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Perturbation of cellular signaling cascades modulated by ionizing radiation and environmental stress

M. Ugolini(*)
on behalf of A. Ottolenghi, G. Babini, G. Baiocco, L. Mariotti and J. Morini

Dipartimento di Fisica, Università di Pavia and INFN, Sezione di Pavia
Pavia, Italy

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Summary. — Cellular signaling plays a central role in the regulation of several cell functions, which can be perturbed by different external stimuli, including environmental stress and ionizing radiation. The dysregulation of intra- and extracellular mechanisms may alter the correct behaviour of cells. The aim of this work was to investigate the activation of strongly interlaced intracellular signaling pathways, following the exposure to low- and medium-doses of X-rays, with a focus on the mechanisms involved in the inflammatory- and apoptotic-related responses. In particular, the temporal dynamics of the ERK1/2 and PKB/AKT pathways and their possible dose dependences were investigated. The presented results indicate a clear dose dependence of such pathways only at early time points, suggesting a fast response of the system to X-rays and the need for further studies at shorter times after exposures.

PACS 87.53.-j – Effects of ionizing radiation on biological systems.
PACS 87.50.-a – Effects of electromagnetic and acoustic fields on biological systems.

1. – Introduction

Intra- and extra- cellular signaling cascades are major components in the regulation of cell functions, such as survival, proliferation, apoptosis and differentiation [1, 2]. The perturbation of pathways involved in these processes by several stressors (*e.g.* ionizing radiation, oxidative stress, etc.) can alter the correct growth of the cell and, in several cases, it can contribute to chemotherapeutic drug resistance [3], proliferation of cancer initiating cells and premature aging [4, 5].

Although recent works have shown evidence of low-dose radiation effects on both extra- and intra-cellular components, the connections among different cell compartments

(*) E-mail: martina.ugolini88@gmail.com

and the mechanisms underpinning cell-to-cell communication are often not fully understood and further investigation is needed [6,7]. In order to simplify the complex response of cells to different perturbations, a "step-by-step" approach is often adopted. Within this approach, one focuses the attention on one single process at a time, evaluating its dose-dependence and temporal dynamics, and trying to reconstruct the complex net of interactions, where the complexity is mainly due to non-linear inter-relationships (*e.g.* negative feedback loops). Intercellular signaling is an example of phenomena where the behaviour of the system cannot be described or explained as the simple result of the sum of each signal involved (reductionist vision). Therefore a *system biology* approach is needed, in order to clarify the complex interactions and the effects of perturbations (such as ionizing radiation) on these interactions.

As a specific cell-to-cell communication study, Mariotti *et al.* have shown a dose dependence of the IL-6 concentration in the medium of *in vitro* cultured human fibroblast cells exposed to γ-rays [8]. IL-6 is an interleukin([1]) acting both as pro- and anti-inflammatory cytokine. Their study has shown that there is an over expression of cytokine for cells irradiated with 0.25 Gy of γ-rays, while at 1 Gy the expression of IL-6 is lower than in sham cells([2]). Such a difference at the extra-cellular level suggests that also intra-cellular mechanisms might be differently perturbed.

Therefore the intra-cellular communication dynamics after ionizing radiation exposures were investigated in this work. In particular the attention was focused on the Extracellular Signal-Regulated Kinase (ERK1/2) and on the Protein-Kinase B (PKB, known also as PKB/AKT) pathways, which are strictly connected to the inflammatory response of cells [9]. ERK1/2 and PKB/AKT are generally activated in response to growth factors or cytokines, present in the extracellular matrix, and they were observed to be misregulated by ionizing radiation exposure [10]. Extra-cellular signals bind to a specific transmembrane receptor, starting an inner cascade of proteins, which leads in turn to the phosphorylation (*i.e.* the activation) of ERK1/2 and PKB/AKT, as illustrated in fig. 1. In the absence of growth stimuli the majority of ERK1/2 and PKB/AKT are confined in the cytoplasm by various regulatory proteins. Upon their phosphorylation, due to an external stimulus, they dissociate from the inhibiting proteins and translocate to the nucleus [11]. Once inside the nucleus ERK1/2 and PKB/AKT can phosphorylate more than one hundred different substrates, therefore they can generate a variety of different biological responses [12].

