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Effect of space radiation on expression of apoptosis-related genes in endometrial cells: a preliminary study

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Abstract

In this paper we present some preliminary results on alteration of gene expression caused by radiation on human endometrial cells. To this purpose, we have studied the modulation of the expression of the bcl-2 gene family in two cell lines following irradiations with low energy protons and gamma-rays from a ^{60}Co. The two epithelial cell strains, namely AN3Ca and HEC1B cells, both obtained from human neoplastic endometrial tissues, grow in culture and continue to maintain some differentiated functions typical of the original tissue. Indeed, these cells, that can be considered as representative of differents stages of cellular transformation of endometrium. Because their epithelial nature and rapid growth, the expression of genes related to the maintenance of the cellular omeostasis, as the pro and anti-apoptotic ones, is expected to be susceptible to changes in environment, including radiation.

The effects have been evaluated in terms of both cell survival and changes in the expression of pro- and anti apoptotic proteins. Even though the data reported above can not be considered complete and/or definitive, nevertheless, in whole, they confirm that these cells may constitute a suitable model system to study, at molecular level, the effects of cosmic radiation on endometrium. Further observation, ensuing from these preliminary data, is that endometrial cells present different sensitivity to radiation in regard to its 'quality' and 'dosage', in accord to the original stage of differentiation.

KEYWORDS: Endometrium, radiation, Bcl-2, Bax.

1. Introduction

Radiation is possibly the principal environmental factor that could affect human beings in space. There, it may act independently or may combine synergistically with microgravity and other factors, to produce adverse outcomes on living organisms. For this reason, the study of the effects of prolonged exposure on the human organism to such an environment is obviously important and need to be faced extensively.

Previous studies on a group of rhesus monkeys have indicated that life expectancy loss from exposure to protons in the energy range encountered in the Van Allen belts and solar proton events can be correlated with dose and energy of the radiation. Wood DH et al. [1-3] have shown that radiation also increases the risk of an abnormal proliferation of the lining of the uterus in females (endometriosis). In view this and other (endocrine or reproductive) possible risks [4-6] of prolonged exposures to radiation of female crew-members in long flight missions, it seemed important to gain knowledge on the effect of radiation on the endometrial tissue. Indeed, since endometrial cells are epithelial in nature, they continuously enter the cell cycle and, consequently, are sensitive to injury induced by radiation. Damage often consists in radiation-induced breaks of single or double strand DNA, dimerization of bases or mutation. Often, but not always, these harms can be repaired through the activation of proper rescue processes. The repair mechanism normally requires the cell cycle arrest and the activation of particular genes, including those governing apoptosis.

Apoptosis or Programmed Cell Death (PCD) is a physiologic mechanism of cellular loss necessary to maintain tissue homeostasis in multicellular organisms. It is an active process that, making use of on both pre-existing and de novo synthesized specific proteins, timely provides to the removal of cells which are altered, in excess or turned to unusable. Important regulators of apoptosis are the proteins of the so-called Bcl-2 family. Among these, the best known components are: Bcl-2, a 26 kDa protein that protects cells against apoptosis in a variety of experimental systems, including radiation; Bcl-XL, whose sequence, structure and function remarkably resembles that of Bcl-2 protein; Bax, a protein that, through dimerization with Bcl-2, antagonizes its action and promotes apoptosis [7, 8].

Since the expression of apoptosis-related genes in epithelial cells is expected to be particularly susceptible to changes in the radiation environment, we have performed some pilot studies on the modulation of the expression of the bcl-2 gene family in endometrial cells, following irradiation with low energy protons and gamma-rays from a ^{60}Co source.

2. Material and Methods

2.1. *Cell lines*

HEC1B and AN3CA endometrial carcinoma cell lines [9-11] were all obtained from American Type Culture Collection (Rockville, MD). Two different histotypes and tumor grade characterize these cells:

Cell strain	Grade	Histological phenotype
HEC1B	G2	endometrioid
AN3CA	G3	adeno-squamous

Cells were cultured according to indications: HEC1B and AN3CA were cultured in MEM (Sigma). All culture media were supplemented with 10% fetal bovine serum, 2 mM glutamine, 0.5 U/ml penicillin and 0.5 μg/ml streptomycin, unless differently specified.

