Effect of osmolarity on glycosaminoglycan production and cell metabolism of articular chondrocyte under three-dimensional culture system

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Abstract

Objectives

This study examined how physiological levels of extracellular osmolarity influence proteoglycan accumulation in articular chondrocytes in a three-dimensional culture system.

Methods

Cells were obtained from metacarpal phalangeal joints of 18-24 month bovine. They were cultured for 6 days in alginate beads at 4 million cells/ml in DMEM containing 6% FBS under 21% O_2. Medium osmolarity was altered by NaCl addition over the range 270-570 mOsm and monitored using a freezing point osmometer. Profiles across intact beads were determined by manual counting using fluorescent probes and transmission electron microscope. Lactate production was measured enzymatically and glycosaminoglycan (GAG) accumulation was measured using a modified dimethylmethylene blue assay. Rate of sulfate GAG synthesis was measured using a standard ^{35}S-sulfate radioactive method.

Results

The cell viability was similar for the high and low osmolarity cultures. However, confocal microscopy showed that the cells were the largest under 270 mOsm and became smaller with increasing osmotic pressure. GAG production was largest in the 370mOsm, and the capacity for GAG production and cell metabolism (lactate production) was low under hypo-osmolarity and hyper-osmolarity, and cell deaths were often observed on electron microscopy.

Conclusions

In our model the prevailing osmolarity was a powerful regulator of GAG accumulation by cultured chondrocytes. These results thus indicate GAG synthesis rates are regulated by GAG concentration, with implications both for the aetiology of osteoarthritis and for tissue engineering.

Key words

Articular chondrocyte, tissue engineering, osmolarity, in vitro, osteoarthritis.

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Introduction

Proteoglycan (PG) accounts for about 7-10% of cartilage tissues, and aggrecan, which is a member of PG representing macromolecules, plays a key role for mitigation of mechanical stress imposed on the cartilage tissues (1). A decrease in proteoglycans, in particular glycosaminoglycans (GAGs), is seen from early changes of osteoarthritis (OA). Among the components of aggrecan, negatively-charged GAG produces a high osmotic pressure in the cartilage tissue, and water is therefore absorbed in the cartilage tissues. As a result, the collagen networks are inflated, and the cartilage tissues acquire elastic resistance characteristic to cartilage tissues to protect from compression force. Thus, the strength of cartilage tissues strongly depends on the density of aggrecan (1-3). Therefore, in order to produce cartilage tissues that can tolerate mechanical force of about 10–20 MPa using the tissue engineering technology, it is necessary to generate sufficient PG (4). GAG production is dependent on the amount of GAGs produced per unit cells, retention in the tissue, and cell density (5). Oxygen concentration (6, 7), pH (8, 9), extracellular osmolarity (10-14), mechanical stress (15-17), and various growth factors (18-22) have been frequently reported to be deeply involved in GAG production and cell growth. There is little information however on the effect of extracellular osmotic change on the accumulation of GAG in long-term culture. First of all, it is important to establish the optimum culture conditions for the generation of cartilagious tissues. In this study, we examine how physiological levels of extracellular osmolarity influence the rate of proteoglycan accumulation in chondrocytes in a three-dimensional culture system."

Materials and methods

Cell isolation

Bovine metacarpal phalangeal joints from 18-24 month old steers were obtained from a local abattoir within 2-3 hours of slaughter and dissected aseptically. Cartilage from 10 feet was used for the experiments described here. The articular cartilage was removed from the joints using a scalpel and the pooled tissue digested enzymatically for 18-20 hours at 37°C in an incubator under 95% air and 5% CO_2. The digestion medium consisted of Dulbecco's modified Eagle's medium (DMEM: cat. no 22320-022 with 25 mM Hepes, 1mM sodium pyruvate, 1000 mg/L glucose, Invitrogen, Carlsbad, CA) containing 1mg/ml collagenase-1 (Sigma-Aldrich, Poole, Dorset, UK), antibiotics/ antimycotics (500 units ml^{-1} penicillin G, 500μg ml^{-1} streptomycin sulphate and 25μg ml^{-1} amphotericin B (Life Technologies Ltd., Paisley, Scotland), made to 370 mOsm with NaCl. After incubation, the digested tissue suspensions were filtered initially through a coarse filter to remove undigested tissue and then through a 20 μm-pore cell-strainer (Sigma-Aldrich, Poole, Dorset, UK). The cells in the filtrate were then washed three times by repeated centrifugation (2500 rpm for 5mins) and resuspension in DMEM. The cell suspension was assessed manually using a haemocytomer and trypan blue exclusion for cell viability and cell number. Only cell preparations with cell viabilities >95% were then used.