Both these pathways are shown to be dysregulated in many tumours, increasing their radioresistance or increasing tumour cell proliferation [13]. All these properties suggest that ERK1/2 and PKB/AKT can be attractive targets for anti-tumoral therapies [14].

2. – Materials and methods

Cell lines

AG01522 normal human fibroblast at passage 3-4 obtained by Coriell Cell Repositories (USA) were used. Cells were incubated at 37 °C in humidified atmosphere of 5% CO_2 as monolayer in T-75 flasks (Greiner-BioOne, Germany) and maintained in alpha Modified

([1]) Interleukins are a subgroup of cytokines mainly involved in controlling the function of the immune system.

([2]) Sham cells are cells which underwent the same mechanical and thermal stress as the irradiated ones, but they did not receive any radiation dose.

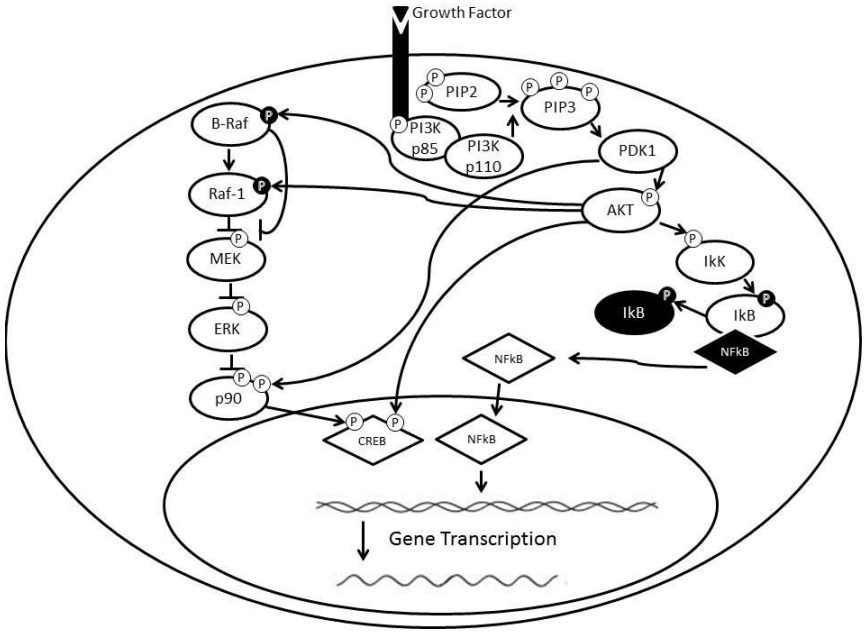

Fig. 1. – An overview of the PI3K/PKB/AKT pathway. Some of the downstream effects of activation of PI3K pathway are shown. PIP2 is phosphorylated by PI3K to create PIP3. PIP3 promotes membrane localization of PDK1, which in turn phosphorylates and activates PKB/AKT. Proteins activated by the PI3K/PKB/AKT pathway are indicated by a clear circle with a black P, while proteins inactivated by this pathway are shown in black circles with white P. Transcription factors, indicated by diamonds, are similarly marked. Arrows from phosphatases to the regulatory phosphates are indicated in open arrows. Figure modified from [4].

Eagles Medium (MEM alpha, α-MEM, Biowest, France) with 10% Fetal Bovine Serum (FBS, Sigma, USA), supplemented with 1% Penicillin 10000 IU/mL (Sigma, USA) and 1% Streptomycin 10 mg/mL (Sigma, USA).

Irradiation Facility

AG01522 monolayers were irradiated at the radiotherapy department of IRCCS S. Maugeri, Pavia, Italy, with different doses of X-rays produced with a 6 MV electron linear accelerator (CLINAC 2100, Varian Medical Systems, USA). A flat and symmetric 20×20 cm radiation field was used. The adopted Dose Range and Dose Rate were 0.5×2 Gy and 3 Gy/min, respectively.

