Effects of Single and Fractionated Irradiation on Natural Killer Cell Populations: Radiobiological Characteristics of Viability and Cytotoxicity *In Vitro*

TENHO HIETANEN[1], MAUNU PITKÄNEN[2,3], MIKA KAPANEN[2,3] and PIRKKO-LIISA KELLOKUMPU-LEHTINEN[1,2]

[1]Department of Oncology, School of Medicine, University of Tampere, Tampere, Finland; Departments of [2]Oncology and [3]Medical Physics, Tampere University Hospital, Tampere, Finland

Abstract. *Background: Natural killer (NK) cells are important in destroying tumor cells. However, they are damaged by radiation therapy. We studied the effects of single and fractionated irradiation on the viability and cytotoxicity of human non-selected NK cells and sub-groups with cluster of differentiation (CD) CD16$^+$ and CD56$^+$ in vitro. Only very few studies dealing with the standard radiobiological parameters for characterizing NK cells exist in the literature. Materials and Methods: NK cell populations were isolated from buffy coats using different methods and irradiated with single doses up to 80 Gy and fractionated doses of 10 or 30 Gy with different numbers of applications and at different intervals. The study end-points were viability using propidium iodide (PI), trypan blue and intracellular adenosine triphosphate (ATP) assays, and cytotoxicity using the ^{51}Cr-release assay. The standard radiobiological parameters α and β of the linear-quadratic (L-Q) model and the mean inactivation dose $\bar{D}$ taken as the area under the curve (AUC) were calculated to characterize the radiosensitivity of different NK cell populations. Results: The AUC values of the 51Cr release data in the dose range of 0-40 Gy were as follows: for non-selected NK cells, 23.6-20.9 Gy; for CD16$^+$ and CD56$^+$ cells, 14.5-13.2 Gy. The AUC values of ATP, trypan blue and propidium iodide methods equally well described the viability of irradiated NK cells. The α/β ratio for cytotoxicity and viability data in the L-Q model corresponded to the acutely responding tissues. Splitting a 30-Gy dose into two fractions applied at different intervals caused a significant rise in ATP levels and cytotoxicity. Dividing the total dose into four doses applied at fixed intervals also resulted in significant elevations of ATP content and cytotoxicity of NK cells at 10 Gy. Conclusion: According to the L-Q method, irradiated NK cells behaved similarly to acutely responding human tissues with respect to cytotoxicity and viability. The AUC proved very useful for comparing the effects of irradiation on NK cells.*

Natural killer (NK) cells were originally defined functionally, based on their ability to mediate non-histocompatibility-restricted cytotoxicity against different tumor target cells (1) including *de novo* (2) and existing tumor cells (3). NK cells can also control the metastatic spread of malignant cells (4). Furthermore, NK cells contribute to the natural resistance against microbial infections, the grafting of bone marrow transplants, and graft *versus* host disease, and regulate the differentiation of hematopoietic and other cells (5). NK cells represent approximately 10-15% of peripheral blood mononuclear lymphocytes. They are characterized phenotypically by possessing CD56 and NKp46 receptors, and comprise of two major sub-groups, CD56$^+$ and CD16$^+$, which have distinctive properties. They do not display the CD3$^+$ T-cell phenotype.

Autologous or allogenic NK cells, alone or activated by interleukin-2, have been used in cancer therapy (6-7). In addition to cytokines current development in therapy has brought potential drugs like lenalidomide and bortezomib, enhanced NK cell function with monoclonal antibodies, adoptive therapy using expanded and activated NK cells, and use of NK-cell chimeric antigen receptors. For recent reviews of NK cell-based immunotherapy, see McDowell *et al.* (8) and Terme *et al.* (9).

Radiotherapy, one of the most common cancer therapies, may impair NK activity (10). The radiosensitivity of NK cells

Correspondence to: Tenho Hietanen, University of Tampere, School of Medicine, Department of Oncology, Lääkärinkatu 1, FI-33520 Tampere, Finland. Tel: +358 0505001863, e-mail: hietanen.tenho.e@student.uta.fi

Key Words: Natural killer cells, natural killer cell subsets, irradiation, viability, cytotoxicity, radiobiological models, enrichment methods.

is controlled by X-linked genes and varies considerably among individuals (11, 12). Radiotherapy effectively inhibits the post-binding stage of NK cell-mediated natural target cell lysis (13). On the other hand, radiotherapy has been used for immune-priming of tumor cells and tumor microenvironment. Therefore, combination of radiotherapy and immunotherapy has been investigated (14).

Only a very limited number of systematic *in vitro* studies are available to document the effects of irradiation, fractionated and single-dose schemes of irradiation, observation time after irradiation and parameters of radiobiological models (10, 15). Radiobiological effects are commonly quantified by the linear-quadratic model with two parameters, linear term α and quadratic term β, which can be determined by fitting the model to the dose–effect data (16). The model enables for the separation of radiation sensitivity for lethal and sublethal radiation injuries and facilitates categorizing the cells as early or late responders. A simpler method in the quantification of the radiosensitivity is to determine the area under the dose–effect curve (AUC) (16). The AUC is commonly used in calculations of the effects of cytotoxic drugs (17, 18).

