Morphological and cytoskeletal aspects of cultivated normal and osteoarthritic human articular chondrocytes after cyclical pressure: a pilot study

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Abstract

Objective

This study investigated the effect of hydrostatic cyclical pressure on the cell ultrastructure and cytoskeleton of normal and osteoarthritis (OA) human cultivated chondrocytes in vitro.

Methods

The different effects of pressurization with sinusoidal waves at a minimum pressure of 1 MPa, a maximum pressure of 5 MPa and a frequency of 0.25 Hz for 3 hrs on normal and OA chondrocytes were assessed by transmission electron microscopy (TEM), scanning electron microscopy (SEM) and immunofluorescence microscopy (IF).

Results

Structural differences exist between normal and OA chondrocytes at the nuclear, cytoplasmic and cytoskeletal level. Pressurization did not alter the normal chondrocytes, but had a beneficial effect on OA chondrocytes, by increasing the number of cell organelles responsible for synthesis activities. IF examination has shown that the distribution of actin protein in normal chondrocytes is polarized on the apical sides of the cellular cytoplasm. However, in OA chondrocytes the signal of the actin protein is not as well defined. Similarly, the localization of the tubulin protein in normal and OA cells also appears to be different. Hydrostatic pressure did not cause any modification in the cytoskeletal organization of the OA chondrocytes.

Conclusion

This study confirms the different morphology, structure and cytoskeletal aspect of normal and OA chondrocytes and the important role played by pressure on cell morphology. The recovery of OA chondrocytes observed by an increase of cytoplasmic organelles does not seem to involve the cytoskeleton.

Key words

Chondrocytes, osteoarthritis, hydrostatic pressure, cytoskeleton.

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Introduction

Articular cartilage is a tissue that is routinely subjected to high and varying loads. The ability to withstand these stresses depends on the structure and composition of the matrix. The matrix is produced by the chondrocytes and therefore it is fundamental that chondrocytes function adequately (1). Under physiological conditions, articular cartilage is subjected 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 (2, 3).

Several *in vitro* studies have shown the importance of articular load as a modulator of cartilage metabolism. In fact, articular load influences the concentration of proteoglycans (PG) and cations, as well as the osmolarity of the cartilage microenvironment (4-7). Chondrocytes respond to intermittent loading by increased PG synthesis, whereas continuous mechanical loading produces the opposite effect. The response is further modified by the magnitude, duration and frequency of loading. Hydrostatic pressure affects proteins, the cytoskeleton and cell organelles. Compression of cartilage results in the deformation of chondrocytes and the extracellular matrix. The physicochemical changes include altered matrix water content, altered fixed charge density, mobile ion concentrations and osmotic pressure (3, 4, 8).

In joint diseases such as osteoarthritis (OA), catabolic processes exceed anabolic processes, leading to a net decrease in matrix material (9). The etiology of OA is only partially understood, but it involves the loading of a joint. How loading is related to the incidence of OA is not yet clear, although some studies have shown that low levels of intermittent fluid pressure, such as those that occur *in vivo* during Ilizarov joint distraction (10), have beneficial effects on joint tissue in OA. This indicates that exposure to pressure might be useful in the treatment of OA.

Our previous *in vitro* studies confirmed the importance of pressurization in chondrocyte metabolism and morphology (11). The cellular cytoskeleton plays a fundamental role in the control of the cellular form and in the differentiation, formation and flow of vesicles (12). Previous studies have shown that high continuous hydrostatic pressure changes the structure of the cytoskeleton in cultivated chondrocytes (13). In this study we investigated the effects of cyclic hydrostatic pressure of a magnitude and frequency close to those that presumably exist in articular cartilage (4) on the ultrastructure of normal and OAarticular chondrocytes cultivated in alginate gels.