Cell culture

The cells were encapsulated in alginate beads (23). Briefly, the washed articular chondrocytes were resuspended uniformly into 1.2% low viscosity alginate (Fluka Biochemika, Gillingham, Kent, UK) at controlled cell densities by gentle pipetting. Microspheres were then formed by expressing the cell suspension through a 21-G needle attached to a 5ml syringe into a 102mM $CaCl_2$ solution. The microspheres were then washed twice with 25ml of 0.9% NaCl solution and washed twice again with 25ml of DMEM. The beads were carefully placed into 48 well culture plates (5 beads per well); in each separate experiment, under each condition tested and at each time point, 3 wells were used for biochemical analysis, 3 for measurement of sulphate incorporation rate and one well for morphological examination of the beads.

The multi-well plates were then cultured for 2, 4 and 6 days at 37°C in DMEM containing 6% foetal bovine serum, 0.5% antibiotic/antimycotic and 0.5% gentamycin (Life Technologies Ltd.,

Paisley, Scotland) at different osmolarities (270, 370, 470, 570 mOsm) under 5%CO_2/95% air. Medium osmolarity was altered by addition of NaCl and was monitored using a freezing point osmometer (Semi-micro osmometer, Knauer, Germany). The medium was changed for 2 days and the lactate concentration in the medium measured.

After incubation, for each condition examined and at each time point, the beads from 3 wells removed from the medium and weighted and dispersed in 3 volumes of citrate buffer[31] containing 5mM cysteine hydrochloride and 0.56 unit/ml papain (Sigma-Aldrich, Poole, Dorset, UK). The beads from another 3 wells per experimental condition were labelled with ^{35}S-sulfate (Amersham Biosciences, Little Chalfont, Bucks, UK) for 4 hours. Cell density and viability were measured manually on 20 ml samples of the resulting solution using a haemocytomer and trypan blue exclusion. The number of cells/bead was used for calculation of lactate production, GAG concentration and of sulphate incorporation rate per million cells. The remaining solution was heated to 67°C overnight in a sealed tube to digest the proteoglycans. Aliquots of the solution were used for measurement of total glycosaminoglycan (GAG) accumulation and sulphate incorporation rate. The cell viability profile was examined across representative beads from the remaining well; other beads from this well were fixed, sectioned and examined morphologically.

Glycosaminoglycan (GAG) content

GAG accumulation was measured using a modified dimethylmethylene blue (DMB) assay (24). The absorbance of aliquots of the bead digest added to the DMB buffer (Serva Feinbiochemica, Heidelberg, Germany) was read at 595 nm using a UV/VIS spectrophotometer (UV-160A, Shimazu, Kyoto, Japan). GAG concentrations were estimated from a standard curve of chondroitin sulphate (Sigma-Aldrich, Poole, Dorset, UK) made up in 0.1% alginate in citrate buffer.

Sulphate incorporation rates

At each time point, beads were resuspended in 0.5 ml DMEM, supplemented with 5μCi ml^{-1} of $^{35}SO_4^{2-}$ (Amersham Biosciences, Little Chalfont, Bucks., UK) and incubated for a further 4 hours at 37°C and 5% CO_2. Radiolabeling was stopped by washing the beads twice in ice-cold PBS supplemented with 2mM $CaCl_2$, 5mM Na_2SO_4 at 4°C and then freezing at -20°C. Labeled alginate beads were defrosted and digested in 0.5ml of PBS and 0.5ml of citrate buffer. The solution was then exhaustively dialyzed to separate the bound and free $^{35}SO_4^2$. The dialysis and tubing were then placed into a scintillation vial and 4ml of scintillant (Liquidscint, LSC-5100, Aloka, Japan) was added. The activity of the dialysate and 20 μl samples of the incubation medium were measured on a beta scintillation counter. Rates of sulphated GAG synthesis were calculated as described previously assuming that the specific activity of the $^{35}SO_4^2$-sulphate was the same in the medium and in the incorporated GAGs.

Lactate dehydrogenase (LDH) assay

Lactic acid production was used as a marker for cell metabolism as glycolysis is the major ATP-generating pathway for chondrocytes (7). Lactic acid production was measured using a commercial kit (Sigma-product no. 735-10) using lactate dissolved in DMEM as a standard.

