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ORIGINAL ARTICLE

Mesenchymal stem cell irradiation in culture engages differential effect of hyperfractionated radiotherapy for head and neck cancers

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Summary

Purpose: *The purpose of this study was to challenge current knowledge on the potential therapeutic advantages of stem cells in radiotherapy by developing an in vitro model of the healthy tissue surrounding or replacing the widely resected tumor. After radical surgery, the start of radiotherapy is often delayed due to wound healing process, with potential loss of the opportunity for treating microscopic disease instead of macroscopic early recurrence. Hyperfractionated radiotherapy, contrary to the standard one, can extend the limits of radical surgery and shorten the gap before the onset of postoperative radiotherapy, with potential improvement in local control.*

Methods: *By using both mesenchymal stem cells and pre-differentiated osteoblasts, cultured in proper pro-osteogenic media after cell irradiation, we investigated both the differences in the response to DNA damage between lineages undergoing differentiation in culture and the intensity of the mineralization process.*

Results: *Ionizing radiation stimulated stem cell proliferation and differentiation at 0.5 Gy and 1 Gy, thus confirming in vitro the clinical results of hyperfractionated irradiation randomized trials in head and neck cancers (HNCs).*

Conclusion: *To our knowledge, this study is the first to investigate the biophysics of low dose gamma irradiation on stem cell culture, focusing on the potential applications in radiation oncology. For advanced oral cavity and oropharyngeal cancers, as radical surgery often implies major bone resection, the use of mesenchymal stem cells as bone reconstruction vectors might shorten the onset of adjuvant hyperfractionated radiotherapy which enhances the mineralization process. As postoperative radiotherapy has recently being revisited for osteosarcoma, this scenario could impact also on bone reconstruction process in this pathology.*

Key words: head and neck cancers, hyperfractionated radiotherapy, mesenchymal stem cell radiobiology

Introduction

HNCs encompass epithelial malignancies that arise in the paranasal sinuses, nasal cavity, oral cavity, pharynx and larynx. Almost all of these epithelial malignancies are squamous cell carcinomas, for which the most important risk factors are tobacco and alcohol consumption or human papillomavirus (HPV) infection [1]. Worldwide, an estimated 650,000 new cases of HNCs are diagnosed each year, with two thirds of these cases occurring in developing countries. In the US and the European Union, HNCs account for 3.2% of all new malignancies and 2.2% of all cancer deaths. Surgery and radiation form the backbone of treatment for these diseases, accompanied by chemotherapy in stages III and IV [2,3].

Despite therapeutic modalities that permit organ preservation, ablative wide surgical resection with reconstruction (osteo-myo-cutaneous flaps) and postoperative radiotherapy are required in many patients. Overall the 5-year survival is only 40% because local or regional disease recur in 30% of patients and distant metastases develop in 25% of them.

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Patients with two or more regional lymph nodes involved, extracapsular spread of disease, or microscopically involved margins of resection have particularly high rates of local recurrence (27-61%) and distant metastases (18-21%) after surgery, with an overall high risk of death within 5 years [4]. Therefore, the chance for cure is low but achievable through an aggressive locoregional treatment that reaches the limits of surrounding tissue biological tolerance. Available evidence suggests that hyperfractionated radiotherapy or the addition of chemotherapy concomitantly with radiation lead to an overall improvement of outcome for these patients [5-10].

For high grade osteosarcomas not involving the extremities (pelvis or facial bones for example) it is often difficult to perform a conservative resection with negative margins. Unresectable tumors and those with close or positive margins may benefit from radiotherapy [11,12].

The ability of stem cells to contribute to the recovery of damaged tissue is the feature that determines stem cell-based treatment plans for a wide array of diseases. However, little is known about the potential therapeutic advantages of stem cells in radiotherapy for patients with HNCs. We developed an *in vitro* model of the healthy tissue surrounding or replacing the widely resected tumor and proved that hyperfractionated radiotherapy, contrary to standard one (1.8-2 Gy/daily fraction) might accelerate the osteogenic differentiation of a bone graft (replacing the mandible or any other resected bone) if mesenchymal stem cells are used, extending the limits of the radical surgery and shortening the gap before the onset of postoperative radiotherapy, with potential improvement in local control.

