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Original article

Bystander effects induced by direct and scattered radiation generated during penetration of medium inside a water phantom

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ABSTRACT

Background: The biological effects of ionizing radiation have long been thought to results from direct targeting of the nucleus leading to DNA damage. Over the years, a number of non-targeted or epigenetic effects of radiation exposure have been reported where genetic damage occurs in cells that are not directly irradiated but respond to signals transmitted from irradiated cells, a phenomenon termed the "bystander effects".

Aim: We compared the direct and bystander responses of human A 549, BEAS-2-B and NHDF cell lines exposed to both photon (6 MV) and electron (22 MeV) radiation inside a water phantom. The cultures were directly irradiated or exposed to scattered radiation 4 cm outside the field. In parallel, non-irradiated cells (termed bystander cells) were incubated in ICM (irradiation conditioned medium) collected from another pool of irradiated cells (termed donor cells).

Materials and methods: In directly irradiated cells as well as ICM-treated cells, the frequency of micronuclei and condensation of chromatin characteristic for the apoptotic process were estimated using the cytokinesis-block micronucleus test.

Results: In all tested cell lines, radiation induced apoptosis and formation of micronuclei. A549 and BEAS-2B cells cultured in ICM showed increased levels of micronuclei and apoptosis, whereas normal human fibroblasts (NHDF line) were resistant to bystander response. In A549 and BEAS-2B cells placed outside the radiation field and exposed to scattered radiation the formation of micronuclei and induction of apoptosis were similar to that after ICM-treatment.

Conclusion: Results suggest that the genetic damage in cells exposed to scattered radiation is caused by factors released by irradiated cells into the medium rather than by DNA damage induced directly by X rays. It seems that bystander effects may have important clinical implications for health risk after low level radiation exposure of cells lying outside the radiation field during clinical treatment.

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1. Background and the aim of the work

Recently, attention in radiobiological studies turned to non-targeted effects, specially to radiation-induced bystander effects. These are induced by signals emitted by directly irradiated cells and manifest in non-irradiated cells as a reduced clonogenic survival,[1,2] cytogenetic damage,[3] apoptosis and modulation of expression of specific proteins.[4,5] The bystander effect has been observed in experiments performed in vitro using very low doses of α-particles[6,7] as well as after conventional irradiation with γ-rays and X-rays.[8] Bystander effects are caused by molecular signals released from irradiated cells and these signals can be transmitted through gap junction intercellular communication and through a medium transfer mechanism. It has been shown that medium collected from irradiated cells (ICM – irradiation conditioned medium) and transferred to non-irradiated cells induce in them genetic changes characteristic for bystander responses.[1] It has been well documented that bystander effects predominate after low dose exposure and saturate at higher doses and cause a deviation from the linear non-threshold (LNT) model in the low dose region of radiation.[3,9,10] In our previous work we compared the dose depth distribution of ionizing radiation in a water phantom with the frequency of occurrence of apoptotic and micronucleated human cells that were exposed to radiation in a beam axis or outside the radiation field. Our measurements showed discrepancy between physical dose distribution observed on different depths in a water environment and biological effects.[11] This discrepancy was especially significant for cells exposed in a beam axis at higher depths and cells placed outside the radiation field during exposure to radiation. The numbers of apoptotic and micronucleated cells were greater than those that should result from the corresponding received dose. Observed effects were explained to result from low dose scattered radiation, since with increasing medium depth as well as distance of beam of axis, its proportion to the incident radiation became greater. In the present work we tested whether bystander effects can play a role in the response of cells to direct irradiation (irradiation in beam axis) and scattered radiation (outside the radiation field). We explored this issue by using the medium transfer technique in which medium of exposed cells (termed donor cells) was transferred to non-irradiated cells (termed bystander cells). The genetic changes (formation of micronuclei and induction of condensation of chromatin characteristic for apoptotic process) in control, directly exposed as well as ICM-received cells were estimated using the cytokinesis-block micronucleus test. This study was presented at ESTRO 29 in Barcelona 12-16.09.2010.

