Original Article

Ionizing Radiation Induces Senescence and Differentiation of Human Dental Pulp Stem Cells

(dental pulp stem cells / ionizing radiation / radiation-induced premature differentiation / dentinogenesis / osteogenesis)

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Abstract. Head and neck cancer is one of the most common cancers in Europe. Many current anti-cancer treatments, including ionizing radiation, induce apoptosis via DNA damage. Unfortunately, such treatments are non-selective to cancer cells and produce similar toxicity in normal cells, including adult stem cells. One of the fundamental properties of an adult stem cell is that it does not have any tissue-specific structures that allow it to perform specialized functions. However, under certain stimuli, unspecialized adult stem cells can give rise to specialized cells to generate replacements for cells that are lost during one's life or due to injury or disease. Nevertheless, specialization of stem cells must be controlled by specific milieu and also initiated at the proper time, making the entire process beneficial for tissue recovery and maintaining it for a long time. In this paper we assess whether irradiated dental pulp stem cells have maintained open their options to mature into specialized cells, or whether they have lost their unspecialized (immature) state following irradiation. Our findings showed radiation-induced premature differentiation of dental pulp stem cells towards odonto-/osteoblast lineages *in vitro.* Matrix calcification was visualized from Day 6 or Day 9 following irradiation of cells expressing low or high levels of CD146, respectively.

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Abbreviations: DPSCs – dental pulp stem cells, DSBs – double-strand breaks, HNOC – head and neck/oral cancer, IR – ionizing radiation, MSCs – mesenchymal stem cells, PLSCs – periodontal ligament stem cells, RT – radiation therapy, SA-β-gal – senescence-associated β-galactosidase, SCs – stem cells, SIPS – stress-induced premature senescence.

Introduction

Adult stem cells (SCs) are undifferentiated cells defined by their ability at the single cell level to both self-renew and differentiate to produce progeny and mature effector cells. Adult SCs are known to be easily capable of differentiating into a variety of specific cell types; in a living organism their differentiation potential reflects the local tissue environment. Native SCs lack tissue-specific characteristics but under the influence of appropriate signals can differentiate into specialized cells with a phenotype and qualities distinct from that of the precursor. In adulthood, SCs play an important role in replacing, replenishing and repairing tissues and cells damaged as a result of aging, injury or the harmful effect of environmental factors. Adult SCs are extremely important in the long-term maintenance of tissue homeostasis throughout life. Indeed, the population of undifferentiated SCs can represent as little as 0.1 % of the total cell population. Adult SCs have been isolated from the brain, bone marrow, peripheral blood, epithelia of the skin and the digestive system, cornea, liver, and pancreas; thus, adult SCs have been found in tissues that develop from all three embryonic germ layers (Pittenger and Marshak, 2001).

Mesenchymal stem cells (MSCs), which belong to adult SCs, were first identified as a residing population

within the stromal compartment of rat bone marrow by Friedenstein and co-workers (Friedenstein et al., 1970). They are characterized by their high proliferative capacity *ex vivo* while maintaining their clonogenic and multi-tissue differentiation potential. Mesenchymal cells natively form connective tissue, including bones, cartilage, adipose tissue, tendons and muscles, and participate in the formation of many craniofacial structures. MSCs have been isolated from bone marrow, periosteum, trabecular bone, adipose tissue, synovium, skeletal muscle and deciduous teeth (Barry and Murphy, 2004).

MSCs sharing biological and stem cell characteristics resembling those isolated from bone marrow were obtained from the dental pulp of permanent teeth. Human dental pulp stem cells (DPSCs) were originally isolated by Gronthos and co-workers (Gronthos et al., 2000) and lately also by the team of Suchanek and colleagues. Extracted third molars, exfoliating/extracted deciduous teeth and teeth extracted for orthodontic treatment are very easily accessible sources of DPSCs (Suchánek et al., 2007). DPSCs were characterized by virtue of their clonogenic abilities, rapid proliferative rates, and capacity to form mineralized tissues both *in vitro* and *in vivo*. Under the influence of specific microenvironment signals, DPSCs are vital for dentine maintenance and the dentine regeneration process in case of injury. Following transplantation into immunocompromised mice, DPSCs had the potential to generate dentine-like tissue. DPSCs were found to be capable of following several cell lineages to ultimately produce terminally differentiated cells such as odontoblasts and osteoblasts. More detailed studies proved the multi-potentiality of DPSCs. This meant that DPSCs were able to form adipocytes, chondrocytes and neural cells in different inductive media (Gronthos et al., 2000, 2002; Karbanová et al., 2011).

Radiation therapy (RT) is an effective form of cancer treatment. At present, at least half of all cancer patients will receive RT at some stage during the course of their treatment (Schneider, 2011). Unfortunately, the direct and indirect interaction of radiation with cells during RT can also cause side effects by damaging normal, healthy cells surrounding the cancer cells. These side effects correlate with the sensitivity of the cells to radiation, the absorbed dose and the time of exposure. Ionizing radiation (IR) induces a variety of DNA lesions in the exposed cells, of which DNA double-strand breaks (DSBs) are considered the most important type of lesion for the biological effects of IR. The molecular pathways that are triggered after radiation exposure within cells act in an integrated fashion to determine a variety of possible responses, whether they be DNA repair, cell cycle arrest, terminal differentiation, accelerated senescence, cell death via apoptosis or necrosis, and adaptive responses – genomic instability or carcinogenesis (Canman et al., 1998).

