Revitalization of Human Bone after Extracorporeal High Hydrostatic Pressure Treatment

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Abstract. *Background: Bone defects due to malignant tumor often lead to repeated surgery or amputation. Thus, a major objective in orthopedic surgery is the extracorporeal devitalization of tumor-bearing bone segments following reimplantation. Extracorporeal irradiation or autoclaving are possible methods even though they may cause severe loss of biomechanical and biological properties. Yet previous studies have shown that high hydrostatic pressure (HHP) allows for complete devitalization of tumor-afflicted bone segments, while the biomechanical and biological properties of bone tissue remained unchanged. The subject of the present study is revitalization of human bone segments after HHP treatment to acquire knowledge about the ingrowth and regeneration of osteoblast-like cells after such treatment. Materials and Methods: Bone pieces of 5 mm^3 were obtained from cancellous bone, taken from human femoral heads of 6 patients undergoing surgery for total hip arthroplasty, and exposed to hydrostatic pressure levels of 0, 300, and 600 MPa for 10 min at 37˚C. Following the HHP treatment, bone segments were coated with primary human bone cells (10,000 cells/segment), cultured for 42 days and cell viability and proliferation quantified at different time points. Results: An adhesion rate of 73.8% on day 1 and an increase in proliferation between day 14 and 42 were determined. Pretreatment of bone segments with 300 and 600 MPa did not affect cell adhesion or proliferation. Histology showed intact cells and new bone formation on the bone specimens; elevated expression of alkaline phosphatase, osteocalcin, and collagen type I was seen by immunohistochemistry. Conclusion: The present study demonstrates, for the first time, the successful revitalization of HHP-treated bone segments. Concerning proliferation and osteogenic differentiation, the findings are a promising demonstration of sufficient osseointegration. Along with previous results, we anticipate that a pressure of a maximum 350 MPa does induce devitalization of malignant bone tumor segments, while HHP treatment of bone matrix up to 600 MPa does not affect osteoconductivity and osteoinductivity.*

Treatment of cancer patients presenting with solid malignant tumor of the bone requires complete resection of the infiltrated tissue, neoadjuvant chemotherapy, and eventually reconstruction. Different treatment modalities have been established for the reconstruction of bone and joint defects, including endoprostheses and spaceholders (1, 2), artificial bone substitutes (3), and allogeneic transplants (4). However, allogeneic transplantation of bones is a matter of dispute since such bones may be contaminated by viruses or bacteria (5), require special awareness of procurement and storage and may cause immunological responses (6, 7). Unfortunately, artificial bone substitutes are not suited for reconstruction of larger bone defects (8).

Since autologous bone grafts are considered as the 'gold' standard (9), an alternative approach for limb reconstruction of tumor-affected bone is extracorporeal irradiation or autoclaving of grafts with subsequent reimplantation. Nevertheless, one has to ensure that all tumor cells are inactivated prior to reimplantation in order to avoid tumor disease recurrence, whereby destruction of bone by irradiation or autoclaving is a matter of concern, associated with the loss of biomechanical and biological properties (6, 7, 10-15).

Therefore, there is a strong need to develop an adequate procedure that can achieve tumor cell inactivation of bone segments, without compromising osteoconductive, osteoinductive and biomechanical properties of the bone. High hydrostatic pressure (HHP) treatment of bone has been convincingly shown to be the *ex vivo* method of choice to inactivate tumor cells leaving the biological and

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biomechanical properties of bone, cartilage, and tendons intact (16). Previous studies have demonstrated that during this treatment, eukaryotic cells including tumor cells or tumor-afflicted bone specimens, are irreversibly impaired (17-20) while the biomechanical and biological properties of bone are preserved (21-23). There is little data as to the revitalization of HHP-treated bone with primary human bone cells, which is why the following study was performed.

The challenge in reconstructive bone surgery is to provide vital specimens with appropriate adaptability to integrate into the surrounding tissue. We now present evidence that revitalization of HHP-treated human bone with primary human bone cells can be achieved in order to accomplish satisfying reconstruction of bone defects.

