Effect of high hydrostatic pressure on biological properties of extracellular bone matrix proteins

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Abstract. In orthopedic surgery, sterilization of bone used for reconstruction of osteoarticular defects caused by malignant tumors is carried out in various ways. At present, to devitalize tumor-bearing osteochondral segments, extracorporal irradiation or autoclaving is mainly used but both methods have substantial disadvantages, for instance, loss of biomechanical and biological integrity of the bone. In particular, after reimplantation, integration of the implant at the autograft-host junction is often impaired due to alteration of osteoinductivity as a result of its irradiation or autoclaving. As an alternative approach, high hydrostatic pressure (HHP) treatment of bone is suggested, a new technology which is in the preclinical testing stage, with the aim to inactivate tumor cells but leaving the biomechanical properties of bone, cartilage, and tendons intact. We investigated the influence of HHP on the major extracellular matrix (ECM) proteins, fibronectin (FN), vitronectin (VN), and type I collagen (Col-I), present in bone tissue, which are accountable for the biological properties within the bone. FN, VN, and Col-I were subjected to HHP ≤600 MPa prior to coating of cell culture plates with these matrix proteins. Thereafter, the capacity of HHP-pretreated FN, VN, and Col-I to affect cell proliferation, cell adherence, and spreading of human primary osteoblast-like cells and the human osteosarcoma cell line Saos-2, was tested. Interestingly, even at HHP ≤600 MPa, all three ECM proteins retained their biological properties because no significant changes were observed between HHP-treated and non-treated FN, VN, and Col-I regarding their biological properties to affect cell adherence, spreading, and proliferation. These data encourage further exploration of the potential of HHP to sterilize tumor-affected bone segments prior to reimplantation. While during this treatment eukaryotic cells including tumor cells will be irreversibly impaired, the bone's biomechanical properties and the biological properties of the ECM proteins FN, VN, and Col-I, respectively, are preserved.

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Introduction

Complete reconstruction of bone defects in patients with malignant bone tumors or osteomyelitis is not always possible. So far, reconstruction of tumor cell-bearing bone is accomplished by two methods: extracorporal irradiation or autoclaving with subsequent reimplantation of the treated bone segment. Such treatments, however, may result in severe alteration of the biomechanical and/or biological properties of the bone segments (1-5). As a consequence, in many cases osteointegration of the reimplanted bone fragment is impaired and subsequent therapeutic treatment is often difficult (6-12). Therefore, it would be of advantage to develop an adequate procedure that could achieve complete tumor cell inactivation of a bone segment but, at the same time, does not compromise the biomechanical, osteoconductive, and osteoinductive properties of the bone, otherwise leading to delayed bone remodeling and unsatisfactory healing. Accordingly, there is an urgent clinical need for alternative methods of bone treatment prior to retransplantation.

HHP is a new technology which is in the preclinical testing stage. Until now, HHP, as an alternative to irradiation or autoclaving, has not been introduced into orthopedic surgery for treatment of tumor afflicted-tendons and/or osteoarticular segments *ex vivo* after *en bloc* resection. In recent studies, the effect of HHP on the viability of normal human cells and on human tumor cells was investigated (13,14). We observed that due to excessive hydrostatic pressure, all of the normal cells as well as the tumor cells were irreversibly damaged and non-viable at 350 MPa while the biomechanical properties of bone, cartilage, and tendons remained unchanged, even at pressures ≤600 MPa (15,16) (Diehl *et al*, 50th Annual Meeting of the Orthopaedic Research Society San Francisco, CA, USA, 2004).

In the present study, we analyzed the effects of HHP on the biological properties of the ECM proteins, fibronectin (FN), vitronectin (VN), and collagen type-I (Col-I), all present in the bone matrix (17) and partially involved in osteoinduction of the bone. Osteoinduction is defined as the ability of the bone matrix to affect adherence, proliferation, and differentiation of certain bone-related cells such as osteoblasts (18). It is well documented that among other proteins such as osteocalcin, osteonectin, bone sialoproteins and the growth factors, the ECM proteins, FN, VN, and Col-I, are partially responsible for the osteoinductive properties of the bone (19-22). FN, VN, and Col-I are multifunctional proteins that are synthesized by many cell types, including osteoblasts, which exert their biological effects through transmembrane cell adhesion receptors of the integrin super-family that physically couple the cell-cytoskeleton to the ECM. Adhesion of cells to ECM proteins via integrin interaction evokes signals that influence numerous cellular responses such as survival, migration, proliferation, and cell differentiation (23,24).