2. Materials and methods

2.1. Cell culture

Human lung carcinoma cells (A549 line), normal bronchial epithelial cells (BEAS-2B line) and normal fibroblasts (NHDF line) were used in the experiments. Cells were grown in a monolayer in tissue culture flasks containing DMEM/F12
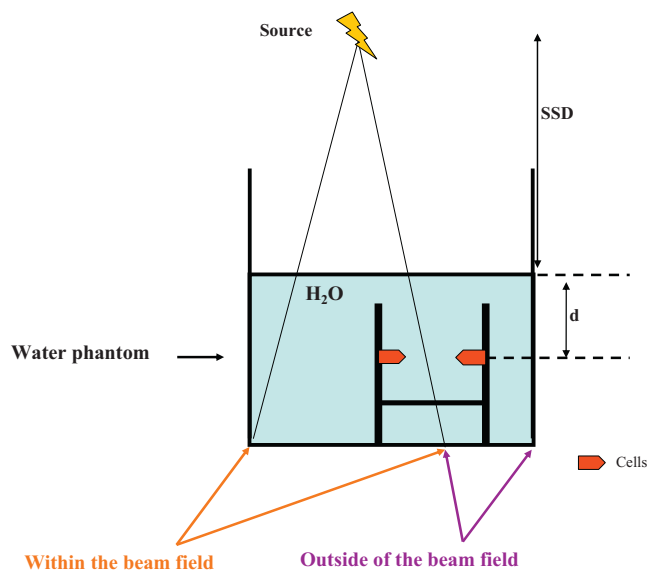

Fig. 1 – The conventional scheme of the irradiation set-up.

medium supplemented with 10% fetal bovine serum (Immuniq) in a humidified atmosphere of 5% CO_2 at 37 °C. Before irradiation, the cells were detached by means of 10 mM EDTA and 0.25% trypsin. Next, 20 μl aliquots of cell suspension, containing approximately 1×10^3 cells, were transferred to Eppendorf's tubes and then the tubes were filled with medium up 0.5 ml so that cells could be irradiated without the presence of air.

2.2. Irradiation of cells

Dose was calculated in a planning treatment system (Eclipse – Varian Medical Systems), algorithm Monte Carlo, on the basis of measurements conducted in a water phantom of 35 cm × 35 cm × 35 cm. The cells in tubes were placed horizontally in a stand and irradiated with 5 Gy defined at the "build-up" depth in a water phantom in two variants: directly on the axis in the beam field or 4 cm outside the field, i.e. with scattered radiation (dose of 0.2 Gy), as shown in Fig. 1. The field size was 15 cm × 15 cm in SSD. Experiments were performed for electron (22 MeV) and photon (6 MV) radiation generated in a linear accelerator Clinac series Varian Medical System, for 300 MU/min accelerator mode and dose of 5 Gy in build-up (3 cm) depth in a water environment.