Cell viability profiles

The cell viability profile across intact beads was determined by manual counting using a Live/Dead assay kit containing two fluorescent probes, Ethidium homodimer-1 and Calcein A (Molecular Probes, Cambridge Biosciences, Cambridge, UK). In order to visualize cells, the beads were cut in half using a blade and soaked in the Live/Dead solution for 1 hour. They were then examined under a Leica TCS SP2 confocal microscope equipped with a 15-milliwatt Krypton argon laser. The number of live (green fluorescence) and dead (red fluorescence) cells were counted manually in the beads. At least 4 fields each containing at least 15 cells was counted in each area. The proportion of live and dead cells was then calculated from pooled results. Furthermore, the diameter of 25 cells was measured under varied osmolarity conditions to investigate the changes of chondrocytes associated with extracellular osmolarity.

Transmission electron microscopy

The beads (n=12) in 3 separate experiments under 4 different osmolarities, were examined under electron microscope. Beads were fixed by immersion in 2.5% glutaraldehyde (0.15M cacodylate buffer, pH 7.2) with 2mM $CaCl_2$, 5mM Na_2SO_4 at 4°C for 4 hours. After fixation, the samples were sliced at approximately 50μm thickness using a cryostat. The specimens were post-fixed in 2% OsO_4 in 0.1M sodium cacodylate buffer, impregnated with 2% uranyl acetate, dehydrated in graded ethanols and embedded in Epoxy resin. For electron microscopy, ultrathin sections contrasted with uranyl acetate and lead citrate were examined under HITACHI H-7000 electron microscope.

Statistical analysis

Unless otherwise stated, data are presented as the mean ± the standard error of the mean (SEM) of at least three separate experiments (n ≥3). Each experiment was carried out in triplicate. Significant differences of prior comparison was determined using a 2-way ANOVA with repeated measures. When interaction was positive, we used Scheffe test. Date were entered into a database and analyzed by using SPSS statistical software, version 14.0.J (SPSS Inc, Chicago, IL). A probability of 5% was considered statistically significant.

Results

Effect of extracellular osmolarity on glycosaminoglycan production and cell metabolism with time in culture

After 2, 4 and 6 days of culture, the chondrocyte viability rate was 90% or higher in all of the 4 osmolarity groups. So, chondrocyte viability was not modified by the difference in extracellular osmolarity. Figs. 1 and 2 give pooled data for four osmolarities from 3 separate experiments for cells cultured by chondrocytes. While total GAG in beads/ml of beads volume increased with the duration of culture, it was greatest in the 370 mOsm

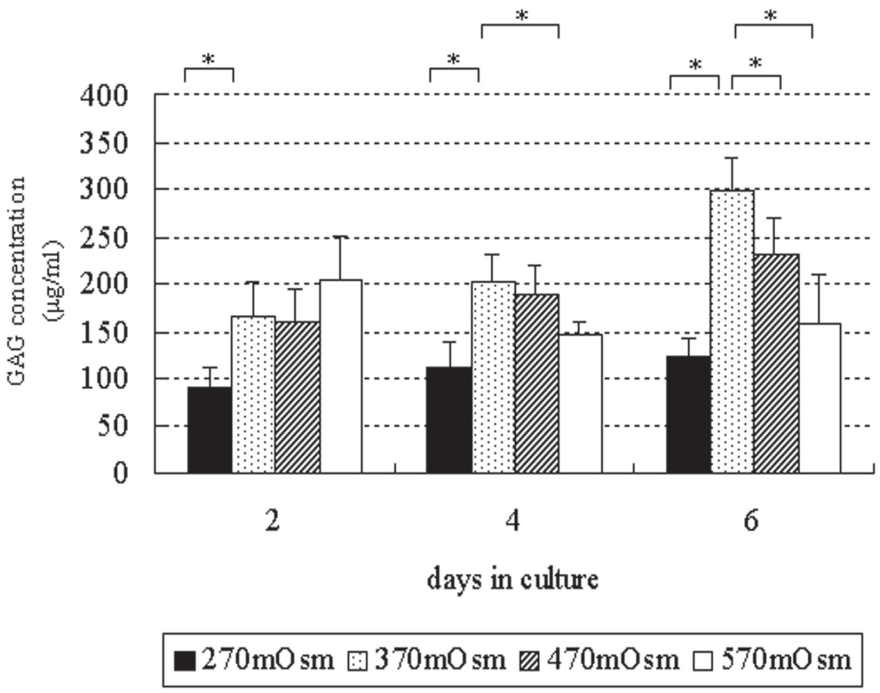

Fig. 1. Effect of extracelluler osmotic change on GAG concentration. This figure gives pooled data for 4 representative osmolarity from 3 separate experiments for cells cultured by articular chondrocytes. GAG accumulation/tissue volume was significantly increased at 370 and 470 mOsm with time in culture. This was the highest in the 370 mOsm group and the lowest in the hypo-osmolarity group (270 mOsm) decreased after 6 days of culture. In the hyper-osmolarity group (570 mOsm), the rate of GAG accumulation/tissue volume was not decreased after 2 days of culture when compared with the 370 mOsm group. Values are mean ± standard error. (Scheffe, *: $p<0.05$).

