ORIGINAL PAPER

Effects of ^{60}Co *γ*-rays on human osteoprogenitor cells

C. I. TOMULEASA[1,2)], V. FORIS[1,2)], OLGA SORIŢĂU[1)], EMÖKE PÁLL[3)], EVA FISCHER-FODOR[1)], V. LUNG-ILLES[2)], IOANA BRIE[1)], PIROSKA VIRÁG[1)], MARIA PERDE-SCHREPLER[1)], I. D. POSTESCU[1)], GABRIELA CHERECHEŞ[1)], OTILIA BARBOS[1)], CORINA TATOMIR[1)]

[1)]*Laboratory of Experimental Radiotherapy and Stem Cell Culture, "Prof. Dr. Ion Chiricuţă" Cancer Center, Cluj-Napoca*

[2)]*Faculty of General Medicine, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca*

[3)]*Department of Obstetrics, Gynecology and Animal Reproduction, University of Veterinary Medicine and Agricultural Sciences, Cluj-Napoca*

Abstract

Background: Radiation therapy is one of the most efficient treatments of neoplastic diseases used worldwide. However, patients who undergo radiotherapy may develop side effects that can be life threatening because tissue complications caused by radiation-induced stem cell depletion may result in structural and functional alterations of the surrounding matrix. This treatment also damages the osteogenic activity of human bone marrow by suppressing osteoblasts, leading to post-irradiation sequelae. Even if widely used in oncology, there is still little information on the fate and potential therapeutic efficacy of electromagnetic rays. *Material and Methods*: We addressed this question using both human mesenchymal stem cells and osteoblasts. Monoclonal antibody characterization identified specific surface markers for stem cells (SSEA-4, CD29, CD105, Oct 3, Nanog and SOX2) and osteoblasts (Osteopontin and Osteonectin). The technique of anti-alkaline phosphatase FITC-staining demonstrated the presence of this specific ectoenzyme. Cells were cultured in complex osteogenic medium (DMEM, 15% fetal calf serum, non-essential amino acids, L-glutamine, dexametazone, ascorbic acid, insulin, TGF-β, BMP-2 and β-glycero-phosphate) after being irradiated at 0.5 Gy, 1 Gy, 2 Gy and 4 Gy using a Theratron 1000 ^{60}Co source. The viability of irradiated cells was assessed using Trypan Blue staining. The comparison between cell lineages after culture in osteogenic media regarding phenotypical characterization and the intensity of the mineralization process included histology stainings (Alizarin Red S, Alcian Blue and von Kossa), and the MTT-based proliferation assay. *Results*: After irradiation, the proliferation and differentiation of osteoprogenitor cells is dose-dependent. *Conclusions*: This study is one among the first papers investigating the biophysics of low-dose gamma-irradiation on stem cell culture, focusing on the potential applications in radiation oncology and various palliative treatments.

***Keywords*:** mesenchymal stem cells, ionizing radiation, differentiation and proliferation, experimental radiotherapy.

Introduction

Ever since first used to treat a patient with Wiskott–Aldrich syndrome [1], bone marrow research has been one of the most important areas of investigation in regenerative medicine. The population of mesenchymal stem cells derived from human bone marrow, having the capability for renewal and differentiation into various lineages, is expected to provide a variety of therapeutic approaches for diseases like bone defects or infarcted myocardium and is being pursued clinically for applications in hematology and oncology, such as aplastic anemia and malignant neoplasia [2–4].

Even so, in today's medicine, approximately 70% of all patients with neoplasic diseases are commonly treated using ionizing irradiation, whereas only 50% receive some form of chemotherapy. Despite its therapeutic value, the adverse side effects can be significant because it may lead to long-term residual damage and acute myelosuppression by inducing apoptosis of hematopoietic cells [5]. The side effects of radiotherapy can be life threatening due to tissue complications determined by radiation-induced stem cell depletion that finally results in structural and functional alterations of the bone matrix. One such complication is osteoradionecrosis, a common sequelae in the medical use of ionizing radiation as part of head and neck cancer treatment [6].

There is little information on the fate and potential therapeutic efficacy of gamma rays on progenitor cells.

We addressed this question using both human adult stem cells and osteoblasts. Cells were cultured in the proper medium to induce *in vitro* proliferation and differentiation, after having undergone low-dose ionizing irradiation.

The possible mechanism of how lose-dose irradiation induces programmed cell death or DNA-repair is discussed.

