Bystander responses in low dose irradiated cells treated with plasma from gamma irradiated blood

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Abstract. There are two specific low-dose radiation-induced responses that have been the focus of radiobiologists’ interest in recent years. These are the bystander effect in non-irradiated cells and the adaptive response to a challenge dose after prior low dose irradiation. In the present study we have investigated if plasma from irradiated blood can act as a “challenge dose” on low dose irradiated reporter epithelial cells (HaCaT cell line). The main aim was to evaluate the overall effect of low dose irradiation (0.05 Gy) of reporter cells and the influence of bystander factors in plasma from 0.5 Gy gamma irradiated blood on these cells. The effects were estimated by clonogenic survival of the reporter cells. We also investigated the involvement of reactive oxygen species (ROS) as potential factors involved in the bystander signaling. Calcium fluxes and mitochondrial membrane potential (MMP) depolarization were also examined as a marker for initiation of apoptosis in the reporter cells. The results show that there are large individual differences in the production of bystander effects and adaptive responses between different donors. These may be due to the specific composition of the donor plasma. The observed effects generally could be divided into two groups: adaptive responses and additive effects. ROS appeared to be involved in the responses of the low dose pretreated reporter cells. In all cases there was a significant decrease in MMP which may be an early event in the apoptotic process. Calcium signaling also appeared to be involved in triggering apoptosis in the low dose pretreated reporter cells. The heterogeneity of the bystander responses makes them difficult to be modulated for medical uses. Specific plasma characteristics that cause these large differences in the responses would need to be identified to make them useful for radiotherapy.

1. Introduction

For the last 20 years a new phenomenon in the area of the radiobiological effects has been described. A radiation response has been found in cells that are not irradiated themselves, but are in the vicinity of irradiated cells, i.e. bystander effects [1, 2, 3]. However, for the low dose range another phenomenon is characteristic that is in conflict with the bystander effect. It is well known that low dose irradiated cells show an increase in radioresistance to a subsequent challenging dose of radiation. This effect is called an “adaptive response” [4, 5]. Both phenomena have been demonstrated for numerous biological endpoints including alteration in gene expression [6, 7], induction of micronuclei [8, 9], reduction in clonogenic survival [1], neoplastic transformation [10], sister chromatid exchanges [3], apoptosis [9, 11, 12], gene mutation [13, 14], chromosomal instability [15], and delayed cell death [16]. The nature of the factors causing these two effects is still unknown. Some studies have suggested that a small protein [2, 17] molecule or long-lived ROS [18, 19] may be potential bystander factors. Emerging data has suggested the involvement of MAPK signal-transduction pathways in the process [20, 21]. MAPK pathways play an important role for cell survival after radiation exposure [22] and...
there are reports of ERK, JNK and p38 activation at low dose α-irradiation and this activation was attenuated by antioxidants, superoxide dismutase (SOD) and catalase [20, 21]. Calcium is a signaling molecule involved in cellular metabolism signal transduction pathways. Changes in intracellular calcium modulate cell functions such as secretion, enzyme activation and cell cycle regulation and can lead to apoptosis [26, 27]. Increased [Ca$^{2+}$] has been shown to cause mitochondrial ROS formation [28]. Lyng et al. [11, 12] have reported calcium fluxes, induction of ROS, and loss of mitochondrial membrane potential in cells exposed to medium from irradiated cells. These findings point to the involvement of Ca$^{2+}$ in bystander processes.

