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Variation of 4 MV X-ray dose rate in fractionated irradiation strongly impacts biological endothelial cell response *in vitro*

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ABSTRACT

Purpose: Even though X-ray beams are widely used in medical diagnosis or radiotherapy, the comparisons of their dose rates are scarce. We have recently demonstrated *in vitro* (clonogenic assay, cell viability, cell cycle, senescence) and *in vivo* (weight follow-up of animals and bordering epithelium staining of lesion), that for a single dose of irradiation, the relative biological effectiveness (RBE) deviates from 1 (up to twofold greater severe damage at the highest dose rate depending on the assay) when increasing the dose rate of high energy X-ray beams.

Material and methods: To further investigate the impact of the dose rate on RBE, in this study we performed *in vitro* fractionated irradiations by using the same two dose rates (0.63 and 2.5 Gy.min⁻¹) of high-energy X-rays (both at 4 MV) on normal endothelial cells (HUVECs). We investigated the viability/mortality, characterized radiation-induced senescence by using flow cytometry and measured gene analysis deregulations on custom arrays.

Results: The overall results enlighten that, in fractionated irradiations when varying the dose rate of high-energy X-rays, the RBE of photons deviates from 1 (up to 2.86 for viability/mortality experiments performed 21 days post-irradiation).

Conclusion: These results strengthen the interest of multiparametric analysis approaches in providing an accurate evaluation of the outcomes of irradiated cells in support of clonogenic assays, especially when such assays are not feasible.

INTRODUCTION

Dose fractionation is a powerful means of enabling healthy tissues to tolerate high doses of exposure. Numerous studies have focused on *in vitro* fractionated irradiations (Terashima et al., 2017; Koyama et al., 2018), with most of the work essentially being based on normo-fractionated schemes (daily fraction of 2 Gy) on cancerous human cells (Brodin et al., 1991; Hedman et al., 2011; van den Berg et al., 2020). Nevertheless, several studies have investigated fractionated irradiation on normal human cells (Boerma et al., 2003; Cervelli et al., 2014) with the dose per fraction usually being up to 2 Gy. But modern radiotherapy uses medical devices (mostly 6-10 MV) that are able to deliver doses up to 20 Gy.min⁻¹, with administrated doses per fraction capable of reaching 15 to 20 Gy for certain kinds of treatments (Golanov et al., 2015; Fast et al., 2019). Relative biological effectiveness (RBE) is classically defined as the ratio of the dose of one reference ionizing radiation relative to the radiation of interest in order to produce the same biological effect. From a general point of view, the ICRP reports that the RBE of X-rays is equal to 1, regardless of the dose rate energy or the energy of the beam when considering photons ranging from 0.1 to 3 MeV (Valentin, 2003). Nonetheless, a clear distinction must be made with very low-energy X-ray beams (in the range of 25-50 kV) for which higher RBE values have been reported (Slonina et al., 2003; Gomolka et al., 2005). Moreover, we recently demonstrated that when using a multiparametric RBE measurement approach, the RBE of photons deviated from 1 when varying the dose rate of high-energy X-rays (Ben Kacem et al., 2020) as well as varying the X-ray energy between 220 kV and 4 MV (Paget et al., 2019).

To take into account these medical considerations, the objective of our study was to investigate the impact of the dose-rate modification on healthy tissues when performing

