

X-irradiation of human bronchial cancer cells causes the bystander effects in normal bronchial cells in vitro

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Using X radiation commonly used in radiotherapy of cancers we investigated bystander interactions between human cells: irradiated A549 bronchial carcinoma human cells and non irradiated BEAS-2B normal bronchial epithelial cells. Non irradiated cells were incubated in medium transferred from irradiated A549 cells (ICM-irradiation conditioned medium) for 48h and next the chromosomal damage and apoptosis were estimated. Conditioned medium collected from irradiated cancer cells induced in non irradiated cells of the same line as well as in BEAS-2B normal cells genetic changes such as micronuclei, chromatid and chromosomal breaks and condensation of chromatin characteristic for processes of apoptosis. Addition of only 1% of conditioned medium to fresh medium was sufficient to induction of bystander response to normal bronchial cells.

The presented results in this study could have implications for human radiation risk and in evaluating the secondary effects of radiotherapy.

Key words: X radiation, bystander effects, A549 cells, normal bronchial epithelial cells, chromosomal damage, apoptosis.

The impact of targeted and untargeted radiation effect in cells increased and improved during the last 20 years. It provides that radiation may interact directly within a cell causing a direct DNA lesions or it may induce a bystander response in non irradiated cells. Bystander effects are produced when the irradiated cells transmit signals, which induce in non irradiated cells death via apoptosis, mutations, chromosomal damage and change in gene expression [1, 2]. It has been suggested that radiation-induced bystander effects, that drives to genomic instability may be involved in induction of second cancer in normal cells after radiotherapy [3, 4]. During radiotherapy not all cancer cells are killed and those survived the radiation exposure, can emit the signal factors and induced bystander response of neighboring or distal normal cells. Most of the studies concerning the bystander effects were conducted on the same cell type. It has been also showed the bystander interaction between cells of different types, for example between glioma cells and fibroblasts co-cultivated together and exposed to α -particles [5]. Recently it was demonstrated that human leukemic K562 cells exposed to X radiation release to medium factors that are able to induction in non irradiated normal human lymphocytes bystander response [6]. In the present work the bystander interactions between irradiated

human A549 bronchial cancer cells and non irradiated normal bronchial epithelial BEAS-2B cells were tested. These 2 cell types were selected as model because the lung cancers are usually cured with radiotherapy. However most of lung cancers arise in the epithelium of the bronchial tree so the study of the sensitivity of bronchial epithelial cells to bystander signals generated from irradiated cancer cells seems to be therefore point of interest.

Materials and methods

Cell culture. Experiments were performed on human lung carcinoma A549 cells and human normal bronchial epithelial BEAS-2B cells. Cells were grown with DMEM/F12 medium supplemented with 10% fetal bovine serum (Immuniq) in a humidified atmosphere of 5% CO₂ at 37°C. The day before experiments cultures were trypsinized and a 50 μ l of cell suspension with approximately 10³/ml of cells were seeded on Petri dish. One hour prior irradiation the medium was changed in cultures.

Irradiation and preparation of conditioned medium. X-irradiation was carried out with a Clinac 600 GMV Machine (Varian) at room temperature. The applied doses were from

0.1 to 4 Gy and the dose rate was 1 Gy/min. After irradiation cells were incubated for 1 h in 37°C and the culture medium (ICM – conditioned medium) was transferred to plastic tubes, centrifuged and filtered prior using in next experiments.

ICM non diluted or diluted with fresh medium in different proportion was added to 24-h cultures. Control cells were incubated in medium collected from non-treated cells. All tested cultures were incubated for 48h because at this point time the maximum frequency of binucleated cells was observed.

Cytokinesis-block micronucleus test. The frequency of micronuclei formation was measured using the cytokinesis-block micronucleus technique [7]. Briefly, after irradiation or transferring of ICM, cytochalasin B (Sigma, 2µg/ml) was added and cultures were incubated at 37°C. After 48h of incubation 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 200 binucleate cells were examined for the presence of micronuclei. The fraction of cells showing condensation of chromatin characteristic for apoptosis processes was also recorded in 1 000 cells in each petri dish. For cell cycle analysis, 400 cells per dish were scored for the presence of one, two, three or more nuclei and the nuclear division index (NDI) was calculated as follows:

$$\text{NDI} = [1\text{N} + (2 \times 2\text{N}) + (3 \times 3\text{N}) + (4 \times 4\text{N})]/400$$

Where 1N is the number of cells with one nucleus, 2N – with two nuclei, 3N – with three nuclei and 4N – with four or more nuclei.

