Different dose rate-dependent responses of human melanoma cells and fibroblasts to low dose fast neutrons

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DIFFERENT DOSE-RATE DEPENDENT RESPONSES OF HUMAN MELANOMA CELLS AND FIBROBLASTS TO LOW-DOSE FAST NEUTRONS

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Abstract

Purpose: To analyse the dose-rate influence in hyper-radiosensitivity (HRS) of human melanoma cells to very low doses of fast neutrons and to compare to the behaviour of normal human skin fibroblasts.

Materials and methods: We explored different neutron dose-rates as well as possible implication of DNA Double-Strand-Breaks (DSB), apoptosis, and energy-provider adenosine-triphosphate (ATP) levels during HRS.

Results: HRS in melanoma cells appears only at a very low dose-rate (VLDR), while a high dose-rate (HDR) induces an initial cell-radio-resistance (ICRR). HRS does not seem to be due either to DSB or to apoptosis. Both phenomena (HRS, ICRR) appear to be related to ATP availability for triggering cell repair. Fibroblast survival after neutron irradiation is also dose-rate dependent but without HRS.

Conclusions: Melanoma cells or fibroblasts exert their own survival behaviour at very low doses of neutrons, suggesting that in some cases there is a differential between cancer and normal cells radiation responses. Only the survival of fibroblasts at HDR fits the Linear-No-Threshold model. This new insight into human cell responses to very low doses of neutrons, concerns natural radiations, surroundings of accelerators, proton-therapy devices, flights at high altitude. Furthermore, ATP inhibitors could increase HRS during high-Linear Energy Transfer (high-LET) irradiation.

Introduction

In human melanoma cells, exposure to fast 14 MeV neutrons induces a hyper-radiosensitivity (HRS) at a very low dose of 50 milliGrays (mGy) when delivered at a very low dose rate (VLDR) of 0.8 mGy/min (Dionet et al. 2000). At doses between 100 mGy and 350 mGy HRS is followed by increased cell survival. HRS is, however, not found at a higher dose rate: it was suggested that the repair machinery was not activated below a threshold dose (Averbeck 2009). HRS appears to be common to several tumour cell types although its exact nature remains unknown (Averbeck 2010), despite recoiled protons are able to give rise to sublethal damages (Alard et al. 2002). In order to find out whether this also holds for neutrons of higher energy we undertook experiments at the 65 MeV facility of the “Université Catholique de Louvain”, UCL (Belgium). HRS was first described with X-Rays (Lambin et al. 1993), then in our study with neutrons (Dionet et al. 2000), and later in studies with protons, alpha particles (Heuskin et al. 2014) and heavy ions (Bohrnsen et al. 2002). In addition, in the same dose range on melanoma cells, but at a higher dose rate, the existence of an initial cell radio-resistance (ICRR) above the survival of controls was suspected. This phenomenon referred to by some authors as “radiation hormesis” on healthy cells (Averbeck 2010) has not been related so far to irradiation dose rate. The present study aimed at understanding how the same low dose of neutrons can have opposite effects on the survival of a cancer cell line as a function of dose-rate. We also investigated if HRS and ICRR could be found in normal fibroblasts. We analysed the involvement of the repair of DNA double-strand-breaks (DSB) and apoptosis. To explain the HRS of melanoma cells at VLDR we assumed that the signal of damage induction was not

transmitted to the repair machinery. Since cell energy is the prime factor in repair process we analysed the energy supply and changes in adenosine-triphosphate (ATP) and adenosine-diphosphate (ADP) levels, the defect of which could be due to a mitochondria impairment.

Materials and Methods

Cell culture

All experiments were done with human melanoma cell line M4 Beu (provided by Institut National de la Santé et de la Recherche: Unité Mixte de Recherche 990, France) (Dionet et al. 2000) (survival fraction at 2 Grays (SF2) of 6MV X-Rays at 1 Gy/min is 35%). Cells were cultured in Minimum Essential Medium supplemented with antibiotics (0.01%), non-essential amino acids (1%), foetal bovine serum (FBS, 10%).

Normal skin fibroblasts K464 (SF2 for 6MV X-Rays at 1 Gy/min is 15.5%) were obtained from a healthy 30-year-old female donor, by surgical skin biopsy (provided by Medical Cytogenetic Laboratory University of Clermont-Ferrand France). Primo-cultures were carried out in Dubelcco's modified Eagle media supplemented with 10% of FBS, 1% of nonessential amino acid, and 1% of pyruvate of sodium and gentamicin.

Melanoma M4 Beu cells were choosen because they are resistant to XRay irradiation used in human therapy. Fibroblasts were chosen to compare the behaviour of cancer and normal cells. Furthermore, in human oncology, this two types of cell lines are neighbours in the skin.

During our experiments K464 were cultured in RPMI-1640 medium (Roswell Park Memorial Institute) supplemented with antibiotics (0.1%), non-essential amino acids (1%), sodium pyruvate (1%), sodium bicarbonate (2%), Hepes (2%) (Hydroxyethyl piperazine ethane sulfonic acid) and FBS (10%). All cell lines were cultured in a humidified incubator at 5% CO2 and 37°C. All culture reagents were purchased from Fischer Scientific (Illkirch, France).

Cell survival

Plating Efficiency (PE)

Melanoma cells were seeded in 25 cm^2 flasks at a number of 500/flask resulting in an easy counted number of colonies in the non-irradiated flasks. Flasks were irradiated 36 hours after cell seeding. After irradiation the medium was changed, and the flasks, filled up with 5 ml of medium, were incubated for 14 days before counting colonies of at least 50 cells. After initial seeding, cells were never trypsinised.

