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Effects of ionizing irradiation and interface backscatter on human mesenchymal stem cells cultured on titanium surfaces

Lisa Printzell[1], Janne E. Reseland[2], Nina F. J. Edin[3], Jan E. Ellingsen[1]

[1]Department of Prosthodontics, Faculty for Dentistry, Institute of Clinical Dentistry, University of Oslo, Oslo; [2]Department of Biomaterials, Faculty for Dentistry, Institute of Clinical Dentistry, University of Oslo, Oslo; [3]Department of Physics, Faculty of Mathematics and Natural Science, University of Oslo, Oslo, Norway

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Radiotherapy to the head and neck region negatively influences the osseointegration and survival of dental implants. The effects of cobalt 60 (^{60}Co) ionizing radiation and the impact of backscatter rays were investigated on human mesenchymal stem cells cultured on titanium surfaces. Bone marrow-derived human mesenchymal stem cells were seeded on titanium (Ti), fluoride-modified titanium (TiF), and tissue culture plastic. Cells were exposed to ionizing γ-radiation in single doses of 2, 6, or 10 Gy using a ^{60}Co source. Density and distribution of cells were evaluated using confocal laser-scanning microscopy, 21 d post-irradiation. Lactate dehydrogenase concentration and the levels of total protein and cytokines/chemokines were measured in the cell-culture medium on days 1, 3, 7, 14, and 21 post-irradiation. Unirradiated cells were used as the control. Irradiation had no effect on cell viability, collagen and actin expression, or cell distribution, but induced an initial increase in the secretion of interleukin (IL)-6, IL-8, monocyte chemotactic protein 1 (MCP-1), and vascular endothelial growth factor (VEGF), followed by a decrease in secretion after 3 or 7 d. Irradiation resulted in secretion of a lower amount of all analytes examined compared with controls on day 21, irrespective of radiation dose and growth surface. Backscattering from titanium did not influence the cell response significantly, suggesting a clinical potential for achieving successful osseointegration of dental implants placed before radiotherapy.

Jan E. Ellingsen, Department of Prosthodontics, Institute of Clinical Dentistry, University of Oslo, PO Box 1109 Blindern, 0317 Oslo, Norway

E-mail: j.e.ellingsen@odont.uio.no

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Patients with head and neck cancer managed by radiotherapy often require osseointegrated dental implants (ODIs) to re-establish oral function and aesthetics. Osseointegrated dental implants are typically made of commercially pure titanium with a smooth or a roughened modified surface, but the latter has become the predominant choice because of its enhanced osseointegration potential (1–4). Studies have shown that radiotherapy targeting the jaws negatively affects the predictability and survival of ODIs (5, 6). There is limited scientific evidence regarding the optimal time-point for implant placement in irradiated jaws but, according to the current literature, secondary placement (i.e., after radiotherapy) is considered the standard approach (5–7). Secondary placement allows evaluation of the oncologic treatment and the cancer prognosis before costly rehabilitation, and provides the dentist with an overview of a patient's individual prosthetic needs and whether the amount of bone available has sufficient volume for implant positioning.

In order to improve the oral health-related quality of life (OHRQoL) among patients with head and neck cancer, it would be favourable to install implants during ablative tumour surgery, prior to radiotherapy (8). With this protocol of primary placement, patients can benefit from oral rehabilitation 1–2 yr earlier than with secondary placement, significantly increasing their QoL (9). This approach may also prevent the need for a second surgical intervention and the use of preoperative hyperbaric oxygen therapy, thus reducing the treatment burden as well as the overall treatment cost. In a systematic review from 2016, only two of 18 relevant publications reported on the primary placement of dental implants (7). In the studies by Korfage *et al.* (10) and Schepers *et al.* (11), the implant survival rates were higher with primary placement of implants than with secondary placement. Moreover, functioning dentures

and OHRQoL were high among patients with no additional comorbidities (10, 11).

The concept of backscattering from titanium during radiotherapy may contribute to some of the reservations about primary installation of dental implants, as the tissues surrounding dental implants receive a higher dose of radiation. However, there is no consensus on whether this is of clinical relevance.

