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Abstract. *Background:* In orthopedic surgery, sterilization of bone used for reconstruction of osteoarticular defects caused by malignant tumors is carried out in different ways. At present, to devitalize tumor-bearing osteochondral segments, extracorporeal irradiation or autoclaving is mainly used, although both methods have substantial disadvantages, e.g. loss of biomechanical and/or biological integrity of the bone and destabilization of the articular surface. In this regard, high hydrostatic pressure (HHP) treatment of bone is a new, advancing technology, now being used in preclinical testing to inactivate tumor cells. To find out if this technique is also suited for extracorporeal inactivation of chondrocytes and chondral tumor cells, the effect of HHP on cell viability and morphology of human chondrocytes / chondrosarcoma cells was investigated in the present study. *Materials and Methods:* SW1353 chondrosarcoma cells and chondrocytes were subjected to HHP in the range of 50 to 350 MPa (10 min, 37°C) and, subsequently, cell viability and cell morphology assessed. *Results:* After exposure at 350 MPa, all HHP-treated chondral cells showed explicit morphological changes, evident by membrane ruffling and bleb formation; chondrosarcoma cells treated this way were irreversibly damaged and not alive. *Conclusion:* We anticipate that, in orthopedic surgery, HHP eventually can serve as a novel, promising technical approach for cell inactivation (including tumor cells) and allow subsequent reimplantation of the osteoarticular autograft.

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Destruction of bone by tumor cells, microorganisms, or trauma is one of the central challenges in orthopedic surgery. Often, for reconstruction of a damaged bone, allogeneic bone transplants are used but the risks of allogeneic transplantation of bones are obvious since such bones may be affected by bacteria, viruses, or tumor cells (1). Therefore, in tumor patients, osteoarticular autografts, after having been subjected to extracorporeal irradiation or autoclaving, are used for limb reconstruction. The presumed advantages of such bone treatment are destruction of tumor cells without disease transmission, while the bone, cartilage and ligamentous tissue are preserved in shape and size. Nevertheless, such treatment has the disadvantage that it may reduce the biomechanical and/or biological integrity of the bone and the mechanical stability of the articular surface (2, 3). High hydrostatic pressure (HHP) is a gentle new alternative to destroy eukaryotic cells, including tumor cells (4-6). In fact, in the non-medical field, HHP is already widely applied to preserve food, thereby avoiding the use of chemical preservatives (7-9).

At present, we are exploring the potential of HHP to impair musculoskeletal tumor tissue cells. To achieve this, immediately after surgery, the resected tissue encompassing bone, cartilage and tendon is subjected to extracorporeal treatment with HHP as high as 600 MPa, in order to destroy tumor cells with the potential to allow subsequent bone reimplantation (5, 6). We observed that, at the high pressure value of 600 MPa, biomechanical properties of bones, tendons, and cartilage (10, 11) remain unchanged. Under these conditions, various normal eukaryotic cells, but also malignant cells such as osteosarcoma and fibrosarcoma cells, were irreversibly damaged (5, 6). Also, we observed that adherently growing osteosarcoma and fibrosarcoma cells are more sensitive to inactivation by HHP than suspension cells; a finding which is very much related to inactivation of tumor cells in bones, where most of the tumor cells are embedded into the bone matrix. In

the present study, we report on the efficient inactivation of cell viability and the accompanying morphological changes of suspended and adherently growing human articular chondrocytes and chondrosarcoma cells.

Materials and Methods

Human cell lines and cell culture conditions. The chondrosarcoma cell line SW 1353, derived from a 72-year-old Caucasian patient (Bank of Biological Material, Genova, Italy), was cultured in L-15 Leibovitz-medium supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (w/v) streptomycin/penicillin, and 1% (w/v) L-glutamine (all Biochrom KG, Berlin). The chondrocytes (Cell-Lining GmbH Berlin, Germany) were cultured in Dulbecco's MEM supplemented with 10% (v/v) FBS, 1% (w/v) HEPES, 1% (w/v) streptomycin/penicillin, and 1% (w/v) L-glutamine. SW 1353 chondrosarcoma cells were seeded in cell culture flasks (Falcon, BD Biosciences, Pharmingen, Germany) at 37°C in a humidified atmosphere without CO₂; chondrocytes were seeded in the presence of 5% (v/v) CO₂. Details of the culture procedure and preparation of suspension cells and adherently growing cells for HHP treatment have been published previously (6).

