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ОРИГІНАЛЬНІ ДОСЛІДЖЕННЯ

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The Effect of Radiotherapy Patients' Blood Plasma on the Apoptosis Rate in Unirradiated Human Leukocytes

Вплив плазми крові пацієнтів після променевої терапії на рівень апоптозу в неопромінених лейкоцитах людини

Мета роботи: Існують суперечливі дані щодо радіаційно-індукованого токсичного «ефекту свідка», опосередкованого плазмою опроміненої крові людини. У зв'язку з цим метою даної роботи було оцінити можливість індукції апоптозу в лейкоцитах периферичної крові людини внаслідок дії плазми крові онкологічних хворих, які отримували променеву терапію (ПТ).

Матеріали і методи: Плазму було виділено з крові 18 хворих на рак тіла матки до і після курсу дистанційної ПТ ^{60}Co на зону малого таза (20 × 2 Гр, 5 сеансів на тиждень на апараті РОКУС-АМ). Лейкоцити, виділені з крові здорового донора, витримували в культурі у присутності плазми крові пацієнток протягом 24 год при температурі 37° С. За контроль правили культури без плазми і культури лейкоцитів, виділених із донорської крові після впливу γ-проміння *in vitro* в дозі 2 Гр. Апоптоз у лейкоцитах вимірювали за допомогою тесту Аннексин V методом проточної цитофлуориметрії.

Результати: Плазма крові онкохворих, отримана як до, так і після курсу ПТ, не викликала ефекту у вигляді підвищення рівня апоптозу у клітинах-відповідачах. На відміну від цього, безпосереднє опромінення призводило до значущого зростання виходу апоптотичних клітин, що перевищувало будь-які флуктуації виживаності клітин-відповідачів, культивованих у присутності плазми крові онкохворих.

Висновки: Визначення можливих немішенних ефектів радіаційного впливу, опосередкованих плазмою крові онкопацієнтів після ПТ, потребує інших тестів замість вимірювання апоптозу в неопромінених нестимульованих лейкоцитах.

Ключові слова: «ефект свідка», променева терапія, апоптоз, плазма крові людини, лейкоцити, цитотоксичні фактори плазми.

Цель работы: Имеют место противоречивые данные о радиационно-индуцированном токсическом «эффекте свидетеля», опосредованном плазмой облученной крови человека. В связи с этим целью данной работы было оценить возможность индукции апоптоза в лейкоцитах периферической крови человека под действием плазмы крови онкологических больных, получавших лучевую терапию (ЛТ).

Материалы и методы: Плазму выделяли из крови 18 больных раком тела матки до и после курса дистанционной ЛТ ^{60}Co на область малого таза (20 × 2 Гр, 5 сеансов в неделю на аппарате РОКУС-АМ). Лейкоциты, выделенные из крови здорового донора, выдерживали в культуре в присутствии плазмы крови пациенток в течение 24 ч при температуре 37°С. Контролем служили культуры без плазмы и культуры лейкоцитов, выделенных из донорской крови после γ-облучения *in vitro* в дозе 2 Гр. Апоптоз в лейкоцитах измеряли с помощью теста Аннексин V методом проточной цитофлуориметрии.

Результаты: Плазма крови онкобольных, полученная как до, так и после курса ЛТ, не вызывала эффекта в виде повышения уровня апоптоза в клетках-респондентах. В отличие от этого, непосредственное облучение приводило к значимому возрастанию выхода апоптотических клеток, которое превышало все флуктуации выживаемости клеток-респондентов, культивированных в присутствии плазмы крови онкобольных.

Выводы: Оценка возможных немишенных эффектов радиационного воздействия, опосредованных плазмой крови онкопациентов после ЛТ, требует применения иных тестов вместо измерения апоптоза в необлученных нестимулированных лейкоцитах.

Ключевые слова: «эффект свидетеля», лучевая терапия, апоптоз, плазма крови человека, лейкоциты, цитотоксические факторы плазмы.

Objectives: There are rather contradictory data concerning radiation-induced, toxic bystander effects mediated by human irradiated blood plasma. Therefore, the aim of the present study was to assess whether cancer patients' blood plasma after radiotherapy can induce an apoptosis in primary human peripheral blood mononuclear cells (PBM).

Material and Methods: Plasma was collected from blood of 18 uterine cancer patients before and after ^{60}Co radiotherapy to the pelvis (20 × 2 Gy, 5 fractions/week). Healthy donor's PBM were separated with Histopaque and held in medium with the test plasma for 24 h at 37 °C. The controls were plasma-free cultures and cultures of PBM separated from the donor's blood given 2 Gy γ-rays *in vitro*. Apoptosis in reporter PBM was measured by the Annexin V test using flow cytometry.

Results: Patients' blood plasma collected either before or after radiotherapy did not produce any apoptotic response above the control level in reporter PBM. By contrast, direct irradiation caused significant apoptotic death in PBM, which yield exceeded any fluctuations of reporter PBM survival caused by patients' plasma.

Conclusions: Other assays instead of apoptosis in unirradiated quiescent leukocytes should be applied for detecting possible untargeted radiation effects mediated by radiotherapy patients' blood plasma.