Methods

Cell isolation and culture

Human bone marrow samples were obtained under general anesthesia from donors undergoing routine hip replacement surgery who had received no growth factors prior to the intervention, in accordance with all aspects of the Declaration of Helsinki [13]. The bone tissue fragments were harvested in complete Dulbecco's modified Eagles Medium (DMEM), with 10% fetal bovine serum (FBS) and afterwards washed several times with phosphate buffered solution (PBS) (all from Sigma Aldrich, St Louis, MO, USA). Mechanically dissected fragments of approximately 2 mm^3 were filtered with 70 nm Filcons meshes (Dako, Glostrup, Denmark) and the cell suspension was centrifuged at 1200 rpm for 10 min. 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 and maintained by incubation at 37° C in 7% CO_2. Cells were subcultured every 3 to 4 days using standard technique. The medium was discarded, the flask washed with PBS and cells were detached with trypsin 0.25% / ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich). DMEM medium supplemented with 10% FBS neutralized trypsin and the cell suspension was centrifuged at room temperature at 1200 rpm for 10 min. The supernatant was removed and cells resuspended, counted and distributed in culture dishes. Following these steps, we isolated mesenchymal stem cells (MSCs) from the iliac crest and osteoblasts both from the iliac crest (ICOs) and the patella (POs). The cell populations were distinguished phenotypically and by using basic histological and immunocytochemical staining techniques.

Stem cell osteogenic differentiation

Induction osteogenic differentiation *in vitro* occurred when MSCs, seeded at a density of 3.1×10^3 cells/cm^2, were cultured until they reached approximately 80% confluence, before passage. The culture was performed in osteogenic differentiation medium DMEM, supplemented with 15% fetal calf serum (FCS), 2 mM L-glutamine, 10 nM dexamethasone, 1% non essential amino acids, 50 μg ascorbic acid, 10 mM β glycerophosphate, 1 μg insulin, 2 ng/ml transforming growth factor β1 (TGF-β1) and 3 ng/ml bone morphogenic protein 2 (BMP-2) (all from Sigma Aldrich).

Immunophenotype characterization

MSCs were identified by pluripotent protein markers of the cell membrane, identified at the 3rd passage, after having been labeled with the following anti-human antibodies: SSEA-4, Oct ¾, Nanog (Santa Cruz Biotechnology, Santa Cruz, CA, USA), CD 29 (β1 integrin), CD105 (Endoglin; Becton Dickinson, Franklin Lakes, NJ, USA) and SOX 2 (R&D, Minneapolis, MN, USA). Cells were fixed in 4% paraformaldehyde (Sigma Aldrich) for 20 min, blocked with bovine serum albumin (BSA) 10% and incubated overnight with primary antibodies diluted 1:50. As secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 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.

We assessed the feasibility of the osteoblasts obtained *in vitro* from human bone marrow adult stem

cells using monoclonal antibodies after a permeabilization step with 0.01% Tween 20 or 1% Triton X-100. Mouse anti-human osteopontin and osteonectin demonstrated the presence of the two specific calcium-binding glycoproteins. Cell nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Cell visualization was possible using an inverted phase Zeiss Axiovert microscope, filtres 488, 546 and 340/360 nm, as previously described by our team [14].

Ionizing radiation methods

Cells were trypsinized when near confluence and resuspended, before being irradiated with doses of 0.5, 1, 2 and 4 Gy, at the Department of Radiotherapy, using a Theratron 1000 ^{60}Co source. To ensure adequate build up and homogeneous irradiation, according to the debit of the source, we calculated the exposure times at 1 cm depth for a 10×10 cm field at source target distance of 100 cm. The dose rate value used was 0.65 Gy/min. Cells were irradiated in suspension instead of monolayer to avoid subjecting irradiated cells to further manipulation stress such as trypsinization, which may have interfered with cell recovery. During the irradiation time, 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. Both control and irradiated cellular populations were further cultured in osteogenic medium, in exactly the same conditions.

Alkaline comet assay

We used the alkaline version of the single cell electrophoresis assay according to Tice's protocol [15]. Briefly, single cells were suspended in agarose and poured on a microscope slide. With the aid of various chemicals all of the non-DNA material was removed. Subsequently, a weak electric field was applied that – in the case of DNA damage –induces a "comet" like structure, because some of the DNA material was moved into the comet tail. When, however, the cells got the opportunity to repair damage before analysis, no or only a small tail was observed. The measures of DNA damage were lesion score (LS) and tail factor (TF) calculated according to Collins' formulas [16].

