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Evaluation of the Interaction between Malignant and Normal Human Peripheral Blood Lymphocytes Under Cocultivation and Separate Cultivation

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Abstract—Using the Comet assay, the peculiarities of the interaction between malignant and normal human peripheral blood lymphocytes under cocultivation and separate cultivation were investigated. A decrease in Tail Moment was observed against an increase in the frequency of cells in the state of apoptosis in the culture of lymphocytes from conditionally healthy volunteers (bystander cells) under the influence of blood cells from patients with CLL (inductor cells). A statistically significant ($p < 0.001$) reduction both in the frequency of cells with high levels of DNA damages and apoptotic activity was established in the population of inductor cells under the influence of the bystander cells. The results obtained indicate the realization of both direct (effect of cells-inductors on bystander cells) and rescue (effect of bystander cells on cells-inductors) TIBE phenomenon.

Keywords: culture of human peripheral blood lymphocytes, tumor-induced bystander effect, Comet assay, DNA damage, apoptosis

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INTRODUCTION

Increased physiological activity of cells undergoing cancer transformation is accompanied by enhanced synthesis of some cytokines (in particular, TNFa, IL6, IL8) and stress messengers (NO, H_2O_2) and the release of microRNAs and DNA fragments into the extracellular space, which can affect normal cells and can directly or mediately alter their gene activity [1]. The consequences of this effect are reminiscent of the result of the interaction between irradiated and nonirradiated cells—bystander and rescue effects, respectively [1–4], by analogy with which it was called tumor-induced bystander effect: TIBE [5]. It is believed that the TIBE phenomenon contributes to the development of secondary malignancies in cancer patients [5, 6] in which risk may be increased after genotoxic chemotherapy or radio-oncotherapy, which is particularly dangerous for pediatric and young cancer patients [7–9]. In turn, the induction of the rescue effect is able to enhance the activation of repair systems in cancer-transformed cells under the influence of intact cells, which can have negative medical consequences [10, 11]. However, there is a probability of increased suppression of malignant cells due to activation of the abscopal effect by T-cell-dependent pathways, especially in response to the combined effect of radiation and immune oncotherapy [1, 12–16].

The relevance of the problem of the interaction of irradiated or nonirradiated human onco-transformed cells with normal cells of another type has stimulated research in this direction [11, 12, 16–18]. Markers of such interaction were micronuclei, cells in the stage of apoptosis, and some molecular genetic indicators. The results of these studies confirmed the reality of manifestation of direct and rescue TIBE. At the same time, the available scientific literature still lacks data on the features of interaction between malignant and

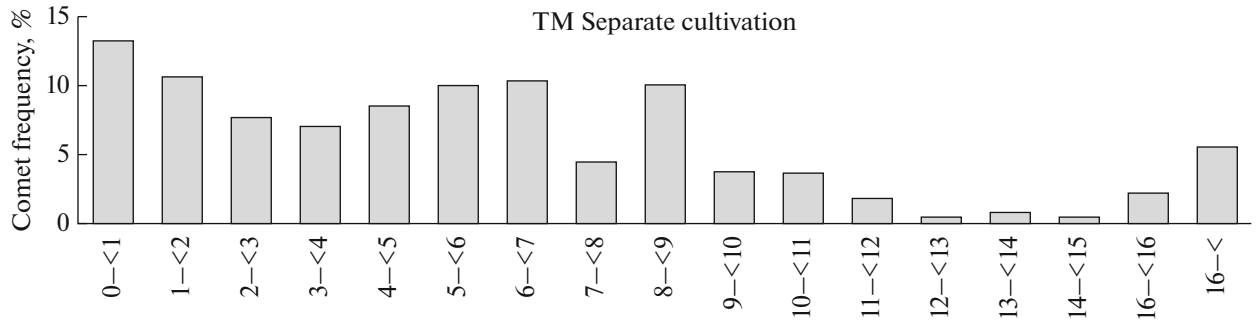

Fig. 1. Distribution of "comets" by TM after separate cultivation of PBL of conditionally healthy individuals.

normal somatic human cells of the same type, in particular, peripheral blood lymphocytes (PBL).