In this study, we report for the first time on the non-significant effect of HHP on the biological properties of the ECM proteins, FN, VN, and Col-I, subjected to HHP of ≤600 MPa.

Material and methods

Cell culture. The human osteosarcoma cell line Saos-2 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM, Biochrom KG, Berlin, Germany) supplemented with heat-inactivated 10% (v/v) fetal bovine serum (FBS), 2 mM glutamine, 1% (w/v) penicillin and streptomycin, and MEM-vitamins (Biochrom) at 37˚C in a humidified atmosphere containing 5% (v/v) CO_2.

Human primary osteoblast-like cells were isolated from cancellous bones originating from patients undergoing hip replacements. Only bone which would otherwise have been discarded was used and its collection was approved by the University Hospital Ethics Committee. Cleaned and minced bone fragments were cultured in 75 cm^2 tissue culture flasks (Falcon, Becton Dickinson, NJ, USA) in calcium-free DMEM (Biochrom) supplemented as described above. After two weeks the medium was replaced by calcium-containing DMEM supplemented with 50 μg/ml ascorbic acid (Sigma-Aldrich, Steinheim, Germany) and 4 ng/ml dexamethasone (Sigma-Aldrich). The passages 2-4 were used and the cells of four patients pooled to level out individual variances. The osteoblastic phenotype of the cultured cells was confirmed by histologic staining for alkaline phosphatase and collagen type-I.

High hydrostatic pressure (HHP) treatment of fibronectin (FN), vitronectin (VN), and type I collagen (Col-I). Human FN was obtained from BD Biosciences (Bedford, MA, USA), Col-1 from Rockland (Gilbertsville, PA, USA), and purified VN was kindly provided by K.T. Preissner, University of Giessen, Germany. Dilutions of 10 μg/ml FN or Col-I and 5 μg/ml VN were prepared in phosphate-buffered saline (PBS) and transferred to 1.2 ml cryogenic vials (Nalgene, Rochester, NY, USA). The vials were tightly sealed with Parafilm® (American National Can, Joplin, MO, USA), vacuum-packed in plastic foil and then placed into the water-filled central cavity of the custom-made high pressure device (Record GmbH, Koenigsee, Germany). The vials were exposed to HHP values of 300 and 600 MPa, respectively. The defined HHP value was reached within a few seconds, the vials kept under pressure for 10 min at room temperature and then relaxed to atmospheric pressure within a few seconds.

