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Bovine articular chondrocyte function *in vitro* depends upon oxygen tension

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Summary

Articular cartilage is a physiologically hypoxic tissue with a proposed gradient of oxygen tension ranging from about 10% oxygen at the cartilage surface to less than 1% in the deepest layers. The position of the chondrocyte within this gradient may modulate the cell's behavior and phenotype. Moreover, the oxygen gradient is likely to be disturbed during joint diseases in which the pO_2 of the synovial fluid declines which may cause changes in chondrocyte behavior and gene expression. Thus, there is a need to understand the chondrocyte's response to different oxygen tensions. We compared the behavior of bovine articular chondrocytes cultured in alginate beads for 7 days in medium maintained at <0.1, 5, 10 or 20% oxygen. The chondrocytes' survival, differentiation, cell division, viability and matrix production were assessed at each oxygen tension and rRNA and mRNA abundance was measured.

Chondrocytes were able to survive under all oxygen tensions for at least 7 days but cells cultured under anoxic conditions were metabolically less active than cells maintained in higher oxygen tensions; this was associated with a decrease in matrix production. In <0.1% oxygen there was a marked decrease in rRNA and mRNA abundance in the cells. There were no differences in cell division or differentiation between any oxygen tensions. These findings indicate that articular chondrocytes can be cultured successfully in the pO_2 range in which they are thought to exist *in vivo* (5–10% pO_2) and are fully active under these conditions. Under anoxic conditions (<0.1% pO_2) function is severely compromised. © 2000 OsteoArthritis Research Society International

Key words: Hypoxia, Oxygen tension, Articular cartilage, Chondrocyte, Alginate bead culture.

Introduction

The deepest layers of articular cartilage are calcified and impermeable to fluid or gas and the tissue has no vascular supply.[1] Thus oxygen, glucose and other nutrients must diffuse into the cartilage at its surface from the synovial fluid. Synovial fluid has a low oxygen tension (~50 mmHg, ~7% pO_2) compared to that of the arterial blood supply (>90 mmHg, >12% pO_2)[2,3] and hence articular cartilage is normally hypoxic compared with vascularized tissue.

There appears to be a gradient of decreasing oxygen tension from the surface of the articular cartilage to the subchondral bone.[3,4] Oxygen diffusing from the synovial fluid is partially used up by chondrocytes in the upper layers so that the oxygen tension is decreased even further in the deeper layers. However, few attempts have been made to measure the oxygen tension in articular cartilage in adult animals. In an analogous tissue, the nucleus pulposus of the canine intervertebral disc, the oxygen tension was 0.3–1.2% oxygen (2–8 mmHg).[5] However, the distance over which diffusion occurs in this tissue is probably greater than in articular cartilage so the pO_2 may be lower than in the deep layer of the latter. Nevertheless, it is likely that the physiological oxygen tension of articular chondrocytes is <10% at the surface and possibly <1% in the deepest layer.[6]

Articular chondrocytes have a characteristic morphology and metabolism depending upon their position in the cartilage.[7–9] This may be due, at least in part, to the position of the chondrocyte within the oxygen tension gradient. Furthermore, the oxygen gradient will be disrupted during disease states. In rheumatoid arthritis, the oxygen consumption of the synovium is elevated and the synovial fluid pO_2 is decreased compared to that of normal tissue (5.6% compared to 7.2% pO_2).[10,11] Additionally, the oxygen-delivering capability of the synovium may be diminished due to disturbance of the blood vessel architecture.[12] Neo-vascularization of inflamed tissue does not relieve the hypoxia probably because of the distribution and disorganization of the new vessels.[13] The posterior articular nerve of the sympathetic nervous system, which regulates blood flow in the synovium, has lower efficacy in the inflamed joint.[11] There is also an accumulation of synovial fluid in the joint which correlates with decreased oxygen tension of the fluid.[14] Thus, although the oxygen gradient is presumably still present across RA cartilage, it must start at a lower oxygen tension and is likely to reach very low oxygen tensions in the basal layer.

In osteoarthritis (OA), degeneration of the articular cartilage causes fibrillations in the tissue. It is probable that the oxygen gradient across the cartilage breaks down as synovial fluid penetrates these cracks. However, it has been suggested that changes in the synovium during OA may alter the oxygen transfer characteristics from the

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capillaries of the membrane to the synovial fluid leading to hypoxia.[15] Microfractures of the subchondral bone may also disrupt the gradient by allowing oxygen to diffuse up to the basal chondrocytes from the subchondral vasculature. However, reduced subchondral blood flow occurs in the osteoarthritic hip[16] making it difficult to assess the overall impact of microfractures.