Based on the above, in the study of the TIBE phenomenon as a model of cancer-transformed cells we selected malignant hematopoietic cells—PBL of patients with chronic lymphocytic leukemia (CLL), since this form of hemoblastosis refers to adiation-associated pathologies increased in Ukraine as a result of the accident at the Chernobyl NPP [19]. Normal PBL of healthy individuals were selected as bystander cells.

The purpose of our study was the investigation of the mutual influence of these cells on each other during their cocultivation using molecular genetic indicators of genome damage.

MATERIALS AND METHODS

Four conditionally healthy volunteers (two males and two females) who denied conscious contact with known or potential mutagens, were included in the comparison group. The group of patients with B-cell CLL consisted of four people (two males and two females) who were examined and treated at the Radiation Hematology Department of the Clinical Radiology Institute of the National Research Center for Radiation Medicine (NAMS of Ukraine). The research included an analysis of 15600 cells, out of which were 8000 lymphocytes of conditionally healthy volunteers and 7600 malignant cells of cancer patients.

Venous blood was collected from patients prior to treatment. The cocultivation of PBL obtained from individuals from both groups was carried out for 48 h using a modified standard micromethod [20] in systems representing two containers separated by a membrane with a pore diameter of 1 μm.

The assessment of the relative level of DNA damage was performed using the method of single cell gel electrophoresis (Comet assay) under neutral conditions. For the preparation of slides, cell lysis, and neutral comet electrophoresis, a conventional technique was used [21]. After electrophoresis, the slides were stained with DAPI (4', 6-diamidino-2-phenylindole) and analyzed under a fluorescence microscope attached to a Canon D1000 camera. Images were processed using ImageJ (imagej.nih.gov) using the OpenComet plugin [22]. The Tail Moment (TM) was used as the main parameter for determination of the relative level of DNA damage [23]. For the assessment of the intensity of apoptosis in cell culture, the frequency of occurrence of "atypical" comets was analyzed. Statistical data processing was performed according to conventional methods [24].

RESULTS AND DISCUSSION

It was found that the individuals from the comparison group did not differ in spontaneous TM levels after separate PBL cultivation (it ranged from 6.28 to 6.60, $p > 0.05$) and mean TM level was 6.46 ± 0.27, which coincides with the results of our previous studies [20].

During the cocultivation of PBL of healthy individuals (bystander cells) with the blood cells of patients with CLL (inductor cells), the range of individual oscillations of TM was in the range from 3.60 to 4.77 and the group average was 4.16 ± 0.80, indicating a statistically significant ($p < 0.001$) decrease of TM in bystander cells. Thus, a decrease in the level of DNA migration into agarose gel in the bystander cells compared with the control cultures (separate cultivation) was recorded.

In order to determine the decrease in the level of TM in PBL of conditionally healthy individuals registered under the influence of cancer-transformed cells, the frequency distribution of comets was evaluated. All comets were divided by TM values into 17 groups (TMs from 0 to 16<). If the TM was equal to the limit value, the "comet" was assigned to the next group (Figs. 1, 2).

As can be seen from Fig. 1, an analysis of such distribution in separate cultured PBL of conditionally healthy individuals showed a predominance of TM cell populations ranging from 0 to 9, corresponding to a low level of genome damage. The distribution of comets among these groups was uniform. The pres-

