Activation of the Nuclear Factor κ B pathway by heavy ion beams of different linear energy transfer

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Abstract

Purpose: Risk assessment of radiation exposure during long-term space missions requires the knowledge of the relative biological effectiveness (RBE) of space radiation components. Few data on gene transcription activation by different heavy ions are available, suggesting a dependence on linear energy transfer. The transcription factor Nuclear Factor κ B (NF- κ B) can be involved in cancerogenesis. Therefore, NF- κ B activation by accelerated heavy ions of different linear energy transfer (LET) was correlated to survival.

Materials and methods: NF- κ B-dependent gene induction after exposure to heavy ions was detected in stably transfected human embryonic kidney 293 cells (HEK-pNF- κ B-d2EGFP/Neo cells carrying a neomycin resistance), using the destabilized Enhanced Green Fluorescent Protein (d2EGFP) as reporter.

Results: Argon (LET 272 keV/ μ m) and neon ions (LET 91 keV/ μ m) had the highest potential to activate NF- κ B, resulting in a RBE of 8.9 in comparison to 150 kV X-rays. The RBE for survival also reached its maximum in this LET range, with a maximal value of 2.

Conclusions: NF- κ B might be involved in modulating survival responses of cells hit by heavy ions in the LET range of 91–272 keV/ μ m and could therefore become a factor to be considered for risk assessment of radiation exposure during space travel.

Keywords: Nuclear Factor κ B, linear energy transfer, heavy ion, space mission, relative biological effectiveness

Abbreviations: bcl-2, B-cell lymphoma 2; bcl-XL, B-cell lymphoma-extra large; CDKN1A, Cyclin-dependent kinase inhibitor 1A; CHO, Chinese Hamster Ovary; CIMAP, Centre de recherches sur les Ions, les Matériaux et la Photonique; d2EGFP, destabilized EGFP; EGFP, Enhanced Green Fluorescent Protein; FBS, fetal bovine serum; GANIL, Grand Accélérateur National d'Ions Lourds; HEK, human embryonic kidney; IAP, inhibitor of apoptosis protein; I κ B α , inhibitor of NF- κ B α ; LARIA, Laboratoire d'Accueil en Radiobiologie avec les Ions Accélérés; LET, linear energy transfer; MeV/n, Mega electron Volt per nucleon; NASA, National Aeronautics and Space Administration (USA); NCRP, National

Council on Radiation Protection and Measurements; NF- κ B, Nuclear Factor κ B; RBE, relative biological effectiveness; Rel, Reticuloendotheliosis; TK, thymidine kinase; TNF- α , tumor necrosis factor α ; TP53, tumor protein p53; DNA-PK, DNA-dependent protein kinase.

Introduction

The radiation environment that astronauts experience during spaceflight differs significantly to that found on Earth (Straube et al. 2010). Besides protons and α -particles, heavier nuclei are part of the radiation field encountered in space. Although heavy nuclei only make up about 2% of the fluence in space, they will account for much of the biological consequences due to their higher linear energy transfer (LET) (Pecaut and Gridley 2010). Primarily late tissue sequelae like genetic alterations, cancer and non-cancer effects are the potential risks resulting from radiation exposure in deep space (Blakely 2000). Due to mass limitations for spacecrafts and production of secondary particles in shielding material, the radiation exposure cannot simply be avoided by radiation shielding of the spacecraft (National Council on Radiation Protection and Measurements [NCRP] 2006). The prolonged exposure to galactic cosmic rays requires extensive further study of the biological effects of these extraordinary radiation qualities and quantities in order to improve the estimation of health effects of radiation exposures on humans during and after exploration missions (Cucinotta et al. 2005, NCRP 2006, Hellweg and Baumstark-Khan 2007).

In the cellular response to radiation, several sensors detect the induced DNA damage and trigger signal transduction pathways resulting in cell death or survival with or without certain amounts of remaining DNA damage (National Aeronautics and Space Administration (USA) [NASA] 2004, Ohnishi et al. 2002). Signal transduction leads to the activation of various transcription factors, resulting in the expression of certain

genes whose protein products are involved in several possible outcomes, such as cell cycle arrest, allocating additional time for repair of the damaged DNA, cellular senescence or different types of cell death, if the damage is too severe and cannot be repaired.