Key words: bystander effect, radiotherapy, apoptosis, human blood plasma, leukocytes, cytotoxic plasma factors.

The phenomenon of the 'bystander effect', i.e. the spatial expanding of initial radiation response, mediated by signals from irradiated to unirradiated part of cell population, was observed in numerous experiments [1]. A comprehensive classification of bystander-type scenarios was recently suggested [2], and in the new paradigm of radiobiology these effects are considered as an important possible mechanism for radiation-induced pathology

in humans [3-5]. In the situation of partial body irradiation, particularly during radiotherapy, the additional mutations or depleting of unirradiated part of the cell population produced via bystander mechanisms might be a source of potential hazard for human health.

For human blood lymphocytes, which are very popular test system in radiobiological research, the bystander signaling *in vivo* naturally occurs via blood plasma, and there are plenty of experimental data on the radiation-induced clastogenic plasma factors causing chromosomal damage in bystander lymphocytes (reviewed in [6, 7]). There are also examples of measuring the effect of irradiated blood plasma on clonogenic survival in other cell types [8, 9]. However, the reports about the possible production of a bystander signal by patients' cells after irradiation either *in vivo* or *ex vivo* contain rather uncertain conclusions [9-11]. Moreover, it remained non-examined whether plasma of irradiated blood might directly induce cell death in quiescent human leukocytes, despite this type of cells has a high natural susceptibility to apoptosis and thus comprises an obvious target for such cytotoxic action in any 'true life' radiation exposure scenario.

To address specifically this question, recently we performed the experiment, in which the potential cytotoxicity of human blood plasma after irradiation *in vitro* was measured in human peripheral blood mononuclears (PBM) by assaying the latter for apoptosis using the Annexin V method [12]. It had been shown, that plasma collected from unirradiated blood or blood exposed *in vitro* to 2-40 Gy γ-rays did not induce early apoptosis or late apoptosis/necrosis in reporter human G_0 PBM. Still, for checking the reproducibility of these *in vitro* data and strengthening their clinical relevance, such conclusions must be validated on material from *in vivo* irradiated individuals. Therefore, in order to finalise this research, the aim of the present work was to assess the yield of apoptosis in unirradiated healthy donor's G_0 PBM cultured in presence of blood plasma taken from cancer patients before and after radiotherapy.

Material and Methods

Radiotherapy patients

Eighteen patients with uterine cancer were selected at the clinic of the Grigoriev Institute for Medical Radiology (IMR) (National Academy of Medical Science of Ukraine), Kharkiv, Ukraine, in May-June 2006. The study was carried out with the approval of the IMR Committee on Ethics in Biomedical Research. Patients' participation was in accordance with the local ethics protocols including their written informed consent. The samples were coded, and anonymised information about their diagnoses and treatment details was provided by the clinicians. The patients were selected in order to achieve minimum variation in age (52-67 y) and tumour grade. According to the "Tumour – Nodules – Metastases" (TNM) classification they had $T_{2a\text{-}c}N_xM_0$ disease. The treatment protocol consisted of post-operative external ^{60}Co therapy without chemotherapy. All received 40 Gy in twenty 2 Gy fractions, 5 per week, at a rate of ~1 Gy min^{-1} to the pelvis using parallel antero-posterior/postero-anterior fields measuring 14×16 or 16×18 cm. The treatment field surface was enclosed by lead blocks, so that total body exposure resulting from scattered radiation did not exceed 1% of the tumour doses.

The blood for separating off plasma for apoptosis testing was taken 1 or 2 days before treatment and again 1 or 2 days after the final fraction. The blood (5 ml) was collected by venopuncture into the sterile syringe containing lithium heparin anticoagulant.

It should be underlined that all subsequent procedures of testing blood plasma against unirradiated reporter PBM were performed using an experimental protocol, which was established as being optimum for such assay in the preceding *in vitro* study [12].

Plasma separation

To separate plasma, patients' whole blood was centrifuged for 15 min at 170 *g*; the supernatant was collected by a syringe, avoiding disturbance of the cell pellet, transferred into Eppendorf tubes, immediately frozen and stored at −20 °C until use. This permitted a number of plasma samples to be tested later in one round, thus avoiding day-to-day variations in reporter cell cultures. Regarding the methodological correctness of such an approach, there are a number of reports [6, 7, 13-16] stating that freezing of plasma does not affect its clastogenic properties.

Cell culture

The reporter cells were healthy donor's peripheral blood mononuclear cells (PBM). Peripheral blood was taken with informed consent and according to the local institutional ethics procedures from a healthy male, age 33 y, with no occupational or medical exposure to any cytotoxic substances or clastogens, except usual dental checks. The blood was collected into Vacutainer™ tubes containing lithium heparin anticoagulant. PBM were separated from whole blood using Histopaque 1077 (Sigma, Poole, UK) according to the manufacturer's standard instructions and washed in Hank's balanced salt solution (Sigma, Poole, UK).