There is a need to understand how chondrocytes behave in different oxygen tensions to assess how their function may change *in vivo* throughout the depth of the cartilage and also during diseases such as OA and rheumatoid arthritis. However, almost all investigations of chondrocytes in cell or tissue culture have been carried out under atmospheric oxygen tension (~21% pO_2). Thus, to assess the effect of lower oxygen tensions, we have cultured isolated articular chondrocytes from the bovine metacarpophalangeal joint in alginate beads under oxygen tensions of <0.1, 5 and 10% to reflect the *in vivo* range, and 20% as in the majority of previous experiments. We used the alginate bead culture system,[17] which allows microscopy of the cells *in situ* and rapid recovery of the cells and cell-associated matrix to analyse the cell biology of the chondrocytes under these varying oxygen tensions.

Materials and methods

ISOLATION OF ARTICULAR CHONDROCYTES

Full-depth cartilage was removed from bovine metacarpophalangeal joints of cows less than 36 months of age following the method of Kuettner *et al.*[18] and was washed in HBSS (Gibco BRL Life Technologies Ltd, U.K.). Cartilage was pooled from 3–6 animals for each experiment. Cartilage strips were digested (1 g tissue/10 ml digest) in 0.5 mg/ml hyaluronidase (Sigma-Aldrich Company Ltd, U.K.) for 10 min, 2 mg/ml trypsin (Sigma) for 40 min and 1 mg/ml type 2 collagenase (Worthington Biochemical Corporation, NJ, U.S.A.) overnight at 37°C. The digested tissue was filtered through a 30 μm nylon mesh into a sterile 50 ml tube and the cells recovered by centrifuging (600 ***g,*** 15 min). The cell pellet was washed in HBSS and resuspended in DMEM culture medium (Gibco). The total cell number was calculated using a haemocytometer.

ALGINATE CULTURE OF ARTICULAR CHONDROCYTES UNDER DIFFERENT OXYGEN TENSIONS

DMEM culture medium [supplemented with 10% (v/v) fetal calf serum (FCS)] and 2.4% (w/v) alginate were added to the cell preparation so that the final concentrations were 7.5×10^6 cells/ml and 1.2% (w/v) alginate. This cell density approximates to that estimated for bovine articular cartilage[19] and is higher than that used in previous alginate cultures.[17,20–23] Beads were generated from 3 ml cells/alginate solution using the method described by Guo *et al.*[17]

CULTURE UNDER DIFFERENT OXYGEN TENSIONS

The gassing and culture apparatus was supplied by Boehringer Mannheim UK Ltd, U.K. Gas of composition 0, 5, 10 or 20% oxygen with 5% carbon dioxide and the balance nitrogen was supplied by the British Oxygen Company. Gases were passed through a Dreschel flask to saturate the gas with moisture. Saturated gas was bubbled through DMEM medium (supplemented with 25 μg/ml ascorbic acid but without FCS) via gas distribution tubes for 30 min. Each alginate bead culture (3 ml) in 10 ml DMEM medium was placed in a siliconized conical flask and water-vapor-saturated gas blown onto the surface of the medium for 10 min. FCS was equilibrated with the appropriate gas mixture by blowing it over the surface for 30 min. Medium (27 ml) and FCS (3 ml) were added to each conical flask. Gas was briefly blown into the flask before it was sealed using rubber stoppers. This was repeated for each gas mixture. The flasks were incubated at 37°C and half of the medium was changed every 2/3 days using medium at the relevant oxygen tension. The atmosphere over the medium was regassed following each medium change. The medium pO_2 was measured at the beginning and end of each experiment using a Mettler Toledo hand-held oxygen meter.

For microscopy, alginate beads were transferred with medium to a 25 cm^2 culture flask which was placed directly onto the microscope platform. Alginate beads are translucent and individual cells can be seen, with no staining, under ×100 or ×200 magnification light microscopy.

