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Radiosensitivity of CD3−CD8+CD56+ NK cells

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ABSTRACT

The effect of lower doses (0.5–3.0 Gy) of gamma radiation on radiosensitivity of CD3−/CD8+ NK cells subpopulation isolated from the peripheral blood of healthy volunteers was studied 48 h after the irradiation. Only a subtle increase in terms of induction of apoptosis (A+ cells), was observed in Annexin positive CD3−/CD8+ NK cells. The assessment of the relative presence of $CD3^-/CD8^+$ NK cells in Annexin negative populations of lymphocytes considerably contributes to the elimination of individual variability and could be useful in biodosimetry.

Living CD3−/CD8+; Annexin negative NK cells were analyzed using five-color flow cytometry 16 h after irradiation by the doses of 1–10 Gy. The study was carried out on NK cells subsets CD3−/CD8−CD16+, CD56 (dim) and CD56 (bright). NK cells characterized with their low-density expression of CD56 (dim) are more cytotoxic and express CD16. Those with high-density expression of CD56 (bright) are known for their capacity to produce cytokines following activation of monocytes but their natural cytotoxicity is low; they are classified as CD16− or CD16 (dim). A dose-depending decrease in the relative presence of CD3−/CD8+ NK cells was observed 16 h after ionizing radiation (1–10 Gy). The decrease was highly pronounced in CD56 (bright) subset of NK cells and this subpopulation was considered as the most radiosensitive one. Unfortunately, the most radiosensitive subpopulation of NK cells – CD56bright cannot be used as a biodosimetric marker due to its insufficient amount in peripheral blood.

1. Introduction

The capability to make diagnostic assessments of radiation exposure is needed to support triage of radiation casualties and medical treatment decisions in military operations or in radiation accidents (Blakely, 2002). Decrease of lymphocyte count in peripheral blood under 50% of initial values at accidentally whole-body irradiated victims during 24 h after irradiation is considered as a marker of significant received dose. Chromosome-aberration-based biomarkers are the gold standard for radiation exposure assessment. This radiation bioassay is characterized by high degree of radiation specificity however several hundreds of cells must be scored for exposure assessment (Blakely, 2002).

Human lymphocytes die in the peripheral blood due to apoptosis after the ionizing radiation (Hertveldt et al., 1997; Philippé et al., 1997; Cornelissen et al., 2002). We reported (Vokurková et al., 2006) that the dose of 7 Gy did not induce apoptosis 6 h after the γ irradiation of lymphocytes separated from the peripheral blood of healthy volunteers in an *in vitro* experiment. Sixteen hours after irradiation, there were 50% of cells Annexin V positive (apoptotic) and nearly a half of them was in an early apoptotic phase (A+/PI−). The late apoptotic phase (A+/PI+) was typical of most of the lymphocytes two days after irradiation. Unfortunately, the population of peripheral lymphocytes is rather heterogenous concerning both their function in the organism (T-, B-lymphocytes, NK cells) and their radiosensitivity.

Natural killer (NK) cells are the main group of lymphocytes responsible for the natural immune response. NK cells have been implicated in several activities *in vivo*, including destruction of tumor cells, resistance to viral infection, and regulation of hematopoiesis. There is no single surface antigen uniquely identifying all the human NK cells to date. They comprise about 10–15% of all circulating lymphocytes. Most of them can be characterized as CD3−/CD56+cells (95%). Small number of peripheral lymphocytes (PBL) expresses both CD56 and CD3 and is capable of non-MNC-restricted cytotoxicity. Most CD56+PBL also express CD16 (80–90%). 30–40% NK cells expresses CD8+. Density of CD8 antigen on NK cells is lower than that on T cells, however, and most CD8+ NK cells express CD8α/CD8α homodimer

Abbreviations: NK, natural killer; PBMC, peripheral blood mononuclear cell; MoAb, monoclonal antibody; PBL, peripheral blood lymphocytes.

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Table 1
CD markers of NK cells in peripheral blood.