An important transcription factor that is known to be activated by ionizing radiation is Nuclear Factor κ B (NF- κ B) (Brach et al. 1991). The NF- κ B/Rel (Reticuloendotheliosis) family (NF- κ B1 (p50/p105), NF- κ B2 (p52/p100), RelA (p65), RelB, and c-Rel) can be activated in response to many agents and is involved in regulation of proliferation, immune system development and performance, inflammation and apoptosis (Barnes and Karin 1997, Sonenshein 1997, Wu et al. 1996). Upon activation, the inhibitor of NF- κ B (I κ B α) that sequesters NF- κ B in the cytoplasm (Zabel et al. 1993), is degraded by several proteases (Alkalay et al. 1995) and the released NF- κ B translocates to the nucleus and binds to κ B DNA motifs (NF- κ B response elements, NRE) initiating gene transcription. NRE have been identified in the promoter or enhancer regions of a number of growth factors, cytokines and adhesion molecules involved in fibrosis and inflammation (Ghosh et al. 1998). In addition, NF- κ B regulates the expression of many genes whose products are involved in the control of cell proliferation and cell death (Baichwal and Baeuerle 1997).

Exposure of human cells to energetic heavy ions allows simulation of an important component of the space radiation field, the heavy ion component of galactic cosmic radiation. Currently, experiments are mostly restricted to high-dose rate experiments due to limited beam time availability. Experiments at heavy ion accelerators with various cell lines and primary cells revealed a strong dependency of the relative biological effectiveness (RBE) for the investigated biological endpoint on the LET of the energetic heavy ions and on ion species, energy and charge (for example, Bird and Burki 1975, Stoll et al. 1995, Wulf et al. 1985, Yatagai 2004). For many biological endpoints, e.g., cell inactivation (for example, Blakely et al. 1979), interphase death (Sasaki et al. 1997), differentiation of fibroblasts (Fournier et al. 2001), mutation induction (for example, Cox et al. 1977), *in vitro* cell transformation (Yang et al. 1985), chromosomal aberrations (Skarsgard et al. 1967) and tumor induction *in vivo* (Alpen et al. 1993), a maximal RBE was reached with an LET from 90–230 keV/ μ m. The RBE for mutation induction was higher compared to the inactivation RBE for all examined LET values (Yatagai 2004).

There are several hints that the RBE for activation of signaling pathways by heavy ions may also depend on LET and ion species. Screening of gene expression in the nematode *Caenorhabditis elegans* suggests an LET or track structure dependence of the gene expression changes (Nelson et al. 2002). In human lens epithelial cells, transcription and translation of Cyclin-dependent kinase inhibitor 1A (CDKN1A, Protein 21^{Cyclin-dependent kinase 2-interacting protein 1, wild-type p53-activated fragment 1}, p21^{CIP1/WAF1}) are both temporally regulated after exposure to 4 Gy of high-energy accelerated iron-ion beams (~150 keV/ μ m) as well as of protons (~1 keV/ μ m) and X-rays, whereby the magnitude and kinetics of the expression enhancement seem to depend on the LET of the radiation (Chang et al. 2005). Such an LET dependency is also

suggested for accumulation of the tumor protein p53 (TP53) (Fournier et al. 2004).

For screening of the cellular NF- κ B response, we developed a recombinant reporter cell line, HEK-pNF- κ B-d2EGFP/Neo L2 (Hellweg et al. 2003). These human embryonic kidney (HEK) cells harbor a plasmid which controls the expression of the destabilized variant of enhanced green fluorescent protein (d2EGFP) (Li et al. 1998) as a reporter molecule by a synthetic promoter containing four NRE tandem copies. HEK cells are a useful tool to study many different processes in human cells, as they can easily be transiently and stably transfected. Many studies, including own investigations, have shown that the NF- κ B pathway is functional in HEK cells (for example, Matsuda et al. 2003, Hellweg et al. 2006, Muscat et al. 2007, Simon and Samuel 2007, Rajagopal et al. 2008). Additionally, HEK cells express neuronal markers similar to that of neuronal stem cells (Shaw et al. 2002, Arnhold et al. 2008) and are therefore of interest for studying basic mechanisms of space radiation-induced cellular effects. In the recombinant cell system, activation of the cellular NF- κ B pathway leads to binding of liberated NF- κ B not only to its target genes but also to the synthetic promoter. Thus, the cells respond with increased transcription of d2EGFP, followed by translation and maturation of the protein. By that, it is possible to quantify NF- κ B-dependent transcriptional activation in response to stimuli simply by measuring the yields of green fluorescence of the reporter protein. In order to overcome problems described for cell-based stable NF- κ B-GFP reporter systems (El-Guendy and Sinai 2008), we have shown for the selected clone that the kinetics of activation of NF- κ B dependent d2EGFP gene expression by tumor necrosis factor α (TNF- α) follows the nuclear translocation of the NF- κ B subunit p65 (Hellweg et al. 2003) and that the response to DNA double-strand break inducing agents is intact (Hellweg et al. 2006). The dose-response curve for cytokines and several chemicals (Hellweg et al. 2006) could thus be followed using the HEK-pNF- κ B-d2EGFP/Neo cells.

