SCIENTIFIC REPORT

Reference code: COST-STSM-P9-02784

NF-κB regulation of Bcl-2 and Bax expression in bystander cells

The aim of the above STSM was to evaluate the involvement of NF Kappa B transcription factor in radiation induced bystander responses. The expression levels and localization of NF Kappa B and two proteins expressed under its control Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic) were studied. Bcl-2 and Bax regulate the apoptotic processes after embedding in the mitochondrial membrane. Mitochondrial membrane potential changes were used as a signal for triggered apoptotic processes in the cells.

We used the common model of bystander experiments – transfer of irradiated cells-conditioned medium (ICCM) on non-irradiated cells. The cells responses were studied on 1 and 24 hour after the ICCM transfer and were compared to direct irradiated cells.

The following methods were employed:

Cell Line and Cell Culture. 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 % CO2, 95% humidity. The cells were split twice a week in new flasks to form monolayer cultures and were plated in T 25 culture flasks or on coverslips in 6 well plates on the day before irradiation. Donor cells were irradiated with a 60Co teletherapy source (dose rate approx. 1,8 Gy/min) with doses 0.05 and 0,5 Gy.

Generation of ICCM. The HaCaT cells were plated in T25 flasks (approx. 5.105 cells per flask) and irradiated. The medium from the donor and the control flasks was poured off 1 h after irradiation. The medium was filtered through 0,22 μm filter to ensure that there are no cells present. After that it was divided into aliquots and stored at -20 ○C. Recipient cells were plated onto coverslips at a concentration of 2.105 cells per coverslip (for immunostaining) or in T25 flasks at a concentration of 3.105 cells per flask (for western blotting) and exposed to ICCM for 1 or 24 h.

Immunofluorescence. The directly irradiated and bystander treated cells were fixed in freshly prepared 4% paraformaldehyde at 1 h and 24 h after exposure. They were washed 3x5 min in PBS and incubated for 3 h in blocking buffer (5% donkey serum, 1% BSA, 0,2% Triton X-

100). After that the cells were incubated overnight at 4○C with NF-κB, Bcl-2 and Bax antibodies (diluted 1:100 in 1% donkey serum, 1% BSA, 0,1% Triton X-100). The cells were washed 3 x 5 min with PBS and incubated with secondary anti- NF-κB, anti-Bcl-2 and anti-Bax antibodies (dilution 1:200) conjugated with fluorescent labels (NorthernLight493, NorthernLight557 and NorthernLight637, all from R&D Systems Inc., MN, USA). Negative controls without primary antibodies were included with every immunofluorescence run.

Western blot analysis. Cells were lysed, centrifuged at 13.103 rpm and protein concentration was determined by the Bradford method. SDS-PAGE was performed on 12% polyacrylamide gels by the method of Laemmli. Equal amount of proteins were loaded from each parallel experiment and after PAGE performance, gels were electroblotted using the semi-dry method onto PVDF membranes. Immuunoblot analysis was performed either with anti- NF-κB, anti-Bcl-2 or anti-Bax monoclonal antibodies (diluted 1:1000 in PBS containing 1% BSA and 0.1 % Tween20). Then the membranes were incubated with peroxidase–conjugated IgG secondary antibodies (dilution 1:10 000) and visualized using a chemiluminescence reaction.

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 2.105 cells per Petri dish) were incubated with ICCM for 6h and washed twice with a buffer containing 130 mM NaCl, 5 mM KCl, 1 mM Na2HPO4, 1 mM CaCl2, 1 mM MgCl2, 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.

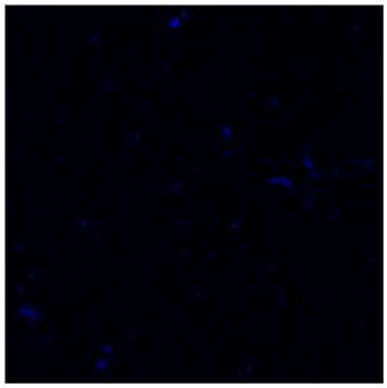
Results.

Immunofluorescence. Immunofluorescence images of directly irradiated cells did not show increased Bax and Bcl-2 levels for both doses 0.05 (Fig. 1A) and B)) and 0.5 Gy (Fig. 1E) and F)) at 1 h and 24 h after irradiation. As reported previously in numerous publications, increased NF Kappa B levels were observed after 1 h in 0,5 Gy irradiated cells. In Fig. 1 G) nuclear translocation of NF Kappa B can be seen in the same cells.

