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ANALYSIS OF RELATIVE TELOMERE LENGTH AND APOPTOSIS IN HUMANS EXPOSED TO IONISING RADIATION

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Background: **Ionizing radiation could modify lymphocyte function via oxidative damage, DNA breaks, and resulting changes of proliferation, apoptosis and cellular senescence, where telomeres may play a critical role.** ***Aim:*** **To study the effect of low-dose irradiation on the telomere length and apoptosis rates in peripheral blood lymphocytes of irradiated persons.** ***Patients and Methods:*** **A study was performed on 83 peripheral blood samples from the Chornobyl clean-up workers, radiation workers exposed under the professional limits at construction works at the "Shelter" object and healthy controls. Bone marrow leukocyte telomere length was estimated in 15 patients with myelodysplastic syndrome secondary to low-dose radiation exposure and 12 age-standardized healthy donors. Relative telomere length was studied by the combination of a fluorescence hybridization *in situ* with PNA probe and flow cytometry, apoptosis — by Annexin-V test.** ***Results*****: A significant relative telomere length decrease has been demonstrated in Chornobyl clean-up workers compared to healthy donors (13.2 ± 0.69 and 18.6 ± 0.73 respectively, $p < 0.05$), and a tendency ($p < 0.1$) in radiation workers. At doses over professional limits an inverse dependency is demonstrated between the relative telomere length and a number of lymphocytes in early stage of apoptosis. In MDS group a tendency of telomere elongation was demonstrated in bone marrow granulocytes in RAEB-t and RAEB as comparing with RA.** ***Conclusion:*** **This study shows telomere shortening after low-dose irradiation and preservation of these changes even 20 years after exposure. Apoptosis induction is possible by the telomere region changes at least in individuals with shorter telomeres. Apoptosis decrease in MDS clonal transformation is associated with a substantially longer telomeres.**

Key Words: **radiation, telomere, apoptosis, Chernobyl, myelodysplastic syndrome.**

The critical function of telomeres consists in adjusting the cellular response to stress and growth stimulation based on previous cell divisions and occurs via progressive reduction of telomere length and respective DNA damage that is activated by terminal chromosome regions not containing telomere sequences [1]. Low-dose ionizing radiation is regarded as one of factors that finally affect life-span via modification of cell functioning through the mechanisms of oxidative stress, DNA damage, cellular senescence, apoptosis and compensatory proliferation [2]. These factors are believed to play a role in genome instability and form a background for radiation-dependent aging. Also telomere shortening may serve as a surrogate marker of tumor progression and seems to be accelerated in allogenic bone marrow transplant recipients [3]. In radiation biology this question is not clearly understood. Neuhof *et al*. [4] suggest telomerase activity to be the radiation modified function, responsible for the damage DNA. Length of telomeres is also associated with enhanced chromosome radiosensitivity that is demonstrated in murine acute myeloid leukemia cell line 7926 [4, 5]. In radiosensitive cells short telomeres might correlate with radiation sensitivity [6]. Such conclusion could be applied to such radiosensitive cells as human lymphocytes and other types of leukocytes. Some other data show that long telomeres, but not telomerase activity seem be factor protecting proliferation activity under radiation exposure [5, 7].

Appearance of radiation senescence in people who suffered due to Chornobyl accident, is among the most debatable aspects of biological effects of irradiation. In some publications the phenomenon of such senescence has been registered in population that lives on radionuclide-contaminated territories, and this phenomenon is believed to be associated with altered immune functions, lymphoadenopathy and lymphopenia, increased incidence of autoimmune thyroiditis and other allergic diseases [8]. Individuals who suffered as a result of the Chornobyl accident, might have genome changes, changes in lineage-associated or functionally active biomolecules, such as lack of CD28 on CD8+ cells, that are experimentally shown to result in lower proliferative activity, decreased IL-2 production, telomere erosion and lesser response to TCR stimulation [9]. It is important to reveal the mechanisms involved in the radiation induced senescence, including the telomere shortening and apoptosis. Due to the emerging data on cell senescence as questionable radiation effect in human body, it is especially important to obtain comparable results using the quantitative approach.