Histology stainings

Before osteogenic culture, immediately after irradiation, cellular viability was evaluated using trypan blue staining and hemocytometer counting. After osteogenic differentiation, the onset of mineral deposition was assessed by von Kossa and Alizarin Red S stainings. 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 a Olympus CKX 41 inverted light microscope, at ×100 and ×200 magnification.

Proliferation assay

To assess the proliferation within two weeks, control and irradiated cells were plated in 96-well flasks in a final volume of 100 μL culture medium per well. Then, at days 5 and 14 post-irradiation, cell proliferation was evaluated using the MTT based cell proliferation assay. After removal of culture media, 1 mg/ml MTT (3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide) was added and cells were kept for 60 min at 37° C. The tetrazole was reduced to formazan by the NAD-dependent dehydrogenase activity of living cell mithochondria to form a purple product. The supernatant was removed and formazan crystals were dissolved after we had added dimethylsulfoxide (DMSO). The color of this biochemical reaction depends on the number of viable cells, measured with a 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: growth %=($A_{irradiation}/A_{control}$)×100. Cell proliferation after irradiation was reflected in higher optical densities in comparison with proliferation of non-irradiated ones [17].

Data analysis

All pictures were processed using Adobe Photoshop CS2 9.0 software. Statistical significance values were obtained using a one-way analysis of variance (ANOVA), with a 95% confidence level using GraphPad Prism 5 statistics program. All experiments were performed in triplicate.

Results

Cell isolation and characterization

A morphologically homogeneous population of fibroblast-like cells with 80% confluence was seen after 10-14 days in DMEM culture. The isolated pluripotent stem cells were positive for various such surface antigens, identified by monoclonal antibodies. The

functional status of MSC was confirmed by standard osteogenic differentiation assay.

Cells derived from human bone tissue adhered after 21-28 days of primary culture. Preosteoblasts, proven to express reliable specific markers like osteonectin and alkaline phosphatase, differentiated into active bone matrix-secreting osteoblasts. These cells, ultimately responsible for depositing organic bone matrix, had a typical cuboidal shape, a large eccentric nucleus with 1-3 nucleoli and rough endoplasmic reticulum and