Non-transformed fibroblasts K464 were unable to give rise to colonies unless they were plated on feeder layer: the autofeeder layer was prepared with fibroblasts irradiated with 20 Gy by a 6 MV X-Ray accelerator. The best PE was obtained by seeding 750 cells per flask on a feeder layer of 2 $x10^3$ feeder cells per cm^2.

Cell proliferation assessment

Proliferation curves were performed to determine the doubling time of each cell line. M4Beu cells, after a 24 hours attachment time, had a doubling time of 24 hours. K464 cells had a doubling time of 40 hours. Irradiation being performed 36 hours after cell seeding, it takes place, for the two cell lines, prior to the end of the first cell population doubling time.

Clonogenic cell survival assays

We established the survival curves for the two cell lines after 6 MV X-rays irradiation at a usual therapeutic dose rate of 1 Gy/min, for comparison between the two radiation modalities.

Each experiment was repeated on 3 separate days in triplicate for each dose (2 different days for normal cells). Survival curves were obtained by combining data from 3 independent experiments.

We verified that the PE in controls was not affected by the irradiation duration : no variation of the PE was noted if control flasks at room temperature (experimental conditions). The PE of M4Beu was 61%, 61%, 58%, 61% at 0, 2h, 3h, 5h, respectively.

Irradiation

Type of neutrons

Fast neutrons were produced at the cyclotron CYCLONE, in Louvain-la-Neuve, by bombarding a 19.6 mm thick Be target with 65 MeV protons [p(65)+Be]. The neutron spectrum has a maximum at 33 MeV and a highest energy of 63.8 MeV. The VLDR was 1.08 mGy/min (± 10%) and the high dose rate (HDR) was 83 mGy/min (±1%). Moderate intermediate dose rates were 7.5 mGy/min (MDR), and 15 mGy/min.

Dosimetry

The dosimetry of irradiation chamber was performed before each experiment (ionization chambers). The cell layer was positioned at the depth of the “build-up” of the beam.

Immunofluorescence analysis

Melanoma cells and fibroblasts were seeded in 9 cm^2 flasks at 150 000 cells.

Thirty six hours after plating, cells were irradiated. Forty-five minutes and 24h after irradiation, cells were fixed with paraformaldehyde 4% and permeabilized with 0.5% Triton X-100 and SAB (Serum Albumin Bovin, Sigma-Aldrich, Saint-Quentin Fallavier, France) 5%. Gamma-H2AX monoclonal antibody (clone JBW301, Millipore, Molsheim,

France) at 1/250 and rabbit anti caspase-3 active (Sigma-Aldrich, C8487) at 1/500 were used in PBS(Phosphate Buffered Saline)/SAB 0.5% solution and incubated overnight at 4°C. Then, cells were saturated with BSA 5%, incubated with FITC (Fluorescein Iso-Thio-Cyanate) mouse antibody (1/250, Invitrogen, A21022, Carlsbad, CA, USA) and Cy3 rabbit antibody (Interchim, Montluçon, France) 1h at 4°C and subjected to image analysis (2 independent experiments). All images were acquired with the fluorescence microscope Leïca SPE using a 40X/1.15 oil immersion objective and analysed using FIDJI software.

ATP and ADP levels

The ATP and ADP levels were determined by bioluminescence with the ApoSensor assay kit (Biovision, Milpitas, CA, US) based on the utilization of luciferase to catalyse the formation of light from ATP and luciferin. ADP levels were determined by its conversion to ATP and the same detection method. Cells were plated 36 hours before irradiation in a 96 well plate. Measures were always performed 10 min after irradiation, with a microplate fluorometer (FluorosKan Ascent FL 2.5, Thermo Fischer Scientific, Waltham, MA, US), in 5 different wells. Optimal cell numbers 5000 cells/well for cancer cells, 750 cells/well for fibroblasts were seeded to overcome background noise. Cells were irradiated in exponential growing phase. ADP and ATP levels were reported (2 independent experiments) in comparison to the level of the control (non-irradiated cells).

Statistical analyses

The survival curves were fitted to the Linear Quadratic model according to the software Kaleidagraph 4.0, and the parameters alpha and beta of the linear quadratic equation were determined ($\log S = -(\alpha d + \beta d^2)$ where log S = survival logarithm and d= radiation dose).

Statistical analysis was performed using Stata 13 software (StataCorp LP, College Station, TX, US). The tests were two-sided, with a Type 1 error set at $\alpha = 0.05$. Random-effects models were used to analyse correlated data 1) by studying fixed effects as measurements of relative ATP and ADP levels, intermediate dose rates and their interaction and 2) taking into account between and within each experiment or replicat variability. On the graphs bars are standard error of the mean (SEM).

Results

HRS occurred in cancer cells at VLDR

HRS was found with 65 MeV neutrons (Figure 1) as previously shown with 14 MeV neutrons (Dionet et al. 2000), despite the large difference between the two neutron spectra: a narrow peak for 14 MeV neutrons, a broad peak for 65 MeV neutrons (average energy 33 MeV and highest energy 63.8 MeV). The survival of melanoma cells after irradiation by low doses of 65 MeV neutrons (pool of three independent experiments) delivered at VLDR or at HDR is shown in Figure 1a: the two survival curves are significantly different (p= 0.047). At each dose evaluation, between the two dose rates, the survival rates are significantly different at 100 mGy and 150 mGy p value being 0.04 and 0.002, respectively. At 150 mGy, the survival rate at VLDR (HRS) is significantly different from control and from the survival rate at HDR with p= 0.001 and p= 0.002 respectively. The cell survival increases at 225 mGy being not different from the control one (p= 0.09). At HDR, survival rates are not statistically different from those of control: at 50, 100 mGy, 150 mGy, p being 0.052, 0.38, 0.17 respectively. At 100 mGy the ICRR is maximal with a cell survival rate of 106% ± 6%. The survival curve shows a HRS at VLDR, and doesn't fit a Linear quadratic (LQ) curve. At HDR the curve fits the LQ curve $\log S = -(\alpha d + \beta d^2)$ in which $\alpha = -0.45$, $\beta = 8.32$.