MSCs of various origin, DPSCs, periodontal ligament stem cells (PLSCs), rat or human bone marrow MSCs treated by IR are considered as resistant to radiation-induced apoptosis (Chen et al., 2006; Schönmeyr et al., 2008; Muthna et al., 2010; Cmielova et al., 2012). Cells respond to IR by cell cycle arrest in G2 phase (Chen et al., 2006; Schönmeyr et al., 2008; Muthna et al., 2010; Cmielova et al., 2012); they do not divide, remain viable and enter into stress-induced premature senescence – SIPS (Schönmeyr et al., 2008; Muthna et al., 2010; Cmielova et al., 2012).

Senescence is a form of irreversible growth arrest that halts proliferation of the metabolically active aging or damaged cells. Stress-induced premature senescence can be induced by treatment with DNA-damaging agents (Robles and Adami, 1998), whereas replicative senescence limits the proliferative life span of normal cells (Chang et al., 1999). The senescence phenotype is characterized by enlargement and flattening of the cell shape, increased granularity and expression of senescence-associated β-galactosidase (SA-β-gal). Cellular senescence in some attributes resembles terminal differentiation, when in both processes it causes cells to irreversibly lose the ability to proliferate in response to mitogenic agents (Wier and Scott, 1986). Both differentiated and senescent cells are growth-arrested and neither can be stimulated to re-enter the cell cycle by any known combination of physiological mitogens, even though such cells can be maintained in this viable, albeit non-replicating state for very long periods of time (Hayflick and Moorhead, 1961; Hayflick, 1965; Pignolo et al., 1998). At the molecular level, both cell differentiation and senescence are associated with an altered pattern of gene expression, much of which is transcriptionally based (Cristofalo et al., 1992). There are also some other theories stating that both differentiation and stress-induced premature senescence could act as tumour suppressive mechanisms (Campisi, 1997; Wangenheim and Peterson, 1998).

The aim of this study was to assess the effects of ionizing radiation on the kinetics of senescence induction and odontogenic and/or osteogenic differentiation of DPSCs. We evaluated spontaneous (i.e. not resulting from stimulation using a lineage-specific differentiation medium) radiation-induced premature differentiation and senescence of DPSCs cultured in a basal stem cell medium in relation to CD146 expression, with the intention to explore whether irradiated stem cells maintain their potency of immature cells for tissue regeneration. In our experiments we used DPSCs expressing high or low levels of CD146 obtained from four healthy donors. CD146 is a transmembrane isoform of the mucin-like glycoprotein (also known as MCAM, Mel-CAM, S-Endo-1, A32 antigen, and MUC18) with ~2-fold difference in expression between tri- and uni-potent clones. CD146 has been previously reported to be up-regulated on highly proliferative human bone marrow MSCs, which are additionally capable of trilineage differentiation (Baksh et al., 2007; Sorrentino et al., 2008). Furthermore, there is a growing body of evidence that CD146 is able to regulate proliferation through transmembrane signalling (Russell et al., 2010). Spontaneous

dentinogenesis and/or osteogenesis was assayed by alizarin red S staining for calcium since dentin and bone matrix are mainly composed of hydroxyapatite (hydroxyapatite forms 65–70 % of dentine and up to 50 % of bone matrix).

Material and Methods

Cell culture, isolation and culture conditions

Dental pulp stem cells were isolated from impacted third molars obtained from healthy donors undergoing tooth extraction for orthodontic reasons. Subjects, or their parents/guardians, gave their written informed consent to use of the extracted teeth following ethical approval by the Ethics Committee of the Medical Faculty in Hradec Králové. The cells were treated with standard operation protocols. In brief, third molars were extracted under aseptic conditions, and dental pulp tissue was isolated in the tissue culture laboratory. Both the tooth and the dental pulp were treated by collagenase enzymes (Sevapharma, Prague, Czech Republic) and dispase enzymes (Invitrogen, Carlsbad, CA) for 70 min. Following centrifugation (600 *g*, 5 min), a cell pellet was obtained. DPSCs were cultivated in 5% CO_2 atmosphere under 37 °C in a low foetal calf serum cultivation medium composed of α-minimum essential medium (MEM; Invitrogen), heat-inactivated 2% foetal calf serum (FCS; PAA, Dartmouth, NH), 10 ng ml^{-1} EGF, 10 ng ml^{-1} PDGF (both from PeproTech, Rocky Hill, NJ), 0.2 mM L-ascorbic acid (Sigma-Aldrich, St. Louis, MO), 2% glutamine, 100 U/ml penicillin/streptomycin, 20 µg/ml gentamycin (all from Invitrogen) and 50 nmol l^{-1} dexamethasone and 10 µl/ml ITS (Sigma). DPSCs were initially cultivated for 5–7 days in culture flasks with Cell$^+$ surface® (Sarstedt, Newton, NC). After that, small colonies were found and using trypsin-EDTA (ethylenediaminetetraacetic acid) (Invitrogen), they were dissociated and reseeded into standard tissue culture-treated flasks (NUNC, Roskilde, Denmark). Each of the following passages were performed after reaching 70% confluence.

The DPSCs used in this study were characterized and their cytogenetical stability and differentiation potential was tested in our previous studies (Suchánek et al., 2007; Suchanek et al., 2009; Karbanová et al., 2011). To control the quality of the cell population, phenotype characteristics (CD45, CD29, CD44, CD73, CD90, CD166 and CD146) were controlled every 1st, 3rd, 7th and 11th passage, whilst morphology and viability were tested at every passage. Karyotype was controlled at passages 5 and 10, and the length of telomeres was measured at passages 0, 2, 5, 7 and 10. No significant changes were detected during the cultivation. Early passages (3rd–7th) were used for the experiments. Dental pulp stem cells were classified as low-expressing or high-expressing based on the level of CD146 expression and the proportion of positive cells described previously (Karbanová et al., 2011).