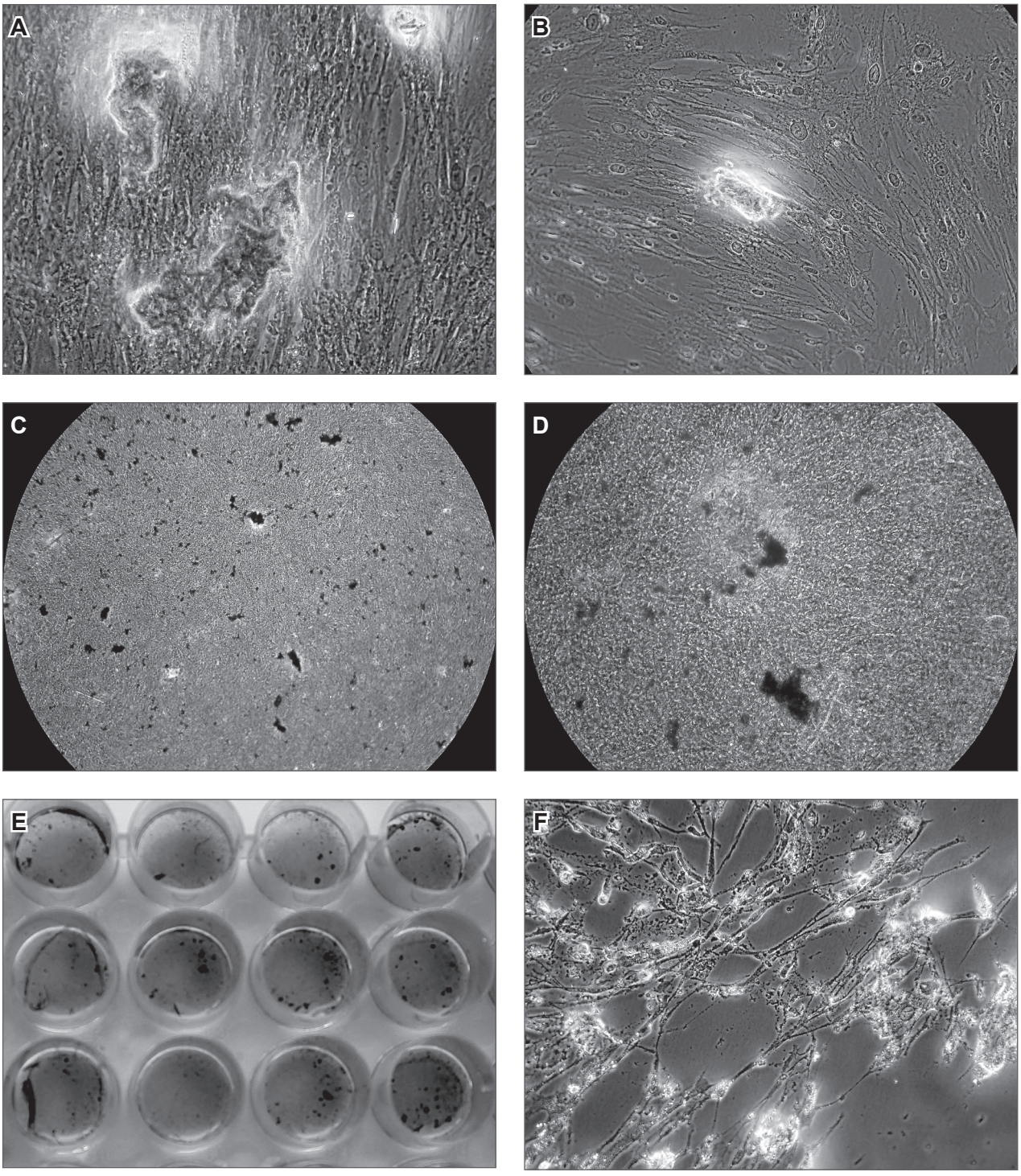
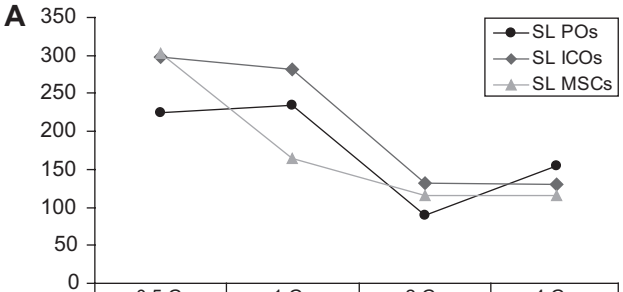
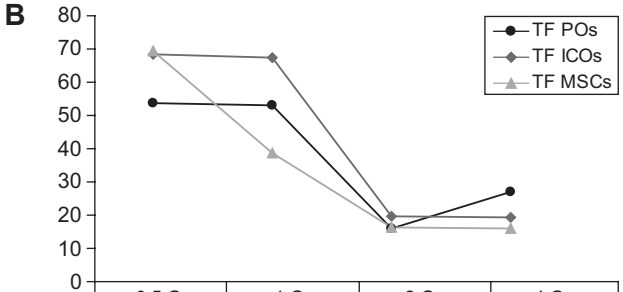


Figure 1. Alizarin S staining **(A, B)** demonstrates bone matrix calcification (×20). The mineral deposits are confirmed by von Kossa staining, both microscopically **(C, D)** (×20) and macroscopically **(E)**. The difference between 0.5 Gy/min and 1 Gy/min **(A, D)** and 2 Gy/min **(B, C)** confirms that low dose ionizing radiation stimulates the mineralization process. *In vitro*, cells have more bone matrix, with greater calcium concentration. Alcian blue is positive due to the presence of chondroitin sulfate glycosaminoglycan in the bone matrix **(F)** (×20).



	0.5 Gy	1 Gy	2 Gy	4 Gy
SL POs	225.2	234	90.33	154.8
SL ICOs	297.6	282	131.6	130
SL MSCs	302	164.8	116	115.2



	0.5 Gy	1 Gy	2 Gy	4 Gy
TF Pos	53.82	53.16	16.2	27.05
TF ICOs	68.74	67.74	19.84	19.55
TF MSCs	69.62	38.97	16.24	15.97

Figure 2. A: Lesion score at different doses of ^{60}Co γ-rays, **B:** Tail factor at different doses of ^{60}Co γ-rays. Data points are the mean for three independent experiments.