Materials and Methods

Bone specimens. Human femoral heads were obtained from 6 patients (3 male and 3 female, aged 33 to 74 years, mean age 56 years) undergoing surgery for total hip replacement. The tissues were collected with the written informed consent of the patient and governed by a Hospital Ethics Committee. Bone biopsies were harvested, placed under the hood of a sterile workbench, and 20 tissue blocks cut (5 mm^3) of each of the bone specimens. Until further use, the tissue blocks were kept in phosphate-buffered saline (PBS).

Cell isolation and culture. The remaining parts of the human femoral head specimens used for preparation of small tissue blocks were minced into small pieces and repeatedly washed with PBS in order to remove blood components. The explants were seeded in culture flasks and incubated with calcium-free Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (v/v) FBS, 1% (w/v) HEPES, 1% (w/v) Dulbecco's vitamins, and 1% (w/v) L-glutamine, at 37˚C in humidified atmosphere of 5% (v/v) CO_2. Media were changed every fourth day. Confluent cell outgrowth was accomplished after 12 days. The adherent cells were trypsinized and then snap-frozen in liquid nitrogen until thawed and cultured for two rounds of 7 days each plus trypsinization.

HHP treatment. Falcon tubes (15 ml) were packed with ten bone specimens and filled with PBS, tightly sealed with parafilm after having eliminated any air bubbles. The sealed vials were placed into the water-filled cavity of a custom-made HHP device (Record Maschinenbau GmbH, Königssee, Germany) and exposed to pressures of 0, 300, and 600 MPa for 10 min at 37˚C (16). The pressure values were reached within a few seconds and then maintained for a defined period of time before reduction to atmospheric pressure within a few seconds.

Seeding procedure. Immediately after decompression bone specimens were placed into 48-well culture plates. Fifty microliters of cell suspension containing 10,000 primary human bone cells were seeded on top of each bone scaffold. Plates were stored at 37˚C in a humidified atmosphere at 5% (v/v) CO_2 for 60 min to allow attachment. Calcium-free DMEM, supplemented with 10% (v/v) FBS, 1% (w/v) HEPES, 1% (w/v) Dulbecco's vitamins, 1% (w/v) L-glutamine, 50 μg/ml ascorbic acid, 10 nM dexamethasone, and antibiotics (penicillin 1000 U/ml, streptomycin 10 mg/ml), was then added until specimens were completely covered with media. Plates were stored for 24 h at 37˚C in a humidified atmosphere at 5% (v/v) CO_2. The next day, each bone specimen was placed into a new well and the remaining osteoblast-like cells attached to the original culture dish wells were counted and subtracted from 10,000 in order to obtain the initial cell adhesion rate. For controls, cells were seeded directly onto the bottom of 6-well dishes without bone specimens. Tissue cultures were maintained for 42 days at 37˚C in a humidified atmosphere at 5% (v/v) CO_2; media were changed every fourth day.

Hexosaminidase assay. At day 1, 14, 28, and 42, bone scaffolds and control wells were analyzed and cell-associated hexosaminidase activity determined in triplicate as a measure of cell proliferation by employing a colorimetric assay according to the protocol of Landegren (24).

Histology and immunohistochemistry. After 6 weeks in culture, scaffolds were fixed with buffered 4% (w/v) paraformaldehyde, decalcified, and embedded in polymethylmethacrylate (PMMA), according to standard procedures. For histological documentation and immunohistochemical assessment of biomarkers, 5 μm-thick sections were cut and processed. Connective tissue was stained according to the trichrome Masson-Goldner staining procedure which combines the staining dyes azophloxine, orange G, and light green SF, whereby muscle fibre, collagenous fibre, fibrin and erythrocytes can be visualized selectively.

Active bone formation was assessed by determining alkaline phosphatase as a product of osteoblast activity in the fixed sections which were incubated for 30 min at room temperature with nitroblue tetrazolium chloride 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (Roche Diagnostics, Penzberg, Germany).