In previous studies, we have shown with this cell system that exposure to accelerated heavy ions (95 MeV/nucleon [MeV/n] - Ar, LET 272 keV/ μ m) results in stronger activation of NF- κ B in human cells than X-ray exposure (Baumstark-Khan et al. 2005). The NF- κ B activation by energetic carbon ions (35 and 75 MeV/n, LET 73 and 33 keV/ μ m, respectively) was comparable to the effect induced by X-rays (Hellweg et al. 2011), suggesting a narrow peak in the LET dependence of NF- κ B activation. As this transcription factor is involved in regulation of apoptosis and immune and inflammatory response, its activation may influence the outcome of a heavy ion-exposed cell and therefore might be relevant for late effects which might occur after prolonged low-dose exposure during an interplanetary mission.

In this work, the influence of the LET of several accelerated heavy ions on activation of NF- κ B dependent d2EGFP gene expression and on cellular survival was investigated. Here, we show that radiation qualities, which are relevant for space missions, are capable of activating the NF- κ B pathway and that the activation threshold is in a space flight relevant fluence range for ions in the LET range of 91–272 keV/ μ m.

Materials and methods

Cell culture

The cell line HEK 293 was established by Graham et al. (1977) from human embryonic kidney cells immortalized by transfection with sheared fragments of adenovirus type 5 DNA. The cells contain only the stably integrated region (E1a and E1b genes) of the human adenovirus genome and do not produce viral particles. Cells were obtained from the American Type Culture Collection (ATCC CRL-1573; now: LGC Standards, Wesel, Germany). Cloning of the plasmid pNF- κ B-d2EGFP/Neo, stable transfection and selection of an appropriate cell clone (HEK-pNF- κ B-d2EGFP/Neo L2) was already described (Hellweg et al. 2003). HEK-pNF- κ B-d2EGFP/Neo L2 and untransfected HEK cells have comparable radiosensitivity to X-rays and to accelerated carbon ions and show no difference in proliferation (Hellweg et al. 2011). Cells were maintained in α -medium (Biochrom KG, Berlin, Germany) containing 1.5 mg/ml G418 and 10% fetal bovine serum (FBS) at standard conditions (37°C, 95% air and 5% CO₂ atmosphere). Cells were passaged every week and seeded in a cell density of 3×10^4 cells/cm² in 80 cm² cell culture flasks (Nunc, Novodirect, Kehl, Germany). Medium was exchanged after four days.

Treatment modalities

For cytokine treatment or irradiation experiments, 3×10^4 cells/cm² were seeded into suitable culture vessels freshly coated with poly-D-lysine (10 μ g/cm², Sigma-Aldrich Chemie, Steinheim, Germany) for 15 min followed by washing with phosphate buffered saline (PBS). For exposure to TNF- α , X-rays, ¹³C, ²²Ne, ³⁶Ar, and ⁵⁸Ni ions, cells were seeded into 25 cm² flasks (Iwaki, Dunn Labortechnik GmbH, Asbach, Germany). For exposure to ²⁰⁸Pb ions, cells were seeded into Petri dishes with a polytetrafluorethylene bottom (thickness 25 μ m, Lumox[®], Greiner bio-one, Frickenhausen, Germany), since these ions could not penetrate the polystyrene bottom of standard culture flasks. Cells were incubated for two days before treatment. Control dishes were sham-treated.

Cytokine treatment

Human recombinant TNF- α was obtained from Sigma-Aldrich Chemie. TNF- α (final concentration 10 ng/ml), was dissolved in PBS, and added in serum-containing medium. Cells were harvested after 18 h for flow cytometry as described below.

X-irradiation

Cells in early exponential growth phase were irradiated at room temperature with 150 kV X-rays generated by an X-ray unit (Müller Typ MG 150, Philipps, Hamburg, Germany) yielding a dose rate of 1 Gy/min.

Heavy ion exposure

Irradiation with accelerated heavy ions was performed at the Grand Accélérateur National d'Ions Lourds (GANIL, Caen, France). Characteristics of the applied beams including the LET in water are shown in Table I. Adherent cells were irradiated in sub-confluent stage and in upright position at room

Table I. Characteristics of the heavy ion beams.