Golgi areas. As matrix deposition continued, osteoblasts became embedded in the cells' secretory product, the osteoid. These osteoid-osteocytes, larger than mature osteocytes, underwent ultrastructural changes on mineralization of the osteoid and decreased protein synthesis and secretion. Newly embedded osteocytes were variable in size and shape in comparison with older, more mature osteocytes.

Stem cell radiation biology

Three days after irradiation, all progenitor cells had begun to exhibit morphological changes typical for the preosteoblastic phenotype. After 6 days in culture, cells showed a polygonal osteoblast-like morphology, and 6 more days later the functional osteoblast secreted bone matrix and formed an ossification nodule before these cells had differentiated into osteocytes. The fully-developed osteoblasts expressed high amounts of alkaline phosphatase, an ectoenzyme anchored to the external surface of the plasma membrane. Cells also expressed positive immunocytology stainings for osteopontin and osteonectin.

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. Even though not observed macroscopically, von Kossa and Alizarin red S staining techniques confirmed that low dose radiation stimulated both stem cell proliferation and the mineralization process. Nevertheless, calcium deposits did not represent a good enough proof that the tissue was indeed bone, so Alcian blue staining confirmed the presence chondroitin sulfate (Figure 1).

DNA damage

Cell lines showed a substantial variability concerning biological parameters of radiosensitivity: basal DNA damage, radiation-induced DNA damage and capacity of repair (Figure 2). Cells irradiated with 0.5 Gy and 1 Gy had higher amounts of DNA damages than others irradiated with 2 Gy and 4 Gy which is shown by the differences between radiosensitivity parameters (SL, TF) obtained performing the Alkaline Comet Assay.

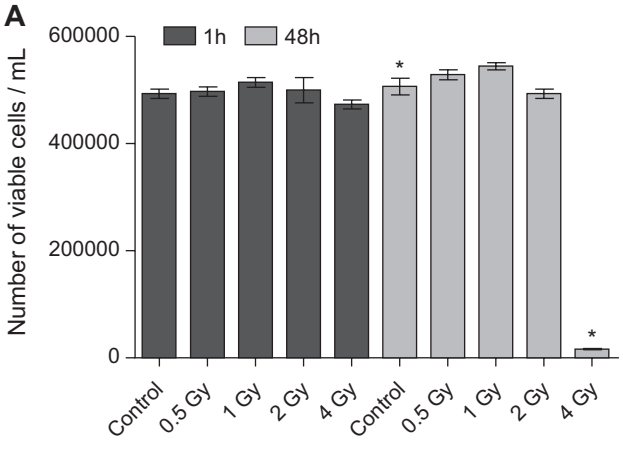
Cellular proliferation

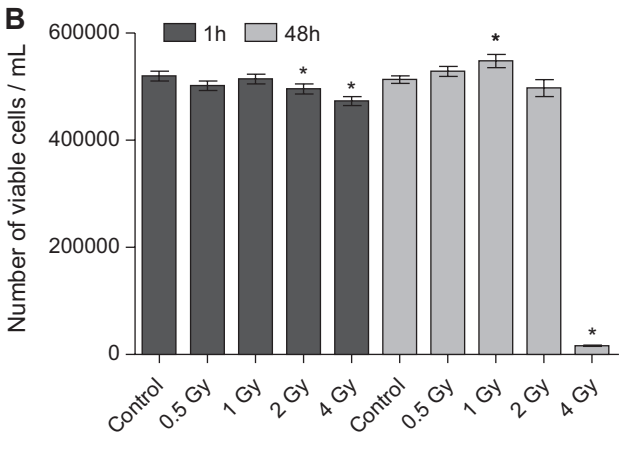
One hour after exposure, the number of viable MSCs, ICOs and POs remained approximately the same compared with the control, with statistically values not significantly different ($p > 0.05$), but after 48 h cells were thought to have repaired DNA lesions and started to divide, confirmed by a trend to significant difference ($p=0.083$). When irradiated at 4 Gy, morphological changes were observed. These changes included beading, membrane blebbing and lifting from the surface of the flask. Even if some cells were viable 1h after exposure to ionizing radiation, after 48 h almost all progenitor cells were dead (Figure 3).

The proliferation of irradiated MSCs, POs and ICOs is stimulated within two weeks after irradiation. Five days after irradiation cells divide and proliferate but then differentiation occurs. Due to this reason 14 days after exposure to ionizing radiation the values that represented controls and irradiated cells were not all that different (Figure 4).