Materials and methods

Cell culture

Normal human articular cartilage was obtained from the femoral heads of 5 subjects with displaced femoral neck fractures (3 females and 2 males), and OA human articular cartilage was obtained from the femoral heads of 5 patients with OA defined by clinical and radiological ACR criteria (14) undergoing surgery for total hip prostheses. The mean age of the group was 31 years (range: 21-40) for normal subjects and 69 years (range 68-74) for OA patients. Normal chondrocytes were obtained from the middle layer of cartilage in the femoral heads, while OA chondrocytes originated from the area adjacent to the OAlesion. OAcartilage was characterized by macroscopic focal fibrillation of the articular surface. Normal cartilage was characterized by a glossy, white, completely smooth surface and a healthy appearance without irregularities.

Immediately after surgery, the cartilage specimens were cut aseptically, minced into 2 mm^2 pieces and sequentially digested by clostridial collagenase (SIGMA, Italy) 1 mg/ml in phosphate buffered saline (PBS) (in mM: NaCl 140, KCl 2.7, NaH_2PO_4 8.1, K_2HPO_4 1.5, pH 7.4) containing 200 U/ml of penicillin, 200 µg/ml of streptomycin and 0.25 µg/ml of amphotericin B. Collagenase digestion was carried out at 37°C for 18 hrs with moderate stirring. Chondrocytes obtained after collagenase digestion were rinsed twice in saline solution A (in mM: 10 HEPES, 140 NaCl, 5 KCl, 5 Glucose, pH 7.4)

and centrifuged for 10 min at 700 g. As shown by Trypan blue viable stain, 90-95% of the recovered cells were alive. Cells were then mixed with alginate (Pronova LVG, Protan, Drammer, Norway) in saline solution A to obtain a final density of 5 x 10^5 cells/ml and 0.75% alginate. Aliquots of 1 ml alginate cell suspension were placed in 35 mm Petri dishes (Costar, Italy) containing a layer of previously polymerized alginate that prevented the cells from sticking to the bottom of the dish. Polymerization was obtained by adding 2 ml of $CaCl_2$ 50 mM in 10 mM HEPES, 0.1 NaCl, pH 7.4 which created a three-dimensional gel. After 5-10 minutes, when gel formation was complete, the excess solution was removed and the cell-containing gel was washed twice with 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). The Petri dishes were maintained in an atmosphere of 5% CO_2 in the air.

Pressurization

Our pressurization system has some special characteristics that have already been described in detail (15). Briefly, this pressure chamber system consists of a hermetically sealed stainless steel cylinder, 400 mm high with an internal diameter of 90 mm. Chondrocytes were cultivated on Petri dishes. After 48 hours the dishes were filled with culture medium and sealed with a covering of Surlyn 1801 Bynel CXA 3048 bilayer membrane (thickness 90 µm; Du Pont, Italy), after expelling all air. Surlyn membrane is partially permeable to O_2 and CO_2, but not to water or other solutions and it is suitable for preserving a stable environment (4).

The membrane was attached to the rim of the Petri dish with Jet Melt 3764 adhesive (3M, Italy). During the experiments the dishes were immersed in preheated (37°C) distilled water. The pressure in the chamber could be adjusted to between 0 and 24 MPa. The loading and unloading periods for cyclic pressure could be freely selected. The chondrocytes covered in culture medium were pressurized by applying sinusoidal waves with a minimum pressure of 1 MPa, a maximum pressure of 5 MPa and a frequency of 0.25 Hz for 3 hrs. Dishes cultivated in the loading chamber but without undergoing pressurization served as controls. After pressurization, the culture medium was removed and the chondrocytes were immediately fixed for transmission electron microscopy (TEM), scanning electron microscopy (SEM) and immunofluorescence (IF).

Transmission electron microscopy (TEM)

Cultures of human chondrocytes were fixed for 2 hrs at 4°C in cold Karnovsky fixative (16), rinsed overnight in 0.1 M pH 7.2 cacodylate buffer and post-fixed for 1 hr at 4°C in 1% buffered OsO_4, dehydrated in a graded series of ethanol and embedded in Epon-Araldite. Ultra-thin sections cut with an LKB III ultramicrotome were collected in copper grids, stained with uranyl acetate and lead citrate, and then photographed using a Philips CM10 electron microscope. We examined at least 100 cells from each group.