For each data point three identical cultures were set up, each comprising 2.0×10^6 cells placed into 4 ml of Eagle's MEM supplemented with 20% heat inactivated fetal calf serum, L-glutamine, 100 IU/ml penicillin and 100 мg/ml streptomycin (all reagents from Invitrogen, Paisley, UK). To these cultures 0.2 ml of plasma assigned for testing was added. Before adding the plasma it was thawed at room temperature for 20 min and centrifuged at 11000 *g* for 1 min for additional sedimentation of any possible cellular material. Cultures were coded and incubated at 37 °C with 5 % CO_2 in air for 24 h.

Apoptosis measurements by the Annexin V assay

After incubation the medium was removed from cultures; the cells were washed three times in cold (4 °C) phosphate buffered saline (PBS) pH 7.4 (Sigma, Poole, UK) and stained with a fluorescein isothiocyanate (FITC)-Annexin V kit (Becton Dickinson, Oxford, UK) according to the manufacturer's instructions. The cells were then run on a flow cytometer (Becton Dickinson FACS Calibur™, 488 nm argon-ion laser) equipped with CellQuest™ software for data acquisition and analysis. FITC-Annexin V positive apoptotic cells were identified using FL1 detector histogram plots. Non apoptotic, early apoptotic, and late apoptotic/necrotic

cell populations were distinguished by simultaneous staining with propidium iodide (PI) and using FL1/FL3 quadrant dot plots.

Fixed gating parameters were used for all samples analysed in order to prevent variations related to flow-cytometric parameters within or between experiments. Two repeated cell staining/ FACS analyses per measurement were performed in each of three replicate cell cultures, which were set up for each data point.

Controls: In vitro irradiated and unirradiated PBM in plasma-free cultures

To check for proper Annexin V staining, negative and positive controls were included into the experiment. These, respectively, were PBM separated from donor's unirradiated blood and blood after exposure *in vitro* to 2 Gy γ-rays, and cultured with no patients' plasma added. For the positive control vacutainers of donor's blood were exposed to ^{60}Co dose of 2 Gy at the dose rate 0.5 Gy min^{-1}. The zero dose control sample, from which the PBM for negative control were obtained, was sham treated and transported identically with the matched irradiated sample. During the period between sampling and PBM separation the irradiated and sham-irradiated blood was kept at 37 °C. Negative and positive control PBM cultures were set up identically to that of plasma-treated cells and assayed in each round of cell staining/FACS analyses performed for plasma-treated cultures.

Statistics

Testing of patients' plasma against healthy donor's PBM was repeated twice, with one month time interval between experiments, each time for the total set of 18 pairs of matched plasma samples collected before and after radiotherapy. A good reproducibility of staining and measuring procedures was judged from very low inter-experiment variations in apoptotic cell outcome in the control and plasma-treated PBM cultures at any particular data point.

In all experimental series each irradiated blood plasma sample was rigorously matched to the unirradiated blood plasma, and tested concurrently. Thus, to minimize any possible influence of the intra-donor variability, statistical analysis of the data was focused on the difference for apoptotic or live cell yields between cultures treated by patients' blood plasma after radiotherapy and their control counterparts containing blood plasma obtained before radiotherapy.

The results of all measurements in this work are presented as mean values and their standard errors (SE) of apoptotic and live cell yields combined from two independent experiments, with two repeated cell staining/FACS analyses per measurement performed in each of three replicate cell cultures, which were set up for each data point. Significance of differences was determined by a Student's *t*-test (paired or unpaired, as indicated in the text), considering the differences to be significant if $p \leq 0.05$.

Results

The cellular changes in human PBM undergoing apoptosis, as detected by FITC-Annexin V/propidium iodide staining with addition of Hoechst 33258 (the latter was used for the visualization of living cells) are presented on Figure 1. The typical profiles obtained by running intact control cells and irradiated cells through the flow cytometer are shown in Figure 2. Cell staining profiles in irradiated cell cultures (positive controls) always contained a distinctive enhanced second peak representing Annexin V (FITC)-positive, apoptotic cells.

In all experimental rounds the reporter PBM cultures set up with patients' plasma collected either before or after radiotherapy showed normal profiles of cell survival; live cell yields varied from 87.4 to 92.9%, and total

