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Dounia Hamdi, François Chevalier, Jean-Emmanuel Groetz, Florent Durantel, Jean-Yves Thuret, et al.. Comparable Senescence Induction in Three-dimensional Human Cartilage Model by Exposure to Therapeutic Doses of X-rays or C-ions. *International Journal of Radiation Oncology, Biology, Physics*, 2016, 95 (1), pp.139 - 146. 10.1016/j.ijrobp.2016.02.014 . cea-01938049

**HAL Id: cea-01938049**

**<https://cea.hal.science/cea-01938049v1>**

Submitted on 28 Nov 2018

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RBE and Particle Therapy Biology

# Comparable Senescence Induction in Three-dimensional Human Cartilage Model by Exposure to Therapeutic Doses of X-rays or C-ions



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Received Sep 30, 2015, and in revised form Jan 6, 2016. Accepted for publication Feb 3, 2016.

## Summary

The relative biological effectiveness of carbon ions compared with X-rays scored 2.6 in primary human articular chondrocytes in

**Purpose:** Particle therapy using carbon ions (C-ions) has been successfully used in the treatment of tumors resistant to conventional radiation therapy. However, the potential side effects to healthy cartilage exposed to lower linear energy transfer (LET) ions in the beam track before the tumor have not been evaluated. The aim of the present study was to assess the extent of damage after C-ion irradiation in a 3-dimensional (3D) cartilage model close to human homeostasis.

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This work was supported by DSV-CEA (AAP Radiobiologie, Direction des Sciences du Vivant, Commissariat à l'Énergie Atomique et aux Énergies Alternatives, Fontenay aux Roses, France) and Conseil Régional de Basse-Normandie (programme Emergence, Caen, France). D.H.H. is supported by Irtelis Graduate Program (DSV-CEA) and a "Ligue contre le cancer" fellowship.

Conflict of interest: none.

Supplementary material for this article can be found at [www.redjournal.org](http://www.redjournal.org).

**Acknowledgments**—The authors thank A.-M. Frelin-Labalme and V. Beaudouin for X-RAD dosimetry and CIMAP physicists E. Balanzat, Y. N'Gnono, and A. Cassimi for IRABAT heavy ions beam calibration and dosimetry (GANIL). We are also grateful to C. Bageot and M. Oger for assistance with IHC-p (Pathology Laboratory, CLCC François Baclesse), M. Guillamin for technical assistance with flow cytometry (UCBN), N. Elie for assistance with imaging (HIQ, UCBN), and B. Bernay at the Protegen Platform (UCBN). We also thank J.-L. Lefaix for critically revising our report.

2-dimensional culture. This was correlated with stronger cellular senescence induction (two-fold). This differential effect was not reflected in the 3-dimensional cartilage model, as both ionizing radiation types induced comparable senescence. Carbon ions seem as safe as X-rays to articular cartilage in the context of radiation therapy.

**Methods and Materials:** Primary human articular chondrocytes from a healthy donor were cultured in a collagen scaffold to construct a physioxic 3D cartilage model. A 2-dimensional (2D) culture was used as a reference. The cells were irradiated with a single dose of a monoenergetic C-ion beam with a LET of approximately 30 keV/μm. This LET corresponds to the entrance channel of C-ions in the shallow healthy tissues before the spread-out Bragg peak (~100 keV/μm) during hadron therapy protocols. The same dose of X-rays was used as a reference. Survival, cell death, and senescence assays were performed.

**Results:** As expected, in the 2D culture, C-ions were more efficient than X-rays in reducing cell survival with a relative biological effectiveness of 2.6. This correlated with stronger radiation-induced senescence (two-fold) but not with higher cell death induction. This differential effect was not reflected in the 3D culture. Both ionizing radiation types induced a comparable rate of senescence induction in the 3D model.