Normal fibroblasts (pool of two independent experiments) (Figure 1b): At VLDR, there is no significant difference from the control: at 50, 100, 150 mGy, p being 0.11, 0.88, 0.13 respectively. At 250 mGy the survival is significantly different from the control one (p=0.001). At VLDR LQ parameters are $\alpha = 0.33$, $\beta = 3.66$.

At HDR, the difference is significant from the control for 100, 150, 250 mGy, p being 0.008, 0.001, 0.001 respectively. So, neither HRS nor clear ICRR is found.

At HDR $\alpha = 2.04$, $\beta = 2.04$, so α/β being equal to 1, at very low doses $\log S \approx -\alpha d$. In this case the survival curve is close to a linear curve.

Fibroblast survival curves are statistically different from those of melanoma cells: at HDR, between the 2 curves, difference p= 0.01; at 100, 150 mGy, p= 0.017 and 0.004 respectively. At VLDR, at 150 mGy p= 0.009. Normal fibroblasts irradiated at VLDR are able to repair most lesions till 150 mGy, suggesting a threshold for this cell type (Figure 1b). On the contrary, cell survival at HDR decreases proportionally with increasing doses up to a significant level (56 %) at 150 mGy, showing no threshold.

The survivals of the two cell lines irradiated with 6 MV X-Rays at a dose-rate of 1 Gy/min (expressed in Grays) are reported in Figure 1c, to show the large differences between the effectiveness of the two radiation modalities (K464: $\alpha = 0.67$, $\beta = 0.13$; M4Beu : $\alpha = -0.06$, $\beta = 0.29$).

DSB are not increased during HRS

The amount of DSB after neutron irradiation in increasing doses is reported in Figure 2. In melanoma cells, pool of two independent experiments (a, b). After 50mGy irradiation, at 45 min there is a statistical difference between foci averages at VLDR and at HDR p= 0.001. At 24 hours there is no significant difference between HDR and VLDR ($p = 0.75$), suggesting that HRS is not due to an increase of unrepaired DSB.

In normal fibroblasts (pool of two independent experiments) (c, d): At 45 min and 24 h after irradiation there is no statistical difference between HDR and VLDR.

Apoptosis is not increased during HRS

The assessment of 24h post-irradiation apoptosis is described in Figure 3. In melanoma cells, pool of two independent experiments (Figure 3a) there are no significantly different responses between VLDR and HDR

In fibroblasts (pool of 2 independent experiments) (Figure 3b) there are no significantly different responses neither between VLDR and HDR nor when compared to melanoma cells.

ATP and ADP decrease during HRS

The 10 min post-irradiation ATP levels are presented in Figure 4, and ADP levels in Figure 5, as a function of 65 MeV neutron doses.

In melanoma cells (Figure 4a), ATP levels in cells irradiated at VLDR are significantly decreased, as compared with non-irradiated cells (100%), to 49%, 28%, 33% at 50, 100 and 150 mGy, respectively ($p < 0.003$). In particular, the ATP amount starts to decrease at 50 mGy and reaches a minimum at 100 mGy, the dose corresponding to HRS. At 150 mGy, the amount is still quite low, when HRS is maximal, and not statistically different than that at 100 mGy. At HDR, ATP levels are also significantly decreased after irradiation with 50, 100 and 150 mGy to 29%, 52%, 27.5%, respectively ($p < 0.01$), as compared with non-irradiated controls. The increase of ATP at 100 mGy is concomitant with the increase in cell survival, when ICRR takes place. This ATP level is significantly different from those at 50 mGy ($p = 0.008$) and at 150 mGy ($p = 0.014$). The ATP level at 50 mGy is significantly lower at HDR than at VLDR

(p= 0.04). At 100 mGy, it is higher at HDR than at VLDR (p= 0.008). At 150 mGy the levels at HDR and VLDR are not statistically different p= (0.47, NS).

Regarding ADP (Figure 5a), the level at VLDR (45%) is significantly lower than the control one (100%) at 100 mGy, when HRS begins (p = 0.002). At 150 mGy, the ADP amount increases to 70% (p= 0.03), when HRS is maximal. At HDR, ADP levels are lower, as compared with non-irradiated cells: at 50,100 and 150 mGy these levels decreased to 50%, 55%, 25%, respectively. The ADP level at 150 mGy is significantly lower at HDR than at VLDR (p= 0.0025).

In melanoma cells, during HRS at VLDR, we found an ATP decrease at 100 and 150 mGy, concomitant with an ADP increase at 150 mGy when HRS occurs.

In normal fibroblasts (Figure 4b) the ATP level at VLDR, decreases significantly in irradiated cells at 50 mGy (p = 0.012) and is also slightly (non-significantly) diminished at 100 mGy (84%) and 150 mGy (73%). In contrast, the ATP level at HDR is increased at 50 mGy (168%, p = 0.015) and 100 mGy (169%, p = 0.09, NS) and decreases at 150 mGy to 68% (p = 0.01).

