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

A number of studies in the field of radiobiology contradict the Linear Non- Threshold model in the low dose range. These observations indicate that there may be more harmful effects for cells exposed to low dose irradiation than would be predicted by extrapolation from high dose data.

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. There are two main theories for the transfer of bystander signals from irradiated to non-irradiated cells. The first is that soluble bystander factors are transmitted through the medium to non-irradiated cells. The second is that bystander factors are transferred from irradiated cells through gap junction intracellular contacts.

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”. The fine balance between these two radiobiological effects operating in the low dose range could affect current risk assessment methods for carcinogenesis at low dose irradiation and could affect doses used in radiotherapy.

The purpose of the present Short-Term Scientific Mission (STSM) is to continue the collaboration in the field of bystander effects of plasma from irradiated peripheral blood between “Radiation and Environmental Science Center”, Focas Institute, DIT, Dublin and the laboratory “Molecular Radiobiology and Epidemiology”, NCRRP, Sofia. This STSM was realized with the kind help and co-operation of Dr. Fiona Lyng, Centre Manager at RESC. The two laboratories have completed a successful STSM project in this area that issued new questions about individual differences in bystander responses. In the present project we investigate the properties of plasma from irradiated blood to act as a challenging dose on low dose irradiated reporter cells. We also extended the number of donors to determine if there was a dependence in the bystander responses of individuals and if they could be grouped in types of responses.

The opportunity to work at the RESC laboratories gave me the chance to be trained in the modern techniques for cell culture maintenance, cell viability evaluation and fluorescence

techniques for detection of metabolic activity and signal transduction pathways. Initiating of these techniques will be very useful in my future work as a radiobiologist at the NCRRP and I would use them in my future research.

The main aim of our project is to evaluate the overall effect of low dose irradiation of reporter cells and the bystander influence of plasma 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. For this purpose we addressed the following questions:

1. Has the influence of plasma from irradiated blood on low dose irradiated cells a positive (adaptive response) or an additive effect?
2. Are cellular signal transduction pathways involved in these effects and are they up- or down- regulated?
3. Are ROS involved in realization of these effects?
4. Could we inhibit these effects by inhibition of ROS and signal transduction pathways?
5. Could we modulate these effects for using them in radiotherapy?

Methods and Materials

Cell Line and Cell Culture. In all experiments HaCaT 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 ^{60}Co teletherapy source (dose rate approx. 1,8 Gy/min) with 0.05 Gy dose.

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 non-irradiated control and 10 ml were 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.

Alamar Blue Microplate assay. This is 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. Six hours after irradiation cells were treated with 100 µl plasma/well diluted with medium 1:4. 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.

Alamar Blue Microassay in the Presence of Signal Transduction Pathways Inhibitors. The experiments were performed to determine the role of these pathways for bystander-mediated effects. 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 diluted plasma/well in the presence of signal transduction pathways inhibitors: ROS inhibitor – SOD (superoxide dismutase) at a final concentration of 100 µg/ml; MAPK (mitogen-activated protein kinase) inhibitor – U0126, inhibitor of the ERK signalling pathway at a final concentration of 20µM; SP600125 − inhibitor of JNK pathway at a final concentration of 10µM.

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 6 h later were incubated with diluted plasma (1 plasma : 4 medium). The cells were kept for 24 h in 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.

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 stabile base line had been established.

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.

Results

Clonogenic assay. The clonogenic assay is an essential method in radiobiology for evaluation of the biological effects of radiation in cell cultures. Recently this assay is frequently used for determination of bystander effects in cell cultures treated with irradiated cell conditioned medium. Our results for clonogenic survival of low dose irradiated HaCaT cells treated with control and irradiated plasma showed the colony-forming ability for cells treated with plasma from donors 1, 2, 3 and 7 to be very similar to non-irradiated controls (for control and irradiated plasma – Tab. 1).

Table 1. Colony forming ability of 0.05 Gy irradiated HaCaT cells treated with blood plasma.

