Effect of Extracorporeal High Hydrostatic Pressure on Cellular Outgrowth from Tumor-afflicted Bone

JOHANNES SCHAUWECKER[1], LILLY WIRTHMANN[1], MANFRED SCHMITT[2], JUTTA TUEBEL[1], URSULA MAGDOLEN[1], REINER GRADINGER[1], WOLFRAM MITTELMEIER[3] and PETER DIEHL[1]

[1]*Department of Orthopedic Surgery and*
[2]*Clinical Research Unit, Department of Obstetrics and Gynecology, Technical University of Munich;*
[3]*Department of Orthopedic Surgery, University of Rostock, Germany*

Abstract. *At present, in orthopedic surgery, the reconstruction of bone defects following resection of malignant tumors is effected by several methods. The irradiation and autoclaving of bone segments are the 2 methods of choice to extracorporeally devitalize the resected tumor-bearing bone segments. An alternative, gentle method of devitalizing bone-associated cells by exposing normal and tumor cells to extracorporeal high hydrostatic pressure (HHP) has been introduced. The aim of this study was to examine the ex vivo effect of HHP on the cell growth of normal and tumor-afflicted freshly-resected small human bone segments. For this, tumor-afflicted human bone segments of 5x5x5 mm in size, obtained during surgery from 14 patients suffering from chondrosarcoma or osteosarcoma, in comparison to bone segments obtained from 36 patients with normal bone, disease were exposed to HHP levels of 0, 150 and 300 MPa for 10 min at 37˚C. Following HHP-treatment, the specimens were placed into cell culture and observed for cell outgrowth up to 50 days. In control samples (0 MPa), rapid outgrowth of cells was observed. HHP-treatment of 150 MPa however, resulted in reduced outgrowth of cells from these bone specimens; at 300 MPa, no outgrowth of cells was detected. Light microscopy and standard histological examination showed morphological changes between control samples (0 MPa) and 150 MPa. Our results suggest that the treatment of tumor-afflicted bone and the associated cartilage by HHP leads to the devitalization of bone cells concomitant with complete impairment of cellular outgrowth, a precondition for re-implantation of the HHP-treated bone.*

Correspondence to: Dr. Johannes Schauwecker, Orthopaedische Klinik und Poliklinik, Technische Universitaet Muenchen, Ismaninger Strasse 22, D - 81675 Muenchen, Germany. Tel: +49-89-4140-2569, Fax: +49-89-4140-4849, e-mail: schauwecker@lrz.tum.de

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In general, malignant tumor disease of bone and soft tissue requires complete resection of the affected tissues, often leading to severe segmental skeletal defects. The consecutive reconstruction of the resected bone, by using synthetic or biological bone matrix replacement substances or metallic bone substitutions and adjuvant chemotherapy, has been established (10, 11). Each of these approaches, however, has its own drawbacks and is often associated with significant functional limitations of the patient and adverse side-effects, so that new innovative strategies are highly desired.

Extracorporeal irradiation or autoclaving of the affected bone segment to be used for re-implantation is an alternative approach to synthetic limb reconstruction (1-4, 16, 18). Nevertheless, one has to assure that all of the tumor cells are inactivated prior to re-implantation in order to avoid disease recurrence. Yet, irradiation or autoclaving of bone segments may cause drastic alterations of the biomechanical and biological properties of the treated tissue, a major concern regarding this type of approach (1-4, 16-18). As a new technology, already in preclinical testing, the administration of short-term high hydrostatic pressure (HHP) to the resected bone segment immediately after surgery offers an alternative to the traditional methods of tumor-affected bone treatment.

