Original

High-dose irradiation of bone chips preserves the *in vitro* activity of bone-conditioned medium

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Abstract: Extracorporeal irradiation sterilizes resected tumor bone used as autografts in reconstruction surgery. Therapeutic irradiation is a standard technique in head and neck cancer therapy that aims to preserve organ function. Bone irradiation has a complex, mostly inhibitory, effect on remodeling and regeneration, although the underlying mechanisms are still not fully understood. It remains unclear if extracorporeal irradiation affects the paracrine-like activity of the corresponding autografts. We recently reported that bone-conditioned medium from autogenous bone chips contains a number of factors that might affect cell activity. In the present study, we investigated the effects of extracorporeal irradiation of porcine cortical bone chips on the activity of the corresponding bone-conditioned medium. The effects of bone-conditioned medium on the expressions of

transforming growth factor-beta (TGF- β) target genes in oral fibroblasts were assessed. Bone-conditioned medium from bone chips exposed to a total radiation dose up to 120 Gy did not affect expressions of TGF- β target genes, including adrenomedullin, BTB/POZ domain-containing protein 11, proteoglycan 4, NADPH oxidase 4, and interleukin 11, in oral fibroblasts. In conclusion, bone irradiation does not alter the capability of the corresponding bone-conditioned medium to provoke a robust fibroblastic cell response *in vitro*. (J Oral Sci 58, 325-331, 2016)

Keywords: radiation; bone-conditioned medium; autologous bone; allogenic bone; bone grafts; bone bank.

Introduction

Extracorporeal irradiation of resected tumor-bearing bone is necessary before such bone can be used as an autograft. This procedure has been used since 1968 and has been successful clinically (1). The resected bone is usually subjected to a total radiation dose of 50 to 300 Gy (2,3), and the dose is as high as 25 kGy for allograft ster-

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ilization (4). Fractionated irradiation is used as definitive or adjuvant treatment of head and neck cancer, especially if organ preservation is desired. Thus, irradiation is frequently combined with chemotherapy and surgical therapy (5). Irradiation of bone is now standard procedure in oral and maxillofacial surgery and in disciplines such as otolaryngology (6) and orthopedics (7), all of which address the oncologic aspects of bone.

Irradiation has complex effects on bone remodeling and repair. Adverse effects include pathologic fractures, continuity defects, and abnormalities in osteosynthesis (8-11). Substantial evidence indicates that irradiation disrupts bone microvasculature (12,13). This may lead to osteocyte apoptosis and a lack of progenitor cells of the mesenchymal lineage, which give rise to osteoblasts and chondrocytes (14-18). The most severe complication is osteoradionecrosis (19,20). It is unclear if microangiopathy is the only cause of impaired bone regeneration and graft consolidation, or if the biological properties of bone are also compromised. Among their biological properties, bone chips exhibit paracrine-like activity and induce substantial changes in transforming growth factor-beta (TGF- β) target genes in oral fibroblasts (21,22).

Autologous bone from cortical sources has a paracrine-like function. Conditioned medium harvested from fresh cortical bone chips could modulate differentiation of mesenchymal cells (21,22). TGF- β targets regulated by bone-conditioned medium (BCM) include adrenomedullin (ADM), pentraxin 3 (PTX3), BTB/POZ domain-containing protein 11 (BTBD11), interleukin 11 (IL-11), proteoglycan 4 (PRG4), and NADPH oxidase 4 (NOX4) (21,22). This gene panel has recently provided a scientific basis for the development of a bioassay to determine paracrine-like activity in bone grafts that have undergone autoclaving, pasteurization, and freezing (23). A previous study assessed the response of oral fibroblasts exposed to supernatants of demineralized bone (24); however, it remains to be determined whether irradiation affects the paracrine-like activity of bone grafts. In this study, we investigated the effects of bone irradiation on the capacity of the corresponding conditioned medium to modulate gene expression in oral fibroblasts.