Our aim in this study was to elucidate the radiobiological effects of single and fractionated gamma and X-ray irradiation on non-selected NK cells, as well as the $CD16^+$ and $CD56^+$ subsets. The study end-points were viability, as measured by propidium iodide (PI), trypan blue and intracellular adenosine triphosphate (ATP) assays, and cytotoxicity, as measured by the 51Cr-release assay. The standard radiobiological parameters α and β, as well as the mean inactivation dose D as the AUC, were calculated to characterize the radiosensitivity of the different NK cell populations.

Materials and Methods

Enrichment of NK cell populations. NK cells were isolated from buffy coats obtained from the Finnish Red Cross Transfusion laboratory with permission of the ethical committee of the Finnish Red Cross Blood Service (customer number 6129, approval number 331/2013, tutkijaluvat@veripalvelu.fi) in accordance to Finnish law. Before blood donation, donors were informed that blood samples that are not required for patient treatment can be used anonymously for research work. Buffy coats were prepared on the day of use.

Non-selected and highly purified $CD56^+$ and $CD16^+$ NK cell populations were enriched using different methods as described previously (submitted to Int J Radiat Biol by Hietanen T., Pitkänen M., Kapanen M., Kellokumpu-Lehtinen P-L., 2015). In brief, we used Ficoll-Paque (method I) and Percoll gradient centrifugation (method II) and a Dynal NK cell negative isolation kit (Dynal Biotech ASA, Oslo, Norway) (method III). The $CD16^+$ and $CD56^+$ subgroups were isolated using flow cytometry (method IV) and magnetic depletion of non-NK cells and positive selection using MACS $CD56^{dim}$ $CD16^+$ and $CD56^{bright}$ CD16- NK cell Isolation Kits (Miltenyi Biotec, Bergisch Gladbach, Germany) (method V). The purity of preparations was confirmed by Giemsa-stained cytocentrifuge preparations and flow cytometry.

Flow cytometric assay. For the phenotypic analysis of enriched NK cells, we used R-phycoerythrin-conjugated mouse monoclonal antibody to human $CD16^+$ and allophycocyanin-conjugated mouse monoclonal antibody to human $CD56^+$ (BD Pharmingen, San Diego, CA, USA) as described elsewhere (19). The cells were analyzed on a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, USA).

Irradiation. The cells were irradiated in an early phase of the study using a ^{137}Cs device (GAMMACELL 2000, Mølsgaard Medical, Risø, Denmark) and later with a clinical radiotherapy accelerator (Varian TrueBeam STx, Varian Medical Systems Inc., Palo Alto, CA, USA) as described in our previous work (submitted to Int J Radiat Biol by Hietanen *et al*. 2015). In viability studies, the radiation dose ranged from 0 Gy to the non-therapeutic dose of 80 Gy to explore the whole dose–response curve for radiobiological calculations. The effects on cytotoxicity were investigated in the dose range from 0 to 40 Gy, according to our previous study (submitted to Int J Radiat Biol by Hietanen *et al*. 2015).

In the split-dose studies, doses of 10 and 30 Gy were selected from the upper and lower linear part of the semi-logarithmic part of the dose–response curve. These doses were split into two, with each dose divided into two fractions applied at intervals from 0 to 24 hours, or split up to three equal fractions applied at 3-hour intervals.

Assessment of viability. Viability was measured by flow cytometry using PI (Sigma Chemical Co., St Louis, MO, USA) as described previously (20). Briefly, isolated lymphocytes were incubated with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies for 30 minutes on ice, washed, and then PI was added at a final concentration of 4 μM. The percentage of PI-stained cells, *i.e*. non-viable cells, was measured using flow cytometry based on the red fluorescence, while viable lymphocytes exhibited green fluorescence.

In the trypan blue exclusion method (21), 0.1 ml of the NK cell suspension and 0.1 ml of 0.2% trypan blue solution were mixed. After 10 minutes, 200 cells were examined under a microscope, and all blue and damaged cells were counted as dead.

Commercial kits from LKB Wallac (Turku, Finland) were used for the intracellular ATP method (22) according to the manufacturer's instructions. The amounts of ATP were then measured using an LKB Wallac 1251 luminometer, and the results were obtained as millivolts and expressed as values normalized to those of non-irradiated controls. Viability assays were performed at different times, from 0 to 72 hours post-irradiation.

Cytotoxicity test. The cytotoxicity of enriched cell populations was measured by the modified ^{51}Cr release assay described by Timonen *et al*. (23) using K-562 cells (LGC Standards GmbH, Wesel, Germany) as a target. The relative cytotoxicity was calculated using the following formula: Cx (%)=((exp.-spont.)/(max.-spont.)) where exp is experimental release; spont. is spontaneous release, and max. is maximal release from K-562 cells lysed with 1% Triton X-100. The spontaneous release was less than 10% of the maximal release. The effector-to-target ratio (E:T) of 12.5:1 used for the cytotoxicity studies was based on pilot studies. The cytotoxicity at this ratio was 52.1±11.5% (SD) of the maximal release. The results were normalized to those of the controls.