MATERIALS AND METHODS

In total 110 individuals were enrolled to a study: 1) control group was composed from 22 healthy individuals with normal within the age limits haematological and immunological indices; 2) the group of irradiated individuals (n = 73) included a) 33 clean-up workers of 1986–1987 at the late period after radiation exposure of 386 ± 92 (M ± m) mSv; b) 10 acute radiation syndrome (ARS) survivors after a high dose radiation exposure of 1430 ± 211 (M ± m) mSv, and c) 30 radiation workers exposed under

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Abbreviations used: ARS – acute radiation syndrome; FISH – fluorescence *in situ* hybridization; MDS – myelodysplastic syndrome; PBL – peripheral bloody lymphocytes; PNA – peptide nucleic. acid; RTL – relative telomere length;

the professional limits during construction works at the "Shelter" object with the dose of external irradiation of 12 ± 0.87 (M ± m) mSv; 3) special comparison group included 15 patients with myelodysplastic syndrome (MDS) that has developed in 8 to 21 years after low-dose radiation exposure during the Chornobyl accident. Mean age of subjects included to control group was 50.1±1.04 (M ± m) years, clean-up workers — 56.2 ± 1.29 years, ARS survivors — 61.1 ± 2.86 years, radiation workers — 47.4 ± 1.41 years. All persons enrolled into the study have given an informed written consent.

Study of the relative telomere length (RTL) was conducted in peripheral blood lymphocytes by the flow-FISH assay, that combines the fluorescent hybridization *in situ* and flow cytometry with a Telomere PNA Kit/FITC (Dako Cytomation, Denmark) using a FITC–conjugated peptide nucleic acid (PNA) probe. This kit is recommended for RTL evaluation in nucleated haematopoietic cells. K562 cells with known telomere length were used as control. Cell line was obtained from the Bank of Cell Lines from Human and Animal Tissues, R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of NAS of Ukraine.

Sample peripheral blood leukocytes were mixed with control cells and distributed to two microcentifuge tubes. The sample DNA was denatured during 10 min. at 82 °C. Hybridization solution without probe was injected to a control tube, and hybridization solution with fluorescein-conjugated PNA telomere probe to a sample tube. Hybridization was performed in the dark at room temperature overnight. The hybridization was followed by two 10-min post-hybridization washes with a Wash Solution at 40 °C. Pellets in the sample and control tubes were resuspended for further flow cytometric analysis. Samples hybridized with the Telomere PNA Probe/FITC exhibit fluorescence signal in FL1, which is higher than the background/autofluorescence signal obtained from the control of the same cells hybridized with the hybridization solution without probe. Cell gating for flow cytometry included cells in G0-G1-phases and gated cells were displayed for analysis in the FL3-height versus FL1-height dot plot. Standard three part differential gating was used for separate analysis of lymphocytes and granulocytes. The RTL value is calculated as the ratio between the telomere signal of each sample and the control cell (K562 cell line) with correction for the DNA index of G0/1 cells by a formula:

$$RTL = \frac{(\text{mean } FL1_{exp.cells} \text{ with PNA}) - (\text{mean } FL1_{exp.\ cells} \text{ without PNA})}{(\text{mean } FL1_{ctrl\ cells} \text{ with PNA}) - (\text{mean } FL1_{ctrl\ cells} \text{ without PNA})} \times 100$$

The differential analysis of apoptosis rate was conducted by the Annexin V-FITC Apoptosis Detection Kit I (BD, USA) by flow cytometry analysis with the use of standard protocol. Shortly, the cells (~1 x 10^6 cells/ml) were twice washed with cold PBS and then in 1X Binding Buffer (0.1 M Hepes/NaOH (pH 7.4) 1.4 M NaCl, 25 mM $CaCl_2$), mixed with 5 µl Annexin V and 5 µl PI, incubated for 15 min at RT in the dark, and analyzed by flow cytometry on a FACScan (BD, USA). Batch analysis of flow cytometry data was performed in Lysis II and WINMDI 2.8 software, statistical analysis of the results was conducted using SPSS 16.0. package.