**Conclusions:** The greater biological effectiveness of C-ions compared with low LET radiation when evaluated in treatment planning systems might be misevaluated using 2D culture experiments. Radiation-induced senescence is an important factor of potential cartilage attrition. The present data should encourage the scientific community to use relevant models and beams to improve the use of charged particles with better safety for patients. © 2016 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## Introduction

Particle therapy with carbon ions (C-ions) has many advantages compared with conventional radiation therapy (photons), owing to their better ballistic features, with a decreased irradiated volume of normal healthy tissue in the beam track. Furthermore, these shallow tissues are irradiated with particles of intermediate linear energy transfer (LET; ~30 keV/μm), upstream from the spread-out Bragg peak (~100 keV/μm). However, owing to its complexity and cost, such treatment today has been mainly limited to cancers close to radiosensitive tissues and/or radioresistant tumors such as chondrosarcoma or osteosarcoma (1). Chondrosarcoma is a malignant bone tumor with cartilaginous differentiation described as chemotherapy- and radiation therapy-resistant. Hadron therapy using protons or C-ions has been successfully used to treat low- and intermediate-grade chondrosarcoma (2). However, the risk assessment of sequelae of healthy tissue such as hyaline cartilage and second malignancies in the beam track of charged particles is not entirely predicted (3).

Moreover, although the side effects and sequelae occurring after conventional therapeutic irradiation have been relatively well described, the cellular mechanisms, especially in mesenchyme-derived cells, are still poorly understood. Studying such mechanisms is even more necessary for C-ion irradiation, considering the tendency toward hypofractionated treatments, which lead to higher doses deposited in healthy tissues (4). It has been hypothesized that the senescence induced by cancer therapy is implicated in the organ deterioration experienced by patients surviving cancer and leading to a poor quality of life (5). Hyaline articular cartilage can be side-exposed to

ionizing radiation (IR), which might induce bone differentiation and senescence (6). The senescence of chondrocytes has been defined as the “age-dependent deterioration of chondrocytes following intrinsic and extrinsic factors” (7). Consequently, these cells develop a senescent secretory phenotype that induces loss of differentiated functions, leading to painful osteoarthritis that can be a major prejudice to a survivor’s quality of life. Several proteins are of interest according to their function in cartilage homeostasis and/or differentiation. The cyclin-dependent kinase inhibitor (CKI) p21 has been associated with cellular senescence in different cell lines (8) and plays a major role in the regulation of long-term radiation damage in mesenchyme-derived tissues (9). Furthermore, H2A.J has recently been described as a novel histone variant accumulating in human senescent cells (10), and cyclooxygenase-2 (COX-2) protein has been associated with chondrocyte differentiation (11). These markers are thus relevant in the analysis of radiation-induced senescence.

The relative biological effectiveness (RBE) is an important parameter for the evaluation of the clinical prescribed dose to the tumor and the maximal admitted dose to healthy tissues. However, the RBE values used in clinics have mainly been extracted from data on cell survival, performed in 2-dimensional (2D) culture and normoxia (partial pressure of oxygen [pO<sub>2</sub>] = 20%). These conditions are dramatically different from human homeostasis in 3-dimensional (3D) models, which can mime more physiologic conditions. Starting from the study by Belyakov et al (12) at Columbia University on bystander signaling in 3D engineered human epidermis, numerous published experiments have included 3D models to study the differential response of tissues to ionizing radiation of different LETs (13-19). Moreover, 3D

culture was shown to confer relative radioresistance to cells in a 3D model of breast epithelial cells (20), probably owing to chromatin modification (21). In addition, cellular clues such as adhesion, apical/basal orientation, mechanical tension, and soluble factor diffusion are radically different in 3D culture compared with monolayer culture, explaining the differential behavior to radiation exposure (22).

The purpose of the present study was to assess senescence induction in human cartilage as a direct response to C-ion irradiation in the context of chondrosarcoma treatment compared with X-rays. We used human articular chondrocytes (HACs) cultured in a 3D cartilage model (3DCaM) (23) and a 2D classic culture model as a control.

Owing to the higher LET, we assumed that C-ions would induce more senescence than would X-rays. However, we found no greater effectiveness with C-ions compared with X-rays in this 3DCaM, in contrast to the 2D culture. On the basis of these endpoints, C-ions seem to be as safe as X-rays for articular cartilage fate during cancer treatment. This evaluation method could be used in the future to estimate the risk of sequelae in healthy tissues during particle therapy.