In previous studies, the effect of HHP on the viability of normal and diseased human cells was described (6, 7, 12). Here, it was observed that excessive hydrostatic pressure, with values up to 600 MPa, did not impair the biomechanical properties of bone (14), tendons (8) or cartilage (5). Moreover, no significant changes in the adhesive or growth-promoting properties of the extracellular matrix proteins fibronectin, vitronectin or collagen-I in mediating cell adhesion due to this treatment could be observed (9). The

Table I. *Results of visual inspection by light microscopy on cultured untreated specimens and HHP-treated (150 MPa and 300 MPa) bone tumor specimens; please note: none = after up to 50 days of culture, no outgrowth of cells was observed.*

Patient no.	Tumor type, nuclear grade	Day of outgrowth of first cells								
		untreated			HHP (150 MPa)			HHP (300 MPa)		
		1	2	3	1	2	3	1	2	3
1	chondrosarcoma, G1	2	2	3	none	none	none	none	none	none
2	chondrosarcoma, G1	5	4	5	13	12	10	none	none	none
3	chondrosarcoma, G1	3	2	2	3	4	3	none	none	none
4	chondrosarcoma, G2	2	2	1	3	5	5	none	none	none
5	chondrosarcoma, G3	1	1	1	3	4	3	none	none	none
6	chondrosarcoma, G3	2	1	1	3	3	4	none	none	none
7	chondrosarcoma, G3	1	2	2	3	3	2	none	none	none
8	osteosarcoma, G1	2	2	2	3	4	5	none	none	none
9	osteosarcoma, G2	3	2	2	none	none	none	none	none	none
10	osteosarcoma, G2	1	1	2	none	none	none	none	none	none
11	osteosarcoma, G2	2	3	3	none	none	none	none	none	none
12	osteosarcoma, G3	2	2	2	none	none	none	none	none	none
13	osteosarcoma, G3	1	1	1	2	2	1	none	none	none
14	osteosarcoma, G3	2	1	2	4	4	3	none	none	none

objective of this study was to examine whether the treatment of resected tumor bone segments by HHP would result in efficient tumor cell damage within the bone, as already shown by us for dispersed normal and tumor cells.

Materials and Methods

Bone biopsies from 14 patients, afflicted with either chondrosarcoma (7 patients) or osteosarcoma (7 patients) with different nuclear grades, were taken during surgery and tissue blocks of 5x5x5 mm in size were prepared, 12 samples each per patient. None of the patients received chemotherapy or irradiation therapy prior to surgery. As a control, trabecular bone chips out of the resected femoral heads were taken from 36 patients undergoing total hip arthroplasty, 12 samples each per patient. The tissues were collected with the written informed consent of the patient and governed by a Hospital Ethics Committee vote.

The resected specimens were placed into 15-ml Falcon tubes (Becton Dickinson Biosciences, Heidelberg, Germany), filled with appropriate medium as described below, and then tightly sealed with Parafilm® (American National Can, Chicago, IL, USA), after elimination of any remaining air in the vials. The tubes were then placed into a water-filled pressure chamber of a custom-made high hydrostatic pressure device (Record Maschinenbau, Koenigssee, Germany) and exposed to pressure values of 0 (normal atmospheric pressure), 150 MPa or 300 MPa for 10 min at 37˚C.

The different high pressure values were reached within a few sec and were sustained for 10 min before the pressure was reduced to atmospheric pressure. Immediately after decompression, the tumor segments were placed into medium-filled 50-ml Falcon culture flasks (Becton Dickinson Biosciences). The 7 osteosarcoma specimens and the 36 normal bone segments were cultured in 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. The 7 chondrosarcoma specimens were cultured in L-15 Leibovitz medium 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 without CO_2. The medium was changed every fourth day.

The outgrowth and shape of the cells were checked daily by light microscopy and were photo-documented for a follow-up period of 50 days. One bone specimen from each patient, 1 HHP-treated and 1 control respectively, was fixed in 4% paraformaldehyde (w/v) after 3 days in culture for histological assessment, followed by a decalcification step and paraffin-embedding, according to standard procedures. Sections of 5-μm thickness were cut and stained using a modified Masson-Goldner trichrome stain.

For immunocytochemical staining, cells, having evaded from non-malignant control tissues cultured on chamber slides, were used on follow-up day 50. The cells were fixed in 4% paraformaldehyde (w/v), air-dried and rinsed with water. Endogenous peroxidase activity was blocked by pretreatment of the slides with 0.3% H_2O_2 in methanol for 30 min at room temperature. Thereafter, the cells reacted with antibodies against osteocalcin (OC) and collagen I (Col-I), respectively. Antibody binding was visualized by use of the avidin-biotin-peroxidase method (Vectastain ABC Elite Kit; Vector Laboratories, Burlingame, CA, USA). For alkaline phosphatase (ALP) activity, fixed cultures were incubated for 30 min with Nitro blue tetrazolium chloride / 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (Roche Diagnostics, Penzberg, Germany).