RESULTS

The performed study has shown a decrease of the mean RTL values of PBL in all groups of radiation exposed individuals (Fig. 1). The most significant decrease was typical for a group of Chornobyl cleanup workers, while in the group of radiation workers differences with control values were less significant. In ARS survivors we have found an increased heterogeneity of RTL values.

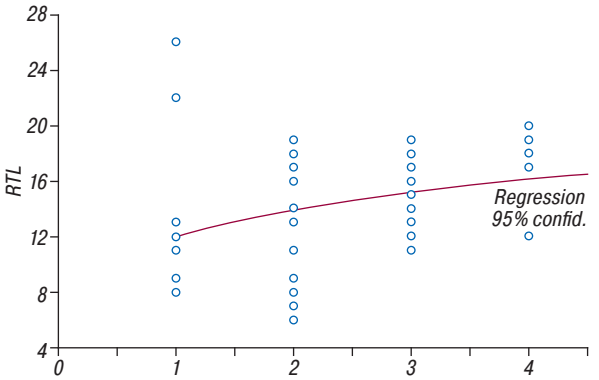

Fig. 1. RTL distribution plot in different groups of patients: 1 — ARS; 2 — Chornobyl clean-up workers; 3 — radiation workers; 4 — control group; Y-axis — RTL (%)

We have studied telomere length in leukocytes from 15 patients with myelodysplastic syndrome (MDS) that was developed after exposure to Chornobyl low-dose radiation. Bone marrow cells were obtained from 7 refractery anemia (MDS-RA) patients, 6 refractery anemia with excess of blasts (MDS-RAEB) and 2 refractery anemia with excess of blasts in transformation to leukemia (RAEB-t) patients and 12 age-matched healthy volunteers. The mean RTL values were higher in MDS-RAEB/RAEB-t patients as compared with MDS-RA (20.02±0.32 *vs* 14.36±0.75). Significant difference in mean RTL values between RAEB-t and control groups was demonstrated (Fig. 2). MDS-RA samples were characterized by high level of apoptosis in granulocytes and lymphocytes and significantly lower RTL index in comparison with that in healthy controls. Comparison of MDS-RA group with other groups of irradiated individuals has shown the absence of differences in spontaneous level of lymphocyte early apoptosis while the late apoptotic cell fraction (AnnexinV+PI+ cells) was significantly larger in RA group. with No difference in apoptosis was shown between exposed and non-exposed MDS-RA subgroups. Mean RTL indices in the radiation-exposed RA subgroup were lower in comparison with other groups except the cleanup workers group. Correlation was found between age and number of cells in early apoptosis, but not between age and RTL. RAEB-t cases have shown a pronounced decrease in the number of cells at all apoptosis stages. These results point on possible relationship between apoptosis induction and the telomere region changes in MDS. RA is associated with cell entry to the late apoptosis stage.

To study radiation-induced early changes of peripheral blood lymphocytes telomere length we have conducted the RTL study in "Shelter" staff exposed to a low

dose exposure during the shift works in 30-km exclusion zone and found a significant inverse correlation between the RTL and a radiation dose (Pearson r = -0,669, $p < 0.049$). The highest numbers of annexinV+PI- cells in early apoptosis stage and annexinV+PI+ cells in late apoptosis stage have been detected in the group of Chornobyl clean-up workers (Fig. 3, Fig. 4).

