Radiotherapy and Oncology 83 (2007) 277–282
www.thegreenjournal.com

Molecular radiobiology

Interrelation amongst differentiation, senescence and genetic instability in long-term cultures of fibroblasts exposed to different radiation qualities

Claudia Fournier[a,*], Marcus Winter[a], Sebastian Zahnreich[a], Elena Nasonova[b], Larissa Melnikova[b], Sylvia Ritter[a]

[a]*Gesellschaft für Schwerionenforschung/Biophysik, Darmstadt, Germany,* [b]*JINR, Dubna, Russia*

Abstract

***Background and purpose*: The goal of the present study was to investigate aging and genetic instability in the progeny of human fibroblasts exposed to X-rays and carbon ions.**

***Materials and methods*: Following irradiation, cells were regularly subcultured until senescence. At selected time-points BrdU-labelling index, expression of cell cycle related proteins, cell differentiation pattern and chromosome aberrations were assessed.**

***Results*: After exposure, an immediate cell cycle arrest occurred followed by a period of a few weeks where premature differentiation and senescence were observed. In all cultures cycling cells expressing low levels of cell cycle inhibiting proteins were present and finally dominated the populations. About 5 months after exposure, the cellular and molecular changes attributed to differentiation and senescence reappeared and persisted. Concurrently, genetic instability was observed, but the aberration yields and types differed between repeated experiments. The descendants of cells exposed to carbon ions did not senesce earlier and displayed a similar rate of genetic instability as the X-ray progeny. For high doses an impaired cell cycle regulation and extended life span was observed, but finally cell proliferation ceased in all populations.**

***Conclusions*: The descendants of irradiated fibroblasts undergo stepwise senescence and differentiation. Genetic instability is frequent and an extension of the life span may occur.**
 Radiotherapy and Oncology 83 (2007) 277–282.

Keywords: Senescence; Differentiation; Genetic instability; X-rays; Carbon ions; Fibroblasts

Normal human fibroblasts are an adequate model system to study early and late radiation effects, in particular with regard to radiosensitivity [1,7,19]. They undergo differentiation along a lineage from mitotically active fibroblasts (MF I, II and III) into postmitotic fibrocytes (PMF), thereby acquiring the capacity to fulfil diverse physiological functions. Concomittant morphological changes are used to distinguish the differentiation stages [2]. The transition into postmitotic stages implies a finite life span that was observed already in the 1960s and is referred to as senescence [10]. Since then, the onset and maintenance of replicative senescence was ascribed to multiple factors, among them an altered expression of cell cycle regulators [3].

Senescence and terminal differentiation can also be induced by DNA damaging agents, such as radiation exposure to ensure the genetic integrity of fibroblasts [16,22]. However, there is increasing evidence that ionising radiation can induce a delayed destabilisation of the genome, considered as a first step towards transformation and tumor formation [11]. Although in human fibroblasts more genetic changes in essential genes are required for neoplastic transformation than in epithelial cells [20], the motivation for the investigation of mechanisms ensuring genetic integrity in fibroblasts is given by the occurrence of mesenchymal tumors [15]. With growing interest in particle therapy, it is highly desirable to investigate these effects for high LET radiation as well.

Materials and methods

Cell culture and irradiation

Normal human fibroblasts (AG1522 C/D, Coriell Institute, Camden, NJ, USA) were cultured in Eagle's MEM (Bio-Whittacker) supplemented with 10% fetal calf serum (Biochrom) as described in [8]. For irradiation confluent monolayers at

0167-8140/$ - see front matter doi:10.1016/j.radonc.2007.04.022

cumulative population doubling levels (CPD) 20—25 were used consisting of >90% G_0/G_1 cells as determined by flow cytometry.

Cells were exposed to 11.4 MeV/u carbon ions or X-rays (250 kV, 16 mA) at GSI, Darmstadt, as reported before [8]. At sample position the ion energy was 9.8 MeV/u (LET = 170 keV/μm). Fibroblasts were reseeded at a density of 6700 cells/cm^2 after 24 h of incubation. Thereafter, cells were regularly passaged and analysed until sensecence.

BrdU labelling, cell differentiation and SA β-galactosidase activity

Incorporation of BrdU was detected after cumulative or pulse labelling (10 μM for 48 and 1 h, respectively) as described in [19]. The differentiation pattern was analysed based on colony size and morphological features [2,6,7], while SA β-galactosidase activity was measured according to [24].