fractionated irradiations. For this purpose, we performed *in vitro* fractionated irradiations on human endothelial cells (from 2 to 20 Gy per fraction) using an RBE multiparametric approach. Thus, we have chosen human umbilical vein endothelial cells (HUVECs) as biological model because i) they are human normal cells, ii) is present in the field of irradiation whatever the organ, iii) injury to the vascular endothelium is among the most common effects of radiotherapy on normal tissues and tumors (Korpela et al., 2014; Guipaud et al., 2018; Venkatesulu et al., 2018), iv) vascular permeability is known to be increased by radiotherapy (Krishnan et al., 1987; Park et al., 2016). Moreover, since endothelial cells are a key driver of late effects of radiation (Rannou et al., 2015; Toullec et al., 2018), we suppose that their behavior *in vitro* could be a good way to evaluate the effects of different irradiation modalities on normal tissues. Confluent monolayers were irradiated for all the assays (viability/mortality, senescence and gene analysis on custom arrays). We set as our reference beam of high energy X-rays (4 MV) at $0.63 \text{ Gy} \cdot \text{min}^{-1}$ on a Linear Accelerator (LINAC) Elekta Synergy Platform. Our beam of interest was set at $2.5 \text{ Gy} \cdot \text{min}^{-1}$ (4-fold) in order to strictly remain at the same energy. For both dose rates, the 4 MV irradiation used on our LINAC remains relevant to clinic as the megavoltage photon beam spectra remains very close to the ones at 6-10 MV (Sheikh-Bagheri et al., 2002). Moreover, all the justifications for using this beam as a reference instead of a Cobalt or Cesium source has been thoroughly argued in one of our previous studies (Ben Kacem et al., 2020) as well as the dose rates, which are in the range of beams used in conventional radiotherapy (Hall et al., 1991). Indeed, several dosimetric studies conducted both on water phantoms or real treatment planning conditions (Kaderka et al., 2012; Jagetic et al., 2015; Yoon et al., 2017) have shown that the lateral dose generally drops down to 10% within 1 cm from the edge field, separately from the kind of radiation and delivery technique. Therefore, in the case of a tumor targeted by a dose-rate up to $6 \text{ Gy} \cdot \text{min}^{-1}$, $0.63 \text{ Gy} \cdot \text{min}^{-1}$ seems to be a relevant dose rate exposure for organs at risks

(OARs) quite close to the planning treatment volume (PTV). For easier interpretation of the data, the different protocols were converted to biological effective dose (BED) (Jones et al., 2001), and must not be confused to biologically equivalent doses which are calculated in 2 Gy equivalents using EQD2 equation (Dale, 1985; Bentzen et al., 2012). In its simplest equation (Jones et al., 2001), BED is a measure of the true biological dose delivered integrating a particular combination of dose per fraction and a total dose to a particular tissue being characterized by a specific α/β ratio (Fowler, 2010). An α/β ratio of 3 was set in this study ($BED_{(3Gy)}$), as this value is classically ascribed to late responding normal tissues (Williams et al., 1985). The overall results obtained in this study clearly indicate that the higher dose rate ($2.5 \text{ Gy}\cdot\text{min}^{-1}$) of high energy X-rays significantly induced higher cell mortality in HUVECs than a 4-fold lower dose rate ($0.63 \text{ Gy}\cdot\text{min}^{-1}$) for $BED_{(3 \text{ Gy})} > 110 \text{ Gy}$ seven days (D7) post-irradiation, and for $BED_{(3 \text{ Gy})} > 44 \text{ Gy}$ twenty one days (D21) post-irradiation. Indeed, RBE value (ratio of viability at 0.63 vs $2.5 \text{ Gy}\cdot\text{min}^{-1}$) was found up to 1.16 (IC not including 1, [1.01; 1.33]) at D7, reaching up to 2.86 (IC not including 1, [2.13; 3.85]) at D21. Moreover, the gene expression of 44 senescence-associated genes was also significantly impacted between the two dose rates at D7 and D21 post-irradiation. Our findings clearly show *in vitro* that the RBE of 4 MV X-rays is not equal to 1 when changing the dose rate in fractionated irradiation. These results also suggest further investigation into the effect of the dose rate in connection with modern radiotherapy.

METHODS AND MATERIALS

In vitro irradiation on LINAC

An irradiation platform delivering 4 MV X-rays at 0.63 and $2.5 \text{ Gy}\cdot\text{min}^{-1}$ has previously been described (Ben Kacem et al., 2020). High-energy X-rays irradiations (4 MV X-rays) were

performed using an Elekta Synergy Platform (Elekta S.A.S. France, Boulogne, France). Irradiations were set at two dose rates (0.63 and 2.5 Gy.min⁻¹) in air kerma free in air, the uncertainty measurement was about 7% for LINAC irradiations at $k = 2$. The irradiation protocols (A to D plus A_{bis} and D_{bis}) (Supplementary Figure S1) were as follows: 1x20 Gy (A), 2x10 Gy (B), 4x5 Gy (C), 10x2 Gy (D), 3x11 Gy (A_{bis}) and 10x2 Gy (D_{bis}), with protocols D and D_{bis} differing in medium changing sequences. The corresponding BED_(3 Gy) for all these protocols are also described in Supplementary Table S1.