Colony-forming ability of low dose irradiated reporter cells treated with blood plasma										
Control	Donor	1	2	3	4	5	6	7	8	9
100%	Control plasma	105,29%	101,59%	94,17%	107,41%	101,59%	133,86%	104,76%	35,98%	29,63%
100%	Irradiated plasma (0.5 Gy)	103,70%	105,82%	103,17%	116,93%	131,75%	129,63%	94,71%	32,80%	38,62%

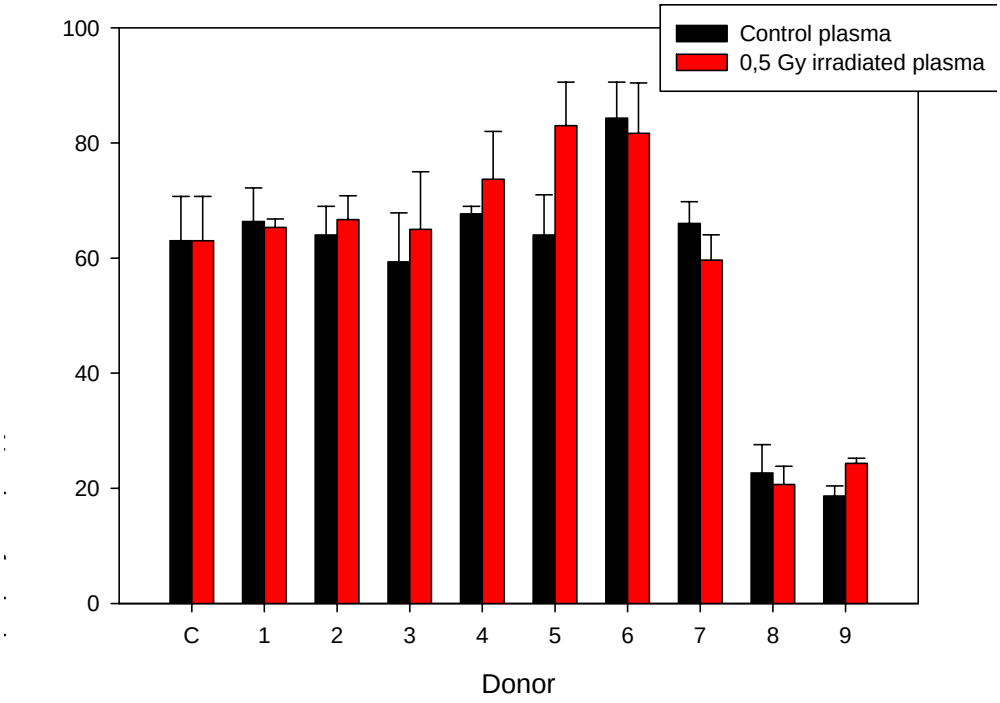

Figure 1. Number of colonies formed after 7 days from plasma treated HaCaT cells.

Cells treated with control and irradiated plasma from donor 8 and 9 had a very low number of colonies. The irradiated plasma from donors 4 and 5 had a stimulating effect on the colony-forming ability 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. More interestingly, the cells treated with plasma from irradiated blood from donors 2, 3, 5, and 6 formed bigger colonies with more cells in each one. This also suggests a stimulating effect from the irradiated plasma bystander factors from these donors on low dose pretreated cells.

Cell viability. The 0.05Gy irradiated cells treated with plasma showed a decrease in cell viability compared to non-irradiated control cells treated with medium (Tab. 2).

The cell viability of reporter cells is presented relative to non-irradiated cells treated only with medium (as controls for treatment with control plasma) or with irradiated medium – controls for the treatment with plasma from the irradiated blood.

Table 2. Cell viability of plasma treated cells, (%) to non-irradiated cells in medium

Cell viability*, %												
Control	Non-irradiated	Irradiated	Donor	1	2	3	4	5	6	7	8	9
Medium	100	86,85	Plasma	87,46	75,82	83,16	83,03	82,92	88,72	83,13	76,19	83,09
Irradiated medium	100	96,36	Plasma from Irradiated Blood	88,88	78,65	81,58	82,81	77,39	87,82	86,76	72,97	81,09

* results are presented relative to non-irradiated controls

The effect is obvious in cells treated with control plasma and in cells treated with plasma from irradiated blood (Fig. 2). There are bystander effects in cells treated with plasma from all donors. The irradiated plasma lowered the cell viability in all cases. There are individual differences in reporter cell responses. The largest decrease in cell viability is in cells treated with plasma from donors 5 and 8. However, this effect is present in cells treated with plasma and in cells treated with plasma from irradiated blood. Possibly, there are individual differences in plasma capacity to influence cell viability and blood irradiation increases these individual plasma effects. The irradiated blood plasma effect on HaCaT cells could be qualified as an additive effect for donor 8.