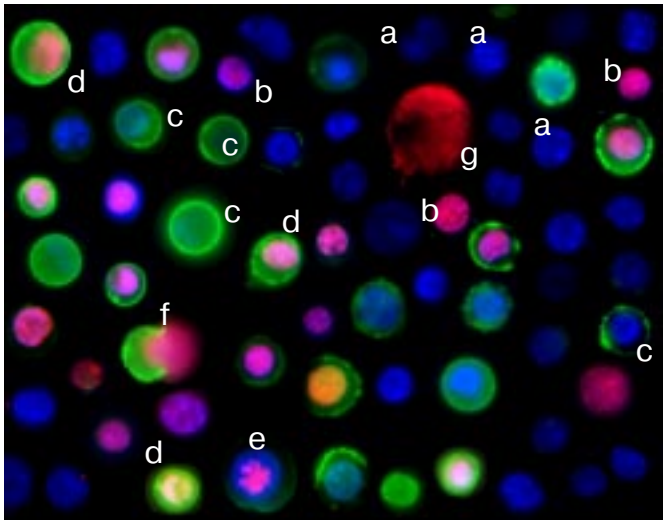

Figure 1. Apoptosis in human peripheral blood mononuclear cells (PBM). Healthy donor's blood was given 2 Gy γ-rays *in vitro*; PBM were separated with Histopaque, held in medium for 24 h at 37 °C and stained with FITC-Annexin V, propidium iodide (PI) and Hoechst 33258. Stained cells were dropped onto slide and subjected to image capturing on the fluorescent microscope Nikon (Japan) equipped with an appropriate filter set and camera. The photo was taken on the triple filter at the magnification ×1000 and acquainted using the Isis image processing system. FITC-labeled Annexin V is expressed on the membrane surface in apoptotic cells; PI accumulates in nuclei of necrotic cells, when the membrane becomes totally compromised. Healthy, non-apoptotic, living cells are FITC-negative and stained only in blue with Hoechst 33258 (a). Annexin V-negative, PI-positive are fully necrotic cells (b). Annexin V-positive, PI-negative are early apoptotic cells (c); Annexin V-positive, PI-positive are late apoptotic / necrotic cells (d). Initial stage of late apoptosis (e), destruction of a membrane in a late apoptotic cell (f) and a post-necrosis destruction of a monocyte (g) are also shown. Note, that staining for flow cytometry does not include Hoechst 33258

Рисунок 1. Апоптоз в лейкоцитах периферичної крові людини. Кров здорового донора опромінювали *in vitro* γ-променями в дозі 2 Гр; лейкоцити виділяли за допомогою Гістопаку, витримували в живильному середовищі протягом 24 год при 37°С і забарвлювали FITC-Аннексином V, пропідій йодидом (ПІ) і Hoechst 33258. Суспензію забарвлених клітин наносили на предметне скло та фотографували за допомогою флуоресцентного мікроскопа Nikon (Японія), оснащеного необхідним набором фільтрів і фотокамерою. Фотографію зроблено на трипл-фільтрі (одночасна візуалізація трьох флуорохромів), при збільшенні × 1000, з розпізнанням зображення за допомогою програмного пакета Isis. FITC-мічений Аннексин V експресується на поверхні мембрани апоптотичних клітин; ПІ накопичується в ядрі некротичних клітин, коли бар'єрна функція мембрани повністю порушується. Нормальні, не-апоптотичні, живі клітини є FITC-негативними і забарвленими у блакитний колір Hoechst 33258 (a). Аннексин V-негативні, ПІ-позитивні є клітинами в некрозі (b). Аннексин V-позитивні, ПІ-негативні клітини — ранній апоптоз (c); Аннексин V-позитивні, ПІ-позитивні клітини — пізній апоптоз / некроз (d). Крім того, показано початкову стадію пізнього апоптозу (e), руйнування мембрани клітини в пізньому апоптозі (f) і постнекротичний розпад моноцита (g). Слід зазначити, що забарвлення для аналізу методом проточної цитофлуориметрії не включало Hoechst 33258