Results

Bone segments from normal bone chips (n=36) and tumor-afflicted chondrosarcoma (n=7), as well as osteosarcoma (n=7), were exposed to hydrostatic pressure up to 300 MPa

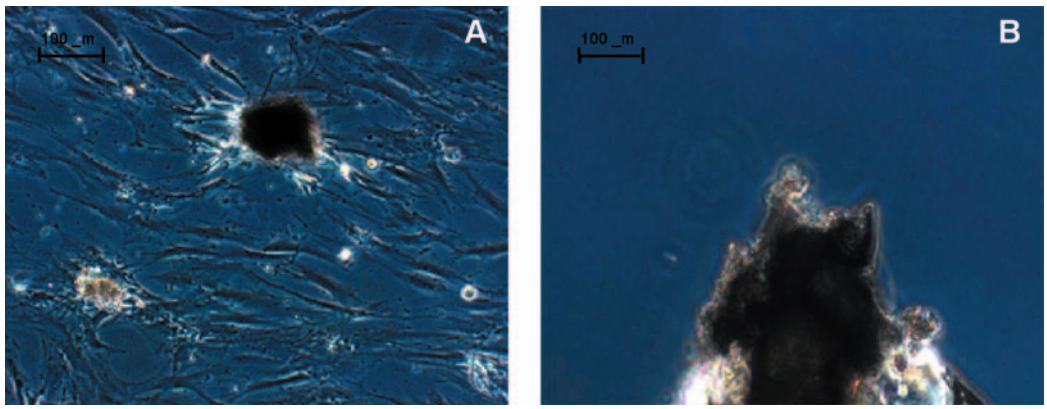

Figure 1. *Outgrowth of cells from an (A) untreated compared to a (B) HHP-treated (300 MPa, 10 min, 37˚C) osteosarcoma-afflicted bone segment with no detectable outgrowth; please note: photo-documentation (A) was performed on follow-up day 14 and (B) on follow-up day 50.*

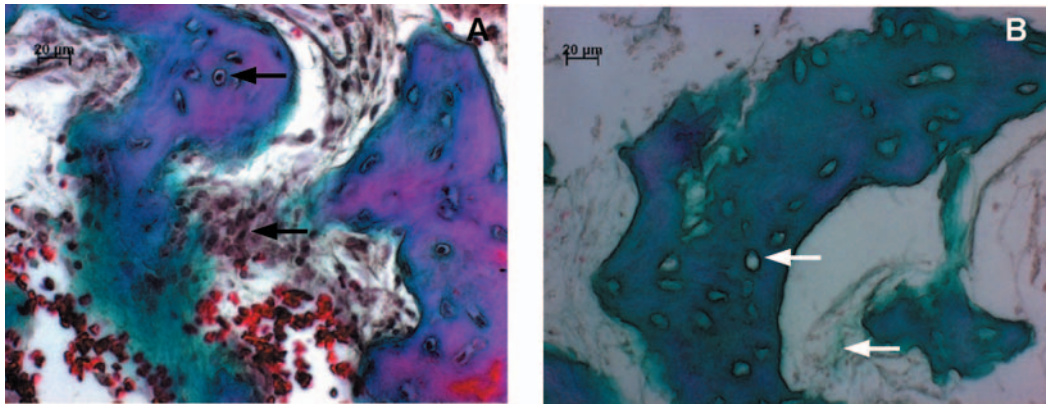

Figure 2. *Decalcified Masson-Goldner-trichrome-stained sections of (A) untreated and (B) HHP-treated (300 MPa, 10 min, 37˚C) osteosarcoma - afflicted bone segment; please note: red cells: erythrocytes, black arrows: cells in bone lacunae and extracellular matrix, white arrows: bone lacunae and extracellular matrix without cells.*

(10 min, 37˚C) and were then put into culture and the outgrowth of cells was observed microscopically for up to 50 days in culture.