## Methods and Materials

### Cell culture, irradiation, and dosimetry

The data reported in the present study were collected from commercially available cells used in compliance with the Declaration of Helsinki. Primary HACs from a healthy Caucasian male donor (model no. 402K-05a) were purchased from CellSystems (Troisdorf, Germany) and maintained in a chondrocyte growth medium (CellSystems). The HACs were amplified in culture flasks and passaged not more than 4 times before irradiation or cell seeding in 3D culture. For the 3D experiments, HACs were grown for 7 days in collagen scaffolds, as previously described (23, 24), to obtain a 3DCaM.

Confluent HACs in 2D culture and 3DCaM were irradiated in physioxia (2% O<sub>2</sub>). We used 75 to 95 MeV/ $\mu$ m monoenergetic C-ions of D1 IRABAT high-energy scanning beam line at the Grand Accélérateur National d'Ions Lourds (GANIL, Caen, France), depending on beam availability. The cells were irradiated in an upright position in 12.5 cm<sup>2</sup> culture flasks (2D) or polypropylene tubes (3DCaM), filled with medium as described previously (24). Because of the scaffold thickness (2 mm), LET distribution was assessed using 2 calculation codes based on the Monte-Carlo method (13), showing <10% difference in the thickness of the scaffold (Fig. E1; available online at [www.redjournal.org](http://www.redjournal.org)). A dose rate of 1 Gy/min ( $\geq 15$  seconds of radiation exposure) was used. Dose homogeneity was estimated before irradiation using ion-track detection on a CR39 detector by Centre de Recherche sur les Ions, les Matériaux et la Photonique (CIMAP), as recently described (25).

For X-ray irradiation, we used the X-RAD iR-225 research X-rays generator (225 kV, 13 mA) from PXI

(North Branford, CT), with a dose rate of 2 Gy/min, as described previously (24). The cells were irradiated horizontally in 12.5 cm<sup>2</sup> culture flasks (2D) or 96-well plates (3DCaM).

Data are expressed as a function of the absorbed dose (Gy), unless stated otherwise (4). The single dose of 2 Gy was chosen, except that the survival curves and sham-treated samples were handled in the same conditions without irradiation. In the present report, we referred to X-rays as low-LET radiation and C-ions as intermediate-LET. All experiments and postirradiation cultures were performed in physioxia.

### Cell survival, cell death, and senescence assays

After irradiation in 2D, the HACs were trypsinized and seeded in 6-well plates (survival assays), T25 culture flasks (cytometry), or Nunc slide flasks (SA- $\beta$ -Gal assay), and this was considered as the initial time of the kinetic. Survival assays were assessed, as described previously (24). In brief, 12 to 16 hours after irradiation, the cells were counted and plated in 6-well plates at low density (100 or 1000 cells) and left untreated for 12 days in physioxia. Only colonies of  $\geq 50$  cells were scored, and the data were fitted to the linear quadratic equation. D<sub>10</sub> (lethal dose for 10% survival), D<sub>37</sub> (lethal dose for 37% survival) and SF<sub>2</sub> (surviving fraction after 2-Gy irradiation) values were determined from the fitted curve.

Hematoxylin-eosin-saffron classic staining was performed, as described previously (24) on paraffin-embedded, transversally cut, 8- $\mu$ m sections of 3DCaM, and images were taken using the Aperio scanscope CS scanner (Leica, Nussloch, Germany).

Cell toxicity was assessed on culture medium using Toxilight (Lonza, Basel, Switzerland), a bioluminescent cytotoxicity assay designed to quantitatively measure the release of adenylate kinase from damaged cells, as described previously (24). In brief, 20  $\mu$ L of thawed cell culture supernatants (2D or 3D) were mixed with 100  $\mu$ L per well of freshly prepared adenylate kinase working solution and incubated for 5 minutes at room temperature before measurement. Digitonin detergent was used as a positive control, and the data were collected in relative light unit.

Apoptosis induction was evaluated in 2D by flow cytometry (sub-G<sub>1</sub> fraction) using 5-ethynyl-2'-deoxyuridine (100  $\mu$ M; Life Technologies, Carlsbad, CA) and propidium iodide in accordance with the manufacturer's instructions, using FlowJo analyzing software (Ashland, OR). Senescence was assessed in 2D culture, as described previously (26), using the SA- $\beta$ -gal assay (Promega, Madison, WI).