The cells incubated with 0,05 Gy ICCM showed the most intensive fluorescence corresponding to high levels of Bax expression after 1 h incubation (Fig. 2 A)). The anti-apoptotic Bcl-2 levels was found to be increased after 1 h exposure to 0,05 Gy ICCM (Fig. 2 B)). Interestingly, the NF Kappa B levels do not increase in the bystander cells. NF Kappa B translocation to the nucleus was not observed in any of the ICCM treated cells. A similar

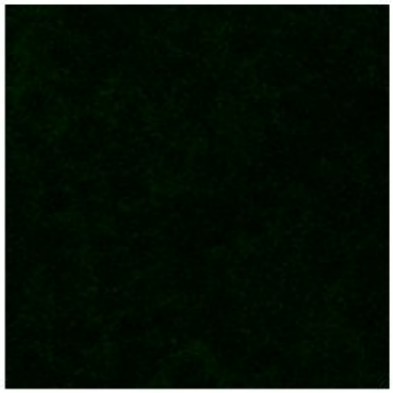
effect was observed for 0,5 Gy ICCM treated cells. Bax expression levels continued to be increased after 24 h for both 0,05 and 0,5 Gy ICCM incubation (Fig. 2 E) and G)). In contrast, the anti-apoptotic Bcl-2 protein expression decreased after 24 h ICCM treatment (Fig. 2 H)).

Western blot analysis. Western blotting was performed with 0; 0,05 and 0,5 Gy directly irradiated and ICCM treated cells after 1 and 24 hours. After developing of the immunoblotted membranes chemiluminescent lines were not visible. Due to the long period necessary for cells to divide to sufficient number for the experiment (3 days and 1 day incubation with ICCM) and the 2 days procedure of Western blotting, we were not able to repeat the western blot for the time of the STSM. It is planned to be repeated in RESC's laboratory and results to be presented in a publication.



A)

B)



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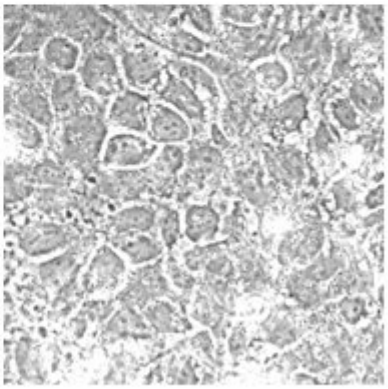

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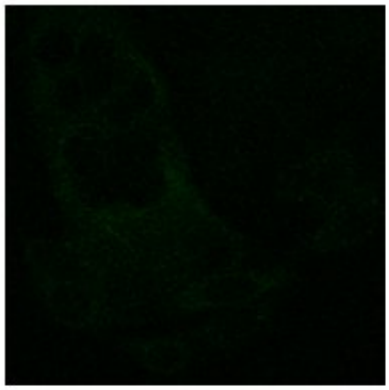

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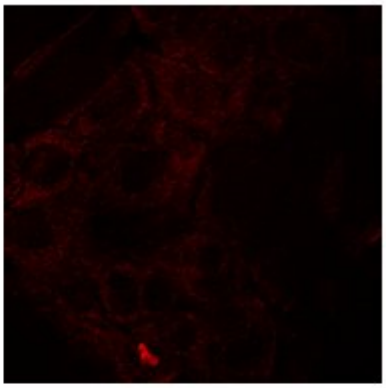

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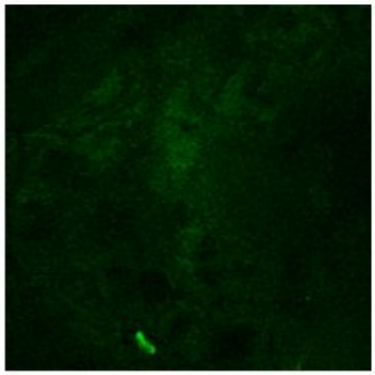
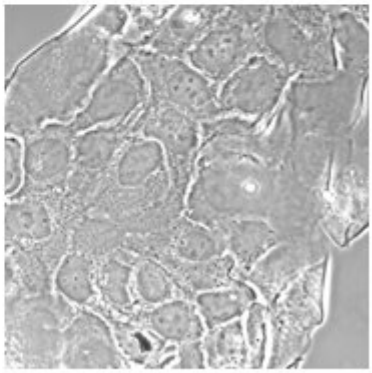
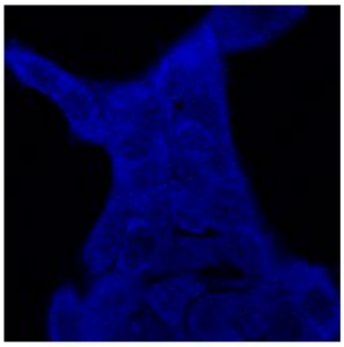


G)