Successful bone repair and regeneration, and thus osseointegration of dental implants, depends on the presence of viable human mesenchymal stem cells (hMSCs) (12). Previous studies demonstrate that the viability of MSCs seems to decrease with increasing radiation dose (13), but FEKETE and colleagues (14) showed that a small fraction of multipotent MSCs survive even 30- and 60-Gy doses of γ-irradiation. The underlying mechanisms of the relatively high resistance of MSCs to ionizing radiation have been studied (15), but the response of hMSCs to ionizing radiation when growing on titanium has not yet been explored. To establish predictable and successful treatment protocols for the oral rehabilitation of patients with head and neck cancer with the use of dental implants installed prior to radiotherapy, more information about the effect of backscattering from the titanium on the hMSCs growing on the surface is required.

The aim of the present study was to investigate the effects of cobalt 60 (^{60}Co) ionizing radiation and surface backscatter on hMSCs cultured on titanium coins.

Material and methods

Titanium disk preparation

Machined cp grade 2 titanium disks were washed in trichlorethylene for 15 min followed by absolute ethanol (100%) in an ultrasonic bath for 15 min, both at room temperature, before being packed and sterilized in an autoclave at 135°C for 20 min. Moderately rough titanium dioxide (TiO_2)-blasted and fluoride-modified cp grade 4 titanium disks (TiF) were premounted on carriers in sealed containers and sterilized by β-irradiation (Dentsply Sirona, Mölndal, Sweden). The titanium disk dimensions were 6.25 × 1.95 mm.

Irradiation

The radiation procedures were carried out at The Norwegian Radium Hospital, Oslo University Hospital. Cells were γ-irradiated using a ^{60}Co source (Theratron 780-C; MDS Nordion, Ontario, Canada). The irradiation field was 40 × 40 cm^2 and the source-to-plate distance was 70 cm. The duration of each irradiation procedure was calculated, taking into account the decay of the ^{60}Co source, to administer exactly 2, 6 and 10 Gy to the sample plates. The plates were placed on a hollow perspex plate transfused with circulating preheated water from a water bath (Grant Instruments, Cambridge, UK) to maintain a constant temperature of 37°C in the medium throughout the irradiation. Dose measurements were carried out using thermoluminescence dosimetry (TLD) (TLD-100; Harshaw TLD Bicrom, Solon, OH, USA). Monte Carlo simulations were conducted to calculate the radiation dose delivered 10–1,000 μm from the titanium surfaces, to determine the degree of backscatter radiation. Thermoluminescence dosimetry on the plastic surface was used as the absolute reference in our calculations.

Experimental design

Bone marrow-derived hMSCs (Lonza, Walkersville, MD, USA) were cultured in Lonza's human Mesenchymal Stem Cell Growth BulletKit Medium (MSCGM catalog no. PT-3001), a serum-containing medium with L-glutamine and GA-1000 which is designed to allow proliferation of human bone marrow-derived mesenchymal cells in an undifferentiated state, at 37°C in a humid atmosphere of 5% CO_2. The cells were cultured at confluence on three different surfaces (Ti, TiF, plastic) with a mean of 1.4×10^5 cells per sample. Cells were allowed to attach to the surface for 24 h before the medium was changed and cells were exposed to ionizing γ-radiation of 2, 6, or 10 Gy. Unirradiated cells were kept as a control, but went through the same handling procedures.

The hMSC growth medium was changed twice per week and consistently 24 h before sampling (days 1, 3, 7, 14, and 21). Media samples were immediately transferred to −20°C and stored at this temperature until required for analyses. The experiment was repeated in three biological replicas, using hMSCs from the same identified donor. Cells in passages 4–7 were used.

Cell morphology

The samples were washed with PBS and fixed with 4% paraformaldehyde (PFA), 21 d post-irradiation. Collagen content and distribution were evaluated in cells cultured on titanium surfaces using a mouse anti-collagen (488) (Invitrogen, Eugene, OR, USA). The samples were co-stained with phalloidin (568) (Invitrogen) and 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St Louis, MO, USA) to reveal intracellular actin and cell-nuclei. Fluorescence microscopy imaging was conducted using an upright Leica SP8 confocal laser-scanning microscope (Leica, Wetzlar, Germany). The cells in the plastic wells were monitored and photographed in an Olympus IX70 inverted microscope (Olympus, Shinjukun Tokyo, Japan) to verify the presence of cells 1, 3, 7, 14, and 21 d post-irradiation.