Regarding the mechanisms of adaptive response, it is thought that a low priming radiation dose may enhance DNA repair ability through p53 and cellular antioxidant activity [29, 30, 31]. The published data show that signals produced by irradiated cells can induce protection against a real dose of ionizing radiation [32, 33]. These authors have also shown that intracellular calcium fluxes precede the induction of responses in bystander cells exposed to signals from irradiated cells [11, 12, 34]. While the response that generally follows exposure to these bystander signals is cell death, this can be protective if it eliminates damaged cells from the population. Both the bystander effect [1] and the adaptive response [23, 24, 25] have been shown to be induced via irradiated cell conditioned medium transfer. Blood plasma is composed mainly of water, blood proteins and inorganic electrolytes. It serves as transport medium for glucose, lipids, amino acids, hormones, metabolic end products and signal molecules. The main aim of our project was to evaluate the overall effect of low dose irradiation of reporter cells and the bystander influence of blood from gamma irradiated blood on these cells. This bystander mediated adaptive response could have implications to lower the dose for radiotherapy by using the active bystander factors in the blood plasma. The role of both calcium signaling and ROS was examined.

2. Methods and Materials

2.1. Cell Line, Cell Culture and Cell Irradiation.

In all experiments HaCaT cells were used as a reporter cell line. The HaCaT cells are immortalized human keratinocytes. They were cultured in Dulbecco’s MEM: F12 (1:1), supplemented with 10% fetal calf serum 1% penicillin-streptomycin solution 1000 IU, 2mM L- glutamine and 1μg/ml hydrocortisone. The cultures were maintained in an incubator at 37° C, 5 % CO$_2$, 95% humidity. The cells were split twice a week in new flasks to form monolayer cultures and were plated in 6 well or 96 well plates on the previous day before irradiation. Cells were irradiated with a $^{60}$Co teletherapy source with 0.05 Gy dose. The dose rate during the experiments was approximately 1.8Gy/min. Normally the source to flask distance is 80cm but for the 0.05 Gy dose, an extended source to flask distance of 170cm was used. TLDs were used to confirm that the appropriate dose was delivered.

2.2. Blood Samples and Plasma Isolation.

The peripheral blood samples were collected from nine healthy donors, approx. 20 ml per donor. The blood from each donor was separated into two tubes – 10 ml as a sham-irradiated control and 10 ml irradiated with 0.5 Gy. Blood plasma was isolated by centrifugation on 1700 rcf for 12 min. The final volume was 3-4 ml plasma per donor.

2.3. Clonogenic Assay

This is a classical method for determination of biological effects in cell cultures after irradiation. Cells were plated in 6-well plates at a concentration of 400 cells per well. They were irradiated with 0.05 Gy and 2 h later were incubated with diluted plasma (1: 4 in medium). The cells were kept for 24 h in the incubator (37° C, 5 % CO$_2$, 95% humidity). After that cells were washed with PBS and fresh medium was added. Cells were incubated for 7 days at the same conditions. Colony-forming ability, colony size and morphology were evaluated after 7 days.

2.4. Alamar Blue Microplate Assay
This is a fluorescent method for cell viability evaluation. It is based on the reduction of the blue form of Alamar blue dye from cellular dehydrogenases and cytochromes to the pink fluorescent form in metabolically active cells. 4000 HaCaT cells were plated per well in 96-well plates and the cells were irradiated with 0.05 Gy. Two hours after irradiation cells were treated with 100 µl plasma/well diluted with medium 1:4 in the presence of an ROS inhibitor – SOD (superoxide dismutase) at a final concentration of 100 µg/ml. The cells were washed with sterile PBS and fresh medium was added after 24 hours. Cell viability was evaluated 96 h after plasma treatment. Cells were incubated with 5% Alamar Blue in DMEM:F12 without phenol red for 3 hours and fluorescence was read at λ_ex 485 nm and λ_em 545 nm using a Tecan Genios microplate reader.