### Western blotting

Cell lysis and Western blotting were performed as described previously (24). In brief, the cells (2D and 3D) were disrupted using glass beads (100- $\mu$ m diameter) and

lysis buffer containing Laemmli buffer at 4°C. For 3DCaM, the entire extracted sample was loaded on precast 4% to 20% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels (Thermo Scientific, Waltham, MA) and was transferred to a nitrocellulose membrane. For 2D samples, approximately 0.15 million cells were used. COX-2 (reference no. 12282), p21 (reference no. 2947), and caspase-3 (reference no. 9662) antibodies were purchased from Cell Signaling (Danvers, MA), glyceraldehyde-3-phosphate dehydrogenase (reference no. 11335232) from Thermo Scientific, and H2A.J was developed and provided by Dr Carl Mann's laboratory. Detections were assessed on X-ray films (GE Healthcare, Little Chalfont, UK) using the enhanced chemiluminescence method.

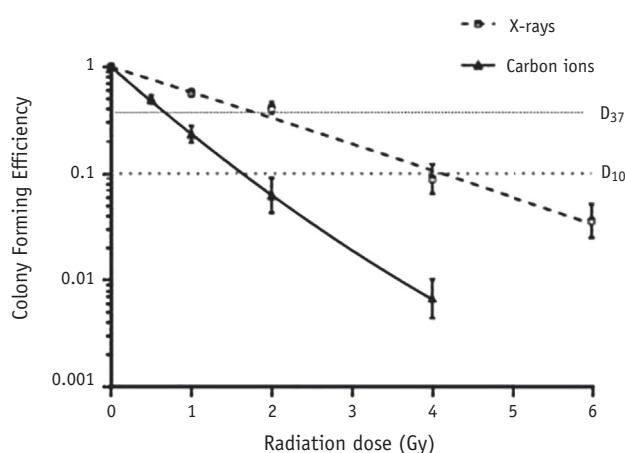
## Statistical analysis

Statistical analysis was performed using Prism (GraphPad, La Jolla, CA), and the statistical testing has been detailed in the figure legends.

## Results

### Survival of HACs after X-ray and C-ion irradiation in 2D culture

We first explored the differential effect of C-ions and X-rays on cell survival. A clonogenic survival assay showed, as expected, a strong dose-dependent effect for both IRs (Fig. 1 and Table 1). The extrapolated  $D_{10}$ ,  $D_{37}$ , and  $SF_2$



**Fig. 1.** Survival curves of human articular chondrocytes (HACs) after X-ray and C-ion irradiation in 2-dimensional culture. HACs were sham-treated or irradiated with the indicated doses of X-rays (square) or C-ions (triangle). The curves (dotted for X-rays, full line for C-ions) represent the surviving fractions of HACs fitted to the linear quadratic model. Data are presented as the mean  $\pm$  standard error of the mean of 2 independent experiments in duplicate. *Abbreviations:*  $D_{10}$  = lethal dose for 10% survival;  $D_{37}$  = lethal dose for 37% survival.

values corresponded to 4.1 Gy, 1.8 Gy, and 33.3% for X-rays and 1.6 Gy, 0.7 Gy, and 6.3% for C-ions, respectively, inferring a RBE of 2.6. In particle therapy with heavy ions, the RBE-weighted absorbed dose refers to a dose that is clinically isoeffective to X-rays (3). In the present study, the samples were irradiated with a physical dose of 2 Gy, corresponding to 5.2 RBE-weighted absorbed dose (C-ions), a value close to the clinical dose used in hypofractionated particle therapy protocols (4).

### Description of the 3DCaM

The effect of the 3D environment on the cellular response to IR was then analyzed using a model (3DCaM) developed for cartilage reconstruction and radiation-biology studies (23, 24). We first checked the cell distribution into the scaffold. At 7 days after seeding, the HACs were distributed homogeneously inside the 3D model (Fig. 2A). Next, we confirmed chondrocyte redifferentiation by measuring COX-2 expression. COX-2 expression has been previously associated with chondrocyte differentiation via p38 activation/NF- $\kappa$ B recruitment (11). In 2D culture, a time-dependent loss of COX-2 expression was measured as a marker of cell dedifferentiation (Fig. 2B). At 7 days after cell seeding in the scaffold, the chondrocytes recovered COX-2 expression, indicating sustainable cell redifferentiation.