For demonstration of osteocalcin (OC) and collagen type I (COL-I) protein expression endogenous peroxidase activity present in fixed sections was blocked by pretreatment with 0.3% H_2O_2 in methanol for 30 min at room temperature. Thereafter, sections were reacted with antibodies against OC (dilution 1:50; Quartett, Berlin, Germany) and COL-1 (dilution 1:25; Biotrend, Cologne, Germany). Antibody binding was visualized by use of the avidin-biotin-peroxidase method (Vectastain ABC Elite Kit; Vector Laboratories, Burlingame, CA, USA) combined with 3-amino-9-ethylcarbazole (Dako, Hamburg, Germany) as chromogen substrate.

Histological staining and immunohistochemical biomarker staining was recorded by light microscopy applying an Axiovert 25 microscope (Zeiss, Jena, Germany) equipped with an Axiocam camera (Zeiss).

Statistics. Statistical analysis employed Student's *t*-test. All results were expressed as the means±standard deviation of assessment of the samples in triplicate. Differences were considered statistically significant at $p \leq 0.05$.

Results

In the present study, we show that on day 1, after seeding 10,000 osteoblast-like cells prepared from donor bones onto pieces of bone specimens, 73.8% of cells adhered to the bone matrix, as assessed by the hexosaminidase activity assay. There was no significant difference observed between control

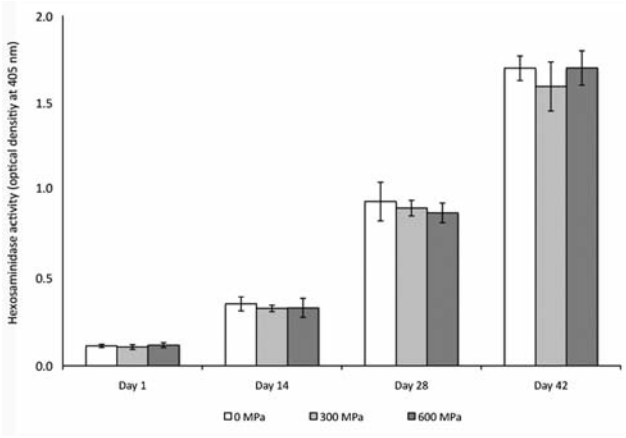

Figure 1. *Hexosaminidase activity determined on day 1, 14, 28, and 42 in pooled (6 patients) primary human bone cells seeded on HHP-pretreated bone specimens placed in microtiter plate wells.*

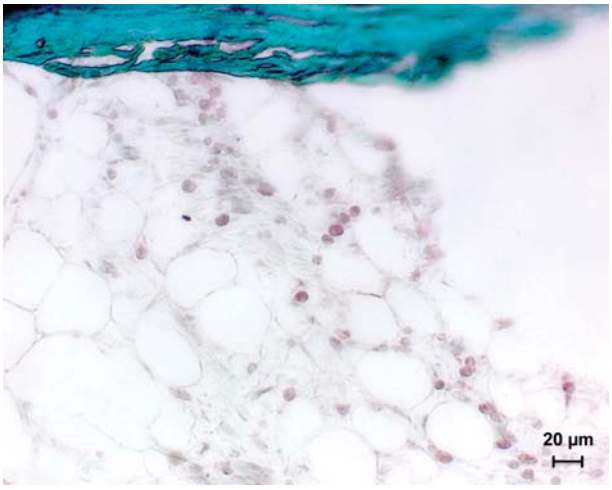

Figure 2. *Histological staining (Masson-Goldner) of HHP-treated (600 MPa) bone specimen colonized with culture pooled bone cells, after 6 weeks in culture. Green: Old bone matrix. Purple: New bone formation.*

experiments without HHP treatment (74.1%) and those treated with 300 MPa (70.0%) or 600 MPa (77.4%). Cell proliferation was noted between day 14 and day 42; again it was similar for HHP values up to 600 MPa (Figure 1).

Histological staining of bone pieces pretreated with different pressure values and seeded with osteoblast-like cells after 6 weeks in culture showed intact cells homogeneously spread on the bone surface; new bone formation also occurred (Figure 2). For this, the bone specimens were assessed for alkaline phosphatase enzymatic activity and for OC and COL-I expression (Figure 3).