Material and Methods

Cell isolation and culture

Human bone marrow from the iliac crest, otherwise discarded during the medical procedure, was obtained from patients undergoing orthopedic surgery for routine hip replacement, under general anesthesia,

in accordance with all legal and ethical laws and having the informed consent of the patients. The bone tissue fragments, rich in adult stem cells, were harvested in complete Dulbecco's modified Eagles's Medium (DMEM), with 10% fetal bovine serum (FBS), and afterwards washed several times with phosphate buffered solution (PBS) (all from Sigma). Mechanically dissected fragments of approximately 2 mm^3 were filtered with 70 nm Filcons meshes (Dako), and the cell suspension was centrifuged at 1200 rpm for 10 minutes. The fibroblast-like adherent cells were grown to approximately 80% confluence in DMEM medium, supplemented with a combination of 1% antibiotics (penicillin and streptomycin), and 10% FBS, then maintained by incubation at 37^0C in 7% carbon dioxide. Cells were subcultured every 3 to 4 days using standard technique.

Following the same protocol with tissue fragments from the patella, we isolated preosteoblasts, bone progenitor cells less cuboidal in shape than classic active osteoblasts.

Stem cell osteogenic differentiation

Inductive osteogenic differentiation *in vitro* occurs when mesenchymal stem cells, seeded at a density of 3.1×10^3 cells/cm^2, are cultured until they reach approximately 80% confluence, before passage. The culture is performed in osteogenic differentiation DMEM medium, supplemented with 15% fetal calf serum (FCS), 2 mM L-glutamine, 10 nM dexametazone, 1% non-essential amino acids, 50 μg ascorbic acid, 10 mM β-glycero-phosphate, 1 μg insulin, 2 ng/ml Transforming Growth Factor β_1 (TGF-β_1) and 3 ng/ml Bone Morphogenic Protein 2 (BMP-2) (all from Sigma) [7].

Immunophenotype characterization

Mesenchymal stem cells are hard to identify even when using state of the art immunochemical technology due to the lack of single definitive markers. These cells are identified by their spindle-shaped morphology, selective adherence to a solid surface, proliferative potential, capacity to differentiate and ability to regenerate connective tissues [8]. Stage specific membrane protein markers are identified in cells at third passage, after being labeled with the following anti-human antibodies: SSEA-4, Oct ¾, Nanog (Santa Cruz Biotechnology), CD29 (β_1-integrin), CD105 (endoglin) (Becton Dickinson) and SOX2 (R&D). Cells are fixated in 4% paraformaldehyde (Sigma) for 20 minutes, blocked with 10% bovine serum albumin (BSA), and incubated overnight with primary antibodies diluted 1:50. As secondary antibodies (Santa Cruz Biotechnology), the cells were stained with fluorescein isothiocyanate (FITC) goat anti-mouse IgG and IgM, phycoerythrin (PE) goat anti-mouse IgG, and Texas Red goat anti-mouse IgG.

The research team assessed the feasibility of the osteoblasts obtained *in vitro* from human bone marrow adult stem cells using monoclonal antibodies, only in this case after a permeabilization step with 0.01% Tween 20 or 1% Triton X-100. Mouse anti-human antibodies also demonstrated the presence of the two specific calcium-binding glycoproteins osteopontin and osteonectin. Cell nuclei are counterstained with 4,6-diamidino-2-phenylindole (DAPI). The same immunophenotype technique was applied to the osteoblasts isolated from the patella. Visualization is possible using an inverted phase Zeiss Axiovert microscope, filters 488, 546 and 340/360 nm.

Ionizing radiation methods

Cells were trypsinized when near confluence and resuspended, before being irradiated with doses of 0.5, 1, 2 and 4 Gy, in cooperation with the Department of Clinical Radiotherapy, using Theratron 1000 as a ^{60}Co source. To ensure adequate build-up and homogenous irradiation, according to the debit of the source, we calculated the exposure times for DSP 100 cm, field 10×10 cm and 1 cm depth. Cells were irradiated in suspension instead of monolayer to avoid subjecting irradiated cells to further stress of manipulation such as trypsinization, which may interfere with cell recovery. During the time of irradiation, control samples were kept outside the ^{60}Co source at the same temperature as the irradiated cultures. To prevent DNA-repair immediately after irradiation, all cell samples were kept on ice and quickly transported to the Laboratory of Experimental Radiotherapy and Stem Cell Culture. All experiments were performed in triplicate.

Histology stainings

Before osteogenic culture, immediately after irradiation, cellular viability was evaluated using Trypan blue staining and hemocytometer counting. The reactivity of this dye is because the chromophore is negatively charged and does not interact with cells unless the membrane is damaged. Therefore, all cells, which exclude the dye, are viable.

After osteogenic differentiation, the onset of mineral deposition was assessed by von Kossa and Alizarin Red S staining. Nevertheless, Alizarin Red S, an anthraquinone derivative, is not strictly specific for calcium and von Kossa staining alone is not sufficient to confirm that mineralization *in vitro* represents bone formation [9]. Thus, Alcian Blue staining confirmed the presence of chondroitin sulfate, a specific bone glycosaminoglycan.

All staining techniques respected current protocols and all histological images were acquired through an Olympus CKX 41 inverted light microscope, at 100× and 200× amplification.