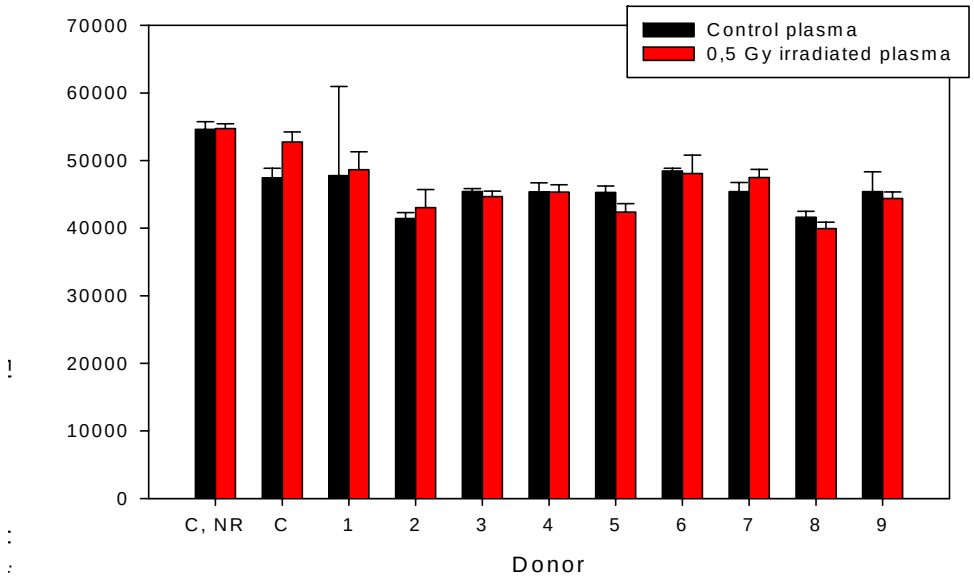

Figure 2. Cell viability of treated with plasma and treated with plasma from irradiated blood 0.05 Gy irradiated HaCaT cells.

Calcium fluxes in the cells. Calcium fluxes were observed in cells treated with plasma from donors 5 and 8, which produced bystander-mediated effects in cell viability experiments. The calcium fluxes in cells are one of the first signals for induction of early processes in apoptosis pathways. Calcium levels in reporter cells do not show significant increase after treatment with plasma from irradiated blood (Fig. 4 and 6). There is a slight increase in the Fluo3/FuraRed ratio in individual cells treated with irradiated plasma 20 s after plasma addition, but they are not significant. The effect is mosaic and there is no uniformity in the responses of the cells from one population. Our observations could not give undoubted support to involvement of calcium signaling in bystander effects. The reduction in cell viability in bystander cells may be due to apoptotic death (in addition to mitotic cell death), but calcium signaling does not appear to be involved in this process.

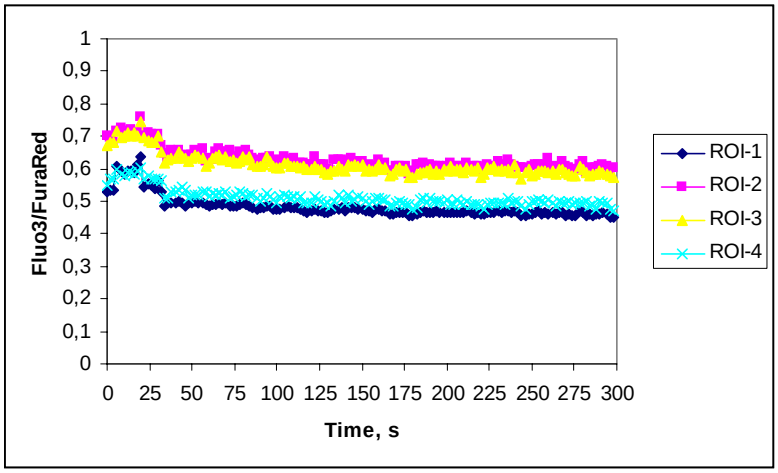

Figure 3. Ratiometric evaluation of Ca fluxes in 0.05 Gy irradiated HaCaT cells treated with control plasma from donor 5 (ROI –Region of Interest).