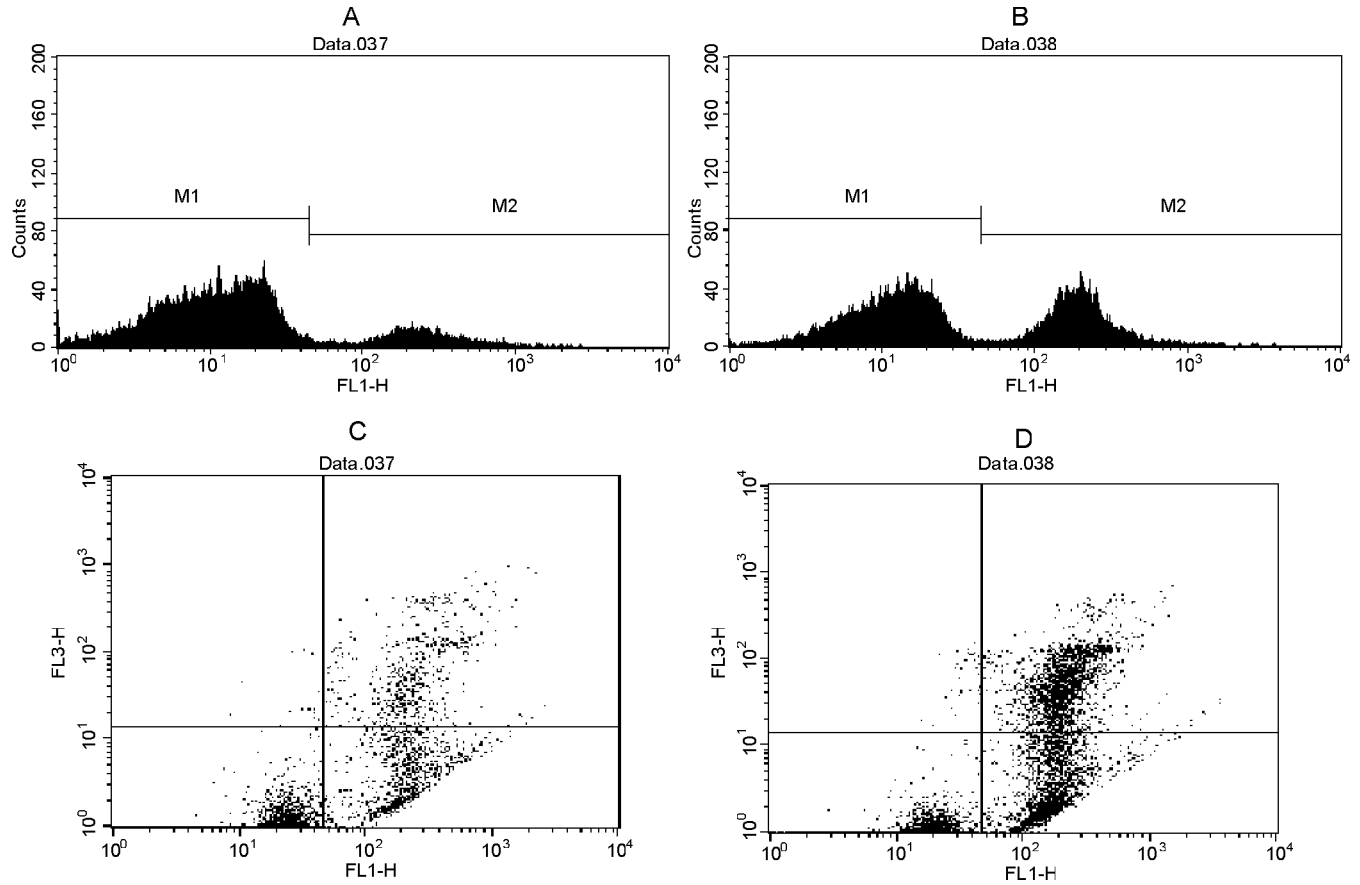

Figure 2. Typical Annexin V / propidium iodide flow cytometry profiles from cultures of unirradiated and irradiated PBM. A and B – histogram plots of Annexin V staining (M1 are FITC-negative, non-apoptotic cells; M2 are Annexin V positive, apoptotic cells); C and D – Quadrant dot plots of Annexin V (FL1) and PI (FL3) staining (lower left Annexin V-negative, PI-negative are healthy living cells; lower right Annexin V-positive, PI-negative are early apoptotic cells; upper right Annexin V-positive, PI-positive are late apoptotic / necrotic cells; upper left Annexin V-negative, PI-positive are fully necrotic). A and C – unirradiated PBM; B and D – PBM irradiated to 2 Gy γ-rays. Note a distinctive second peak (M2) in Fig. 2B compared with Fig. 2A, and a respective shift of cells from the lower left to lower and upper right parts of the graph in Fig. 2D compared with Fig. 2C, representing the accumulation of early apoptotic and late apoptotic/necrotic cells, respectively, in irradiated PBM culture

Рисунок 2. Типові профілі, отримані на проточному цитофлуориметрі при аналізі культур неопромінених і опромінених лейкоцитів крові людини, із забарвленням клітин Аннексином V / пропідій йодидом.
А і В — гістограми розподілу клітинної популяції за забарвленням Аннексином V (М1 — FITC-негативні, не-апоптотичні клітини; М2 — Аннексин V-позитивні, апоптотичні клітини); С і D — розподіл клітин між квадрантами за сумісним забарвленням Аннексином V (FL1) і ПІ (FL3): зліва внизу — Аннексин V-негативні, ПІ-негативні нормальні, живі клітини; зліва вгорі — Аннексин V-позитивні, ПІ-негативні клітинни на ранній стадії апоптозу; справа вгорі — Аннексин V-позитивні, ПІ-позитивні клітини на пізній стадії апоптозу; зліва вгорі — Аннексин V-негативні, ПІ-позитивні клітини в некрозі. А і С — неопромінені лейкоцити; В і D — лейкоцити з крові, підданої дії γ-променів *in vitro* в дозі 2 Гр. Слід відмітити виразний другий пік (М2) на рисунку 2В у порівнянні із рисунком 2А, та відповідний перерозподіл клітин з нижнього лівого до правих верхнього і нижнього квадрантів на рисунку 2D порівняно із рисунком 2С, що відбиває накопичення клітин у ранній і пізній фазі апоптозу в культурі опромінених лейкоцитів

(early plus late) apoptotic cell yields ranged from 6.7 to 10.8%. These values were consistent with plasma-free unirradiated control. With all patients, the reporter PBM survival in cultures with blood plasma after radiotherapy was similar to that with plasma before radiotherapy.

Individual results, combined and averaged from two repeating experiments, are shown in Figure 3, and the mean data for the whole group are presented in Table 1, where the cytotoxic effect produced by direct γ-irradiation of donors' blood *in vitro* is given for comparison.

