THE EFFECT OF CHRONIC EXPOSURE ON THE FOXP3 CONCENTRATION IN LYSATES OF THE MITOGEN-STIMULATED MONONUCLEAR CELLS

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Disruptions of the Treg differentiation and functioning processes can play one of the crucial roles in the pathogenesis of radiation-induced malignant neoplasms in residents of the Techa riverside villages, who were chronically exposed in the low-to-medium dose range with predominant damage to the red bone marrow (RBM). This study aimed to determine the effect of radiation exposure, gender, age at the time of examination, and ethnicity on concentration of FOXP3 protein in lysates of mitogen-stimulated peripheral blood mononuclear cells in chronically exposed individuals in the period of cancer effects development. The main group consisted of 30 people aged 67–80 years, predominantly female and Turks. The comparison group included 10 unexposed individuals of similar age, gender, and ethnicity. In the main group, the mean dose to RBM was 867 mGr, to the thymus and peripheral lymphoid organs — 125 mGr. After 24-hour *in vitro* PHA stimulation, mononuclears were lysed, and the concentrations of the total protein and FOXP3 (using quantitative enzyme immunoassay) were measured. Among the different dose groups, there were no significant differences in FOXP3 concentration in mitogen-stimulated mononuclears (prior to the stimulation: 0 pg/ml in the comparison group and 3.50 ± 1.50 (0–27.19) pg/ml in the main group at $p = 0.349$; after the stimulation, respectively: 1.54 ± 1.51 (0–15.16) pg/ml and 9.71 ± 3.86 (0–77.92) pg/ml, $p = 0.512$). The variability of individual values is slightly higher in the main group than in the comparison group. Preliminary results allow concluding that the dose to RBM, thymus and peripheral lymphoid organs, age at the time of examination, gender, and ethnicity have no statistically significant effect on the concentration of FOXP3 protein in the lysates of the mitogen-stimulated peripheral blood mononuclear cells of chronically exposed people.

Keywords: chronic radiation exposure, the Techa River, intracellular concentration, FOXP3 transcription factor, peripheral blood mononuclear cells, Phytohemagglutinin

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ВЛИЯНИЕ ХРОНИЧЕСКОГО ОБЛУЧЕНИЯ НА КОНЦЕНТРАЦИЮ БЕЛКА FOXP3 В ЛИЗАТАХ МИТОГЕН-СТИМУЛИРОВАННЫХ МОНОНУКЛЕАРОВ КРОВИ

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Нарушения процессов дифференцировки и функционирования Treg могут быть одним из важнейших звеньев в патогенезе радиационно-индуцированных злокачественных новообразований у людей из когорты реки Течи, хронически облученных в диапазоне малых и средних доз с преимущественным поражением красного костного мозга (ККМ). Целью работы было определить влияние радиационного воздействия, пола, возраста на момент обследования и этнической принадлежности на концентрацию белка FOXP3 в лизатах митоген-стимулированных мононуклеарных клеток периферической крови у хронически облученных людей в период реализации канцерогенных эффектов. Основную группу составили 30 человек в возрасте 67–80 лет, среди них преобладали женщины и лица тюркской этнической группы. В группу сравнения вошли 10 необлученных человек аналогичного возраста, пола, этнической группы. В основной группе средняя доза облучения ККМ составила 867 мГр; тимуса и периферических лимфоидных органов — 125 мГр. После 24-часовой стимуляции ФГА *in vitro* мононуклеары лизировали, измеряли концентрацию общего белка и количественным иммуноферментным анализом — концентрацию FOXP3. Концентрация белка FOXP3 в митоген-стимулированных мононуклеарах статистически значимо не различалась у людей из разных дозовых групп (до стимуляции: 0 пг/мл в группе сравнения и 3,50 ± 1,50 (0–27,19) пг/мл в основной группе при $p = 0,349$; после стимуляции, соответственно: 1,54 ± 1,51 (0–15,16) пг/мл и 9,71 ± 3,86 (0–77,92) пг/мл, $p = 0,512$). Вариабельность индивидуальных значений несколько выше у людей из основной группы, чем в группе сравнения. По предварительным результатам, статистически значимого влияния дозы облучения ККМ, тимуса и периферических лимфоидных органов, возраста на момент обследования, а также пола, этнической принадлежности на концентрацию белка FOXP3 в лизатах митоген-стимулированных мононуклеаров периферической крови хронически облученных людей не выявлено.