Cell viability

The cytotoxic effects of radiation were evaluated by measuring lactate dehydrogenase (LDH) activity in cell-culture medium after 1, 3, 7, 14, and 21 d of incubation. The LDH activity was determined in 50 μl of culture medium using a cytotoxicity detection kit (Roche Diagnostics, Mannheim, Germany), and absorbance was measured spectrophotometrically at 490 nm using an ELx800 Absorbance Reader (BioTek Instruments, Winooski, VT, USA). The LDH activity in the medium of irradiated cells was calculated relative to the activity in media from unirradiated controls at the same timepoints.

Total protein concentration in cell-culture medium

The concentration of total protein in cell-culture medium was measured using the Pierce BCA Protein Assay Kit

(Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA). The protein concentrations were determined with reference to standards of bovine serum albumin using the Standard Test Tube Protocol. The range of the standard curve was 20–2,000 μg ml^{-1}.

Changes in mineral deposition

Alkaline phosphatase (ALP) activity was quantified by measuring the cleavage of *p*-nitrophenyl phosphate (pNPP) (Sigma-Aldrich) into a soluble yellow end-product, which was detected by spectrophotometry at 405 nm. The concentration of ALP in test samples was determined by comparison with a standard curve (ranging from 0 to 6,000 pM) created using calf intestinal alkaline phosphatase (CIAP; Promega, Madison, WI, USA) standards.

Cytokine secretion

Multianalyte profiling was performed using the Luminex 200 system (Luminex, Austin, TX, USA). The concentrations of the secreted cytokines epidermal growth factor (EGF), eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte–macrophage colony-stimulating factor (GM-CSF), interferon alpha 2 (IFN-α2), interferon gamma (IFN-γ), interleukin (IL)-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, IL-1ra, IL-1a, IL-1b, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, interferon gamma-induced protein 10 (IP-10), monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein 1-alpha (MIP-1-α), macrophage inflammatory protein 1-beta (MIP-1-β), tumor necrosis factor alpha (TNF-α), tumor necrosis factor beta (TNF-β), and vascular endothelial growth factor (VEGF) were measured in culture media using the HCYTOMAG-60K-PX29 kit (EMD Millipore, Billerica, MA, USA) according to the manufacturer's protocol. Minimum detectable concentrations for the selected cytokines varied between 0.4 and 26.3 pg ml^{-1}. The data were analysed using XPONENT 3.1 software (Luminex). The measurements were performed with samples harvested on days 1, 3, 7, 14, and 21 with four biological replicas. The experiment was repeated three times.

Statistical analysis

Statistical analyses were conducted using SIGMAPLOT (V 13.0 for Windows; Systat, Chicago, IL, USA). The effects of irradiation (2, 6, or 10 Gy) on hMSCs growing on three different surfaces (Ti, TiF, plastic) were analysed. The effects were always compared with unirradiated control cells on the same surfaces at the same time points of incubation, using two-sided Student's *t*-test when data sets were normally distributed. If data sets were not normalized, the Mann–Whitney *U*-test was conducted. Values of $P \leq 0.05$ indicated a significant difference. Results were calculated and are presented as a percentage of the values obtained for unirradiated controls.

Results

The Monte Carlo simulations demonstrated escalated radiation doses of > 40%, 10–20 μm from the titanium surface and of 15%, 1 mm from the titanium surface, compared with the plastic surface (Fig. 1).

Cell viability and morphology

Cell density and distribution, and production of actin and collagen were not significantly different between irradiated and non-irradiated hMSCs. Irradiated cells had a slightly larger nucleus and a shadow-like background, compared with non-irradiated cells on the same surface (Fig. 2). No ALP activity above the detection limit was measured in cell-culture medium from irradiated or non-irradiated cells (data not shown).

Lactate dehydrogenase activity measured in the cell-culture medium showed no clear pattern of dose-dependent cytotoxicity of the irradiated cells, except that hMSCs on the TiF surface displayed a significantly reduced LDH activity on day 21, at all doses of radiation (Fig. 3). Irradiation did not induce changes in total protein levels in the cell-culture medium compared with non-irradiated controls (data not shown).

Cytokine secretion

Of the 29 cytokines and growth factors analysed in the cell-culture medium, IL-6, IL-8, MCP-1, and VEGF were quantified at levels above the set detection limit.