2.2. *Irradiation*

HEC1B and AN3CA cells were irradiated with low energy protons at the radiobiological facility installed at the 3MV TTT-3 Tandem accelerator in Naples, and with gamma-rays from a ^{60}Co source at the local Medical School. To this purpose cells were directly seeded and grown (to semiconfluence) in special containers purposely designed to this scope (see below).

Irradiation with monoenergetic proton beam at terminal voltage of 2.5 MV, and the energy at the top of the cell monolayer was 4.86 MeV (LET 8.35 keV/μm). Irradiation of cells was accomplished in the growing containers (2 cm height glass cylinders with 1 cm inner diameter) whose bottom was constituted by a 3 μm thick mylar foil, glued with special non toxic adhesive (araldite).

In the gamma-ray experiment, cells were exposed directly in Falcon 25 cm^2 flasks at the ^{60}Co source.

In both cases the cells were irradiated when were in a growth semiconfluent condition.

2.3. *Western blot analysis*

Total cell proteins preparations were obtained lysing cells by 1mM EDTA, 0.2% Triton X 100, 1 μg/ml aprotinin, 170 μg/ml phenylmethylsulfonyl-fluoride. Protein concentration was measured by the Bio-Rad protein assay.

Polyacrylamide gels (15 or 7.5%) were prepared essentially as described by Laemmli [12].

Molecular weight standards were from Pharmacia. Proteins separated on the polyacrylamide gels were blotted onto nitrocellulose filters (Hybond-C pure Amersham) according to the procedure of Bittner et al. [13].

The actual total amount of proteins transferred on nitrocellulose was estimated by scanning (Discover Pharmacia Scanner equipped with a Sun Spark Classic Workstation) after staining with Ponceau Red. Filters were washed and immunologically revealed by incubating with specific antibodies. Specific secondary antisera, conjugated with peroxidase (Amersham; diluted 1:2,000), were let to react with primary immunoglobulins. Peroxidated immunoglobulins were finally revealed by ECL Western blotting detection reagent (Amersham) and quantitatively estimated by scanning. Corrections for total protein loading were made when necessary.

Antibodies were purchased from Santa Cruz Biotechnologies (namely anti-Bcl-XL(S-18), anti-Bax(N-20), anti-Bcl-2(C-100) and anti-PARP (H250)).

2.4. *Survival assay*

After each dose of proton irradiation cells were trypsinized, diluted to get 100 cells/dish, and plated in five 60mm Petri dishes. The ‘plating efficiency’ was evaluated by plating ten dishes from non-irradiated cells.

Dishes were incubated for 7 days, then fixed and stained in crystal violet. Colonies containing more then 50 cells were considered as survivors.

3. Results and Discussion

Cell sensitivity to external injury depends on the cell cycle phase. Peak sensitivity occurs during G2 and M phases; reduced sensitivity in G1 and minimal in the late S phase. For this reason, cells with a high turnover rate, as epithelial endometrial cells, are particularly vulnerable to radiation. In the normal individual, cells are capable of repair sublethal radiation injury. Breaks of a single strand of DNA are rapidly repaired using the intact strand as template. (Double strand breaks are normally more difficult to repair). Similarly, when ionizing radiation causes dimerization or point mutation, the defect may be excised and repaired in most instances. In rapidly dividing populations, small genetic defects may compatible with survival. This fact may led to DNA changes, leading ultimately to measurable effects including programmed cell death (apoptosis) or aberrant proliferation (neoplastic transformation). Indeed, it is becoming clear that deregulation of the delicate equilibrium between cellular proliferation and death events may be the cause of the development and progression of several human pathologies including malignant and/or preneoplastic changes (as endometriosis). Indeed, the specific contribution of *bcl-2* family genes in tumor pathogenesis and progression has been widely analyzed in neuronal and lymphoid system. These contributes, have been, in general, less characterized in epithelia; in this regard only a few observations in non-malignant versus malignant tissues have been reported in details [14-16].