HHP treatment. Small, flexible 2.3-ml vials, either containing suspension cells or two microtiter plate wells with adherently growing chondrocytes or chondrosarcoma cells, were filled with culture medium, tightly sealed with parafilm (American National Can, Chicago, USA) after having eliminated any air bubbles. Then these sealed vials were placed into a water-filled pressure chamber of a custom-made high hydrostatic pressure machine (RECORD Maschinenbau GmbH, Königssee, Germany) (5, 6) and exposed to pressure values ranging from 50 to 350 MPa (10 min, 37°C). The different pressure values were reached within a few seconds. After the 10-min incubation period, the pressure was relaxed to atmospheric pressure within a few seconds. Immediately after decompression, suspended cells were checked for viability by Trypan blue exclusion. Those cells, initially growing adherently on a fibronectin-coated surface, which were detached while exposed to HHP, were recovered from the supernatants, counted and then subjected to a viability check by Trypan blue exclusion testing; still adherently growing cells were detached by exposure to 0.01% (w/v) trypsin-EDTA (Gibco, Paisley, UK), resuspended in DMEM and then subjected to a viability check by Trypan blue exclusion testing as well.

Flow cytometry. After exposure to HHP in suspension, chondrocytes and chondrosarcoma cells were cultured for another 24 h at 37°C, adherent cells detached by trypsin-EDTA, and subsequently these cells, resuspended in Dulbecco's MEM, combined with non-adherent suspension cells present in the culture supernatant. Afterwards, these cells were subjected to a viability check applying propidium iodide (PI) staining (2.5 µg/ml propidium iodide for chondrosarcoma cells and 20 µg/ml for chondrocytes), according to the manufacturer's protocol (Roche, Penzberg, Germany). Therefore, the cell suspension was centrifuged (5 min), the cell pellet resuspended in 100 µl of PI-containing incubation buffer, and then incubated in the dark (20°C, 15 min). After the addition of another 500 µl of pure incubation buffer, the percentage of damaged cells was determined by means of flow cytometry (FACSCalibur, BD, Biosciences, USA). Cells which stained with DNA intercalating propidium iodide were considered non-viable.

Light microscopy. Human chondrocytes and SW 1353 cells were grown adherently in fibronectin-coated (10 µg/ml) microtiter culture plates until reaching 80% confluence and then subjected to HHP ≤200 MPa (10 min, 37°C), while still being attached to the fibronectin-coated surface. Immediately after decompression, the cells were examined by light microscopy (Axiovert 25, Zeiss, Jena, Germany).

Confocal laser scanning microscopy (CLSM). Human chondrocytes and SW1353 cells, after having been detached from culture flasks by trypsin-EDTA, were placed into culture medium-filled cryovials and exposed to the same HHP treatment as described above. Untreated cells and cells exposed to HHP ≤200 MPa (10 min, 37°C) were examined visually for changes in cell morphology using a Zeiss Axiovert 35 microscope attached to a Leica confocal laser scanning unit (Leica, Heidelberg, Germany) (12) equipped with differential interference contrast microscopy (Normarski Optics).

Statistics. The Mann-Whitney-U-Test was used to assess the significance of difference between test groups. The significance level was set to 0.05.

Results

We observed a severe impact of excessive HHP on cell viability and morphology of human chondrocytes and chondrosarcoma cells. At HHP values of 350 MPa for both the suspended and the adherent state of the cell lines, cell death was achieved within 10 min (37°C) (Figure 1). Regarding the adherently growing cells, at this pressure, no adherently growing cells were detached from the layer of the substratum. Interestingly, adherently growing chondrocytes / chondrosarcoma cells were less resistant to HHP than suspended cells; for the adherent cell lines, cell death was achieved at an HHP of 200 MPa. Chondrosarcoma cells in suspension, but not in their adherently growing state, proved to be more resistant to HHP (100% dead at 350 MPa) than suspended chondrocytes (100% dead at 250 MPa). This transition is also reflected by light scatter and DNA analysis of suspended cells (by flow cytometry) after 200 MPa HHP treatment (Figure 2). At 200 MPa, almost all cells were stained with propidium iodide, indicating interaction of nuclear DNA of dead cells with this dye due to this treatment (Figure 2). At 200 MPa, explicit morphological changes, assessed by forward and side-scatter light analysis, were apparent for chondrocytes and chondrosarcoma cells (Figure 2).