Ion species	Energy (MeV/n) ^a		LET (keV/ μ m) in H ₂ O	Penetration depth (μ m)
	beam	on target ^b		
Carbon (¹³ C)	75.0	71.4	34.2	15120
Neon (²² Ne)	80.0	74.5	91.2	10000
Argon (³⁶ Ar)	95.0	83.8	271.5	6336
Nickel (⁵⁸ Ni)	75.0	54.1	905.9	2092
Lead (²⁰⁸ Pb)	29.2	19.5	9674.0	368

^aMeV/nucleon; ^bEffective irradiation energy at the cell monolayer after the energy losses in two detectors, the exit window, ~ 1 cm air and the bottom of the culture vessel (1200 μ m polystyrene or 25 μ m polytetrafluoroethylene foil).

temperature. Therefore, flasks and Petri dishes were completely filled with α -medium and closed. Petri dishes were wrapped with parafilm. Dose rates were adjusted to ~ 1 Gy/min. Dosimetry was performed by the Centre de recherches sur les Ions, les Matériaux et la Photonique (CIMAP) / Laboratoire d'Accueil en Radiobiologie avec les Ions Accélérés (LARIA) staff at GANIL, yielding the fluence in particles/cm² (P/cm²). To convert fluence (F) to the absorbed dose, the following formula was applied (Wulf et al. 1985):

$$\text{Dose [Gy]} = 1.6 = 10^{-9} \times \text{LET [keV}/\mu\text{m}] \times F [\text{P}/\text{cm}^2] \quad (1)$$

Colony forming ability

For survival experiments with X-rays and heavy ions, cells were seeded and irradiated as described previously. Immediately after irradiation cells were trypsinized and re-plated in six Petri dishes per dose. The cell density in the dishes was adjusted to compensate for the plating efficiency of the cell line and for the anticipated lethal effect of the radiation in order to allow growth of about 50 colonies per dish.

The cells were incubated for 21 days without medium change. The resulting colonies were stained with crystal violet (1 mg/ml in 3% formaldehyde solution, Sigma-Aldrich Chemie). Only colonies containing more than 50 cells were scored as survivors. The experiments were performed with six dishes per dose and repeated if beam time was available.

All data from the irradiated samples were fit by least-squares linear regression analysis to $\ln S = \ln(\text{PE}_D/\text{PE}_{D=0})$ versus dose, where the natural logarithm of S is the survival and PE are the plating efficiencies of treated (PE_D) or untreated cells (PE_{D=0}).

Flow cytometry

For measurement of d2EGFP, cells were harvested 18 h after radiation or cytokine treatment by trypsinization and fixed with 3 ml cold 3.5% formaldehyde in PBS. For flow cytometric analysis, cells were centrifuged and resuspended in PBS. Forward and sideward scatter and green fluorescence (FL-1) of 20,000 cells from the samples were measured in a FACScan (Becton Dickinson, San Jose, CA, USA) with an argon laser (488 nm) as excitation source. The markers d2EGFP⁽⁻⁾ and d2EGFP⁽⁺⁾ were set by means of untreated and TNF- α treated cells (Figure 2A). For comparison of NF- κ B-dependent d2EGFP expression after exposure to different radiation qualities, the percentage of d2EGFP⁽⁺⁾ cells in the treated samples was normalized to the percentage of the d2EGFP⁽⁺⁾ cells in the corresponding mock-treated controls.

Calculation of relative biological effectiveness

The relative biological effectiveness (RBE) was calculated according to the formula:

$$\text{RBE} = \frac{\text{Absorbed dose of reference radiation inducing biological effect [Gy]}}{\text{Absorbed dose of test radiation inducing biological effect [Gy]}} \quad (2)$$

Fluences were converted to energy dose according to Equation (1). The reference radiation was 150 kV X-rays.

Survival

The D_0 , the reciprocal of the slope in the linear range of the survival curves, was used for RBE calculation. The D_0 was converted to absorbed dose for heavy ion exposure. The RBE for survival was calculated as follows:

$$\text{RBE}_{\text{Survival}} = \frac{D_0 \text{ reference radiation [Gy]}}{D_0 \text{ energetic heavy ion [Gy]}} \quad (3)$$

Activation of NF- κ B dependent d2EGFP gene expression

Using Sigma Plot 2002 for Windows Version 8.02 (Systat Software GmbH, Erkrath, Germany), a regression was adapted to each dose effect curve of NF- κ B dependent d2EGFP gene expression activation, based on the regression coefficient. The doses initiating a doubling of the d2EGFP fluorescence in HEK-pNF- κ B-d2EGFP/Neo L2 cells 18 h after irradiation were derived from the regression curves. These doses were used as indicator of the threshold for activation of NF- κ B dependent d2EGFP gene expression. The RBE for NF- κ B dependent d2EGFP gene expression was calculated by dividing according to formula (2) the absorbed dose [Gy] of the reference radiation that elicited a doubling of d2EGFP fluorescence 18 h after irradiation by the absorbed dose [Gy] of energetic heavy ions inducing the same biological effect.