RECOVERY OF CELLS FROM ALGINATE BEADS

The contents of each flask were placed in a 50 ml tube, allowed to settle and the medium removed until the total volume was 25 ml. An equal volume of 110 mM sodium citrate solution (sparged with the appropriate gas mixture) was added to give a final concentration of 55 mM sodium citrate. The beads were incubated at 37°C with agitation until the alginate gel had solubilized (<10 min). The cells were then collected by centrifugation, the supernatant removed, and counted after resuspending them in HBSS. Viability of the recovered chondrocytes was assessed using Trypan blue (Sigma) following the manufacturer's instructions.

Cells released from alginate beads are associated with an attached pericellular coat of extracellular matrix. To estimate differences in extracellular matrix deposition in different culture conditions, HBSS (1 ml) was added to the pellet using a 1 ml pipette and the pellet resuspended. The total volume was carefully measured using a 2 ml pipette and the pellet volume was calculated. Since cell numbers were constant (see below), differences in cell pellet volume after culture under different pO_2 were taken as a measure of deposition of extracellular matrix around the cells.

MEASUREMENT OF rRNA YIELD

Total RNA was extracted from 2.1×10^7 cells using the Ultraspec II total RNA isolation kit (Biotecx, TX, U.S.A.) and resuspended in 30 μl DEPC-treated water. Aliquots of RNA (7 μl) were run on an agarose gel containing ethidium bromide. rRNA yields were compared by taking an image of the gel on a UV transilluminator using a UVP video camera and a Mitsubishi video copy processor. Images were scanned using an Epsom scanner fitted with a reflective lightsource and saved as *Adobe PhotoShop* TIFF files, 1:1 scale and 300 dpi resolution. Band intensity of the combined 18S and 28S bands was assessed using NIH Image processing software.

RT-PCR TO GENERATE PROBES

RT-PCR primers were designed for bovine aggrecan (forward—CACCAACGAAACCTATGACGTGTAC, reverse—AAACGTGAAAGGCTCCTCGG) and β-actin (forward—GTGGGGCGCCCCAGGCACCA, reverse—CTCCTTAAT GTCACGCACGATTTC). Superscript II Reverse Transcriptase (Gibco) was used to generate cDNA following the manufacturer's instructions. cDNAs were amplified using Taq polymerase (Gibco) following the manufacturer's instructions. PCR products were purified using Qiaquick PCR purification columns (Qiagen Ltd, U.K.). The PCR products were sequenced using the original primers to confirm identity.

To generate a 'mixed-transcript probe', low stringency RT and PCR reactions were carried out on the total RNA using 20-mer arbitrary primers following the method described by Welsh *et al.*[24] but without incorporating a radioactive nucleotide in the reaction.

cDNA amplified from rRNA was donated by L. Buluwela.

NORTHERN HYBRIDIZATION SLOT BLOTS

Up to 25 ng of probe DNA was randomly labeled with α^{32}P-dCTP using the *redi*prime random primer labeling kit (Amersham Pharmacia Biotech, Bucks, U.K.). Total RNA (2 μg) was bound to Hybond N^+ nylon membrane (Amersham Pharmacia Biotech) using a Minifold II slot blot apparatus (Schleicher and Schuell, Germany). Probe hybridization was carried out with Rapid-hyb buffer (Amersham Pharmacia Biotech) following the manufacturer's instructions. After washing, the nylon membrane was wrapped in Saran wrap. Kodak Bio-Max MR film was exposed to the membrane overnight at −70°C.

Autoradiographs were scanned directly with an Epsom scanner fitted with a transmission lightsource. Images were saved as *Adobe PhotoShop* TIFF files, 1:1 scale and 300 dpi resolution. The band absorbance was assessed using NIH Image analysis software. Units of measurement (pixel density) are arbitrary. The band absorbance was normalised against a β-actin control.

STATISTICAL ANALYSIS

Results were tested for statistical significance using *InStat Version 2.01* software. Repeated measures ANOVA is analogous to the paired *t*-test and the Tukey–Kramer multiple comparisons test is similar to the Student–Newmans–Keuls test.

Results

CELL SURVIVAL AND DIVISION

Cells were recovered from the alginate beads after 7 days' incubation in different pO_2s. The total cell number per flask was counted and viability examined. Cells recovered from alginate showed 90–100% viability regardless of the oxygen tension of the culture medium. Each flask initially contained 2.1×10^7 cells and the same number was recovered after 7 days with no significant difference between any oxygen tensions.