group and lowest in the 270 mOsm group during culture ($p<0.05$). The total GAG in beads/ml of beads volume was greatest in the 370 mOsm group, being 0.298±0.035 (mean ± SEM) mg/ml at day 6 (Fig. 1). It was lowest in the 270 mOsm group than in the other osmolarity groups, being 0.122±0.019 mg/ml. In the high osmolarity group at 570 mOsm, the total GAG in beads/ml of beads volume was greater than that in the 370 and 470 mOsm group after 2 days of culture, while the percentage of increased diminished subsequently until day 6 ($p<0.05$). The cell cultured at 370 and 470 mOsm were thus more active and accumulated significantly more GAG than cells cultured at 270 and 570 mOsm with time.

Figure 2A shows the effect of extracellular osmolarities on lactate production by chondrocytes, a marker for total energy production. The rate of lactate production per live cell significantly decreased with time in culture ($p<0.05$). Lactate production was significantly decreased in hypo-osmolality (270 mOsm) group compared with the other

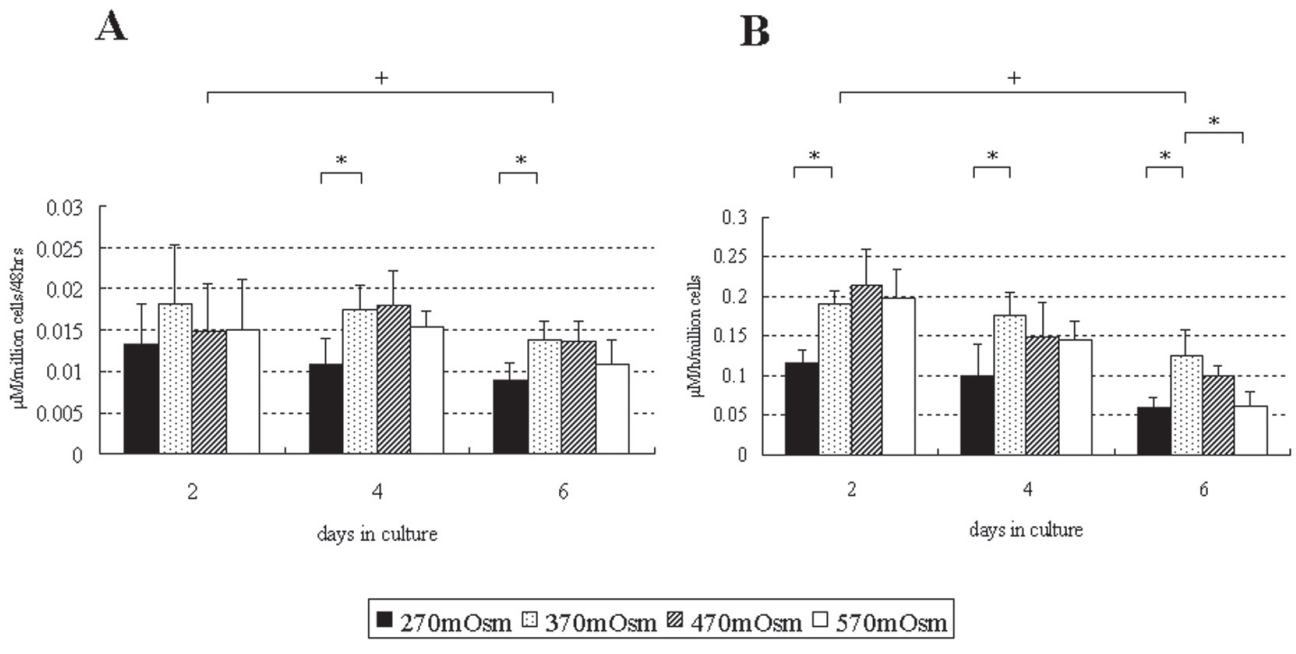

Fig. 2. Effect of extracellular osmotic change on lactate production rate (**A**) and ^{35}S-sulphate incorporation rate (**B**). This figure gives pooled data for 4 representative osmolarity from 3 separate experiments for cells cultured by articular chondrocytes. (**A**) The rate of