Ionizing radiation

Exponentially growing human dental pulp stem cells were suspended in a basic medium at a maximal concentration of 1×10^5/ml. Aliquots of cell suspension were plated into 75 cm^2 flasks or well-plates (TPP, Trasadingen, Switzerland or NUNC) and after reaching 50% confluence, generally 24 h later, they were irradiated at room temperature using a ^{60}Co γ-ray source (Chisotron Chirana, Prague, Czech Republic) at a distance of 1 m from the source with a photon dose-rate of 1 Gy/min. Dosimetry was performed using an ionization chamber (Dosemeter PTW Unidos 1001, Serial No. 11057, with ionization chamber PTW TM 313, Serial No. 0012; RPD Inc., Albertville, MN); the set was validated by the Czech Metrology Institute (CMI) – Inspectorate for Ionizing Radiation (IIR), Protocol No. 9011-OL-U4124/2005). Media were replaced every 2–3 days. After irradiation, flasks or well-plates were incubated in 5% CO_2 atmosphere under 37 °C and cells were collected following various time periods for analysis. Non-irradiated control cells were handled in the same way, except that irradiation was omitted.

Growth curve and cell viability

Human dental pulp stem cells expressing high or low levels of CD146 were seeded into Falcon flasks (TPP, Trasadingen, Switzerland or NUNC) at a density of 1×10^5/ml and irradiated with a dose of 6 Gy after 24 h. For nine subsequent days, cell counting was performed using the Z2-Counter (Beckman Coulter, Miami, FL). Cell viability was determined on the 9th day after irradiation using the Vi-CELL Viability Analyzer (Beckman Coulter) based on the trypan blue reagent. Control groups of non-irradiated cells were also examined.

Detection of senescence-associated β-galactosidase activity

Microscopy: The cells in 50% confluence were irradiated in chamber slides (Thermo Fisher Scientific, Pardubice, Czech Republic). SA-β-galactivity was detected using a Senescence β-galactosidase Staining Kit (Cell SignalingTechnology, Danvers, MA) according to the manufacturer's instructions. Control cells were handled in the same way, except that irradiation was omitted.

Flow cytometry: We used the fluorogenic substrate 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C_{12}FDG) (Invitrogen). C_{12}FDG is a substrate which, when hydrolysed by SA-β-gal to C_{12}-fluorescein, becomes fluorescent and membrane impermeable. Subconfluent cells seeded in 75 cm^2 Falcon flasks were exposed to ionizing radiation. The activity of SA-β-gal in irradiated and sham-treated cells was analysed according to the method published by Debacq-Chainiaux et al. (2009). Single-cell suspension was prepared by a 5-min exposure to 0.25 trypsin-EDTA solution (Sigma Aldrich); inactivation of the enzyme was done using a

medium containing 20 % FCS; and the required amount of cells was used. The C_{12}-fluorescein signal was quantified in the FL1 (530/540 nm) detector of the flow cytometer CyAn ADP (Beckman Coulter) and β-galactosidase activity was presented as the mean of C_{12}-fluorescein fluorescence intensity. Experiments were performed in hexaplicate.

Alizarin red S staining visualization and quantification

To detect and quantify radiation-induced premature differentiation of DPSCs towards odonto-/osteoblast lineages *in vitro*, cells were irradiated and subsequently cultured in a basal medium before analysis. Media were replaced every 2–3 days. After 6 or 9 days of culturing in a basal medium, six Petri dishes per group were stained histochemically to determine mineralization. To assess osteo-/dentinogenic differentiation, cells were stained for calcium content in mineralized nodules formed by DPSCs using the alizarin red S staining method as described by Stanford et al. (1995). Briefly, after 6 or 9 days, cells were washed with PBS and fixed in ice cold 70% ethanol for at least 1 h. Ethanol was removed and the cells were rinsed with distilled water and stained with 2% alizarin red S (Sigma-Aldrich) dissolved in distilled water with pH adjusted to 4.2 for 10 min at room temperature. Stained cells were further processed by five rinses with distilled water, followed by a wash in PBS to reduce any nonspecific alizarin red S stains and finally photographed digitally at 100× magnification.

Quantitative analysis of alizarin red S staining was performed by extracting the dye from the stained monolayer of cells and determining its optical density at 450 nm. Alizarin red S (2%)-stained cells were incubated with 10% acetic acid for 30 min to release bound alizarin red S into the solution. The solution was neutralized with 10% ammonium hydroxide (all reagents from Sigma-Aldrich) and the absorbance of alizarin red S was measured at 450 nm using the microplate reader Tecan Infinite 2000 (Tecan Group, Männedorf, Switzerland). Results were expressed as the arithmetic means of six independent experiments, and bars indicated SD. Absorbances were normalized to 1×10^6 cells.

Statistics

The descriptive statistics of the results were calculated and the charts were made in either Microsoft Office Excel 2003 (Microsoft, Redmond, WA) or GraphPad Prism 5 biostatistics (GraphPad Software, La Jolla, CA). In this study, all the values were expressed as arithmetic means with SD of a hexaplicate, unless otherwise noted. The significant differences between the groups were analysed by Student's *t*-test and P values ≤ 0.05 were considered statistically significant.