Discussion

The complex head and neck anatomy and vital physiological role of the tumor-involved structures dic-





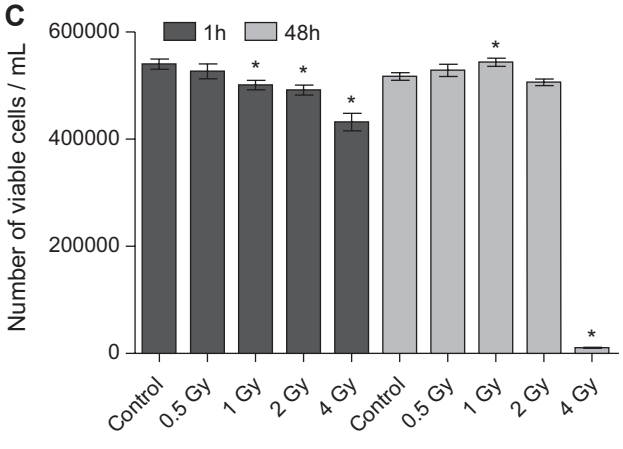
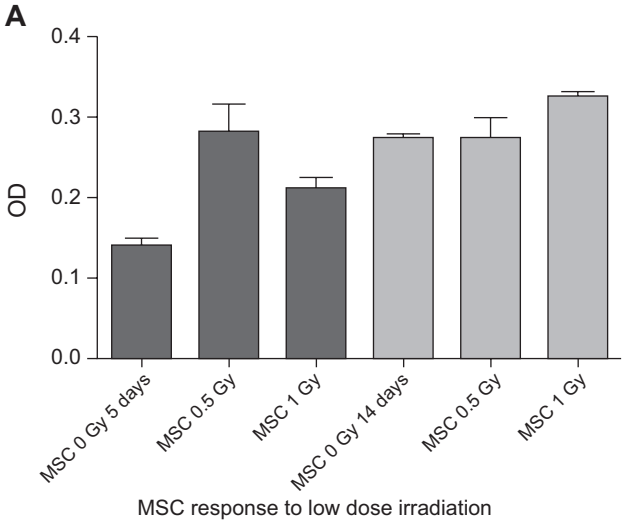
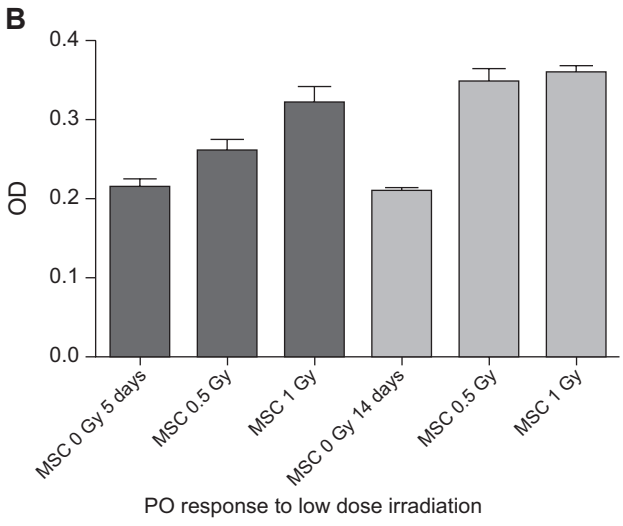


Figure 3. Trypan blue staining for number of viable cells: mesenchymal stem cells **(A)**, patella osteoblasts **(B)** and iliac crest osteoblasts **(C)**. One and 48 h after irradiation, trypan blue was used to measure the number of viable cells. Results were calculated as the number of viable cells/mL. Statistically significant values were obtained using a one-way analysis of variance, with a 95% confidence level (marked with*).