lactate production per live cell decreased with time in culture (+: $p<0.05$, 2-way ANOVA with repeated measures among 2, 4 and 6 days). After 4 and 6 days of culture, lactate production was clearly decreased under hypo-osmolarity (270 mOsm) compared with other levels of osmolarity. (Scheffe, *: $p<0.05$). (**B**) Sulphate incorporation rates fall with time in culture (+: $p<0.05$, 2-way ANOVA with repeated measures among 2, 4 and 6 days). Values are mean ± standard error. It was clearly decreased in the hypo-osmolarity (270 mOsm) groups compared with the 370 mOsm groups during culture. After 6 days of culture, lactate production was clearly decreased under the 570 mOsm groups compared with the 370 mOsm groups. (Scheffe, *:$p<0.05$)

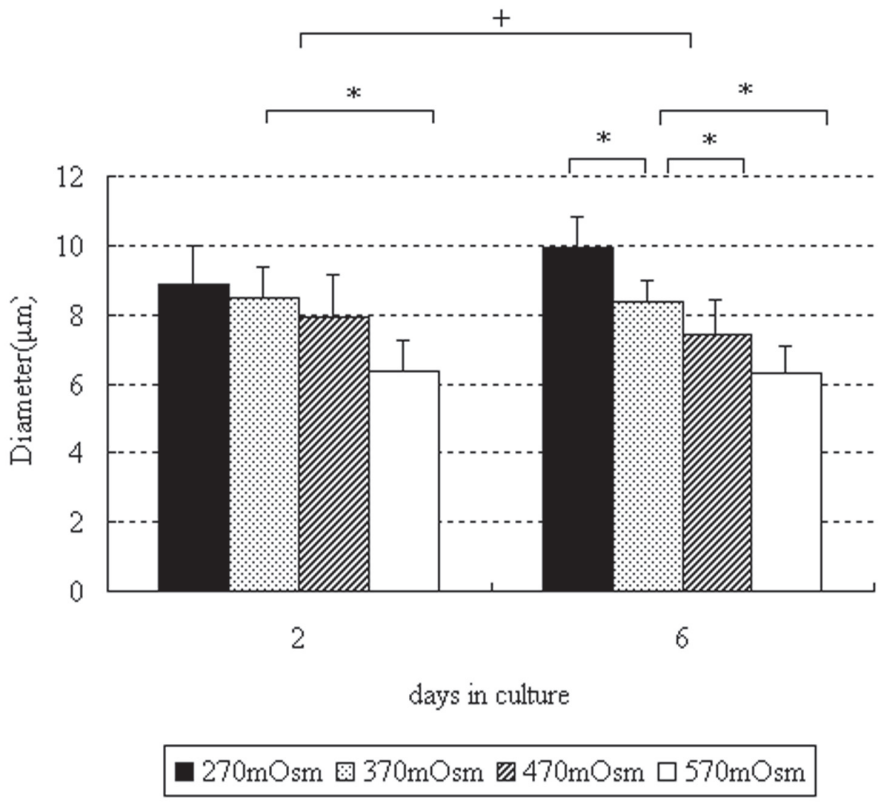

Fig. 3. Effect of extracelluler osmotic change on diameter of chondrocytes. The diameter of 25 cells was measured under varied osmolarity conditions. On confocal microscopy, the viability of chondrocytes was also 90% or more in all osmolarity groups after 2 and 6 days of culture. However, the cell diameter increases with time in culture (+: $p<0.05$, 2-way ANOVA with repeated measures among 2 and 6 days). After 6 days, cells were largest under 270 mOsm and gradually decreased became smaller with increasing osmolarity. (Scheffe, *: $p<0.05$). Values are mean ± standard error.

