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Dounia H. Hamdi, François Chevalier, Jean-Emmanuel Groetz, JI Lefaix, Yannick Saintigny. Analysis of targeted effects of ionizing radiations on reconstructed human healthy cartilage and chondrosarcoma. 60th annual meeting of the Radiation Research Society, Sep 2014, Las Vegas, United States. . hal-04248682

**HAL Id: hal-04248682**

**<https://hal.science/hal-04248682>**

Submitted on 18 Oct 2023

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# Analysis of targeted effects of ionizing radiations on reconstructed human healthy cartilage and chondrosarcoma

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### Abstract

Chondrosarcoma, second most common primary bone sarcoma, is resistant to chemotherapy and conventional radiotherapy (X-rays). The sole alternative to mostly incapacitating surgery is Hadrontherapy. Indeed, the benefit of better ballistic and high efficiency of hadrons (proton and carbon ions) for cancer treatment was demonstrated since two decades. However, if the better ballistic of protontherapy is already successfully used worldwide, hadrontherapy with carbon ions stay underused and raise some concerns about likely sequelae for patients.

Since one decade, 3D models are used in radiobiology to approach tissue and tumor microenvironment. In fact, some recent studies have shown that vascular damages could play an important role in tissue response to radiation exposure. Except for cartilage, which is the only avascular tissue of human body, all 3D models available are developed without vascular compartment and, per se, have key restraint in their use as relevant model for radiobiology.

By using collagen matrix, physioxia (2% O<sub>2</sub>) and well defined chondrosarcoma cell lines or primary chondrocytes from healthy donors, we developed a pertinent *in vitro* 3D model for radiobiology studies. With this engineered cartilage and the corresponding 2D cells as an internal control, we study the impact of ionizing radiations on cell fate. We use a physical dose of 2 Gy and radiations at different LETs (Linear Energy Transfer): X-rays (0.2 keV/μm) as radiation exposure control and heavy ions (30-40 keV/μm or 80-90 keV/μm) to mimic energy distribution in healthy cartilage and the tumor during hadrontherapy with carbon ions.

As expected, results show that, 2D cells are more resistant to X-rays than carbon ions (clonogenic assay, RBE from 2 to 3.5). Furthermore, both 2D primary chondrocytes and chondrosarcoma cell line are more resistant to 30-40 keV/μm irradiations than 80-90 keV/μm irradiations for the same physical dose. However, 3D models cell survival (necrosis and viability) do not show such differences. Thus, it may explain some discrepancies between canonical radiobiology studies and clinical data. We are currently analyzing samplings from 2D and 3D systems to investigate protein expression kinetics implicated in cell damage responses at different LETs.

### Context

■ Radiolinduced arthritis in patients treated by conventional radiotherapy and growth arrest in pediatric patients treated by conventional radiotherapy.

■ Chondrosarcoma: Resistant to chemotherapy and conventional radiotherapy. Hadrontherapy: alternative to surgery (side effects reported)

■ Cartilage: Chondrocytes + Extracellular Matrix (ECM = Collagen) Avascular tissue (1-3 % O<sub>2</sub>)

### Project

■ Understand healthy cartilage and chondrosarcoma early responses to ionizing radiations in a 3D cellular model close to human homeostasis (physioxia).

■ Build *in vitro* references in X-rays and hadrons to optimize a TPS for Hadrontherapy

■ One physical dose (2 Gy), different LETs to mimic a conventional vs non-conventional radiotherapy

### ARCHADE facility: Advanced Resource Center for HADrontherapy in Europe

■ ARCHADE is dedicated to create a European Centre for research, development and training in ion beam therapy, nuclear physics and radiobiology.

■ Validation of accelerator prototype dedicated to therapeutic use in a collaboration frame.

■ To develop an industrial transfer in the field of fabrication and use of medical accelerators.

■ Beam lines devoted to fundamental research in biology and physics.