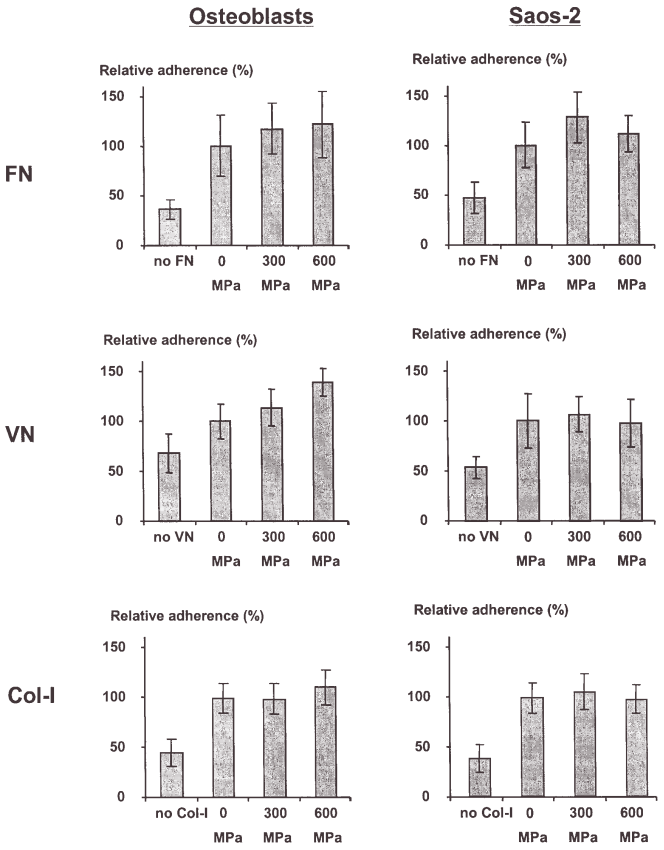

Figure 1. Adhesion of osteoblast-like cells and Saos-2 tumor cells to native or HHP-treated ECM proteins, FN, VN, and Col-I. Wells of 96-well microtiter plates were coated with ECM proteins, FN (3 μg/ml), VN (0.1 μg/ml), and Col-I (0.1 μg/ml), in PBS, control wells received PBS only. Another set of wells received ECM proteins which had been pre-treated with HHP (300 or 600 MPa, 10 min, 20˚C). Then osteoblast-like cells (800 cells/well) and Saos-2 cells (6000-10000/well) were added and allowed to attach and spread to the wells. After 1.5 h at 37˚C of incubation in DMEM/1% (w/v) BSA, non-adherent cells were removed and adhesion assays were performed to determine the relative number of attached cells. Numbers of cells, adherent to non-treated ECM proteins, FN, VN, or Col-I were set to 100%. The data presented are mean values of four independent experiments.

Cell adhesion and cell proliferation tests. For coating of Break-Apart Module-96-well microtiter plates (Nunc, Wiesbaden, Germany), 50 μl of untreated or HHP-treated FN, VN, and Col-1 were applied per well and incubated at 4˚C overnight at different concentrations (FN: 3 μg/ml, VN and Col-I: 0.1 μg/ml). Cells only attach efficiently to this type of microtiter plate if the wells are coated with adhesion promoting factors such as the ECM proteins, VN, FN, or Col-I. After coating with these proteins, the wells were washed twice with PBS and then incubated for 1 h at room temperature with 1% (w/v) BSA (bovine serum albumin, fraction V, Sigma-Aldrich) in DMEM to block residual uncoated sites of the wells.

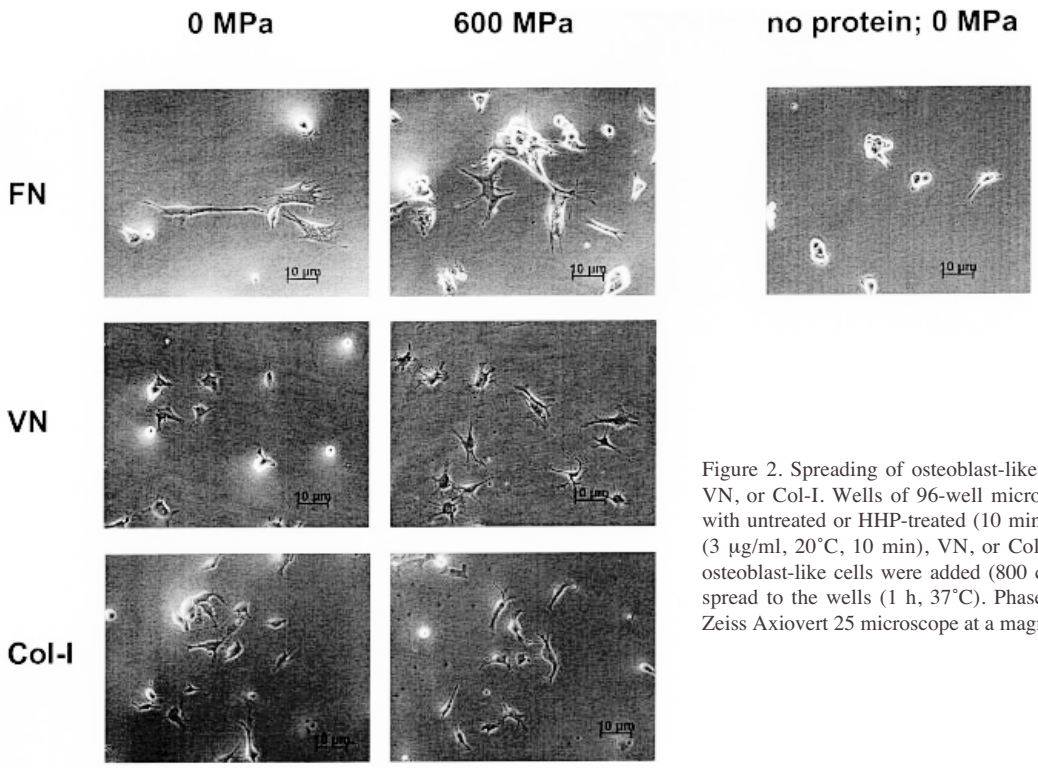

Figure 2. Spreading of osteoblast-like cells on native or HHP-treated FN, VN, or Col-I. Wells of 96-well microtiter plates were filled with PBS or with untreated or HHP-treated (10 min, 20˚C, 600 MPa) ECM proteins FN (3 µg/ml, 20˚C, 10 min), VN, or Col-I (0.1 µg/ml, 20˚C, 10 min). Then osteoblast-like cells were added (800 cells/well) and allowed to attach and spread to the wells (1 h, 37˚C). Phase contrast images were taken with a Zeiss Axiovert 25 microscope at a magnification of x100.