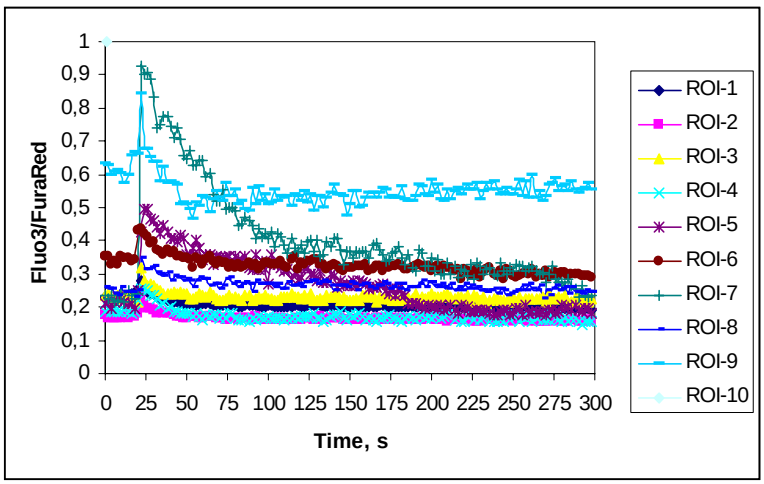

Figure 4. Ratiometric evaluation of Ca fluxes in 0.05 Gy irradiated HaCaT cells treated with irradiated plasma from donor 5.

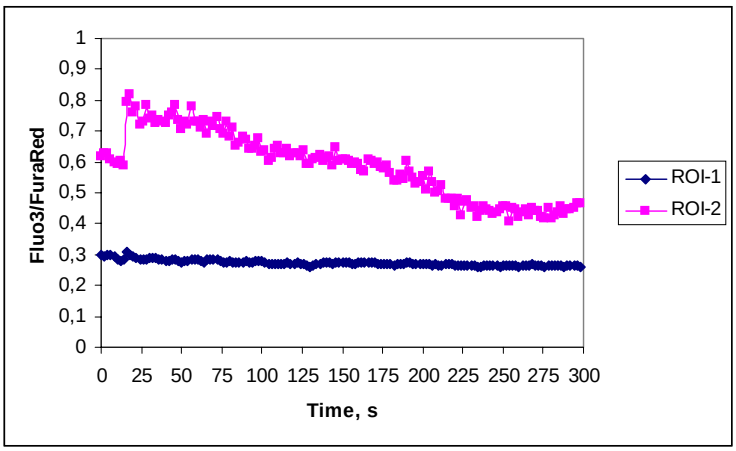

Figure 5. Ratiometric evaluation of Ca fluxes in 0.05 Gy irradiated HaCaT cells treated with control plasma from donor 8.

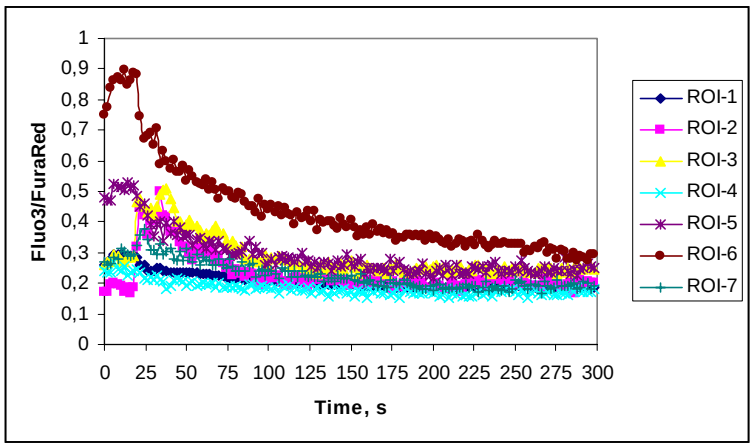

Figure 6. Ratiometric evaluation of Ca fluxes in 0.05 Gy irradiated HaCaT cells treated with irradiated plasma from donor 8.

Mitochondrial membrane potential. The cultures plated for MMP determination for the donors 1, 3, 5 and 9 did not attach to the glass bottoms of the Petri dishes so only results for donors 2, 6, 7 and 8 are presented. Decreased fluorescence indicates depolarization of the mitochondrial membrane potential. The mean fluorescence intensity for control and irradiated plasma is shown in Fig.7.