MTT assay

A known number of cells are cultured in osteogenic media on a 96 well plate. Using the MTT cell viability assay, we evaluated the post-irradiation proliferation potential of human mesenchymal stem cells and osteoblasts.

After the removal of culture media, 1 mg/mL MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, is added and cells are kept for 60 minutes at 37^0C. The tetrazole is reduced to formazan by the NAD-dependent dehydrogenase activity of living cell

mitochondria, to form a purple product. The supernatant is removed and formazan crystals are dissolved after we add dimethyl-sulfoxide (DMSO). The color of this biochemical reaction depends on the number of viable cells, measured with an ELISA TECAN Sunrise reader, at 492 nm. The obtained values are units of optical density and the percent of viable cells is calculated using the formula:

$$\%\text{Growth} = (A_{\text{irradiation}}/A_{\text{control}}) \times 100.$$

Cell proliferation after irradiation is reflected in higher optical densities, in comparison with non-irradiated ones

Data analysis

All images were processed using Adobe Photoshop CS2 9.0 software. Trypan blue staining for number of viable cells data is expressed as the mean ± standard deviation (SD) for separate experiments and were analyzed by Student's *t*-test, with $p<0.05$ considered statistically significant. Microsoft Excel 6.0 software was used for data representation and statistical analysis. For MTT assay statistical significance values were obtained with a one-way analysis of variance (ANOVA) with a 95% confidence level using Graph Pad Prism 5 statistics program.

Results

Cell culture and immunocytochemical characterization

A morphologically homogenous population of fibroblast-like cells with 80% confluence was seen after 10–14 days in DMEM culture (Figure 1).

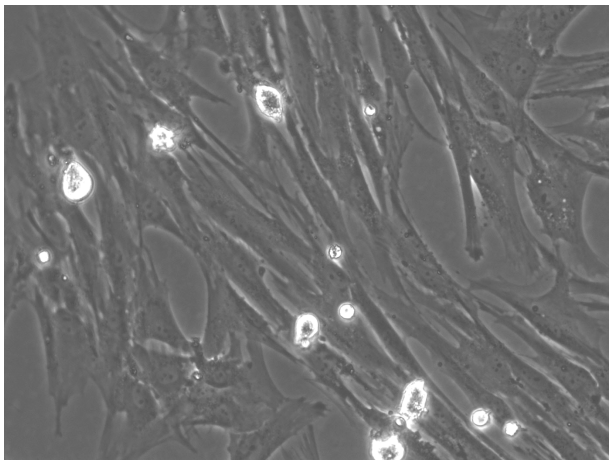

Figure 1 – *Morphology of human mesenchymal stem cells, day 13 of primary culture.*

Even if the first methods described to isolate bone marrow, mesenchymal stem cells (BM–MSC) were based on their capacity to adhere to plastic flasks and because stem cells do not have single definitive immunological markers, current assays rely on the presence of various specific proteins on the cellular membrane. The isolated pluripotent stem cells are positive for such surface antigens, identified by monoclonal antibodies (Figure 2). The functional status of MSC was confirmed by standard osteogenic differentiation assay.

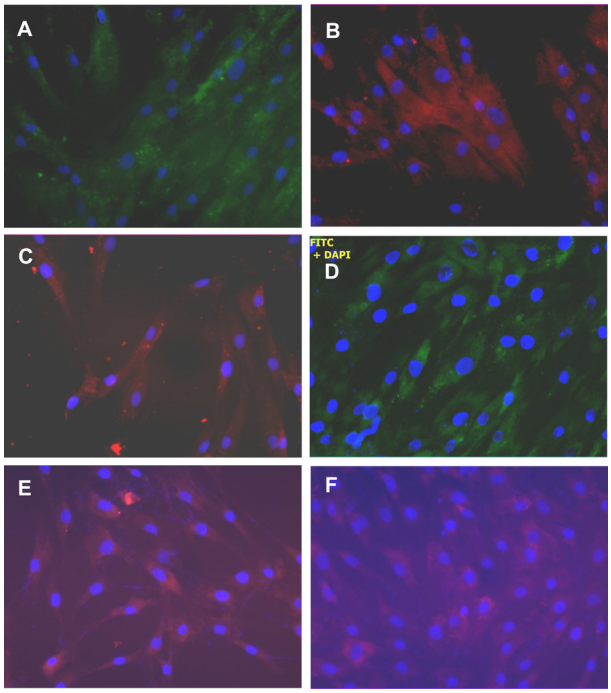

Figure 2 – *The cultured hBM–MSC have positive immunological staining for Nanog (FITC+DAPI) (A), Oct ¾ (FITC+DAPI) (B), SOX2 (TR+DAPI) (C), SSEA-4 (FITC+DAPI) (D), CD29 (PE+DAPI) (E), and CD105 (PE+DAPI) (F).*

Cells derived the patella adhered after 21–28 days of primary culture. Preosteoblasts, proven to express reliably specific markers like osteonectin and alkaline phosphatase, differentiate into active bone matrix-secreting osteoblasts. These cells, ultimately responsible for depositing organic bone matrix, have a typically cuboidal shape, a large excentric nucleus with one to three nucleoli and rough endoplasmatic reticulum and Golgi areas. As matrix deposition continues, osteoblasts become embedded in the cells' secretory product, the osteoid. These osteoid-osteocytes, larger than mature osteocytes, undergo ultrastructural changes on mineralization of the osteoid and decrease protein synthesis and secretion. Newly embedded osteocytes may be variable in size and shape in comparison with older, more mature osteocytes (Figure 3).