The outgrowth of cells from normal bone was detected between days 2 and 7 (median 4). Similarly, in untreated tumor specimens, a rapid outgrowth of tumor cells was observed between days 1 and 5 (median 2) (Table I, Figure 1A). The outgrowing cells of normal bone reacted positively for the osteogeneic markers ALP, OC and Col-I, demonstrating their bone tissue origin. Upon histological assessment, the modified Masson-Goldner-stained sections of the bone specimens showed intact tissue displaying numerous normal or tumor cells, both in the cell lacunae and around the bone matrix (Figure 2A).

At 150 MPa, cell outgrowth from normal bone chips was observed in about 75% of the HHP-treated non-malignant specimens, with an average first cell appearance on day 13, demonstrating a delayed and diminished outgrowth of cells evading from the normal bone upon HHP-treatment. A corresponding observation was made with the tumor tissue specimens where fewer cells had evaded at later time-points compared with the untreated tumor specimens. There was no outgrowth of cells at all in 5 samples (36%). In the remaining 9 samples (64%), outgrowth was observed, but delayed, mostly on day 3 and, at the latest, on day 13 (median 3) (Table I).

Most interestingly, no outgrowth of normal cells or tumor cells from the cultivated bone specimens was detected at 300 MPa (Table I, Figure 1B). The histological examination of decalcified bone from normal and tumor segments showed no intact cells, but empty cell lacunae and a morphologically-unchanged bone matrix (Figure 2B).

Discussion

In tumor patients, the re-implantation of an autologous bone segment following irradiation or autoclaving has several advantages over allograft bone transplants or implanted prostheses (2, 3, 13, 15). However, autologous bone transplants undergoing such treatment may show severe biomechanical and biological alterations and, therefore, are of limited use with respect to re-incorporation after re-implantation. These unsatisfactory results call for adequate procedures that will allow tumor cell inactivation of a bone segment, without compromising its biomechanical or biological properties.

In previous preclinical studies, we investigated the viability of adherently growing (6, 12) and suspended (6, 7) human normal cells and human tumor cell lines which were subjected to HHP between 50 and 350 MPa. At 350 MPa, all of the cells were damaged and nonviable, indicating that normal cells and tumor cells can be irreversibly impaired by excessive HHP.

To date, no information has been available on the effects of HHP on normal bone and tumor-afflicted bone segments regarding cell viability and cellular outgrowth. The present results reveal that, like single normal cells and tumor cells, tumor bone specimens do not tolerate HHP as high as 300 MPa. At this pressure no outgrowth of normal or tumor cells was detected. This new finding is promising with respect to the rapid devitalization of tumor-associated cells and the subsequent possibility of re-implantation of the once tumor-bearing bone segment.

It is worth mentioning that normal bone seems to be slightly more sensitive to HHP than tumor-afflicted bone or single tumor cells. This is in accordance with our previous finding that normal tissue cells, such as fibroblasts and osteoblasts, are less resistant to HHP than cultivated tumor cells (6, 7). Still, adherently-growing tumor cells were considerably more sensitive to HHP than tumor cells detached from the growth-supporting surface (6).

In the present investigation, no outgrowth of cells was detected after a short-term exposure to 300 MPa. This is in line with the histological examination of the specimens, both at the light microscopic and scanning electron microscopic levels, demonstrating a considerable morphological change with evidence of cell membrane disruption (unpublished data). These findings are in accordance with recent investigations of HHP-treated adherently-growing tumor cells examined by flow cytometry and confocal laser scanning microscopy (12).

In conclusion, we have demonstrated, for the first time, that the cell outgrowth of tumor-afflicted bone and cartilage segments can be efficiently blocked by extracorporeal HHP. These investigations complement our recent data on the exposure of freshly excised bone (14), tendons (8) and cartilage (5) to excessive HHP, which showed that this physical treatment did not significantly change the biomechanical or biological properties of these tissues. These findings raise the hope that HHP can eventually be used in orthopedic surgery as an alternative technique for treating resected tumor-afflicted bone, in order to devitalize cancer cells and allow autologous re-implantation. Before that goal is reached, however, further *ex vivo* and also animal studies are required.

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