The Influence of Perforin Expression on the Sensitivity of LAK/NK Killing Against Various Tumor Target Cells

Zahra Amirghofran

Immunology Department, Medical School, Shiraz University of Medical Sciences, Shiraz, Iran

ABSTRACT

Background: Perforin is known to be important in cytolytic activity mediated by natural killer (NK) cells. **Objective:** To study the relationship between the efficiency of NK and lymphokine-activated killer (LAK) cells activity, and the expression of perforin and HLA class I molecules. **Methods:** LAK cells were generated by in vitro culturing of human peripheral blood lymphocytes (PBLs) in the presence of human recombinant interleukin-2 (rIL-2). Cytotoxic activity was measured at different intervals of activation by MTT colorimetric assay using different human tumor cell lines. Immunocytochemical staining of molecules was performed on LAK/NK cells using specific monoclonal antibodies and Biotin-conjugated anti-immunoglobulin. **Results:** LAK/NK killing against Fen and two other cell lines, KB and Scaber showed that at day 9 and 15 of activation, 57% to 60% and 45.5% to 92.5% of Fen cells were killed at different E/T ratios. At the same time, the maximum percent killing against Scaber and KB cell lines was 47.3 and 54.3 at 5/1 ratio, respectively, showing that Fen cells were more sensitive than the two other cells. Time-course experiments using Fen cell line demonstrated 60.0, 83.9 and 34.8 percent killing at days 9, 15 and 22 at 10/1 E/T ratios. When other E/T ratios were investigated, a similar profile was observed. The maximum activity was at day 15 and 5/1 E/T ratio (92.5%). In immunocytochemical staining of activated LAK cells, 75.9% to 86.3% of LAK cells expressed HLA class I molecules. Perforin expression changed from 30.3% at day 7 to 42.7% at day 17 followed by a decrease to 27.9% at day 24. **Conclusion:** These data indicate that perforin expression is closely correlated with NK/LAK killing activity.

Keywords: HLA, LAK/NK activity, Perforin

Corresponding author: Dr. Zahra Amirghofran, Immunology Department, Medical School, Shiraz University of Medical Sciences, Shiraz, Iran. Tel/Fax: (+) 98 711 2304069, (+) 98 711 2359847, P.O. BOX: 71345-1798, e-mail: amirghz@sums.ac.ir

INTRODUCTION

Natural killer (NK) cells are innate effector lymphocytes necessary for defense against stressed, virus infected and tumor cells (1-3). These cells are distinct from cytotoxic T lymphocytes (CTLs) in their circulation patterns, profile of surface markers, receptor repertoire and their ability to discriminate self from non-self (4-6). CTLs recognize a specific peptide epitope presented by the major histocompatibility complex (MHC) class I molecules on the target cells, whereas NK cells lyse targets that express no or low MHC class I molecules (7-8). A main pathway used by NK cells to eliminate pathogenic cells is via exocytosis of cytoplasmic granule toxins in the direction of the target cell, delivering a lethal hit of cytolytic molecules. Amongst these toxins, perforin, a membrane-disrupting protein, has shown to induce apoptosis of the target cell (9-10). The molecular mechanisms of the NK cell-mediated cytolysis have been studied by several investigators (11-13). As the killing activity of LAK/NK cells relies on the expression of molecules like perforin, in the present study the concomitant expression of perforin and MHC antigens on IL-2-activated cells was compared with the efficiency of LAK/NK killing.

MATERILAS AND METHODS

Cell Lines and Culture Conditions. Cell lines including Fen, Scaber (bladder carcinoma cell lines) and KB (head and neck tumor cell), were grown to confluence in growth medium (RPMI 1640 supplemented with L-glutamine (2mM), 10% heat inactivated fetal calf serum, penicillin (100 u/ml) and streptomycin (100 µg/ml) all from (Sigma, St Louis, USA). Adherent tumor target cells (T) were washed with sterile PBS, and then 2 ml of trypsin EDTA (Sigma) was added. The flask was incubated for 5 to 10 minutes. Then the trypsin was inactivated by adding an equal volume of the medium. After centrifugation, the cell count was performed using trypan blue and the concentration of tumor cells adjusted to 10^5 cells/ml. One hundred µl of cell suspension were added into each well of flat-bottomed micro culture plate (Nunclone, Denmark). The plates were incubated for 24 hours at 37 ºC in a 5% CO2 incubator to allow cells to adhere to the plate.