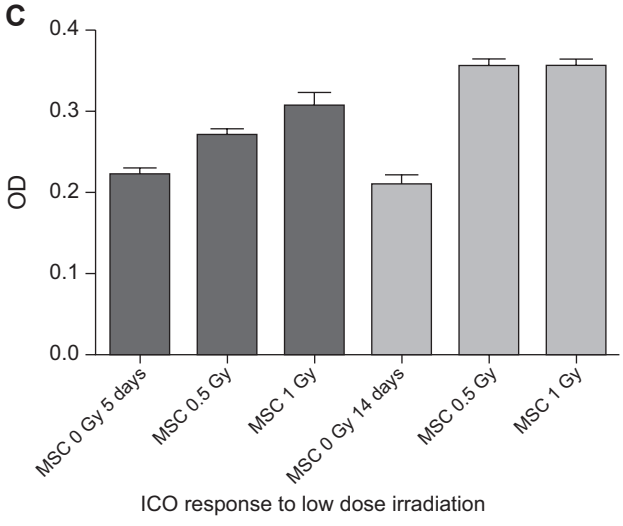


Figure 4. The MTT assay shows that stem cells are stimulated immediately after irradiation, but once they differentiate into osteoblasts the proliferation potential drops **(A)**. The ionizing radiation also stimulates the proliferation of pre-differentiated osteoblasts within the first two weeks **(B, C)**, confirming the results obtained for stem cells. OD: optical density, MSC: mesenchymal stem cells, PO: patella osteoblasts, ICO: iliac crest osteoblasts.

tate that the goals of treatment are not only to improve survival outcomes, but also to preserve organ function. Surgery is the basic standard treatment for this kind of cancers, but is frequently limited by the anatomical extent of tumor and desire to achieve organ preservation. Advances in microsurgical free tissue transfer for reconstruction of surgical defects have made major reconstructive procedures common, helping in the resection of locally advanced tumors. Selective neck dissection is a reasonable procedure for both clinically uninvolved necks, which can harbor micrometastasis, and for clinically N1 disease. Most surgeons accept cases with invasion of the carotid artery, base of the skull or paravertebral musculature as unresectable [18].

As primary treatment, radiotherapy alone results in high tumor control and cure rates for some HNCs, like early-stage glottic, base of tongue or tonsillar cancers with accurate imaging like CT, MRI or PET that improve three dimensional tumor delineation. Conventional radiation therapy for the primary tumor and gross lymph nodes is typically given in daily fractions of 1.8-2 Gy, 5 days a week, up to a total dose of 70 Gy over 7 weeks. Higher dose per fraction have been attempted for early-stage laryngeal cancer with excellent results and no increase in late toxic events. Modern oncology addresses tumor cell kinetics and exploits differences in damage repair between healthy and tumor cells. Two major fractionation variants that make it possible for multiple fractions per day to be delivered have been tested: hyperfractionation and accelerated fractionation [19,20].

Hyperfractionation was designed to improve effectiveness by delivering 2-3 fractions every day with a reduced dose per fraction, usually 1.10-1.25 Gy, reducing late effects without increasing late toxicity, despite an increased total dose. Accelerated fractionation was designed to increase radiation dose intensity by delivering 1.6-1.8 Gy more than once every day, in a reduced time period compared with hyperfractionation, but maintaining the same or slightly reduced dose of conventional radiation treatment.

Cells and tissues recover from irradiation effects in a variety of ways. Greenberger et al. confirm that surviving cells within irradiated tissues and those in adjacent unirradiated tissue, particularly in the primitive or stem cell compartments, are induced to proliferate and repopulate areas in the tissue that were depleted by ionizing radiation killing. In addition, stem cell or progenitor populations from outside the irradiated field migrate into the damaged tissues and facilitate repopulation or replenishment of tissue function [21]. Stem cell populations involved in regeneration of irradiated tissue (epithelial progenitor cells for example in the irradiated oral cavity of a patient with a squamous cell carcinoma) include those which home to sites in the vacated tissue microenvironment, depleted by radiotherapy.

Recovery from acute irradiation effects for HNCs (such as mucositis, increased secretions, dysphagia, loss of taste, hoarseness caused by laryngeal oedema, and dermatitis) occurs both at the cellular level and at the tissue level. Cellular recovery is realized by restauration of antioxidant pools through biochemical synthesis of glutathione and up-regulation of antioxidant enzymes [22]. Tissue recovery is possible due to stem cell mediated repopulation through both proliferation of *in situ* cells and by migration via the circulation of progenitor cells from distant sites.

Because radiation therapy involves the art of balancing the recurrence of cancer due to under treatment against severe damage to local tissues due to overtreatment, results so often fall short of desired success rates. Altered radiation schemes allow the total dose of 70 Gy, lethal for cancer cells, while improving at the same time the therapeutic ratio by favoring normal tissue over cancer. The *in vitro* data obtained in our laboratory confirms the stimulation of proliferation and differentiation of normal bone progenitor cells by low dose ionizing radiation.