2.5. Ratiometric Measurements of Calcium.
Intracellular calcium levels were determined using two calcium-sensitive dyes. Fluo 3 exhibits an increase in green fluorescence upon binding to calcium, whereas Fura Red exhibits a decrease in red fluorescence upon binding to calcium. The ratio Fluo 3/Fura Red is an indicator of intracellular calcium levels. Cells were plated in glass bottomed 35mm Petri dishes (conc. 1.10^5) and incubated overnight. Cultures were irradiated and washed twice with a buffer containing 130 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM CaCl_2, and 1 mM MgCl_2 (pH 7.4). Cells were loaded with the calcium-sensitive dyes by incubation with 3 µM Fluo 3 and 3 µM Fura Red AM esters for 1 h in the buffer at 37°C. Subsequently, the cultures were washed three times with buffer. Fluo 3 and Fura Red were excited at 488 nm, and fluorescence emissions at 525 nm and 660 nm were recorded simultaneously using Zeiss LSM 510 confocal laser microscope. The plasma was added 10 s after a stable base line had been established.

2.6. Measurements of Mitochondrial Membrane Potential (MMP).
MMP was measured using rhodamine 123 a green fluorescent dye that accumulates in active mitochondria with high membrane potential. Cultures (approx 1.5.10^5 cells) were irradiated and washed twice with a buffer containing 130 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM CaCl_2, 1 mM MgCl_2, and 25 mM HEPES (pH 7.4). Cells were loaded with 5 µM Rhodamine 123 for 30 min in the buffer at 37°C. Then the cultures were washed three times with buffer. Rhodamine 123 was excited at 488 nm and fluorescence emission at 525 nm was recorded.

3. Results

3.1. Clonogenic assay.
The clonogenic assay is the gold standard method in radiobiology for evaluation of the biological effects of radiation in cell cultures [35]. Recently this assay has been frequently used for determination of bystander effects in cell cultures treated with irradiated cell conditioned medium [1, 2]. Cells exposed to only the priming dose of 0.05 Gy showed approx. 20% reduction in survival compared to the sham irradiated controls. The percentage survival for cells pretreated with the low priming dose and subsequently treated with plasma from donors 1, 2, 3 and 7 was similar to the cells exposed only to the priming low dose irradiation (figure 1). Cells treated with control and irradiated plasma from donor 8 and 9 had a very low percentage survival. The irradiated plasma from donors 4 and 5 had a stimulating effect on the survival of low dose pretreated cells whereas both control and irradiated plasma from donor 6 showed a stimulatory effect. The bystander plasma factors from these donors acted as challenging dose for the low dose irradiated reporter cells and a radioadaptive type response was observed. Interestingly, the cells treated with plasma from irradiated blood from donors 2, 3, 5, and 6 formed bigger colonies than the cells treated with control plasma with more cells in each colony. This also suggests a stimulating effect from the irradiated plasma bystander factors from these donors on low dose pretreated cells.

3.2. Cell viability.
The cells exposed to the low priming dose of 0.05 Gy showed approx. 15% reduction in cell viability compared to the sham irradiated controls. We used superoxide dismutase (SOD), a well known ROS
scavenger, to determine if superoxide was involved in the bystander responses to plasma from irradiated blood in the low dose irradiated reporter cells.

Figure 1. Percentage survival of HaCaT cells pretreated with a priming dose of 0.05 Gy and exposed to control and irradiated plasma from different donors. % survival of unirradiated HaCaT cells and HaCaT cells exposed only to the low priming 0.05 Gy dose is also shown. These cells were also subsequently exposed to medium to control for the effects of adding the challenge dose of donor plasma.

A large heterogeneity in responses of low dose irradiated reporter cells to treatment with plasma in the presence of the ROS scavenger was observed (figure 2). Interestingly, it appeared that ROS were involved in the radioadaptive response seen with irradiated plasma from donors 4 and 5 as no stimulation of viability was observed when the cells were exposed to the irradiated plasma in the presence of SOD. Similarly, no stimulatory effect was observed in the cells exposed to control and irradiated plasma from donor 6 in the presence of SOD.