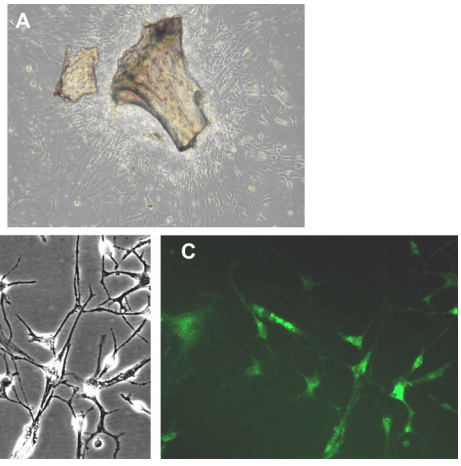

Figure 3 – *(A) Cells migrated from a patella bone fragment, week 5 of culture; (B) Osteoblast morphology, white light microscopy, ×200, (C) Osteopontin-FITC fluorescence staining, ×200.*

γ-Rays effects on MSC-differentiation into mineralized osteoblasts

In order to investigate whether low doses of ionizing radiation influences the ability of MSCs to differentiate into mineralized osteoblasts, both control and irradiated cellular populations were further cultured in osteogenic medium, in exactly the same conditions.

After three days in culture, the Runx2-protein acts as a scaffold for nucleic acids and regulatory factors involved in skeletal gene expression and all progenitor cells begin to exhibit morphological changes typical for the preoosteoblastic phenotype.

After six days in culture, cells show a polygonal osteoblast-like morphology. Six more days later, the functional secrete bone matrix and form an ossification nodule before these cells differentiate into osteocytes.

The fully developed osteoblasts express high amounts of alkaline phosphatase, an ectoenzyme anchored to the external surface of the plasma membrane.

Alkaline phosphatase plays an important role in bone mineralization, a metabolic process that lags behind matrix production and cells express positive immunocytology staining for osteopontin and osteonectin (Figure 4).

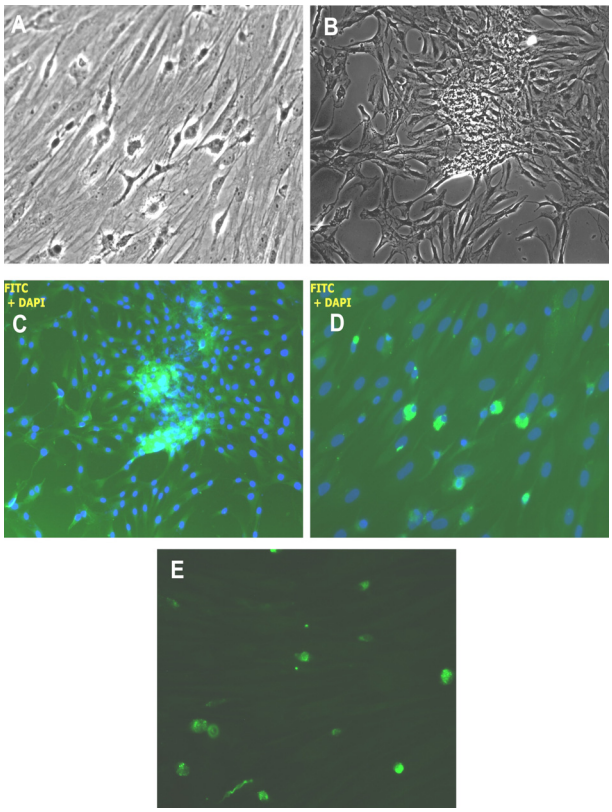

Figure 4 – ***(A) Osteoblast morphology, day 6 in osteoblast-differentiation medium; (B) Osteoblast ossification nodule, day 12 of osteoblast-differentiation medium; (C) Anti-alkaline phosphatase staining (FITC/DAPI, ×100, Zeiss Axiovert microscope, filter 488 nm); (D) Osteopontin (FITC+DAPI); (E) Osteonectin (FITC+DAPI).***

The onset of the mineralization process was also demonstrated by specific histological stainings. Alizarin Red S and von Kossa have specific binding sites for calcium. Nevertheless, calcium deposits do not represent a good enough proof that the tissue is indeed bone, so Alcian Blue staining confirmed the presence chondroitin sulfate (Figure 5).