Materials and Methods

BCM from irradiated bone

The bone samples used in this study were obtained within 6 h of death from adult pigs (Metzgerei Balsiger, Wattenwil, Switzerland). A "bone scraper" (Hu-Friedy, Rotterdam, the Netherlands) was used to collect bone chips from the buccal surface of the mandibular cortical bone. All samples were then stored in sterile plastic dishes

containing Dulbecco's modified Eagle medium (DMEM) supplemented with antibiotics and antimycotics (all from Invitrogen Corporation, Carlsbad, CA, USA). All experiments were conducted using bone chips harvested from at least three independent preparations. Using a ^{137}Cs gamma ray unit (Best Theratronics, Ottawa, Canada), we subjected the harvested bone chips to γ -irradiation (dose rate: 1 Gy/min; total dose: 0, 15, 30, 60, or 120 Gy) administered at room temperature under normoxic conditions. A ratio of 5 g of bone chips (wet weight) per 10 mL of culture medium was used to prepare the conditioned medium (21). The BCM was collected in the form of liquid medium containing growth factors released from autogenous bone chips after incubation (24 h at 37°C) in a humidified atmosphere containing 5% carbon dioxide. The medium was filtered until sterile (pore diameter, 0.22 μm ; Merck Millipore, Billerica, MA, USA) and kept frozen at -80°C. The stocks were thawed immediately before each experiment.

Irradiated BCM and irradiated recombinant human TGF- β 1

Conditioned medium prepared from fresh bone chips and medium including 10 ng/mL recombinant human TGF- β 1 (ProSpec, Ness-Ziona, Israel) were irradiated with 0, 15, 30, 60, or 120 Gy in the manner described above.

Primary mesenchymal cells

After wisdom tooth extraction, human bone and palatal and periodontal ligament fibroblasts were harvested from the tissue grafts of two independent donors, as previously reported (21,22). The protocol was approved by the Ethical Committee of Vienna (EK Nr. 631/2007). Both cell types were incubated in a humidified atmosphere at 37°C in growth medium consisting of DMEM, 10% fetal calf serum (FCS; Invitrogen), and antibiotics. Cells were plated at 30,000 cells/cm² with either 20% BCM, 20% irradiated BCM, irradiated medium including 10 ng/mL recombinant TGF- β 1, or serum-free medium alone for 24 h before gene expression analysis.

Quantitative RT-PCR analysis

A High Pure RNA Isolation Kit (Hoffmann-La Roche, Basel Switzerland) was used to extract the cellular RNA. A Transcriptor Universal cDNA Master kit (Hoffmann-La Roche) was used to perform reverse transcription, and a FastStart Universal SYBR Green Master kit (Hoffmann-La Roche) was used to perform polymerase chain reaction (PCR) amplification on a 7500 Real-Time PCR System (both Applied Biosystems, Life Technologies Corp., Carlsbad, CA, USA). The probe sequences used

Table 1 Primer sequences for human cells

Genes	Forward primer	Reverse primer
hADM	GGACATGAAGGGTGCCTCTC	TGTTTCATGCTCTGGCGGTAG
hPTX 3	TGTATGTGAATTTGGACAACGAA	CATTCCGAGTGCTCCTGAC
hBTBD11	CAAAGCACTCCTCTCCAGCA	AATGAGCAGTGACTCTGGGC
hIL-11	TGCACCTGACACTTGACTGG	AGTCTTCAGCAGCAGCAGTC
hPRG4	CGACGCCCAATGTAAGAAGT	GGTGATGTGGGATTATGCACT
hNOX4	TCTTGGCTTACCTCCGAGGA	CTCCTGGTTCTCCTGCTTGG
h β -actin	CCAACCGCGAGAAGATGA	CCAGAGGCGTACAGGGATAG
mALPL	AGAACCCCAAAGGCTTCTTC	TCATGATGTCCGTGGTCAAT
m β -actin	CTAAGGCCAACCGTGAAAAG	ACCAGAGGCATACAGGGACA

Fig. 1 Expression of TGF- β -regulated genes in oral fibroblasts after irradiation of bone chips (total dose, 0-120 Gy). RT-PCR was used to assess relative mRNA levels of (A) ADM, (B) PTX3, (C) BTBD11, (D) IL-11, (E) PRG4, and (F) NOX4 in human oral fibroblasts exposed to BCM obtained from different processed bone chips irradiated with a total dose of 0 to 120 Gy. * $P < 0.05$ vs. 0 Gy. The data represent findings from three different BCM preparations from three donors of oral fibroblasts ($n = 9$).

are shown in Table 1. The mRNA levels were calculated by normalization to the housekeeping gene beta actin, using the $\Delta\Delta C_t$ method.