Scanning electron microscopy (SEM)

Cultures of human chondrocytes in alginate were fixed for 2 hrs at 4°C in cold Karnovsky fixative (16), washed in cacodylate buffer 0.1 M pH 7.2 overnight, post-fixed in 1% buffered OsO_4 in veronal acetate buffer for 2 hrs and, after brief washing in cacodylate buffer 0.1M, placed in citrate pH 7.4 to remove the alginate. The chondrocytes were dehydrated in a graded series of ethanol, 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 a Philips SEM 505. At least 200 chondrocytes from each group were evaluated.

Immunofluorescence microscopy (IF)

Human articular cartilage from healthy and OA subjects was digested as described above. Normal and OAchondrocytes washed in PBS were smeared on glass slides, air dried, and fixed for 15 min in methanol at –20°C. The specimens were rehydrated in PBS, treated with 5% normal goat serum (NGS), diluted 1:20 in PBS-0.1% bovine serum albumine (BSA) to avoid unspecific staining, and then incubated in anti-actin monoclonal antibody (Sigma, Italy) diluted to 1:500 with PBS – 0.1%BSA – 1%NGS or in anti-tubulin monoclonal antibody (Sigma, Italy) diluted to 1:500 with PBS – 0.1%BSA – 1%NGS for 2-3 hrs at room temperature (or overnight at 4°C). After thorough rinsing in PBS, they were incubated for 1 hr at room temperature in FITC-conjugated – goat antimouse IgG diluted to 1:1000 in PBS 0.1% – BSA1% – NGS. Controls for secondary antibody were carried out by omitting the primary antibodies. All slides were then washed for 30 min in PBS, mounted in PBS-glycerol 1:10 containing 5% propyl-gallate (to reduce fluorescence fading), and examined under a Leitz Aristoplan microscope with fluorescence and Olympus BH-2 optics. Photographs were taken with a Kodak TMAX 400. The same procedures were also performed on cultivated chondrocytes exposed to cyclical hydrostatic pressure. At least 100 chondrocytes from each group were evaluated.

Morphometric and statistical analysis

For the morphometric studies, we analyzed sections of four different blocks from each group. For standardization and comparison of the different groups, only medially sectioned chondrocytes were investigated; 100 chondrocytes were selected using the nucleous/cytoplasm ratio as the selection criterion. Our analysis was based on an established method for ultrastructural quantitative evaluation of changes in chondrocytes (17). The number of mitochondria and Golgi bodies, expressed as the mean ± SD, were used as parameters.

Because a normal distribution is not present in all cases, the statistical analyses for differences between the study groups are performed entirely with distribution-free non-parametrical tests. Morphometrical parameters are tested

with the Mann-Whitney U test. Values of $p < 0.05$ were considered as significantly different.

The presence of actin and tubulin signal was estimated by the same researcher in three degrees: absent, limited, or intense (18). This is expressed per study group as a percentage referred to the total number of cells studied.

Results

Structural and morphological examination by TEM and SEM showed some differences between normal and OA chondrocytes.

In the normal chondrocytes (Fig. 1A) the nucleus appeared bright and eu-chromatic. The cytoplasm contained many cell organelles. Smooth endoplasmic reticulum and rough endoplasmic reticulum were abundant with many adjacent secretory vesicles. The mitochondria were numerous and regularly shaped. The Golgi bodies were arranged in flattened cisternae and also were surrounded by many vesicles. Secretory vesicles as a sign of the synthetic and secretory activities of the cell were detectable in all compartments of the cytoplasm. Lysosomes, glycogen deposits and lipid droplets were present as described in normal chondrocytes (17).

In OA chondrocytes (Fig. 1B) the nucleus had a dark, densely heterochromatic appearance. The cell organelles, smooth and rough endoplasmic reticuli, Golgi bodies, mitochondria and vesicles were significantly reduced compared to normal chondrocytes.