Cell Culture

Human umbilical vein endothelial cells (HUVECs, C2519A) from Lonza were cultured and seeded following the procedure widely described in our previous works (Paget et al., 2019; Ben Kacem et al., 2020).

Viability/Mortality (trypan blue)

At each endpoint, supernatant was collected, and the cells were trypsinised and added to the respective supernatant. Manually counting method was fully described in our previous works (Paget et al., 2019; Ben Kacem et al., 2020).

Senescence (C₁₂FDG)

Seven days after the last fraction of irradiation (Supplementary Figure S1), senescence experiments were performed by following Debacq-Chainiaux *et al.* protocol (Debacq-Chainiaux et al., 2009). Samples processing, gating strategies, use of positive and negative controls and data analysis procedures have been widely detailed in our previous works (Paget et al., 2019; Ben Kacem et al., 2020).

RT-qPCR (custom TLDA)

Seven or 21 days after the last fraction, HUVECs were harvested and samples processing was performed strictly following the same protocol as we have previously published in the literature (Paget et al., 2019; Ben Kacem et al., 2020). The TLDA list of genes has been detailed in our previous works as well (Paget et al., 2019; Ben Kacem et al., 2020), and the full list can be found in Supplementary Table S2. These 44 genes are involved in the following pathways: cell survival (BBBC3, BIRC5, FAS, ...), cell cycle (CDK1, CDKN1A, CDKN1B, CDKN2A, CDKN2D, CDKN2B, ...), cell signalisation (ACTA2, IGFBP3, IGFBP5, IGFBP7, NOTCH1, ...), inflammation (IL1B, IL6, IL8, CSF2, CSF3, ...) and tissue remodelling (MMP7, MMP10, PAI, ...). The analysis of the data was performed using the ExpressionSuite software (ThermoFisher Scientific).

Statistical Analysis

Statistical analysis

For an irradiation condition consisting on “n fractions of d Gy”, the biologically effective dose (BED) (Jones et al., 2001), based on linear quadratic cell survival model, is defined as:

$$BED = n \times d \times \left(1 + \frac{d}{\alpha/\beta} \right)$$

In this study, we converted the different fractionation conditions into a unified continuum BED formalism in order to investigate intra and inter dose-rate effects.

Moreover, for a direct comparison of the two dose rate effects, we defined the RBE for each endpoint by considering $0.63 \text{ Gy} \cdot \text{min}^{-1}$ as a reference condition:

$$\frac{RBE_{ref\ vs\ 2.5Gy.min^{-1}}}{RBE_{ref\ vs\ 0.63Gy.min^{-1}}} = \frac{\frac{X_{ref}}{X_{2.5Gy.min^{-1}}}}{\frac{X_{ref}}{X_{0.63Gy.min^{-1}}}} = \frac{X_{0.63Gy.min^{-1}}}{X_{2.5Gy.min^{-1}}} = RBE_{0.63\ Gy.min^{-1}\ vs\ 2.5Gy.min^{-1}}$$

Cell viability

Let n_j designate the number of viable cells remaining 7 or 21 days after a radiation exposure to BED_j Gy and n_0 the number of viable cells in the control sample at the same time point.

We modelled the log ratio $LR_j = \log\left(\frac{n_j}{n_0}\right)$ as a flexible function on BED_j through the regression:

$$LR_j^{7\ or\ 21\ days} = \beta_{2.5\ Gy.min^{-1}}(BED_j) + \chi_{0.63\ Gy.min^{-1}} \times \beta_{0.63\ Gy.min^{-1}\ vs\ 2.5\ Gy.min^{-1}}(BED_j) + \varepsilon_j$$

Where $\beta_{2.5\ Gy.min^{-1}}$ and $\beta_{0.63\ Gy.min^{-1}\ vs\ 2.5\ Gy.min^{-1}}$ represent two penalized B-spline functions, and $\chi_{0.63\ Gy.min^{-1}}$ a dummy variable indicating a cell irradiation under 0.63 Gy. min⁻¹ dose rates and ε_j error terms.