Statistics

Each experiment was repeated up to five times depending on the availability of beam time with one to six replicates each. Beam time is very restricted and it was not possible to repeat the experiments in independent beam times for every ion analyzed in this work. In order to account for different numbers of replicates and repeats, the standard error was calculated. Means, standard errors and significance levels in the *t* test were calculated with Microsoft® Office Excel 2003 (Microsoft Deutschland GmbH, München, Germany). The *t*-test can be used even with small sample sizes. Regression analyses were performed using SigmaPlot 2002.

Results

The effect of different energetic heavy ions on the NF- κ B pathway was studied in human embryonic cells. Heavy ion fluences resulting in absorbed doses between 0.2 and 60 Gy, depending on the anticipated killing effect of the examined ion (Stoll et al. 1995), were selected for the experiments.

Survival of human embryonic cells after X-ray and after heavy ion exposure

There are no significant differences in the survival of untransfected HEK cells and stably transfected

HEK-pNF- κ B-d2EGFP/Neo clone L2 cells after X-irradiation and after carbon ion exposure (Hellweg et al. 2011).

High-LET radiation exposure of HEK cells results in purely exponential survival curves (Figure 1A). Based on fluence, the cell killing potential strongly increases with LET up to 272 keV/ μ m. The increase of the killing effect from 272–9674 keV/ μ m is less prominent. Based on absorbed dose, heavy ions with an LET of 34–91 keV/ μ m are most efficient in cell killing, while radiation qualities with an LET above or below this range are less efficient in cell killing (Figure 1B). Lead ions with an extremely high LET are least efficient in reducing cellular survival. Exposure to X-rays resulted in a slightly shouldered survival curve (Figure 1B).

Figure 1. Clonogenic survival after exposure of HEK-pNF- κ B-d2EGFP/Neo cells to X-rays or to heavy ions, as fluence-effect curves (A) and dose-effect curves (B). HEK-pNF- κ B-d2EGFP/Neo cells were exposed to 150 kV X-rays or to accelerated carbon, neon, argon, nickel or lead ions (A, B). Survival was determined by the colony forming ability test (CFA). Irradiated cells were seeded in Petri dishes. Grown colonies were fixed and stained after 14–21 days incubation. Data of one to five experiments were combined (X-rays: 5; ^{36}Ar : 3; ^{13}C , ^{58}Ni , ^{208}Pb : 2; ^{22}Ne : 1); bars show standard error of up to 30 Petri dishes (5–6 per dose per independent experiment). P/cm² = particles/cm², MeV/n = MeV/nucleon.

The D_0 indicating the fluence necessary to reduce survival of HEK cells to 37% first decreases, then increases with increasing LET. The D_0 ranges between 2.5×10^7 P/cm² (1.4 Gy) for ¹³C ions (75 MeV/n), $\sim 2.2 \times 10^6$ P/cm² (3.1 Gy) for nickel ions and 1.2×10^6 P/cm² (18.2 Gy) for lead ions. These fluences result in average in 35.5, 3.1 and 1.5 hits per cell nucleus, respectively.

Induction of NF- κ B dependent d2EGFP gene expression by exposure to different radiation qualities

Previous experiments with TNF- α and argon ions revealed that the maximal d2EGFP fluorescence (3–6 h are necessary for d2EGFP translation, transcription and maturation) occurs 12–24 h after treatment (Hellweg et al. 2003, 2005, Baumstark-Khan et al. 2005). Therefore, 18 h was chosen as intermediate time point. HEK-pNF- κ B-d2EGFP/Neo cells responded to TNF- α (10 ng/ml) with a strong increase of d2EGFP fluorescence ($\sim 60\%$ positive cells at 18 h, Figure 2A).

As argon ions strongly activate the NF- κ B pathway (see below), fluorescence histograms of d2EGFP expression are shown in detail for this ion. After exposure with 95 MeV/n argon ions (Figure 2B), an increase in d2EGFP fluorescence is observed already after exposure to the lowest fluence. Part of the cell population shows a very strong fluorescence increase, and this subpopulation increases dose-dependently. The rest of the population shows only a small increase in fluorescence which is slightly dose-dependent.