Markers of NK cells	% of NK cells
CD3+/CD56+	5
CD3−/CD56+	95
CD56+/CD16+	80–90
CD3−/CD8+	30–40
CD3−/CD8+/CD16+/CD56+	25–40

(Robertson and Ritz, 1990). A large number of CD8+ cells in peripheral blood does not express CD3 and exhibits cell surface phenotypes typical of natural killer cells i.e. CD3 negative cells expressing CD56 and CD16. Most CD8bright cells are known to express CD3 and CD8dim cells that are believed to be CD3− NK cells (Simpson et al., 2007; Campbell et al., 2008). Cytotoxic T lymphocyte co-express the T cell receptor, CD3 and MHCI restricted antigen CD8. Campbell et al., 2008 proved that 25% of all CD8 cells were CD3 negative, CD8dim cells and expressed the NK-cell markers CD16 and CD56. The minor population of NK cells with high density of CD56 is CD56bright/CD16dim and is able to produce cytokines following the activation of monocytes, but its natural cytotoxicity is low (Cooper et al., 2001). (Table. 1)

Previously, we compared (Vokurková et al., 2006) the presence of separated populations CD3+/CD8+ (cytotoxic T-lymphocytes), CD3+/CD4+ (helper T-lymphocytes) and CD3−/8+ (NK cells) in the lymphogate of A− cells after the irradiation with the single dose of 5 Gy *in vitro*. NK cells were considerably affected. The irradiation decreased the initial value to 50% 16 h after the dose, while there were only a few CD3−/8+ cells remaining after 48 h.

In the present study, we focused on two problems: 1/Is it possible to use decrease in live (A−) /CD3 /CD8+ subset as a biodosimetric marker? 2/How different subsets of CD3−/CD8+ NK cells − CD16+, CD56bright and CD56dim react? We studied apoptosis induction and decrease in live CD3−/CD8+ NK cells after irradiation by the doses in the dose range of 0.5–10 Gy and radiosensitivity of CD3−/CD8+ NK cells subsets in the dose range of 1–10 Gy.

2. Materials and methods

2.1. Peripheral blood mononuclear cells (PBMC)

Peripheral blood mononuclear cells were isolated from volunteers' heparinized blood by centrifugation on the Histopaque-1077 (Sigma-Aldrich, Czech Republic) according to manufacturer's instructions. After double washing in cold PBS the cells were resuspended in Iscove's modified Dulbecco's medium (Sigma-Aldrich, Czech Republic) supplemented with 20% fetal bovine serum (PAA Laboratories GmbH, Austria), 2 mM glutamine (Sigma-Aldrich, Czech Republic), 100 UI/ml penicillin (Sigma-Aldrich, Czech Republic) and 0.1 mg/ml streptomycin (Sigma-Aldrich, Czech Republic) at the density of 5×10^5 cells/ml. The cell suspension was maintained in an incubator (37 °C, humidified atmosphere with 5% CO_2) till irradiation (2–3 h).

2.2. In vitro irradiation

Cells in 10 ml aliquots were transferred into 25 cm^2 flasks (Nunc) and irradiated at room temperature using ^{60}Co gamma -ray source. The doses of 0.5–5 Gy were delivered with a dose-rate of 0.4 Gy/min at a distance of 1 m from the source, for the dose of 10 Gy 1.5 Gy/min dose rate and a distance of 0.5 m from the source were used. Immediately after the irradiation the flasks were placed into a 37 °C incubator with 5% CO_2 and aliquots of cells were collected at various time intervals after irradiation for analysis.

2.3. Five-color flow cytometry

The cells were washed in ice-cold washing and staining buffer (PBS containing 0.2% gelatin and 0.1% NaN_3) and kept in cold during subsequent processing to prevent further apoptosis development. The cells were then superficially immunostained with the mixture of anti CD56-PE − clone NKH-1, antiCD8-ECD − clone T8, antiCD3-PC5 − clone UCHT1, and antiCD16-PC7 − clone 368, Immunotech, Marseille, counterstained with FITC labeled Annexin V (A) (annexin kit Dako, Demmark) and five color analysis was performed on the Cytomics 500 flow cytometer (Beckman-Coulter). The minimum of 50 000 events was analyzed in each sample and the results were evaluated using the CXP Analysis Version II analytical software.