Immunocytochemistry Assay (ICC) and ImageJ Analysis

For the immunocytochemistry assay (ICC), 10^5 cells were seeded 24 hours prior to the irradiation on a cover-glass. Thirty minutes, 2, 4, 6 and 8 hours after the irradiation, cells were fixed for 15 minutes at room temperature with 4% parafolmaldehyde in PBS (Phosphate Buffered Saline). Afterwards, in order to detect p-S6([3]), p-ERK1/2

([3]) S6 is a ribosomial protein used to detect cell nuclei.

and p-PKB/AKT, cells were stained through the *Signaling Nodes Multiplex IF Kit* (Cell Signaling, USA), following the protocol as described by the manufacturer. Biological duplicates of each condition were collected and 10 pictures from each slides were recorded through a CCD camera (RETIGA 2000R, QImaging, Canada) coupled with the fluorescent microscope (IX51, Olympus, Japan). All the images were collected using a 40× objective magnification.

In order to process and evaluate the images, a semi-automatic procedure was developed. Such a procedure was based on a script, purposely written from scratch using ImageJ, a free source, Java-based image processing program developed at the National Institutes of Health (USA). The script converts the fluorescent image of the cell nuclei to a 8-bit grayscale image by linearly scaling from I_{Min}–I_{Max}([4]) to 0–255. Once the Region Of Interests (ROIs), *i.e.* the cell nuclei, are detected, the corresponding ROI coordinates are recorded. Subsequently, the image concerning the investigated protein is split into three 8-bit grayscale images containing the red, green and blue channels of the original. Only the channel of interest is considered, in particular the red channel for p-PKB/AKT and the green one for p-ERK1/2, while the ROIs of the nuclei are overlapped to the selected protein image. Then the ratio

$$R = \frac{N - C}{N + C}$$

is calculated, where N and C are, respectively, the nuclear and cytoplasmic integral fluorescence intensities. The R ratio, rather than the simple $\frac{N}{C}$ ratio, has been chosen because it ranges from (-1) for fully cytoplasmic, to $(+1)$ for fully nuclear localization, and it does not depend on absolute intensities, since cytoplasmic and nuclear values are normalized according to their sum [15].

Enzyme-Linked ImmunoSorbent Assay (ELISA Assay)

Cells were seeded in T-75 flasks in order to reach 90% confluence on the day of irradiation. In order to quantify the p-ERK1/2 and the p-PKB/AKT inside the nuclear and cytoplasmatic cell compartments a *Nuclear Extraction Kit* (Cayman Chemical Company, USA) was used. Both nuclear and cytoplasmic concentration of p-PKB/AKT and p-ERK1/2 were determined with the *Signaling Nodes Multi-Target Sandwich ELISA Kit* (Cell Signaling, USA) performed on extracts from 2 Gy and sham irradiated samples collected 70 minutes after X-ray exposures. Protein concentrations were measured in terms of absorbance with a microplate reader (DV990win6, GDV, Italy) at 450 nm wavelength. Results were divided by the total protein mass of the corresponding sample, which was measured using a *BCA Protein Quantification Kit* (Abcam, United Kingdom).

3. – Results

The Immunocytochemical analysis of the sham and 0.5 Gy irradiated cells showed a dose-dependence of both p-ERK1/2 and p-PKB/AKT R ratios at 30 minutes after irradiation (fig. 2). The value of R was found to increase as the dose increased, corresponding to a higher migration of these proteins to the nucleus.

([4]) I_{Min} and I_{Max} are the lowest and highest thresholds of fluorescent intensity.