The characteristics of the isolated non-selected NK cell populations, including the viability, purity and cytotoxicity, and those of the $CD16^+$ and $CD56^+$ subsets, were presented in our

Table I. *Linear-quadratic model parameters of the cytotoxicity data of the irradiated natural killer cells obtained using the ^{51}Cr-release method (dose range from 0 to 40 Gy). The cytotoxicity test was performed 18 h post-irradiation. Standard errors of the parameters (±SE) and p-values are given. CD16$^+$ enriched with methods IV and V were combined, as well as CD56$^+$ cells.*

Enrichment method	α (Gy^{-1})	± SE	β (Gy^{-2})	±SE	*p*-Value
Method I	0.03199	0.008	–2.57E-05	0.000	0.000
Method I *	0.0313	0.003	0	0	1
Method II	0.01362	0.030	0.00164	0.001	0.002
Method III	0.01532	0.001	0.00106	0.000	0.000
CD16$^+$	0.08985	0.029	–7.55E-04	0.001	0.009
CD16$^+$ *	0.0732	0.011	0	0	1
CD56$^+$	0.0885	0.032	–3.66E-04	0.001	0.008
CD56$^+$ *	0.0807	0.011	0	0	1

*When β was negative and assigned a value of zero, a new α value was re-determined.

Table II. *Area under the survival curve (AUC) values calculated for the ^{51}Cr-release data of natural killer cells 18 h post-irradiation.*

Enrichment method	AUC (Gy)
Method I	23.6
Method II	24.1
Method III	20.9
Methods IV+V: CD16$^+$	14.5
Methods IV+V: CD56$^+$	13.2

previous paper (submitted to Int J Radiat Biol by Hietanen *et al*. 2015). CD16$^+$ cells enriched using methods IV and V were equally cytotoxic. The same is also true for CD56$^+$ cells. Therefore, we combined the CD16$^+$ cells and CD56$^+$ cells for further cytotoxicity analyses. These sub-populations were called CD16$^+$ and CD56$^+$, respectively.

Calculation of the radiobiological parameters. The following standard mathematical radiobiological models were used to quantify the radiation dose–response curves: I: The linear-quadratic model $S=e^{-(\alpha D+\beta D^2)}$, where S represents the surviving fraction, and D is the radiation dose in Gy (24). The linear-quadratic model has two inactivation parameters, the linear term α (Gy^{-1}) and the quadratic term β (Gy^{-2}). When negative values were obtained for β, and thus biologically meaningless, the negative values were assigned a value of zero, and the linear-quadratic model was re-fitted as suggested by Fertil *et al*. (25). II: The mean inactivation dose $\bar{D}(Gy)=\int S(D)dD$ represents the area under the survival curve (AUC) in linear coordinate representation (16), where S(D) represents the survival probability and D the radiation dose. The AUC was not calculated from the fitted linear-quadratic model but by direct integration of the dose–response curve in question.

The two models were fitted to the experimental data using the OriginPro 2015 program (OriginPro Corporation, Northampton, MA, USA).

Culture conditions. Cells were cultured as described in our previous work (submitted to Int J Radiat Biol by Hietanen *et al*. 2015). The effector NK cells and target K-562 cells were cultured in RPM-1640 medium (Orion Diagnostica, Vantaa, Finland) supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories, Irvine, Scotland), L(+)-glutamine (0.3 g/l) (Fluka, Buchs, Switzerland) and gentamycin (20 μg/ml) (Flow Laboratories). The experiments were conducted at 37˚C in a humidified air atmosphere containing 5% CO_2.

Basis of data presentation and statistical analysis. For statistical calculations, the SPSS 15.0 statistical package (SPSS Inc., Chicago, IL, USA) was used. The results are presented as the mean±SD or standard error (SE). Analysis of variance (ANOVA) with post hoc testing using Bonferroni's modification of the *t*-test or non-parametric tests such as the independent-samples Mann–Whitney *U*-test, the Kruskal–Wallis test and Spearman's rho test were applied. Differences with a *p*-value of 0.05 or less (two-sided) were considered significant. The results of the PI, ATP, trypan blue and ^{51}Cr-release assays were normalized to those for the non-irradiated controls.

Results

Effect of single-dose irradiation on the killing capacity of NK cells. The killing capacity of the irradiated NK cells enriched using the methods I-V was measured using the 51Cr-release assay. The results showed that the cytotoxicity of the cells enriched using method I remained highest up to 40 Gy (Figure 1). The parameters of the linear-quadratic model (α and β) based on these data in Figure 1 are presented in Table I. The β values of the cytotoxicity data were positive only with methods II and III, resulting in α/β-ratios of 8.3 Gy and 14.4 Gy, respectively, which are characteristic for acutely responding tissues. The other β values were negative and replaced with 0, and re-fitted, as described above. For these cases, the new α values representing the linear part of the α/β model were added to Table I. The α values were 0.03 Gy^{-1} for non-selected NK populations and 0.07 and 0.09 Gy^{-1} for CD16$^+$ and CD56$^+$ cells, respectively.

The 18 hours post-irradiation AUC values for the cytotoxicity data obtained with the irradiation doses ranging from 0 to 40 Gy (Figure 1) were between 21 and 23 Gy for the non-selected NK cells enriched using methods I-III (Table II), whereas the AUC was approximately 13-14 Gy for CD56$^+$ and CD16$^+$ cells combined.

However, there were no significant differences between the populations (p=0.714).