2.3. Conditioned medium transfer

To obtain the conditioned medium (ICM), we employed the described technique[1] and the experimental protocol is presented in Fig. 2. Briefly, donor cells that had been placed in a water phantom and irradiated, were transferred to plastic dishes and incubated for 1 h in 37 °C in a normal culture medium (5 ml). Then, the ICM was collected, centrifuged and filtered through a 0.22 μm pore filter to ensure that no cells were present in the transferred medium. The filtrate was immediately added to non-irradiated (bystander) cells. Control cell cultures were incubated in a normal medium.
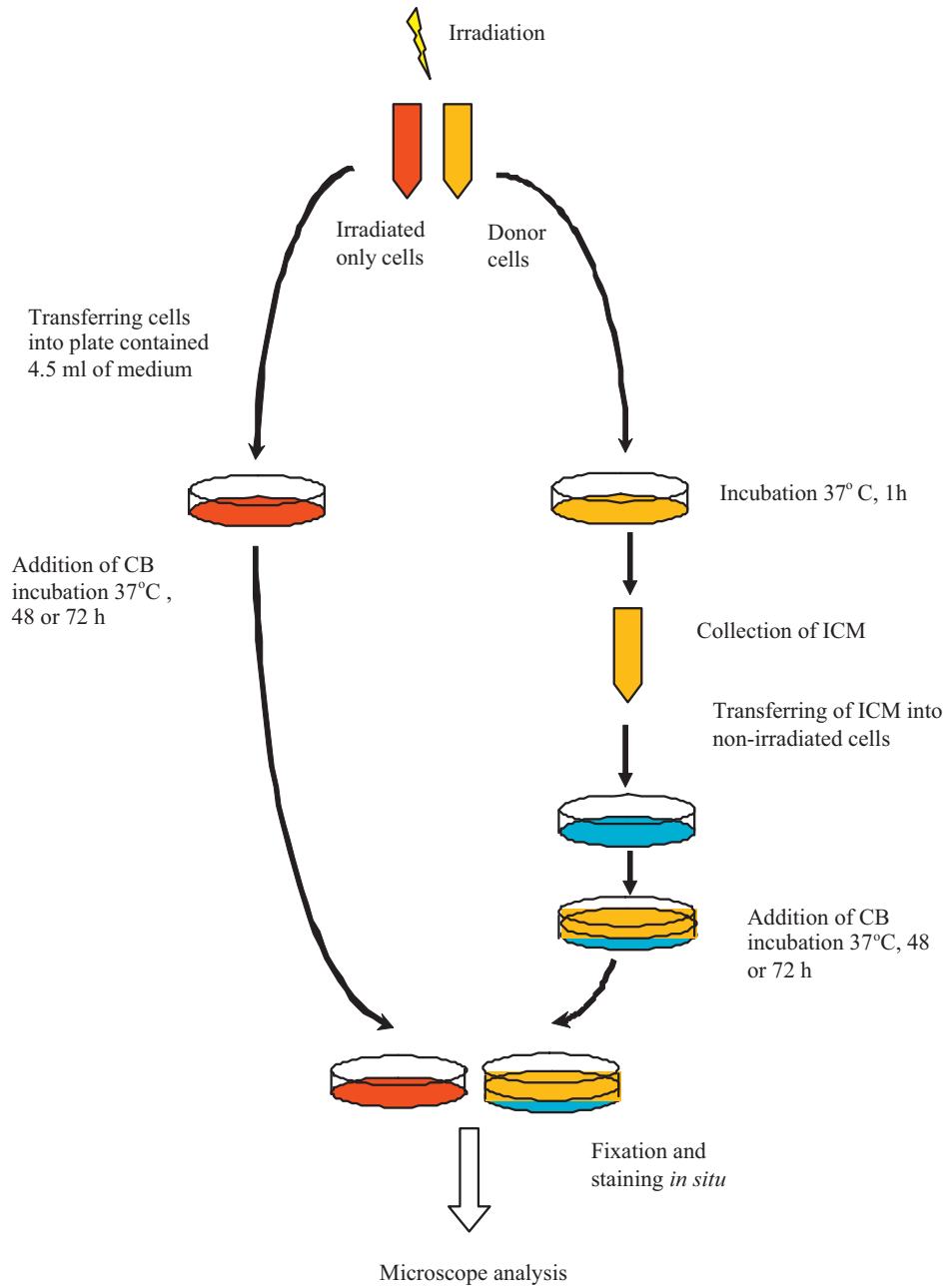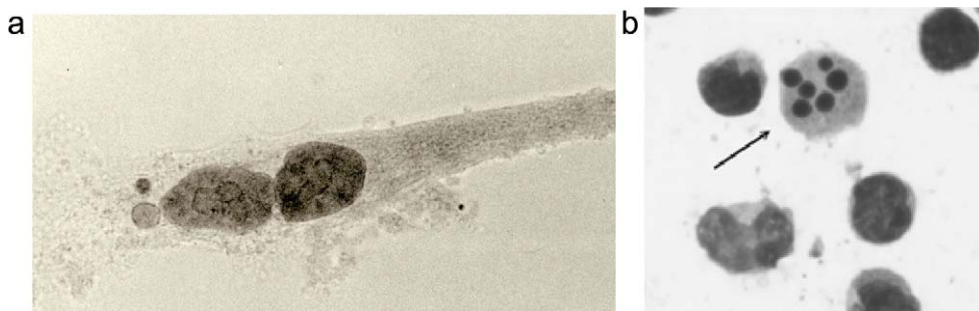
Fig. 2 – Preparation of conditioned medium (ICM).

Fig. 3 – Images of damaged cells scored 2 days after irradiation or icubation in ICM. (A) Micronucleated cell and (B) apoptotic cell.