In vitro, irradiated functional MSCs started to exhibit morphologic changes specific for the pre-osteoblastic phenotype after 3 days and showed a polygonal osteoblast-like morphology after 5 days. This clearly represents an improvement in the healing process of patients that have undergone radical resection of the tumor. After surgery, the start of radiation therapy is most often delayed because of slow wound healing. A significant shorter post-ablative recovery time will allow oncologists to fight cancer at a different level, by treating microscopic disease instead of macroscopic early recurrence. Due to the fact that all experiments were performed in triplicate, we may say with no doubt that doses of 0.5 and 1 Gy will stimulate stem cell proliferation and differentiation, confirming the possibility of delivering altered radiation schemes in HNC.

The use of MSCs radiobiology in radiotherapy is confirmed by the experiments of François et al. [23]. In a study of MSCs quantitative distribution after irradiation damage, it was found that local irradiation not only induces homing of stem cells at exposed sites, but also promotes their widespread engraftment to multiple organs. Inflammation due to irradiation can activate the molecular pathways that increase the release of tissue chemokines, which attract stem cells to areas where they may home, proliferate and differentiate. This is the case of the α-chemokine stromal-derived factor (SDF-1α), which binds exclusively to CXCR4 receptor, also

known as CXCL12 [24]. The CXCR4-SDF-1α axis explains why after local irradiation the levels of human MSCs engraftment increased not only at the sites of injury, but also in all distant organs and tissues tested outside the irradiation field. In a different set of experiments, using a non-human primate model submitted to mixed γ-neutron irradiation and infused with green fluorescent protein-labeled non-human primate MSCs, Chapel et al. concluded that stem cells engrafted preferentially in regenerating tissues, with important implications in the preservation of targeted tissues [25].

Because the radiation dose that can be given to a tumor is often limited by the concomitant dose that adjacent normal tissue receives during the process, the radiation oncologist has 3 basic approaches to improve upon this. One is to somehow make the tumor more sensitive or the normal tissue more resistant, by the use of radiosensitizers or radioprotectors. The second one is gene therapy approaches for stem cell protection. Greenberger indicates that antioxidant gene therapy using manganese superoxide dismutase plasmid liposomes may provide organ-specific radiation protection associated with the delay or prevention of acute and late toxicity [26]. Evidence has suggested that manganese superoxide dismutase transgene expression in cells of the organ microenvironment contributes to radioprotection, but incorporating this knowledge into designs of novel approaches for stem cell protection is not immediately applicable. The last approach is the use of altered fractionation, shown to be effective by several randomized trials and recently confirmed by a large meta-analysis [27-29]. The last hypothesis has the huge advantage of integrating stem cells as tissue reconstruction vectors, as shown by our study, with the potential to shorten the onset of adjuvant hyperfractionated accelerated radiotherapy and enhance the mineralization process.

Another potential use of ionizing radiation is the very challenging plastic and reconstructive surgery following curative resection for mandibular osteosarcoma. The combination of a vascularized and an extracorporeally-irradiated bone autograft may be a useful reconstructive technique for massive bone defects arising after ablative oncological surgery because it brings together the biological properties provided by the vascularized bone graft and the mechanical endurance of the irradiated bone graft. The hypothesis is confirmed by Muramatsu et al. that after a wide *en-bloc* resection and curettage of the tumor from the resected bone, treatment is followed by extracorporeal irradiation, vascularized bone grafting from the fibula or scapula and re-implantation of the irradiated bone into the recipient, before fixation with plates and screws [30].

Conclusion

In the last few years, the ability of MSCs to increase hematopoietic recovery and contribute to tissue repair has been of major public interest because of the huge potential in future medicine. In an effort to complete this endeavor, we built an *in vitro* model in which both osteoblasts and adult stem cells were submitted to ionizing radiation in order to investigate the biology of the healthy tumor-surrounding tissue of a patient undergoing radiation-treatment for HNCs, for example. We confirmed the efficiency of altered radiation schemes in comparison with the conventional ones because they introduce the concept of radiation-stimulated stem cell-based recovery from radio-induced damage of the surrounding tissue and permit better recovery from the acute and also late complications of the oncological treatment.

Advances in basic research and application of both genomic and proteomic profiling are expected to provide powerful methods for the individualisation of treatment approaches in cancer. By combining radiotherapy with stem cell research we may find a way to decrease treatment-related toxicity for cancers where adjuvant radiotherapy after radical surgery is mandatory for cure.

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