### *In vitro* reconstituted human 3D cartilage

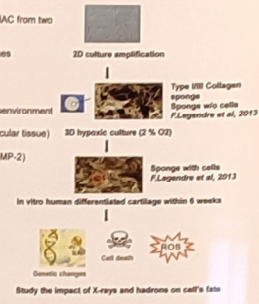
■ Human primary cells (HAC from two male healthy donors)

■ Chondrosarcoma cell lines

■ Collagen = natural microenvironment

■ Physioxia; 2% O<sub>2</sub> (avascular tissue)

■ Differentiation factors (BMP-2)



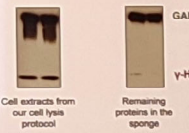
Patent from Université de Caen, P. Galéra Lab: "Procédé d'obtention *in vitro* ou *ex vivo* de chondrocytes différenciés à des fins de thérapie cellulaire du cartilage" N° 10 57883

### Lab protocol developments for radiobiology studies

#### A powerful protein extract protocol

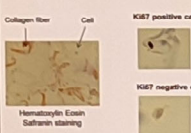
Our cell lysis protocol enables a good protein extraction efficiency even for intracellular proteins.

SW1353 cells were cultivated in 3D for 7 days. SDS-PAGE was performed on protein extracts.



#### An IHC-p protocol to evaluate cell proliferation after irradiation

SW1353 cells were cultivated in 3D for 7 days, fixed in PFA 4% then paraffin-embedded. 8 μm sections were cut with a microtome. HES staining and immunohistochemical (IHC) analysis were performed.



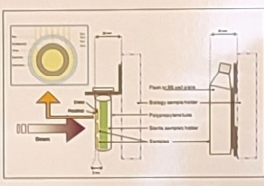
The ToxLight™ BioAssay Kit from Lonza is a bioluminescent, non-destructive cytotoxicity assay kit designed to measure the release in the medium of adenylate kinase from dying (necrotic) cells.

*In vitro* engineering of human 3D chondrosarcoma: a preclinical model relevant to hadrontherapy. Hamdi,DH et al, in preparation

### Irradiation facility and set up



Source of heavy ions: GANIL, European Heavy Ion Accelerator

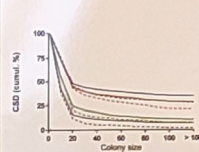


Heavy ion irradiation set up: 2D vs 3D

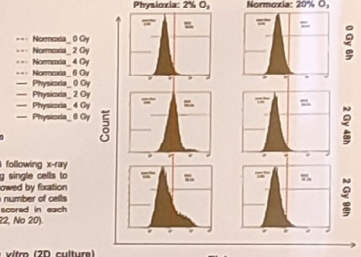
LET	Ions	Energy (MeV/n)	LET (keV/μm) Apical side	LET (keV/μm) Distal side
Intermediate	<sup>12</sup> C	75	36.7	30.4
	<sup>16</sup> O	75	36.6	30.0
High	<sup>28</sup> Si	95	85.5	82.2
	<sup>40</sup> Ar	50	96.2	122.9

LET distribution profile in the collagen sponge. (PHITS 2.64 (Particle and Heavy Ion Transport code, Japan).

### Physioxia vs Normoxic irradiation conditions



Colony size distributions (CSD) of SW1353 following x-ray irradiation. CSD was determined by allowing single cells to form colonies over the course of 11 days, followed by fixation and crystal violet staining and counting of the number of cells in individual colonies (800 colonies were scored in each condition). (Saintigny et al, MCB 2002, Vol. 22, No 20)



Oxygen level plays a key role in *In vitro* (2D culture) cell proliferation and response to X-rays:

■ After X-Rays exposure at 2 Gy, Physioxia show a significant resistance for chondrosarcoma.

■ Persistence of a stressed cell sub-population 96h post-X-ray irradiation in physioxia conditions.