Thus, by considering the 0.63 Gy. min⁻¹ dose rate irradiation as a reference, the comparison in time and dose between the viable cells under the two dose rates is driven by the function

$\beta_{0.63\ Gy.min^{-1}\ vs\ 2.5\ Gy.min^{-1}}$:

$$\frac{Cell\ Viability\ 0.63\ Gy.min^{-1}}{Cell\ Viability\ 2.5\ Gy.min^{-1}} = \exp\left(\beta_{0.63\ Gy.min^{-1}\ vs\ 2.5\ Gy.min^{-1}}(BED)\right)$$

Gene expression

The measured HUVEC transcriptional profiles for each gene at each time point (after 7 and 21 days) can be viewed as a noisy discretiation of a continuous BED-dependent process:

$$-\Delta C_{T_i} = \alpha(BED_i) + \chi_{0.63\ Gy.min^{-1}} \times \beta(BED_i) + \varepsilon_i$$

where $-\Delta C_{T_i}$ represents the opposite of the measured ΔC_T at BED_i ($1 \leq i \leq n$),

$\chi_{0.63\ Gy.min^{-1}}$ is a dummy variable indicating the type of dose rate (zero for 2.5 Gy.min⁻¹

and one for $0.63 \text{ Gy}\cdot\text{min}^{-1}$), α and β represent two penalized B-spline functions and ε_i an error sampled from a zero expectation distribution. Thus, the function $\beta(BED)$ can be viewed as a log-fold change (LFC) of a gene expression profile between the two dose rates for a given BED.

Flow cytometry data analysis

The dose and dose-rate profile variations of the log-mean Beta Galactosidase antibody [FITC] intensities was modelled as a flexible spline function in dose:

$$\Delta C_i^{FITC} = \alpha^{FITC}(BED_i) + \chi_{0.63 \text{ Gy}\cdot\text{min}^{-1}} \times \beta^{FITC}(BED_i) + \varepsilon_i$$

where ΔC_i^{FITC} represents the difference between the exposed (BED_i) and the none exposed mean FITC intensities, α^{FITC} and β^{FITC} represent two penalized B-spline functions, $\chi_{0.63 \text{ Gy}\cdot\text{min}^{-1}}$ a dummy variable indicating the type of dose rate ($0.63 \text{ Gy}\cdot\text{min}^{-1}$ or $2.5 \text{ Gy}\cdot\text{min}^{-1}$) and ε_i is an error sampled from a zero expectation distribution. Thus, for a given BED, the function $\beta^{FITC}(BED)$ captures the rate-dose modulation of the FITC intensities.

RESULTS

Cell viability

At D7 post-irradiation, cell viability counting using the trypan blue method (Figure 1.A) showed no significant differences between protocol A ($BED_{(3\text{Gy})} = 154$) and all the other protocols at $0.63 \text{ Gy}\cdot\text{min}^{-1}$, although a slight decreasing trend of cell viability could be observed when BED was increasing. On the other hand, at D7 post irradiation, a significant decrease of cell viability was observed at $2.5 \text{ Gy}\cdot\text{min}^{-1}$ when $BED_{(3\text{Gy})}$ was increasing. A statistical representation of $(0.63 \text{ Gy}\cdot\text{min}^{-1})/(2.5 \text{ Gy}\cdot\text{min}^{-1})$ cell viability ratios and associated

statistical results is shown in Figure 1A (lower panel), for which the cell viability was significantly higher at the lowest dose rate for $BED_{(3Gy)}$ above 115 Gy. Supplementary Table S3 reports calculated viability RBE values at D7, ranging up to 1.45 (IC not including 1, [1.17; 1.80]). In terms of protocols having the same $BED_{(3Gy)}$, no significant difference in cell viability was observed for either of the dose rates at D7 (Supplementary Figure S2.A.)