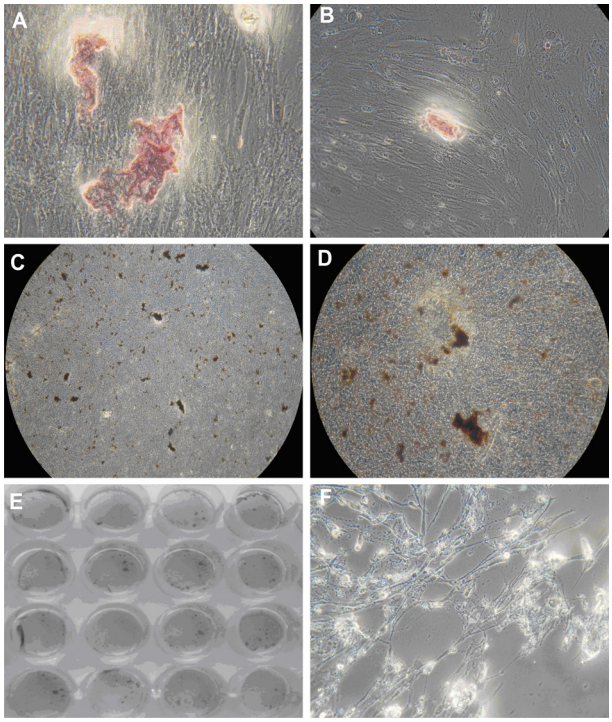

Figure 5– ***Alizarin S staining (A, B) demonstrates bone matrix calcification. The mineral deposits are confirmed by von Kossa staining, both microscopically (C, D) and macroscopically (E). Alcian Blue is positive due to the presence in the bone matrix of the chondroitin sulfate glycosaminoglycan (F).***

Cell proliferation assessment

Immediately after irradiation, Trypan Blue staining was used to estimate the number of viable cells at 0.5 Gy, 1 Gy, 2 Gy and 4 Gy.

One hour after exposure, the number of viable MSCs and POs remained approximately the same as that of the control, with statistical values not significantly different (p>0.05), but after 48 hours cells are thought to have repaired DNA-lesions and having started to divide. This is confirmed by statistics, by significantly different values (p=0.083). When irradiated at 4 Gy/min., morphological changes were observed. These changes include beading, membrane blebbing and lifting from the surface of the flask. Even if some cells were viable one hour after exposure to ionizing radiation, after 48 hours almost all progenitor cells were dead (Figure 6).

The proliferation of irradiated hMSC and hPOs is stimulated within two weeks after irradiation. Five days after radiation treatment, cells divide and proliferate, but then differentiation occurs. Due to this very reason 14 days after exposure to ionizing rays the values that represent controls and irradiated cells are not all that different (Figure 7).

During the experiments, cells irradiated at 2 Gy were the same number as the control group.

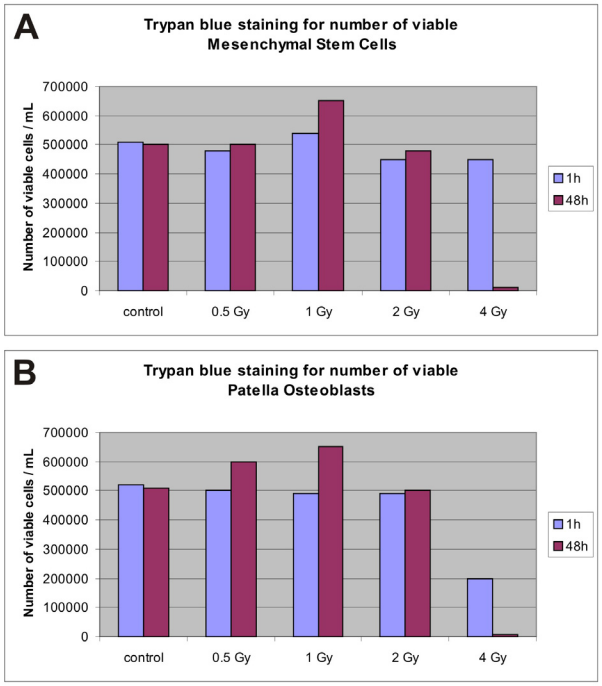

Figure 6 – ***Trypan Blue staining for number of viable cells: MSC (A) and patella osteoblasts (B). One and 48 hours after irradiation, Trypan Blue was used to measure the number of viable cells. Results were calculated as the number of viable cells/mL and p<0.01 vs. control. Data was obtained from three separate sets of experiments.***