### Differential senescence induction in 2D HACs and 3DCaM after X-ray and C-ion irradiation

Depending on the radiation quality, irradiation dose, or cell type, IR can induce several types of cell death (27). Necrosis and apoptosis were analyzed in 2D HACs, with no significant induction until 96 hours after irradiation (Fig. E2; available online at [www.redjournal.org](http://www.redjournal.org)). In the 3DCaM, cellular toxicity analysis showed no sign of IR-induced necrosis until 96 hours after irradiation (Fig. E3A; available online at [www.redjournal.org](http://www.redjournal.org)). Moreover, the analysis of caspase-3 protein expression in either 2D culture or 3DCaM after irradiation did not show any sign of radiation-induced apoptosis (Fig. E2C and Fig. E3B, respectively; available online at [www.redjournal.org](http://www.redjournal.org)). Together, these data suggest that X-rays and C-ions did not induce cell death in either 2D HACs or 3DCaM.

Senescence has been suggested as a potential mechanism to explain low-LET-induced cartilage attrition and osteoarthritis in the clinic (6). We investigated the differential senescence induction of C-ions versus X-rays in 3DCaM and 2D HACs. For 2D HACs, the SA- $\beta$ -gal assay was used as a senescence marker. Despite the assay's limitations, it is, to date, the most widely used senescence marker (26). Moreover, it has been previously described as a good indicator of cellular senescence in articular chondrocytes after X-ray irradiation, in association with other markers (28). The percentage of SA- $\beta$ -gal-positive cells scored 18.5%  $\pm$  2.9%, 32.5%  $\pm$  4.3%, and 57.8%  $\pm$  0.4%

**Table 1** Radiation survival curve characteristics for HAC cells cultured in 2D model

Radiation quality	Energy	LET (keV/μm)	$\alpha$ (Gy <sup>-1</sup> )	$\beta$ (Gy <sup>-2</sup> )	R <sup>2</sup>	D <sub>10</sub> (Gy)	RBE <sub>10</sub>	D <sub>37</sub> (Gy)	RBE <sub>37</sub>	SF <sub>2</sub> (%)
X-rays	225 kV	<1	0.542 ± 0.118	0.004 ± 0.022	0.723	4.1	-	1.8	-	33.3
Carbon ions	75-95 MeV/a	~33	1.512 ± 0.205	-0.064 ± 0.057	0.794	1.6	2.6	0.7	2.6	6.3

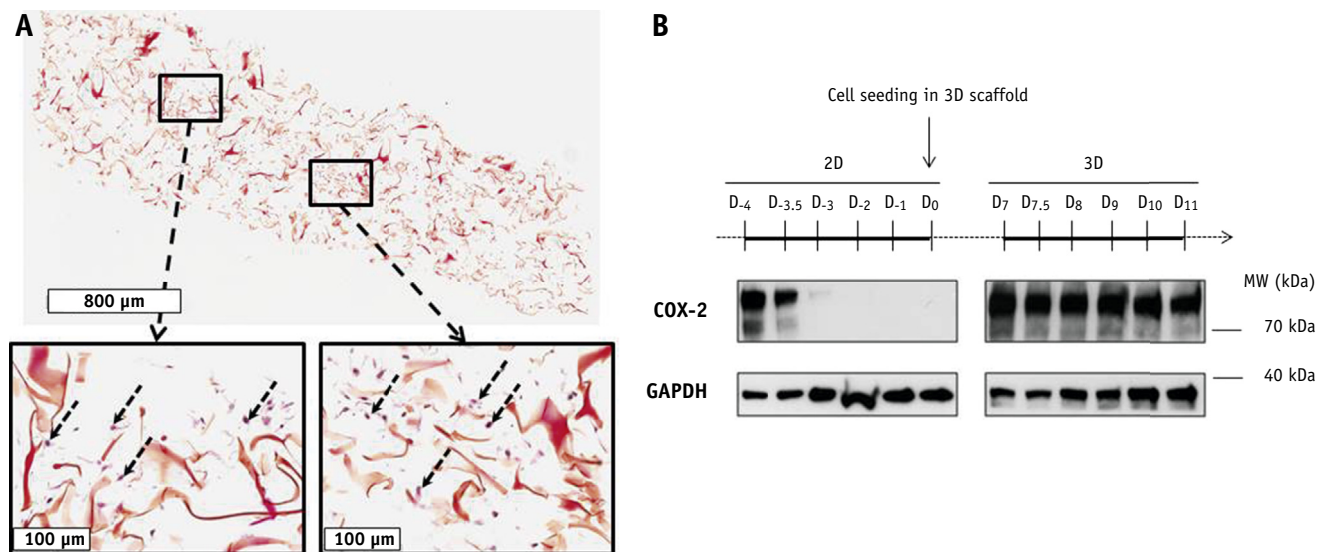
Abbreviations: 2D = 2-dimensional; D<sub>10</sub> = lethal dose for 10% survival; D<sub>37</sub> = lethal dose for 37% survival; HAC = human articular chondrocyte; LET = linear energy transfer; RBE = relative biological effectiveness; SF<sub>2</sub> = surviving fraction after 2-Gy irradiation.