The difference in the number of mitochondria and Golgi bodies between normal and OA chondrocytes was significantly lower ($p < 0.01$) in OAchondrocytes (Table I). However, there was an increase in lysosomes, glycogen accumulation and fat vacuoles, reflecting the degeneration of the chondrocytes.

After pressurization the normal chondrocytes maintained their shape and ultrastructure (Fig. 2A). In OA chondrocytes the application of pressure partially restored many of the characteristic cytoplasmic structures (Fig. 2B).

The number of mitochondria and Golgi bodies did not change after pressurization in normal cells, whereas in the OA cells their numbers (Table I) increased significantly ($p < 0.01$).

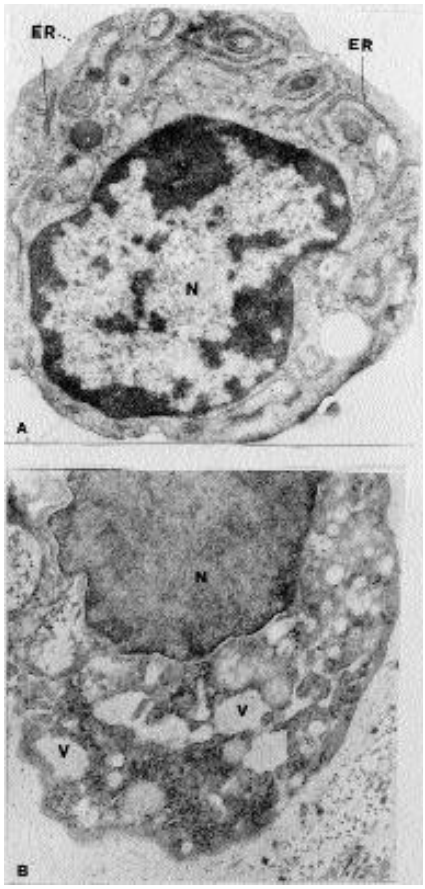

Fig. 1. Ultrastructure of human cultured chondrocytes. (**A**) Normal active chondrocyte with pronounced rough endoplasmic reticulum (ER); the nucleus (N) contains normally condensed chromatin (x 19000). (**B**) OA chondrocyte with dilatation of the cisternae of the rough endoplasmic reticulum and many vacuoles (V) in the cytoplasm; nucleus (N) (x 13500).

Morphological examination of the cell surface by SEM showed control chondrocytes with many cytoplasmic processes of the chondrocyte membrane reflecting intense biosynthetic activity (Fig. 3A).

Chondrocytes from OA patients subjected to pressurization were similar to normal chondrocytes (Fig. 3B).

Cytoskeletal examination by IF of normal and OA chondrocytes before pressurization (Fig. 4A and 4B) showed that the distribution of actin protein in normal chondrocytes yields a clearly polarized fluorescent signal on the apical sides of the cellular cytoplasm. In OA cells, the actin protein signal was not as well defined and it appeared to be diffused throughout the cytoplasm or else limited to the periphery of the cells (Fig. 4D and 4E). The reduction in the number of cells showing the regular intense polarization in OA (3%) compared to normal (75%) chondrocytes was evident and significant (Table II).

The localization of the tubulin protein appeared to be different between normal chondrocytes, in which the fluorescent signal was more or less uniformly localized at the periphery of the cells (Fig. 4C), and OA cells in which the tubulin signal was sometimes absent (Fig. 4F). The reduction in the number of OA cells showing the uniform intense peripheral signal (3%) compared to normal chondrocytes (80%) was evident and significant (Table II).

The localization of both the actin and tubulin proteins in OA and normal chondrocytes after the same pressurization was similar. The localization of the actin protein in normal chondrocytes exposed to pressurization (Fig. 4H) seemed unchanged. The signal appeared well-defined and no differences were observed compared to the same chondrocytes at basal conditions. The actin localization in OA chondrocytes (Fig. 4L) also did not appear to be changed after pressurization. In normal

Table I. Number of organelles in normal and OAchondrocytes under basal conditions and after pressurization.