Moreover, we noticed an increase in cell proliferation of these attached bone cells between day 14 and day 42 (Figure

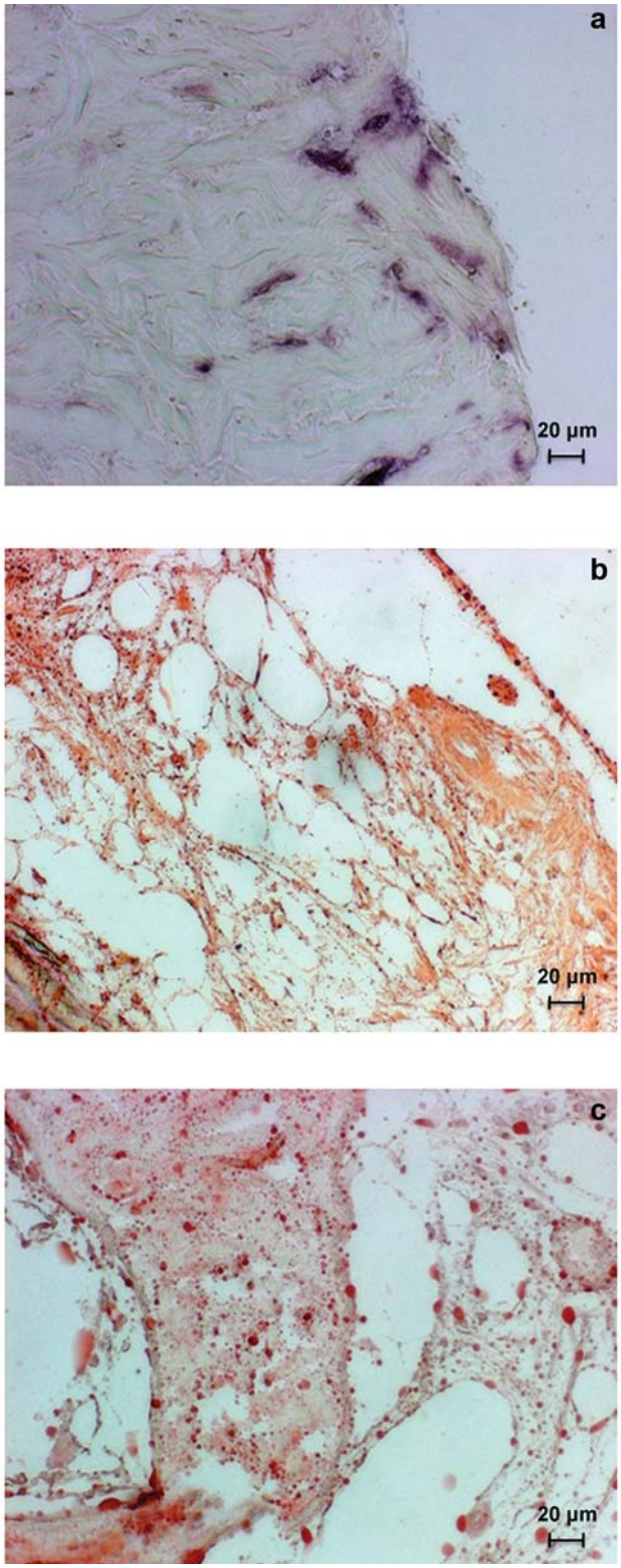

Figure 3. *Alkaline phosphatase activity stain (a), and immunohistochemical staining for osteocalcin (b) and collagen type I (c) of HHP-treated (600 MPa) bone specimen colonized with culture pooled bone cells, after 6 weeks in culture.*

1); histological examination confirmed the presence of a multilayer of bone cells on the HHP-treated bone specimens (Figure 2).