The data from 2 experiments (duplicate) were fitted to the linear quadratic model, and the indicated parameters were determined from the fitted curve, as previously described (24).

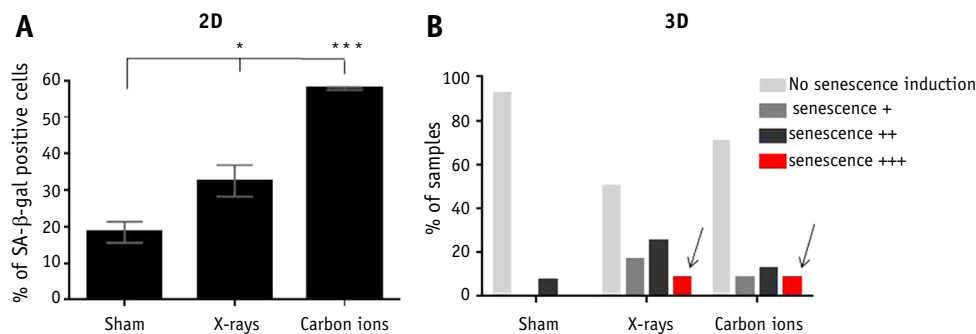
in the sham-treated, X-ray-, and C-ion-irradiated samples, respectively (Fig. 3A). This result suggests that C-ions were twice as effective in inducing senescence compared with X-rays. This value was quite similar to the extrapolated RBE from the survival curves.

Because the SA-β-gal assay was not feasible in the 3DCaM, we selected a combination of 3 proteins involved in senescence signaling for cell fate monitoring. The expression of COX-2, p21, and H2A.J proteins was analyzed from 1 to 96 hours after sham-treatment or irradiation. The data from all Western blots stemming from 3 independent experiments are represented in Figure E4 (available online at [www.redjournal.org](http://www.redjournal.org)). For each sample, a positive score (+) was assigned to protein expression if upregulated for p21 and H2A.J or downregulated for COX-2. The whole analysis is presented in Figure E5 (available online [www.redjournal.org](http://www.redjournal.org)). The experimental data displayed no correlation between senescence induced by radiation as measured here (both radiation qualities) and the time of occurrence after irradiation. Each 3DCaM can

be considered as an independent sample, which would imply the stochastic induction of early stages of senescence within hours after exposure to radiation. Consequently, the percentage of senescent (+/+/+/+) or nonsenescent models was determined by protein scoring, as described, regardless of the time of induction (Fig. 4). The y-axis represents the percentage of 3DCaM with a specific protein expression pattern related to the total of analyzed samples in the indicated group. Gray histograms (+ and ++) correspond to 3DCaMs expressing 1 or 2 senescence markers. The proportion of samples expressing 1 or 2 senescence markers was greater in the irradiated groups (X-rays and C-ions) compared with the sham-treated group. Furthermore, if we consider only the strongest scoring (+++), irradiation of 3DCaM induced senescence in 0% of sham-treated, 8.3% of X-ray-, and 8.3% of C-ion-irradiated samples (Fig. 3B). Thus, according to these results, a single dose of C-ions (~30 keV/μm) seemed to induce no more harmful effects than did X-rays in the 3DCaM, in contrast to the 2D culture data.