	Basal conditions		After pressurization	
	Normal	OA	Normal	OA
Mitochondria	7.2 ± 2.6	3.6 ± 1.9*	7.8 ± 2.8	6.7 ± 2.1*
Golgi bodies	2.8 ± 1.2	1.6 ± 0.9	2.9 ± 1.1	2.3 ± 1.3

*$p < 0.01$ (Mann-Whitney U test) OA basal conditions versus normal basal conditions; OApressure versus OAbasal conditions.

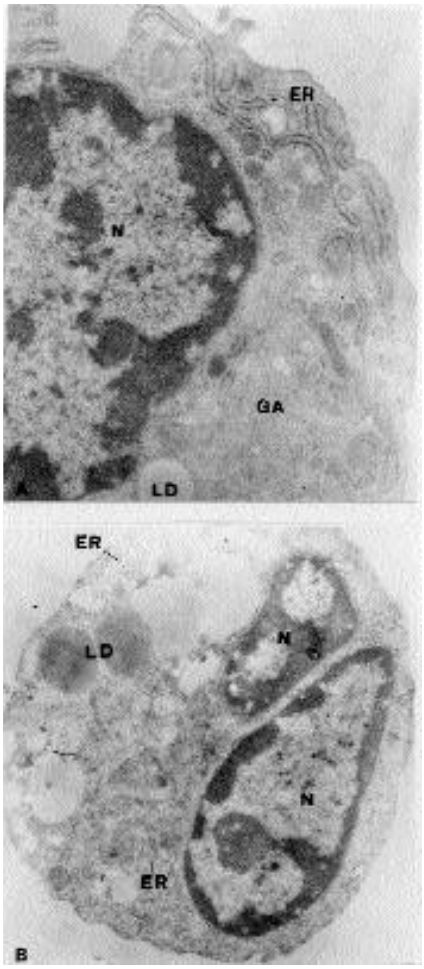

Fig. 2. Ultrastructure of human cultured chondrocytes after pressurization. (**A**) Normal active chondrocyte with abundant rough endoplasmic reticulum (ER), Golgi apparatus (GA) with flattened cisternae arranged in a stack and few lipid droplets (LD); nucleus (N) (x 21000). (**B**) OA chondrocyte displaying enhanced synthesis activity reflected in an increase in cell organelles. Nucleus (N), endoplamic reticulum (ER), lipid droplets (LD) (x 7500).