Multiparameter analysis of apoptosis and flow-cytometric characteristic in live lymphocyte subsets was carried out as described earlier (Vokurková et al., 2006). PBL isolated from 6 donors were analyzed for the survival (A−PI−) of lymphoid populations defined by CD3−/CD8+, CD3−/CD8+/CD16+ or CD3−/CD8+/CD56dim and CD3−/CD8+/CD56bright surface immunophenotyping.

Multiparameter flow cytometry offers several possibilities how to study apoptosis development in irradiated lymphocyte preparations.

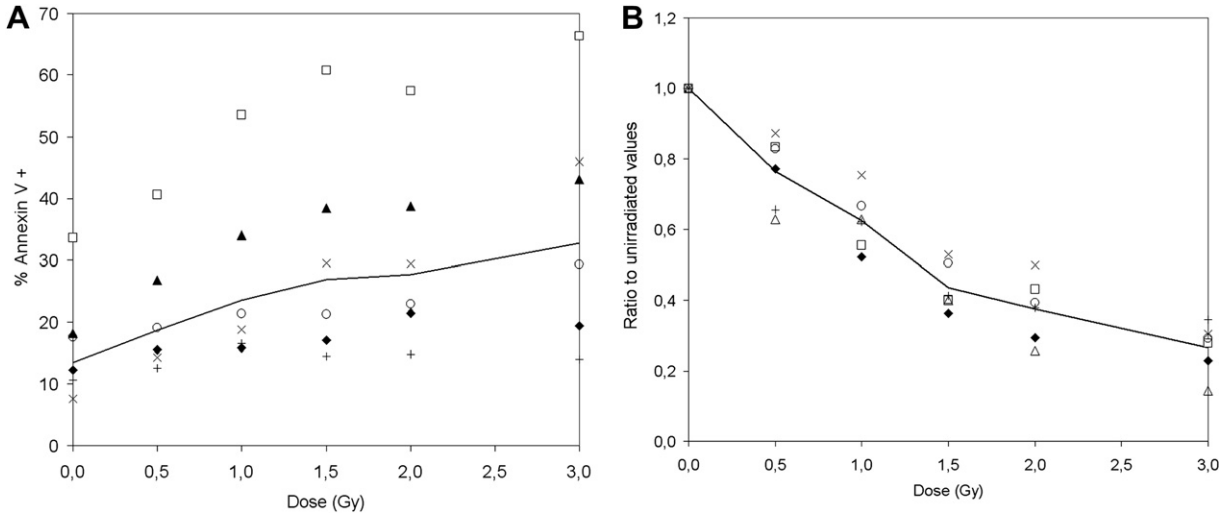

Fig. 1. A − Increase in number of apoptotic NK cells (CD3−/CD8+) 48 h after irradiation by the doses 0.5–3 Gy. B − Decrease of relative abundance of NK cells in population of live lymphocytes 48 h after irradiation by the dose 0.5–3 Gy. The points represent the values of 6 samples, the line connects the means.