**Fig. 2.** (A, Top) Representative image of paraffin-embedded, hematoxylin-eosin-saffron-colored 8-μm section of 3-dimensional (3D) cartilage model showing a homogenous cell distribution in the 3D scaffold. (A, Bottom) Magnified images of top image. The collagen fibers shown as pale red, and the cells, indicated by dotted arrows, in violet. (B) Western blot analysis of cyclooxygenase-2 (COX-2) protein in human articular chondrocytes (HACs) showing cell dedifferentiation during amplification in 2-dimensional (2D) and cell differentiation in 3D model. Glyceraldehyde-3-phosphate dehydrogenase was used as a loading control. Abbreviation: MW = molecular weight.



**Fig. 3.** Ionizing radiation induced senescence in 2-dimensional (2D) human articular chondrocytes (HACs) and 3D cartilage model (3DCaM). (A) Cells were sham-treated or irradiated with X-rays or carbon ions in 2D culture. SA-β-gal testing was performed 96 hours after the initial time of the kinetic. Data are presented as the mean ± standard error of the mean of 2 independent experiments in duplicate after counting ≥200 cells/sample (\* $P < .05$  and \*\*\* $P < .001$ , by 1-way analysis of variance with Tukey's post test compared with the sham-treated sample). (B) Cells were sham-treated or irradiated with X-rays or carbon ions in 3D culture and collected 1 to 96 hours after initial time of the kinetic. Western blot analysis of H2A.J, p21, and cyclooxygenase-2 (COX-2) proteins was used to score senescence induction in the samples. Data are presented as a percentage of senescent (++++) or nonsenescent models, regardless of the time after treatment. The y-axis represents the percentage of 3DCaM with a specific protein expression pattern related to the total of analyzed samples in the indicated group (total of 14 samples for the sham-treated group, 24 samples for the X-ray-irradiated group, and 24 samples for carbon ion-irradiated group). "No senescence induction" corresponded to H2A.J-negative samples. "Senescence +" relates to samples expressing H2A.J only. "Senescence ++" corresponds to samples expressing H2A.J and either p21 induction or COX-2 downregulation. "Senescence +++" indicated with arrows, corresponds to samples expressing H2A.J and both p21 induction and COX-2 downregulation. The whole qualitative analysis is presented in Figs. E4 and E5 (available online at [www.redjournal.org](http://www.redjournal.org)).

## Discussion

The purpose of the present study was to assess the putative senescence of healthy articular cartilage reconstructed in vitro (3DCaM) after therapeutic C-ion irradiation compared with the same physical dose of X-rays. Monolayer 2D culture of HACs was used as a control.

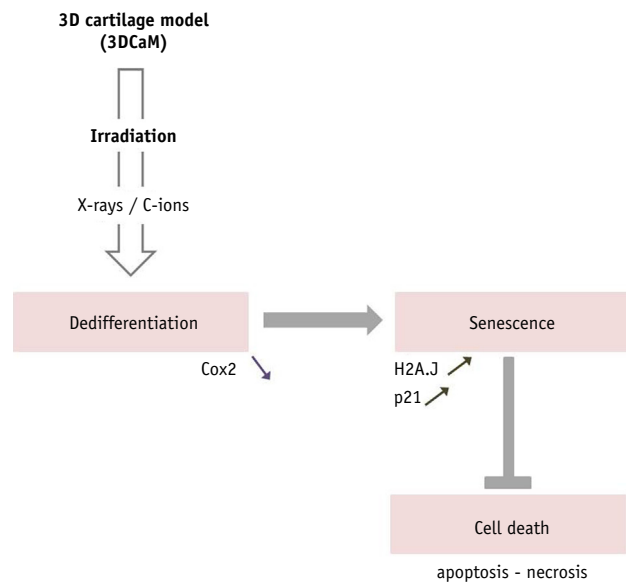
Very few studies have been published on the survival of HACs after IR exposure (6). The data analysis of  $D_{10}$  survival after X-ray irradiation (4.1 Gy) showed a rather higher value (2.7 Gy) than previously reported (29). This might have resulted from a differential oxygen tension in culture media. Our cells were cultured and treated under cartilage physiologic oxygen tension ( $pO_2 = 2\%$ ), instead of atmospheric oxygen tension ( $pO_2 = 20\%$ ), as previously proposed (29). Moreover, a putative interindividual variability in radiosensitivity between human cell donors could explain in part such a discrepancy between studies. The RBE ( $D_{10}$ ) calculated from our data (2.6) was rather smaller than that found with normal human fibroblasts (3.29) in our laboratory using the same irradiation facility (30). However, it was in the range of a previously published database consisting in 855 in vitro survival experiments after ion and photon irradiation (31).