Generation of NK /LAK Cell. Peripheral blood mononuclear cells obtained from healthy individuals were isolated by density gradient centrifugation using Ficoll-hypaque. After washing, the concentration of the cells was adjusted to 10^6 cells/ml. The cells were transferred into 25 cm^2 flask and stimulated with 100 µl of rIL-2 (Serotec, UK) for 72-96 hours at 37 ºC, and were fed every 3 days with 2 ml of medium plus 100 µl of rIL-2. The activated cells were washed and resuspended at required density to be used as effector (E) cells.

Cytotoxicity of LAK/NK Cells. NK /LAK cell activities of the freshly isolated cells were examined by the standard 3 (4,5-dimethylthiazoyl-2, 5-diphenyltetrazolium bromide (MTT) assay using different tumor target cells. To determine the sensitivity of the tumor cells to LAK/NK killing, effector cells were added to each well with E/T ratios of 50/1 to 0.65/1 (each in four replicates). Two series of four replicates were

considered as LAK cell alone and target cell alone. The plates were incubated for 3 hours. All non-adherent and residual effector cells were washed off the plate with medium. After the incubation period, 10µl of MTT labeling reagent (0.5 mg/ml, Sigma) were added into each well. The plate was incubated for 4 hours and then 100µl of acidified isopropanol were added into each well. The absorbance of the samples was measured using an ELISA reader at 570 nm and 630 nm as the reference wavelength and then the percentage of killing was determined.

Immunocytochemistry for Expression of Perforin and HLA Molecules. Effector cells were cytocentrifuged and cell slide preparations were dried at room temperature for at least two hours and fixed in acetone for 5 minutes. They were then placed in PBS/hydrogen peroxide for 5 minutes. 20µl of the mouse monoclonal anti perforin and anti HLA class I (W6/32) (Dako, Denmark) were added and the slides were incubated for one hour. The unbound antibodies were removed by washing in PBS for 10 minutes and 20µl of rabbit anti-mouse Biotin-conjugated antibody (Dako) diluted 1:50 in PBS were dispensed and incubated for one hour as before. The slides were washed and 20µl of 1:50 diluted Avidin–HRP (Dako) were added onto the slides. Following an incubation, the slides were washed and color was developed by incubating in DAB/hydrogen peroxide solution. The slides were washed and counterstained with hematoxylin.

RESULTS

Sensitivity of LAK/NK Killing Against Various Tumor Targets. The LAK/NK killing was compared against Fen and two other cell lines, KB and Scaber. As shown in Table 1, at day 9, 57% to 60% of Fen cells were killed at different E/T rations. At day 15, the range of killing was 45.5% to 92.5%. Corresponding results for Scaber, at

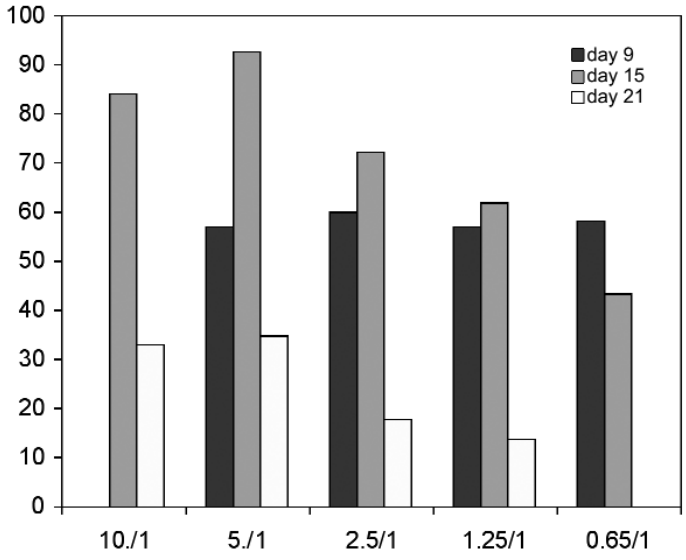

Figure 1. Time course of LAK/NK killing against Fen target

day 15 was 24.9% to 47.3% and it was 43.4% to 54.3% for KB cells at day 9. The maximum percent killing against Scaber and KB cells was 47.3 and 54.3 at 5/1 ratio, respectively. At 25/1 ratio the percent killing for Fen and KB cells were 68.5 and 53.2, respectively. Thus, Fen cells were 1.3 times more sensitive than KB cells. Fen cells were also 2.3 times more sensitive than Scaber cells at 10/1 ratio.

