 42°C over 1 h. The stress response and apoptosis were assayed by HSP70 accumulation and DNA fragmentation, respectively.

We used nonspecific caspase inhibitor Z-VAD-FMK (25 μM) and HSP70 synthesis inhibitor quercetin (50 μM).

The cells were lyzed in a buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 25% glycerol, and 0.01% bromophenol blue. The proteins were separated on 7.5% polyacrylamide gel and transferred on a PVDF membrane using a buffer consisting of 48 mM Tris, 380 mM glycine, 0.1% SDS, and 20% methanol (pH 8.3). Nonspecific binding to the membrane was blocked with 5% milk. The blots were consecutively incubated with primary anti-HSP70 antibodies (Stressgen, SPA-810, 1:2000) and secondary anti-mouse antibodies conjugated to peroxidase (Santa Cruz, sc-2005, 1:3000). The blots were detected with a Santa Cruz kit (sc-2048).

DNA content was measured as described elsewhere [6]. 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 generated a specific subdiploid peak in fluorescence histograms.

The experiments were performed at least in 5 repetitions. The results were analyzed by ANOVA and Student's *t* test.

RESULTS

Z-VAD-FMK in specified doses had no effect on DNA integrity in macrophages, but significantly promoted DNA fragmentation during HS (Table 1). These data show that caspase inhibitor Z-VAD-FMK can potentiate apoptosis in macrophages induced by not only microbial agents [5], but also physical factors (HS). Therefore, HS-induced apoptosis in macrophages is a caspase-independent process.

We assumed that Z-VAD-FMK potentiates HS-induced apoptosis in macrophages due to the inhibition of intracellular protective systems. This hypothesis was tested in respect to the most important endogenous system with antiapoptotic function (HS proteins). HS was followed by moderate activation of HSP70 synthesis over the first 24 h (Fig. 1). Z-VAD-FMK did not activate HSP70 synthesis, but considerably increased the intensity of this process during HS. Z-VAD-FMK probably initiates the development of abnormalities in cells, which nonspecifically induces HSP70 synthesis. On the other hand, Z-VAD-FMK can inhibit caspases cleaving HSP70 during HS.

TABLE 1. Effect of Caspase Inhibitor Z-VAD-FMK on the Number of Cells with Fragmented DNA 72 h after HS (*M*±*m*)

Conditions	Percentage of cells with fragmented DNA
Normal	3±1
HS	9±2*
Z-VAD-FMK	5±2
HS+Z-VAD-FMK	41±5+
HS+Z-VAD-FMK+quercetin	67±8°

Note. $p<0.05$: *compared to normal; +compared to HS; °compared to HS+Z-VAD-FMK.

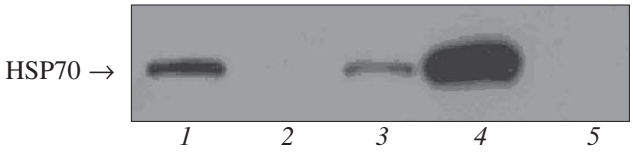

Fig. 1. Effect of caspase inhibitor Z-VAD-FMK on HSP70 synthesis in macrophages induced by heat shock (HS, Western blotting): 50 ng HSP70 marker (*1*), control (*2*), HS (*3*), HS+Z-VAD-FMK (*4*), and Z-VAD-FMK (*5*).

These data show that proapoptotic changes in macrophages produced by the caspase inhibitor Z-VAD-FMK were preceded by accumulation of HSP70. At first glance it would seem that our results are contradictory to published data on antiapoptotic activity of HSP70 in other cells. However, the HSP70 synthesis inhibitor quercetin in a dose of 50 μM prevented HS-induced accumulation of HSP70 and potentiated DNA fragmentation in macrophages cocultured with Z-VAD-FMK after HS (Table 1). Therefore, activation of HSP70 synthesis in macrophages cocultured with Z-VAD-FMK after HS is a protective reaction suppressing caspase-independent apoptosis.

The mechanism for proapoptotic activity of the caspase inhibitor Z-VAD-FMK in macrophages is unknown. Our results indicate that proapoptotic activity of Z-VAD-FMK is associated with activation of HSP70 synthesis.

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