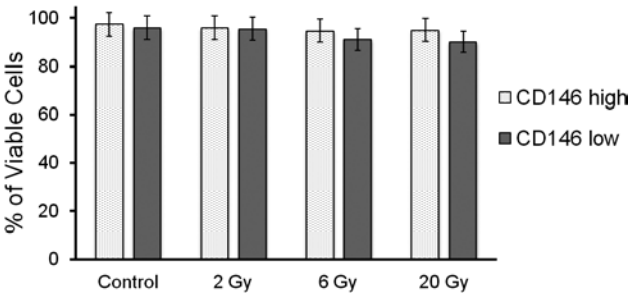

Fig. 1. The viability of human dental pulp stem cells nine days following irradiation by 2, 6 and 20 Gy
Dental pulp stem cells expressing high or low levels of CD146 were seeded in a basal stem cell medium and irradiated after 24 h with a dose of 2, 6 and 20 Gy. The percentage of viable cells was determined by Vi-Cell using trypan blue exclusion staining nine days following irradiation. Data represent the mean ± SD of a hexaplicate (N = 6). No statistically significant difference between the irradiated group and the non-irradiated control group was observed.

Results

Viability and proliferation of DPSCs

Irradiated dental pulp stem cells were viable during the entire examined intervals. There were no statistically significant differences in viability between groups of irradiated and control cells nine days following irradiation. The human DPSCs expressing high or low levels of CD146 remained viable even after exposure to a dose of 20 Gy (Fig. 1). Irradiation of DPSCs by the dose of 6 Gy strongly inhibited growth of the cells. The rate of growth was markedly lower as compared with non-irra-

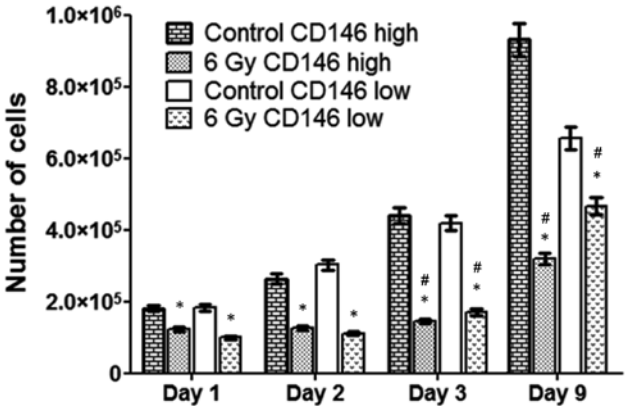

Fig. 2. Total number of human dental pulp stem cells at different time points after irradiation
Dynamics of cell proliferation, determined as the total number of cells during nine days after irradiation. Values from six different experiments are shown as the means ± SD. * – significantly different as compared to control cells at corresponding time intervals (P ≤ 0.05). # – significantly different to the respective group of 6 Gy-irradiated sample of cells expressing high or low levels of CD146 on the 2nd day (P ≤ 0.05).

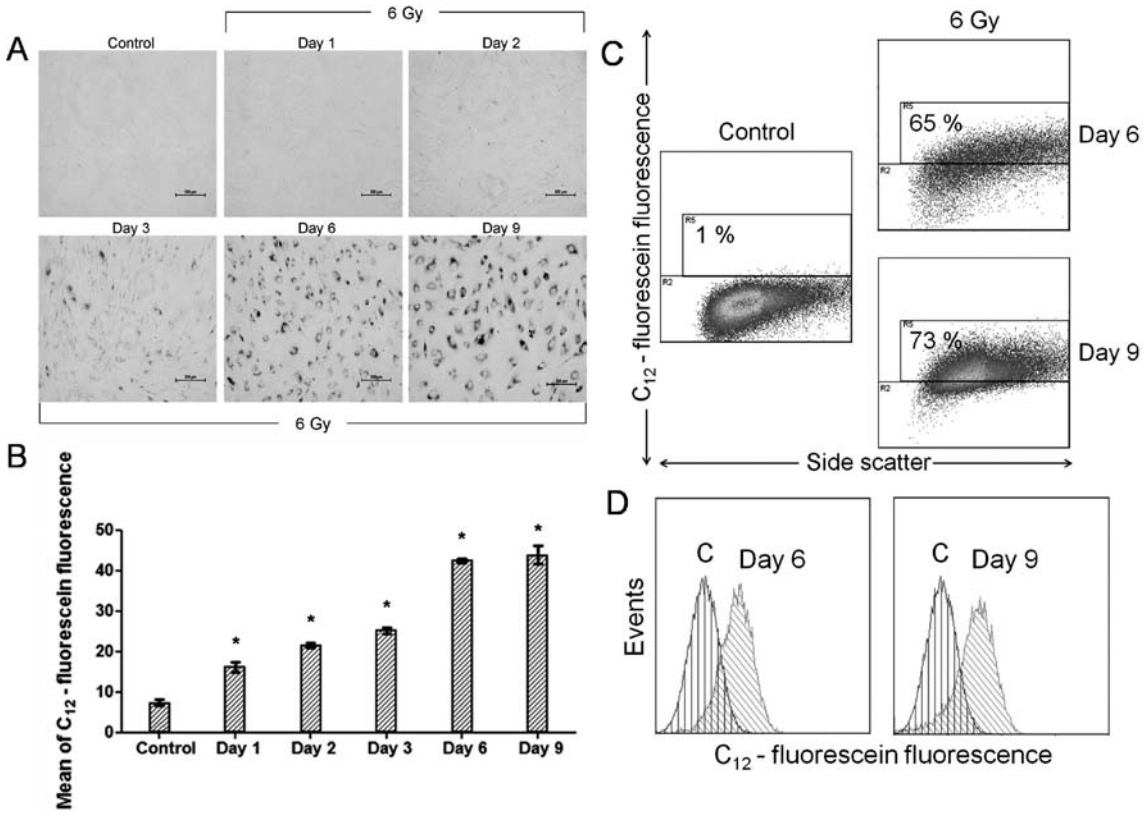

Fig. 3. Time-dependent kinetics of senescence induction in human dental pulp stem cells expressing high levels of CD146 irradiated with a dose of 6 Gy

(A) Human dental pulp stem cells were irradiated in chamber slides and SA-β-galactosidase activity was detected histochemically at the desired intervals. Fig. A shows representative projections of control and irradiated samples obtained by microscopy.