Influence of E/T ratios and Length of IL-2 Activation on the Efficiency of LAK/NK Killing. In order to determine the efficiency of LAK/NK killing, the cells were cultured in the presence of IL-2 and the activity of cells in different intervals was determined. Fen cells were used as the tumor target for this experiment. As it can be

Table 1. Killing activity of LAK/NK cells against tumor target cells at different duration of exposure to IL-2 and E/T ratios. Results for four distinct time courses are shown. E/T ratio demonstrates effector and target respectively

Cells	Day	E/T ratio	Target+Efector*	% Killing
Fen	9	20/1	0.46± 0.07	57
		10/1	0.42± 0.05	60
		5/1	0.41± 0.01	57
		2.5/41	0.43± 0.01	58
Fen	12	50/1	0.25± 0.02	72.4
		25/1	0.28± 0.02	68.5
		12.5/1	0.29± 0.02	66.9
		6.25/1	0.30± 0.03	65.4
		3.12/1	0.36± 0.02	56.1
Fen	15	10/1	0.17± 0.02	83.9
		5/1	0.19± 0.02	92.5
		2.5/1	0.21± 0.02	72.3
		1.25/1	0.24± 0.01	61.7
		0.65/1	0.28± 0.02	45.5
Fen	21	20/1	0.42± 0.02	33
		10/1	0.40± 0.04	34.8
		5/1	0.46± 0.01	17.6
		2.5/1	0.44± 0.01	13.7
KB	9	50/1	0.58± 0.01	43.4
		25/1	0.50± 0.02	53.2
		12.5/1	0.49± 0.02	54.3
		6.25/1	0.55± 0.02	47.1
		3.12/1	0.58± 0.02	43.4
Scaber	15	10/1	0.39± 0.03	36.2
		5/1	0.39± 0.01	47.3
		2.5/1	0.40± 0.02	33.5
		1.25/1	0.42± 0.02	28.4
		0.65/1	0.42± 0.02	24.9

* Data is presented as Mean ± SD of optical density.

seen in Table 1, Fen cells were killed after 9 days of IL-2 activation by as much as 60%. As the time of IL-2 activation increased, the killing efficiency changed. Thus, the percent killing at 9, 12, 15 and 22 days at 10/1 E/T ratios were 60.0, 66.9, 83.9 and 34.8, respectively. When other E/T ratios were investigated, a similar profile emerged. As, at 5/1 E/T ratio the percentage killing was 57.0, 92.5 and 17.8. Corresponding data for 2.5/1 ET ratio at 9, 15 and 22 days was 58.0, 72.3 and 13.7, respectively. The maximum activity was observed at day 15 and 5/1 E/T ratio (92.5%). At the same time, percent killing at 0.65/1 E/T ratios declined to 45.5, showing a fall in killing activity with decrease in E/T ratio (Fig. 1).

Expression of Perforin and HLA Class I. Activated LAK cells were assessed using immunocytochemical staining and the percentage of positive cells was determined. As it is shown in Fig. 2, 75.9% to 86.3% of LAK cells expressed HLA class I molecule. Staining of the cells with anti-perforin antibody showed an increase in the expression of perforin from 30.3% at day 7 to 42.7% at day 17 followed by a decrease to 27.9% at day 24.

