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Effect of Recombinant Heat Shock Protein 70 of Mycobacterial Origin on Cytotoxic Activity and Immunophenotype of Human Peripheral Blood Mononuclear Leukocytes

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Recombinant heat shock protein of mycobacterial origin with a molecular weight 70 kDa in concentration 0.1 μg/ml *in vitro* activates cytotoxic activity of mononuclear lymphocytes from peripheral blood of healthy donors against K-562 human erythroblastic leukemia cells sensitive to natural killers. In other concentrations (1.0 μg/ml and 10 μg/ml) this heat shock protein did not significantly affect functional activity of mononuclear leukocytes. Expression of CD4, CD25, CD16 and CD56 on membrane of mononuclear leukocytes was also studied.

Key Words: *heat shock protein; natural killers; cytotoxic activity*

Heat shock proteins (HSP) located on the surface of tumor cells enhance cytolytic activity of natural killers by interacting with high-sensitivity receptors of natural killers (CD94, CD56) [1,2]. It was hypothesized that surface HSP are the target (danger signal) for natural killers. However, there is still no unambiguous concept about the influence of HSP on functional activity and immunophenotype of lymphocytes; it also remains unclear whether HSP can be considered as activators of innate immunity effectors.

Here we studied the role of recombinant HSP with a molecular weight 70 kDa (HSP-70) on cytotoxic activity of natural killers and immunophenotype of mononuclear leukocytes (MNL) from human peripheral blood.

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MATERIALS AND METHODS

We used genetically engineered HSP-70 obtained from *Mycobacterium tuberculosis* and subsequently cloned in *Escherichia coli*. The substance contained lypopolysaccharides (LPS) in a concentration 0.266 μg/ml. LPS content was measured in gel clot LAL (Limulus amebocytes lysate) using a PYROGENT® test (CAMBREX Bioscience).

Human erythroblastic leukemia cell line K-562 sensitive to natural killers was used. K-562 cells were cultured in complete culture medium containing RPMI-1640 medium and supplemented with 10% fetal serum, glutamine, and penicillin-streptomycin.

Peripheral blood MNL were isolated from peripheral blood of healthy donors stabilized with heparin (25 U/ml) on a one-step Ficoll gradient (Pharmacia, 1.077 g/cm^3) by centrifugation at 400*g* for 30 min. MNL forming an interphase ring were collected with a

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pipette and washed 3 times with medium 199 (10-fold volume). After each washout, the cells were precipitated by centrifugation at 200*g*.

MNL isolated from peripheral blood were resuspended in complete culture medium at concentration 10^6 cells/ml. Then HSP-70 (in concentrations of 0.1, 1, and 10 μg/ml) or IL-2 (Proleukine, Chiron, in concentration 10,000 U/ml) was added. The cells were incubated at 4.5% CO_2 and 37°C for 3 days.

Lymphocyte cytotoxic activity was measured using K-562 cells, sensitive to natural killers. Tumor cells (10^4 cells/ml) were incubated in complete culture medium with MNL and HSP-70 in concentration 0.1 μg/ml, 1 μg/ml, and 10 μg/ml (1:5, 1:2, 1:1 target/effector ratio) in 96-cell flat-bottom microtitration plates (Costar) for 18 h. Then vital dye MTT (Sigma) was added to the wells and the percent of tumor cells lysis (cytotoxicity rate) was calculated by absorption measured on a Multiscan MS (Labsystems).

Expression of surface MNL markers was detected using monoclonal antibodies to the corresponding antigens (Caltag Laboratories). The results were analyzed by flow cytofluorometry using a FACScan flow cytometer (Beckton Dickinson). MNL were used for investigation of differentiation antigens CD4 and CD25 (T-cell markers), CD16 and CD56 (natural killer markers). Cell population gate was established on the basis of combination of forward and side light scatter and cell dimentions. Per gate 10,000 events were calculated.

The results were statistically processed using WINMDI 2.8.