Chondrocyte cell division was also assessed by light microscopy. Chondrocytes are immobile in alginate and are generally suspended singularly. Although it is not feasible to count the cells resident in alginate beads, it is possible to observe whether cells remain single or whether there are nests of doublets in the bead. The latter would be indicative of cell division. After seven days in culture there were few (<1%) 'twinned' chondrocytes indicating that little or no division of chondrocytes occurred in alginate under any oxygen tension.

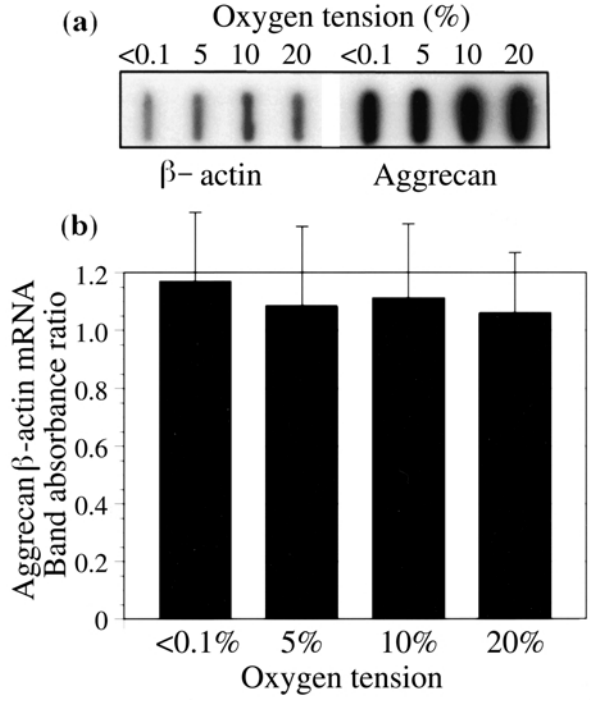

Fig. 1. (a) Autoradiograph of a slot blot using total RNA from chondrocytes cultured for 7 days under different oxygen tensions. The slot blots were probed for aggrecan and β-actin. (b) Quantitation of aggrecan mRNA: β-actin mRNA ratio under different oxygen tensions. Figures are mean±S.E.M. (N=6). Repeated measures ANOVA shows P>0.05.

CELL DIFFERENTIATION

Chondrocytes maintained their spherical shape in the alginate beads under all oxygen tensions after 7 days of culture. This shape is characteristic of differentiated chondrocytes.[25,26] The expression of aggrecan, a phenotypic marker for chondrocytes, was maintained under all oxygen tensions (Fig. 1). RT-PCR for COLIA1 mRNA did not detect the presence of transcripts at any oxygen tension, indicating maintenance of chondrocyte phenotype (data not shown).

DE NOVO MATRIX PRODUCTION

After 7 days' culture under different pO_2, cells maintained with 10% pO_2 had the maximum pellet volume (Fig. 2). The pellet volume was significantly lower in lower oxygen tensions and marginally lower in 20% pO_2 cultures.

METABOLISM

The pH of the medium prior to cell culture was pH 7.4 at all oxygen tensions. After 7 days under different oxygen

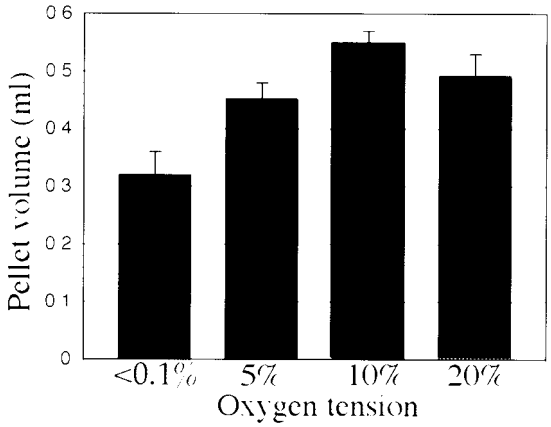

Fig. 2. Cell pellet volume for 2.1×10^7 chondrocytes after seven days in culture under different oxygen tensions. Results are mean±S.E.M. (N=5). Repeated measures ANOVA shows P<0.0001. Tukey–Kramer multiple comparisons test gave the following P values: <0.1% vs 5%, P<0.001; <0.1% vs 10%, P<0.001; <0.1% vs 20%, P<0.001; 5% vs 10%, P<0.001; 5% vs 20%, P<0.05; 10% vs 20%, P<0.01.

tensions, the media from the last 48 h of culture were collected and the pH tested (Table I). Decreases in pH are likely to be due to accumulation of lactate in the medium and indicate the levels of anaerobic metabolism of glucose by the cells. There was no significant difference in medium pH between cultures maintained in 5–20% pO_2. Anoxic cultures (i.e. <0.1% pO_2) showed a smaller change towards acid pH, suggesting a lower rate of glycolysis in these cells than at higher pO_2. These differences, though small, were observed consistently in five separate experiments.