The ADP level at VLDR (Figure 5b) is non-significantly decreased at 50 mGy (84%, NS), but this decrease becomes highly significant at 100 mGy (37%, p = 0.0001). At HDR, the ADP level decreases at 50 mGy (58%, p = 0.01) and then increases to 133% at 100 mGy, but this increase is not significant (p = 0.12).

Of note, ATP and ADP levels in absolute values are much lower in fibroblasts than in melanoma cells. Mean ATP level (arbitrary units) was 105 In control fibroblasts

vs 842 in melanoma cells, and corresponding mean ADP levels were 137 vs 323 ($p < 0.003$ for both ATP and ADP).

Intermediate dose rates between HDR and VLDR

The effect of intermediate dose rates were also studied on M4Beu (Figure 6). At a moderate dose rate (MDR) of 7.5 mGy/min, between the HDR (when the ICRR takes place) and the VLDR (when the HRS takes place), we note the absence of ICRR and of HRS. At 100 mGy the survival rates at MDR and HDR are 96,7±6,5 and 105,5±6,4 respectively. At 150 mGy the survival rates at MDR and VLDR are 88,2±6,5 and 61,3±8,7 respectively. A second intermediate dose rate of 15 mGy/min was analysed: the survival curve (not shown) is not different from the HDR one.

HRS is obvious only at the VLDR of 1 mGy/min and maximum at 150 mGy. At MDR (7.5 mGy/min), there is no statistically significant ICRR or HRS. This dose rate is regarded as the intermediate dose rate when a switch occurs between dose rates leading to ICRR and the one leading to HRS. More accurate dose rates, between 1 and 7.5 mGy/min, could be explored to improve the result.

Discussion

We found that human melanoma cells show HRS after irradiation with low doses of neutrons at a very low dose rate. The range value of cell survival nadirs (HRS), 50 and 150 mGy, for 14 and 65 MeV neutrons, respectively, depends on several parameters: the relative biological effectiveness (Gueulette et al. 1996), the spectrum shape and experimental features. Furthermore HRS has been reported with several types of high Linear Energy Transfer (LET) particles (Heuskin et al. 2014) and heavy ions (Bohrnsen et al. 2002).

Survival curves obtained give a new insight into “Linear-No-Threshold” model (LNT). The LNT concept is the basis of international rules of radioprotection (Monson 2006). It assumes that from the smallest radiation dose, the radiation-induced lesion frequency proportionally increases with increasing doses.

So, the survival curve of this cancer cell line does not fit the LNT model in this dose range for the two dose rates tested. The ICRR suspected in melanoma cells may be related to the ability of tumours to react to external stresses.

At HDR the survival curve of normal fibroblasts, at very low doses, fits a linear curve. This result seems to be in line with the LNT concept. A recent paper by Averbeck (Averbeck 2010) points out the particular effects of low doses. In many cases, doses lower than 100 mGy induce biological responses much more variable than the responses obtained with higher doses (>1 Gray) and exert non-linear effects. Apparently, HRS is not only due to a failure to recognize DNA double-strand breaks (Wykes et al. 2006). Also failures in apoptosis (Krueger et al. 2007) and G2- checkpoint

arrests (Krueger et al. 2010), as well as bystander effects (Ryan et al. 2009) and intra- and extracellular signalling (Edin et al. 2013) may be involved. The discrepancy of responses between normal fibroblasts and melanoma cells may be explained by the integrity of the repair system in normal cells and its deficiency in cancer cells. The subject is related to the dose dose-rate effectiveness factor (DDREF) that quantifies the effects of low doses and low dose rates on cell survival (Monson 2006). Few lethal lesions may not be detected by cellular sensor mechanisms and may not give rise to DNA damage response signalling and repair or apoptosis.

In our study, neither the results on DSB induction nor those on apoptosis can explain the HRS at VLDR. Theoretically autophagy cannot explain an acute cell death that would take place only at two very low doses (100 and 150 mGy) (Altmeyer et al. 2011).

HRS at VLDR and ICRR at HDR found with M4Beu, may be related to available cell energy. For triggering enzymatic repair, radiation energy deposition in the cell can be expected to stimulate a sensor, and to provide enough energy to activate efficient repair. A cell has no energy reserves and fits its ATP production to repair lesions (Bradbury et al. 2000). In our hypothesis the ATP variations may precede the changes in survival. The lack of ATP at 100 mGy, which may be responsible for the absence of repair, occurs prior to the maximal of cell death (150 mGy). The slight ATP increase at 150 mGy, may then activate the DNA repair system, but at the same time the repair of the DSB induced may use up most of the available energy and thus limit repair of other organelles. At HDR cell survival exceeds that of the control presumably because there

is enough energy to allow also the repair of cytoplasmic lesions while DSB repair is taking place. This is in agreement with the ICRR reported in several cell lines (Feinendegen 2005; Averbeck 2010). The discrepancy between survival rates at VLDR/HDR disappears at higher doses because the number of DSB increases as DNA repair is progressively overwhelmed.

In non-irradiated fibroblasts, ATP and ADP levels are much lower than those of melanoma cells, probably because their metabolisms are slower and less variable. In irradiated fibroblasts, ATP levels at VLDR are globally decreased, whereas they are rather increased at HDR, probably in response to higher cell damage evidenced by the survival curve. ADP levels are mostly decreased at VLDR and HDR.