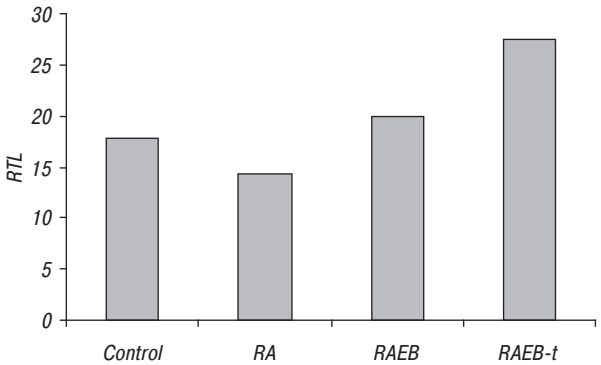

Fig. 2. Mean RTL indices in MDS group

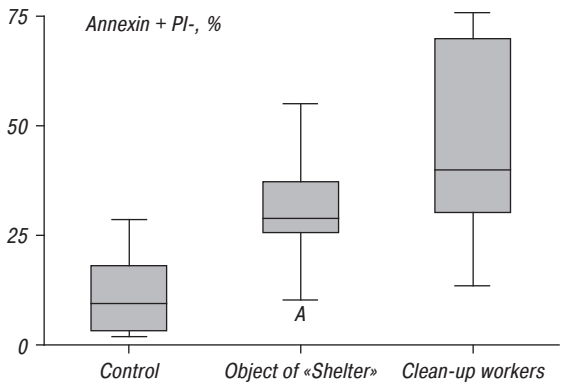

Fig. 3. Number of lymphocytes (%) in early stage of apoptosis in different groups of patients

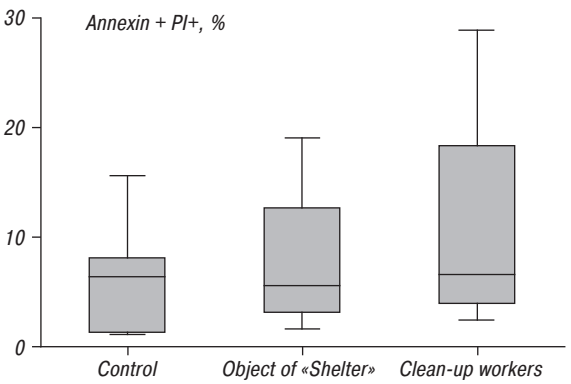

Fig. 4. Number of lymphocytes (%) at late stage of apoptosis in different groups of patients

The data on the relationship between the RTL values and apoptosis rate in PBL are presented in Fig. 4. The groups of persons with higher doses of irradiation are characterized by the declined RTL. For annexin+PI- fraction, however these changes are of an opposite type. The lower RTL values are associated with the higher apoptosis levels, especially notable in a group of clean-up workers (Fig. 5). Such type of response with a reduction of telomere length together with oxidative cell damage and genome instability might result in elimination of defective cell by apoptosis.

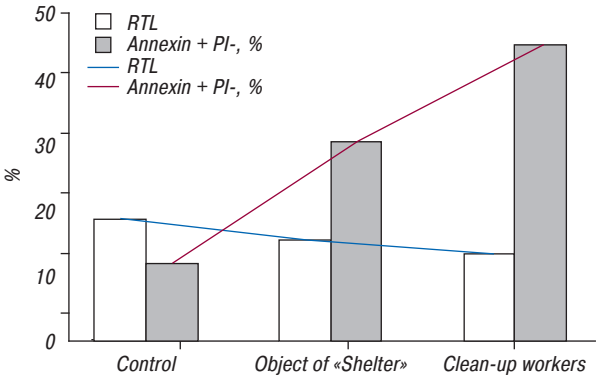

Fig. 5. Dependence between the RTL values and number of cells in apoptosis in different groups of patients