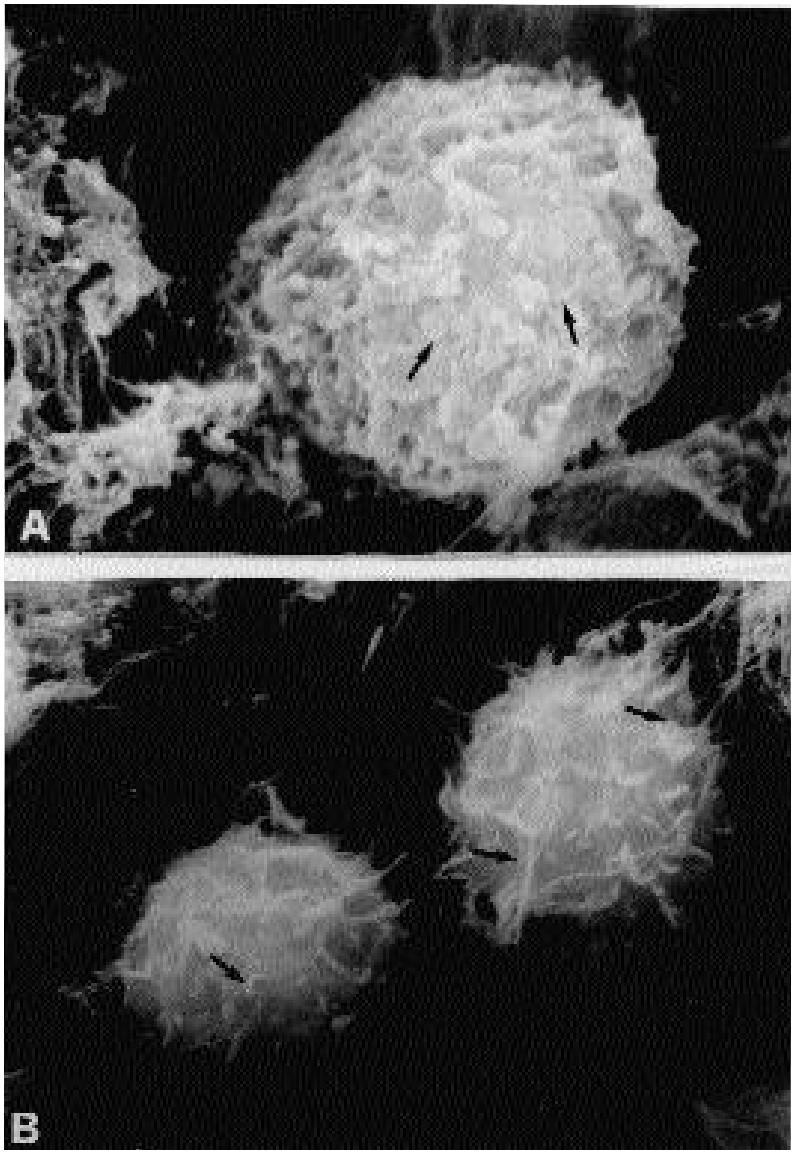

Fig. 3. SEM micrographs of human cultured chondrocytes. (**A**) Normal chondrocyte with numerous superficial processes (**arrows**) (x 5500). (**B**) OAchondrocyte after exposure to pressurization shows a reduction in the number of processes and the presence of superficial vesiculation (**arrows**) (x 7500).

chondrocytes (Fig. 4I) and OA chondrocytes (Fig. 4G) the distribution of the tubulin antibody after pressure did not appear to be particularly different from that seen in the chondrocytes under basal conditions.

Table II reports data relating to the percentage of cells showing the signal of both antibodies evaluated after pressurization in both types of chondrocytes.

The percentage of cells showing intense polarity after incubation with anti-actin antibody and intense peripheral localization after incubation with anti-tubulin antibody did not increase in the OAcell group.

Discussion

Human articular chondrocytes were cultured in alginate, currently the preferred technique (19), since such culturing closely resembles the *in vivo* situation and can be used to obtain reliable morphological and metabolic assessments (11, 20). Articular load modulates the chondrocyte metabolism and morphology in a complex fashion (3, 4, 11, 21) and cultured chondrocytes offer the possibility to evaluate the effects of hydrostatic pressure *in vitro.*

Our data show that there are significant structural differences between normal and OA chondrocytes *in vitro* at both the nuclear and cytoplasmic levels. Normal cells are rich in synthetic and secretory cell organelles, while the cytoplasms of OA cells are poor in these organelles and they contain many vacuoles and glycogen deposits. Recently, other authors have shown altered mitochondria respiratory activity in OA chondrocytes (22). These changes represent an intracellular biochemical–enzymatic imbalance which affects the chondrocyte during the OA process (9). The ultrastructural difference between normal and OAchondrocytes explains the different reactions of the two types of chondrocytes when