After the induction of DSB chromatin relaxation and restructuring, necessary for giving repair proteins access to the DNA lesions, is energy consuming (Clapier and Cairns 2009). One major cytoplasmic target of radiation is mitochondria, the ATP producing power units (Clemencon et al. 2013). The ADP-ATP system is oscillating around an equilibrium but is leading to cell dysfunction in case of genotoxic stress (Clemencon et al. 2013). The mitochondrial DNA may be particularly vulnerable to radiation. We can hypothesize that, after VLDR radiation, the DNA damage density is too low and so unable to trigger repair, while the same energy deposition (acute dose) at HDR induces in a short time a sufficient amount of DNA damages to activate repair. During HRS, in melanoma cells, our results suggest a lack of ATP production. At the same time, the ADP increase may indicate the inability to convert ADP to ATP. This is compatible with a mitochondrial impairment.

In radioprotection low doses of high LET radiations such as neutrons, delivered at very low dose rate are of societal and environmental importance because of the presence of such particles in natural radiation, in the neighbourhood of medical accelerators and proton-therapy. A major problem concerns (Brenner and Hall 2008) the role of neutron low doses in the occurrence of secondary cancers after proton-therapy. Also, flights at high altitude (Yasuda et al. 2009) and in space are of concern (Tsetlin and Deshevaia 2003; Spurny et al. 2007). It is widely recognized that total body irradiation with low doses of high-LET particles (Gridley et al. 2002; Gridley and Pecaut 2006) and exposure to heavy ions (Tucker et al. 2004) may constitute a risk to human health. However, during Hadron-therapy they can be very effective against tumour growth (Suetens et al. 2014). ATP depletion also inhibits the growth of tumour cell lines (Lu et al. 2000), and chemicals decreasing ATP levels have radiosensitizing effect (Kumar et al. 1993), whereas exogenous ATP is protective against neutron irradiation (Szeinfeld and De Villiers 1992). In line with our present findings, it would be of interest to use molecules, which interfere with normal mitochondrial energy metabolism and ATP production, in order to understand the mechanism of HRS. These results may have an interest in human therapy. Nevertheless, taking into account the duration of an irradiation at VLDR, such a therapy at best would be reserved to total body irradiation. Animal experiments should be necessary to assess this hypothesis.

We demonstrate here a hyper-radiosensitivity response of human melanoma cells to very low doses of fast neutrons of 65 MeV when applied at very low dose rate. We hypothesize that the radiation-induced lesions at VLDR are not taken care of because of

the absence of appropriate DNA damage signalling towards repair enzymes due to the lack of energy. Conversely, at HDR, an ICRR is seen. This may rely on stimulation of energy metabolism, pushing some cells to progress in cell cycle and may well be linked to the aggressiveness of the tumour cells. From a radiobiological point of view our interest is to explore the different pathways of ATP production during the HRS phenomenon.

In human normal fibroblasts such mechanisms were absent, although survival levels remained radiation dose rate dependent. Very low doses delivered at VLDR had no measurable effects till 150 mGy, but at HDR cells were killed proportionally with increasing doses, without survival curve shoulder.

Finally, at VLDR melanoma cells present very sentitive to neutron radiation, while normal fibroblasts are able to repair damages. At HDR, melanoma cells react by an initial radio-resistance while normal cells are proportionally killed with increasing doses. These findings suggest a challenge to the validity of the “Linear-No-Threshold” model for different cell types. Each cell type (cancer cells or normal cells) may exert its own survival behaviour and response at very low doses of radiations, depending on the radiation dose rate and cell energy status.

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Authors' contribution

C.D. supervised the project and designed the experiments. G.M., V.S., M.M-B., E.C., and C.D. have made the experiments and analysed the data. J-M. D. performed the radiation experiments B.P. performed statistical analysis. C.D., M.M-B., J.G., D.A., A.T., E.C., and P.V. wrote the manuscript.

Competing financial interests

The authors certify that there is not any competing financial interest.

Captions

Figure 1: Survival curves of melanoma cells (a) and normal fibroblasts (b) as a function of 65 MeV neutrons dose delivered at High Dose Rate (83 mGy/min: empty squares) or at Very Low Dose Rate (1.08 mGy/min: black dots). For a given experiment, each point is the mean of colony number counted in 3 flasks. Survival curve of melanoma cells (Error bars indicate the standard error of the mean for N = 3 independent experiments) and normal fibroblasts (Error bars indicate the standard error of the mean for N = 2 independent experiments) as a function of 6 MV X-Rays delivered at 1 Gy/min (c) (Error bars indicate the standard error of the mean for N = 6 independent experiments).

Figure 2: Analysis of DSB (average foci/cell): DSB were measured by γ-H2AX foci detection 45 min (grey columns) and 24 hours (black columns) after irradiation as a function of 65 MeV neutrons dose. At HDR (83 mGy/min) (a,c), foci in non-irradiated and irradiated cells were recorded at the same time. At VLDR (1.08 mGy/min) (b,d), foci in non-irradiated cells were counted at each time-point corresponding to irradiation duration. Error bars indicate the standard error of the mean for N = 2 independent experiments.

Figure 3: Analysis of apoptosis. Fluorescence expression of active caspase-3 measured 24 hours after irradiation, recorded as a function of 65 MeV neutron dose at High Dose Rate (HDR 83 mGy/min) (grey columns), and at Very Low Dose Rate (VLDR: 1.08 mGy/min) (empty columns). Error bars indicate the standard error of the mean for N = 2 independent experiments.