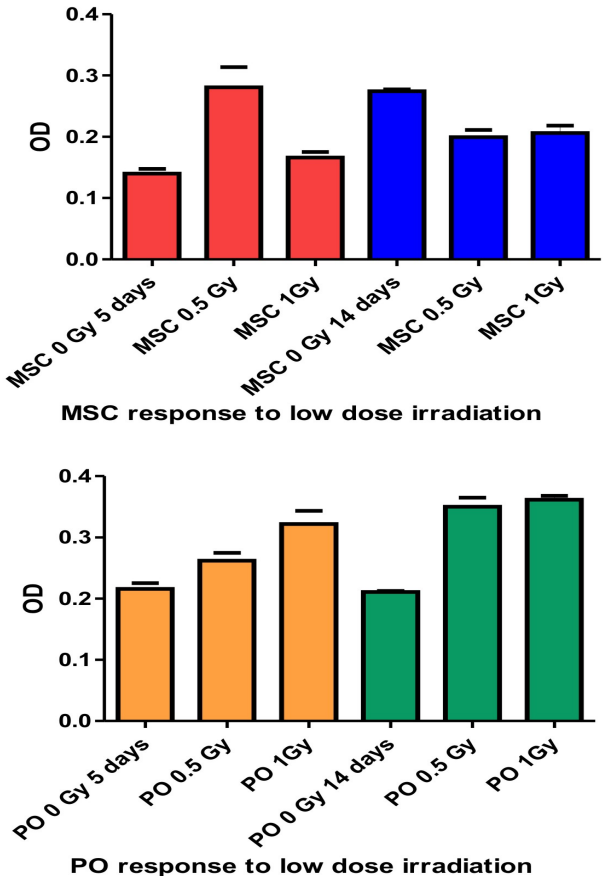

Figure 7 – ***The MTT-assay shows that stem cells are stimulated immediately after irradiation, but once they differentiate into osteoblasts, the proliferation potential drops.***

Discussion

To our knowledge, few studies have looked at the *in vitro* differences in the response of human mesenchymal stem cells and osteoblasts to low-dose electromagnetic radiation [10–13]. In the present research paper, we describe the effects of radiation administered from an external source. The effects caused by internal radiation because of incorporation of radioactive substances are beyond the scope of this article. Radiation effects of radioactive elements deposited in tissues are, however, essentially similar to those of external radiation.

According to Scheven BA *et al.* [14], radiosensitivity appears to vary among cell lines or even along one differentiation lineage. Furthermore, the external conditions, type of irradiation and dose rate may influence the response of cells to external irradiation. The radiosensitivity of a specific tissue is directly proportional to its mitotic activity and inversely proportional to the differentiation degree of its cells. As most tissues are composed of heterogeneous cell populations, the response to ionizing radiation will depend on the relative sensitivities of the various cell types, indirect effects of the result of functional interplay between different cells and the effects of the microenviroment and supporting matrix. Because of this very reason, in order to fully comprehend the dynamics of bone tissue, we analyzed the properties of both human mesenchymal stem cells and osteoblast cells, cultured in osteogenic medium, after surviving a sublethal exposure to ionizing radiation. Some aspects of this problem are closely related to the characteristics of stem cell kinetics in general, but others have a more specific radiobiological significance.

Bone is a specialized form of connective tissue that, like other connective tissues, consists of cells and extracellular matrix. The feature that distinguishes bone from other connective tissues is the mineralization of its matrix, which produces an extremely hard organ capable of providing support and protection. The mineral is calcium phosphate, in the form of hydroxyapatite crystals. The process of new bone formation, osteogenesis, is essential to normal bone function. It requires a population of renewable osteoprogenitor cells responsive to molecular stimuli, capable of becoming bone-forming cells. Osteoprogenitor cells are derived from mesenchymal stem cells in the bone marrow that have the potential to differentiate into many different cell types, including fibroblasts, chondroblasts or adipocytes. The key factor that triggers differentiation of progenitor cells is a transcription factor called Core Binding Factor α1, a protein that prompts the expression of genes characteristic to the phenotype of the osteoblasts. Other such small regulatory metabolic factors are Bone Morphogenic Proteins (BMPs). BMPs also induce the differentiation of mesenchymal stem cells into osteoblasts, the bone-producing cells. Recombinant human BMP7, also known as Osteogenic Protein 1, is for example, now used clinically to induce bone growth after orthopedic surgery [15–18].

Almost as soon as the biological effects of ionizing radiation were discovered, attempts were made to explain some of their basic characteristics in terms of the physical process of energy absorption in the irradiated tissues. Gamma irradiation from ^{60}Co sources was used to terminally sterilize bone allografts for many years. It affects the mechanism and biological properties of bone allografts by degrading the collagen in bone matrix [19, 20]. Specifically, gamma rays split polypeptide chains. Irradiation causes release of free radicals via radiolysis of water molecules that induces cross-linking reactions in collagen molecules, causes the disruption of normal molecular structures, and damages the biological target. The main target of ionizing radiation is the DNA-molecule and damage is particularly expressed when the nucleic acid molecule is duplicated. Because radiation-induced changes in DNA-strands may involve wide areas, cell death is generally described to interference with proliferation. An exception to this, however, is the interphase death occurring in non-dividing small lymphocytes and thymocytes after low radiation doses. In radiobiology, the term "radiosensitivity" is used to indicate the reproductive death of proliferating cells.