2.4. Cytokinesis-block micronucleus test

After irradiation, the cells were transferred from Eppendorf's tubes into plastic dishes (50 mm diameter), supplemented with up to 5 ml of the culture medium and incubated at 37 °C. The micronucleus test was performed according to the standard procedure.[12] The cytochalasin B was added to medium of irradiated and ICM-treated cultures to a final concentration of 2 μg/ml and cells were incubated for 48 h prior to fixation. The cells were fixed in situ with a cold solution of 1% glutaraldehyde (Sigma) in phosphate buffer (pH = 7.5) and stained by Feulgen reaction. At least 500 binucleate cells (Fig. 3A) were examined for the presence of micronuclei under microscope. The fraction of cells showing condensation of chromatin characteristic for the apoptosis process (Fig. 3B) was also recorded in 1000 cells in each plate.

Published data concerning the bystander effect have commonly classified apoptotic cells on the basis of morphological criteria.[13] In all our experiments the frequency of apoptosis was assessed by microscopic observation. In selected cell cultures, we additionally performed annexin V-FITC flow-cytometry analysis. Both of these methods showed a similar tendency as the one we presented earlier.[14]

2.5. Statistical analysis

Student's t-test was used to determine the significance of differences (in terms of the number of micronuclei formed and induction of apoptosis) between cells irradiated and non-irradiated, as well as between cells cultured in a normal medium and in ICM.

3. Results and discussion

We compared the biological responses of cells incubated in ICM with those of cells exposed directly to radiation. It is well documented that following irradiation cells release molecular signals which can induce genetic changes in non-irradiated cells distant from the target cells. This phenomenon, defined as "bystander effect" may reflect the occurrence of at least two separate mechanisms for the transmission of signals from irradiated cells to non-irradiated ones. One line of evidence indicates that the bystander effect is dependent on intracellular communication through gap junctions; the other explanation is that irradiated cells secrete cytokines or other signaling factors into the medium (referred to as ICM – irradiated conditioned medium).[1,15] Genotoxic action of ICM is inactivated by heating,[1] which suggests that molecular factors released from irradiated cells are proteins. In our control experiments no changes in the number of micronucleated or apoptotic cells were observed when the non-irradiated cells were cultured in medium irradiated in the absence of cells (data not shown), suggesting that genetic changes in these cells are mediated by factors released by irradiated cells into the medium.

Fig. 4 shows the frequency of micronucleated A549 cells that were exposed to electron and photon radiation or incubated in ICM collected from donor cells irradiated in the same conditions. The number of damaged cells increased over the
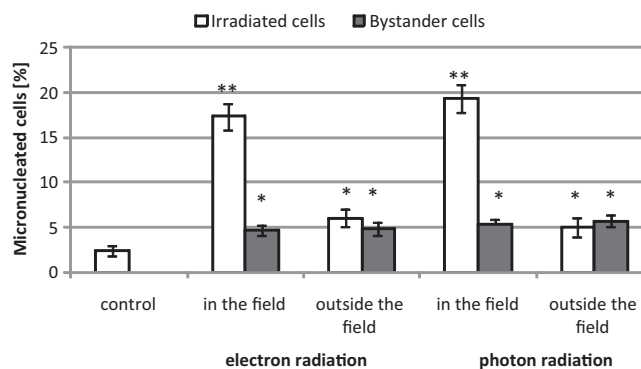

Fig. 4 – The percentage level of micronucleated A549 cells in directly irradiated or incubated in ICM cultures. Significant at *p < 0.01 and **p < 0.001 compared with control.

value of control not only in cells directly irradiated in a beam axis but also in cells exposed to scattered radiation. In cultures incubated in ICM the frequency of micronucleated cells increased nearly two-fold in comparison with the control cells incubated in a normal medium. The ICM collected from cells irradiated with photon and electron radiation caused a significant increase in the frequency of damaged cells and induced the same number of micronuclei, independent of the radiation dose and the positioning with respect to beam axis during exposure.