Figure 4: ATP levels in melanoma cells (a) and in fibroblasts (b) as a function of 65 MeV neutrons dose delivered at HDR (83 mGy/min) (grey columns) and at VLDR (1.08 mGy/min) (empty columns). Error bars indicate the standard error of the mean for N = 2 independent experiments (5 different microplate wells for each).

Figure 5: ADP levels in melanoma cells (a) and in fibroblasts (b) as a function of 65 MeV neutrons dose delivered at HDR (83 mGy/min) (grey columns) and at VLDR (1.08 mGy/min) (empty columns). Error bars indicate the standard error of the mean for N = 2 independent experiments (5 different microplate wells for each).

Figure 6: Histogram of melanoma cells as a function of 65 MeV neutrons dose delivered at three different dose rates. VLDR (1.08 mGy/min); HDR (83 mGy/min); MDR (7.5 mGy/min). At MDR (7.5 mGy/min) there is neither HRS nor ICRR. For a given experiment, each point is the mean of colony number counted in 3 flasks. Error bars indicate the standard error of the mean for N = 3 independent experiments for VLDR and HDR.

References

Alard JP, Bodez V, Tchirkov A, Nenot ML, Arnold J, Crespin S, Rapp M, Verrelle P, Dionet C. 2002. Simulation of neutron interactions at the single-cell level. Radiat Res 158: 650-656.

Altmeyer A, Ignat M, Denis JM, Messaddeq N, Gueulette J, Mutter D, Bischoff P. 2011. Cell death after high-LET irradiation in orthotopic human hepatocellular carcinoma in vivo. In Vivo 25: 1-9.

Averbeck D. 2009. Does scientific evidence support a change from the LNT model for low-dose radiation risk extrapolation? Health Phys 97: 493-504.

Averbeck D. 2010. Non-targeted effects as a paradigm breaking evidence. Mutat Res 687: 7-12.

Bohrnsen G, Weber KJ, Scholz M. 2002. Measurement of biological effects of high-energy carbon ions at low doses using a semi-automated cell detection system. Int J Radiat Biol 78: 259-266.

Bradbury DA, Simmons TD, Slater KJ, Crouch SP. 2000. Measurement of the ADP:ATP ratio in human leukaemic cell lines can be used as an indicator of cell viability, necrosis and apoptosis. J Immunol Methods 240: 79-92.

Brenner DJ, Hall EJ. 2008. Secondary neutrons in clinical proton radiotherapy: a charged issue. Radiother Oncol 86: 165-170.

Clapier CR, Cairns BR. 2009. The biology of chromatin remodeling complexes. Annu Rev Biochem 78: 273-304.

Clemencon B, Babot M, Trezeguet V. 2013. The mitochondrial ADP/ATP carrier (SLC25 family): pathological implications of its dysfunction. Mol Aspects Med 34: 485-493.

Dionet C, Tchirkov A, Alard JP, Arnold J, Dhermain J, Rapp M, Bodez V, Tamain JC, Monbel I, Malet P, et al. 2000. Effects of low-dose neutrons applied at reduced dose rate on human melanoma cells. Radiat Res 154: 406-411.

Edin NJ, Sandvik JA, Vollan HS, Reger K, Gorlach A, Pettersen EO. 2013. The role of nitric oxide radicals in removal of hyper-radiosensitivity by priming irradiation. J Radiat Res 54: 1015-1028.

Feinendegen LE. 2005. Evidence for beneficial low level radiation effects and radiation hormesis. Br J Radiol 78: 3-7.

Gridley DS, Pecaut MJ. 2006. Whole-body irradiation and long-term modification of bone marrow-derived cell populations by low- and high-LET radiation. In Vivo 20: 781-789.

Gridley DS, Pecaut MJ, Nelson GA. 2002. Total-body irradiation with high-LET particles: acute and chronic effects on the immune system. Am J Physiol Regul Integr Comp Physiol 282: R677-688.

Gueulette J, Beauduin M, Gregoire V, Vynckier S, De Coster BM, Octave-Prignot M, Wambersie A, Strijkmans K, De Schrijver A, El-Akkad S, et al. 1996. RBE variation between fast neutron beams as a function of energy. Intercomparison involving 7 neutrontherapy facilities. Bull Cancer Radiother 83 Suppl: 55s-63s.

Heuskin AC, Michiels C, Lucas S. 2014. Low dose hypersensitivity following in vitro cell irradiation with charged particles: Is the mechanism the same as with X-ray radiation? Int J Radiat Biol 90: 81-89.

Krueger SA, Joiner MC, Weinfeld M, Piasentin E, Marples B. 2007. Role of apoptosis in low-dose hyper-radiosensitivity. Radiat Res 167: 260-267.

Krueger SA, Wilson GD, Piasentin E, Joiner MC, Marples B. 2010. The effects of G2-phase enrichment and checkpoint abrogation on low-dose hyper-radiosensitivity. Int J Radiat Oncol Biol Phys 77: 1509-1517.

Kumar A, Kimura H, Aoyama T. 1993. Radiosensitization of human lung fibroblasts by chemical that decrease ATP levels. J Radiat Res 34: 195-203.

Lambin P, Marples B, Fertil B, Malaise EP, Joiner MC. 1993. Hypersensitivity of a human tumour cell line to very low radiation doses. Int J Radiat Biol 63: 639-650.

Lu X, Errington J, Chen VJ, Curtin NJ, Boddy AV, Newell DR. 2000. Cellular ATP depletion by LY309887 as a predictor of growth inhibition in human tumor cell lines. Clin Cancer Res 6: 271-277.