Compared with controls, irradiation with 2 Gy enhanced the secretion of IL-6 from hMSCs growing on Ti and TiF surfaces on day 1. Cells on the Ti surface maintained elevated levels of IL-6 until day 7, while cells on the TiF surface gradually decreased secretion of IL-6 from day 3 with significantly reduced levels on days 14 and 21. Cells growing on the plastic surface had a reduced secretion of IL-6 on day 21.

Irradiation with 6 Gy stimulated increased secretion of IL-6 from hMSCs growing in the Ti and plastic surfaces, on day 7; followed by reductions on all surfaces on day 14 and 21, but this was significant only for the TiF and plastic surfaces.

Irradiation with 10 Gy increased IL-6 secretion only from cells growing on the Ti surface (days 1 and 7), while significant reductions in IL-6 secretion were seen from cells growing on the TiF surface on days 14 and 21 and from cells growing on the plastic surface on day 21 (Fig. 4A, Table S1).

Compared with controls, irradiation with 2 Gy enhanced the secretion of IL-8 from hMSCs growing on the Ti surface on day 1. From day 7, reductions in IL-8 secretion were recorded for cells growing on all surfaces.

Irradiation with 6 and 10 Gy induced production of elevated levels of IL-8 from cells growing on the Ti surface on day 3, but these results were not significant because of the wide spread in data. Significant reductions in IL-8 secretion were induced after 10 Gy of irradiation from hMSCs growing on all surfaces on days 7, 14, and 21 (Fig. 4B, Table S2).

Compared with controls, irradiation with 2 Gy enhanced the secretion of MCP-1 from hMSCs growing on the Ti surface on day 1, and decreased the secretion of MCP-1 from cells growing on the TiF surface on day 14.

After 6 Gy of irradiation, a decrease in MCP-1 secretion was seen for cells growing on the TiF surface on day 1 and for cells growing on the plastic surface on

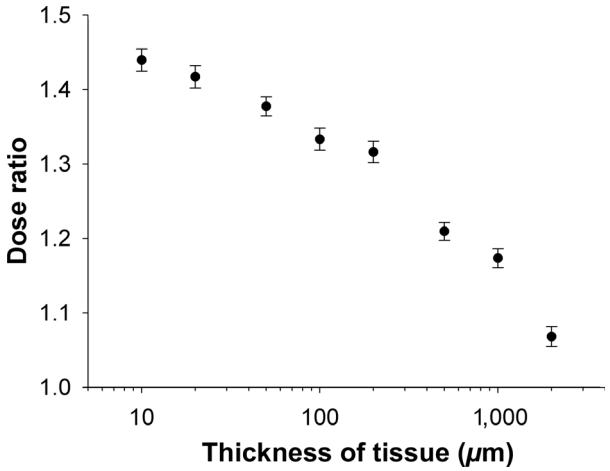

Fig. 1. Monte Carlo simulation of relative increase in radiation dose, as a result of backscatter to tissue, 10–1,000 μm from the titanium surfaces.

day 3. On day 21, hMSCs growing on all surfaces exhibited reduced levels of MCP-1, but this was only significant for TiF and plastic surfaces.

Irradiation with 10 Gy reduced the secretion of MCP-1 from cells growing on the TiF surface on days 1, 14, and 21. Cells growing on Ti and plastic surfaces also exhibited reduced levels of MCP-1 on day 21, but this was significant only for the plastic surface (Fig. 4C, Table S3).

Compared with controls, irradiation with 2 Gy enhanced the secretion of VEGF from hMSCs growing on Ti and TiF surfaces on day 1, followed by reductions in VEGF secretion from cells growing on all surfaces on days 7, 14, and 21.

After 6 Gy of irradiation, the secretion of VEGF from hMSCs growing on the Ti surface enhanced dramatically on day 3, but because of the wide spread in data, this result cannot be considered as significant. Significant reductions in VEGF secretion were detected for cells growing on the Ti surface on days 14 and 21, on the TiF surface on days 7, 14, and 21, and on the plastic surface on days 3, 14, and 21.

Irradiation with 10 Gy enhanced VEGF secretion from hMSCs growing on Ti and plastic surfaces on day 1, followed by significant reductions in VEGF secretion from cells growing on all surfaces on days 14 and 21 (Fig. 4D, Table S4).