(B) Subconfluent human dental pulp stem cells were irradiated in tissue culture flasks (75 cm^2) and SA-β-gal activity was quantified by flow cytometry at the desired intervals. The results in the graph represent the arithmetic mean of the C_{12}-fluorescein fluorescence intensity depending on the time after irradiation; error bars indicate SD for N = 6 independent experiments. * – significantly different as compared to control cells ($P \leq 0.05$).

(C) Flow-cytometric data are shown as bivariate histograms (dotplots) of the C_{12}-fluorescein fluorescence intensity on the log scale (y axis) vs. the side scatter channel SSC (x axis).The numbers in the corner depict the percentage of cells with bright fluorescence. The histograms shown are from one of six representative experiments.

(D) Flow-cytometric data are shown as C_{12}-fluorescein fluorescence single-parameter overlay histograms. The fluorescence intensity of control (C) and irradiated samples at desired intervals is represented on the abscissa, in arbitrary units, and the relative number of cells is represented on the ordinate. The histograms shown are from one of six representative experiments.

diated controls ($P \leq 0.05$, Student's *t*-test, *). During the first two days after irradiation cells did not proliferate, at three days they had recovered and they started to grow in number. The total numbers of DPSCs in the irradiated groups were higher at three and nine days after irradiation, contrary to two days post irradiation ($P \leq 0.05$, Student's *t*-test, #). The recovery process during proliferation was more successful for CD146 low-expressing cells, compared to CD146 high-expressing cells (Fig. 2).

Kinetics of senescence induction in DPSCs

In order to measure the kinetics of senescence induction in human DPSCs after irradiation we used chromogenic and fluorogenic substrates. Senescence-associated β-galactosidase activity was monitored by flow cytometry using a fluorogenic substrate after lysosomal alkalinization, with the aim to permit measurements in live cells and to increase the sensitivity of senescence determination. Using microscopy and a chromogenic substrate we visualized SA-β-gal activity in the monolayer of cells cultured in chamber slides. Three days after having been exposed to γ radiation, SA-β-gal activity was rarely detected by microscopy. In a later period of six days, an insoluble blue cleavage product occurred diffusely within all DPSCs expressing high or low levels of CD146, respectively, whereas it was absent in non-irradiated control cells (Figs. 3A and 4A).

For quantification of the increase in SA-β-gal activity, flow-cytometric detection was used. We observed a statistically significant ($P \leq 0.05$, Student's *t*-test, *) increase of SA-β-gal activity (measured as the mean of C_{12}-fluorescein fluorescence intensity) on Day 1 after

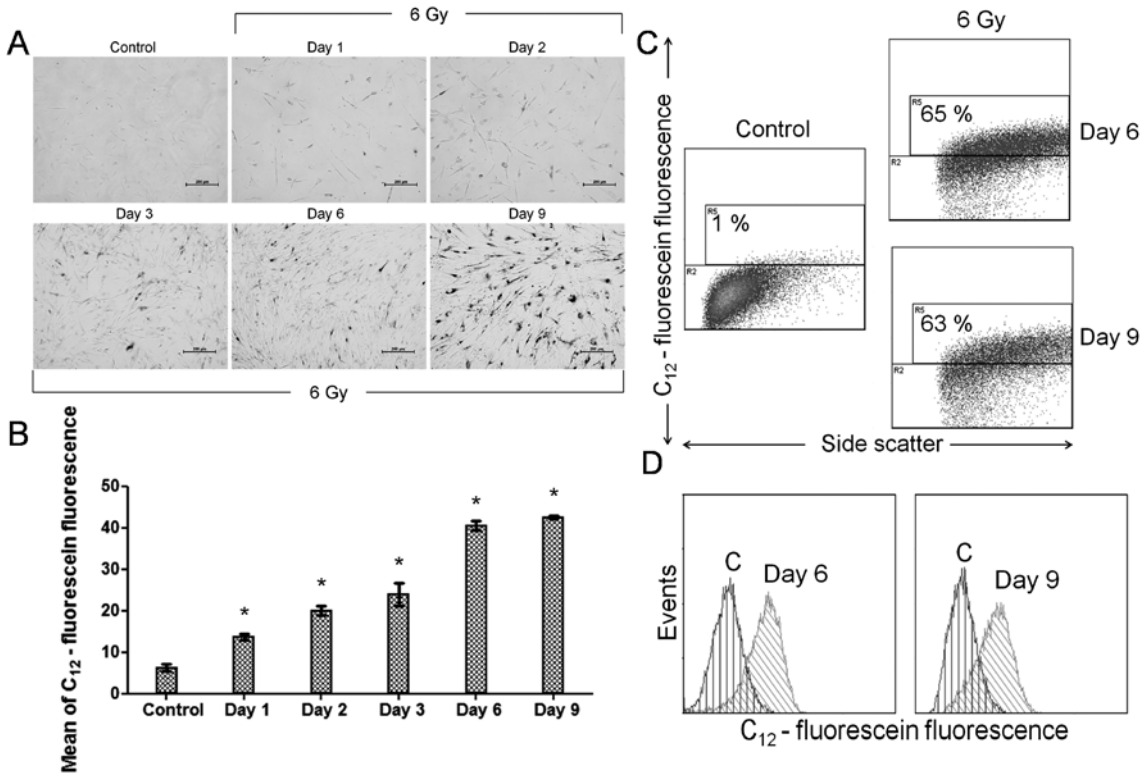

Fig. 4. Time-dependent kinetics of senescence induction in human dental pulp stem cells expressing low levels of CD146 irradiated with a dose of 6 Gy

(A) Human dental pulp stem cells were irradiated in chamber slides and SA-β-galactosidase activity was detected histochemically at the desired intervals. Fig. A shows representative projections of control and irradiated samples obtained by microscopy.