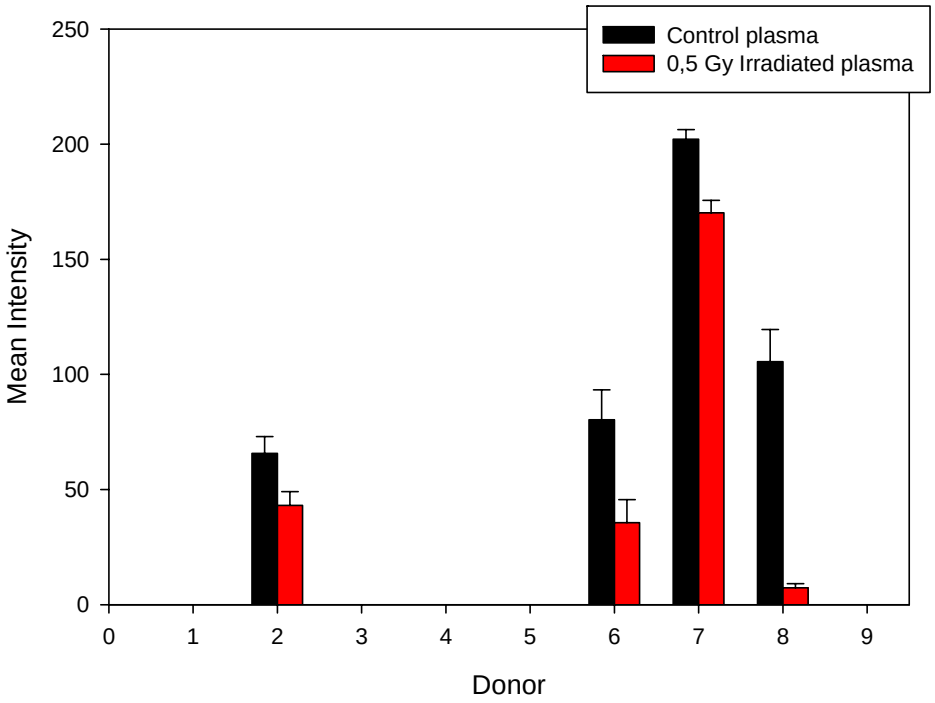

Figure 7. Mitochondrial membrane potential as mean intensity of Rhodamine 123 fluorescence.

For comparison of MMP changes between donors, the results were presented relative to controls, treated with plasma from the same donor (Tab. 3).

Table 3. Decrease of mitochondrial membrane potential in treated with plasma from irradiated blood 0.05 Gy irradiated reporter cells

Decrease of mitochondrial membrane potential				
Donor	2	6	7	8
Reduction in Rhodamine 123 fluorescence intensity (%)*	65,68±5,94	44,33±10,04	84,19±5,43	6,91±1,82

* presented relative to results for control plasma

In all cases there is decrease in MMP. 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 are observed for MMP between donors 7 and 8. Donor 7 has decrease to 84,19%, in comparison with donor 8 – to 6,91%, or almost total loss of MMP. The results for donor 8 for cell 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. However, there was no increase in calcium fluxes in these cells. The Ca^{2+} could not be identified as early signals triggering apoptosis in the low dose pretreated reporter cells. This may indicate a different pathway not involving calcium.