Interestingly, X-ray and C-ion irradiation were not able to induce either necrosis or apoptosis in either 2D or 3D

culture. This result is similar to those from previous studies using X-rays in primary rabbit articular chondrocytes (28) or ex vivo culture of porcine and human (32) articular cartilage, supporting the relative radiation resistance of chondrocytes, regardless of the radiation quality. Necrosis results typically from a high magnitude of stress after either apoptosis or cellular senescence. Depending on the cell lines, it can be triggered by low or very high doses of IR (27). Moreover, human mesenchymal cells (precursors of chondrocytes), in contrast to arthritic chondrocytes, do not undergo radiation-induced apoptosis, probably owing to the efficient activation of the DNA damage response pathways (6, 33).

Furthermore, our results have shown that C-ion irradiation induced twice as much SA-β-Gal-positive cells in 2D culture compared with X-ray irradiation (Fig. 3A). This explains, at least in part, the greater effectiveness of C-ions compared with X-rays in reducing cell survival in 2D culture (Fig. 1) and extensively defined previously (34). However, the greater effectiveness of C-ions compared with X-rays, as described, has not been reproduced in 3D culture conditions.

Chondrosenescence has been described as a hypertrophic cell cycle arrest associated with a loss of differentiation. Hypertrophic arrest, in contrast to quiescence ( $G_0$  arrest), is a cell cycle arrest beyond the restriction point ( $G_1$  or  $G_2$ ) in the presence of activation of mitogenic



**Fig. 4.** Model of radiation-induced chondrosenescence. Irradiation of 3DCaM with X-rays or C-ions induced p21 and H2A.J protein upregulation associated with a down-regulation of cyclooxygenase-2 (Cox-2). This phenotype was associated with radiation-induced senescence and loss of differentiation. The induction of senescence was most probably responsible for cell death resistance.

pathways. It is usually associated with high levels of CKIs such as p21 and can be caused by contact inhibition or DNA damage (34). To evaluate the potential radiation-induced senescence in 3DCaM, we analyzed the expression of 3 proteins of interest: p21 (CKI), responsible for the maintenance of cell cycle arrest (8); the histone H2A.J, recently discovered to accumulate in senescent cells (10); and COX-2 protein, associated with the differentiation of chondrocytes (11).

We have shown in the present report that irradiation of 3DCaM induced an overexpression of p21 and H2A.J proteins, combined with downregulation of COX-2, in nearly 8% of the samples (if we consider only the highest scoring samples). These results show a comparable senescence induction in an in vitro 3D model of human cartilage after a single dose of low- or intermediate-LET radiation (detailed model described in Fig. 4). Hence, the biologic effects measured in 2D cultures, such as the higher effectiveness of C-ions compared with X-rays, might be overexaggerated compared with the reality of clinics, especially concerning healthy tissues and primary cell analysis. Thus, the present study is the first to investigate the differential effect of C-ions versus X-rays in human articular cartilage using a 3DCaM, which could be useful to assess the RBE of therapeutic beams in healthy tissues and tumors.

In our study, we monitored cellular senescence until 96 hours after irradiation, which corresponds to the early stage of the phenomenon and could explain the relatively

low measured percentage (8%). A major furtherance would be to extend the kinetic to weeks after irradiation to assess long-term senescence induction after C-ion and X-ray irradiation. Moreover, the study of the alteration of osteogenesis and chondrogenesis in human mesenchymal stem cells by C-ion beams in this collagen scaffold could be useful for evaluating the extent of the bone and/or joint defects observed after radiation therapy as it has been performed previously using X-rays (33).

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