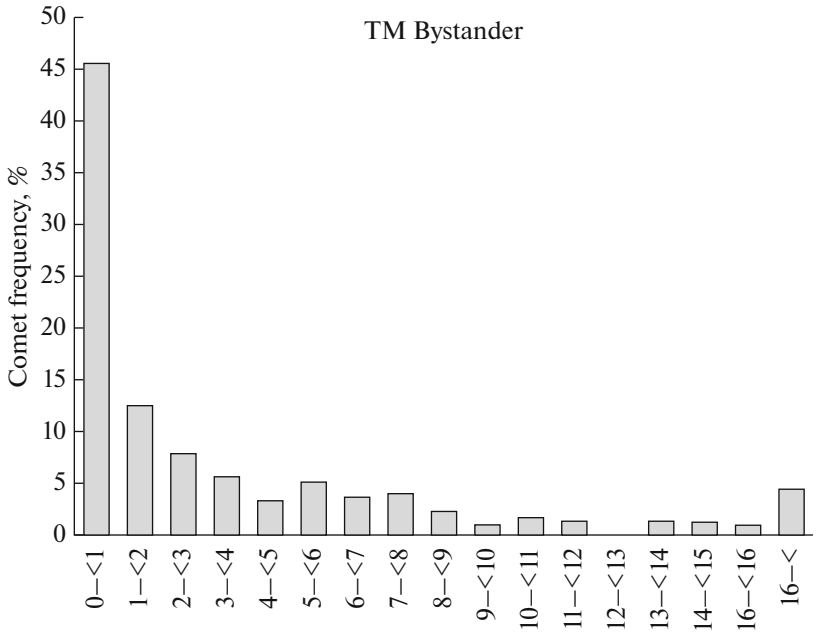

Fig. 2. Distribution of "comets" by TM after the cocultivation of PBL of conditionally healthy individuals with malignant blood cells of patients with CLL.

ence of a cell pool with a TM value of 16 (last group) was recorded.

Joint cultivation of normal and malignant PBL (Fig. 2) revealed a statistically significant ($p < 0.001$) increase in the frequency of comets of the first group (TM < 1).

One of the features of comet electrophoresis under neutral conditions is the lack of DNA migration into the agarose gel from cells in the S stage of the mitotic cycle [20]. These cells form the group with TM from 0 to 1. Thus, considering that the PBL cultures are conditionally synchronized, the result may be associated with the presence of a pool of intensely damaged cells with triggered sensitive checkpoint S/G_2, which resulted in their delay at this stage of the cell cycle.

It is known that in the malignant PBL of patients with CLL a significant increase in cytokine synthesis is detected which is one of the key elements in triggering the radiation-induced bystander effect [25, 26]. Probably, the influence of the onco-transformed cells also leads to the development of similar processes in a nonirradiated bystander cell. In response to the action of stressors from inductor cells (cytokines, miRNAs, DNA fragments), the synthesis of secondary stress messengers with an active form of oxygen (NO, H_2O_2) increases in bystander cells [1, 27], leading to the formation of single-DNA strand breaks and, consequently, the development of genomic instability [28]. As a result, cells with a high level of unrepaired genomic damage do not pass the checkpoint at the S stage of the cell cycle. The obtained result confirms the effect of malignant hematopoietic cells of CLL patients on the stability of the PBL genome of healthy individuals, e.g., the realization of the TIBE phenomenon.

The data on the analysis of the frequency of DNA damage in the blood cells of patients with CLL during their cocultivation and separate cultivation with PBL of conditionally healthy individuals are represented in Figs. 3 and 4.

As can be seen from the data shown in Fig. 3, in a separate cultivation of patient cells it was dominated by "comets" with a maximum value of TM > 16, indicating a high level of genomic instability in cancer-transformed cells. At the same time, during the cocultivation of cells of CLL patients with PBL of conditionally healthy individuals (Fig. 4), a statistically significant ($p < 0.001$) decrease in the frequency of comets with TM > 16 and an increase in the population of cells with TM from 0 to 6 was revealed. The obtain data suggest the occurrence of an effect that is similar in its action to the radiation-induced rescue effect, when there is an increase in the repair processes in the inductor cells under the influence of bystander cells [1, 27, 28]. However, not all cells in the blood of CLL patients were in a state of malignant transformation; therefore, the obtained result raised the question: which of the cell populations is involved in these processes: onco-transformed or normal? When the enhancement of repair processes occurs in malignant cells, does it increase their resistance? The answer to these questions requires further research that will provide an understanding of the potential risk of secondary malignancies in cancer patients and its prevention.