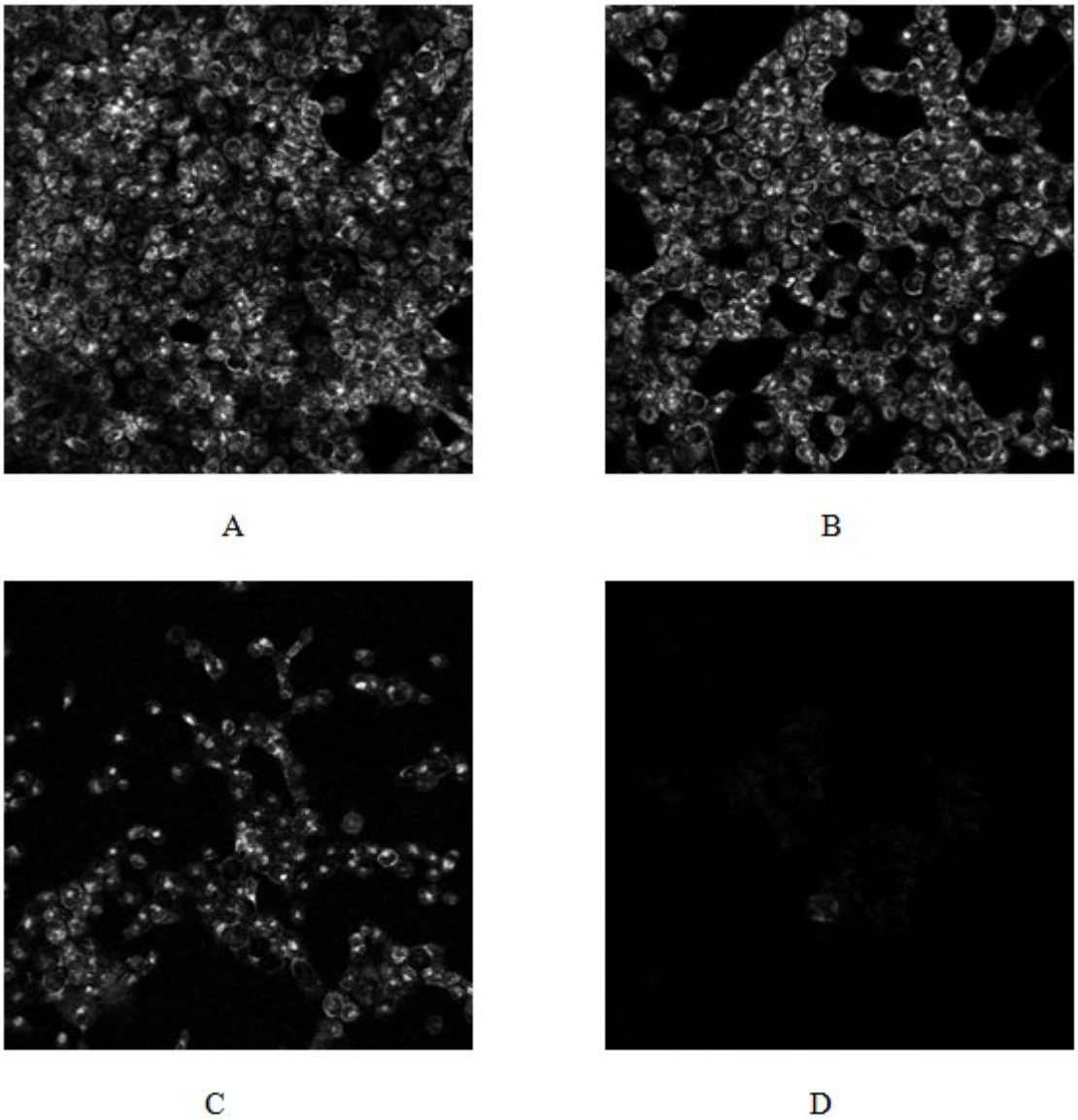

Figure 8. Fluorescent images of mitochondria membrane potential, 6 h after plasma treatment. A. Treatment with control plasma from donor 7; B. Identical levels to control of mitochondrial membrane potential in treated with irradiated plasma from donor 7 cells; C.

Control and irradiated plasma (D) from donor 8 treated cells. There is an obvious decrease in mitochondrial membrane potential 6h after treatment with the plasma from irradiated blood.

Cellular viability in the presence of inhibitors of signal transduction pathways. In our first series of experiments we used superoxide dismutase (SOD), a well known ROS scavenger to determine if superoxide was involved in bystander responses to plasma from irradiated blood in low dose irradiated reporter cells.

Table 4. Celullar viability of 0.05 Gy irradiated HaCaT cells in the presence of signal transduction pathways inhibitors.

Cell viability in the presence of signal transduction pathways inhibitors*							
Donor	Control,NR cells	Control plasma+SOD	Irradiated plasma +SOD	Control plasma+ERK inh.	Irradiated plasma +ERK inh.	Control plasma+JNK inh.	Irradiated plasma+JNK inh.
1	100,00%	71,71%	66,63%	9,07%	7,99%	10,22%	2,35%
2	100,00%	88,93%	75,75%	9,22%	7,45%	9,31%	9,76%
3	100,00%	59,32%	50,77%	6,90%	6,02%	9,26%	14,38%
4	100,00%	67,42%	68,70%	6,71%	9,32%	9,13%	8,39%
5	100,00%	57,19%	52,73%	7,04%	6,71%	10,64%	8,92%
6	100,00%	52,93%	56,71%	5,35%	7,15%	12,93%	8,43%
7	100,00%	52,08%	37,96%	8,35%	7,08%	11,97%	11,01%
8	100,00%	29,41%	28,71%	4,01%	3,85%	8,27%	7,11%
9	100,00%	21,66%	31,62%	3,03%	2,86%	6,48%	1,92%