RESULTS

Effect of HSP-70 in various concentrations and target cell/effector ratio on spontaneous cytotoxicity was characterized (Table 1). HSP-70 in a concentration 0.1 μg/ml (group 1) induced maximum cytotoxic activity MNL against K-562 cells, while two other concentrations (1 μg/ml and 10 μg/ml, groups 2 and 3, respectively) did not significantly affect MNL cytotoxicity. Maximum cytotoxic effect (death of 97% tumor cells) was obtained upon stimulation of MNL with HSP-70 at 1:5 target/effector ratio. The same effect was observed in the positive control group after addition of lymphokine-activated killers (LAK; group 4) to tumor cells. Reduction of the target/effector ratio to 1:2 and 1:1 led to a decrease in cytotoxic activity against tumor cells.

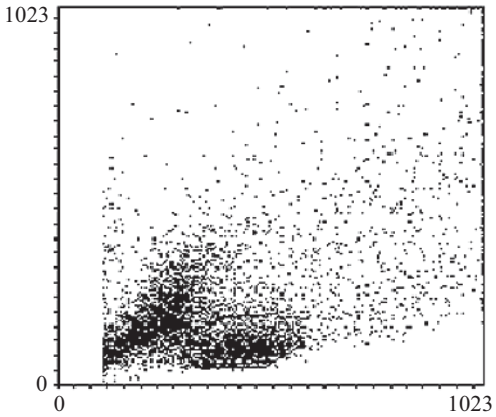

Fig. 1. FACS-analysis of MNL cells population activated with HSP-70 in concentration 0.1 μg/ml. The outlined area indicates gate (area of analyzed cells). Horizontal axis: side scatter (arb. units); vertical axis: forward scatter (arb. units).

Immunophenotype of MNL activated by HSP-70 was investigated (Figs. 1 and 2). We showed that HSP-70 promotes expression of CD25 and CD56 molecules on MNL membrane and does not affect the expression of CD4 and CD16 antigens.

Enhanced expression of CD56 and CD25 antigen on MNL attests to increased content of natural killers and activated lymphocyte forms in the population of lymphocytes stimulated with HSP-70. This can explain increased functional activity of MNL treated with HSP-70 against human erythroblastic leukemia cells K-562 sensitive to natural killers.

HSP can appear on the surface of tumor cells and serve as a danger signal for natural killers, effectors of congenital immunity [1]. This mechanism promotes

TABLE 1. Effect of HSP-70 on MNL Cytotoxic Activity

Group	Substance	Target/effector ratio, %		
		1:1	1:2	1:5
1	HSP, 0.1 μg/ml	66±4.3*	84±4.1*	97±4.2*
2	HSP, 1.0 μg/ml	43±2.4	53±5.8	73±6.1
3	HSP, 10 μg/ml	39±1.7	50±2.9	69±5.1
4	LAK	71±3.2*	89±2.8*	99±4.0*
5	Control (MNL)	46±4.3	71±4.2	80±5.1

Note. *$p<0.05$ compared to the control.