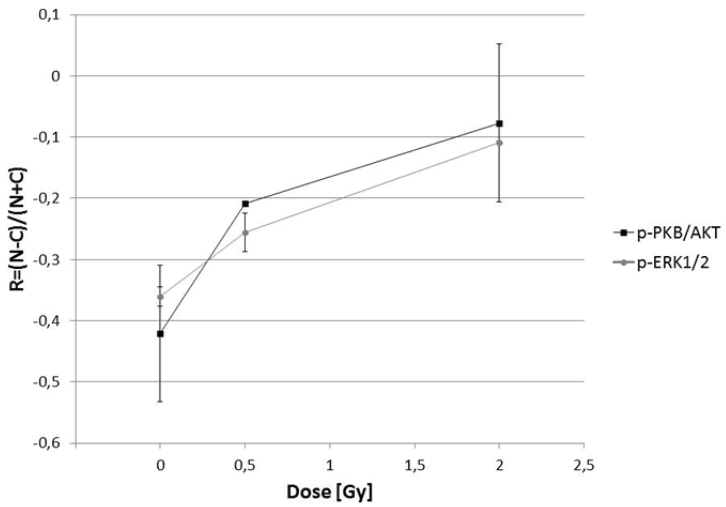

Fig. 2. – p-ERK1/2 and p-PKB/AKT R ratio as a function of dose at 30 minutes after irradiation. All the irradiation conditions are represented (0–0.5–2 Gy). Lines connecting experimental points are drawn to guide the eye.

The temporal dynamics from 30 minutes to 8 hours for the irradiated samples were also measured, showing a return to the non-irradiated perturbed equilibrium (sham condition). For both ERK1/2 and PKB/AKT this process seemed to be slower for 2 Gy irradiated cells than for the 0.5 Gy condition. The complete temporal dynamics of R for ERK1/2 is shown in fig. 3: indeed the R value measured for the 2 Gy irradiated samples at 8 hours after irradiation was found to be higher than the corresponding values for the sham and 0.5 Gy conditions, but no significant dose-dependence at late time points (2, 4, 6 and 8 hours) could be observed. Such results suggest that the activation of these proteins might be very fast, and further studies at shorter times are needed to better understand the perturbed dynamics.

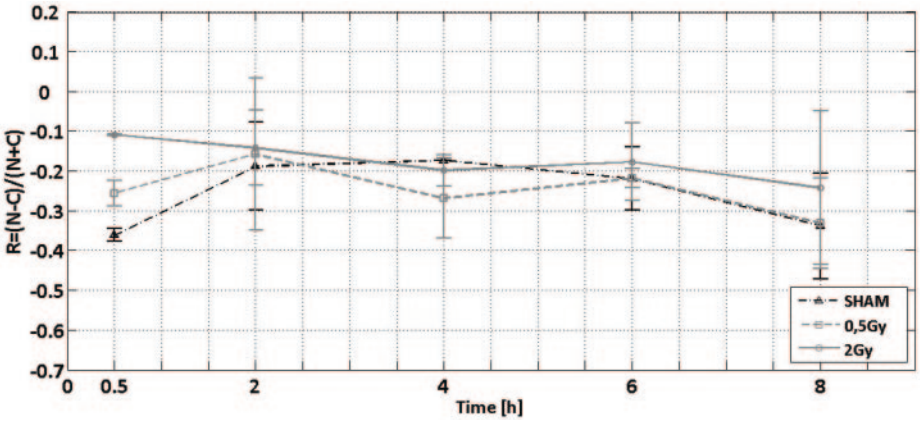

Fig. 3. – Temporal dynamics of p-ERK1/2 R ratio. All the irradiation conditions are represented (0–0.5–2 Gy). Lines connecting experimental points are drawn to guide the eye.