Fig. 5 shows the increase in the number of cells showing condensation of chromatin in both directly irradiated and ICM-received A549 cells. In directly irradiated cells, the number of apoptotic like cells increased nearly 3-fold in comparison with the number of spontaneous apoptosis. In cells exposed to scattered radiation, we observed nearly a 2-fold increase of the number of apoptotic cells compared to the control. A significant increase of the number of apoptosis was also observed in ICM-received cells. The level of induced apoptosis was similar in bystander cells incubated in ICM irrespective of the kind of irradiation of donor cells. The results for the micronuclei induction in irradiated and ICM-treated BEAS-2B cells are presented in Fig. 6. In directly irradiated cells the number of micronuclei increased about 10-fold over the control, whereas in cells exposed to scattered radiation we
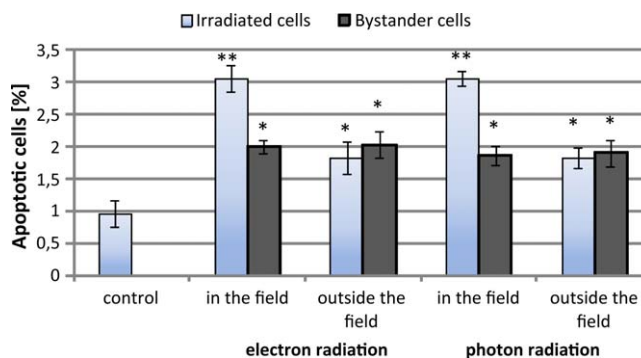

Fig. 5 – The percentage level of apoptotic A549 cells indirectly irradiated or incubated in ICM cultures. Significant at *p < 0.01 and **p < 0.001 compared with control.
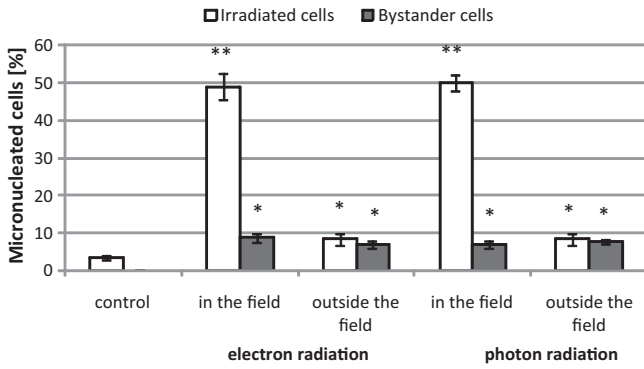
Fig. 6 – **The percentage level of micronucleated BEAS-2B cells in directly irradiated or incubated in ICM cultures. Significant at *$p < 0.05$ and **$p < 0.001$ compared with control.**

observed a 2-fold increase. The ICM obtained in different kind of irradiation induced also a two-fold increase of the number of chromosomal damage compared to non-treated control. As shown in Fig. 7, irradiation in a beam axis caused about a 4-fold increase in the number of apoptotic BEAS-2B cells. Over a 2-fold increase in the number of apoptosis was observed in cells exposed outside the radiation field as compared with the control. ICM collected from donor cells exposed to photon and electron radiation, both direct and scattered, induced a comparable increase in the number of apoptotic cells.

Figs. 8 and 9 show the results for irradiated and ICM-treated normal human fibroblasts (NHDF). We observed a significant increase in comparison with the control in the frequency of micronuclei in directly irradiated cells but no significant change in the level of micronuclei in cells exposed to scattered radiation (Fig. 8). There were also no differences in the number of micronuclei between control and ICM-received cells. Fig. 9 indicates that the number of apoptotic cells increased nearly 2-fold over the spontaneous level in directly irradiated cells. In cells exposed to scattered radiation, the level of apoptotic like cells increased over the control value but these differences were not significant. ICM did not affect the frequency of apoptotic cells in any treatment kind.