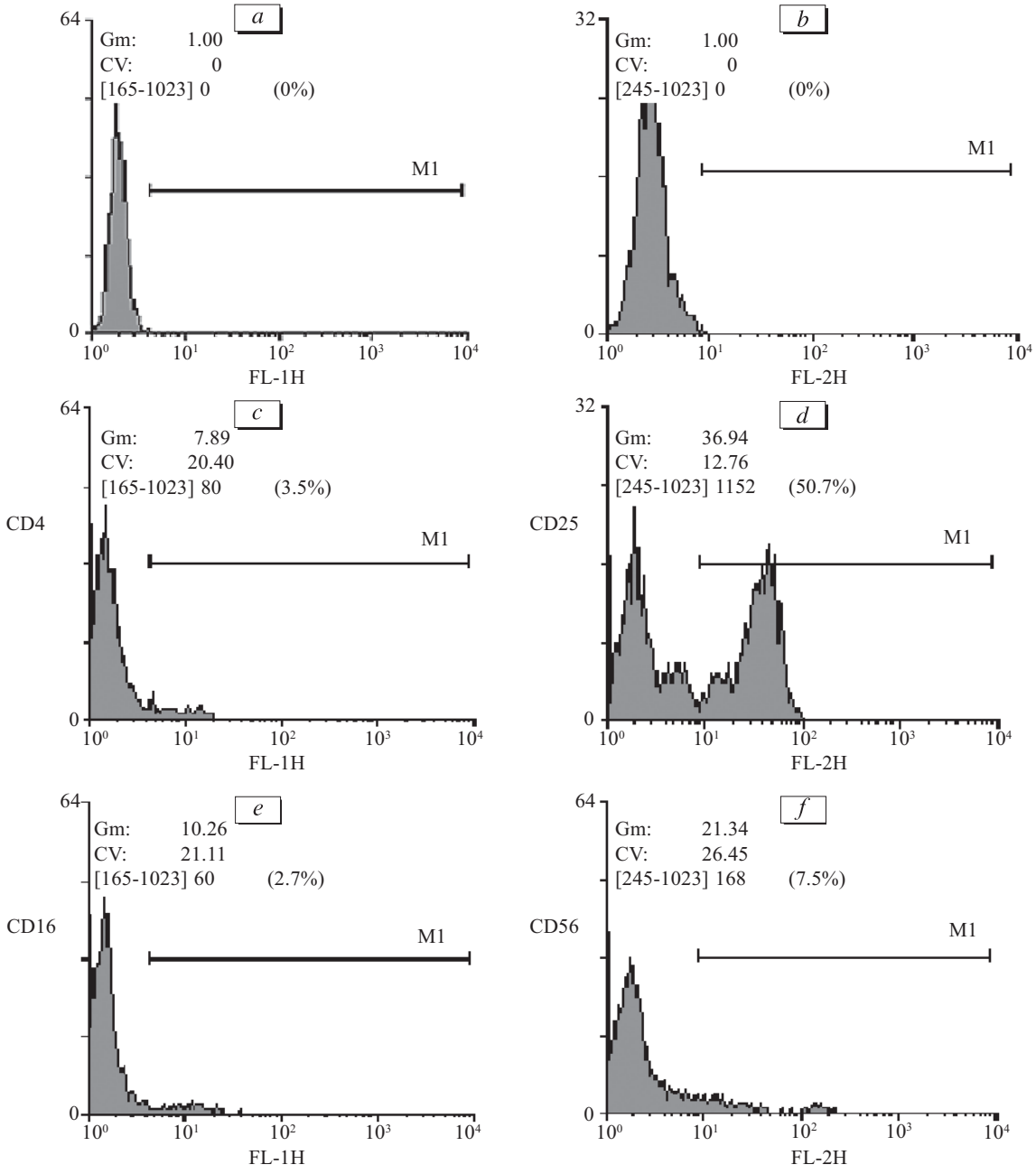

Fig. 2. Histograms demonstrating the expression of surface markers labeled with monoclonal antibodies. CD: differentiation antigens; Gm: geometric mean deviation of the signal; CV: coefficient of variation; FL-1H: FITC-labeled cells; FL-2H: phycoerythrin (PE)-labeled cells; in square brackets: recorded signals, after square brackets: mean signal value, in parentheses: proportion of cells expressing the antigen; M1: values differing from the control. Horizontal axis: intensity of signal for the given parameter (arb. units), vertical axis: number of events (arb. units). *a, b*: MNL control for FITC staining (*a*) and PE staining (*b*), *c-f*: MNL+HSP-70 0.1 μg/ml, FITC staining (*c, d*) and PE staining (*e, f*); differentiation genes CD4 (*c*), CD25 (*d*), CD16 (*e*) and CD56 (*f*).

recognizing of tumor cells by natural killers. Therefore, lysis of tumor cells can be enhanced by activation of natural killers by recombinant HSP [3].

Thus, HSP-70 in a concentration of 0.1 μg/ml efficiently enhances MNL activity against natural killers at 1:5 target/effector ratio. The obtained marked cytotoxic effect (97%) corresponded to the effect of LAK on tumor cells (positive control, group 4) and surpassed the effect of intact MNL (negative control, group 5). At target/effector ratios of 1:2 and 1:1, HSP-70 less markedly increased cytotoxic activity of MNL, but it significantly surpassed that in the control group.

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