At D21 post-irradiation, cell viability counting using the trypan blue method (Figure 1.B) only showed differences between protocol A ($BED_{(3Gy)} = 154$) and protocol C ($BED_{(3Gy)}=53$) at $0.63 \text{ Gy}\cdot\text{min}^{-1}$, although a slight decreasing trend in cell viability could be observed when BED was increasing. On the other hand, at D21 post irradiation, a significant decrease in cell viability was observed at $2.5 \text{ Gy}\cdot\text{min}^{-1}$ when $BED_{(3Gy)}$ was increasing. A statistical representation of $(0.63 \text{ Gy}\cdot\text{min}^{-1})/(2.5 \text{ Gy}\cdot\text{min}^{-1})$ cell viability ratios and associated statistical results is shown in Figure 1B (lower panel), for which the cell viability was significantly higher at the lowest dose rate for $BED_{(3Gy)}$ above 44 Gy. Viability RBE values at D21 were calculated and reported in Supplementary Table S4, ranging up to 2.86 (IC not including 1, [2.13; 3.85]). In terms of protocols having the same $BED_{(3Gy)}$, no significant difference in cell viability was observed for either of the dose rates at D21 (Supplementary Figure S2.B.), except between protocols A and A_{bis} at the highest dose rate ($2.5 \text{ Gy}\cdot\text{min}^{-1}$).

Senescence

At D7 post-irradiation, senescence was measured by $C_{12}FDG$ for both dose rates. We found significant differences between the two dose rates for $BED_{(3Gy)} > 150 \text{ Gy}$ (protocol A) (Figure 2). The senescence RBE values were calculated for the whole BED continuum and are reported in Supplementary Table S5, ranging up to 0.49 (IC not including 1, [0.26; 0.91]). For protocols having equal $BED_{(3Gy)}$, a significant difference was observed only at the lower dose rate $0.63 \text{ Gy}\cdot\text{min}^{-1}$ (Supplementary Figure S2) between protocols A and A_{bis}

(BED_(3Gy) = 154 Gy), while a significant difference was observed for both dose rates between protocols D and D_{bis} (BED_(3Gy) = 33 Gy) (Supplementary Figure S2).

Senescence-associated gene expression clustering

Based on data obtained from RT-qPCR gene expression measurements of 44 senescence-associated genes (customized Taqman Low-Density Assay (TLDA)) (Supplementary Table S2), an unsupervised gene expression hierarchic clustering was established. An Excel datasheet hosting all the CT values used for the modelling is provided as Supplementary File. For each end-point (D7 and D21), the clustering was performed according to the similarity strength in terms of shape and significance domain of the delta fold changes viewed as functions (curves) of BED_(3Gy). At D7 post-irradiation, six clusters of expression were obtained taking into account the 36 statistically differentially expressed genes according to the dose rate (Figure 3.A). At D21 post-irradiation, significantly differentially expressions according to the dose rate were found for 30 genes hosted on 7 different clusters (Figure 3.B).

DISCUSSION

RBE measurements are commonly based on clonogenic assays (Puck et al., 1956), which remains a widely common tool to evaluate the radiosensitivity/resistance of cells (Dunne et al., 2003; Matsui et al., 2019), more specifically in the case of cancerous cells. Nevertheless, the clonogenic assay, considered as the gold standard, was not performed in the present study as HUVECs, which are normal human cells, are very sensitive to irradiation. Although HUVECs are able to form colonies (Helm et al., 2016; Paget et al., 2019; Ben Kacem et al., 2020), it has been widely described in literature that the survival fraction of HUVECs after irradiation to doses above 4 Gy is close to 0 (Helm et al., 2016; Dos Santos et al., 2018; Paget et al., 2019; Ben Kacem et al., 2020). Furthermore, several authors also

