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EFFECT OF HYDROSTATIC PRESSURE ON MORPHOLOGICAL AND ULTRASTRUCTURAL ASPECTS OF NORMAL AND OSTEO-ARTHRITIC HUMAN ARTICULAR CHONDROCYTES

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INTRODUCTION

Articular cartilage is a connective tissue routinely subjected to high and varying loads. Under physiological conditions articular cartilage is subject to cycles of loading which control the matrix through the metabolic activity of chondrocytes. These loads alter the extracellular physical environment of the chondrocyte in a complex manner (1-3).

Articular cartilage cycles between a resting hydrostatic pressure of around 0.2 MPa and pressures of 4-5 MPa during normal walking, and pressure can rise to nearly 20 MPa during some activities (4).

During joint loading, chondrocytes in articular cartilage are first exposed to a hydrostatic pressure gradient which is followed by deformation and fluid flow (1,3).

In articular diseases such as osteoarthritis (OA), the catabolic process exceeds the anabolic process, leading to a net decrease in matrix material (5). The aetiology of OA is only partly understood, although the loading of a joint is certainly involved. The intense use of joint and traumatic forces may increase the risk for OA (6). The way in which loading is related to the incidence of OA is not clear.

Several studies have been undertaken in order to investigate the changes

in the cartilage under different loading conditions, and attempts have been made at mimicking physiological loading and normal joint use. Some studies showed that OA cartilage was stimulated (in terms of proteoglycan synthesis) by low physiological hydrostatic pressure which had no effect on normal cartilage (7,8).

In this study we have investigated the effects of hydrostatic pressure on the cell ultrastructure in human articular chondrocytes cultivated in alginate gels.

MATERIALS AND METHODS

Cartilage - Human articular cartilage was obtained from the femoral heads of OA subjects undergoing surgery for total hip prostheses; normal cartilage was obtained from the femoral heads of subjects treated for traumatic events. Normal and OA cartilage was taken from the middle layer of the cartilage from three donors. The mean age of the groups was 31 years (ranging from 21-40) for normal subjects and 69 years (ranging from 68-74) for OA subjects.

Normal cartilage was designated by a glossy, white, completely smooth surface and a healthy appearance without irregularities. OA cartilage was designated by macroscopic focal fibrillation of the articular surface.

Normal and OA cartilage were cut aseptically, carefully excluding underlying bone, minced into 2 mm^2 pieces and sequentially digested by 1 mg/ml clostridial collagenase in phosphate buffered saline (PBS) for 18 hours incubation at 37°C.

Alginate culture - Alginate is an unbranched linear copolymer of 1-4 linked ß-D-mannuronic acid and α-L-guluronic acid. Alginate forms gel in the presence of divalent cations (particularly Ca^{+2}); the gels can be conveniently used to entrap the cells for "in vitro" growth. The gels can then be easily dissolved by sequestering the gelling ion by citrate, EDTA or phosphate.

Cells obtained after collagenase digestion were rinsed twice in saline (A) (in mM: 10 HEPES, 140 NaCl, 5 KCl, 5 Glucose, pH 7.4).

They were then mixed with an alginate (Pronova LVG, Norway) solution

in the saline (A), to obtain a final density of 10^6 cell/ml and 0.75% alginate. Aliquots of 1 ml of the cell suspensions in alginate were plated in 35 mm Petri dishes, and were allowed to form gels by the addition of 2 ml of 50 mM $CaCl_2$ in 10 mM HEPES, 0.1 M NaCl, pH 7.4.

After 5-10 min, when gel formation was completed, the excess solution was removed and the cell-containing gel was washed twice with the saline (A) and then overlaid with a medium containing 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 50 μg/ml ascorbate in Dulbecco's minimum essential medium (DMEM).

Petri dishes were maintained in an atmosphere of 5% CO_2 in air at 37°C for 48 hours.

Pressurization system - Our pressurization system is a pressure chamber consisting in a hermetically sealed stainless steel cylinder, with a height of 400 mm and an internal diameter of 90 mm (9).

Chondrocyte cultures were cultivated on Petri dishes, and after 48 hours the dishes were filled with medium and sealed with a covering of Surlyn 1801- bynel CXA 3048 bilayer membrane (thickness 90 mm), after all air was excluded.

The membrane was attached to the rim of the Petri dish with Jet Melt 3764 adhesive. During the experiments the dishes were immersed in prewarmed (37°C) distilled water. The pressure in the chamber could be set between 0 and 24 MPa. The loading and unloading periods in the cyclic pressure mode could be selected at any time.