RNA ABUNDANCE

RNA yield from cells cultured under different oxygen tensions was assessed by electrophoresis (Fig. 3). The intensity of the 28S and 18S rRNA bands was assessed using NIH Image analysis software. The rRNA yield did not differ significantly between oxygen tensions after 2 days [Fig. 3(a)]. After 7 days of culture the rRNA yield was significantly reduced under <0.1% oxygen compared to higher oxygen tensions [Fig. 3(b)]. The marked decrease in rRNA after seven days of culture in pO_2 <0.1% suggests that degradation of these molecules occurs in prolonged anoxic conditions, possibly in the absence of synthesis.

Table I

*pH of the conditioned medium after 7 days under different oxygen tensions. Results are the mean±S.E.M. (*N=*5). Repeated measures ANOVA shows* P=*<0.0001. The Tukey–Kramer multiple comparisons test gave the following* P *values: <0.1% vs 5%* P=*<0.001, <0.1% vs 10%* P=*<0.001, <0.1% vs 20%* P=*<0.001. All other pairs of oxygen tensions gave* P=*>0.05*

Oxygen tension of medium	pH
<0.1%	7.10±0.02
5%	6.90±0.01
10%	6.88±0.01
20%	6.87±0.01

Probes were made from cDNA reverse transcribed from rRNA and β-actin mRNA. rRNA abundance was compared to β-actin abundance by Northern hybridization slot blot (Fig. 4). Repeated measures ANOVA showed that there was no significant difference in the rRNA:β-actin ratio in chondrocytes cultured in different oxygen tensions for seven days. Thus, there must be a decrease in β-actin mRNA in anoxic chondrocytes which is in proportion to the decrease in rRNA in these cells.

The average abundance of a number of mixed transcripts was tested. Bovine chondrocyte RNA was reverse transcribed and then amplified by PCR in low stringency reactions with arbitrary primers; similar reactions incorporating a ^{32}P nucleotide amplify between 30 and 200 transcripts (unpublished data). The PCR products were purified and used to make a labeled probe. A slot blot probed with this mixed mRNA probe and a β-actin probe showed that there was no significant difference between the mixed mRNA:β-actin ratios at different oxygen tensions (Fig. 5). Thus overall mRNA abundance in anoxic cells decreases in proportion to the decrease in β-actin and rRNA.

Discussion

Bovine chondrocytes from young but skeletally mature animals (18–36 months of age) were used for this study. The articular cartilage is avascular at this age so these chondrocytes would be exposed only to oxygen diffusing into the tissue from the synovial fluid *in vivo*. *In vitro* the chondrocytes were able to survive under all oxygen tensions between <0.1% and 20% oxygen for at least seven days and possibly for much longer. This distinguishes their behavior from that of most other cell types and suggests that extreme hypoxia, as might occur in rheumatoid arthritis, would not lead to tissue failure due to chondrocyte death. Hyperoxia is toxic to most cells, particularly anaerobes (for examples, see refs 27–29) yet oxygen tensions higher than those present *in vivo* are not apparently detrimental to the articular chondrocyte.

We investigated chondrocyte behavior under distinctly different oxygen tensions (<0.1, 5, 10%), encompassing the physiological range rather than over smaller pO_2 intervals. This was to maximize the possibility of measuring changes in chondrocyte behavior at different pO_2. Cell number did not change significantly under any oxygen tension after 7 days' culture. 'Twinned chondrocytes' in alginate, indicating recent cell division, were present, but only in small numbers, under all oxygen tensions. Previous studies have shown that chondrocytes cultured in alginate beads under atmospheric oxygen show a small amount of cell proliferation for the first 10 days of culture but then proliferate no further.[21–23] This increase in cell number may be attributable to the initial cell density being lower than that used here; the higher cell density better reflected the *in vivo* cell density.