support the fact that the linear quadratic modelling used in clonogenic assay is inappropriate for high dose per fraction (Kirkpatrick et al., 2008; Kirkpatrick et al., 2009). Therefore, based on this point, and the range of doses used in the design of our fractionation protocols, the application of a multiparametric biological assay appears particularly appropriate. Moreover, such methods and approaches have been previously validated on HUVECs for single doses of irradiation comparing X rays from different energies (Paget et al., 2019) and dose rates variation (Ben Kacem et al., 2020). Finally, irradiation at low dose rates can be concomitant to DNA damage repair and apoptosis mechanisms at molecular and cellular levels. Thus, to minimize these facts, a “30-minute limit” of irradiation was applied in our experimental design (corresponding to the 20 Gy irradiation at $0.63 \text{ Gy} \cdot \text{min}^{-1}$). Indeed, the climax of γ -H2AX foci occurs 30 minutes after irradiation (or stress) as widely described in the literature and is extensively used to monitor DNA double-strand breaks and their repair (Redon et al., 2009; Mariotti et al., 2013; Sharma et al., 2015). Moreover, γ -H2AX foci is a very sensitive method for the measurement of DNA double-strand breaks (Redon et al., 2011), even for low doses (5 to 50 cG) (Jakl et al., 2020), but it is also a method predictive of *in vivo* genotoxicity (Tsamou et al., 2012). Also, a recent meta-analysis of DNA double-strand break response kinetics (Kochan et al., 2017) has shown that the accumulation of most proteins (among 79 double-strand break repair proteins at sites of DNA damage) starts immediately after damage induction, continues in parallel and peaks within 15-20 minutes.

Fractionated irradiation protocols can be quite complex to design *in vitro*. Indeed, they must take into account i) the radiosensitivity of the cells, ii) the cell culture duration, which can reach several weeks (especially in the case of normal cells to avoid, for example, a non-negligible part of replicative senescence within cell populations), and iii) the delay between each fraction of irradiation. Furthermore, it has been well described that a prolonged delivery time decreases the radiobiological effects for cancerous cells (Ogino et al., 2005; Jiang et al.,

2013; Kim et al., 2014) and several studies have also focused on the estimation of the cell response in fractionation radiotherapy using different methods derived from a linear quadratic model (Nikzad et al., 2015). Nevertheless, most of this work was focused on cancerous cells and did not take into account the case of high doses per fractions. To evaluate *in vitro* the impact of the dose rate on fractionation protocols with high dose per fraction, we performed our experiments on HUVECs monolayers synchronised by confluence prior to irradiation (Paget et al., 2019; Ben Kacem et al., 2020), hosting at least 70-80% of the cells in the G1 phase. This step was a prerequisite to properly compare the biological outcomes, as the cell cycle phase can strongly affect the results after irradiation and more or less drives radiosensitivity of cells (Yau et al., 1980; Pawlik et al., 2004; Mori et al., 2018). Terashima *et al.* previously performed fractionated irradiation at various dose rates (1, 1.5 and 2 Gy.min⁻¹) (Terashima et al., 2017). Even though their work was performed i) on cancerous cells (instead of normal human cells in the present study), ii) at a lower total dose (2 Gy instead of up to 20 Gy in the present study), and iii) had a very short delay between each fraction (1 min instead of at least one day in this study), Terashima *et al.* found that the higher dose rate induced a higher mortality (Terashima et al., 2017), which fully corroborates with our data obtained on HUVECs in the present study. However, no significant effects on viability and apoptosis were reported for HUVECs exposed to low doses (up to 0.5 Gy) of fractionated irradiation (Cervelli et al., 2014). This could be explained by the gap between the total doses studied within the two works (40-fold higher in the present work) but also strongly linked to end-point timing (D7 and D21 post-irradiation in our study, as opposed to up to 72 h post-irradiation for Cervelli *et al.* (Cervelli et al., 2014)). Indeed, when focusing on RBE calculations, we found for protocol B (2x10 Gy) an RBE $(0.63/2.5 \text{ Gy.min}^{-1}) = 1.04$ (not significant, IC including 1, [0.90; 1.20]) and 1.17 (significant, IC not including 1, [1.02; 1.35]) at D7 and D21, respectively (Supplementary Table S3 and S4). From a general point of