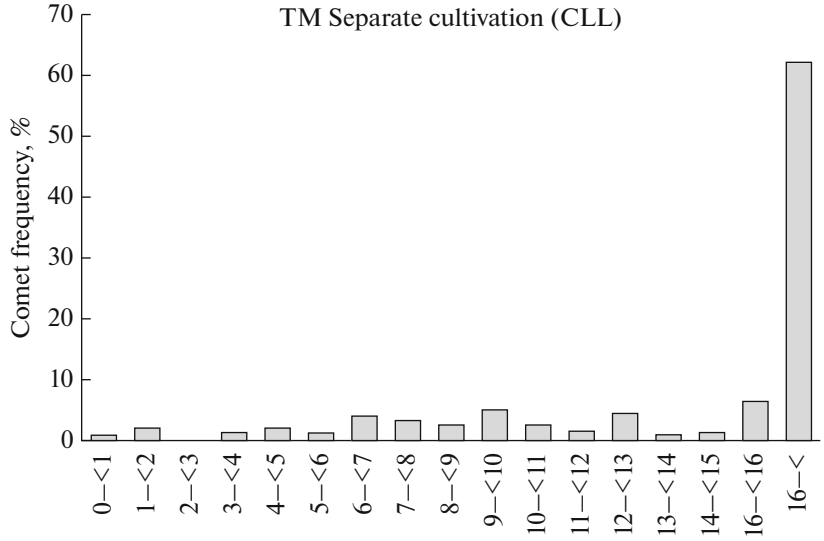

Fig. 3. Distribution of "comets" by TM after the separate cultivation of cells of patients with CLL.

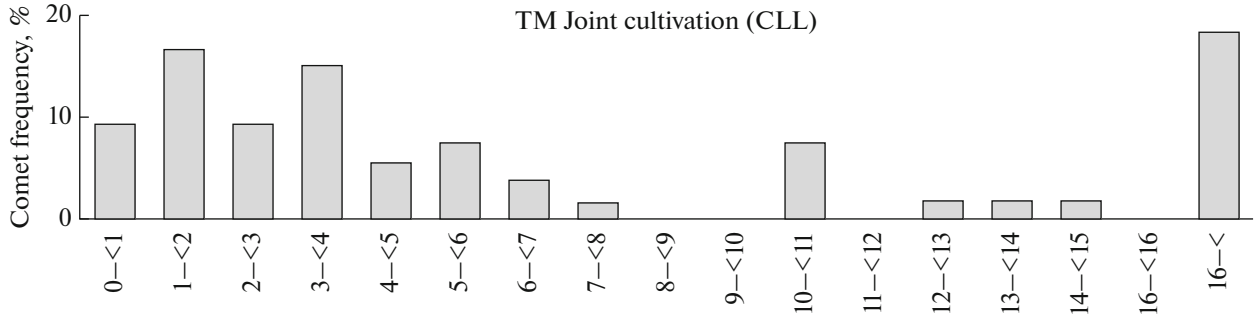

Fig. 4. Distribution of "comets" by TM after the cocultivation of blood cells of patients with CLL with normal PBL of conditionally healthy individuals.

Data on apoptogenic activity in PBL culture of conditionally healthy individuals and patients with CLL are represented in Fig. 5.

During the separate cultivation of PBL of individuals in the comparison group, the spontaneous level of apoptotic activity ranged from 1.11 ± 0.67 to 2.89 ± 1.33 per 100 cells ($p > 0.05$) and the mean value was 2.00 ± 0.60 per 100 cells, which is consistent with our previous data [20]. In the PBL culture of CLL patients a statistically significant ($p < 0.05$) increase of individual cell frequencies in the state of apoptosis with a range of individual oscillations of 3.11 ± 1.01 to 6.33 ± 1.33 per 100 cells, respectively, and their group average level (4.94 ± 1.33 per 100 cells) compared to the corresponding PBL cultures of conditionally healthy individuals was observed.