### Radiation Survival Curves Characteristics for low, intermediate or high LET exposure

SW1353	LET	D <sub>01</sub> (Gy)	D <sub>02</sub> (Gy)	SF <sub>2</sub> (%)	RBE (D <sub>01</sub> )	RBE (D <sub>02</sub> )
Carbon ions	Low	6.2	3.6	64.6	-	-
	Intermediate	2.7	1.7	26.1	2.3	2.1
	High	1.0	0.5	< 1	6.3	7.6
<sup>28</sup> Ne ion	Low	0.9	0.5	< 1	6.6	7.0
	Intermediate	1.1	0.5	2.0	1.9	1.9
	High	0.5	0.2	< 1	3.9	3.9
<sup>40</sup> Ar ion	Low	0.6	0.3	< 1	3.5	3.4
	Intermediate	2.5	1.1	16.1	2.2	2.7
	High	0.6	0.3	< 1	3.5	3.4

Chondrocytes and Chondrosarcoma are 2 times more sensitive to Carbon ions compared to X-rays  
Chondrocytes and Chondrosarcoma are 4 to 6 times more sensitive to high LET ions compared to X-rays  
The first HAC donor seems to be more radiosensitive than the second one. However, RBE values of carbon ions are similar.

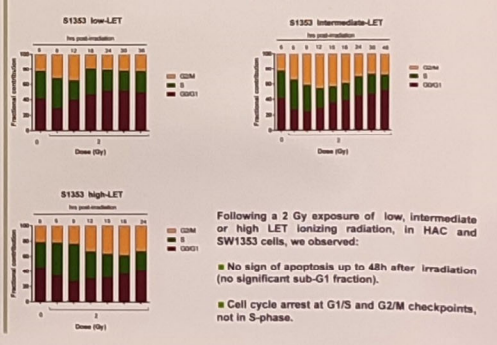
### 2D vs 3D cell viability

SW1353	Positive control	Low LET	Intermediate LET	High LET
2D	7.3	1.2	0.7	ND
3D	5.5	1.0	0.6	1.1

Colony-forming efficiency (CFE) was determined by plating 100 or 1,000 chondrocytes or chondrosarcoma irradiated cells (SW1353) in six-well plate. The cells were grown for 11 days and then fixed and stained with crystal violet prior to the determination of the fraction of cells plated that were able to form colonies of 50 cells or more.

No sign of cellular necrosis until 48h after 2 Gy irradiation with low, intermediate or high LET ionizing radiation.

### 2D cell cycle analysis



Following a 2 Gy exposure of low, intermediate or high LET ionizing radiation, in HAC and SW1353 cells, we observed:

- No sign of apoptosis up to 48h after irradiation (no significant sub-G1 fraction).
- Cell cycle arrest at G1/S and G2/M checkpoints, not in S-phase.

### γ-H2AX, p21 and p-p53 induction in 2D chondrosarcoma cells



Cells were cultivated in 2D in physioxia (2% oxygen), irradiated with 2 Gy of low, intermediate or high LET ionizing radiation at sub-confluency then harvested at the indicated time in hours. Cell extract were performed by cell disruption and lysis with 600 mM NaCl. Blot revelation was done by ECL Classico or Crescendo (Millipore).

γ-H2AX induction starts as soon as 1 hour post-irradiation. 96 hours after, it remains higher than the non-irradiated control.

p21 level increases constantly post-irradiation and reaches a plateau at 48 hours.

p53 phosphorylation starts as soon as 1 hour post-irradiation with intermediate or high LET ionizing radiation and disappears 48 hours later. Whereas, no detectable p-p53 level was observed following a low-LET irradiation with the same dose.

### Conclusions / Perspectives

- Tissue physioxia is a key factor of radiation biology studies
- 3D vertues (basa/apical organisation, metabolism, cell exchange) versus 3D vices (experimental set up, protocols and limited end-points)
- No significant difference in LET distribution profile in the collagen sponge
- RBE of intermediate and high LET ionizing radiations, in our cell types, are comparable to the literature (2 and 4-6 respectively).
- At 2 Gy, we observed no sign of necrosis nor apoptosis until 48 hours post-irradiation (low, intermediate or high LET). Cellular senescence is a privileged hypothesis.
- The first analyzed Western blot show different protein expression profiles between different LET exposures, at least in 2D chondrosarcoma cells.



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