Under normal cell culture conditions, adherently growing chondrosarcoma cells and chondrocytes exhibit a typical spread monolayer cell morphology (Figure 3). When these adherent cells were subjected to an HHP pressure value of 150 MPa (10 min, 37°C), cell morphology and cell adherence pattern changed significantly. Adherent cells retracted from their spindle-like morphology and acquired a rounded, ruffled cell morphology (Figure 3). At HHP >200 MPa, all cells were disrupted and just a few cell fragments could be detected (figure not shown). Detached, suspended cells were round before HHP treatment (Figure 4). HHP of 200 MPa (10 min, 37°C) effected a severe cellular change. Cell membranes were

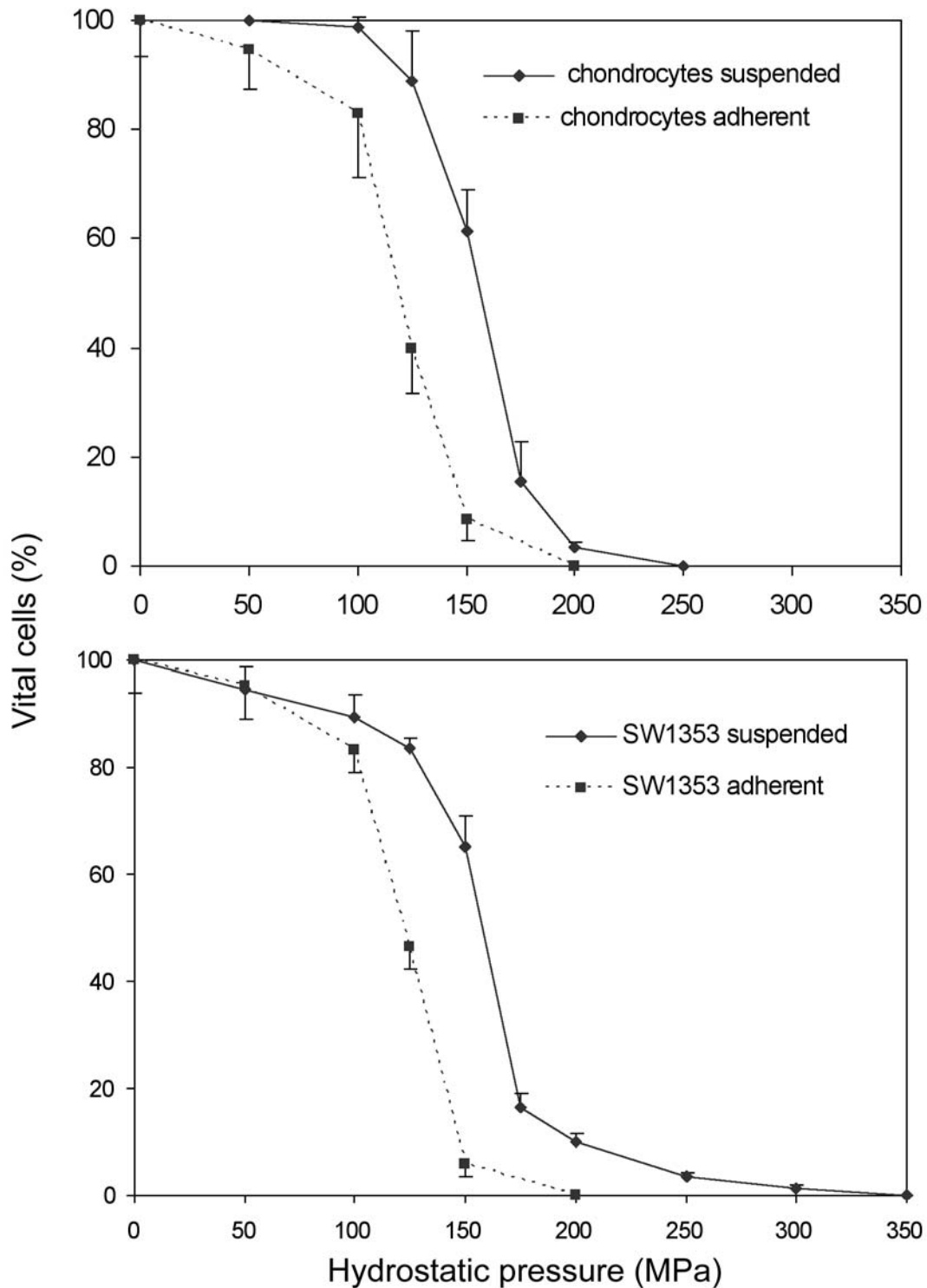


Figure 1. Effect of HHP on human chondrocyte and chondrosarcoma cell viability. Human chondrocytes and chondrosarcoma cells (SW1353) were grown adherently in fibronectin-coated microtiter culture plates. One set each of chondrocytes and chondrosarcoma cells was detached from culture flasks and then these suspension cells subjected to HHP (10 min, 37°C) whereas the other set of cells was treated by HHP while still being attached to the fibronectin-coated culture plate surface. After HHP treatment, cell viability was determined by the Trypan blue exclusion test. Note that a higher susceptibility for cells treated by HHP while being attached to the fibronectin-coated surface in comparison to cells treated in suspension is demonstrated and that SW1353 chondrosarcoma cells were less sensitive to HHP than chondrocytes when treated in suspension. Values are means \pm standard deviation of at least four independent experiments.