Alkaline phosphatase

ST2 cells were incubated in serum-free medium with and without 20% BCM and investigated by histochemical staining of alkaline phosphatase after 3 days. Cells were fixed with 4% formaldehyde and incubated with a substrate solution containing naphthol AS-TR phosphate and fast blue BB salt (Sigma). After rinsing with distilled water, cultures were imaged. For the respective gene expression analysis, the cell line was exposed to 20% BCM for 24 h.

Statistical analysis

All experiments were repeated at least twice, and the data

are reported as mean and standard deviation. Analysis of variance (ANOVA) and post-hoc testing with the paired t -test were used for the analyses. Statistical significance was defined as a P value of <0.05 .

Results

Irradiation of bone chips had little effect on the activity of the conditioned medium

First, we investigated the effect of irradiation of freshly prepared bone chips on the activity of the corresponding conditioned medium. As shown in Fig. 1, irradiation of fresh bone chips had no effect, at any dose, on the expressions of the TGF- β target genes ADM, BTBD11, IL-11, PRG4, and NOX4 in oral fibroblasts in the corresponding conditioned media. However, there was a significant effect on PTX3 expression ($P > 0.05$). BCM prepared from fresh bone and bone irradiated with a dose up to

Fig. 2 Expression of TGF- β -regulated genes in oral fibroblasts after irradiation of BCM (total dose, 0-120 Gy). RT-PCR was used to assess relative mRNA levels of (A) ADM, (B) PTX3, (C) BTBD11, (D) IL-11, (E) PRG4, and (F) NOX4 in human oral fibroblasts exposed to irradiated BCM. No significant difference was observed for any target gene. The data represent findings from three different BCM preparations from three donors of oral fibroblasts ($n = 9$).

Fig. 3 Expression of TGF- β -regulated genes in human oral fibroblasts after exposure to irradiated recombinant TGF- β 1. RT-PCR was used to assess relative mRNA levels of (A) ADM, (B) PTX3, (C) BTBD11, (D) IL-11, (E) PRG4, and (F) NOX4 in human oral fibroblasts exposed to irradiated recombinant TGF- β 1. No significant difference was observed for any target gene. The data represent findings from three different BCM preparations from three donors of gingival fibroblasts ($n = 9$).

120 Gy exhibited a substantial decrease in ADM expression and large increases in BTBD11, IL-11, PRG4, and NOX4 expressions. PTX3 expression was also inhibited by BCM from fresh bone and irradiated bone. However, PTX3 gene expression was significantly higher in BCM prepared from bone irradiated with a dose of 120 Gy than in fresh bone ($P = 0.044$). These data show that bone maintains its paracrine-like activity even after exposure

to 120 Gy of radiation.

Irradiation had no effect on BCM or recombinant TGF- β 1 activity

To further assess the effect of irradiation *in vitro*, conditioned media prepared from fresh bone chips and from recombinant TGF- β 1 were exposed to a radiation dose up to 120 Gy. As was the case in our analysis of bone chips,

Fig. 4 Expression of differentiation markers in ST2 cells after exposure to BCM from irradiated bone chips (total dose, 0-120 Gy). (A) RT-PCR was used to assess relative mRNA levels of AP of ST2 cells exposed to BCM obtained from different processed bone chips irradiated with a dose up to 120 Gy. No significant difference was observed. (B) Alkaline phosphatase histochemical staining of ST2 cells after exposure to BCM obtained from different processed bone chips irradiated with a dose up to 120 Gy.

irradiation of BCM and recombinant TGF- β 1 had little effect on expressions of ADM, PTX3, BTBD11, IL-11, PRG4, and NOX4 in oral fibroblasts ($P > 0.05$; Figs. 2, 3). These results further indicate that bone chips retain paracrine-line activity after irradiation.