Quantification of proteins

Western blot analysis with subsequent immunodetection was performed for the overall quantification of cell cycle inhibiting proteins in confluent cells as described elsewhere, including loading correction, references for the densitometric evaluation of the film signals and compensation for nonlinear film responses [8]. The following monoclonal mouse antibodies were used: anti-TP53 (Oncogene), anti-Ser15-phosphorylated p53 (Cell Signaling Technology), anti-p21 (Transduction laboratories), anti-p16 (Neomarkers) and anti-α-tubulin (Sigma). The ratios of irradiated samples were normalised to the ratio of the corresponding control sample for each timepoint.

Immunofluorescence (IF) staining was performed using the same antibodies. Details of the staining procedure and the analysis of fluorescence intensities on the level of single cells are described in [9].

Cytogenetic studies

For cytogenetic analysis metaphase spreads were prepared according to standard techniques and stained with Giemsa. Three independent experiments were performed (X-ray: 2, 8, 16 Gy; carbon ions: 2, 4 Gy). At least 100 metaphases were analysed per time-point and the chromosome number and the yield of chromosome-type and chromatid-type aberrations were scored [19].

Statistical analysis

The number of independent experiments is indicated in each figure. If not stated otherwise, the data points of parallel experiments were averaged and the standard deviation or the standard error of the means (SEM) was calculated.

Results

The fate of irradiated human fibroblasts was studied over 7 months. According to our results, the cellular response has been divided into three phases: the weeks after exposure (phase I), an intermediate period of several months (phase II) and a final period when almost all cells of the population had reached senescence (phase III).

Cell cycle arrest, premature differentiation and expression of markers of senescence after exposure (phase I)

The cumulative BrdU-labelling index 2 days postirradiation revealed a dose-dependent decrease in the number of labelled cells demonstrating a cell cycle arrest in the initial G_0/G_1 phase (Fig. 1a). Carbon ions were more effective than X-rays resulting in a relative biological effectiveness (RBE) of 4.1. Because of this higher effectiveness, dose ranges for carbon ions were chosen as 0—4 Gy, whereas X-ray experiments were performed in the range of 0—16 Gy. BrdU-pulse labelling at 5 and 19 days after irradiation showed that the proliferation index for high doses was still low. However, with increasing time, the differences between carbon ion and X-ray exposure became smaller (Fig. 1b and c).

In two experiments, the differentiation pattern was analysed. As shown in Fig. 2a, a dose dependent premature transition from cells with high mitotic activity (MF II) to cells with low activity (MF III) and finally to postmitotic stages was observed after the second subcultivation 9 days after irradiation. This shift of the differentiation pattern was less pronounced after further subcultivation (i.e. 35 days), indicating that cells of the mitotically active stages dominated the populations (Fig. 2b). Remarkably, the descendants of 16 Gy X-ray irradiated cells either consisted of nearly 100% MF III and stopped proliferation during phase I (not shown), or a subpopulation of cells continued proliferation (indicated in Fig. 2b). The progenies of cells irradiated with carbon ions consisted of more MF II cells than expected when comparing doses leading to the same initial cell cycle delay, in line with the higher number of cells entering S-phase (shown above).

To assess whether the cell cycle arrest observed after exposure leads to replicative senescence, the overall expressions of the senescence-related cell cycle regulators p53, p21 and p16 were quantified by Western blot analysis (Fig. 3). The level of p53 protein increased, peaking at 8 days after exposure and then declined to control levels. A 3- to 5-fold induction of p21 protein was reached between 8 and 21 days in the progeny of both X-ray and carbon ion irradiated cells. This induction persisted (>2-fold) longer only for the descendants of cells irradiated with 16 Gy of X-rays. Similar to the accumulation of p53 protein, the levels of p21 protein were higher following X-rays compared to the initial isoeffective doses as determined from the cell cycle delay. Compared to p21 protein, the induction of p16 protein was delayed and lower. The induction of p16 was observed for both radiation qualities, but earlier in the progeny of X-ray irradiated cells. The general trend of a transiently enhanced mean expression level of cell cycle inhibitors described above was confirmed by the observation of a 2- to 3-fold increase in the number of cells displaying an enhanced activity of senescence related β-galactosidase (SA β-gal). The number of SA β-gal positive cells declined to control levels (7.5 ± 2.5% cells) at 35 days (data not shown).