Discussion

In the present study, human bone marrow-derived MSCs appeared to tolerate therapeutic single doses of ionizing radiation up to 10 Gy. Cells were irradiated at confluence; hence, a high degree of proliferation was not expected because of cellular contact inhibition (16). Thus, radiation-induced responses could be compared with unirradiated controls based on similar cell numbers in the different groups.

Regarding cytotoxicity, no doses of radiation enhanced LDH activity or induced any considerable changes in total protein concentration in the cell-culture medium. The small changes observed are probably physiologically negligible. However, although radiation may not lead to cell death, damage to DNA can cause inhibition of mitosis and lead to permanent cell-cycle arrest or senescence (17, 18).

Previous reports have shown that MSCs possess relatively high radioresistance properties. Nicolay *et al.* (19) found that photon (10 Gy) and carbon ion (4 Gy) irradiation did not alter cellular morphology, adhesion, or the migration abilities of MSCs. The MSCs were able to repair DNA double-strand breaks, and the differentiation potential was unaffected by either form of radiation. Fekete *et al.* (14) showed that after exposure to high-dose γ-irradiation (30 and 60 Gy), the surviving subpopulations of MSCs continued to proliferate up to 120 d after radiation, although this occurred more slowly than in unirradiated controls. The MSCs maintained a tri-lineage differentiation capacity (adipocytes, chondrocytes, and osteoblasts) following an extended period of cultivation. However, irradiation changed the cytokine secretion profile in the cells, and they became prematurely senescent (14).

One identified characteristic of senescent cells is large morphology, unlike the spindle-like shape of proliferating, untransformed cells (20). Correspondingly, compared with unirradiated controls, larger nuclei in irradiated cells were observed in this study. Another observation was a diffuse backmesh on the Ti surfaces of irradiated cells. Even though osteogenic differentiation was not induced by the medium, we investigated if the shadow-like background could be caused by extracellular mineralization. However, no differences in ALP activity in the cell-culture medium between irradiated and unirradiated cells were found.

Exposure of a monolayer of cells to 10-Gy irradiation, as in this study, is considered a high dose. In the clinic, the protocol for conventional radiotherapy is fractionated doses of 2 Gy, to enable healthy cells to recover between doses. As shown in a large animal model, pig mandibles exposed to fractionated radiation with a total dose of 18 Gy showed no changes in proliferative capacity and the osteogenic differentiation potential of isolated MSCs. On the other hand, when the pigs received 18-Gy irradiation as a single dose, the MSCs exhibited reduced osteogenic differentiation capacity, and for the first 2 wk after irradiation the rates of proliferation were diminished (21).

Information on the effects of irradiation on hMSCs cultured on titanium implants *in vitro* is lacking. Huang *et al.* (22) exposed bone marrow stromal cells (BMSCs) from rats to γ-irradiation of 2, 4, and 8 Gy, before the cells were seeded on sandblasted/acid-etched or polished titanium specimens. They found that radiation reduced cell proliferation, adhesion, and spreading, as well as osteogenic and adipogenic differentiation capacity, in a dose-dependent manner (22). The experimental design of Huang and colleagues illustrates the clinical situation of when implants are installed in irradiated tissue. In this study, hMSCs were seeded on titanium surfaces before being exposed to radiation, in order to illustrate the