DISCUSSION

In present study, the sensitivity of LAK/NK killing against Fen, Scaber and KB epithelial cell tumor targets was determined. The experiments were performed either at day 9 or at day 15 of IL-2 activation and E/T ratios range of 50/1 to 0.65/1. In the case of Scaber, similar to that of Fen cells, there was a dose response curve between E/T ratios and the extent of target killing. However, there was a significant difference in the absolute susceptibility of the two lines to killing, i.e. the maximum percent killing against Scaber was much less than that for Fen cells (47.3% *vs.* 92.5% at 5/1 ratios). Similarly, in the case of KB cells, the maximum percent killing of LAK cells

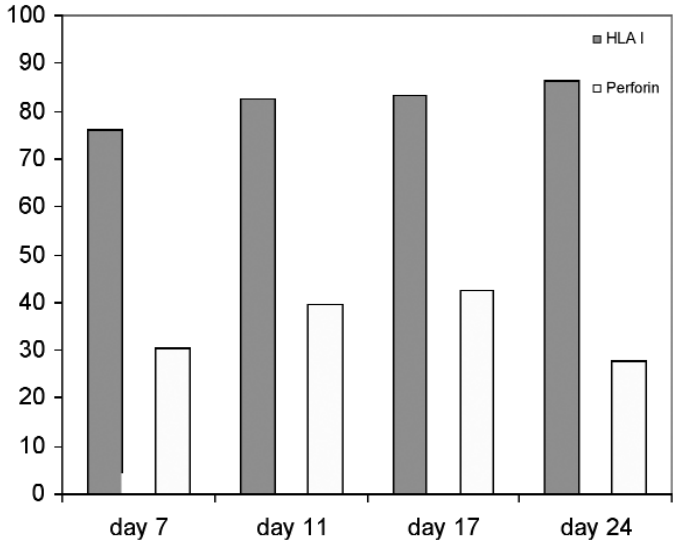

Figure 2. Expression of molecules on LAK/NK cells at different durations of exposure to IL-2

against KB at 25/1 ratio was 53.2% *vs.* 68.5% for Fen cells. It is worth mentioning that LAK/NK killing against KB and Scaber cells in all E/T ratios was less than 55%. These data revealed that Fen cells were more sensitive than the other two cell lines. In previous studies, Fen cells have been shown to be more susceptible to killing by NK/LAK effector cells compared with conventional target cells, i.e. K562 and Daudi, indicating that it could be replaced by the conventional approach for assessment of NK/LAK activity (14-15). Our results showed that Fen cell line was more sensitive than K562 and Daudi targets and could replace the conventional approach for assessment of NK/LAK activity.

Fen cells were used to investigate the influence of the time of IL-2 activation on the efficiency of LAK/NK killing. As shown, these cells were killed after 9 days of IL-2 activation by as much as 60%. At this time, alterations of E/T ratios had a little influence on such a killing. As the time of IL-2 activation increased, the efficiency of killing changed, indicating the time dependency of LAK/NK activity. In addition, the change in E/T ratios also influenced the efficiency of LAK killing. It can be seen that as the E/T ratios decreased from 10/1 to 5/1, there was a marked increase in the efficiency of LAK/NK activity. Further decrease in E/T ratios resulted in a dose related decrease in the tumor target killing. The overall data seemed to indicate that 15 days at E/T ratio of 5/1 was the most efficient approach to achieve maximum killing.

In order to find the relationship between the expression of perforin and HLA molecules and the activity of LAK/NK cells, the expression of perforin and HLA class I antigen on IL-2- activated cells were studied. It is known that virtually all of the measurable cell-mediated cytotoxicity delivered by cytotoxic T lymphocytes and natural killer cells comes from either the granule exocytosis pathway or the Fas pathway (16-21). The granule exocytosis pathway utilizes perforin to traffic the granzymes to appropriate locations in target cells, where they cleave critical substrates that initiate DNA fragmentation and apoptosis (22). Immunocytochemical staining performed in our study revealed that as the time of LAK/NK exposure to IL-2 was raised, the percent of perforin positive cells increased. Thus, the highest level of expression was observed at day 17 when the LAK cells were the most efficient. This was followed by a general decrease in perforin expression, indicating a close correlation between the efficiency of NK/LAK killing and the expression of perforin.

In terms of HLA class I, expression at days 11 to 24 was seen in more than 80% of cells and this remained more or less constant througout, and there was no major alteration in class I antigen expression.

In conclusion, this study demonstrated that perforin was functionally important effector cell surface molecule expressed by LAK cells and suggested that enhanced perforin expression may be important for development of fully activated LAK effector cell in the presence of IL-2.

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