Radiation experiments using low-LET ionizing radiation (150 kV X-rays) and moderate LET (75 MeV/n ¹³C ions) show a dose-dependent increase of NF- κ B dependent d2EGFP fluorescence (Figure 3A).

The comparison of the activation of NF- κ B dependent d2EGFP gene expression by radiation of different LET reveals the maximal potency for neon and argon ions (Figure 3B). In average, 2.4 argon ion hits per cell nucleus (0.7 Gy) result in doubling of NF- κ B dependent d2EGFP gene expression. For nickel and lead ions, a significant increase of fluorescence occurs at doses above 3.6 Gy and 10.6 Gy, respectively (Figure 3C). Nickel ions activate NF- κ B dependent d2EGFP expression to an extent comparable to X-rays, and lead ions have a lower activating potency than X-rays (Figure 3C). Much higher fluences are required for maximal induction of NF- κ B dependent d2EGFP gene expression in HEK cells. Depending on the radiation quality, the maximal activation is provoked by energy doses above 10 Gy (carbon, neon, argon), above 15 Gy (nickel, X-rays) or above 60 Gy (lead). After argon ion exposure, the number of d2EGFP⁽⁺⁾ cells decreases after reaching the maximum (Figure 3B), while after neon, nickel and lead ion exposure, the maximum is not reached in the examined dose range (Figure 3B, 3C).

Relative biological effectiveness of different energetic heavy ions

The RBE for survival was calculated from the D_0 . For NF- κ B activation, the dose in Gy exposure to which resulted in doubling of the d2EGFP fluorescence was chosen for RBE calculation, as not all curves reached a clear maximum, precluding the determination of the dose that initiated maximal response. The maximal biological effect was observed

Figure 2. Activation of NF- κ B-dependent d2EGFP gene expression by tumor necrosis factor α (TNF- α) and by high LET radiation, as detected in stably transfected HEK cells from d2EGFP reporter gene expression controlled by a promoter containing four NRE and the minimal promoter of thymidine kinase (TK) (HEK-pNF- κ B-d2EGFP/Neo clone L2). Cells were harvested and fixed for flow cytometry 18 h after treatment. Histogram overlays of representative flow cytometric analyses are shown in A and B. As positive control for activation of NF- κ B dependent d2EGFP gene expression, cells were treated with 10 ng/ml TNF- α (A). An overlay of d2EGFP fluorescence histograms of cells exposed to ³⁶Ar ions (95 MeV/n, LET 272 keV/ μ m) is shown in B. Fluences were converted to absorbed doses according to Equation (1). P/cm² = particles/cm².

between 91 and 272 keV/ μ m for the examined endpoints survival and activation of NF- κ B dependent d2EGFP gene expression (Figure 4). The extent of the RBE strongly depends on the nature of effect. The highest relative biological effectiveness for induction of NF- κ B dependent d2EGFP gene expression is 8.9 (Figure 4B), for reproductive cell death only around 2 (Figure 4A).

Discussion

In this study, we show that energetic argon ions (LET 272 keV/ μ m) and neon ions (LET 91 keV/ μ m) are most effective

Figure 3. Dose effect relationships for activation of NF-κB-dependent d2EGFP gene expression after exposure to X-rays or to various energetic heavy ions, as detected in stably transfected HEK cells by the reporter gene d2EGFP controlled by the promoter containing four NRE and the minimal promoter of TK (HEK-pNF-κB-d2EGFP/Neo L2). Cells were harvested and fixed for flow cytometric analysis 18 h after treatment. Doses (Gy) were calculated from fluences (P/cm^2) according to Equation (1). Means and standard deviations of one to five independent experiments (X-rays: 5; ^{36}Ar : 4; ^{13}C , ^{58}Ni : 2; ^{22}Ne , ^{208}Pb : 1) with 3–6 samples per dose are shown. Significance compared to the mock-irradiated control: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (for X-rays only shown in A). MeV/n = MeV/nucleon.

in activating NF-κB dependent d2EGFP gene expression in human embryonic kidney cells. Relative biological effectiveness for survival reduction also peaks in this LET range, but the RBE is much lower.