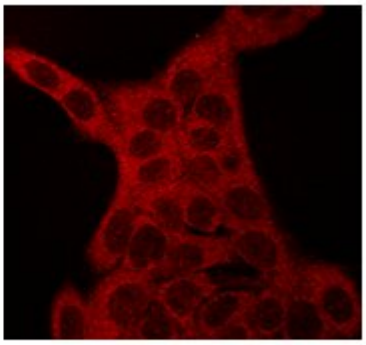


H)

Figure 1. Immunofluorescence images of 0,05 Gy irradiated cells for Bax (A); Bcl-2 (B), NF Kappa B (C) and phase contrast image of the cells (D). Immunofluorescence of 0,5 Gy irradiated cells: Bax (E), Bcl-2 (F), NF Kappa B (G) and phase contrast image (H).



A)



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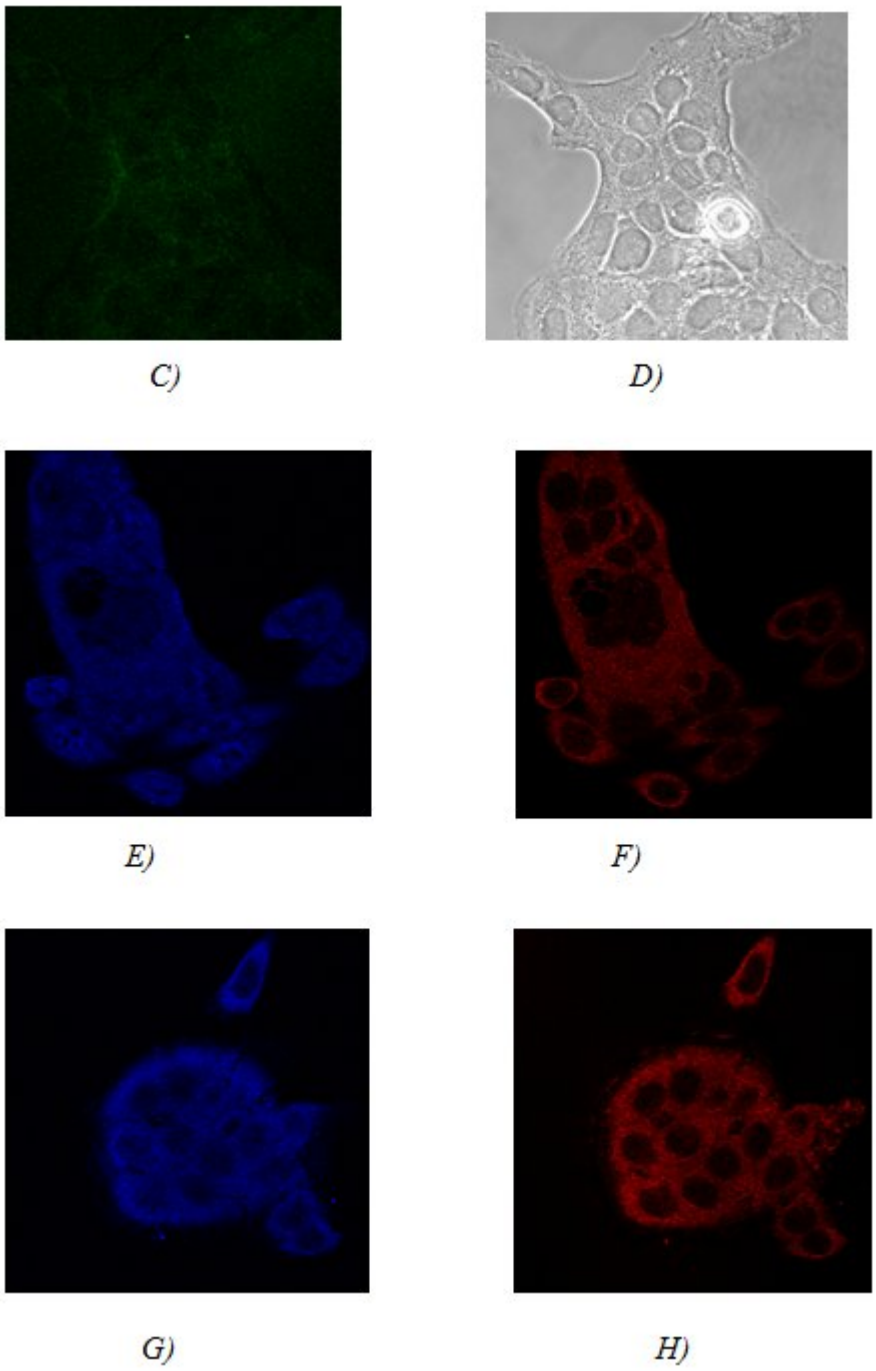

Figure 2. Immunofluorescence images of 1 h 0,05 Gy ICCM treated cells for Bax (A); Bcl-2 (B), NF Kappa B (C) and phase contrast image of the cells (D). Immunofluorescence images of 24h 0,05 Gy ICCM incubated cells: Bax (E), Bcl-2 (F) and 1 h 0,5 Gy ICCM treated cells Bax (G) and Bcl-2 (H).