Monson RRea. 2006. Molecular and cellular repsonses to ionizing radiation - General aspects of dose-reponse relationships. Health Risks from Exposure to Low Levels of Ionizing Radiation: BEIR VII Phase 2. Washington, The National Academies Press**:** 43-44.

Ryan LA, Seymour CB, Joiner MC, Mothersill CE. 2009. Radiation-induced adaptive response is not seen in cell lines showing a bystander effect but is seen in lines showing HRS/IRR response. Int J Radiat Biol 85: 87-95.

Spurny F, Ploc O, Dachev T. 2007. On the neutron contribution to the exposure level onboard space vehicles. Radiat Prot Dosimetry 126: 519-523.

Suetens A, Moreels M, Quintens R, Chiriotti S, Tabury K, Michaux A, Gregoire V, Baatout S. 2014. Carbon ion irradiation of the human prostate cancer cell line PC3: a whole genome microarray study. Int J Oncol 44: 1056-1072.

Szeinfeld D, De Villiers N. 1992. Radioprotective properties of ATP and modification of acid phosphatase response after a lethal dose of whole body p(66MeV)/Be neutron radiation to BALB/c mice. Cancer Biochem Biophys 13: 123-132.

Tsetlin VV, Deshevaia EA. 2003. [Influence of chronic exposure to low doses of space ionizing radiation on the character of formation of microbial assemblage in the habitat of orbital station]. Radiats Biol Radioecol 43: 172-175.

Tucker JD, Marples B, Ramsey MJ, Lutze-Mann LH. 2004. Persistence of chromosome aberrations in mice acutely exposed to 56Fe+26 ions. Radiat Res 161: 648-655.

Wykes SM, Piasentin E, Joiner MC, Wilson GD, Marples B. 2006. Low-dose hyper-radiosensitivity is not caused by a failure to recognize DNA double-strand breaks. Radiat Res 165: 516-524.

Yasuda H, Yajima K, Sato T, Takada M, Nakamura T. 2009. Responses of selected neutron monitors to cosmic radiation at aviation altitudes. Health Phys 96: 655-660.

Figure 1

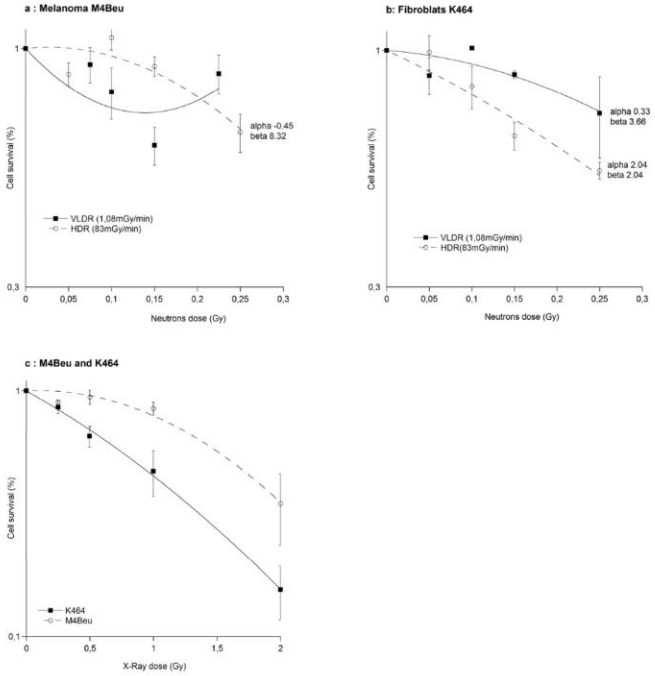

a : Melanoma M4Beu
Cell survival (%)
1
0,3
0
0,05
0,1
0,15
0,2
0,25
0,3
Neutrons dose (Gy)
alpha -0.45
beta 8.32
VLDR (1,08mGy/min)
HDR (83mGy/min)
b: Fibroblats K464
Cell survival (%)
1
0,3
0
0,05
0,1
0,15
0,2
0,25
0,3
Neutrons dose (Gy)
alpha 0.33
beta 3.66
alpha 2.04
beta 2.04
VLDR (1,08mGy/min)
HDR(83mGy/min)
c : M4Beu and K464
Cell survival (%)
1
0,1
0
0,5
1
1,5
2
X-Ray dose (Gy)
K464
M4Beu

Figure 2

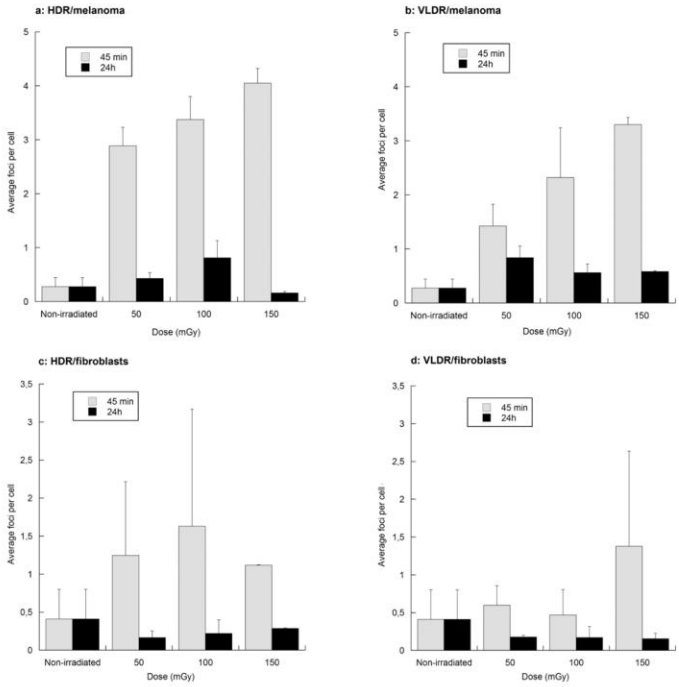

a: HDR/melanoma
b: VLDR/melanoma
c: HDR/fibroblasts
d: VLDR/fibroblasts
45 min
24h
Average foci per cell
Non-irradiated
50
100
150
Dose (mGy)