In human cancer, A549 cells and normal bronchial epithelial BEAS-2B cells we observed for medium-mediated
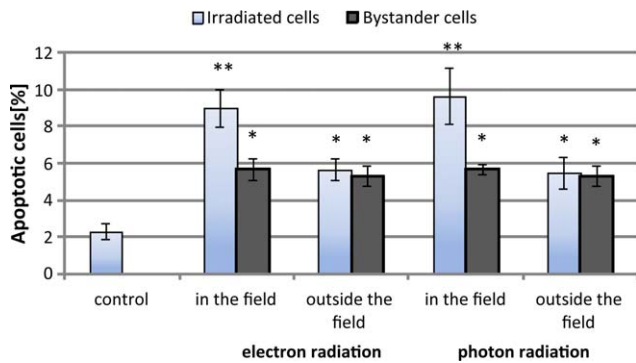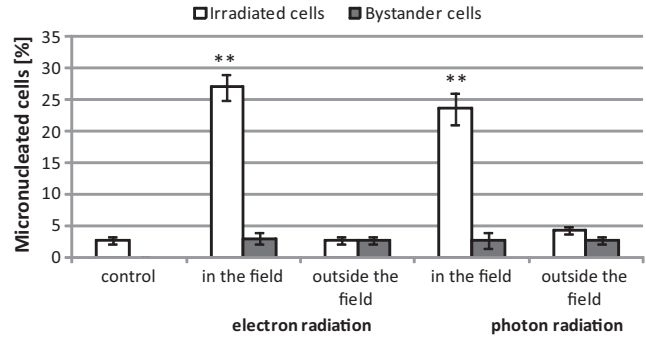

Fig. 8 – **The percentage level of micronucleated NHDF cells in directly irradiated or incubated in ICM cultures. Significant at **$p < 0.001$ compared with control.**

bystander effects that were independent of radiation type and radiation dose. When these cells were exposed to scattered radiation, the formation of micronuclei and induction of apoptosis were similar to that caused by ICM, suggesting that genetic changes are caused by factors released by irradiated cells into the medium rather than by radiation-induced ionization of DNA. Otherwise, normal human fibroblasts (NHDF) were resistant to the radiation-induced bystander effect. It has been shown that the bystander effect is cell specific since it was observed in epithelial cells,[1,8,16] leukemia cells,[17] glioma cells[18] and primary human fibroblasts,[13] but not in fibroblasts line MSU1.1.[1] The above described results and observations from the present work indicate that normal human fibroblasts are resistant to radiation-induced bystander signals, while normal epithelial cells are sensitive to these signals. These data suggest that epithelial cells can be more sensitive to induction of genetic alterations leading to cancer development than fibroblasts. This suggestion can be confirmed by published data indicating that over 90% of human cancers arise from epithelial tissues.[19] Although bystander effects may have potential disadvantages, such as risk of late organ damage or induction of secondary tumors,[9,20] they may also deliver benefits to clinical radiotherapy, including more effective killing of cancer cells. It is especially important in modern radiotherapy techniques, such as intensity modulated radiation therapy (IMRT) that aims at reducing the radiation dose to
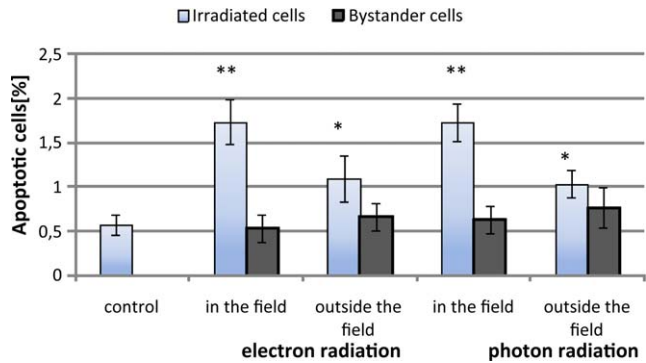

Fig. 7 – **The percentage level of apoptotic BEAS-2B cells in directly irradiated or incubated in ICM cultures. Significant at *$p < 0.0.01$ and **$p < 0.001$ compared with control.**

Fig. 9 – **The percentage level of apoptotic NHDF cells in directly irradiated or incubated in ICM cultures. Significant at *$p < 0.001$ and **$p < 0.01$ compared with control.**

normal tissues.[21,22] It has been demonstrated that bystander effects predominate after low dose exposure and saturate at higher one and can be induced when a few cells in a population are irradiated with dose of about 0.1 Gy.[1,16,18]

4. Conclusion

Genetic changes observed in cells exposed to scattered radiation outside the field were at least in part induced by low dose irradiation. Additionally, low-dose scattered irradiation induces, among other things, the bystander effect and this effect can predominate the response of cells exposed outside the radiation field. The genetic damage of cells exposed outside the radiation field can be caused by factors released by irradiated cells into the medium rather than induced directly in DNA by X rays. It seems that bystander effects may have important clinical implications for health risk after low level radiation exposure of cells lying outside the radiation field during clinical treatment.

Acknowledgment

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