* relative to non-irradiated controls

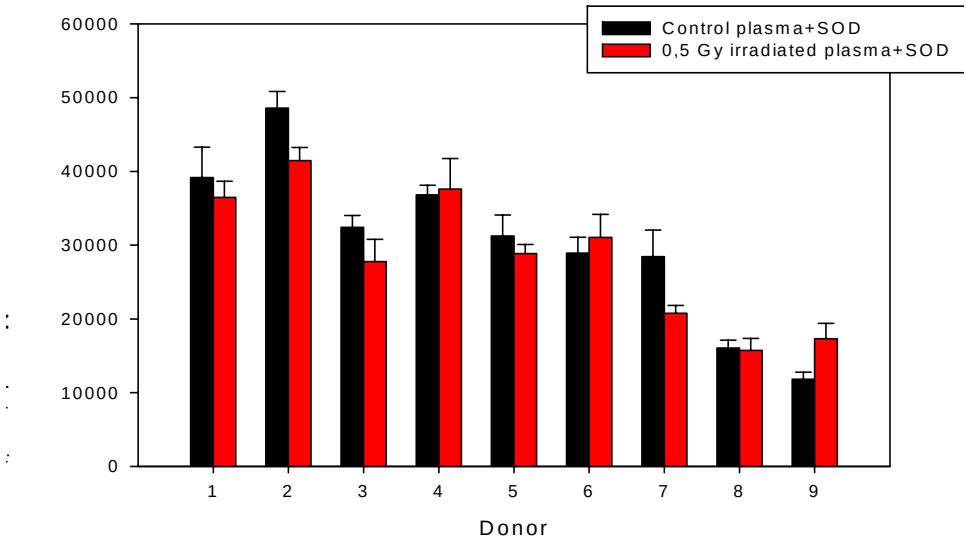

Figure 9. Cellular viability in the presence of SOD for plasma treated 0.05 Gy irradiated HaCaT cells.

A large heterogeneity in responses of low dose irradiated reporter cells to treatment with plasma in the presence of ROS scavenger SOD was observed. Surprisingly, incubation of the cells either with irradiated and non-irradiated blood plasma in the presence of SOD reduced to a large extent their viability, compared with non-irradiated controls (Tab. 4 and Fig. 9). It appears that the bystander cytotoxic factors are not ROS, because a positive effect after ROS inhibition was not observed. The loss of viability is largest in the cells treated with plasma from donor 8 and 9. It is most likely that blood cells from these donors secrete toxic factors for HaCaT cells that are transmitted through the blood plasma.

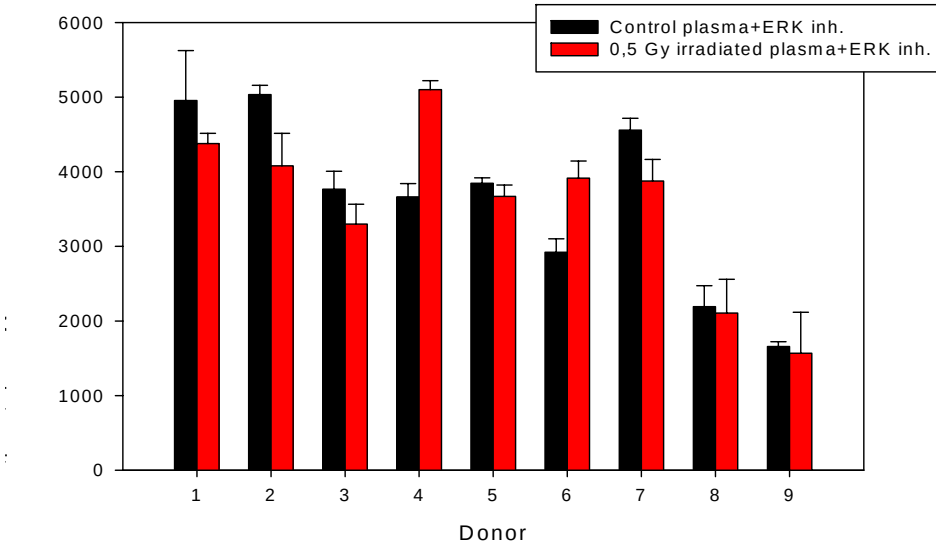

Figure 10. Cellular viability in the presence of ERK inhibitor for plasma treated 0.05 Gy irradiated HaCaT cells.

The results for the cell viability of low dose pretreated HaCaT cells in the presence of ERK signal transduction pathway showed a dramatic loss of viability (Tab. 4 and Fig. 10). ERK, one of the MAP kinase signal transduction pathways, is recognized as a pro-survival pathway. Possibly its inhibition blocks the transmission of signals that could rescue the cells from apoptotic death. In general this process could have a positive effect for tissues and organisms, because it is a way for removing cells with gene mutations or potentially lethal damage. The cells treated with plasma from donors 8 and 9 show again very low levels of metabolic activity in the presence ERK inhibitors, either for the control and for the irradiated plasma. Interestingly, the cells treated with plasma from irradiated blood from donors 4 and 6 exhibit higher levels of cellular viability in the presence of the ERK inhibitor. Results for

these cells from the clonogenic assay show a radioadaptive response to treatment with plasma from irradiated blood after low dose irradiation. It appears that the ERK signal transduction pathway is not involved in the transmission of these radioadaptive signals from irradiated blood to reporter cells.