The ability of endometrial cells to differentially express pro/antiapoptotic genes in distinct stressing conditions is currently under investigation in our laboratory [17-19]. To this purpose we routinely use a model system that is constituted by a number of cultured human endometrial cells originally derived from a human endometrial carcinoma (two of which are presented here). In this framework we have collected the responses in terms of gene expression alteration under the action of different radiation sources to which cells have been exposed. In this regard, two sets of experiments were designed. In the first cells were irradiated with low energy protons; in the second the cells were irradiated with gamma rays using a 60Co source. The energy used ranged between 0 and 10 Gy for the protons and between 0 and 1.6 Gy for gamma rays.

The effects were measured in terms of changes in the expression of some proteins, related to resistance or sensitivity to apoptosis and measure of ability to survive. Most of the data shown in the bar plots are the averages from triplicate (sometimes duplicate) independent measurements.

3.1. *HEC1B endometrioid cells*

3.1.1. IRRADIATION WITH PROTONS - Cells were irradiated at 1, 5, and 10 Gy (triplicate samples). After irradiation cells were put at 37°C for additional 4 hours before cell lysis and protein extraction. At doses of 1 Gy we have noticed a detectable up regulation of Bcl-2 (Fig 1, panels a and b, upper picture) and concomitant PARP fragmentation (panel b of Figure 1, lower picture). While the up regulation of Bcl-2 would be interpreted as an increase in cell resistance to apoptosis, the appearance of the 85kDa of PARP fragment would indicate an ongoing apoptotic process. These observations may indicate, besides an alteration in gene expression, that after irradiation the cell population undergoes a kind of selection. Accordingly, significant part of the cell population acquires resistance (up-regulation of Bcl-2), while the remaining undergoes an apoptotic fate.

At higher energy both Bcl-2 and PARP resume the original pattern, indicating that at these energies cells can not actively respond possibly progressing immediately to necrosis.

Cell survival curve (not shown here) are not in contrast with this interpretation.

3.1.2. IRRADIATION WITH GAMMA RAYS - Cells irradiated at doses of 0.2-1.6 Gy revealed a small Bcl-2 and a significant Bax up regulations. The Bax up regulation is maximal at 0.4 Gy (Fig. 2) where also PARP fragmentation (ongoing apoptosis) is evident (see insert of panel b). Above 0.4 Gy, necrosis is the only detectable process. The survival curve (panel b) is in accord with this picture.