Bone cell differentiation after 6 weeks in culture was verified by staining HHP-treated, bone cell populated bone tissue for alkaline phosphatase activity, which is an indicator for osteoblast-like cells and thus an indicator for the formation of new bone tissue (Figure 3a) (25). In addition, in the bone, the peptide hormone OC is manufactured exclusively by osteoblasts, and thus is a biomarker for the bone formation process (26). As shown in Figure 3b, OC was expressed in the seeded bone cells attached to the HHP-treated bone specimens. COL-I is a fibrillar collagen found in most connective tissues, including cartilage and bone (26). Immunohistochemical examination of bone specimens treated with increasing pressure rates up to 600 MPa showed no significant difference in COL-I expression (Figure 3c).

Discussion

The aim of the present study was to demonstrate the efficacy of *ex vivo* revitalization of HHP-treated bone specimens with primary human bone cells. Extracorporeal irradiation and autoclaving are possible methods for sterilization of tumor-bearing bone segments. However, both methods suffer from disadvantages due to insufficient biomechanical and biological properties of the bone subsequent to irradiation or autoclaving (5, 11, 12). Since the food industry successfully demonstrated the use of HHP for preserving food and beverages, medical application of this technology was envisioned, for instance the use of extracorporeal HHP as an alternative approach for tumor devitalization of bones.

Our previous studies investigated the influence of HHP on biological and biomechanical properties of bone explants (16-23, 27). Owing to the effect of elevated pressure treatment, eukaryotic cells including tumor cells are irreversibly impaired at pressure values exceeding 350 MPa (17-19), while at pressure values up to 600 MPa, biomechanical properties of bone, tendon and cartilage remain nearly unchanged (21-23). As a result, extracorporeal treatment with HHP inhibits cell outgrowth of tumor-afflicted bones and cartilage (20). However, HHP treatment did not significantly affect adherence, spreading, or proliferation of human osteoblast-like cells on major extracellular matrix proteins present in bone tissue, such as fibronectin, vitronectin, and type I collagen (27).

Subsequent to our previous experiments, the present study was designed to demonstrate whether colonization of explanted HHP-treated human bone by osteoblast-like cells and their subsequent proliferation was affected by HHP pretreatment. The excellent adhesion rate of 73.8% along with continuous cell proliferation up to 42 days and the proofed osteogenic differentation that HHP treatment of human bone specimen does not affect the osteoconductive and osteoinductive properties. For successful ingrowth of bone cells into mid-size bone defects, competent osteogenous cells are required for successful colonization of the connecting surface of the bone to achieve effective remodelling of the bone defect (28).

For extracorporeal irradiation or autoclaving of tumor-afflicted bone, clinical, radiographic and biomechanical protocols are available (29, 30). However, *in vitro* investigations of revitalization of irradiated or autoclaved bone tissue are lacking. The present results demonstrate effective adhesion and proliferation of primary human bone cells onto HHP-devitalized human bone specimens. Compared to other tissue engineering procedures mentioned in the literature, the bone cell adhesion rate of 73.8% after HHP treatment is very promising (31). Published long-term *in vitro* follow-up results demonstrating the effect of scaffolds such as sterilized bone matrices or synthetic implants on human osteoblast-like cells are rare. As an example of the few reports available in the literature, similar to our findings, Klein *et al*. showed comparable long-term proliferation rates and osteogenic differentiation of human osteoblast-like cells seeded on titanium surfaces over a duration of 21 days in culture (32).

Lastly, the present study demonstrates, for the first time, the successful revitalization of HHP-treated bone specimens with human osteoblast-like cells. Regarding bone cell proliferation and osteogenic differentiation, the results are indicative of sufficient osseointegration by employing this gentle mode of bone sterilization. In accordance with our previous results, we anticipate that short-term HHP treatment up to 600 MPa causes no alterations in bone matrix which would impair its osteoconductivity and osteoinductivity, while a pressure level of 350 MPa is sufficient to induce devitalization of malignant bone tumor segments.

In summary, these and the present findings suggest that HHP could be used clinically for *ex vivo* bone tumor devitalization with increased and accelerated bone graft integration in order to allow autologous reimplantation of previously tumor cell-affected bone segments.

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