Pressurization procedures - The chondrocytes in Petri dishes were exposed to cyclic pressurization according to sinusoidal waves (minimum pressure 1 MPa and maximum pressure 5 MPa) at 0.25 Hz frequency for three hours. During the other loading procedures the cells were exposed to continuous pressure (24 MPa) for three hours. Some dishes which did not undergo pressurization served as controls.

After pressurization, the culture medium was removed and the cells in alginate gel were fixed immediately for transmission electron microscopy (T.E.M.) and for scanning electron microscopy (S.E.M.).

Transmission Electron Microscopy (T.E.M.) - Human articular cartilage

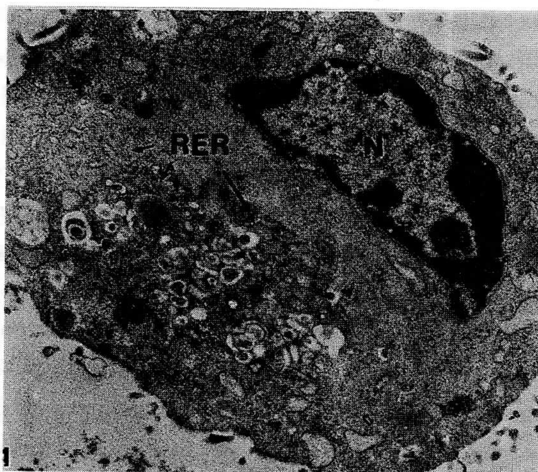
from the middle layer was processed for transmission electron microscopy (T.E.M.) immediately after it was obtained from femoral heads of normal and OA patients. A portion of the chondrocytes obtained from these cartilage samples was cultivated as described above. All samples were fixed for 2 hours at 4°C in cold Karnovsky fixative rinsed overnight in 0.1 M pH 7.2 cacodylate buffer and postfixed for 1 hour at 4°C in 1% buffered OsO_4, dehydrated in a graded series of ethanols and embedded in Epon-Araldite. Ultrathin sections cut with an ultramicrotome LKB III were collected on copper grids, stained with uranyl-acetate and lead citrate and photographed with a Philips CM10 electron microscope. We observed at least 50 cells from each group for evaluation.

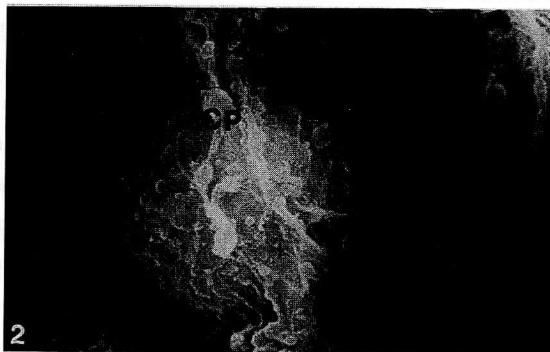
Scanning Electron Microscopy (S.E.M.) - Chondrocyte cultures were fixed in Karnovsky fixative for 3 hours at 4°C, washed in cacodylate buffer 0.1 M at 4°C overnight, postfixed in OsO_4 1% in veronal acetate buffer for 2 hours and, after a brief wash in cacodylate buffer 0.1 M, and placed in citrate pH 7.4 to remove the alginate. The chondrocytes were dehydrated in a graded series of ethanols, placed in tert-butanol and frozen at 0°C before drying by sublimation of the tert-butanol in a vacuum chamber. The samples were sputter coated with gold and observed in Philips SEM 505. We observed at least 100 cells from each group for evaluation.

RESULTS

Normal and OA cartilage was immediately fixed, processed and examined by T.E.M. and S.E.M. In normal subjects chondrocytes showed nuclei which were differently shaped with euchromatic chromatin and a nucleolus was occasionally present. The cytoplasm contained a variable amount of organelles: smooth endoplasmic reticulum was abundant, rough reticulum appeared to have abundant secretory material, mitochondria were regularly shaped, and the Golgi apparatus had flattened cisternae arranged in a stack. A number of different vesicles could occur elsewhere in the cytoplasm: lysosomes were present as well as glycogen deposits and lipid drops. In OA subjects, chondrocytes showed nuclear chromatin