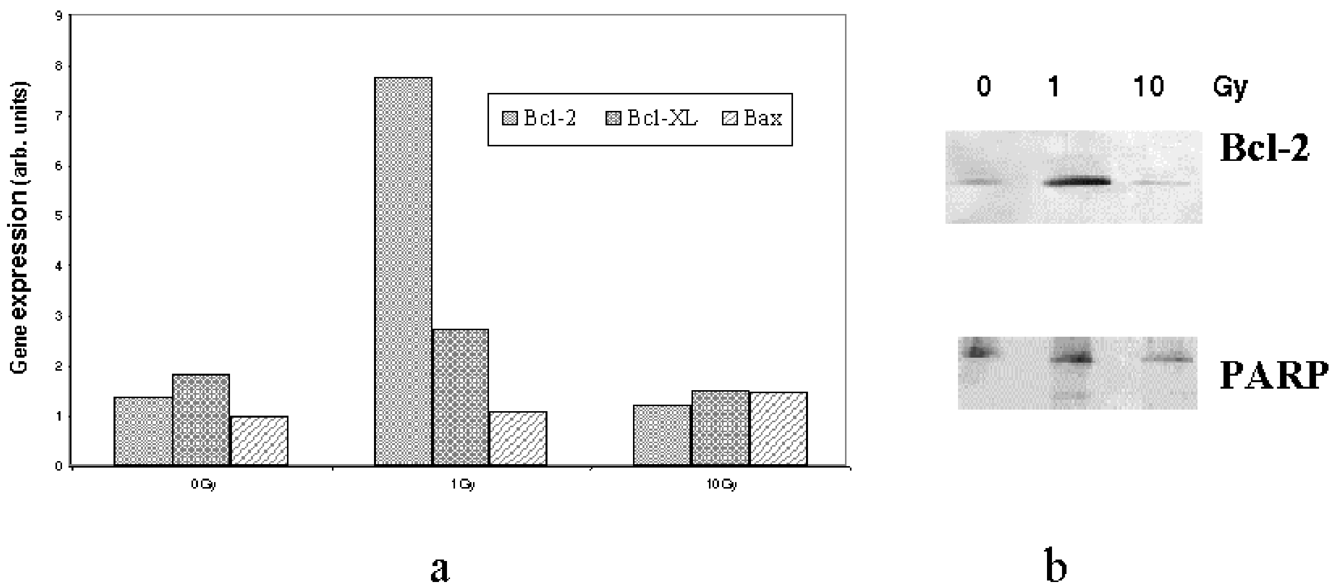

Fig. 1 – Panel a. After irradiation, HEC1B cell lysates were analyzed by western blotting (see Methods). The levels of protein expression were evaluated by densitometry and represented as relative protein amounts (arbitrary units). Panel b, upper. Relative expression of Bcl-2 at 0, 1, 10 Gy. Note the up-regulation of Bcl-2 at 1 Gy. Panel b, lower. Typical profile (western blotting) of PARP fragmentation. Note the appearance of a second, fast migrating electrophoretic band of ~85 kDa at 1 Gy.

HEC1B (gamma rays)