Effect of single-dose irradiation on the viability of NK cells. For detailed viability studies, irradiated, non-selected NK cells enriched using method II were used. The viability was measured using trypan blue exclusion, PI and ATP measurements. The data are presented in Figure 2. The

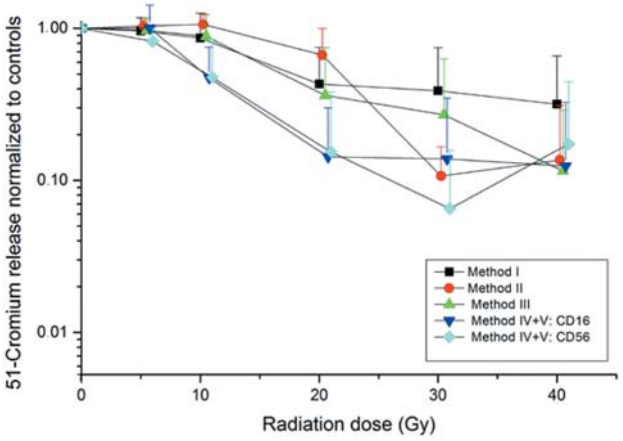

Figure 1. *Natural killer cells enriched using methods I-V were irradiated using doses ranging from 0 to 40 Gy. The cytotoxicity was measured by the ^{51}Cr-release assay 18 hours post-irradiation. The α (Gy^{-1}), β (Gy^{-2}) and the area under the survival curve (Gy) values were determined from the data of these curves. Error bars represent the standard deviation of the mean.*

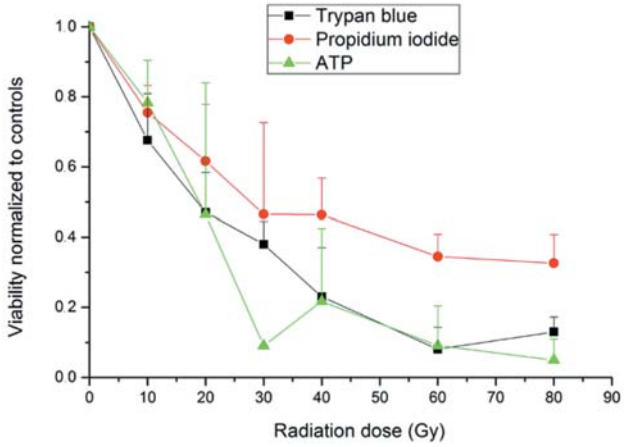

Figure 2. *Viability of non-selected natural killer cells enriched using method II irradiated with a single dose (from 0 to 80 Gy). The viability was measured by trypan blue exclusion, propidium iodide and ATP methods 18 h after irradiation. The results are expressed as fractions of the non-irradiated controls. The α (Gy^{-1}), β (Gy^{-2}) and the area under the survival curve (Gy) values were determined from the data of these curves. Error bars represent the standard deviation of the mean.*

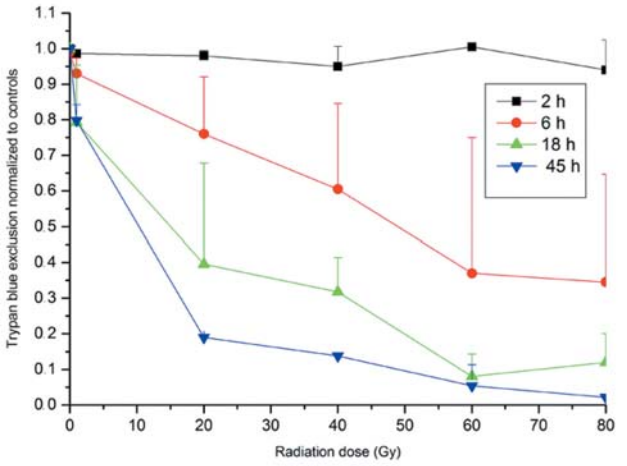

Figure 3. *The long-term effects of single irradiation doses up to 80 Gy on the viability of non-selected natural killer cells were studied by the trypan blue exclusion test 2, 6, 18 and 45 h after irradiation. Error bars represent the standard deviation of the mean.*

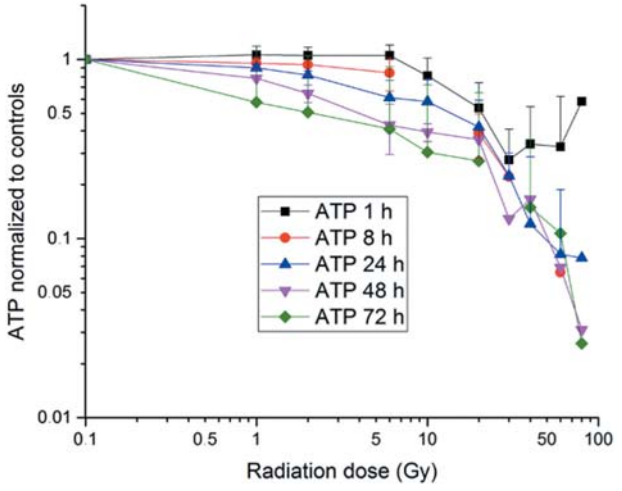

Figure 4. *ATP content of irradiated natural killer cells as a function of irradiation dose and time post-irradiation. Non-selected NK cells were irradiated with single doses ranging from 0 to 80 Gy. The ATP content of cells was measured at time points from 0 to 72 h post-irradiation. Error bars represent the standard deviation of the mean.*

corresponding α and β values are presented in Table III. Only the ATP data resulted in positive β values. The α/β-ratio was 39.6 Gy, corresponding to the acutely responding tissues. For trypan blue exclusion and PI data, the refitted α values were 0.040 Gy^{-1} and 0.019 Gy^{-1}, respectively.

The AUC values obtained with the trypan blue exclusion, PI and ATP methods were 29.9 Gy, 25.6 Gy and 16.8 Gy, respectively. All of these methods were equally good to describe NK cell death caused by irradiation (p=0.387).