The data were combined from two experimental rounds, and PBM survival was compared in 18 pairs: plasma after *versus* before radiotherapy. The mean difference in percentage of live cells was +0.21±0.34% (Student's paired t=0.620; p>0.05). Individually it varied from –2.28 to +3.56%, being negative in 8 cases and positive in 10; and for 16 it fell within ±2%. No meaningful correlation was detected at the individual level when the effects produced by plasma taken before and after radiotherapy were plotted against each other: For the percentages of total (early plus late) apoptotic cells in matched pairs the linear correlation coefficient was r = -0.058; p >0.05. In the averaged data the mean yield of apoptotic cells in PBM cultured with patients'

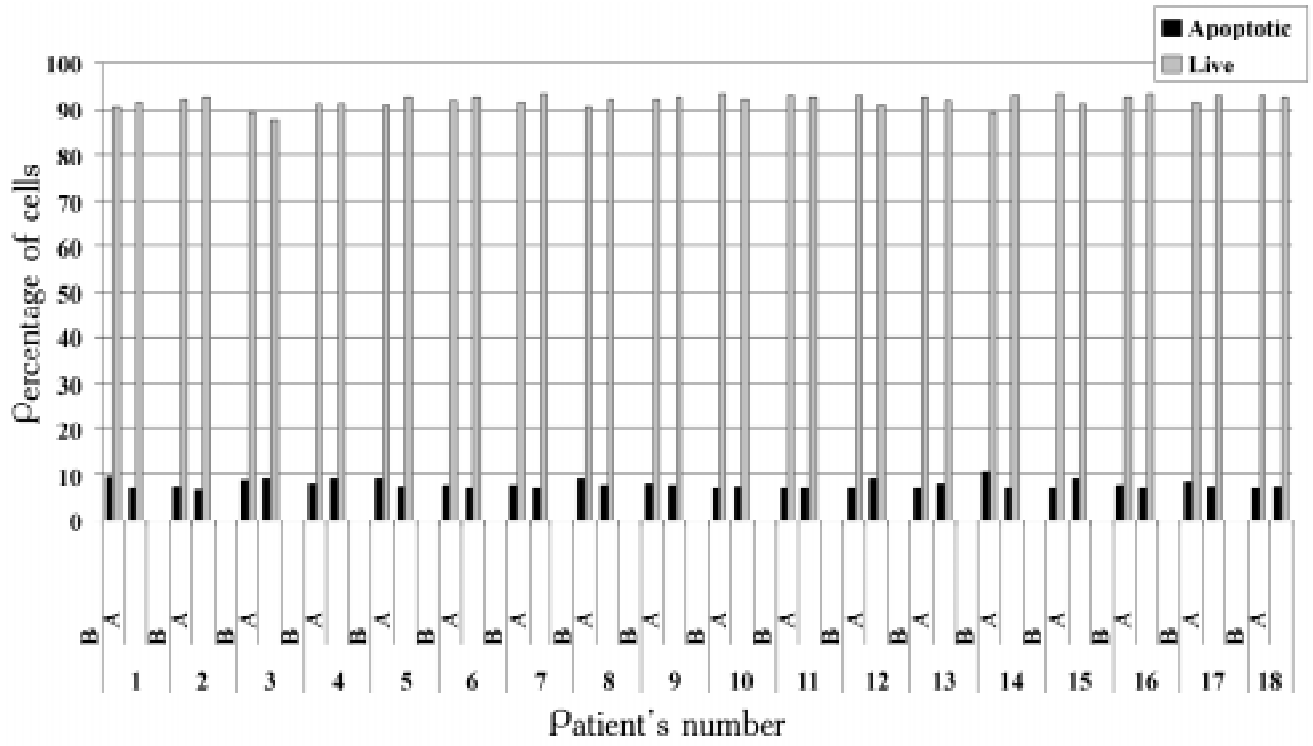

Figure 3. The interphase cell survival measured by Annexin V test for apoptosis in healthy donor's peripheral blood mononuclears cultured for 24 h in presence of blood plasma of 18 uterine cancer patients sampled before and after a standard course of external γ-radiotherapy (20 fractions x 2 Gy). Data represent mean values for two repeating series of testing for each plasma sample

Рисунок 3. Інтерфазна виживаність клітин, оцінена за допомогою тесту Аннексин V на апоптоз в лейкоцитах здорового донора, культивованих протягом 24 год у присутності плазми крові 18 хворих на рак тіла матки до і після стандартного курсу дистанційної γ-терапії (20 сеансів по 2 Гр). Дані представляють середні значення для двох повторних серій експерименту із кожним зразком плазми

Table 1

Averaged results of testing plasma of 18 uterine cancer patients before and after a radiotherapy course (40 Gy γ-rays) against healthy donor's peripheral blood mononuclears compared with plasma-free control and effects of direct irradiation of blood in vitro *to 2 Gy γ-rays*

Усереднені результати тестування плазми крові 18 хворих на рак тіла матки до і після курсу променевої терапії (40 Гр, γ-промені) щодо здатності спричинити апоптоз у лейкоцитах крові здорового донора, у порівнянні з контрольними культурами без плазми та ефектом прямого опромінення крові in vitro *γ-променями в дозі 2 Гр*