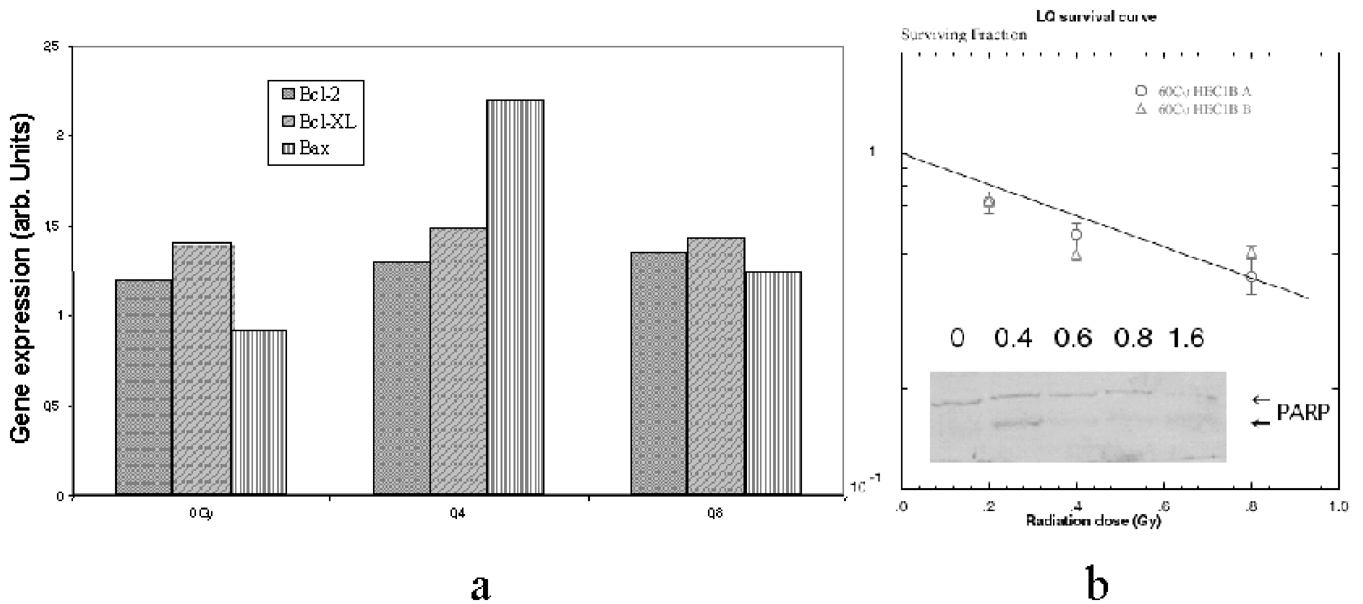

Fig. 2 – Panel a. After irradiation, HEC1B cell lysates were analyzed by western blotting (see Methods). The levels of protein expression were evaluated by densitometry and represented as relative protein amounts (arbitrary units). Panel b. Survival curve at different radiation dosages. The insert shows a typical profile (western blotting) of PARP fragmentation. Note the appearance of a second, fast migrating electrophoretic band of ~85 kDa at 0.4 Gy only.

AN3CA (protons)

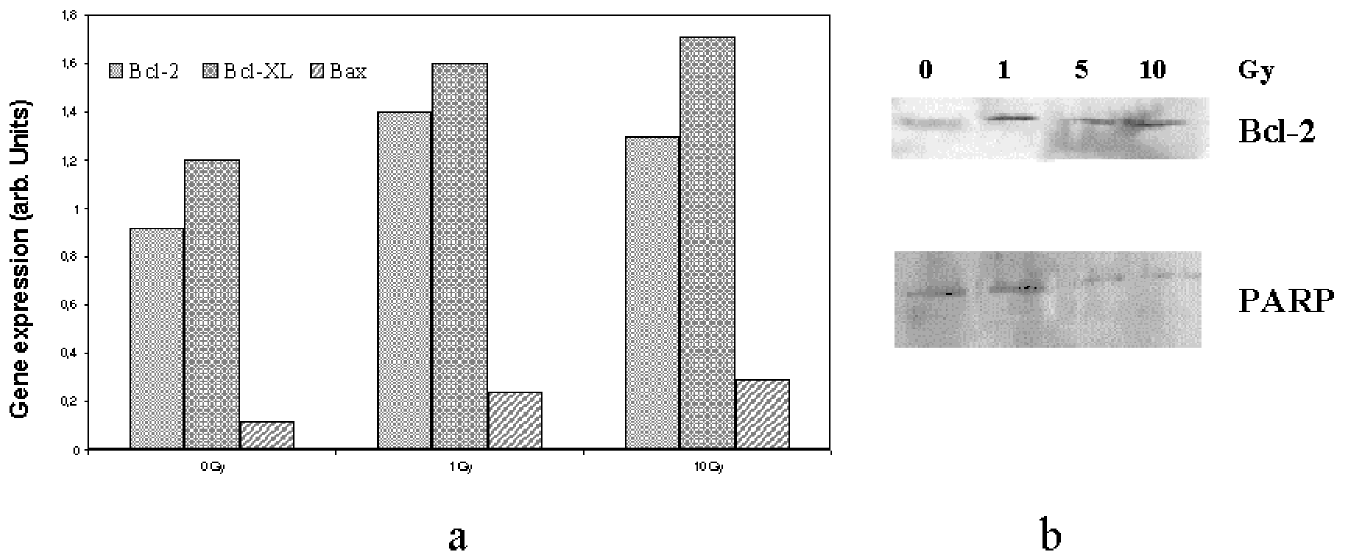

Fig. 3 – Panel a. After irradiation, AN3Ca cell lysates were analyzed by western blotting (see Methods). The levels of protein expression were evaluated by densitometry and represented as relative protein amounts (arbitrary units). Panel b, upper. Relative expression of Bcl-2 at various energies (as indicated). Panel b, lower. Typical profile (western blotting) of PARP fragmentation. Note the absence of appearance of a second, fast migrating electrophoretic band of ~85 kDa at any energy and the fading of the band at higher energies.

3.2. *AN3Ca adenosquamous cells*

3.2.1. IRRADIATION WITH PROTONS - Cells irradiated at doses of 1-10 Gy reveal modest up regulation of Bcl-2 and Bcl-XL (Fig. 3, panels a and b, upper picture). Above 1 Gy no further changes are evident. AN3CA do not show any sign of apoptosis up to 1 Gy; above this dose an intense and rapid necrosis is evident as suggested by the progressive band fading (Fig. 3, panel b lower picture). The survival curve (not shown) is in accord with this representation.