The proportion of dead NK cells was evaluated up to 45 h post-irradiation using the trypan blue exclusion method (Figure 3). Two hours after irradiation, practically no effect was observed on viability within the dose range from 0 to 80 Gy.

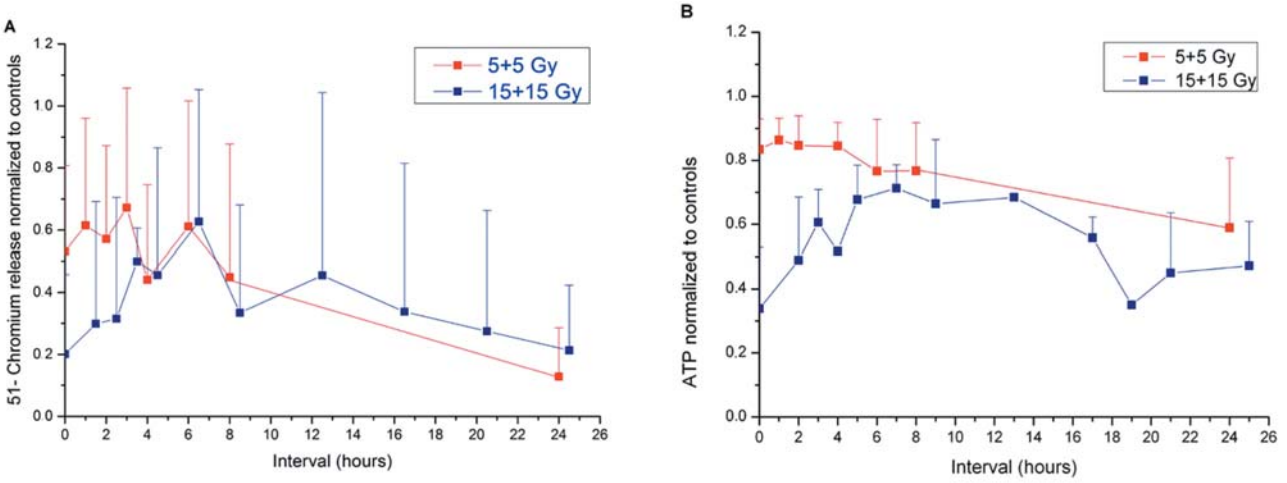

Figure 5. *Natural killer cells enriched using method II were irradiated with total doses of 10 and 30 Gy split into two fractions. The interval between fractions ranged from 0 to 24 h. The radiation effect was measured with ^{51}Cr release (A) and ATP (B). Error bars represent the standard deviation of the mean.*

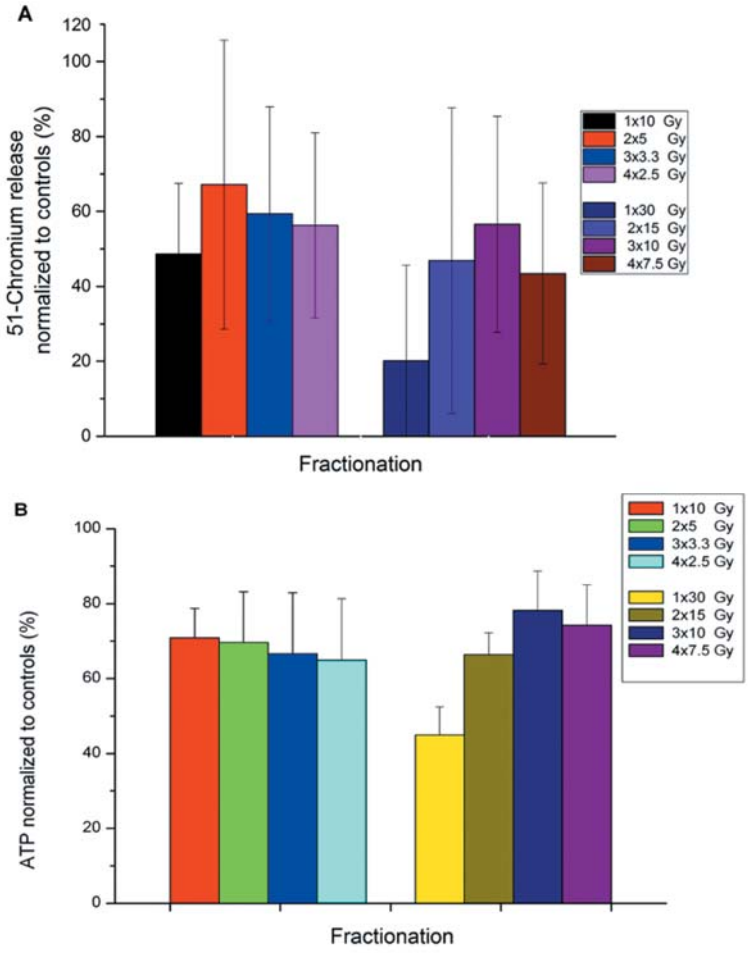

Figure 6. *Natural killer cells enriched using method II were irradiated with doses of 10 Gy and 30 Gy. The target-effector ratio was 1:12.5. The doses were split into several fractions applied at 3-h intervals. ^{51}Cr-release (A) and ATP (B) and were measured and normalized to those of the controls. Error bars represent the standard deviation of the mean.*

Table III. *The linear-quadratic model applied to the survival data of irradiated natural killer cells enriched using method II. Viability was measured using trypan blue exclusion, propidium iodide and ATP methods 18 h after irradiation. The standard errors of the parameters (SE) and p-values are given.*

	Isolation method	α (Gy^{-1})	SE	B (Gy^{-2})	SE	*p*-Value
Trypan blue	II	0.0478	0.011	−2.71546E-4	0.0003	0.000
Trypan blue*		0.0397	0.004	0	0	1
Propidium iodide	II	0.0287	0.002	−1.8565E-4	0.000	0.000
Propidium iodide*		0.0187	0.0018	0	0	1
ATP	II	0.0109	0.006	2.7524E-4	0.000	0.000

*Negative β values were replaced by zeros, and the linear-quadratic model was refitted.