The cocultivation of malignant hematopoietic cells of patients with CLL (inductor cells) and PBL of conditionally healthy volunteers (bystander cells) revealed multidirectional processes: statistically significant increase in the average frequency of cells in the state of apoptosis in a population of bystander cells (7.8 ± 0.97 per 100 cells, $p < 0.001$) but a decrease in this parameter in inductor cells (2.40 ± 0.63 per 100 cells, $p < 0.01$).

It is known that the occurrence of the radiation-induced bystander effect is accompanied by an increase in the frequency of apoptosis among the bystander cells [30]. It is possible that the established change in apoptotic activity in PBL of conditionally healthy individuals during cocultivation with cells of patients with CLL is related to the response to this effect, despite the active participation of tumor necrosis factor TNF-α and other cytokines in the development of the classical bystander effect [1] and taking into consideration the enhanced synthesis of these substances by cancer-transformed cells of CLL patients [25, 26, 29].

Particular attention is drawn to the results on the reduction of apoptotic activity in the culture of cells of

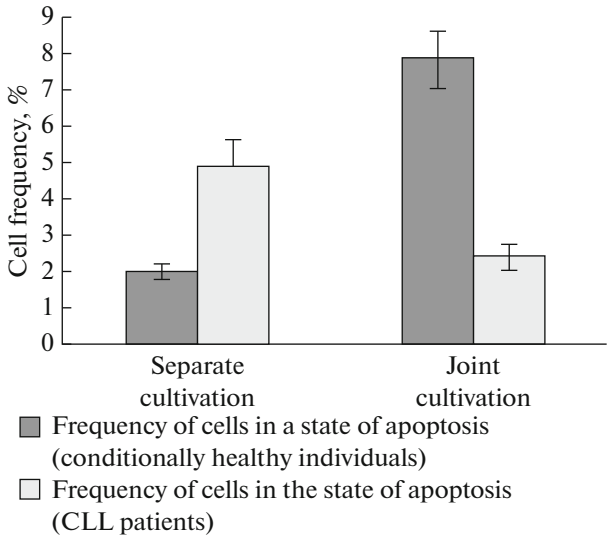

Fig. 5. Comparison of apoptotic cell frequencies after separate cultivation and cocultivation of the cells of CLL patients and PBL of conditionally healthy individuals.

CLL patients during cocultivation with PBL of conditionally healthy individuals. A statistically significant ($p < 0.01$) decrease in the average group frequencies of cells in the state of apoptosis from 4.94 ± 0.67 per 100 cells (separate cultivation) to 2.40 ± 0.63 per 100 cells (cocultivation) may be associated with the activation of repair processes. A possible explanation for the results is the activation of the NF-<B dependent pathway of apoptosis blocking in CLL patients [31], which was caused by the effect of PBL of conditionally healthy individuals. This assumption needs further validation.

The obtained results indicate that there is a mutual influence of normal and oncologically transformed human somatic cells and confirm the possibility for induction of direct and rescue TIBE phenomena.

CONCLUSIONS

The cocultivation of hematopoietic cells of CLL patients with intact peripheral blood lymphocytes of conditionally healthy individuals leads to an increase in the level of DNA damage and an increase in the frequency of apoptosis in normal cells, similar to the direct bystander effect. The cocultivation of hematopoietic cells of CLL patients with intact peripheral blood lymphocytes of conditionally healthy individuals led to a decrease in genomic instability and inhibition of apoptosis in induction cells, similar to the rescue bystander effect.

FUNDING

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflict of interest.

Statement of compliance with standards of research involving humans as subjects. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the World Medical Association Declaration of Helsinki (2008) and ethical principles adopted by the First National Congress of Ukraine on Bioethics (2001). Informed consent was obtained from all individual participants involved in the study.

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