Measurements of Mitochondrial Membrane Potential (MMP). The results for MMP changes (Fig. 3) confirmed the previous observations of the RESC's scientists after bystander

experiments on HaCaT cells. MMP is decreased both in 0,5 Gy (Fig. 3 B)) directly irradiated and 0,5 Gy ICCM treated (Fig. 3 D)) cells.

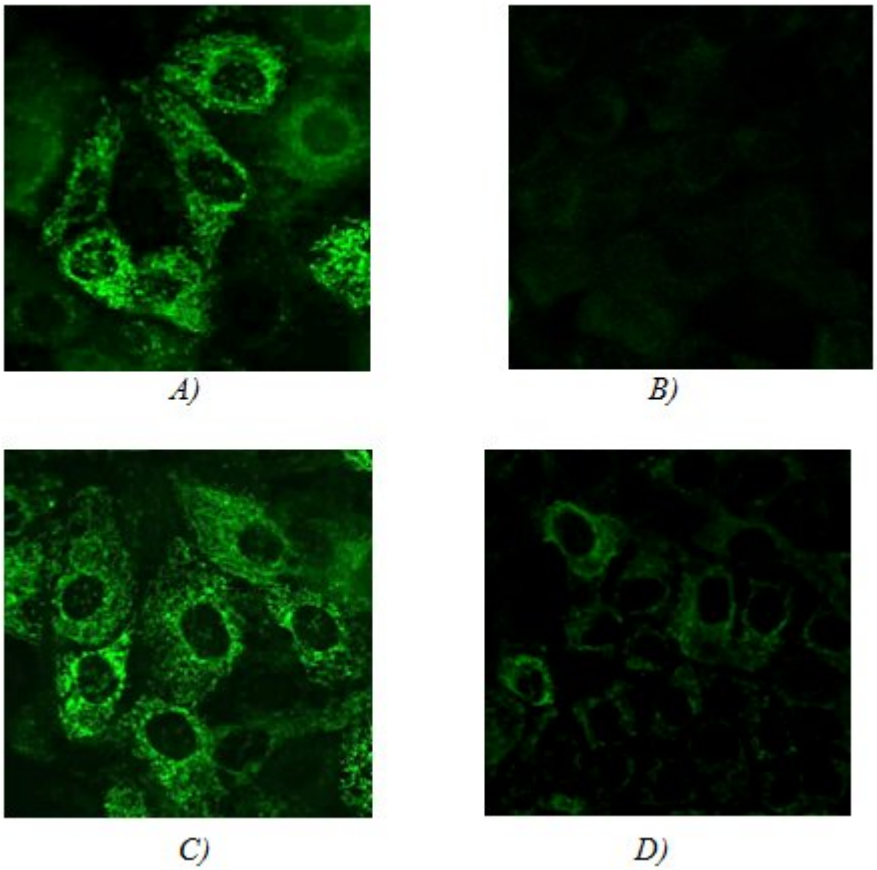

Figure 3. Mitochondrial membrane potential changes. Control (A), 0,5 Gy irradiated (B), Bystander control medium incubated (C), and 0,5 Gy ICCM treated (D) cells.

Discussion.

The results confirmed NF Kappa B activation after direct irradiation with 0,5 Gy. However, bystander induced NF Kappa B activation was not observed in the bystander cells. The bystander cells showed increased levels of cytoplasm located pro-apoptotic Bax protein that was maximally increased at 1 h after ICCM incubation. The decrease in MMP of the same cells is possibly caused by bystander induced apoptotic processes in the cells triggered from Bax embedding in the mitochondrial membranes. Interestingly, at same time, in 0,05 Gy ICCM incubated cells, high Bcl-2 expression level were observed. But that effect probably can not be due to NF Kappa B transcription regulation, as no nuclear translocation of the transcription factor was detected. All our results point to possible Bax-mediated apoptotic death in bystander cells after 0,05 and 0,5 Gy ICCM treatment. There was no evidence for NF

Kappa B involvement in this process. But the results can be described only as preliminary and the western blot analysis needs to be repeated to prove the changes in protein levels observed using immunofluorescence staining.

We plan to continue the work on western blot analysis to confirm the results. Afterwards, they will be presented in a collaborative RESC, Focas Institute, DIT, Dublin and LMRE, NCRRP, Sofia future publication on radiation induced bystander responses.