which was differently heterochromatic and moving near the plasma membrane. Smooth endoplasmic reticulum was less evident, as well as the Golgi apparatus, showing a reduction in secretory activity with respect to normal chondrocytes. A dilatation of the cisternae of most of the rough reticulum was evident. The mitochondria seemed to be well shaped and the cytoplasm appeared abundant in lysosomes, glycogen deposits and lipid droplets. The plasma membrane maintained its integrity but often lost its processes. Chondrocytes from normal and OA patients were cultivated in alginate gels and placed in our pressurization system to study the effects of hydrostatic pressure "in vitro", with the objective of reproducing in the laboratory the pressures corresponding to the articulations at rest, during standing, walking and running, and to offer the possibility of studying the ultrastructural changes of the cells during these activities. A portion of the samples was exposed to cyclic pressurization according to sinusoidal waves at 0.25 Hz frequency for three hours, another portion was exposed to continuous pressure for three hours, and the last portion was cultivated without any pressure. All specimens were observed by T.E.M. and by S.E.M. No ultrastructural changes were noted in the normal chondrocytes exposed to physiological pressure, nor were any noted in those not exposed to any pressure. Cultivated chondrocytes from normal cartilage showed the same good state of health before and after pressure (figs. 1,2). Cytoplasmic organelles maintained a normal morphology, a large quantity of rough reticulum was present. Chondrocytes from OA subjects cultivated without any pressure showed a cytoplasm which was extremely vacuolated and almost devoid of Golgi apparatus and of the other typical organelles (fig. 3). However, when these cells were placed under physiological pressure they showed a partial recovery of secretory activity demonstrated by the presence of rough reticulum and glycogen vesicles (figs. 4,5). All the samples exposed to continuous pressure (24 MPa) for three hours showed a morphological worsening in both by T.E.M. and S.E.M. studies. In fact, after continuous pressurization, sections of normal and OA chondrocytes showed nuclei with heterochromatic chromatin almost picnotic in OA; the





Figs. 1,2 - Healthy human chondrocytes before (fig.1, T.E.M. X9.000) and after (fig. 2, S.E.M. X5.200) physiological pressure. Cells show a normal ultrastructure and a good morphology. (N) nucleus; (RER) endoplasmic rough reticulum; (CP) cytoplasmic processes.

Golgi apparatus, and rough and smooth endoplasmic reticuli were disorganized or completely lost. The cytoplasm was full of vesicles and vacuoles. The morphology of some cells was also altered to a more retracted form. S.E.M. observations confirmed these data: vesiculation was also clearly evident in the tridimensional aspect and the chondrocytes were devoid of cytoplasmic processes and the surrounding matrix (fig. 6).

DISCUSSION

Ultrastructural studies showed remarkable differences between normal and OA human chondrocytes at a nuclear and cytoplasmatic level. The healthy cells present a good quantity of rough reticulum and the Golgi apparatus had more large and numerous cisternae than the OA cells. In fact, in these cells the cytoplasm did not present an intense activity and it was almost devoid of organelles; many different vacuoles were also seen in tridimensional analysis by S.E.M. These results may indicate that the OA chondrocytes are phenotypically different from normal chondrocytes, since they react differently to the same stimulus. The differences in the phenotype of OA chondrocytes compared to normal chondrocytes may be responsible for the different effects of pressure on normal and OA cartilage. Our results suggest that negative structural changes are not present when healthy cultivated cells are placed under physiological pressure, however, the OA chondrocytes can gain positive effects from this condition: the presence of endoplasmic reticulum and of glycogen vacuoles can demonstrate a moderate recovery of metabolic activity. All the samples exposed to continuous pressure (24 MPa) for three hours showed an altered morphology observed by T.E.M. as well as by S.E.M. The damage was more evident in the OA cells. We can affirm that physiological pressure did not alter the healthy cell ultrastructure and, on the contrary, that it stimulated the OA cell which may benefit from it. Moreover, high pressure forces healthy and OA cultivated chondrocytes towards retracted forms that demonstrate a serious suffering of the cells.

In this work we studied the morphological and ultrastructural aspects of normal and osteoarthritic (OA) human articular chondrocytes cultivated in alginate gel for 48 hours. After this period the chondrocytes in Petri dishes were exposed to cyclic pressurization (minimum pressure 1 MPa and maximum pressure 5 MPa) at 0.25 Hz frequency for three hours. In other loading procedures the cells were exposed to continuous pressure (24 MPa) for three hours. Some dishes were not pressurised and these served

as controls. The cells were then fixed for transmission electron microscopy (T.E.M.) and for scanning electron microscopy (S.E.M.). No ultrastructural changes were observed in normal chondrocytes exposed at physiological pressure. OA cells placed under physiological pressure showed a partial recovery on morphological and ultrastructural aspects. Normal and OA samples exposed to continuous pressure (24 MPa) showed a morphological worsening in both T.E.M. and S.E.M. studies.