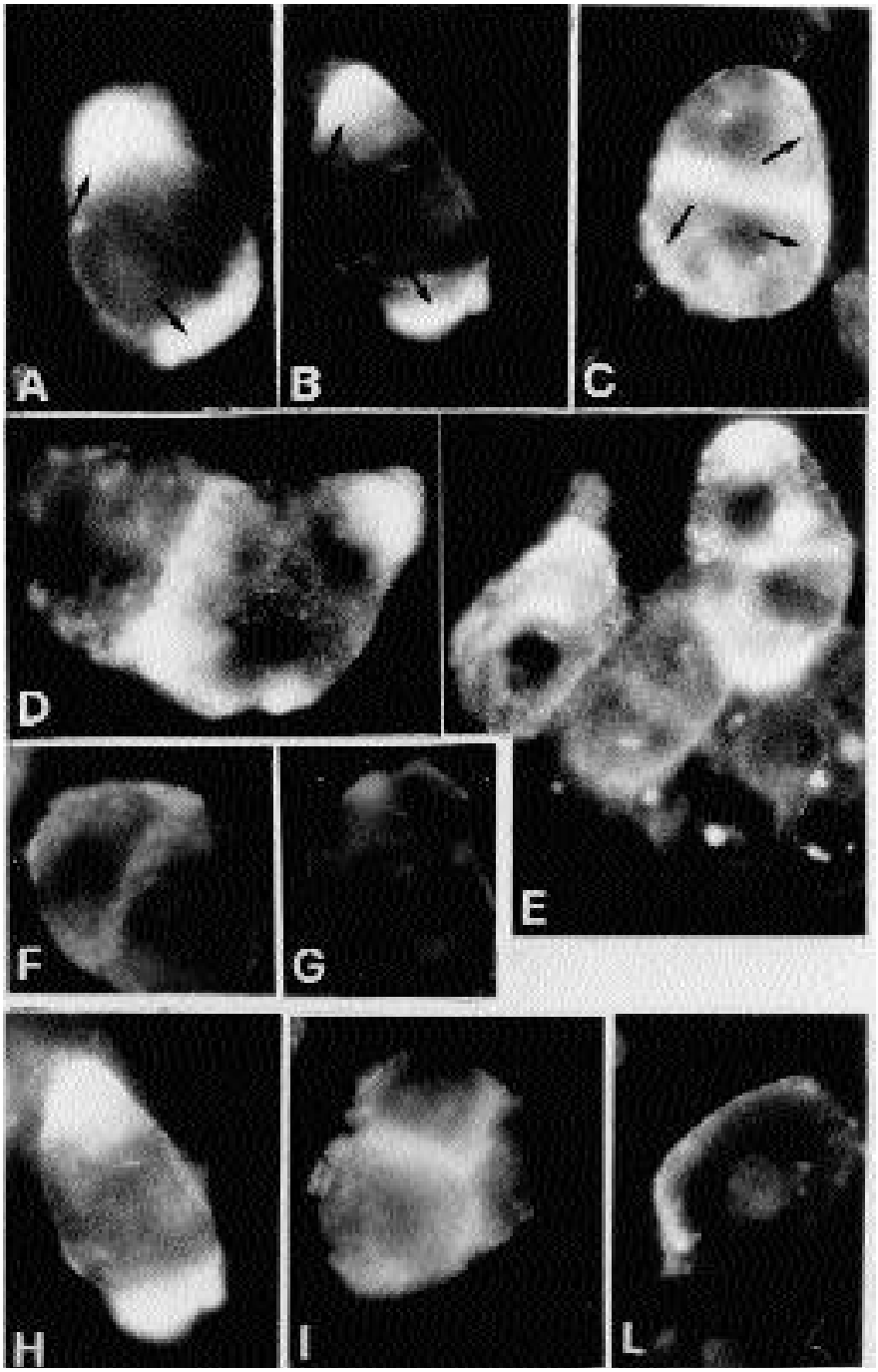

Fig. 4. UVmicrographs of human cultured chondrocytes. (**A**) and (**B**) Normal chondrocytes incubated with anti-actin antibody show intense fluorescence at the polarity of the cytoplasm (**arrows**). (**C**) Normal chondrocyte incubated with anti-tubulin antibody shows fluorescent staining limited to the thin layer at the periphery of the cell, adjacent to the plasma membrane (**arrows**). (**D**) and (**E**) OAchondrocytes incubated with anti-actin antibody show no clear polarization signal. (**F**) OAchondrocyte incubated with anti-tubulin antibody, in which the signal is almost absent. (**H**) and (**I**) Normal chondrocytes incubated with anti-actin (**H**) and anti-tubulin (**I**) antibodies show no change in localization after exposure to pressurization. **G** and **L**: OA chondrocytes incubated with anti-actin (**L**) and anti-tubulin (**G**) antibodies show no change in localization after exposure to pressurization. (A-G x 900)

subjected to the same stimuli. Articular cartilage is constantly subjected to loads, dependent upon body weight and muscular tension. The load also varies according to posture and physical activity. Hydrostatic pressure is one of the many factors on which the function of articular cartilage, subjected to load, is dependent (2, 4).