Chromosomal aberrations test. BEAS 2B cells were incubated in fresh or conditioned medium collected from irradiated A549 cells for 48h. Colcemid (Sigma, 0.02µg/ml) was added for 2h prior to harvesting to accumulate mitotic cells. Following hypotonic treatment in 75 mM potassium chloride for 15 min. cells were fixed in three changes of a 3 : 1 methanol : acetic acid mixture and placed on cold microscope slides. Two slides were prepared for each culture. Air-dried preparations were stained with 10% Giemsa solution for 10 min. For each experimental data point, 100 well-spread metaphases were analysed for chromatid and chromosomal aberrations. Induced aberrations were mainly chromatid and chromosomal breaks and fragments. Gaps were excluded from the analysis. The mitotic index was also calculated in 1 000 cells per slide.

All experiments were repeated three times. Data were analysed using the T-test.

Results

Direct and bystander response of A549 bronchial cancer cells. In first part of experiments we tested the genetic change in A549 cells that were directly exposed to X radiation or incubated in conditioned medium transferred from another pool of irradiated and then one hour incubated cells of the same line. The dose-response for the frequencies of micronuclei formation in both, directly irradiated or ICM-received cells are presented in Fig.1.

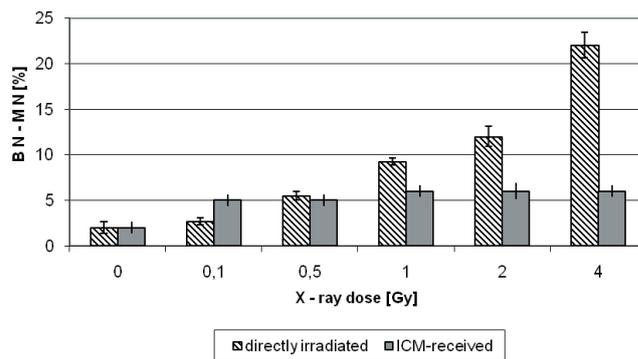


Figure 1. Dose response for frequencies of MN formation in directly irradiated or ICM-received A549 cells. Results are the means of four independent experiments \pm SD.

The data shows that in directly irradiated cells the number of micronucleated and binucleate cells increase with enhancing dose of X radiation. At 4 Gy the number of micronucleated cells increase to 10 times in comparison with non-irradiated control. The bystander response of A549 cells was also observed. At 0.1 Gy bystander response was over two fold higher than response of cells to direct irradiation but there was no further increase in the number of micronucleated cells with increasing dose.

Fig. 2 presents dose-response for apoptosis induction in directly irradiated or ICM received A549 cells.

The number of apoptotic-like cells increase in dose-dependent manner in directly exposed to X radiation cells. At 4 Gy the number of apoptotic cells increased nearly two-fold in comparison with the level of spontaneous apoptosis. The results of parallel determination of effect of ICM-treatment indicate that below 0.5 Gy, bystander response predominate but then it saturate.

Bystander response of BEAS-2B normal bronchial cells. The ability of conditioned medium collected from irradiated A549

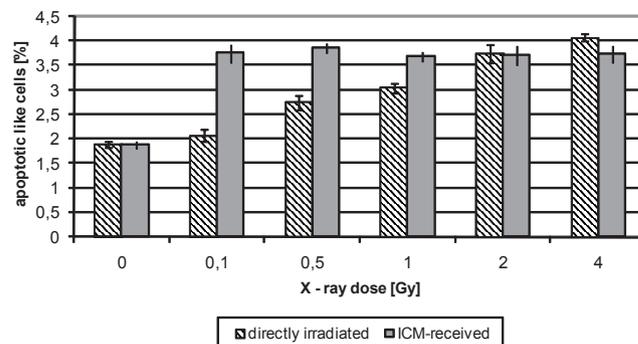


Figure 2. Dose response for frequencies of apoptosis induction in directly irradiated or ICM-received A549 cells. Results are the means of four independent experiments \pm SD.

Table 1. The effect of dilution of ICM collected from irradiated cancer cells (A549) on the medium-mediated bystander response of normal cells (BEAS-2B)

Composition of culture medium (%)		Frequency of cells (%)			
Fresh medium	Conditioned medium (ICM)	micronucleated (MN-CB)	apoptotic (AP)	with chromosomal aberrations (CA)	
100	0	3.34 ± 0.38	1.86 ± 0.09	1.33 ± 0.11	
99	1	9.33 ± 1.22	3.82 ± 0.40	11.00 ± 1.02	
90	10	9.00 ± 0.92	3.50 ± 0.29	10.6 ± 1.16	
50	50	9.66 ± 0.98	3.83 ± 0.44	10.2 ± 0.91	
0	100	9.00 ± 1.24	3.66 ± 0.33	11.3 ± 1.34	

Means ± SD of three experiments are shown.