view, the differences linked to the dose rate can be explained by a higher induction of cytotoxic effects (i.e. cell mortality, apoptosis, cell cycle arrest, etc.) when the dose rate increases. Indeed, the literature has described cases of hypersensitivity to fractionated low-dose radiation exposure such that an increase of cytotoxic effects is observed when increasing the dose rate on cancerous cells (Smith et al., 1999; Dey et al., 2003; Gupta et al., 2011; Terashima et al., 2017). Moreover, we previously published that an increase in the dose rate leads to, after a single dose irradiation on HUVECs, an increase in mortality and senescent cells (Ben Kacem et al., 2020). Thus, instead of the widely used biomarker X-GAL, we also performed staining with $C_{12}FDG$ to verify a possible impact of the dose rate on radiation-induced senescence *in vitro* on HUVECs after fractionated irradiations. Unfortunately, by using this assay we were not able to identify any significant differences according to the dose rate except for $BED_{(3Gy)} > 150$ Gy. Thus, we found a $RBE (0.63/2.5 \text{ Gy} \cdot \text{min}^{-1}) = 0.5$ at $BED_{(3Gy)} = 154$ Gy, for which the data are very close to those obtained in one of our previous studies for the same exposure (Ben Kacem et al., 2020). Nevertheless, while we were able to identify, at D7 post-irradiation, significant differences according to the dose rate for doses above 2.4 Gy (Ben Kacem et al., 2020), it is clear that such an approach ultimately does not work *in vitro* for long-term fractionated protocols, even though the cytometric method measuring senescence associated-beta-galactosidase activity has been described as superior in discriminating between degrees of senescence in different populations of fibroblasts (Noppe et al., 2009). Moreover, it has been reported in the literature that i) cell contact can accelerate replicative senescence (Munro et al., 2001; Ho et al., 2011), ii) senescence can be induced by long-term cultivation (Chow et al., 1996; Fridlyanskaya et al., 2015), and iii) long-term quiescent fibroblast cells can transit into senescence (Marthandan et al., 2014). Thus, this could partly explain why we were not able to find any significant differences according to the dose rate for protocols with long-term cultures. To support these facts, when comparing the

two same long-term protocols (protocols D and D_{bis}, both 10 x 2 Gy), differing only with respect to medium changing schedule, (Supplementary Figure S1), we found significant differences for both dose rates in senescent positive cells (Supplementary Figure S2), demonstrating that cell culture medium changing schedules can strongly impact the results. Indeed, at 0.63 Gy.min⁻¹, the delta of Log₁₀ FITC (control vs irradiated) was reduced by approximately 80% between D vs D_{bis}, while it was reduced by approximately 73% at 2.5 Gy.min⁻¹ between protocols D vs D_{bis}, showing that the variation is the same regardless of the dose rate. This clearly states that the baseline rate was impacted by the cell culture conditions. Moreover, when looking at the RBE (0.63/2.5 Gy.min⁻¹), we found a value of 0.85 but that was not significant (IC including 1, [0.2927; 1.8433]). These facts combined suggest that the C₁₂FDG approach can be affected by long-term cultures, such as in the case of fractionated protocols. This also strengthens the prerequisite of an own control for each irradiation scheme to properly interpret the data and to potentially identify a reduction in sensitivity of this assay.

In order to bypass this lock of the senescence measurement by flow cytometry, we decided to further investigate the impact of the dose rate on senescence by gene deregulation analysis. From the selected custom arrays, our statistical analysis sorted 36 and 30 genes statistically differentially expressed according to the dose rate at D7 and D21 post-irradiation, respectively (Figure 3.A and B., respectively). From a general standpoint, this fact highlights a dose rate effect during fractionated irradiation. Interestingly, Boerma *et al.* have reported that fractionated irradiation leads to minor changes in the expression of specific cytokines endothelial cells but also in cardiac myocytes and fibroblasts as well (Boerma et al., 2003). Moreover, contrary to the classic unsupervised analysis of gene deregulation (Paget et al., 2019), our modelling approach (refer to the section of statistical analysis) enables sole detection of the significant differences between the two dose rates according to a range of

BED_(3Gy). Such modelling could be a useful tool in the future in identifying key genes in cellular responses impacted by varying dose rates or, to a lesser extent, when comparing two qualities of irradiation.

CONCLUSION

By showing a clear effect of the dose rate in several fractionated protocols *in vitro*, our study emphasises that the approach of our multiparametric radiobiological assay can be a robust and sensitive method to evaluate the cellular response, especially in the case of normal cells at very high dose of irradiation. This study also clearly raises the question of the importance of the dose rate in fractionated protocols in radiotherapy when using high energy X rays, even though our findings should be confirmed by *in vivo* experiments. For example, compared to increased conventional flattened outputs, the use of flattening filter free (FFF) technology instantaneously increases the dose-rate of X-ray pulses by a factor up to 6 (Georg et al., 2011; Feofanova et al., 2014). Interestingly, recent work carried out by Laurent *et al.* showed no influence of a high dose rate in FFF technique on the anti-tumour immune response in a CT26 murine colorectal tumour model (Laurent et al., 2020). Nevertheless, this study also raises the question about what could have happened in the normal tissues, for which further experiments are needed.