Figure 3

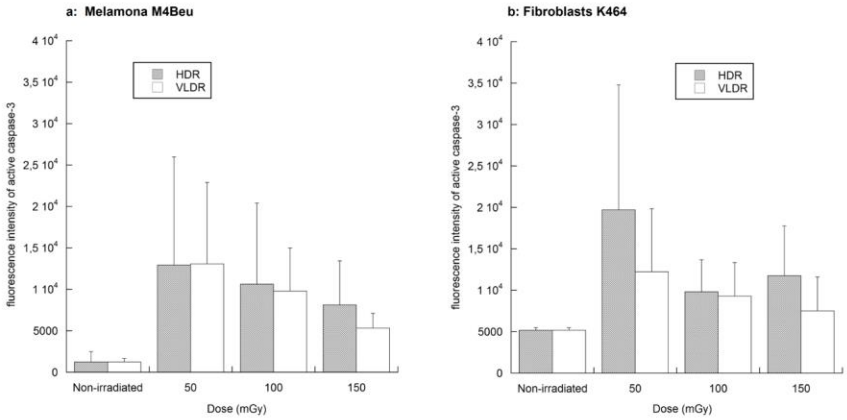

a: Melamona M4Beu
b: Fibroblasts K464
HDR
VLDR
fluorescence intensity of active caspase-3
4 10⁴
3,5 10⁴
3 10⁴
2,5 10⁴
2 10⁴
1,5 10⁴
1 10⁴
5000
0
Non-irradiated
50
100
150
Dose (mGy)

Figure 4

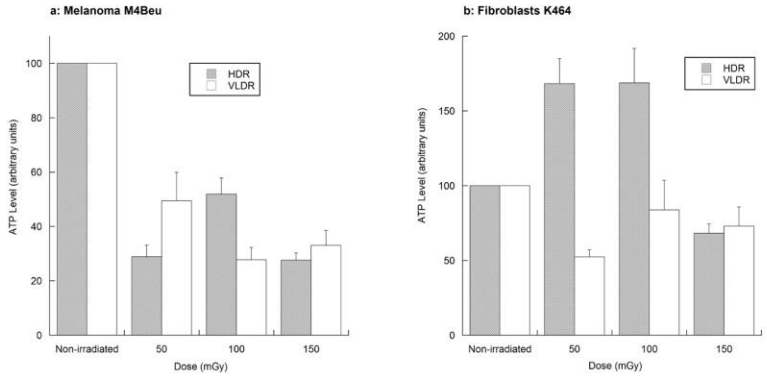

a: Melanoma M4Beu
ATP Level (arbitrary units)
100
80
60
40
20
0
HDR
VLDR
Non-irradiated
50
100
150
Dose (mGy)
b: Fibroblasts K464
ATP Level (arbitrary units)
200
150
100
50
0
HDR
VLDR
Non-irradiated
50
100
150
Dose (mGy)

Figure 5

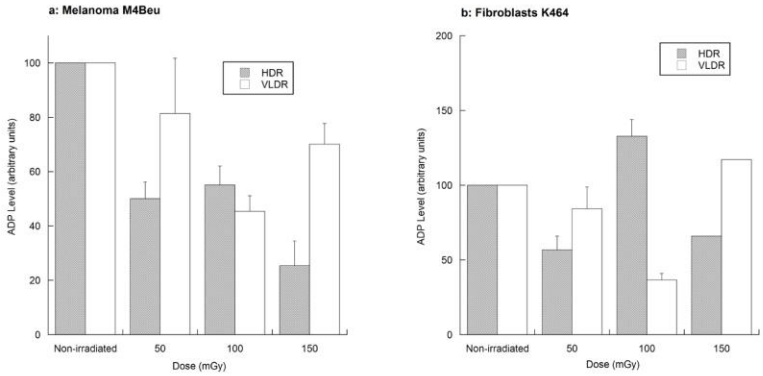
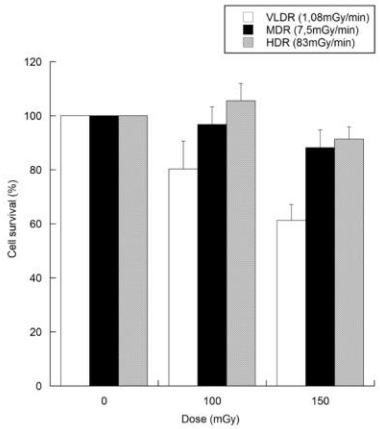

a: Melanoma M4Beu
ADP Level (arbitrary units)
HDR
VLDR
Non-irradiated
50
100
150
Dose (mGy)
b: Fibroblasts K464
ADP Level (arbitrary units)
HDR
VLDR
Non-irradiated
50
100
150
Dose (mGy)

Figure 6


VLDR (1,08mGy/min)
MDR (7,5mGy/min)
HDR (83mGy/min)
120
100
80
60
40
20
0
Cell survival (%)
0
100
150
Dose (mGy)