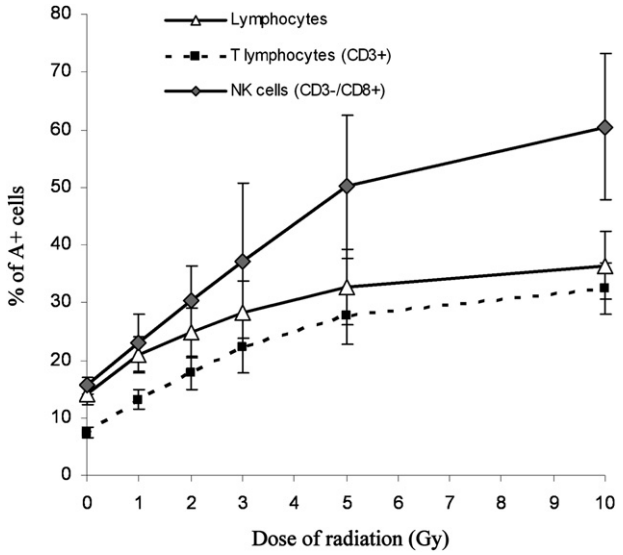

Fig. 2. Dose dependent increase in apoptosis induction 16 h after *in vitro* irradiation of lymphocytes isolated from peripheral blood of healthy donors by doses 1–10 Gy. The values represent the mean from 6 samples ± SD (standard deviation).

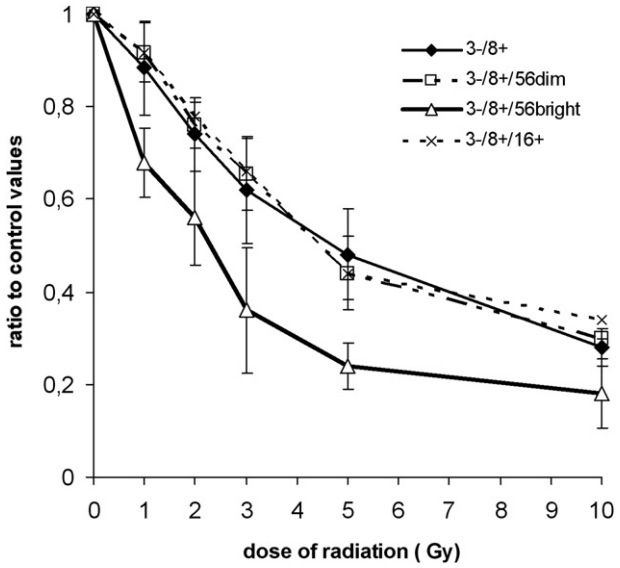

Fig. 3. Dose-dependent decrease in abundance of NK cells in population of Annexin V negative cells 16 h after irradiation by the doses 1–10 Gy. NK cells subpopulations were detected as CD3−/CD8+, CD3−/CD8+/CD16+, CD3−/CD8+/CD56bright or CD3−/CD8+/CD56dim. The values represent the mean from 6 samples ± SD (standard deviation).

In the second part of our work we have continued with the analysis of intact (A−/PI−) cells in an attempt to identify radiosensitivity of two subpopulations of CD3−/CD8+ NK cells − CD56bright and CD56dim and prove if there is a dose-depending decrease in the intact lymphocyte population of NK cells.

2.4. Statistical analysis

All data were expressed as the mean ± SD (standard deviation). For all statistical analyses, values of $P < 0.05$ were considered significant. Statistical analyses were done by one way and two way analysis of variance with repeated measures. For multiple comparisons of NK cells Scheffé's post hoc test was used.

3. Results

The first part of our experiments focused on possible to use of changes in CD3-/CD8+ subset as a biodosimetric marker in the dose range 0.5–3 Gy (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 Gy). We followed both the induction of apoptosis (Fig. 1A) and the relative amount of living (A−) CD3−/CD8+ NK cells (Fig. 1B) 48 h after *in vitro* exposure. The amount of apoptotic cells (A+) increased after irradiation but there was a great individual variability between samples (Fig. 1A). However, statistical evaluation by one way analysis of variance using Scheffé's post hoc test confirmed statistically significant increase in apoptosis induction in the range of the whole curve.

The experiments with living (A−) cells gave us better results. The relative amount of NK cells in the population of living lymphocytes does not vary to a great extent (Fig. 1B). Also in this curve the statistically significant decrease in CD3−/CD8+ NK cells was detected. This might allow us to predict the dose of γ radiation but it is conditioned by the knowledge of relative NK cells presence before the exposure.