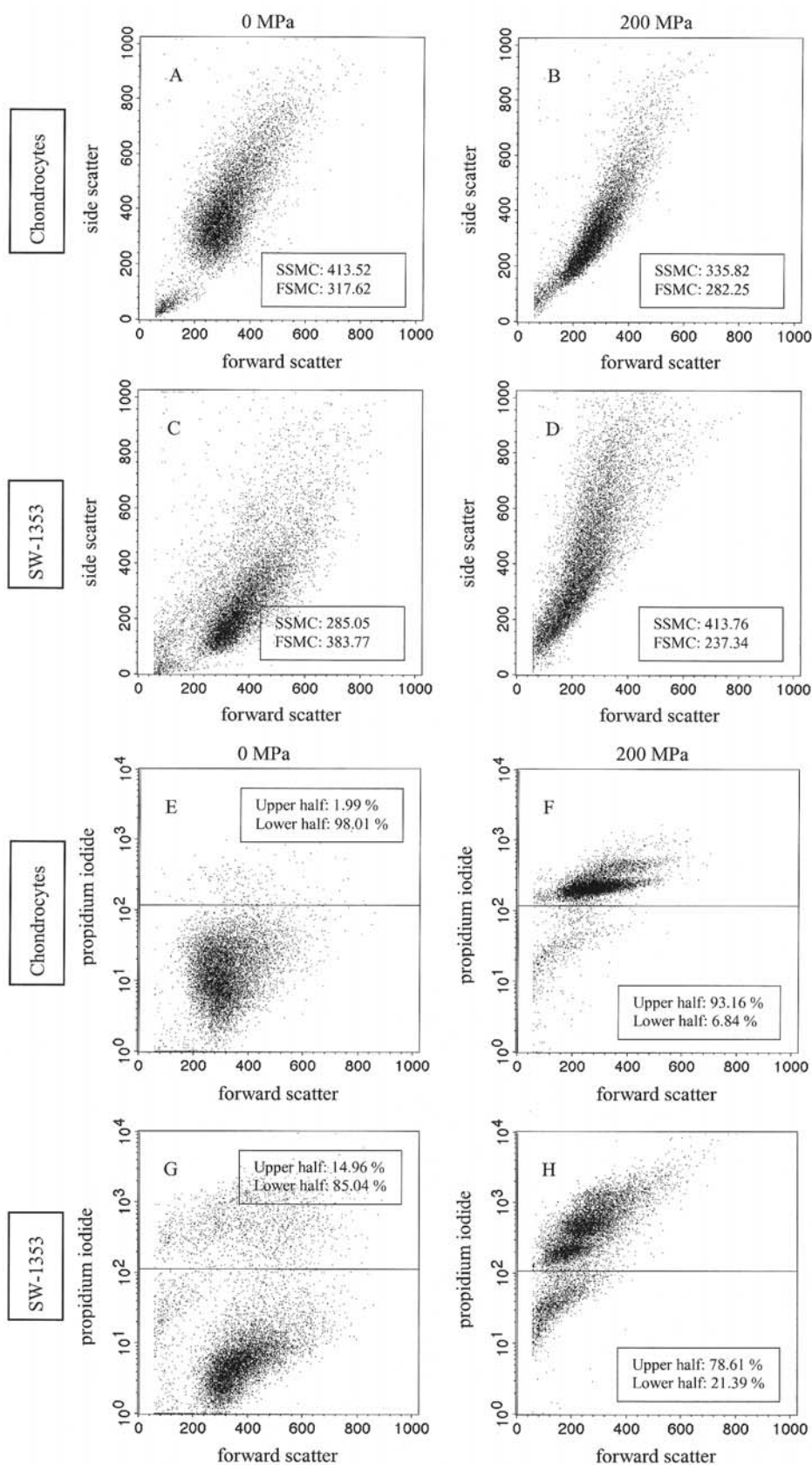


Figure 2. HHP-induced cell death in human chondrocytes and chondrosarcoma cells. Human chondrocytes (A,B,E,F) and SW1353 chondrosarcoma cells (Figure 4 C,D,G,H) were detached (suspension cells) from culture flasks and subjected to HHP of 200 MPa (10 min, 37°C). After another 24-h cultivation under normal conditions, cells were detached, and subsequently stained with propidium iodide (PI) and then examined by flow cytometry. At HHP of 200 MPa almost all of the cells were stained with PI, indicating cell death effected by HHP. Using flow cytometry, at 200 MPa severe morphological changes and declining of cell size of the chondrocytes and chondrosarcoma cells were detected by light scatter analysis.