Cell survival increases as soon as DNA-synthesis begins and falls sharply as soon as DNA-synthesis is completed. If the survival depends on the rate of DNA-synthesis and if this were the only factor controlling it, maximum survival should occur in the S-phase. The kinetics of DNA-repair is different during the various phases of the cellular cycle. During mitosis, the cell is most sensitive because it lacks the ability to repair radiation damage.

Irradiated osteoblasts undergo G2-cycle arrest and the molecules that regulate the cell cycle change.

Szymczyk KH *et al.* [21] concluded that activation of the radiation-induced arrest sensitizes osteoblasts to agents that mediate apoptosis through a mitochondrial-dependent death pathway. There is an increase in p53-transcription, a raised level of MDM2 dephosphorylation, an elevation in p21 and GADD153 protein levels. Since these proteins are concerned with the regulation of the cell cycle, the observed changes in expression would be expected to disturb cyclin-activity and cause G2-arrest.

Mature cartilage and bone tissue, due to a slow turnover rate, proved to be very radio-resistant, but this is not the case of progenitor cells because of the high turnover rate [22]. Radiation damage will be almost immediately visible. Bones have a small growth factor, but regenerative capacity. Radiation may cause a retarded dose-dependent diminution of cells, but during the phase of increased compensatory proliferation and accelerated expression of radiation, injury becomes obvious.

Acutely, at doses of radiation smaller than 2 Gy, cells repair most sublethal DNA-damage successfully and radiosensitivity is therefore determined by the induction of non-reparable lesions. Cells irradiated at 2 Gy demonstrate similar results to controls, while doses above 4 Gy demonstrate significant radiation toxicity and death, rendering them inadequate for the study.

Conclusions

Ideally, the radiation dose used for the study of normal cells *in vitro* should be similar to the clinical dose *in vivo,* but hypoxia and intercellular contact are believed to contribute to the differences between experimental laboratory studies and clinical ones.

While correlations have been made, for example, between the *in vitro* sensitivity of squamous cell carcinoma to radiation and its clinical response, it is equally as important that a definitive relationship is established between the present study and the therapeutic or palliative potential. This because radiation therapy involves the art of balancing the recurrence of cancer due to undertreatment against severe damage to local tissues due to overtreatment.

Results too often fall short of desired success rates and yet the therapist is continually tantalized to the likelihood that a slight shift of therapeutic ratio favoring normal tissue over cancer would have a profoundly beneficial effect.

Thus, the application of cell cycle kinetics to radiotherapy is one huge hope for improving the therapeutic ratio.

Acknowledgements

This experiment was carried out at the "Prof. Dr. Ion Chiricuță" Institute of Oncology, Cluj-Napoca, Romania, due to the collaboration between the Departments of Cancer Immunology, Biochemistry Research and Radiotherapy.

We gratefully acknowledge the Department of Orthopedic Surgery, "Iuliu Hațieganu" University of Medicine and Pharmacy, Cluj-Napoca, especially Professor Tomoaia and Doctor Benea, for supplying bone marrow harvests.

The work was financially supported through a Grant of the Romanian Research Ministry, Contract No. 41–050/14.09.2007.

References

[1] BACH F. H., ALBERTINI R. J., JOO P., ANDERSON J. L., BORTIN M. M., *Bone-marrow transplantation in a patient with the Wiskott–Aldrich syndrome*, Lancet, 1968, 2(7583):1364–1366.

[2] QUARTO R., MASTROGIACOMO M., CANCEDDA R., KUTEPOV S. M., MUKHACHEV V., LAVROUKOV A., KON E., MARCACCI M., *Repair of large bone defects with the use of autologous bone marrow stromal cells*, N Engl J Med, 2001, 344(5):385–386.

[3] TOMA C., PITTENGER M. F., CAHILL K. S., BYRNE B. J., KESSLER P. D., *Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart*, Circulation, 2002, 105(1):93–98.

[4] HOROWITZ M. M., *Current status of allogeneic bone marrow transplantation in acquired aplastic anemia*, Semin Hematol, 2000, 37(1):30–42.

[5] MA J., SHI M., LI J., CHEN B., WANG H., LI B., HU J., CAO Y., FANG B., ZHAO R. C., *Senescence-unrelated impediment of osteogenesis from Flk1+ bone marrow mesenchymal stem cells induced by total body irradiation and its contribution to long-term bone and hematopoietic injury*, Haematologica, 2007, 92(7):889–896.

[6] GAL T. J., MUÑOZ-ANTONIA T., MURO-CACHO C. A., KLOTCH D. W., *Radiation effects on osteoblasts in vitro: a potential role in osteoradionecrosis*, Arch Otolaryngol Head Neck Surg, 2000, 126(9):1124–1128.