(B) Subconfluent human dental pulp stem cells were irradiated in tissue culture flasks (75 cm^2) and SA-β-gal activity was quantified by flow cytometry at the desired intervals. The results in the graph represent the arithmetic mean of the C_{12}-fluorescein fluorescence intensity depending on the time after irradiation; error bars indicate SD for N = 6 independent experiments. * – significantly different as compared to control cells ($P \leq 0.05$).

(C) Flow-cytometric data are shown as bivariate histograms (dotplots) of the C_{12}-fluorescein fluorescence intensity on the log scale (y axis) vs. the side scatter channel SSC (x axis). The numbers in the corner depict the percentage of cells with bright fluorescence. The histograms shown are from one of six representative experiments.

(D) Flow-cytometric data are shown as C_{12}-fluorescein fluorescence single-parameter overlay histograms. The fluorescence intensity of control (C) and irradiated samples at desired intervals is represented on the abscissa, in arbitrary units, and the relative number of cells is represented on the ordinate. The histograms shown are from one of six representative experiments.

irradiation independently of the CD146 status. The mean of C_{12}-fluorescein fluorescence continually increased until six days post irradiation and then reached a plateau. This increase was homogenous across the entire population of irradiated cells (Figs. 3B, C, D and 4B, C, D). The increase in SA-β-gal activity was accompanied by changes in cell morphology. The cells became flattened, enlarged (Figs. 3A and 4A) and more granular, which was observed via a shift in flow-cytometric side scatter (Figs. 3C and 4C).

Radiation-induced premature differentiation of DPSCs

Furthermore, we investigated whether irradiated DPSCs have maintained open their options to mature into differentiated effector cells based on the hypothetical microenvironment of the tissue, or whether they have lost their immature (naive) state after irradiation. To examine radiation-induced differentiation of DPSCs cultured in a basal stem cell medium, the odontogenic/osteogenic response of DPSCs was used as an experimental model, with respect to their natural differentiation capacity *in vivo*. Matrix calcification was visualized using alizarin red S staining at Day 6 and Day 9 following irradiation for cells expressing high or low levels of CD146 in comparison with non-irradiated control cells which were stained in the same way. Irradiated DPSCs expressing high levels of CD146 showed an increase in alizarin red S staining after nine days. DPSCs expressing low levels of CD146 proved positive for alizarin red S staining earlier in the group of irradiated cells (after six and nine days) as compared with cells expressing high levels of CD146 (Fig. 5A).

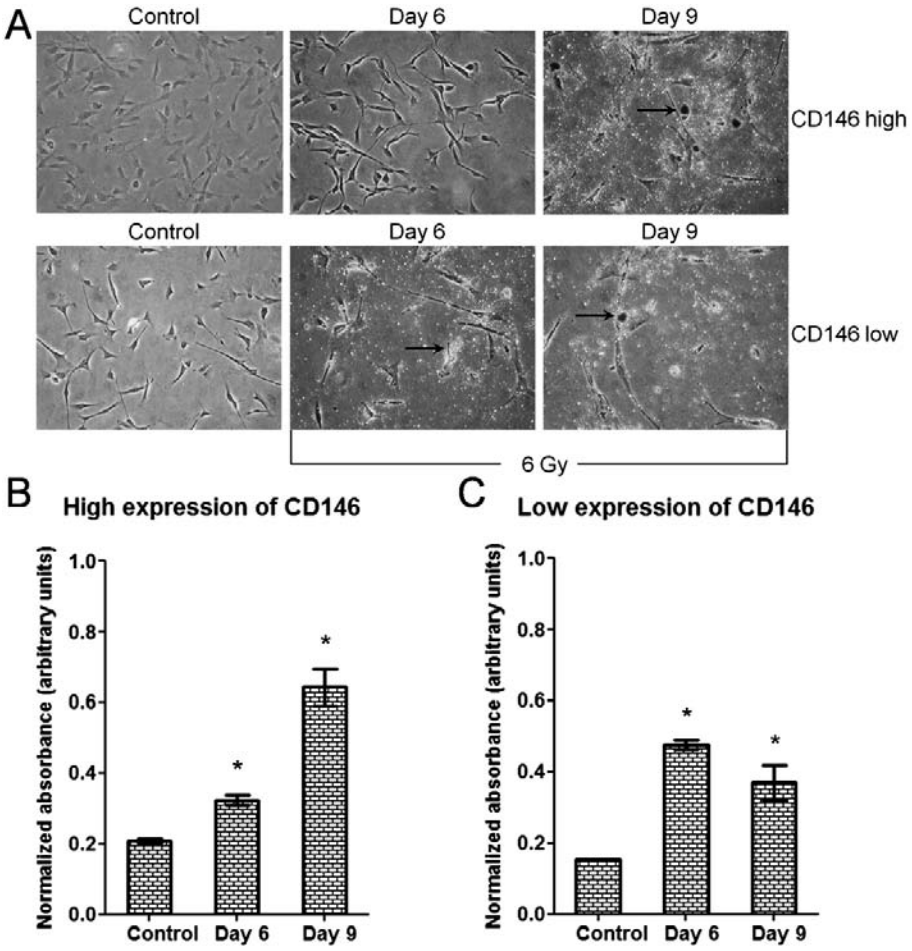

Fig. 5. Osteogenic differentiation of human dental pulp stem cells expressing high or low levels of CD146 cultured for six and nine days following irradiation