In the following experiments, the dose range included higher doses up to 10 Gy of radiation (1, 2, 3, 5 and 10 Gy), therefore the interval 16 h after the *in vitro* irradiation was used. We observed a dose-dependent increase in the presence of apoptotic CD3−/CD8+ NK cells and T lymphocytes (CD3+) (Fig. 2).

Further we studied response of different subsets of CD3−/CD8+ NK-cells (Fig. 3). CD3−/CD8+ NK-cells were divided according to the amount of CD16 and CD56. We proved dose-dependent decrease in number of CD3−/CD8+ and similar decrease in CD3−/CD8+/CD16 and CD3−/CD8+/CD56dim. The decrease in NK cells was statistically significant as computed by two way analysis of variance by Scheffés post hoc test. The evaluation also revealed that CD3−/CD8+/CD56bright subpopulation is significantly more radiosensitive in comparison to other evaluated subpopulations. However, even in the unirradiated samples CD56bright subpopulation represents less then 5% of CD3−/CD8+ NK cells (data not shown).

4. Discussion

We and others have previously demonstrated that superficial phosphatidyl serine exposure at the early stage of apoptosis is rapidly followed by cell shrinking and decreased surface marker expression (Hertveld et al., 1997; Vokurková et al., 2006). In this work we show that individual differences in apoptosis induction in the separate irradiated groups (each consisted of 6 donors) were considerable and therefore the differences in average apoptosis induction in lymphocytes by particular doses were not significant. The analysis of the cell phenotype in the A+/PI− compartment has not provided any significant dose-dependent differences in the number of cells with the selected phenotype. Moreover, since such cells represent only transitional apoptotic population with as yet unknown lifetime and their frequency depends on the gradually decreasing number of intact cells, the analysis of the A+/PI− cells cannot represent a reliable biodosimetric parameter under our experimental conditions.

In our previous experiments we proved that no significant difference in radioresistance between CD4−/CD8+ and CD4+/CD8− lymphocytes was observed during the first day of cultivation, but 48 h post irradiation CD4+/CD8− subset prevailed (Vokurková et al., 2006).

In this work we used five color flow-cytometric analysis (A, CD3, CD8, CD16 and CD56) and looked for the changes in NK cells subpopulations 16 h after exposure to ionizing radiation. We observed concordant dose-depending decrease in NK cells subpopulations CD3−/CD8+, CD3−/CD8+/CD16+, CD3−/CD8+/CD56dim. Decrease in the subpopulation CD3−/CD8+/CD56bright was significantly higher after all doses compared to the

subpopulations discussed above. Unfortunately, our experiments also revealed that the subpopulation CD56bright represents a minority between CD3−/CD8+ NK cells and cannot be used as a valid biodosimetric marker. We can claim that the subpopulation CD56bright is more radiosensitive in comparison with CD56dim. It could play a certain role in immune response after irradiation knowing that CD56dim NK cells are more naturally cytotoxic then CD56bright and express higher levels of CD16 than CD56bright NK cell subset which is CD16dim (Fehniger et al., 2002). On the contrary, the CD56bright subset has the capacity to produce abundant cytokines following activation of monocytes. Romagnani et al. (2007) proved that peripheral blood CD56dim NK cells have shorter telomeres than peripheral blood and lymph node derived CD56bright NK cells. The results suggest that CD56bright/CD16− and CD56dim/CD16 NK cells correspond with sequential steps of differentiation and support the hypothesis that secondary lymphoid organs can be sites of NK cell final maturation and self-tolerance acquisition during immune reaction.

Against all expectations the measurement of apoptosis of CD3−/CD8+ NK cells initiated by low doses of radiation (0.5–3.0 Gy) 48 h after exposure also failed due to great individual variability. However, the evaluation of relative presence of living NK cells in the peripheral lymphocytes population could be taken as a good marker of radiation dose used in the range of 0.5–3.0 Gy in an *in vitro* experiment.

Conflict of interest

None declared.

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