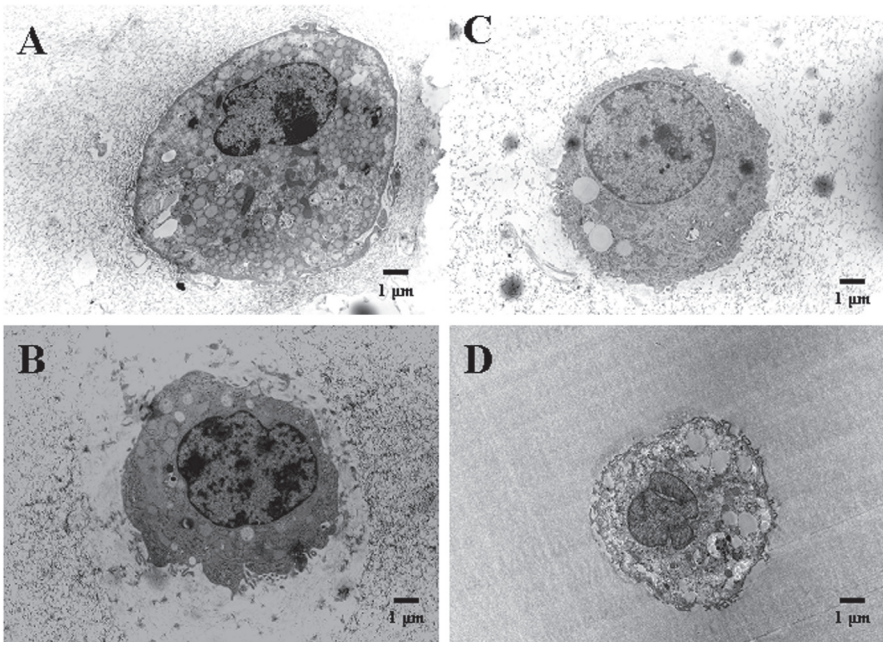

Fig. 4. Electron micrograms of chondrocytes in the centre of the beads under varied osmolarity conditions. **A.** under 270 mOsm, **B.** under 370 mOsm, **C.** under 470 mOsm, **D.** under 570 mOsm, At 270 mOsm, the cell was swelling with numerous vacuoles and cytoplasmic organelles destroyed were visible. This cell undergoing oncosis were seen. At 370 and 470 mOsm, all cells appeared viable. At 570 mOsm, the cell and nuclei was reduced in size and chromatin condensation was visible in the nuclei.

groups ($p<0.05$). Thus, cell metabolism was decreased with the duration of culture, but metabolic hypofunction persisted under hypoosmolarity.

Similarly the rate of sulphate incorporation per live cell was the highest in the 370 mOsm group during culture, and was decreased with an increase in extracellular osmolarity ($p<0.05$) (Fig. 2B). It was the lowest in the hypo-osmolarity (270 mOsm) group during culture ($p<0.05$). The cells cultured at 370 and 470 mOsm were more active significantly more sulphate incorporation per live cell than cells cultured at 270 and 570 mOsm. The rate of sulphate incorporation fell more steeply than lactate rates with time in culture.

Cell viability across the beads

After 6 days of culture, the survival rate of cells was 90.3±8.7, 93.3±11.5, 94.4±9.6, and 93.3±11.5% (mean ± SEM) respectively in the 270, 370, 470, and 570 mOsm groups. The percentage of live and dead cells in sections was similar for the high and low osmolarity cultures. However, confocal microscopy showed that the chondrocytes were greatest in the 270 mOsm group and diminished gradually along with the increases in osmolarity. The diameters of cells measured obviously decreased in the higher osmolarity groups ($p<0.05$). After 6 days of culture, the chondrocyte diameter was 9.9±0.9, 8.4±0.6, 7.4±0.9, and 6.3±0.8 μm (mean ± SEM) respectively in the 270, 370, 470, and 570 mOsm groups. The cell diameter was already established by 2 days of culture. The cell diameter increase with time in culture ($p<0.05$).

Under transmission electron micrographs of chondrocytes, at 370 and 470 mOsm all cells appeared viable, with large nuclei, dotted with chromatin and abundant rough en doplasmic reticulum (Fig. 4B, C). The cells appeared active throughout the beads. In the beads cultured at 270 mOsm, however, all cells were swelling with numerous cytoplasmic vacuoles and lipid droplets (Fig. 4A). Many cells had blebbing and these cells undergoing oncosis were seen. Under 570 mOsm, many cells were reduced in size and blebbing was visible in the nuclei (Fig. 4D). Some cells