Thereafter, the viability decreased almost linearly up to the dose of 60 Gy, followed by a plateau up to 80 Gy. Consequently, all values in the radiobiological models were strongly dependent on the selected time point after irradiation. The decrease in viability as a function of time is also reflected by the AUC for the trypan blue exclusion data. After 2, 6, 8 and 45 h, the AUC values were 39.5 Gy, 33.5 Gy, 20.4 Gy and 12.5 Gy, respectively. Conversely, the AUC values for living NK cells changed from 69.5 Gy to 10.6 Gy in 48 h.

The ATP content of non-selected NK cells was measured up to 72 h after irradiation using doses from 0 Gy to 80 Gy. The dose range of 0-1 Gy produced practically no changes in the ATP content 2 h after irradiation (Figure 4). From 1 Gy to 6-8 Gy, an elevation of ATP levels was seen (108.9±6.5%). This effect disappeared within 4-6 h. A steep fall was observed with doses between 10 and 40 Gy. Thereafter, a plateau was reached up to doses of 60 Gy and over. During the time course, the ATP levels dropped very rapidly within 1-2 h, followed by a slow and continuous fall for up to 48 h, after which there was hardly any further decrease.

Effects of fractionated irradiation at different intervals on the cytotoxicity and viability of NK cells. When splitting the total dose into two equal fractions applied at different intervals (1, 2, 3, 4, 8, 12, 16, 20 and 24 hours), ^{51}Cr-release measurements at 10 Gy resulted in non-significant changes (Figure 5A). At 30 Gy, a continuous rise of ^{51}Cr release was observed up to 6 h. However, the rise was not significant (p=0.664). A slow decline in values was observed over longer intervals.

The fractionation did not affect the ATP levels of NK cells significantly at the 10-Gy dose (p=0.063). However, at 30 Gy, changing the fraction interval from 0 to 6 hours caused a progressive 2.1-fold rise (p=0.002). After 12 h, a slow decrease was observed until 24 h (Figure 5B).

Effects of fractionated irradiation with different numbers of fractions on the cytotoxicity and viability of NK cells. The total dose of 10 Gy was divided into fractions of 2×5 Gy, 3×3.3 Gy, 4×2.5 Gy applied at 3-h intervals. The cytotoxicity of NK cells was significantly elevated when 10 Gy was divided into two fractions (p=0.04). Splitting 30 Gy into several (up to three) fractions resulted in a 2.7-fold rise in 51 Cr release (Figure 6A). However, the increase was not significant (p=0.130). The radiation effects were still detectable after 3 days, although to a lesser extent.

When the total dose of 10 Gy was divided into fractions of 2×5 Gy, 3×3.3 Gy, 4×2.5 Gy applied at 3-h intervals, ATP levels were not significantly affected. However, when 30 Gy was divided into multiple fractions (2×15 Gy, 3×10 Gy, 4×7.5 Gy), a steady rise in the ATP levels was observed up to 1.4-fold that of the controls at 3 fractions (p=0.001) (Figure 6B).

Discussion

The aim of this work was to characterize the radiosensitivity of non-selected NK cells, and $CD56^+$ and $CD16^+$ sub-groups using viability and cytotoxicity as end-points. For viability, we used several methods, including trypan blue exclusion, PI and ATP methods. These methods describe different aspects of cell death. According to our results, they describe NK cell viability and death equally well. Our cytotoxicity and viability data were strongly dependent on the irradiation dose, time after irradiation and NK cell-enrichment methods.

We applied mathematical models developed for clonogenic dividing cells to describe the radiation dose–survival relationship to functional data such as cytotoxicity and viability. NK cells do not divide without biological response modifiers (26). The α/β ratio is currently used in practical radiotherapy to calculate radiobiological effects, particularly for different fractionation schemes. It is also useful in describing functional data (27, 28). In our experiments, the α/β ratio of cytotoxicity data measured by the ^{51}Cr release assay was relatively high and of the same order as for acutely responding tissues. The same is true for the viability data obtained by the ATP, PI and trypan blue exclusion methods.

The mean inactivation dose $\bar{D}$ is also applicable to non-dividing cell populations and produces more consistent results than other parameters (such as α/β) when comparing

repeated experiments and different cell lines. It quantifies the radiation reactions well over the whole dose range (16). It is recommended by the International Commission on Radiation Units & Measurements to characterize the radiosensitivity of mammalian cells (29). The cytotoxicity AUC values of NK cells enriched by methods I-V were identical even if the cells were non-selected or highly purified.

In some instances, fractionated irradiation enhanced the viability measured by the ATP method and the killing capacity of NK cells *in vitro*. In clinical situations, where daily fractions from 1.8 to 4 Gy are typically used, these results may have some clinical importance.