Dental pulp stem cells expressing high or low levels of CD146 were seeded in a basal stem cell medium and after 24 h irradiated with a dose of 6 Gy. The cells were subsequently cultured and the differentiation response was assessed through alizarin red S staining for calcium after six and nine days. Irradiated dental pulp stem cells generated calcified nodules (arrows). Experiments were performed in hexaplicate and photographs from representative chambers are shown. The photographs were not analysed statistically **(A)**. The alizarin red S (2%)-stained cells were incubated with 10% acetic acid for 30 min to release bound alizarin red S into the solution. The solution was neutralized with 10% ammonium hydroxide and the absorbance of alizarin red S was measured at 450 nm using a microplate reader for CD146 high **(B)** and CD146 low **(C)** expressing cells. Control cells were handled in exactly the same way as the irradiated cells except that they were not irradiated. Columns represent the arithmetic means of six independent experiments, bars indicate SD (N = 6). * – significantly different as compared to control cells ($P \leq 0.05$). Absorbances were normalized to 1×10^6 cells.

Quantification of matrix mineralization was performed by leaching of the alizarin red S stain and measurement of its absorbance at 450 nm. Values were normalized to the cell number (1×10^6 cells). Quantitative determination of alizarin red S staining in irradiated cultures of DPSCs showed a statistically significantly ($P \leq 0.05$, Student's *t*-test, *) higher Ca^{2+} accumulation than that of non-irradiated controls. In irradiated DPSCs expressing high levels of CD146 the amount of the alizarin red S stain continually increased until Day 9. However, in irradiated DPSCs expressing low levels of CD146 the amount of the alizarin red S stain peaked six days post irradiation (Figs. 5B and 5C).

Discussion

Over 140,000 patients in Europe are diagnosed each year as having cancer of the head and neck, resulting in approximately 65,000 deaths per year. Cancers of the oral cavity and pharynx are the most common type of head and neck cancer. It occurs predominantly in an older population, occurring in males more than in females at all sites. In absolute numbers, head and neck/oral cancer (HNOC) is the sixth most frequent cancer worldwide (Ferlay et al., 2010). At present, RT belongs to the treatments of choice for cancers that are diagnosed early (Chen et al., 2010). Unfortunately, various site-specific complications may develop, depending on the treatment volume, fractionation, and prescribed dose

during RT. Head and neck RT can irreversibly injure oral mucosa, vasculature, muscle, and bone, resulting in xerostomia, rampant dental caries, trismus, soft tissue necrosis, and osteonecrosis (Otmani, 2007). Since DPSCs are shown to reside in the perivascular regions of the pulpal cavity, recovery after RT vitally requires association and cooperation with stem cells, including DPSCs (Téclès et al., 2005). Thus, in this paper we studied the effects of ionizing radiation on human DPSC differentiation and senescence, because alterations in the stem cell pool and unique stem cell properties in terms of the potential for cellular differentiation may contribute to poor recovery of craniofacial tissues after head and neck RT. Our hypothesis reflects the facts that human DPSCs have responded during injury by differentiation into terminally differentiated odontoblast-like cells, which synthesize reparative tubular dentine (sometimes referred to as osteodentine) *in vivo* (Gronthos et al., 2000, 2002; Sloan and Waddington, 2009). In a later study, DPSCs demonstrated the ability to further differentiate along odontogenic, myogenic and adipogenic pathways *in vivo* after transplanting cells/collagen scaffold constructs into subcutaneous tissue of immunocompromised mice (Zhang et al., 2008).

It has been known for years that contrary to haematopoietic stem cells (Milyavsky et al., 2010), mesenchymal stem cells are resistant to radiation-induced apoptosis (Chen et al., 2006; Schönmeyr et al., 2008; Muthna et al., 2010; Cmielova et al., 2012). Previous experiments showed that DPSCs cultured in a basal stem cell medium respond to IR by cell cycle arrest in the G2 cell cycle phase, apoptosis is not induced, cells remain viable, but enter into stress-induced premature senescence (Muthna et al., 2010). Although post-irradiation survival rates of DPSCs are vital for cellular function, the reduced amount of naive dental pulp stem cells and their radiation-induced premature differentiation may contribute to a reduced recovery of radiation-injured tissues. We thus undertook a study to assess the kinetics of senescence induction and osteogenic differentiation.

As in the study of Muthna et al. (2010), we did not demonstrate any statistically significant decrease of cell viability nine days after irradiation by the doses of up to 20 Gy. The majority of DPSCs remained viable, irrespective of the CD146 status, even after exposure to a lethal dose of 20 Gy. Irradiated DPSCs were strongly inhibited in proliferation contrary to non-irradiated controls during those nine days. Similar observations describing the loss of the ability to proliferate after irradiation were published based on experiments with human mesenchymal stem cells isolated from bone marrow (Chen et al., 2006; Cmielova et al., 2012), rat mesenchymal stem cells isolated from bone marrow (Schönmeyr et al., 2008), human DPSCs (Muthna et al., 2010) or human periodontal ligament MSCs (Cmielova et al., 2012). Although the total number of cells was lower in the group of irradiated cells, they recovered within two days after irradiation by 6 Gy in culture and started to proliferate, at least partially. Proliferation kinetics was delayed during the entire experiment. Three and nine days after irradiation by 6 Gy, the number of cells was significantly higher than at two days. These observations are in conformity with results published in papers studying human DPSCs (Muthna et al., 2010) and human mesenchymal stem cells derived from periodontal ligaments (Cmielova et al., 2012). Interestingly, the rate of cell growth seems to be higher for cells expressing high levels of CD146 against those expressing low levels of CD146.