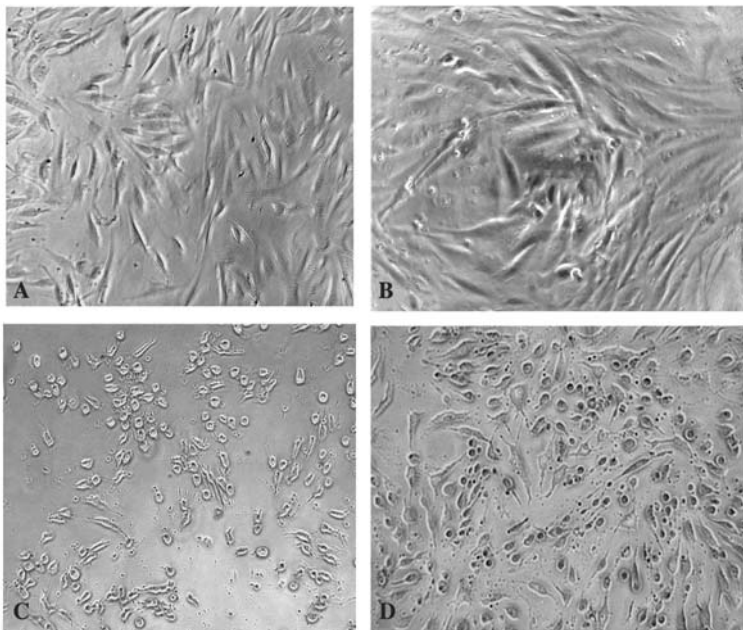


Figure 3. HHP-induced changes in adherent human chondrocytes and chondrosarcoma cells. Human chondrocytes (A,C) and SW 1353 chondrosarcoma cells (B,D) were grown adherently in fibronectin-coated microtiter 96-well culture plates and then subjected to 150 MPa HHP (10 min, 37°C) while still being attached to the fibronectin-coated surface. Immediately after decompression, the cells were subjected to light microscopy analysis. At this pressure, cells did not detach from the culture plate surface but displayed a rounded cell morphology. A,B) no HHP; C,D) plus HHP.