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1) URBAN J.P.G., Br. J. Rheumatol., 1994, 33, 901-908.
2) HALL A.C., URBAN J.P.G., GEHL K.A., J. Orthop. Res., 1991, 9, 1-10.
3) VAN KAMPEN G.P.J., VAN DE STADT R.J., Joint Loading, John Wright and Sons, Bristol, England, 1987.
4) HODGE W.A. et al., Proc. Natl. Acad. Sci. USA, 1986, 83, 2879-2883.
5) HAUS G., FASSBENDER M.D., Am. J. Med., 1987, 83, 17-22.
6) BRANDT K.D., LOHMANDER L.S., DOHERTY M., Osteoarthritis, Medical Publications, Oxford, 1998.
7) LAFEBER F., VELDHUIJZEN J.P., VANROY J.L.A.M., HUBER-BRUNING O., BIJLSMA J.W.J., Br. J. Rheumatol., 1992, 31, 437-442.
8) VAN VALBURG A.A., VAN ROY H.L.A.M., LAFEBER F.P.J.G., BIJLSMA J.W.J., J. Rheumatol., 1998, 25, 515-520.
9) NERUCCI F., FIORAVANTI A., CICERO M.R., SPINELLI G., MARCOLONGO R., In Vitro Cell. Dev. Biol., 1998, 34, 9-10.

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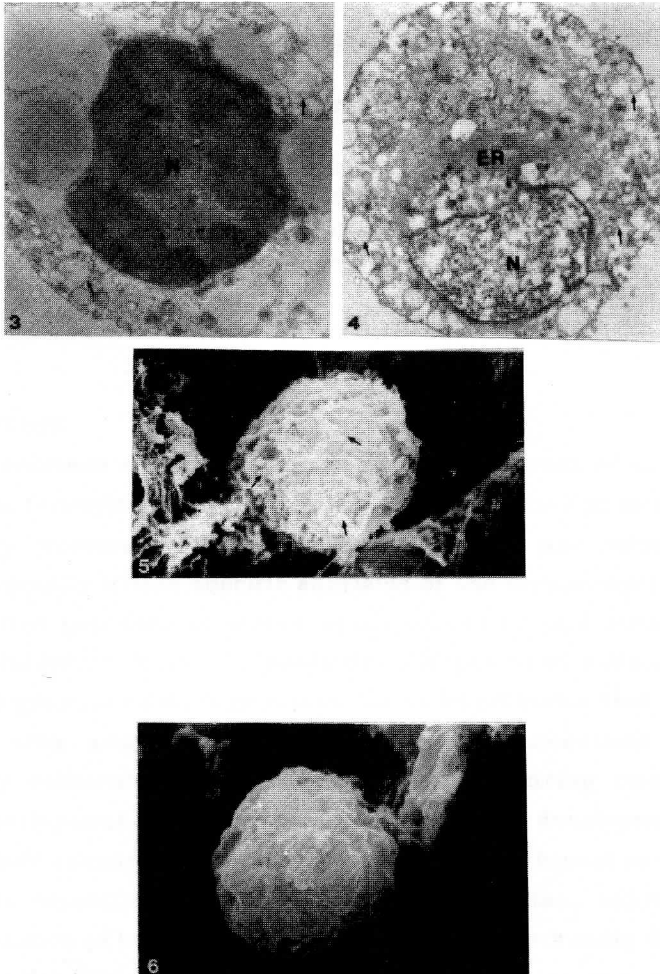

Figs. 3-5 - Cultivated human OA chondrocytes before (fig. 3, T.E.M. X12.000) and after (fig. 4, T.E.M. X7.500; fig. 5, S.E.M. X5.800) physiological pressure. The OA cytoplasm appears extremely vacuolated (arrows) but in fig.4, cytoplasmic organelles are present. (N) nucleus; (ER) endoplasmic reticulum.

Fig. 6 - Cultivated human OA chondrocyte after continuous pressure (24 MPa) observed by S.E.M. The cell is devoid of cytoplasmic processes and depressed areas are present in its surface. X5.600.