Figure 2. Percentage viability of HaCaT cells pretreated with a priming dose of 0.05 Gy and exposed to control and irradiated plasma from different donors in the presence of 100 µg/ml superoxide dismutase. % viability of unirradiated HaCaT cells and HaCaT cells exposed only to the low priming 0.05 Gy dose is also shown. These cells were also subsequently exposed to medium to control for the effects of adding the challenge dose of donor plasma.
3.3. Calcium fluxes

Calcium fluxes were observed in cells treated with plasma from donors 5 and 8, which produced bystander-mediated effects in the cell survival experiments. Calcium fluxes in cells are one of the first signals for induction of early processes in apoptosis pathways [26, 27, 36]. Some increases in calcium levels in reporter cells were observed after treatment with plasma from irradiated blood (figure 3). However, not all cells showed the calcium increases as observed previously by Lyng et al [11]. This may be due to an adaptive response to the low priming dose as has been reported previously by Maguire et al [33].

Figure 3. Ratiometric measurement of calcium fluxes in HaCaT cells pretreated with a low priming 0.05 Gy dose and subsequently exposed to plasma from donor 5 (top panel – (a)) and donor 8 (bottom panel – (b)). The open symbols show the response to addition of unirradiated plasma while the closed symbols are representative of different responses observed after addition of irradiated plasma.

3.4. Mitochondrial membrane potential

Decreased fluorescence which indicates depolarization of the mitochondrial membrane potential was observed after treatment with plasma from irradiated blood (figure 4). The MMP decrease may be an early event in the apoptotic process. However, there are emerging data suggesting that, depending on the model of apoptosis, the loss of MMP may not be an early signal for apoptosis, but may be a consequence of the apoptotic-signaling pathway. Very large individual differences were observed for MMP between different donors. For example, cells exposed to irradiated plasma from donor 7 showed a decrease in fluorescence to 84% of the control, in comparison to a decrease to 7% of the control for donor 8 (figure 4). The results for donor 8 for cell survival / viability in irradiated plasma treated
reporter cells are the lowest in the donor group. From the substantial decrease in MMP we could assume apoptotic death in HaCaT cells caused from factors in the irradiated plasma from donor 8. As calcium fluxes were also observed following exposure to irradiated plasma from donor 8, these could be early signals triggering apoptosis in the low dose pretreated reporter cells.

Figure 4. Fluorescence images of HaCaT cells stained with rhodamine 123, which indicates the mitochondria membrane potential, 6 h after treatment with (a) control plasma from donor 7; (b) irradiated plasma from donor 7, (c) control plasma from donor 8 and (d) irradiated plasma from donor 8.
4. Discussion

From the experimental data obtained we could confirm our prior unpublished observations for large individual differences between the bystander effects of irradiated blood plasma from different donors on reporter cells. The use of irradiated blood plasma as a challenging dose for low dose irradiated reporter cells showed a large heterogeneity in cell responses to plasma from individual donors. Two main groups of reporter cell responses can be proposed – radioadaptive (to the irradiated blood plasma from donors 4, 5 and 6) and additive or bystander-mediated cytotoxicity (donor 8 and 9).

These effects may be the result of activation of different signal transduction pathways, accordingly to the individual characteristics of blood cells and blood plasma. There are reports from other authors that observed bystander-mediated radioadaptation of cells in vicinity of low dose irradiated cells [33, 37, 38, 39, 40]. Irradiation of those cells resulted in enhanced cell growth [37, 38] or increased radioresistance [39, 40]. Our experiments used the plasma from irradiated blood as a challenging dose. The results showed that ROS appear to play a role in blood plasma bystander-mediated responses in reporter cells, particularly for donors 4, 5 and 6.

These data give perspectives for future research in the field of bystander effects from irradiated blood plasma. The individual differences suggest specific plasma components to be responsible for the observed effects. Analysis of the blood plasma and blood cells characteristics for each donor before irradiation would be required to identify these specific factors. Identification of these factors could be of high importance for the risk assessment for nuclear power plant workers, who are exposed to low dose irradiation at low dose rates.

A thorough analysis of plasma components and individual characteristics could provide an answer to the question of why there are such large individual differences in the bystander responses and how they could be modulated for use in medical practice.

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