When the effects of ionizing radiation on the kinetics of senescence induction were analysed and quantified by flow cytometry, we discovered a statistically significant increase of SA-β-gal activity already one day after irradiation irrespective of the CD146 status. The results were cross-verified using the histochemical method of staining for SA-β-gal. Employing microscopic detection we observed an increase of SA-β-gal activity as soon as three days after irradiation by 6 Gy, followed by a massive increase on Day 6 and Day 9. The discrepancy between the results of flow cytometry-based quantification and microscopic detection of SA-β-gal activity is most likely caused by the different methodological approach of these two assays. Microscopy is based on the staining of glass slides using a chromogenic substrate followed by heat fixation, contrary to the flow cytometry-based method using a fluorogenic substrate, which *in situ* of living cells forms C_{12}-fluorescein based on β-galactosidase activity after lysosomal alkalinization. Formation of fluorescent molecules capable to emit fluorescence after excitation will probably cause higher sensitivity of flow cytometry. Notwithstanding the novelty of the method (Debacq-Chainiaux et al., 2009), our observations are in agreement with other studies determining SA-β-gal activity in mesenchymal stem cells by flow cytometry (Schellenberg et al., 2011). Almost identically to our results, Schönmeyr et al. (2008) proved an increase in the SA-β-gal intensity of staining in rat bone marrow MSCs three and seven days after irradiation by 7 and 12 Gy. An increase in SA-β-gal activity was detected in DPSCs irradiated by 20 Gy 3–13 days post irradiation (Muthna et al., 2010). A recent study proved an increase of SA-β-gal activity in bone marrow and periodontal ligament MSCs six and 13 days following irradiation by 20 Gy (Cmielova et al., 2012). These findings are in agreement with SIPS induced by sub-cytotoxic doses of genotoxic stress in other cell types. Lung, skin, embryonic fibroblasts, melanocytes and endothelial cells undergo SIPS by these treatments (Suzuki and Boothman, 2008). However, the results published by Chen and colleagues contrast our conclusions. These authors did not detect any increase in SA-β-gal activity in bone marrow MSCs five days after irradiation by 9 Gy (Chen et al., 2006).

In experiments comparing the odontogenic/osteogenic differentiation of DPSCs expressing high or low levels of CD146 subjected to or excluded from the irradiation procedure, the subconfluent layer of stem cells grown in a basal stem cell medium were irradiated with

a single dose of 6 Gy. The control group was processed under the same conditions excluding γ irradiation. Based on the quantification of alizarin red S staining, DPSCs showed a statistically significant increase in mineralization as early as six days post irradiation. The robust mineralization, which we were able to visualize as a calcified nodule, was captured by a phase contrast microscope six days post irradiation for CD146 low-expressing cells and nine days post irradiation for CD146 high-expressing cells. The radiation-induced differentiation described in this study is consistent with the findings of a previous study on irradiated bone marrow-derived rat mesenchymal stem cells cultured in media without lineage-specific differentiation supplements. Schönmeyr et al. (2008) reported an increase in the expression of genes associated with bone differentiation in irradiated cells. The expression of Runt-related transcription factor 2, alkaline phosphatase and osteocalcin was up-regulated seven and 14 days post irradiation by 7 and 12 Gy. Bone differentiation was confirmed by histochemical staining for alkaline phosphatase, demonstrating an increase of staining in irradiated cells seven days post irradiation as compared with controls. Recent study has demonstrated the importance of spontaneous osteogenic differentiation of bone marrow MSCs *in vivo* for bone marrow recovery after sublethal total body irradiation of mice by 4 Gy. A shift in bone marrow MSC osteogenic differentiation properties accompanied by an increase in alkaline phosphatase activity and osteoblastic marker expression was observed *in situ* three days after irradiation of mice. The results of Poncin et al. (2012) indicate that mouse bone marrow MSCs are relatively radioresistant in case of 4 Gy irradiation and suggest that these cells proliferate during bone marrow recovery *in vivo*. The controversial effect of ionizing radiation was observed in irradiated human MSCs by Xiaohui et al. (2010). A middle dose (4 Gy) of irradiation caused morphological changes in MSCs, as well as an alteration in both the proliferation and differentiation potentials of cells without further specifications by the authors. Contrary to our results, a dose of 4 Gy of ^{60}Co γ radiation markedly decreased cell viability assayed by trypan blue exclusion staining (Xiaohui et al., 2010).

In conclusion, our findings suggest that ionizing radiation initiates spontaneous odontogenic/osteogenic differentiation of DPSCs and that the cells become senescent without affecting their viability. It seems that the loss of tissue function resulting from a lack of regenerative capacity after DNA damage may not only be due to an insufficient number of viable adult stem cells within the tissues, but also due to other phenomena such as spontaneous differentiation and senescence in the stem cell pool. This fact is especially interesting since MSCs are known to actively migrate to compartments comprised of non-stem cells and interact with them upon injury (Mauney et al., 2010). To our knowledge, this is the first work studying radiation-induced differentiation of DPSCs. The data published so far reported increase in spontaneous differentiation of irradiated rat bone marrow MSCs cultivated in media without lineage-specific differentiation supplements (Schönmeyr et al., 2008). This finding could have profound implications in the field of radiation biology/oncology, although further studies are still required to clearly address the underlying mechanisms involved in the multi-factorial and multi-step events leading to the loss of the multi-tissue regenerative potential of DPSCs after irradiation.

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