Ключевые слова: хроническое радиационное воздействие, река Теча, внутриклеточная концентрация, фактор транскрипции FOXP3, мононуклеарные клетки периферической крови, фитогемагглютинин

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Today, the search for markers of individual radiosensitivity in the context of realization of long-term effects of radiation exposure is an urgent problem for modern radiobiology [1–3]. Radiation-induced carcinogenesis is one of the most significant effects of human exposure to ionizing radiation; its pathogenetic mechanisms are being actively investigated. There are various cohorts of people that run an increased long-term oncopathology risk after radiation exposure: survivors of atomic bombings [4], liquidators of radiation accidents [5], professionals working with sources of radiation [6], population living in radiation-contaminated areas [7, 8]. A particular cohort that belongs to this list are the residents of the Techa riverside villages. For them, the risks of morbidity and mortality from malignant tumors and leukemias are increased [9].

In the context of ensuring the optimal medical monitoring for persons running an increased risk of malignant neoplasms (MN), it is important to identify and verify the markers of predisposition to the development of radiation-induced oncopathology that enables optimization of approaches to the formation of high-risk groups in cohorts of people affected by radiation exposure [2]. Immunity indicators, primarily those characterizing the state of T-cells in the long term after radiation exposure, can be considered as such markers. For example, many of the mentioned residents of the Techa riverside villages had chronic radiation syndrome (CRS), and 65 years after the start of the exposure, they exhibited decreasing absolute numbers of $CD3^+$, $CD4^+$ and $CD8^+$ lymphocytes in peripheral blood, and increasing serum concentrations of IL4 and TNFα, compared to people of the same age and gender who received comparable doses but had no CRS in their medical histories [10].

In the context of pathogenetic mechanisms of radiation-induced carcinogenesis, a heterogeneous subpopulation of T-regulatory cells (Treg or $CD3^+CD4^+CD25^+FOXP3^+$ lymphocytes [11]) is particularly interesting. The FOXP3 transcription factor is specific to Treg; it supports control of differentiation and functioning of this subpopulation of lymphocytes [12]. It may be feasible to establish the level of this protein as one of the potential markers of predisposition to radiation-induced human oncopathology in the long term after chronic radiation exposure that predominantly involved damage to the central organ of hematopoiesis, the red bone marrow (RBM). The *FOXP3* gene dominantly controls the function of Treg, and its continuous expression guarantees that these cells fully preserve their suppressive ability [11]. The FOXP3 transcription factor represses IL2 transcription, increases expression of CD25 and other Treg markers. The mechanisms of Treg-mediated suppression are still a subject of discussion, but it is known that the regulation of FOXP3 protein expression is crucial for the control over immune responses, including antitumor immune surveillance [12].

Normally, FOXP3 protein interacts with the key transcription factors of T-lymphocytes, including NFAT, NFkB and AML1/Runx1, and others. Transcriptional and epigenetic regulation enables control over the *FOXP3* gene expression; a change therein entails alterations in the phenotype of T-cells and their functions [12]. In humans, expression of the *FOXP3* gene in most $CD4^+$-T-cells can be caused by prolonged stimulation of the T-cell receptor, with most $FOXP3^+$ T-cells having a low level of FOXP3 factor [12]. Transcription of the *FOXP3* gene can be initiated in effector T-cells upon antigen recognition during inflammation [13]. Due to their ability to inhibit antitumor immunity, Tregs promote development and progression of tumors. High infiltration of tumor tissue by Treg cells is associated with poor survival rate among patients with various types of MN [14]. The regulatory functions of Tregs infiltrating a tumor are realized via the COX2/PGE2 signalling pathway [15]. Currently, Tregs are being actively investigated as potential targets for oncotherapy [14], but there are few studies that cover modulating effects of ionizing radiation on the Treg cells' phenotype and functions, including expression of the *FOXP3* gene, concentrations and functional activity of the FOXP3 transcription factor [16].

The plasticity of Treg subpopulation, participation of the FOXP3 transcription factor in Treg differentiation, as well as the role of regulatory T-cells in radiation-induced carcinogenesis underpin the relevance of counting the FOXP3 protein in peripheral blood mononuclears donated by people from the Techa river cohort.

This study aimed to investigate the effect of radiation exposure, gender, age at the time of examination, and ethnicity, on concentration of the FOXP3 protein in lysates of mitogen-stimulated peripheral blood mononuclear cells sampled from chronically exposed residents of the Techa riverside villages at the time of realization of carcinogenic effects.

METHODS

We studied the blood samples donated by permanent residents of the Techa riverside villages that were chronically exposed to low dose rate radiation mainly from bone-seeking radionuclides (targeting RBM) at the premises of the Urals Research Center for Radiation Medicine of the FMBA of Russia. The dose of radiation received by each patient was assessed using the TRDS-2016 dosimetry system [17].