Culturing conditions for donor's reporter peripheral blood mononuclears (PBM)	Percentage of cells in PBM cultures,(Mean ±SE)* %				
	Earlyapoptosis (FITC+/PI−)	Late apoptosis (FITC+/PI+)	Necrosis (FITC−/PI+)	All apoptotic cells (FITC+)	Live
Plasma-free control	5,45±0,46	3,37±0,19	0,24±0,13	8,81±0,27	90,95±0,40
With patients' plasma before RT	5,56±0,20	2,44±0,15	0,43±0,10	8,00±0,25	91,57±0,27
With patients' plasma after RT	5,25±0,21	2,35±0,14	0,62±0,21	7,60±0,20	91,78±0,31
Directly irradiated PBM,plasma-free	11,76±2,07 [b]	5,85±1,85	1,39±0,54	17,61±3,90[a]	81,00±3,53 [b]

Notes. RT – radiotherapy. * – Averaged values and standard errors (SE) of the mean were calculated for 2 independent experiments. Statistically significant difference for mean values between directly irradiated cells and plasma-free control or cultures with patients' blood plasma: [a] – ($p<0,05$); [b] – ($p<0,01$) by Student's unpaired *t*-test.

Примітки. RT — променева терапія. * — Значення середнього і стандартна похибка (SE) обчислені для двох незалежних, повторних серій експерименту. Статистично вірогідна різниця за середніми значеннями частоти апоптотичних клітин між опроміненими клітинами і контролем без плазми чи культурами лейкоцитів із плазмою крові пацієнток: [a] — ($p < 0,05$); [b] — ($p < 0,01$) за *t*-критерієм Стьюдента для незв'язаних вибірок.

plasma after radiotherapy was indistinguishable from that with plasma taken before irradiation.

In contrast to a minor influence of patients' blood plasma on bystander cell survival, the direct 2 Gy γ-irradiation of PBM in blood produced, as expected, a significant elevation of apoptosis yield within the given cell culturing time of 24 h. Clear changes in cell staining profiles, comprising a noticeable reduction of the proportion of surviving PBM and a distinctively enhanced second peak representing Annexin V (FITC)-positive, apoptotic cells were observed after direct irradiation. In this series the mean total apoptotic cell yield was in-

creased above the matched background level by (8.80±2.02) % (Student's paired t=4.36; p<0.01). Thus, the magnitude of cell-killing effect directly induced by radiation exceeded any fluctuations of survival caused by irradiated or unirradiated plasma in reporter unirradiated PBM.

Discussion

Several experimental studies employing various cell types, including human leukocytes demonstrated that the soluble factors released from irradiated cells can induce apoptosis in bystander reporter cells [17-23]. Meanwhile, in order to assess properly the actual risk from the bystander effect in 'true life' radiation exposure scenarios, its magnitude must be estimated in test-systems that reproduce *in vivo* conditions as closely as possible[2]. A natural media for transmitting a possible bystander signal to leukocytes is plasma. The data found in the literature showed that plasma of *in vitro* irradiated donors' blood or radiotherapy patients' blood produced a large heterogeneity in responses in cycling bystander cells assayed by a clonogenic survival test, and in some cases a triggering of apoptosis was noted [8, 9]. However, human leukocytes, being normally in a quiescent, G_0 state, also show rather high susceptibility to apoptosis. From this a hypothesis arised that plasma factors of irradiated blood might induce apoptosis in unirradiated reporter leukocytes, thus causing an additional depletion of the cell population after irradiation. The present study was designed to clarify this after *in vivo* therapeutic irradiation.

To the best of our knowledge, it is the first investigation where quiescent, non-proliferating PBM were used as reporters for measuring the apoptosis rate changes caused by blood plasma taken from patients before and after radiotherapy. If plasma-mediated cytotoxicity had been detected, then a clinically applicable test might be developed for identification of individuals with this source of risk among radiotherapy patients, in order to predict and prevent potential abscopal effects and excessive normal tissue damage. It should be underlined that in present work the test-system applied for measuring a possible cytotoxic effect of irradiated blood plasma in reporter PBM contained optimum experimental conditions, which were constructed on the basis of the preceding *in vitro* study [12]. That was in contrast to some other studies, which involved cell culture characteristics that were explicitly unfavorable for reporter cells, or did not correspond to any realistic pathological scenario or normal homeostasis in human tissues, or were irreproducible in clinical practice.

In the present study, blood plasma collected before and after radiotherapy from 18 uterine cancer patients did not produce any apoptotic response above the control level in reporter PBM. In our previous experiment plasma separated from unirradiated blood or blood irradiated *in vitro* to 2-40 Gy γ-rays also did not induce early apoptosis or late apoptosis/necrosis in reporter PBM, whereas, as expected, direct irradiation caused significant and dose-dependent apoptotic death [12]. The absence of cytotoxic or clastogenic effects in unirradiated bystander cells was systematically observed in other studies, employing different end-points [24-30]. Also there are several examples of cytogenetic testing failure to demonstrate clastogeneity of human blood plasma after either *in vitro* or *in vivo* irradiation, including after radiotherapy [11, 31-33]. Thus, despite numerous reports about the presence of distinct cytotoxic or clastogenic bystander effects, including those produced by irradiated blood plasma [7], a result obtained in the present work was not very surprising.