Cells were detached from the cell culture flask surface by treatment with 0.01% (w/v) trypsin-EDTA (Gibco, Invitrogen, Paisley, UK), harvested by centrifugation, and washed in PBS. The cells were resuspended in medium and seeded at a density of 800 cells (osteoblasts) and 6,000-10,000 cells (Saos-2) per well (37˚C). As serum contains considerable amounts of ECM proteins, FN and VN, serum-free medium (DMEM) containing 1% (w/v) BSA was used. After attachment of the cells to the microtiter plate wells, serum-free medium was replaced by 2% (v/v) fetal bovine serum (FBS) containing medium (DMEM) for Saos-2 cells and 10% (v/v) FBS containing medium for the osteoblast cells, respectively. Adhesion experiments were stopped after 1.5 h, and rate of cell proliferation analyzed after 2 and 4 days (Saos-2) and after 7 and 14 days (osteoblast-like cells).

For quantification of cell number, the cell's lysosomal hexosaminidase activity was determined by use of an enzymometric colorimetric assay (25). Spreading of cells on VN, FN, or Col-I-coated microtiter plates was examined by light microscopy, 1 h after cell seeding, applying the Axiovert® 25 microscope (Zeiss, Jena, Germany). Photographs were taken using a Zeiss Axiocam® camera at a magnification of x100.

Results and Discussion

At present, extra-corporal irradiation or autoclaving is mainly used to sterilize tumor-bearing osteochondral segments to be used for reconstruction of osteoarticular defects (1-4). Yet, both methods have substantial disadvantages, for instance, loss of biomechanical and biological integrity of the bone (4,12,26). As an alternative approach, a new technology, high hydrostatic pressure (HHP) treatment of bone is tested in preclinical studies with the aim to inactivate tumor cells in the bone but leaving the biomechanical properties of the bone, cartilage and tendons intact (15,16). Pressure levels ranging from 300-600 MPa are required to destroy tumor cells and normal cells (13). However, HHP may exert its effect not only on tumor cells and normal cells present in the bone but also on bone matrix proteins, including the extracellular matrix proteins, FN, VN, and Col-I, which are also involved in osteoinductive processes taking place within the bone matrix (17,18).

In order to investigate this, FN, VN, and Col-I were subjected to HHP ≤600 MPa (10 min, room temperature) prior to coating of cell culture plates with these ECM proteins. Thereafter, the capacity of HHP-pretreated FN, VN, and Col-I to affect cell proliferation, adherence, and spreading of two different cell types; human primary osteoblast-like cells and human osteosarcoma Saos-2 cells, was tested. Interestingly, the capacity of FN, VN, and Col-I to mediate adhesion of osteoblast-like cells and Saos-2 cells was not impaired by pretreatment with HHP ≤600 MPa (Fig. 1). Exemplarily, the spreading pattern of osteoblast-like cells on the non-treated and HHP-treated ECM proteins was examined (Fig. 2). Irrespective of pretreatment with HHP ≤600 MPa, the cells adhered equally well to FN, VN, and Col-I, with extensive cell spreading shown on FN which was less pronounced on VN and Col-I as underlying growth substrate. Coating of culture plate wells with FN, VN, or Col-I promoted proliferation of osteoblast-like cells and Saos-2 cells in a time-dependent manner (measured ≤14 days for osteoblast-like cells and ≤4 days for Saos-2 cells), compared to cells kept in non-coated wells (Fig. 3). Pretreatment of these ECM proteins by HHP ≤600 MPa did not diminish their cell proliferation capacity towards these two types of cells.