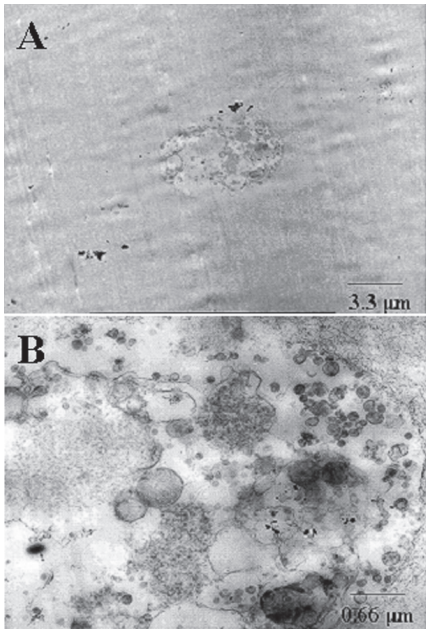

Fig. 5. Electron micrograph of a chondrocyte under 570 mOsm. The cell was reduced in size (**A**) and fragmentation of the nuclei was visible (**B**). These pictures show apoptosis characterized by condensation and fragmentation of the nuclei.

undergoing apoptosis were seen. Cells with condensed and fragmented nuclei and condensed chromatin (apoptotic bodies) and with cytoplasmic organelles destroyed were visible (Fig. 5).

Discussion

Articular cartilage represents avascular tissue and has poor ability for tissue restoration. A decrease in PG, especially GAG, is noted from the early stage of cartilage degeneration and is a major factor precipitating cartilage abrasion and arthralgia that are seen in osteoarthrosis (25, 26). Chondrocytes essentially owes it to concentration gradient to receive nutrition and oxygen supply from the surrounding tissues and evacuate metabolic products (16). Cartilage tissues have osmolarity of 350-450 mOsm (27, 28), pH of 6.8-7.1 (8), and oxygen concentration of 1-6% (29, 30). So, the chondrocyte extracellular environment is relatively hypoxic, unlike the case of other tissues. And also, articular chondrocytes are exposed to a unique osmotic environment, which varies throughout the depth of cartilage, and in response to mechanical loading or pathological conditions. It is said that such a harsh environment suppresses chondrocyte differentiation and maintains the nature characteristic to chondrocytes.

Osmotic environment of cells in cartilage tissues is altered significantly by loading and morbid conditions. The cartilage tissues sustain static load and prolonged cyclical loading all the time, and osmolarity imbalance occurs in the articular cartilage. To overcome osmotic imbalance and acquire new equilibrium, fluid is exuded from the tissue, and the PG level, cation level, and osmotic pressure are increased as a result. The chondrocytes always sustains high osmotic pressure. When loading is removed the tissue, fluid is slowly absorbed in turn, and the normal osmotic status is recovered. Hopewell *et al*. (28) investigated the effect of extracellular osmolarity on chondrocytes cultured in alginate beads. Their study showed decreased sulphate incorporation rate for the cells incubated at high osmolarity for 4 hours, recovery of sulphate incorporation rate for the cells incubated at high osmolarity for 24 and 48 hours, and a higher sulphate incorporation rate than the original level for the cells incubated further. Based on these results, they indicated that chondrocytes are sensitive to osmolarity and are able to adjust for high osmolarity during short time. Bush *et al*. reported a single impact caused temporal and spatial changes to in situ chondrocyte viability with cell shrinkage occurring in the majority of cells. However, chondrocyte shrinkage by raising medium osmolarity at the time of impact protected the cells from injury, whereas swollen chondrocytes were markedly more sensitive. These data showed that chondrocyte volume could be an important determinant of the sensitivity and response of *in situ* chondrocytes to mechanical stress. And also, Erickson *et al*. (13) indicated that osmotic stress causes significant volume change in chondrocytes and may activate an intracellular second messenger signal by inducing transient increases in intracellular calcium ion. Palmer *et al*. (12) measured the aggrecan promoter activity and mRNA levels using bovine monolayer chondrocytes subjected to hyper-osmotic loading for different time periods from 1 minute to 24 hours. They concluded the hyper-osmotic loading regulates aggrecan gene expression and cell size in isolated. Thus, mechanical compression of cartilage is associated with a rise in the interstitial osmotic pressure, which can alter cell volume and activate volume recovery pathways. In this study, the cells incubated at 370 mOsm produced the greater amount of GAG, and the cells incubated at high osmolarity for 2 days showed a similar trend for GAG production to the results of Hopewell's experiment (28) using isolated articular cartilage. However, the cells incubated further for 6 days produced a lower amount of GAG in the condition of high osmolarity and showed the profile of cell death (apoptosis) under electron microscope. Thus, this study indicates that chondrocytes is unable to adjust for such non-physiological conditions lasting for a long time and this phenomenon plays a critical role in the development of cartilage degeneration and resultant OA.