[7] TITORENCU I., JINGA V. V., CONSTANTINESCU E., GAFENCU A. V., CIOHODARU C., MANOLESCU I., ZAHARIA C., SIMIONESCU M., *Proliferation, differentiation and characterization of osteoblasts from human BM mesenchymal cells*, Cytotherapy, 2007, 9(7):682–696.

[8] KALAJZIĆ I., KALAJZIĆ Z., WANG L., JIANG X., LAMOTHE K., SAN MIGUEL S. M., AGUILA H. L., ROWE D. W., *Pericyte/ myofibroblasts phenotype of osteoprogenitor cell*, J Musculoskelet Neuronal Interact, 2007, 7(4):320–322.

[9] BONEWALD L. F., HARRIS S. E., ROSSER J., DALLAS M. R., DALLAS S. L., CAMACHO N. P., BOYAN B., BOSKEY A., *von Kossa staining alone is not sufficient to confirm that mineralization in vitro represents bone formation*, Calcif Tissue Int, 2003, 72(5):537–547.

[10] AHMAD M., SAMPAIR C., NAZMUL-HOSSAIN A. N., KHURANA N., NERNESS A., WUTTICHAROENMONGKOL P., *Therapeutic doses of radiation alter proliferation and attachment of osteoblasts to implant surfaces*, J Biomed Mater Res A, 2008, 86(4):926–934.

[11] LI J., KWONG D. L., CHAN G. C., *The effects of various irradiation doses on the growth and differentiation of marrow-derived human mesenchymal stromal cells*, Pediatr Transplant, 2007, 11(4):379–387.

[12] SAWAJIRI M., NOMURA Y., BHAWAL U. K., NISHIKIORI R., OKAZAKI M., MIZOE J., TANIMOTO K., *Different effects of carbon ion and gamma-irradiation on expression of receptor activator of NF-kB ligand in MC3T3-E1 osteoblast cells*, Bull Exp Biol Med, 2006, 142(5):618–624.

[13] NGUYEN H., MORGAN D. A., FORWOOD M. R., *Sterilization of allograft bone: effects of gamma irradiation on allograft biology and biomechanics*, Cell Tissue Bank, 2007, 8(2):93–105.

[14] SCHEVEN B. A., WASSENAAR A. M., KAWILARANG-DE HAAS E. W., NIJWEIDE P. J., *Direct and indirect radiation effects on osteoclast formation in vitro*, Bone Miner, 1987, 2(4):291–300.

[15] ROSS M. H., PAWLINA W., *Histology: A text and atlas with correlated cell and molecular biology*, 5th edition, Lippincott Williams & Wilkins, Philadelphia, 2006, 202–237.

[16] SAMEE M., KASUGAI S., KONDO H., OHYA K., SHIMOKAWA H., KURODA S., *Bone morphogenetic protein-2 (BMP-2) and vascular endothelial growth factor (VAGF) transfection to human periosteal cells enhances osteoblast differentiation and bone formation*, J Pharmacol Sci, 2008, 108(1):18–31.

[17] HO A. M., MARKER P. C., PENG H., QUINTERO A. J., KINGSLEY D. M., HUARD J., *Dominant negative BMP5 mutation reveals key role in BMPs in skeletal response to mechanical stimulation*, BMC Dev Biol, 2008, 8:35.

[18] BENNETT K. P., BERGERON C., ACAR E., KLEES R. F., VANDENBERG S. L., YENER B., PLOPPER G. E., *Proteomics reveals multiple routes to the osteogenic phenotype in mesenchymal stem cells*, BMC Genomics, 2007, 8:380.

[19] WAGENAAR-MILLER R. A., ENGELHOLM L. H., GAVARD J., YAMADA S. S., GUTKIND J. S., BEHRENDT N., BUGGE T. H., HOLMBECK K., *Complementary roles of intracellular and pericelullar collagen degradation pathways in vivo*, Mol Cell Biol, 2007, 27(18):6309–6322.

[20] MANOLAGAS S. C., *Death and birth of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis*, Endocr Rev, 2000, 21(2):115–137.

[21] SZYMCZYK K. H., SHAPIRO I. M., ADAMS C. S., *Ionizing radiation sensitizes bone cells to apoptosis*, Bone, 2004, 34(1):148–156.

[22] FRANZ-ODENDAAL T. A., HALL B. K., WITTEN P. E., *Buried alive: how osteoblasts become osteocytes*, Dev Dyn, 2006, 235(1):176–190.

Corresponding author:
Olga Sorițău, MD, Laboratory of Experimental Radiotherapy and Stem Cell Culture, "Prof. Dr. Ion Chiricuță" Cancer Center, 34–36 Republicii Street, 400015 Cluj-Napoca, Romania; Phone +40751–026 189, e-mail: osoritau@hotmail.com

Received: January 29th, 2009

Accepted: July 24th, 2009