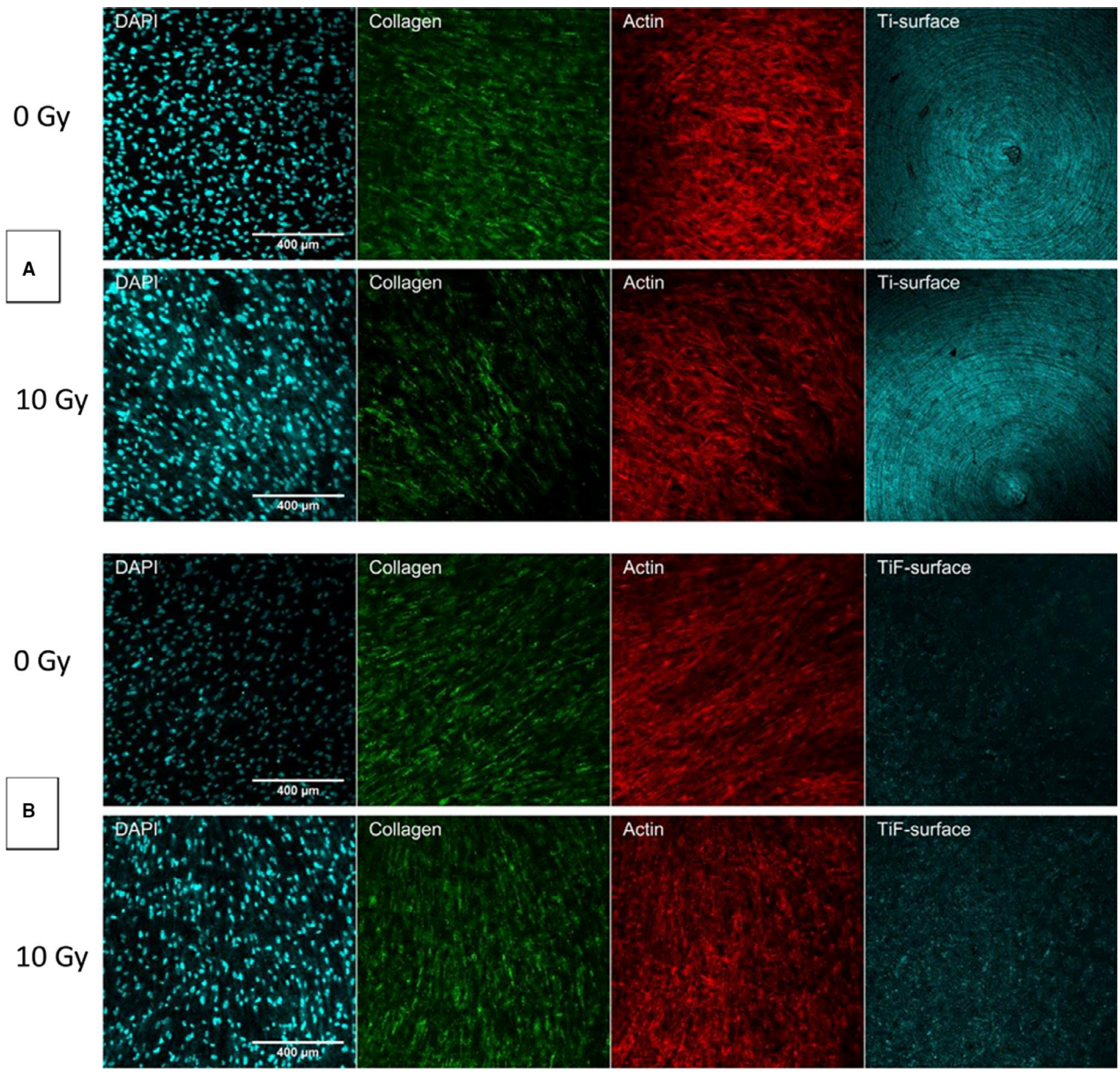

Fig. 2. Morphology of human mesenchymal stem cells (hMSCs), 21 d post-irradiation (0 and 10 Gy), on a titanium (Ti) surface (A) and a fluoride-modified titanium (TiF) surface (B). The two surfaces are processed independently because of different autofluorescence.