Maroundas *et al*. (27) investigated the osmotic pressures in articular sections extending to the sagittal sections and reported that the osmotic pressure in the articular cartilage is about 370-400 mOsm and were decreased in the degenerated articular cartilage. Urban *et al*. (10) incubated chondrocytes isolated from the articular cartilage in commercially available DMEM solutions set at 250-270 mOsm of osmolarity for 2 hours. Their experiment showed that the chondrocytes swelled by about 30-40% in the above osmolarity condition and chondrocytes incubated in a medium set at 350-400 mOsm for osmolarity were most close to the size of chondrocytes in the intact tissues and synthesized the highest amount of PG. In this study, the chondrocytes produced the highest amount of GAG in the osmolarity condition of 370 mOsm after 2 and 6 days of culture. The amount of GAG production was obviously lower in the low osmolarity cultures than in the culture at the optimal osmolarity close to that in the normal cartilage. On electron microscopy of chondrocytes cultured under varying levels of osmolarity, cells under 370 mOsm generally showed normal nuclei and cytoplasm, while cells under hypo-osmolarity presented oncotic changes, with cellular swelling

and destructed organelles of the cytoplasm. On the other hand, cells cultured under hyper-osmolarity were reduced in size and some cells underwent apoptosis. Manjo *et al*. (31) reported oncosis is a form of cell death accompanied by cellular swelling, organelle swelling, blebbing, and increase membrane permeability. They also showed that necrosis can occur after both forms (oncosis and apoptosis) of cell death. Therefore, its mechanism is based on failure of the ionic pumps of the plasma membrane induced by the changes of extracellular osmotic environment. Thus, our physiological and morphological study showed the articular chondrocytes is unable to adjust for such non-physiological conditions lasting for a long time and this phenomenon plays a critical role in the development of cartilage degeneration and resultant OA.

The osmotic pressure in the cartilage tissues is obviously higher than the plasma osmolarity (about 280 mOsm), and chondrocytes exist in the extracellular environment different from that of other tissues. This study indicated that adjustment of osmolarity is very important for the culture of chondrocytes. At cell densities found *in vivo* (standard conditions) in the cartilage tissue viz, 4 million cells/ml and GAG concentration in beads cultures was 0.298 mg/ml at 370 mOsm in 6 days. Assuming that the initial production rate is maintained and that there is no loss of GAG, it is calculated that >1000 days of culture is necessary to produce a GAG concentration equal to the *in vivo* GAG concentration of 7% per wet weight (viz. 70 mg/ml). That is, it is suggested that chondrocytes need to be cultivated at the cell density of 4 x 10^6 cells/ml for more than 1 year in order to construct cartilage tissue in the GAG concentration of about 70-100 mgs/ml, which is equal to the GAG concentration in the normal cartilage tissues, using cell culture technology. It has been suggested that about 2-3 fold amount of GAG can be produced using growth factors such as transforming growth factors (TGF) (18, 20, 21) and insulin growth factors (IGF) (19, 21). Even if such growth factors are used, more than 100 days of culture is thought to be necessary. Furthermore, it has been reported that turnover of GAG in the cartilage tissue takes about 2-3 years (32). So, GAG is slowly synthesized in the biological condition, and a long time is necessary to construct articular cartilage with adequate mechanical strength even if cells are maintained in active status by three-dimensional culture.

At present, the target diseases of treatment utilizing bioengineering (tissue engineering) such as chondrocytes implantation are local lesions such as traumatic cartilage defect and osteochondritis dissecans (33-36). For regeneration of extensive degenerated cartilage in OA, it is necessary to secure a large amount of chondrocytes for implantation. If a large graft is implanted, a nutritional problem may occur as explained above. In addition, the subchondral bone needs to be healthy to obtain the normal function of articular cartilage such as dispersion of load. However, lesion of OA is not localized in the cartilage layer but involves the subchondral bone (*e.g*., osteosclerosis). The osteosclerosis which is the subchondral bone covers the bone-cartilage junction with age and tries to close the nutritional route through these vascular systems (37, 38). Therefore, even if regeneration of cartilage is achieved by means of hyaline cartilage, the regenerated cartilage may sustain overload and may be degenerated again unless the mechanical environment is modified together.

Conclusion

In our model, the prevailing osmolarity was a powerful regulator of GAG accumulation by cultured chondrocytes. Changes in osmolarity have the potential to disrupt cartilage matrix homeostasis. In view of the long culture times which appear necessary to achieve the required GAG composition *in vitro*, achievement of an *in vivo* concentration before implantation of a construct may be unrealistic and possibly an unnecessary goal for tissue engineered cartilage of OA.

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