3.2.2. IRRADIATION WITH GAMMA RAYS - Cells irradiated at doses of 0.2-1.6 Gy reveal a scarcely significant Bcl-2 up regulation, which reaches its maximum at 0.2 Gy (Fig. 4 panel b, upper). Unfortunately we do not have information regarding both Bax and Bcl-XL since relative samples were lost. AN3Ca cells do not undergo apoptosis, even at low energy since we could not detect signs of PARP proteolysis at any radiation dose (Fig. 4, panel b, lower). These data seem to suggest a cellular high radio-sensitivity: above 0.2 Gy, all cells experience a rapid and intense necrosis. The cell survival curve is excellent accord with this view (Fig. 4, panel a).

4. Conclusions

Even though the data reported above are unfinished and need to be refined, nevertheless, in whole, they confirm that HEC1B and AN3Ca cells may constitute a suitable model system to study at molecular level, the effects of cosmic radiation on endometrium.

The principal information that ensues from our data, is that endometrial cells exhibit different sensitivity to radiation in regard not only to the radiation dosage, and radiation type but also in accord to the relative 'state' of differentiation. This particular meaning of the higher resistance showed by the 'more' differentiated HEC1B cells, compared to the more malignant AN3Ca, however, merits further attention.

Acknowledgements

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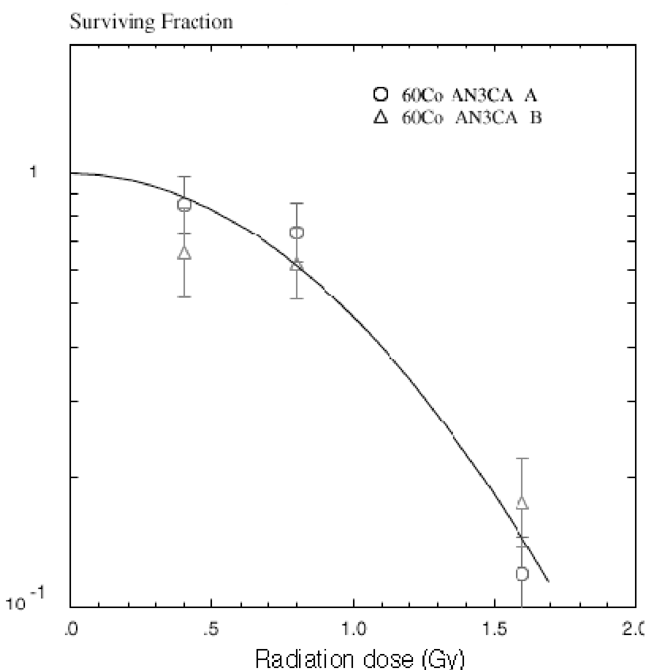
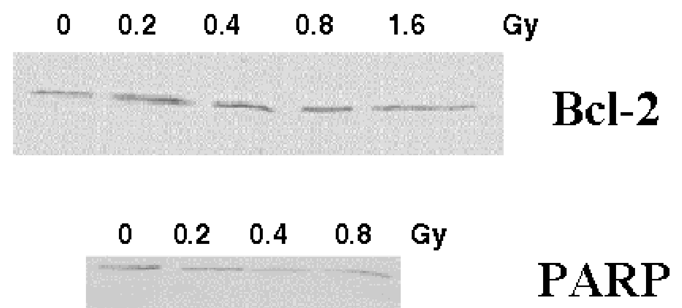
AN3CA(gamma rays)



a



b

Fig. 4 – Panel a. Survival curve of AN3Ca cells after irradiation at the indicated energies. Panel b, upper. Relative expression of Bcl-2 at the indicated energies. Panel b, lower. Typical profile (western blotting) of PARP fragmentation. Note the absence of appearance of a second, fast migrating electrophoretic band of ~85 kDa at any energy and the fading of the band at higher energies.

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