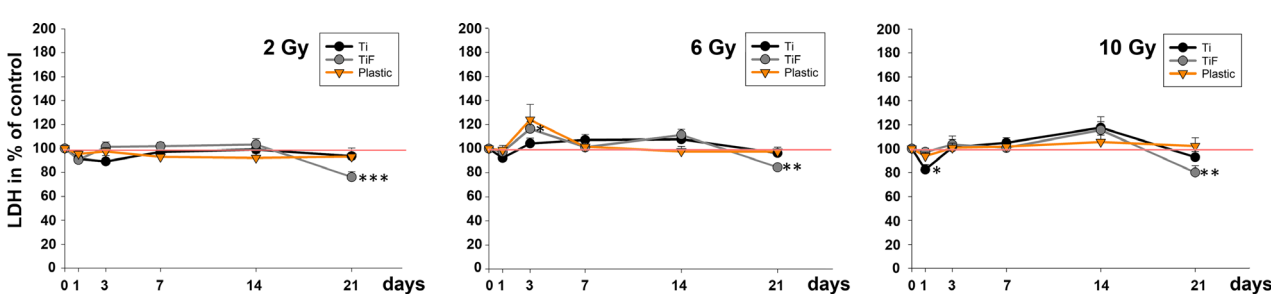

Fig. 3. Lactate dehydrogenase (LDH) activity in the cell-culture medium of human mesenchymal stem cells (hMSCs) cultured on titanium (Ti), fluoride-modified titanium (TiF), and plastic surfaces, after single doses of 2, 6, or 10 Gy irradiation. LDH activity was measured 1, 3, 7, 14, and 21 d post-irradiation. Data are given as mean ± SD ($n = 12$), as a percentage of the values obtained for non-irradiated controls. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