The results of this study demonstrate for the first time that the biological functions of the ECM proteins, FN, VN, and Col-I, also present in the bone matrix, are not deteriorated after HHP treatment *in vitro* regarding their cell proliferation, spreading, and adherence capacity. This may be due to the fact that, in contrast to irradiation or autoclaving, high hydrostatic

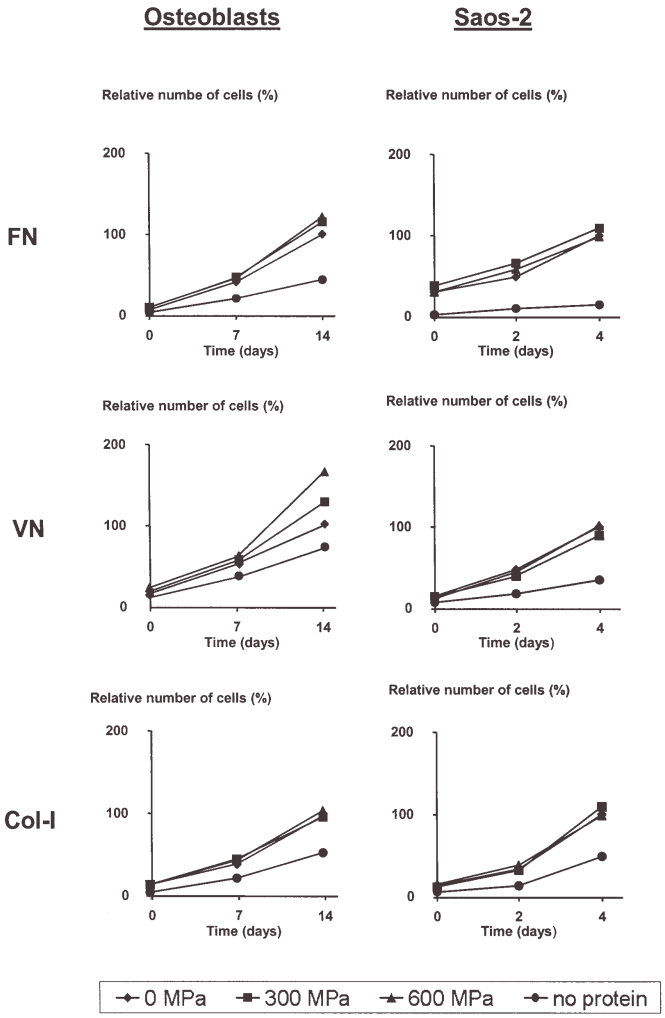

Figure 3. Proliferation of osteoblast-like cells and Saos-2 tumor cells after attachment and spreading on non-treated or HHP-treated FN, VN, and Col-I. Wells of 96-well microtiter plates were filled with PBS or with untreated or HHP-treated (10 min, 20˚C, 300 and 600 MPa, respectively) ECM proteins FN (3 μg/ml, 20˚C, 10 min), VN, or Col-I (0.1 μg/ml, 20˚C, 10 min). Then osteoblast-like cells (800 cells/well) and Saos-2 cells (6000-10000/well) were added and allowed to attach and spread to the wells (1 h, 37˚C). The hexosaminidase test was applied at day 0, 2, and 4 (Saos-2) or day 0, 7 and 14 (osteoblast-like cells), respectively, to assess for the rate of cell proliferation. The proliferation rate for Saos-2 determined at day 4 and for osteoblast-like cells at day 14 was set to 100%. The data presented are mean values of four independent experiments.

pressure does not exert damaging effects on covalent molecular bonds (27). As a result, natural compounds such as flavors, aromas, and other pharmacologically active molecules are not destroyed by HHP (27) while some multimeric proteins disintegrate at HHP <300 MPa (29). Other proteins, however, are not affected, even at pressures ≤1,000 MPa (28). Conformational changes of proteins are known to be induced by HHP, due to interaction of water molecules with the target molecules, eventually leading to alterations of the intra-molecular non-covalent architecture (28,29).

Generally, the effects of HHP on the structure and function of a defined protein cannot be predicted and therefore may vary considerably, depending on the extent and duration of pressure, temperature, buffer conditions, and the molecular characteristics of a protein. Several enzymes, for instance, are deactivated by HHP while others are not affected by HHP treatment or show an increase in biological activity (28,29).

We have already shown in previous studies that excessive HHP ≤600 MPa damages normal and tumor cells but does not lead to any significant alteration of the biomechanical properties of the bone, which is true for freshly resected cortical and trabecular bone chips but also for articular cartilage and Achilles tendons (15,16). Regarding their biological properties towards osteoblast-like cells and the tumor cell line Saos-2, our results provide evidence that the investigated ECM proteins, FN, VN, and Col-I, are also not affected by HHP ≤600 MPa. As FN, VN, and Col-I are also involved in mediating adhesion and migration of endothelial cells during wound repair and angiogenesis (30), we anticipate that resistance of these ECM proteins towards HHP might also assist in the neovascularization process necessary to occur in the reimplanted HHP-treated bone graft.

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