Clonogenic survival

The survival curves' exponential course without shoulder after heavy ion exposure conforms to previously published data for the survival of human mammary epithelial cells (Durante et al. 1995), human kidney cells (T-1) (Yang et al. 1977), melanoma and Chinese Hamster Ovary (CHO) cells (Bird and Burki 1975) and CHO V79 cells (Stoll et al. 1995). The analysis of cell death of glioblastoma cell lines after exposure to high LET carbon beams by the colony forming ability test revealed an LET dependent decrease in survival fraction (Tsuchida et al. 1998). Such an LET dependence of reproductive cell death was also observed in human fibroblasts (Hamada et al. 2006). Also in this work, the fluences necessary for cell inactivation were dependent on the LET of the examined heavy ion beams. The higher the LET, the lower were the fluences to achieve the same killing effect. Based on dose, the relationship is more complex, with a maximal killing

effect in the range of 34–91 keV/μm, as the fluences are weighted with the respective LET for conversion to absorbed dose according to Equation (1).

Activation of the NF-κB pathway by different energetic heavy ions

Our first study examining the effects of heavy ions on the NF-κB pathway revealed that high LET argon ions (272 keV/μm) activate the sequence of all events in the NF-κB pathway from DNA binding via transcription to protein translation and maturation (Baumstark-Khan et al. 2005). Here, the LET dependence of this cellular response to heavy ion exposure was examined. Argon ions (272 keV/μm) have the maximal potency to activate NF-κB dependent d2EGFP gene expression in HEK cells. Neon ions (91 keV/μm) have a strong potency for NF-κB activation, and the maximal response was not reached in the examined dose range.

In line with the results presented here, other groups report NF-κB translocation after exposure of normal human monocytes (MM6 cells) to 0.7 Gy of ^{56}Fe ions using a DNA binding assay (Natarajan et al. 2002). This clearly indicates that high LET iron ion exposure induces rapid and persistent NF-κB

Figure 4. Relative Biological Effectiveness (RBE) of energetic ions of different LET in inducing reproductive cell death (A), as measured by colony forming ability, and in activating NF- κ B dependent d2EGFP gene expression (B). 150 kV X-rays were used as reference radiation and RBE was calculated according to Equation (2). The D_0 as descriptor of the survival curves was converted to energy dose for heavy ion exposure (A). The fluences eliciting a doubling of the NF- κ B-dependent fluorescence signal were converted to absorbed dose (B). Means and standard errors for one to five beam times per radiation quality with six replicates are shown (Number of beam times: X-rays: 5; ^{36}Ar : 4, ^{13}C , ^{58}Ni , ^{208}Pb : 2; ^{22}Ne : 1). *Significant difference to the X-ray induced cellular response with $p < 0.05$, **with $p < 0.01$, or ***with $p < 0.001$. MeV/n = MeV/nucleon.

activation. This activation of NF- κ B was shown to be mediated through phosphorylation of I κ B α and the subsequent proteasome-dependent degradation pathway. The iron study only revealed binding of NF- κ B to its consensus sequence of 5'-GGGACTTTCC-3', and not transcriptional activation. Mitra et al. (2004, 2005) determined that the expression of the transcription factor NF- κ B itself is influenced by ^{16}O and ^{12}C ion exposure (LET not published) in CHO V79 cells. Exposure to ^{16}O ions first decreased p65 expression as detected by Western blot 0.5 and 2 h after irradiation, with a recovery slightly over the initial cellular p65 content 4 h after

irradiation (Mitra et al. 2004). NF- κ B p65 expression was enhanced by ^{12}C ion exposure 8 h after 0.1 Gy and already 3 h after 1 Gy (Mitra et al. 2005). Together with the half-life of the reporter protein d2EGFP, this enhanced NF- κ B availability might explain the sustained NF- κ B dependent d2EGFP gene expression which was observed 18 h after heavy ion exposure. Up-regulation of NF- κ B activating cytokines such as interleukins and tumor necrosis factor might also play a role in perpetuating the NF- κ B activation. The DNA-dependent protein kinase (DNA-PK) and phosphatidylinositol kinase 3 (PI3 kinase) inhibitor Wortmannin (10 $\mu\text{mol/l}$) enhanced the ^{12}C ion-induced expression of NF- κ B (Mitra et al. 2005). In oral squamous cell carcinoma cells, a microarray analysis after carbon and neon ion (LET $\sim 75 \text{ keV}/\mu\text{m}$; 1, 4, 7 Gy) irradiation revealed no significant gene expression changes in the NF- κ B signaling pathway (Fushimi et al. 2008). Whether the lack of inducible NF- κ B activation is specific for these cancer cells, e.g., because of a constitutively highly active NF- κ B pathway (Nakayama et al. 2001, Tamatani et al. 2001, Bindhu et al. 2006), or if methodological differences are responsible for the contrary results, it remains to be determined.