Our pressurization system allowed us to study the effect of hydrostatic pressure on chondrocytes *in vitro*. The aim of this study was to reproduce the pressure corresponding to the joints at rest and during standing, walking and running, and to study the ultrastructural changes of chondrocytes during these activities (11,15).

The results suggest that no changes occur when normal chondrocytes are placed under pressure of a magnitude and frequency close to those to which articular cartilage are subjected. OA chondrocytes, however, are positively affected, showing a partial restoration of their cytoplasmic organelles. This data is in agreement with the results of an earlier study, in which we showed an increase in the production of PG in OAchondrocytes subjected to the same pressure. OA chondrocytes are more sensitive to hydrostatic pressure than normal chondrocytes (23).

The results of IF examination have shown that there is a different localization of the two cytoskeletal proteins in chondrocytes. This localization is dependent upon the different roles of the two cytoskeletal proteins within the cell. Actinic apical polarization is fundamental to maintaining the form of the cellular membrane and interactions with the extracellular matrix. The peripheral integrity of the microtubulins is important for ensuring the correct localization and distribution of cytoplasmic organelles (12, 24). The different organization of the cytoskeletal structure of OA cells compared to normal cells shows that the correct cytoskeletal actinic and tubulinic arrangement is altered during the course of OA. This alteration affects a series of phenomena, including cellular differentiation, formation and flux of vesicles, synthesis of the extracellular matrix molecules, adhesion and cellular migration (13, 24-26). The cellular cytoskeleton also plays a fundamental role in the migration and proliferation of chondrocytes; these processes are known to be essential for cartilage repair (26). Our cytoskeletal analyses of cultivated chondrocytes demonstrated that there is no recovery of a physiological magnitude after hydrostatic pressure. Cytoskeletal structures might

Table II. Percentage of cells showing a regular signal after incubation with anti-actin (polarization signal) and anti-tubulin (uniform peripheral signal) antibodies.

		Basal conditions		After pressurization	
		N	OA	N	OA
Actin	Absent	7%	70%	8%	72%
	Limited	18%	27%	20%	20%
	Intense	75%	3%	72%	8%
Tubulin	Absent	5%	65%	6%	61%
	Limited	15%	25%	12%	27%
	Intense	80%	10%	82%	12%

not be normally assembled in OAcartilage and they remain the same after pressure stimulation. A potential mediator of cartilage damage such as nitric oxide (NO) could have disrupted the integrin complex (25) that conditioned the release of PG and glycoprotein as well as chondrocyte migration, adhesion and cytoskeletal assembly (26).

Our data confirmed the occurrence of important ultrastructural modifications and a different cytoskeletal organization in OA chondrocytes compared to normal chondrocytes.

Exposure to low magnitude intermittent hydrostatic pressure does not modify healthy chondrocytes, as already shown (27), but it has a beneficial effect on the ultrastructure of OA chondrocytes. Increased anabolic activity is reflected in an increase in cell organelles, such as mitochondria and the Golgi apparatus. This effect has also been demonstrated in PG synthesis (4-7, 11).

The pressure level used in this study does not cause any modification in the cytoskeletal organisation of the OA chondrocyte. This indicates irreversible damage to the cytoskeleton and, consequently, the loss of all functions related to this structure (25, 26). More studies are needed, most importantly to determine whether the modifications that we found are related to the OA process or if they could be due to the age of the donor. Several questions remain to be answered in order to determine the factors that are responsible for the cytoskeletal damage which occurs during OA pathology, and whether pharmacological and/or physical treatment could prevent and/or reverse the cytoskeletal alterations.

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