Author Contributions

M.B.K., M.D.S., V.B., G.T. and V.P.: Acquisition, analyses and interpretation of data. M.A.B.: statistical analyses, help in writing the manuscript. B.L.G, A.F, O.G. and F.M.: Critical review of the manuscript. V.P.: Writing the manuscript. V.P. and F.M.: Conception, design and supervising the project.

Disclosure statement

The authors have read the journal's policy and have no competing interests. B.L.G. is employee at Electricité de France, Cap Ampère, Saint-Denis, France. This work was supported by Electricité de France EDF (Groupe Gestion Projet Radioprotection). This does not alter the authors' adherence to all the Radiation Research policies on sharing data and materials.

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FIGURE LEGENDS

Figure 1. Cell survival at 0.63 and 2.5 Gy.min⁻¹ at D7 and D21 post-irradiation. (A) Upper panels: cell survival D7 post-irradiation of HUVECs irradiated at 0.63 Gy.min⁻¹ (pink curve) and 2.5 Gy.min⁻¹ (blue curve). Lower panel: the associated RBE curve (0.63 Gy.min⁻¹/2.5 Gy.min⁻¹) is defined as the ratio of cell viability for a given BED_(3Gy) (thick black line) and its associated bootstrap confidence intervals (two fine black lines). The green arrow represents the range of BED_(3Gy) wherein the value of RBE is significantly different from 1 (p<0.0001). (B) Upper panels: cell survival D21 post-irradiation of HUVECs irradiated at 0.63 Gy.min⁻¹ (pink curve) and 2.5 Gy.min⁻¹ (blue curve). Lower panel: the associated RBE curve is defined as a ratio of cell viability for a given BED_(3Gy) (thick black line) and its associated bootstrap confidence intervals (two fine black lines). The green arrow represents the range of BED_(3Gy) wherein the value of RBE is significantly deviates from 1 (p<0.0001). Each curve represents the mean of three independent experiments.

Figure 2. Senescence (C₁₂FDG) (Flow Cytometry) analysis A) Example of flow cytometry measurements obtained at D7 post-irradiation for negative control, control and 20 Gy

irradiations at $0.63 \text{ Gy}\cdot\text{min}^{-1}$ and $2.5 \text{ Gy}\cdot\text{min}^{-1}$. Each bi-parametric representation (Size (FSC)/C₁₂FDG (FITC)) represents one independent experiment for at least 5×10^4 living cells. (B) Each curve (purple and blue for 0.63 and $2.5 \text{ Gy}\cdot\text{min}^{-1}$, respectively) represents the delta of Log_{10} FITC compared to its own control conditions for the corresponding fractionation protocol. Each curve represents the mean of four independent experiments based on at least 5×10^4 living cells for each experiment. (C) The curve represents the delta log_{10} FITC (0.63 - $2.5 \text{ Gy}\cdot\text{min}^{-1}$) as a function of the $\text{BED}_{(3\text{Gy})}$. The green arrow represents the range of $\text{BED}_{(3\text{Gy})}$ where this delta is significant between the two dose rates (from 150 to 154 Gy).

Figure 3. RT-qPCR gene expression clustering. (A) Gene clusters at D7 post-irradiation, with each curve representing the delta of fold changes for one gene (0.63 - $2.5 \text{ Gy}\cdot\text{min}^{-1}$). The genes were grouped into six clusters according to their expression tendencies as a function of the $\text{BED}_{(3\text{Gy})}$. Only the significantly differentially expressed genes in the delta fold change (36 among the 44 measured) are represented. (B) Gene clusters at D21 post-irradiation. The genes were grouped into seven clusters according to their expression tendencies as a function of the $\text{BED}_{(3\text{Gy})}$. Only the significantly differentially expressed genes in the delta fold change (30 among the 44 measured) are represented.

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