Acheva et al. [8] tested plasma of healthy donors' blood given 0.5 Gy γ-rays *in vitro* against a keratinocyte cell line pre-irradiated to 0.05 Gy γ-rays. They observed a large individual variability in the plasma action on the low dose irradiated reporter cells. Among 9 individuals, whose plasma was tested, there were two cases of cytotoxicity and one case of stimulatory effect produced by plasma from both unirradiated and irradiated blood, and also two cases of cell growth stimulation, caused particularly by plasma from irradiated blood. In 8 out of 9 cases, the difference between the effects produced by unirradiated and irradiated blood plasma was insignificant, and no cytotoxicity caused specifically by irradiated blood plasma was detected.

In the work of Seymour and Mothersill [9], radiotherapy patients' plasma showed very large individual variation in its effect on clonogenic survival of human keratinocytes. Among 19 samples collected at the start of radiotherapy, in 9 cases patients' plasma caused a decrease and in 8 cases – a promotion of proliferation in the reporter cells. Later, plasma of only 4 out of 9 individuals sampled midway during radiotherapy and 6 weeks after completing the course markedly reduced a proliferation of reporters, and in other 4 cases a sti-

mulation effect of irradiated blood plasma was detected. Interestingly, in almost all cases sampled after radiotherapy the plasma produced an opposite effect to that observed before treatment, and again plasma toxicity can not be related to radiation exposure as a cause.

In present work the fluctuations of apoptosis yields in reporter PBM cultured with different patients' plasma were rather small, that was in contrast to other reports stating a high heterogeneity of the effects produced by plasma or medium conditioned by irradiated cells taken from several individuals [8-11, 34]. This dissimilarity could be most probably attributable to the different measured end-points, e.g. apoptosis yield *versus* clonogenic survival, and particularly the types of reporter cells, actively proliferating keratinocytes *versus* quiescent PBM, mainly G_0 lymphocytes. However, this discrepancy does not change the main conclusion about the absence of specific radiation-induced cytotoxicity of human blood plasma towards unirradiated bystander cells. Noteworthy, Lindholm et al. [11] similarly concluded that interindividual variations in the patient plasma-induced chromosomal aberrations and γ-H2AX foci in reporter cells were not associated with the radiotherapy, since patient-to-patient differences in plasma-induced effects were observed within a patient group with the same treatment regimen.

A comparison of experimental conditions in several studies of radiation-induced plasma factors showed that quiescent or cycling status of reporter cells, depletion of culture medium in free radical scavengers, using a purified clastogenic fraction of plasma obtained by its centrifugation through cut-off filters, plasma concentration in reporter cell culture or time interval between exposure and collecting patients' blood do not influence the fact of absence of the damaging bystander effect [8, 9, 11, 32]. Terzoudi et al. [30] did not observe additional chromosomal damage induced in either G_0 or cycling lymphocytes *via* bystander mechanism and suggested that "specific conditions are required for the generation of bystander responses and that these conditions apparently were not satisfied in our experiments. The fact that specific and as-yet not well-characterized conditions are required for the development of bystander responses is also indicated by the relative irreproducibility of the effect and the partly contradictory data available in the literature". Simultaneously, Blyth and Sykes [2] underlined that if an *in vitro* system does not correspond to any realistic human exposure scenario, then this should also be disclosed and the relevance of any findings to *in vivo* bystander effect should be justified. Generally, from data available in the literature a conclusion can be made that clastogenic or cytotoxic properties of blood plasma can be considered as a result of acting some other factors, apart from radiation, or artifacts due to very special, unphysiological culture conditions [8, 9, 11, 12, 31-33].

Considering the absence of direct cytotoxicity of irradiated blood plasma, more attention should be paid to regular reports that soluble factors released from irradiated cells can initiate signaling cascades in reporter cells, due to which the latter acquainted enhanced proliferation, radioresistance and adaptive response to radiation (briefly reviewed in [12]). Therefore, the number of assays with radiotherapy patients' plasma should be expanded, e.g. by including measurements of the cycling rate of reporter lymphocytes pushed from a quiescent, G_0 state into proliferation, as such approach earlier provided valuable data in the elegant experiment of Lloyd and Moquet [35]. If plasma-mediated "nourishing" effect would be observed in proliferating human leukocytes, then it's possible clinical implications in radiotherapy patients should be studied extensively.

Conclusion

Apoptosis measurements using the Annexin V test showed that plasma collected from blood of 18 uterine cancer patients before or after their radiotherapy (20 fractions of 2 Gy g-rays) did not produce any early apoptosis or late apoptosis/necrosis above the control level in quiescent healthy donor's PBM, whereas direct radiation caused significant apoptotic death. The magnitude of cell-killing effect directly induced by irradiation to 2 Gy g-rays exceeded any fluctuations of reporter cell survival caused by patients' plasma either before or after irradiation. Other assays instead of apoptosis in unirradiated quiescent leukocytes should be applied for detecting possible untargeted radiation effects, including protective or stimulatory effects, mediated by radiotherapy patients' blood plasma. However the experimental conditions must be constructed carefully in order to maintain a clinical relevance of such tests.

Conflict of interest statement

The author declares no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations that could

inappropriately influence this work. The author alone is responsible for the content and writing of the paper.

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