Before donating the blood, all patients underwent a medical examination in accordance with the established procedure. The inclusion criteria were: absence of acute inflammatory diseases, absence of exacerbations of chronic inflammatory diseases; absence of renal or hepatic insufficiency. The exclusion criteria were: acute cerebral circulation disruption incidents or traumatic brain injuries within three months before the study; confirmed oncological and autoimmune diseases; courses of hormone, antibiotic, chemo- and (or) radiotherapy; medical procedures using ionizing radiation within six months before the study.

The exposed group (main group) comprised 30 persons aged 67–80 years, the mean age being 72.4 ± 0.5 years. The mean accumulated RBM dose in this group was 876 ± 136 mGr, with the values ranging from 87 to 3716 mGr. The

Table. Concentrations of FOXP3 transcription factor in MNC lysates after mitogenic stimulation

Concentration, pg/ml		Comparison group, RBM: less than 0.07 Gy, *n* = 10	Main group, RBM dose: (0.07–3.72) Gy, *n* = 30	Subgroups of the main group, RBM dose:		
				from 0.07 to 0.49 Gy, *n* = 10	from 0.50 to 0.84 Gy, *n* = 10	from 0.85 to 3.72 Gy, *n* = 10
FOXP3 after 24 hours of incubation	without PHA	0	3.50 ± 1.50 (0–27.19)	2.63 ± 2.63 (0–26.33)	6.78 ± 3.52 (0–27.19)	1.09 ± 0.78 (0–7.32)
	with PHA	1.54 ± 1.51 (0–15.16)	9.71 ± 3.86 (0–77.92)	5.97 ± 4.88 (0–48.84)	13.42 ± 8.00 (0–77.92)	9.73 ± 7.28 (0–73.2)

Note: The data are presented as M ± SE (min–max).

mean dose accumulated by thymus and peripheral lymphoid organs was 125 ± 20 mGr, the range of values spanning from 28 to 446 mGr.

To study dose dependencies, we divided the main group into three subgroups similar in age, gender and ethnic makeup but different in the RBM doses received: minimal (0.07 through 0.49 Gy), moderate (0.50 through 0.84 Gy) and high (0.85 through 3.72 Gy). These subgroups were called dose groups; there were no statistically significant differences in qualitative characteristics between them and the comparison group.

The comparison group consisted of 10 people aged 63–82 years, none of whom was exposed to radiation in the context of industrial activities. The mean age in this group was 71.2 ± 2.0 years, mean accumulated RBM dose — 27 ± 4 mGr (values from 15 to 49 mGr), and the mean dose accumulated by thymus and peripheral lymphoid organs — 12 ± 3 mGr (values from 2 to 34 mGr).

Women were more numerous in both groups: 73.3% (22 persons) in the main group and 90.0% (9 people) in the comparison group. Eighty percent (24 individuals) of the main group participants were of Turkic origin, and in the comparison group this value was 70.0% (7 people). We found no significant differences in the age, gender, and ethnic composition of the main and comparison groups.

Fasting blood samples (4 ml) were taken under a standard protocol [18], from the ulnar vein, in the morning, into vacuettes containing sodium heparin. The fraction of mononuclear cells (MNCs) was isolated at the density gradient of 1.077 g/cm^3 (Biolot; Russia), washed twice with the modified Dulbecco′s phosphate buffered saline (Biolot; Russia). The incubation of MNCs lasted 24 hours at 37.0 ± 0.5 °C; for the process, we used the RPMI-1640 medium (HEPES 25 mM, $NaHCO_3$ 24 mM) (Paneco; Russia) with vitamins (Paneco; Russia) and L-glutamine 2 mM (Paneco; Russia), to which 10% fetal calf serum (Biolot; Russia) was added. Phytohemagglutinin-P (PHA) (Paneco; Russia) was added to the test sample of MNCs at the final concentration of 20 μg/ml, and the control sample of MNCs was completed with purified water in the amount equal to that of the mitogen solution. After stimulation, MNCs were precipitated, supernatant removed, and the samples were stored at minus 80 °C until the next stage. The thawing temperature was 2–8 °C; we induced hemolysis of erythrocyte impurities with a cooled ammonium chloride solution with pH 7.2–7.4 [19]. MNCs were washed with a cold phosphate-salt buffer (pH 7.4) (Sigma-Aldrich; USA). The concentration of cells was estimated using the Countess II FL (Thermo Scientific; USA) cell counter. We lysed the MNCs by freezing the samples three times at minus 20 °C and then defrosting them at room temperature as per recommendations of the manufacturer of the enzyme immunoassay test system (ELISA). Total protein content in cell lysates was determined in reaction with bicynchonicic acid (Merck test system; USA) in a 96-well tablet, with the help of a Lazurite analyzer (Dynex Technologies Inc.; USA). We relied on quantitative ELISA (Blue gene test system; China) to establish the content of FOXP3 transcription factor in the samples; the counting enabled by the same analyzer and followed by recalculation of the result for 1 μg of total protein in the sample. SigmaPlot software (demo version; SYSTAT Software, USA) was used for statistical data analysis. The normality of frequency distribution in the samples was checked with the help of the Kolmogorov-Smirnov test. The actual distribution was abnormal in all samples. For maximum clarity (the median value is zero in cases when concentration of the FOXP3 transcription factor in all or most of the samples is below the minimum detection limit of the ELISA system), we expressed the descriptive statistics data as arithmetic mean (M), error of mean (m) and a range of values (min–max). Datasets were compared using the Mann Whitney U-test, and for quality indicators we used the chi-squared test. Spearman's rank correlation coefficient enabled correlation analysis; the differences or relationships were considered significant at 95% confidence level.