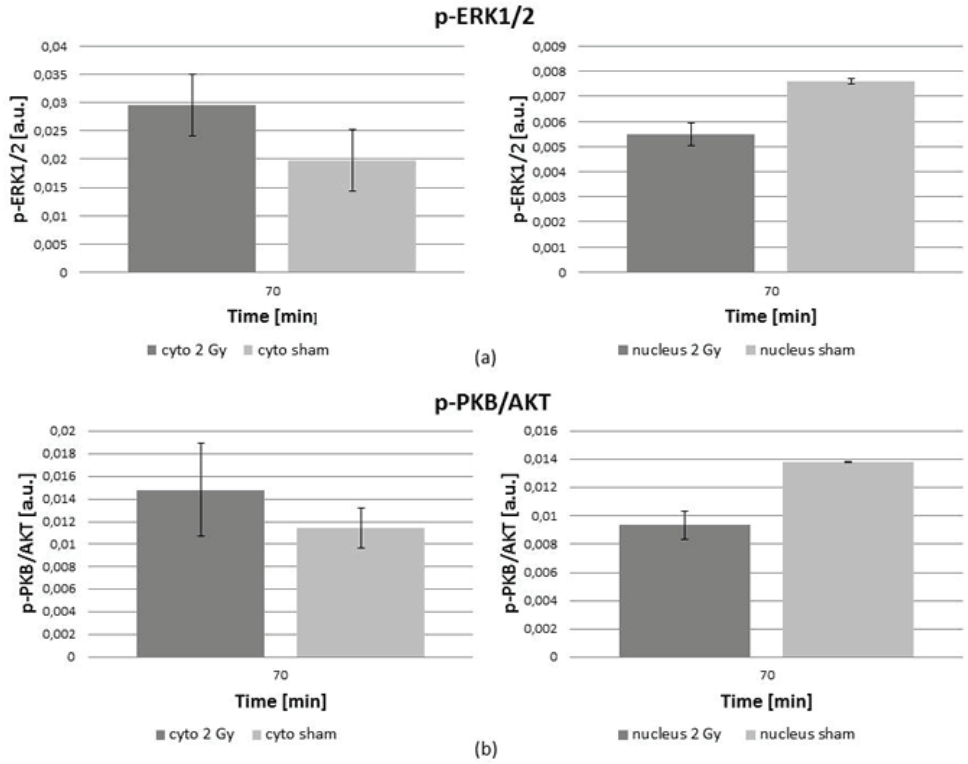

Fig. 4. – (a) Cytoplasmic (left) and nuclear (right) absorbance of p-ERK1/2 divided by the total protein mass of the sample. (b) Cytoplasmic (left) and nuclear (right) absorbance of p-PKB/AKT divided by the total protein mass of the sample. Two of the irradiation conditions are represented (0–2 Gy).

Since the significant dose-dependence observed at 30 minutes after irradiation seemed to disappear already at 2 hours, a further investigation at an intermediate time (70 minutes) was carried out through ELISA assay. The results obtained for sham and 2 Gy irradiated cells collected at 70 minutes after irradiation are shown in fig. 4. For both ERK1/2 and PKB/AKT data showed a clear difference for the nuclear compartment between the sham and 2 Gy conditions, while the difference in the cytoplasmic absorbance was measured to be less significant.

4. – Conclusions

The aim of this work was to analyze the perturbation of intracellular signaling pathways induced by ionizing radiation. In particular the effects of low dose ionizing radiation on the temporal modulation of ERK1/2 and PKB/AKT were investigated. Low and moderate doses of X-rays (0–0.5–1–2 Gy) were delivered to the cells and the activation of the proteins was investigated through immunocytochemistry. Such a technique was used to evaluate p-ERK1/2 and p-PKB/AKT temporal dynamics inside the nuclear and cytoplasmic compartments. In order to analyze the fluorescent images, a semi-automatic procedure was purposely developed with ImageJ. The ratio $R = (N - C)/(N + C)$ was calculated for each protein, irradiation condition and time point. The imaging analysis

of AG01522 showed a dose dependence of R for both the investigated proteins at 30 minutes after irradiation. The p-ERK1/2 and p-PKB/AKT R ratios were found to increase with increasing dose, indicating a migration of such proteins to the nucleus. Furthermore the complete temporal dynamics showed a slow return to the non-perturbed equilibrium conditions, and this process seemed to be slower for 2 Gy irradiated cells than for the 0.5 Gy or sham conditions. These results suggest that the activation of these proteins is very quick, and therefore further measurements at shorter time are needed.

Finally, the ELISA assay allowed to evaluate the relative amount of p-ERK1/2 and p-PKB/AKT both in the nuclear and cytoplasmic compartment. For both proteins a clear difference for the nuclear compartment between the sham and 2 Gy conditions was measured, while the difference in the cytoplasmic absorbance was found to be less significant.

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