ATP measurements are used in radiation biology to reflect the radiation damage to ATP synthesis (30). In our pilot work, we measured the intracellular energy production by determining the intracellular ATP, ADP and AMP levels of NK cells by high-performance liquid chromatography. The results indicated that ATP production is stimulated by low-dose irradiation and inhibited by doses higher than 10 Gy; concomitantly, ATP turnover rises. These findings support a hypothesis of mitochondrial excitation at lower radiation dose levels followed by damage at higher doses. The anti-oxidative capacity of the cells seems to be exhausted, and the elimination of radiation-induced radicals seems to be inhibited. Thus, intracellular ATP may reflect the ability of irradiated NK cells to survive.

There are very few radiobiological characteristics of NK cells described in the literature that are comparable with our data. Many reports on the radiobiological characteristics of B- and T-lymphocytes have been published but the comparison of these data is limited because radiobiological data of this type are clearly dependent on factors such as the enrichment and assay methods used, time points of measurements after irradiation and cell cycle (31, 32). In our pilot study, $CD3^+$ T-cells were more radioresistant than $CD16^+$ NK cells, as defined by AUC ($\bar{D}$) values. Our data confirm the findings of Rana *et al*. (13).

In modern radiotherapy, the daily dose fraction most commonly is 2 Gy, delivered at a dose rate ranging from 4 to 6 Gy/min. Even single doses of approximately 10 Gy have been used (33). According to our results, this dosage should cause only little instantaneous reduction of the NK cell cytotoxicity. However, the long-term influence of radiotherapy on NK cells also depends on the total dose, fractionation scheme used and location of the radiation fields (34). *In vivo*, several factors, including biological response modifiers such as interferons and interleukins, alter the radiosensitivity of different NK cell properties (35).

Conclusion

The linear-quadratic model and mean inactivation dose as the AUC were applied to viability and cytotoxicity data of different non-selected and highly purified $CD56^+$ and $CD16^+$ human NK cell populations. The viability and cytotoxicity data were typical for acutely responding human tissues. The AUC method was very useful when comparing different NK populations. When using the linear-quadratic model, the quadratic component β was sometimes negative, and only the linear component α was useful. No *in vitro* post-irradiation recovery of viability or cytotoxicity was detected.

Conflicts of Interest

The Authors declare no conflicts of interest.

Acknowledgements

This work was supported by grants from the Finnish Cancer Society, Paulo Foundation and Emil Aaltonen Foundation. We thank Miss Eija Isolehto for excellent technical assistance; Timo Metsä-Ketelä, Ph.D., for assistance with the high performance liquid chromatography; Tuomo Timonen, Antti Ojala and Kai Krohn for fruitful discussions; Jouni Vuorinen, M.Sc. and Heini Hautala, M.Sc. for the statistical calculations; Antti Vanhanen for technical assistance in irradiating NK cells; and Anatoly Malyguine, Jenni Lappiniemi and Jyrki Sivula for assistance with flow cytometric studies.

References

1 Pross HF and Baines MG: Spontaneous human lymphocyte-mediated cytotoxicity againts tumour target cells. I. The effect of malignant disease. Int J Cancer *18*: 593-604, 1976.

2 Warner JF and Dennert G: Effects of a cloned cell line with NK activity on bone marrow transplants, tumour development and metastasis *in vivo*. Nature *300*: 31-34, 1982.

3 Takasugi M, Mickey MR and Terasaki PI: Reactivity of lymphocytes from normal persons on cultured tumor cells. Cancer Res *33*: 2898-2902, 1973.

4 Hanna N and Burton RC: Definitive evidence that natural killer (NK) cells inhibit experimental tumor metastases *in vivo*. J Immunol *127*: 1754-1758, 1981.

5 Robertson MJ and Ritz J: Biology and clinical relevance of human natural killer cells. Blood *76*: 2421-2438, 1990.

6 Curti A, Ruggeri L, D'Addio A, Bontadini A, Dan E, Motta MR, Trabanelli S, Giudice V, Urbani E, Martinelli G, Paolini S, Fruet F, Isidori A, Parisi S, Bandini G, Baccarani M, Velardi A and Lemoli RM: Successful transfer of alloreactive haploidentical KIR ligand-mismatched natural killer cells after infusion in elderly high risk acute myeloid leukemia patients. Blood *118*: 3273-3279, 2011.

7 Rosenberg SA: Immunotherapy of cancer by systemic administration of lymphoid cells plus interleukin-2. J Biol Response Mod *3*: 501-511, 1984.

8 McDowell KA, Hank JA, DeSantes KB, Capitini CM, Otto M and Sondel PM.: NK Cell-based Immunotherapies in Pediatric Oncology. J Pediatr Hematol Oncol *37*: 79-93, 2015.

9 Terme M, Ullrich E, Delahaye NF, Chaput N and Zitvogel L: Natural killer cell-directed therapies: moving from unexpected results to successful strategies. Nat Immunol *9*: 486-494, 2008.

10 Dean DM, Pross HF and Kennedy JC: Spontaneous human lymphocyte-mediated cytotoxicity against tumor target cells--III. Stimulatory and inhibitory effects of ionizing radiation. Int J Radiat Oncol Biol Phys *4*: 633-641, 1978.