RESULTS

The table below shows the results of quantification of the FOXP3 transcription factor in lymphocyte lysates after 24-hour incubation with PHA and without mitogen.

Comparing the main group, the three dose groups and the comparison group, we identified no significant differences in concentrations of the FOXP3 protein in MNC lysates incubated for 24 hours with PHA and without mitogen. The respective values were as follows: main group — $p = 0.349$ before stimulation, $p = 0.512$ after stimulation; three dose subgroups — $p = 0.706$, $p = 0.257$, $p = 0.450$ before stimulation, and $p = 0.940$, $p = 0.326$, $p = 0.597$ after stimulation (ascending order by the RBM dose).

In the groups of chronically exposed individuals with different accumulated doses, intracellular concentration of the FOXP3 transcription factor after *in vitro* mitogenic stimulation of the MNCs was slightly higher than in the comparison group.

Analyzing dose dependencies, we found no significant relationships between concentration of the FOXP3 transcription factor in the MNC lysates (stimulated (SR = 0.13; $p = 0.414$) and not stimulated (SR = 0.18; $p = 0.263$) with mitogen for 24 hours) and RBM and thymus/peripheral lymphoid organ doses (before stimulation: SR = 0.23, $p = 0.183$; after stimulation: SR = 0.09, $p = 0.602$).

We detected no effect of gender (before stimulation: SR = –0.08, $p = 0.609$, after stimulation: SR = –0.03, $p = 0.856$), age at the time of examination (before stimulation: SR = 0.02, $p = 0.915$; after stimulation: SR = 0.11, $p = 0.484$), ethnicity (before stimulation: SR = 0.05, $p = 0.767$; after stimulation: SR = –0.01, $p = 0.966$) on the studied indicators neither in the main group nor in the control group. Spearman's rank correlation was used for this analysis.

DISCUSSION

Radiation-induced carcinogenesis implies long-term realization of the effects. The reason behind this specificity is the complex of factors of non-radiation nature that affect the exposed individual, including the MN risk factors. A body that received sublethal doses activates compensatory adaptive mechanisms, which, when they function adequately, prevent oncotransformation of normal cells [10]. Immunocompetent cells are the main effectors of antitumor immune surveillance; MN pathogenesis largely depends on the disruptions of their activity [20].

The Techa floodplain was contaminated with radionuclides as a result of the Mayak Production Association activity; practically healthy residents of that area have been exposed to low dose rate ionizing radiation for many years and in the long term, they have persistent changes in the immune status, with such in the T-cell component of the immunity being most drastic. Accordingly, previous studies have reported decreased quantities of peripheral blood leukocytes (mainly because of neutrophils and lymphocytes), higher lysosomal activity of neutrophils, some suppression of the intracellular oxygen-dependent monocyte metabolism [1], and inclination of the

cytokine system towards a pro-inflammatory response [21].

On the one hand, regulatory T-cells can directly inhibit activity of cytotoxic T-cells, and on the other hand, they can be recruited or induced by the oncotransformed cells and cells of the tumor microenvironment, which allows them to avoid attack from the immune system. Treg lymphocytes can interfere with the activation and differentiation of $CD4^+$ and $CD8^+$ T cells, induce reactivity against autologous and tumor antigens [11].