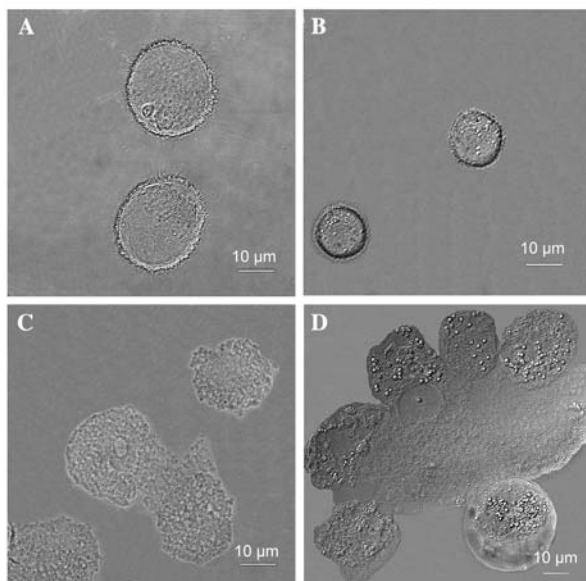


Figure 4. HHP-induced morphological changes in suspended human chondrocytes and chondrosarcoma cells. Human chondrocytes (A,C) and human chondrosarcoma cells (SW1353) (B,D) were detached from culture flasks and then this suspension subjected to HHP (10 min, 37°C): A,B) no HHP; C,D) plus 200 MPa. Immediately after decompression, the cells were subjected to confocal laser scanning microscopy using differential interference contrast microscopy (Nomarski optics). At 200 MPa, we observed a severe impact of HHP on both cell lines; cell membranes were disrupted and subcellular components disintegrated (C,D).

disrupted and subcellular components disintegrated, findings which are in accordance with the drastic change in light scatter properties of the HHP-treated cells as shown by flow cytometry analysis (Figure 2).

Discussion

So far, reconstruction of tumor cell-bearing osteochondral segments has been accomplished by two methods mainly: extracorporeal irradiation or autoclaving with subsequent reimplantation of the treated segment. Both methods, however, suffer from major drawbacks, *e.g.* loss of biomechanical and/or biological integrity (1-4, 13-17) and degenerative arthropathy (18). Accordingly, there is an urgent clinical demand for alternative ways of treating tumor-afflicted osteoarticular segments prior to reimplantation. HHP is such a new technology, now in preclinical testing in order to explore whether such a technique is suited for extracorporeal inactivation of tumor cells present in cartilage, bone, tendon, or connective tissue, while leaving the biomechanical properties unchanged (10, 11).

In a previous study on different human cell lines, we provided evidence that malignant tumor cells are irreversibly damaged by excessive HHP (5, 6). So far, no information was available on the effect of HHP on chondrocytes and chondrosarcoma cells regarding cell viability. Therefore we investigated the effect of increasing HHP (0-350 MPa) on cell morphology and viability of these cell lines, either still being attached to wells of a fibronectin-coated culture plate or to tumor cells after having been detached from the culture flask surface.

The present results revealed that adherent and detached (suspended) chondrocytes and chondrosarcoma cells do tolerate high pressure conditions ≤ 100 MPa but then, exceeding this pressure value, a sharp decrease in viability (tested by Trypan blue uptake) over a relatively narrow pressure, in the range of an additional 50-100 MPa, occurred.

Adherently growing chondrocytes and chondrosarcoma cells demonstrated equivalent pressure tolerance. On the other hand, suspended chondrocytes are more sensitive to HHP than chondrosarcoma cells. This is in agreement with previous findings that suspended normal bone and tissue cells like fibroblasts and osteoblasts were less resistant to HHP than tumor cells (5).

In support of these results, intense staining of chondral cells with propidium iodide was demonstrated at 200 MPa. This staining is the result of immediate irreversible damage of the cells at this pressure value. This is in agreement with the Trypan blue uptake and with morphological changes at 200 MPa, demonstrating disruption, blebbing, ruffling, and clumping of the cells.

Irrespective of the adherence/suspension state, all of the cells were irreversibly impaired at HHP >350 MPa, 10 min; 37°C. This is in agreement with our previous observation with tumor cells, that adherence of cells to fibronectin, an extracellular matrix protein, did not delay HHP-mediated cell death (6); a fundamental result for the inactivation of tumor cells in cartilage, where most of the tumor cells are embedded into the extracellular matrix and not present in suspension. In conclusion, we have demonstrated that both chondrocytes and chondrosarcoma cells are irrisistant to short-term HHP exposure of 350 MPa (10 min). At this pressure value, chondral cells were irreversibly damaged, irrespective of whether they were treated in suspension or in the adherent state. Further to that result, by exposing freshly excised bones, tendons, or cartilage to HHP of 300 to 600 MPa (10, 11), we experienced that their biomechanical properties remained unchanged, whereas both the malignant and non-malignant tissue cells were destroyed. Such finding are very important in orthopedic surgery regarding reconstruction of tumor-bearing osteoarticular segments.

These results give hope that, in orthopedic surgery, HHP will eventually be used as a new and gentle way of treating tumor-afflicted osteoarticular segments to irreversibly damage tumor cells in order to allow autologous reimplantation.

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