11 Brovall C and Schacter B: Radiation sensitivity of human natural killer cell activity: control by X-linked genes. J Immunol *126*: 2236-2239, 1981.
12 Schacter B, Hansal S, Arno J and LeVine MJ: Polymorphic radiation sensitivity of human natural killer activity: possible role of DNA strand breakage. Hum Immunol *14*: 49-58, 1985.
13 Rana R, Vitale M, Mazzotti G, Manzoli L and Papa S: Radiosensitivity of Human Natural Killer Cells: Binding and Cytotoxic Activities of Natural Killer Cell Subsets. Radiat Res *124*: 96-102, 1990.
14 Shahabi V, Postow MA, Tuck D and Wolchok JD: Immune-priming of the tumor microenvironment by radiotherapy: rationale for combination with immunotherapy to improve anticancer efficacy. Am J Clin Oncol *38*: 90-97, 2015.
15 Hietanen T, Pitkänen M, Aaran R and Leinikki P: Interleukin IL-2 (IL-2) clearly reduces the effect of radiation on natural killer cells (NK cells): interferons are less active. *In*: Frontiers in Radiation Biology (Rikeis E, ed.). New York, VCH, pp. 77-82, 1990.
16 Kellerer AM, Hall EJ, Rossi HH and Teedla P: RBE as a function of neutron energy. II. Statistical analysis. Radiat Res *65*: 172-186, 1976.
17 Calvert AH, Newell DR, Gumbrell LA, O'Reilly S, Burnell M, Boxall FE, Siddik ZH, Judson IR, Gore ME and Wiltshaw E: Carboplatin dosage: prospective evaluation of a simple formula based on renal function. J Clin Oncol *7*: 1748-1756, 1989.
18 Mazumdar M, Smith A, Tong WP and Motzer RJ: Calvert's Formula for Dosing Carboplatin: Overview and Concerns of Applicability in High-Dose Setting. J Natl Cancer Inst *92*: 1434-1436, 2000.
19 Patrikoski M, Sivula J, Huhtala H, Helminen M, Salo F, Mannerstrom B and Miettinen S: Different culture conditions modulate the immunological properties of adipose stem cells. Stem Cells Transl Med *3*: 1220-1230, 2014.
20 Malygin AM, Somersalo K and Timonen T: Promotion of natural killer cell growth *in vitro* by bispecific (anti-CD3 x anti-CD16) antibodies. Immunol *81*: 92-95, 1994.
21 Pappenheimer AM: Experimental studies upon lymphocytes: i. The reactions of lymphocytes under various experimental conditions. J Exp Med *25*: 633-650, 1917.
22 Kangas L, Gronroos M and Nieminen AL: Bioluminescence of cellular ATP: a new method for evaluating cytotoxic agents *in vitro*. Med Biol *62*: 338-343, 1984.
23 Timonen T, Ortaldo JR and Herberman RB: Characteristics of human large granular lymphocytes and relationship to natural killer and K cells. J Exp Med *153*: 569-582, 1981.
24 Kellerer AM and Rossi HH: A generalized formulation of dual radiation action. Radiat Res *178*: AV204-13, 2012.
25 Fertil B, Dertinger H, Courdi A and Malaise EP: Mean inactivation dose: a useful concept for intercomparison of human cell survival curves. Radiat Res *99*: 73-84, 1984.
26 Timonen T, Ortaldo JR, Stadler BM, Bonnard GD, Sharrow SO and Herberman RB: Cultures of purified human natural killer cells: growth in the presence of interleukin 2. Cell Immunol *72*: 178-185, 1982.
27 Neriishi K, Nakashima E, Akahoshi M, Hida A, Grant EJ, Masunari N, Funamoto S, Minamoto A, Fujiwara S and Shore RE: Radiation Dose and Cataract Surgery Incidence in Atomic Bomb Survivors, 1986-2005. Radiol *265*: 167-174, 2012.
28 Fowler JF: Dose response curves for organ function or cell survival. Br J Radiol *56*: 497-500, 1983.
29 ICRU: Quantitative Concepts and Dosimetry in Radiobiology, Report No. 30. Bethesda, Maryland, USA, International Commision on Radiation Units and Measurements, 1979.
30 Skog S, Tribukait B and Sundius G: Energy metabolism and ATP turnover time during the cell cycle of Ehrlich ascites tumour cells. Exp Cell Res *141*: 23-29, 1982.
31 Cole J, Arlett CF, Green MH, Harcourt SA, Priestley A, Henderson L, Cole H, James SE and Richmond F: Comparative human cellular radiosensitivity: II. The survival following gamma-irradiation of unstimulated (G0) T-lymphocytes, T-lymphocyte lines, lymphoblastoid cell lines and fibroblasts from normal donors, from ataxia-telangiectasia patients and from ataxia-telangiectasia heterozygotes. Int J Radiat Biol *54*: 929-943, 1988.
32 Kwan DK and Norman A: Radiosensitivity of human lymphocytes and thymocytes. Radiat Res *69*: 143-151, 1977.
33 Schultheiss TE: The radiation dose-response of the human spinal cord. Int J Radiat Oncol Biol Phys *71*: 1455-1459, 2008.
34 McGinnes K, Florence J and Penny R: The effect of radiotherapy on the natural killer (NK)-cell activity of cancer patients. J Clin Immunol *7*: 210-217, 1987.
35 Hietanen T, Kellokumpu-Lehtinen P and Pitkanen M: Action of recombinant interferons and interleukin 2 in modulating radiation effects on viability and cytotoxicity of large granular lymphocytes. Int J Radiat Biol *67*: 119-126, 1995.

Received June 24, 2015
Revised July 22, 2015
Accepted July 24, 2015