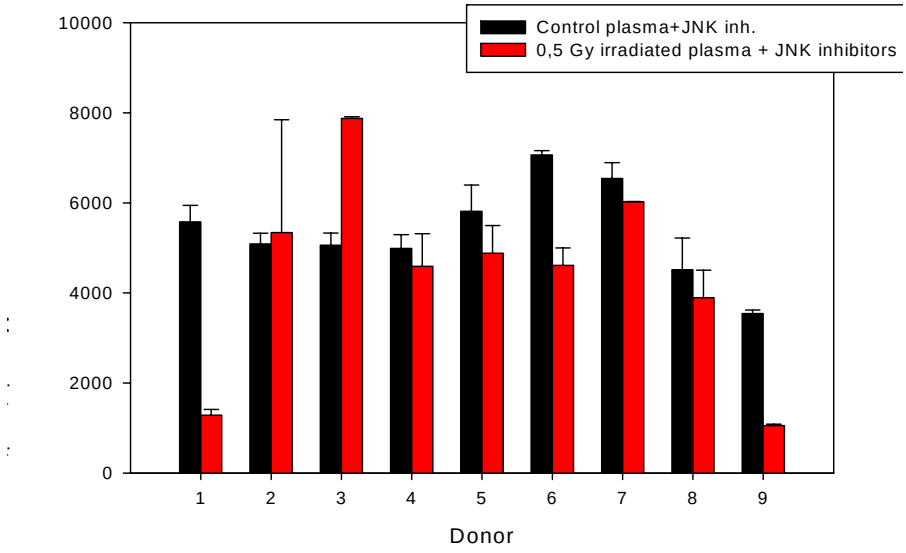

Figure 11. Cellular viability in the presence of JNK inhibitor for plasma treated 0.05 Gy irradiated HaCaT cells.

The viability of 0.05 Gy irradiated cells in the presence of the JNK signal transduction pathway inhibitor is very low compared to non-treated with inhibitors controls (Tab. 4 and Fig. 11). There is tendency for irradiated plasma to lower the cell viability of reporter cells. The inhibition of the JNK pathway, which is known as pro-apoptotic, leads to reduced cellular viability in irradiated blood plasma treated cells from donors 4, 5 and 6. The cells treated with blood plasma from these donors showed a radioadaptive response for the clonogenic assay. It is possible that the JNK signal transduction pathway assists the stimulating bystander signals generated in the irradiated blood. The inhibition of JNK pathway may block the transmission of the bystander signals from irradiated blood plasma to reporter cells.

The cellular viability in the presence of JNK inhibitors for cells treated with irradiated plasma from donor 1 and 9 is statistically significant very low compared to controls. The irradiated plasma from donor 3 in the presence of the same inhibitor has contradictory effect –

stimulates cellular viability. It is another example for the heterogeneity of cellular responses to the bystander stimuli.

Conclusions

From the experimental data obtained during the project we could confirm our prior 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. The radioadaptive response after treatment with plasma from donors 5 and 6 is supported by the increased colony-forming ability and larger colony size for reporter cells treated with irradiated plasma. Probably, there is involvement of the JNK signal transduction pathway in the radioadaptive effects. Although JNK is thought to be part of signaling that triggers apoptosis in the cells, the overall effect for the cell population could be positive. Its activation in low dose pretreated cells by irradiated plasma bystander factors could eliminate the cells with mutations and potential lethal damage in the genome and this could lead to survival of the most adaptive cells with better clonogenic abilities.

The results showed that ROS did not appear to play a role in bystander-mediated responses in reporter cells. The ROS scavenging by SOD did not show a positive effect on cell viability. Probably, ROS are not involved in the bystander effects of plasma from irradiated blood.

We could summarize the results as follows:

1. There are large individual differences between bystander effects in low dose irradiated reporter cells, depending on the donor plasma.
2. The observed effects in these cells generally could be divided into **two groups: adaptive responses and additive effects.**
3. There was no evidence for ERK signal transduction pathway involvement in these effects. Perhaps **JNK signal transduction pathway plays a significant role for the bystander responses in the low dose irradiated reporter cells.**

4. Probably ROS are not involved in bystander effects in low dose pretreated reporter cells.
5. **The heterogeneity of 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.

These data gave perspectives for future researches in the field of the 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 doses 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.