The appearance of two populations in the d2EGFP histogram after argon ion exposure might be explained by two different processes: The population with the strong fluorescence increase shows high d2EGFP expression in response to binding of liberated NF- κ B to the synthetic promoter of the stably transfected reporter construct. The subpopulation showing only a very small shift towards higher fluorescence intensities might result from radiation-induced cell cycle arrest, during which d2EGFP continues to accumulate without 'dilution' by cell division. Such a shift was described for UVC-irradiated stably transfected Chinese Hamster Ovary Cells that constitutively expressed EGFP (Baumstark-Khan et al. 1999). The threshold for the d2EGFP $^{(+)}$ cells was set above this subpopulation, in order to exclude possibly unspecific d2EGFP fluorescence.

As activation of the NF- κ B pathway is supposed to play a role in the negative regulation of apoptosis, survival of cells with residual DNA damage might thus be favored. Even if this happens only in a small fraction of irradiated cells, it might be important for the initiation or promotion of cancer. Such effects which may be related to better survival of exposed cells through transitory induction of genes involved in maintaining DNA fidelity and in modulating cell-cycle progression and cell-death, would contribute considerably to astronauts' radiation risk, especially at low doses (Williams et al. 1999).

Enhanced expression of cytokines and extracellular matrix proteases which are regulated by NF- κ B after heavy ion exposure might also contribute to the remodeling of extracellular matrix that is observed after particle irradiation (NCRP 2006). Activation of NF- κ B by high radiation doses (1–10 Gy) could contribute to the inflammatory response observed, e.g., in the developing brain after radiation exposure (Monje et al. 2003).

Relative biological effectiveness of different energetic heavy ions

In this work, the maximal biological effect for the two examined endpoints peaks between 91 and 272 $\text{keV}/\mu\text{m}$. As mentioned

in the *Introduction*, other groups locate maximal biological effectiveness of energetic heavy ions in the range of 90–230 keV/μm.

The maximal relative biological effectiveness strongly depends on the nature of the effect and the cell type. As found in this work, the RBE for activation of NF-κB dependent d2EGFP gene expression is around 9, for reproductive cell death only around 2. The RBE for reproductive cell death is very similar to the RBE for DNA double-strand break induction (~1.8) by energetic heavy ions (Löbrich et al. 1996, Whalen et al. 2008). The RBE for survival also depends on the survival level used for its calculation, and its maximum was described to range between 2 and 4 (Blakely et al. 1984, Alpen et al. 1993, Tsuruoka et al. 2005, Hamada et al. 2006). For LET values above 900 keV/μm, RBE values for reproductive cell death drop below 1, which is in line with observations by Tolkendorf and Eichhorn (1983), Wulf et al. (1985) and Fournier et al. (2001). This overkill effect is explained by an increase in the degree of clustering of the initial physical damage within the target area with an increasing LET. It is seen as cause for the appearance of non-hit cells and the decrease in the mean number of lethal damage per unit dose, resulting in the decrease of RBE for cell killing (Mehnnati et al. 2005).

Assuming that NF-κB activation promotes survival, it can be hypothesized that the extreme capacity of energetic heavy ions in the LET range of 90–300 keV/μm to activate NF-κB's transcriptional effects might be responsible for the lower relative effectiveness in cell killing observed in this range. Above 300 keV/μm the overkill effect possibly results in a decrease of the relative biological effectiveness.

Under these aspects, one can ask how can activated NF-κB influence the survival of heavy ion-irradiated cells? Possible pro-survival genes that might be transcribed at a higher rate are the inhibitor of apoptosis protein genes (IAP), and other anti-apoptotic genes such as bcl-2 (B-cell lymphoma 2), bcl-XL (B-cell lymphoma-extra large) and survivin. Preliminary experiments by Gong et al. have shown that high-LET radiation has a higher potential to activate survivin expression compared to low-LET radiation (Gong et al. 2007, Jin et al. 2008).

The small numbers of particle tracks per cell nucleus – in the narrow LET range where the RBE reaches its maximum – which are necessary to activate NF-κB suggest that NF-κB activation might be relevant for judging the biological effects of heavy ion exposures during long-term space missions. Availability of more sensitive methods to screen NF-κB activation in large sample numbers might enlarge this LET range.

The LET dependence of NF-κB activation found in this work might suggest that a differential activation of cellular signaling pathways by different radiation qualities could contribute to the underlying causes of the LET dependency of biological endpoints such as cell inactivation and mutation or chromosomal aberration induction.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Notice of correction

This paper published online on 6 July 2011 contained an error. Figure 1 A was published twice instead of Figure 1B. This current version of the paper is the correct version. The publisher would like to apologize for the inconvenience caused.