In the tumor microenvironment, Tregs induction and differentiation occurs from T-lymphocytes with a strong immunosuppressive function, which suppress antitumor immunity and thus support tumor appearance and development. Tregs from the tumor microenvironment, in turn, can suppress the function of immune effector cells (using various mechanisms); they play an important part in the tumor's effort to elude immune surveillance [22–25]. Such Tregs can secrete TGFß, IL10, and IL35 [26], which inhibit antitumor immune response, suppress antigenic presentation in the dendritic cells as well as the T-helper function, and generate tumor-specific $CD8^+$ cytotoxic T-lymphocytes. The expression of IL10 and IL35 cytokines differs among subpopulations of the tumor microenvironment Treg cells; synergistically, they promote depletion of intratumor T-cells by regulating the expression of several inhibitory receptors [27]. Tregs are capable of direct cytolysis of other cells through secretion of perforin and granzymes, and they also synthesize and produce cyclic adenosine phosphate, thus affecting the metabolism of other cells [11, 14].

Data from mice experiments show that the share of tumor and splenic Tges grows after local irradiation at doses of 10 and 20 Gy, and the dose of 1.25 Gy (whole irradiation) brings down the total amount of $CD4^+$*FOXP3*$^+$-Treg in the lymph nodes [28].

In humans, ionizing radiation decreased the viability of human $CD4^+$ lymphocytes, and this effect is dose-dependent. There is evidence of a higher radioresistance of Tregs compared to $CD4^+$ lymphocytes, as well as of a dose-dependent reduction of expression of the *FOXP3* gene in Treg when the received doses are 0.940 Gy and 1.875 Gy. Compared to regular $CD4^+$ lymphocytes, natural (nTreg) and TGFß-induced (iTreg) regulatory T-cells exhibit increased resistance to radiation at a dose of 10 Gy. Forty-eight hours after exposure to this dose, the expression of *FOXP3* gene decreases in nTreg and in iTreg (more pronounced). After *in vitro* irradiation, the expression of *FOXP3* gene in iTreg goes down, but it does not affect differentiation into T-helpers of the first or second type. In $CD4^+CD25^+$-iTreg, the expression of the *T-BET* gene involved in the differentiation of cells into first type T-helpers was low before and after irradiation at a dose of 10 Gy, and the expression of the *GATA3* gene involved in the differentiation of lymphocytes into second type T-helpers decreased 48 hours after such exposure. Irradiation changes the expression of characteristic iTreg molecules. Exposed to ionizing radiation, iTregs increase the expression of *LAG-3* gene, decrease that of CD25 and CTLA-4 molecules and the ability of the cells to inhibit proliferation of $CD3^+CD8^+$ lymphocytes weakens [16].

The data from this study allow assuming functional integrity of the peripheral blood Tregs (in practically or conditionally healthy individuals) in the long term; inter alia, they preserve the ability to differentiate effected by the *FOXP3* gene and its key transcription factor, FOXP3 protein. The possible reason therefor is the high adaptive potential of the human immune and hematopoietic system, which realizes in the long term after chronic low-intensity radiation exposure with RBM as the primary target [1, 10, 21]. However, the presented data disallow excluding the possibility of aberrant local immune responses with participation of Tregs at the MN initiation stage in the exposed patients from the high oncological risk cohort. This problem requires a more thorough investigation.

The results of this study are generally consistent with the current scientific knowledge; its contribution thereto is information about the reaction of MNCs isolated from the peripheral blood of the chronically exposed people to *in vitro* mitogenic stimulation at the time of realization of carcinogenic effects of radiation in the studied population cohort.

CONCLUSIONS

Comparison of the main (chronically exposed) and comparison (not exposed) groups revealed no significant differences in the concentrations of the FOXP3 transcription factors in MNC lysates after 24 hours of incubation with mitogen and without PHA. Likewise, we found no evidence of the effect of RBM doses and doses to thymus and peripheral lymphoid organs to intracellular concentrations of the FOXP3 transcription factor in human peripheral blood MNCs after mitogen stimulation. There were no significant correlations between gender, age at the time of examination, ethnicity of the examined people and concentration of the FOXP3 protein in lysates of mitogen-stimulated MNCs. In the samples from practically healthy chronically irradiated people, we registered high variability of intracellular concentrations of the FOXP3 transcription factor after *in vitro* MNCs stimulation by mitogen, which indirectly confirms the hypothesis of presence of latent and, apparently, compensated functional changes in the mature immunocompetent cells. This indicator can be considered as one of the potential markers enabling assessment of individual radiosensitivity under chronic exposure to low and moderate doses with RBM as the primary target organ. It is necessary to further study the key transcription factors involved in differentiation of immunocompetent cells and ensuring their normal functioning in chronically irradiated population, as well investigate their role in radiation-induced carcinogenesis.

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