DISCUSSION

According to literature data, some dualism exists between telomere length and cancer development. Longer telomeres are associated with an extensive tumor growth and poor prognosis [10, 11]. A question appear, is it possible to consider such process as an increased replication senescence, or radiation-induced cellular senescence? In previous studies telomeres in a group of 181 individuals were associated with *in vitro* radiosensitivity as the group with short telomeres presented higher frequencies of ionizing radiation-induced micronuclei when compared to the long telomeres group and the individual telomere length is regarded as biomarker of individual chromosome instability upon exposure to ionizing radiation [12]. Later study hasn't shown differences in cell cycle regulatory proteins (cyclins A, B1, D1, E, and Ki67) and telomere length in radiation-induced and sporadic papillary thyroid carcinoma. Coming from the theory of cellular senescence, which is bound to reduction of telomere ends of chromosomes and our findings, the two ways of realization of cellular cycle could be probable: cells can be eliminated by apoptosis, or move to a "senile" phenotype. The last type could be of some positive importance, as far as cellular senescence decreases the proliferation rates and thus blocks carcinogenesis [13, 14]. *Vice versa*, an accumulation of genetic errors at senescence could also lead to cell transformation. The other way is a programmed cellular death. Realization of apoptosis includes a cascade of reactions, in which one of main roles is played by *TP53* gene and its product — p53. However, in cells with p53 mutations genome instability is increased, which promotes malignant transformation. Malignant cells overexpress telomerase that elongates telomeres and allows for plentiful divisions. Telomere elongation is a common molecular feature of advanced malignancies, but there is some evidence suggesting that short telomeres and concurrent chromosomal instability contribute to malignant cell transformation [11, 15].

Our data on MDS are in consistence with work of P. Scheinberg *et al*. [16]. In a cohort of 183 patients with severe aplastic anemia receiving immunosuppressive therapy, telomere length was unrelated to response but was associated with risk of relapse, clonal evolution to myelodysplasia, and overall survival. Authors suggest that telomere assessment may be of potential

diagnostic value in myeloprolipherative disease. In our study none of remarkable differences in telomere length have been demonstrated between MDS-RA and age-standardized controls. Specific diagnostic value of RTL seems disputable. In tumor pathology the low leukocyte telomere length with the associated cell subset changes seem to be a sign of a decreased anti-tumor surveillance as a factor promoting clonal growth. Therefore, low RTL values in clean-up workers peripheral blood lymphocytes possibly display a genetic instability in a late period after irradiation, as well as the development of cellular aging and potential susceptibility to oncologic transformation. There are suggestions that telomere shortening might be the main or even the sole mechanism of the natural and radiation aging [17].

Our study shows that there is a substantial change in telomere length as in the early and also in the late period after radiation exposure and these changes are related to changes of apoptosis rates. This suggestion is confirmed by other studies using the advances in the molecular pathology of hematopoietic stem cells which indicate that there exist a fine balance between various extrinsic and intrinsic signaling pathways in preserving the self-renewal and proliferative capacity of stem cells. Extrinsic signaling involves a microenvironment niche factors such as neighboring stromal cells, osteoblasts, and adipocytes secreting cytokines, chemokines, and metalloproteinases; intrinsic regulation involves Wnt/hedgehog/Notch signaling, DNA damage-induced epigenetic alterations, telomere shortening, and early senescence [18].

Data of our and other studies at the early period after radiation exposure demonstrate that ionising radiation induces a complex response including not only DNA, but a set of epigenetic changes, oxidative damage, membrane viscosity and expression of surface antigens molecules accompanied by somatic mutations in exposed cells [19].

The lowest RTL indices in a group Chernobyl clean-up workers after low-dose exposure were consistent with linear dose-effect dependency. At recovery after radiation exposure in ARS survivors we have found an increased heterogeneity of RTL. This has to be explained in future. One of the explanations is a stage switching of reparative mechanisms, and possibly telomerase and genes regulating T-loop. Previous experimental studies [10] and study of cancer patients give some support to such point of view, demonstrating different gene involvement in response to low-, middle and high-dose radiation. Influence of the heterogeneity of gene reactions triggering telomere shortening at different radiation doses and rates on anti-cancer immune control and cancer induction or promotion have to be elucidated in future.

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