BCM from irradiated bone did not suppress alkaline phosphate in ST2 cells

The ST2 mouse mesenchymal cell line is a suitable model to study changes in alkaline phosphatase activity (21). As shown in Fig. 4, conditioned medium prepared from different processed bone chips exposed to a radiation dose up to 120 Gy had no effect on suppression of the alkaline phosphatase gene at the RNA level ($P > 0.05$) or the results of histochemical staining.

Discussion

Extracorporeal irradiation is now routinely used in clinical practice for treatment of head and neck cancer (5). Bone irradiation causes undesirable side effects leading to unfavorable bone remodeling and repair (8-11) and, in the worst case, osteoradionecrosis (16). In the present study, we exposed cortical bone chips to a gamma radiation dose up to 120 Gy and investigated the subsequent effects on the activity of TGF- β target genes. In addition, we assessed the effects of irradiation on BCM and recombinant TGF- β 1. We found that irradiation did not affect the activity of TGF- β target genes in oral fibroblasts of various origins. Our main finding was that bone chips exposed to as much as 120 Gy of radiation retained their paracrine-like activity *in vitro*.

What is known regarding the effect of gamma ster-

ilization on the activity of TGF- β and its superfamily members? Radiation exposure induces latent TGF- β activation (25,26). Other studies reported that the osteo-inductive potential of bone morphogenetic proteins of various origins was preserved after sterilization at 25 kGy (27,28). Growth factors react differently to gamma irradiation: gamma sterilization alters the properties of insulin-like growth factor-1 (29), whereas the TGF- β superfamily is resistant to high doses of radiation. Our data suggest that gamma irradiation does not activate, but rather maintains, the intrinsic activity of BCM to induce changes in gene expression. Thus, BCM cannot be further activated by low or moderate doses of gamma irradiation.

This study is the logical continuation of our previous research on the paracrine-like function of bone chips *in vitro* (21,22,30). We previously reported that sterilization of bone chips abolished BCM activity (23). Heating bone chips to 80°C, and freezing to -80°C, maintained BCM effects on TGF- β target gene expression (23). However, in an analysis of the effects of antiseptic solutions used to treat bone chips, hypochlorite substantially impaired the activity of the corresponding BCM (31). These past and present results indicate that the manner in which bone chips are sterilized greatly influences the paracrine-like activity of conditioned medium *in vitro*. Theoretically, sterilization with gamma irradiation is preferable to autoclaving of bone grafts. However, it remains to be determined whether the present *in vitro* observations are relevant for processing of bone grafts for *in vivo* applications.

The present bone chips were harvested within 6 h

of death; thus, the effect of time on the paracrine-like activity of these bone chips after irradiation therapy is unclear. The cells within the bone matrix, which are mainly osteocytes, begin to die after the death of the host (32), and the effect this might have on the harvesting of bone chips is unclear. In contrast, commercially available bone chips from human donors typically undergo sterilization many hours or even days later (33). Under such conditions, bone cells would no longer be vital, and it is possible that processing destroyed osteocytes embedded in bone that otherwise might contribute to the paracrine activity of the tissue (34). Therefore, the effect of time on paracrine-like activity is unclear.

In vitro, irradiation did not greatly affect the paracrine-like activity of bone chips or alter the effects of BCM or recombinant TGF- β 1 on cell activity. However, it is possible that the effects of irradiating bone chips or conditioned medium with doses as high as 120 Gy are not relevant in vivo or clinically. Moreover, despite their usefulness in bioassays, oral fibroblasts are not the usual primary target cells for paracrine-like factors from cortical bone chips. Thus, it may be necessary to further evaluate the present model by using an osteogenic cell culture system. Furthermore, one of the main problems of irradiation is a significant decrease in angiogenesis (17,18,35,36). It may therefore be worthwhile to include other cell types related to the endothelial lineage and the expression of factors that control angiogenesis, such as the vascular endothelial growth factors.

In summary, our results indicate that exposing bone chips to as much as 120 Gy of radiation had no significant effect on paracrine-like activity, as indicated by expression of TGF- β target genes. This finding supports the use of gamma sterilization in bone banks.

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Conflict of interest

The authors report no conflicts of interest related to this study.

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