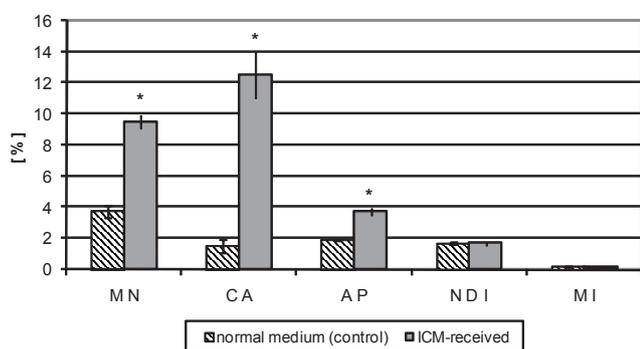


Figure 3. Induction of micronuclei (MN), apoptosis (AP), chromosomal aberrations (CA), change in nuclear division index (NDI) and mitotic index (MI) in BEAS-2B cells incubated in normal (control) or conditioned medium (ICM) collected from irradiated A549 cells. Results are the mean of the four independent experiments ± SD.

* significant at $p < 0.01$ (T-test) compared with control cells.

cancer cells to induction of chromosomal damage and apoptosis in non irradiated normal BEAS 2B cells were examined. Data presented in Fig.3 shows that ICM increased over two-fold the number of micronucleated cells in comparison with controls receiving fresh medium.

Over 10-fold increase in the number of chromosomal aberrations was also noted in cells growing in ICM. A minor but significant increase of fraction of apoptotic-like cells was observed in cells incubated in ICM in comparison with control cells that were incubated in fresh medium.

While ICM induced chromosomal damage and apoptosis in non irradiated cells it did not change the proliferative capacity of these cells. The nuclear division index as well as mitotic index was also calculated. The values of both of them were similar in cells growing in ICM and fresh medium.

The effect of dilution of ICM on the medium-mediated bystander response of normal cells were examined. For this purpose ICM collected from irradiated cancer A549 cells was mixed with fresh medium in different proportion and added to non irradiated BEAS 2B cells. In these cells the number

of chromosomal damage and induction of apoptosis were recorded. Table 1 shows that the number of damaged cells did not change with dilution of ICM. In cultures incubated in medium contained 100% as well as 1% of ICM the number of damaged cells was comparable.

Discussion

The bystander interactions between irradiated cancer cells and non-targeted normal cells take the important role in cancer risk associated with medical exposure. In present study we investigated bystander interactions between irradiated human lung carcinoma A549 cells and non-irradiated normal bronchial epithelial BEAS-2B cells. The results presents that cancer cells exposed to X-radiation commonly used in radiotherapy are able to release into medium factors leading to induction of apoptosis and chromosomal damage in non-targeted cells of the same line as well as in BEAS-2B normal bronchial cells. The observed increase in the number of unstable chromosomal aberrations (chromosomal and chromatid breaks) in normal cells incubated in ICM is specially unsafe effect, since these damage are responsible to induction genomic instability [8]. The loss of genomic stability is accepted as being one of the most important aspects of cancer development [9, 3]. It has been reported that genomic instability can be mediated through bystander signals interactions via secretion from irradiated cells of NO/ROS that activate of same long-lived bioactive cytokines [10, 11].

The results indicate that the medium-mediated bystander effect observed in A549 cancer cells was independent of radiation dose. This relationship has been described in several papers [1, 12]. However, the observed dilution of conditioned medium up to 1:100 with fresh medium caused induction of the same bystander response of normal non-irradiated cells as non-diluted ICM. According to published data the amount of signal molecules needed to activate the bystander response is very low. It has been demonstrated that irradiation of a single human T lymphocyte with a charged particle leads to a significant induction of chromosomal damage in the all bystander population [8]. It has been also described

that a single cell irradiated with a He² particle could produce a significant increase in micronuclei induction in the neighbouring non-irradiated cell population [5]. Cited above data and our present result suggest that tumor cells that survive radiotherapy can emit soluble factors into environment and these factors present even at small amount can be able to induce genetic changes in distal normal tissues. The observation could have significant implications for human radiation risk and for evaluating the secondary effects of radiotherapy. Because radiotherapy is an important treatment option for patients suffering from lung cancer the observed effects should be taken into consideration in both treatment planning and clinical practice.

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