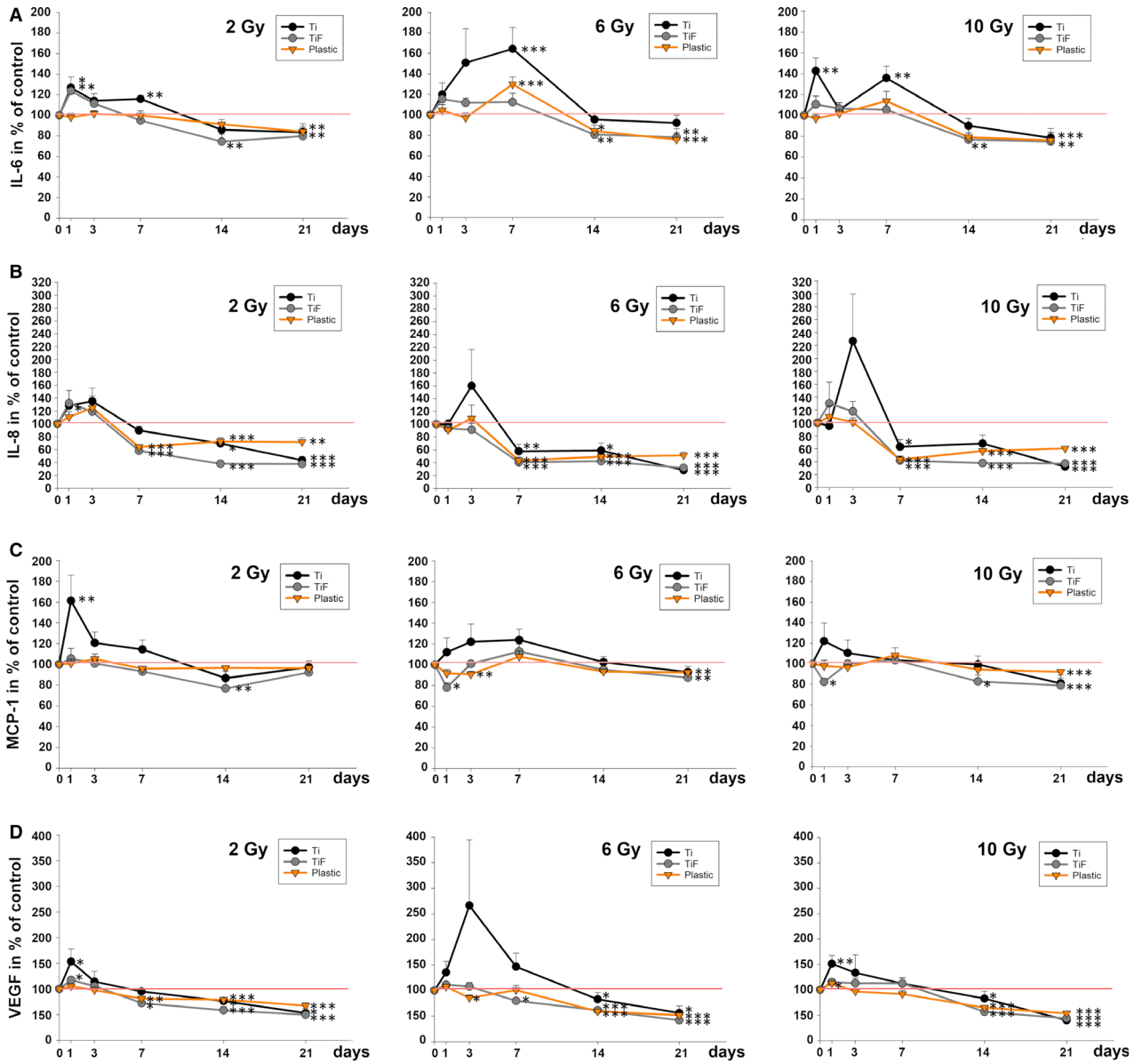

Fig. 4. Secretion of the cytokines and growth factors interleukin (IL)-6 (A), IL-8 (B), monocyte chemotactic protein 1 (MCP-1) (C), and vascular endothelial growth factor (VEGF) (D), into cell-culture medium by human mesenchymal stem cells (hMSCs). The hMSCs were cultured on titanium (Ti), fluoride-modified titanium (TiF), and plastic surfaces after single doses of irradiation (2, 6, or 10 Gy), and the levels of cytokines and growth factors were calculated 1, 3, 7, 14, and 21 days post-irradiation. Data are given as mean ± SD (n = 12), as a percentage of the values obtained for non-irradiated controls. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

clinical approach of when dental implants are installed prior to radiotherapy.

Studies have shown that cells located 1-2 mm from the titanium implant surface may receive a dose of radiation that is between 15% (23) and 36% (24) higher compared with that received by plastic, when using a ^{60}Co source. As backscattered rays from titanium are of a very short range, and because of the 1 mm thickness of the TLD, we conducted Monte Carlo simulations (25) to calculate the radiation dose delivered to the cell layer of 10-20 μm attached to the Ti surface. The calculations showed a theoretical increase in radiation dose of more than 40% to the cells. Rosengren *et al.* (26) investigated the theoretical effect of backscatter rays on human colon carcinoma cells and embryonic hamster cells growing on titanium compared with plastic surfaces. The cells were irradiated with doses of 0-10 Gy with either ^{60}Co photons or range-modulated protons. Cells grown on titanium expressed the same colony-forming capacity and survival as controls grown on plastic. The results were dose-independent, and the increased risk of impaired osseointegration as a result of backscatter radiation was judged to be minimal (26).

Regarding the cytokine secretion from irradiated hMSCs growing on the different surfaces, cells on the

Ti surface exhibited the highest levels of IL-6, IL-8, MCP-1, and VEGF. However, the high secretion levels from cells on the Ti surface decreased with a steeper curve, resulting in lower secretions of all the analytes from irradiated cells on day 21 compared with controls, irrespective of radiation dose and growth surface. Surprisingly, hMSCs growing on plastic did not seem to be significantly more functional post-irradiation compared with cells growing on titanium, despite the higher radiation dose caused by backscattering. One exception was the secretion of IL-8, regarding which cells growing on plastic exhibited marginally higher secretion on day 21 compared with cells growing on titanium surfaces.

A viable stem cell will naturally produce high levels of immune-response factors after any trauma or external influence, such as irradiation. Correspondingly, we found a marked impact of radiation on the cytokine profile of hMSCs. The reduction in cytokine secretion observed in all irradiated cells towards the end of the observation period is presumably a consequence of fewer functional cells or a small reduction in cell numbers.

Some limitations to this study should be noted. To substantiate the assumptions of a low degree of apoptosis, an apoptosis assay could have been performed. Bcl-2 expression or the activation of caspases are two of many methods to detect markers of apoptosis. Furthermore, the expression of p53 or fluorescent detection of senescence-associated beta galactosidase activity, could confirm the hypothesis of senescent cells.

The objective of this study was, however, to evaluate if the hMSCs responded differently when irradiated during growth on titanium compared with plastic as a result of backscattering. Independent of dose, we found no marked differences in cell response between the surfaces. The results may suggest a clinical potential for achieving successful osseointegration of dental implants placed prior to radiotherapy. Future studies are needed to assess the impact of backscatter irradiation on other cells involved in the osseointegration of dental implants.

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Conflicts of interest – One of the authors, J. E. Ellingsen, has a patent licensing agreement with Dentsply Sirona Implants.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Concentration (pg ml^{-1}) of IL-6 to the media from hMSC

Table S2. Concentration (pg ml^{-1}) of IL-8 to the media from hMSC

Table S3. Concentration (pg ml^{-1}) of MCP-1 to the media from hMSC

Table S4. Concentration (pg ml^{-1}) of VEGF to the media from hMSC