The chondrocytes retained their differentiated phenotype under different oxygen tensions. The cells maintained a spherical shape under all oxygen tensions and continued to express chondrocyte-specific aggrecan. Chondrocytes cultured in alginate beads at atmospheric oxygen tensions have previously been shown to remain chondrocytic for several months.[23] These data show that chondrocytes do not require hypoxia to maintain their phenotype.

The pH of the medium dropped less in cultures maintained at <0.1% oxygen than in cultures maintained under higher oxygen tensions. A similar effect has been seen in

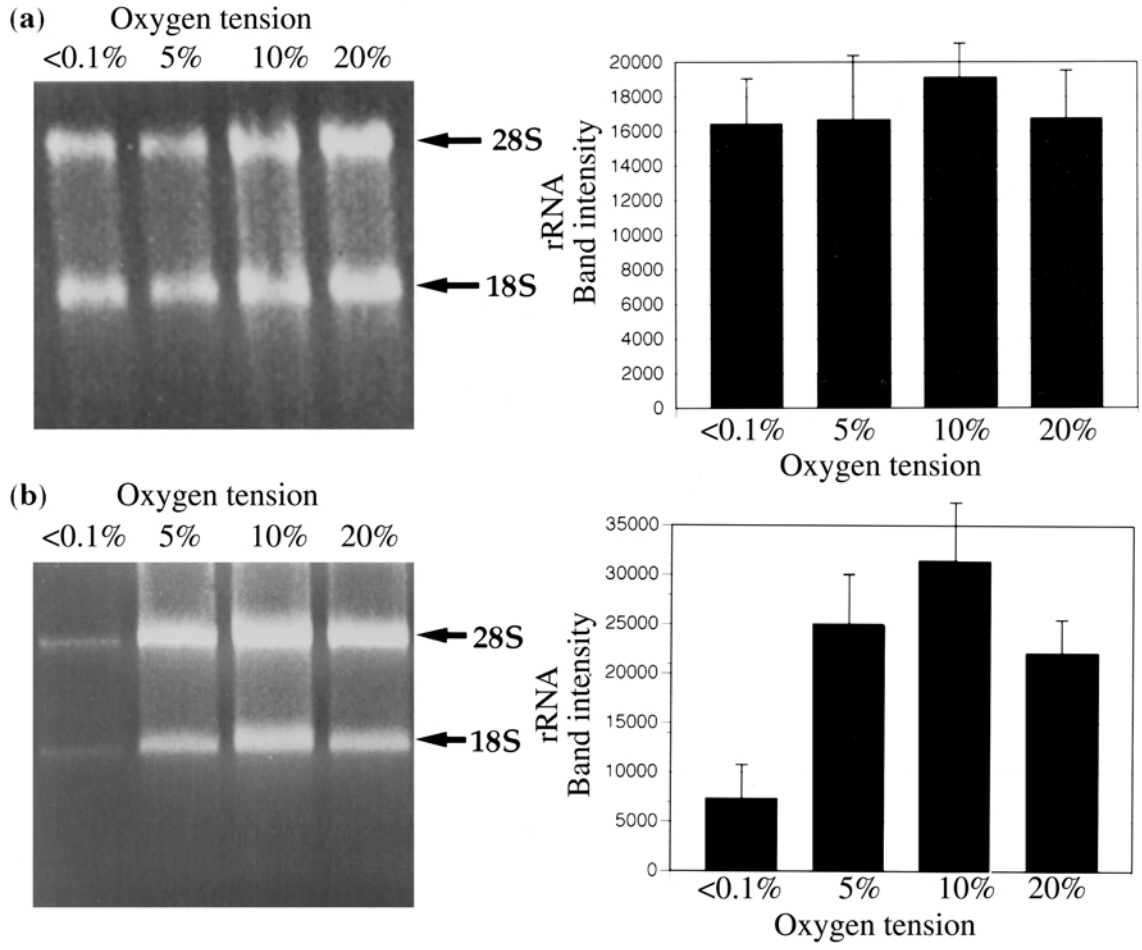

Fig. 3. Agarose gels of 18S and 28S rRNA isolated from chondrocytes cultured for (a) 2 and (b) 7 days under different oxygen tensions. The rRNA yield was assessed by image analysis. Results are mean±S.E.M. (2 days, N=3; 7 days, N=4). Repeated measures ANOVA shows P=0.05 (2 days) and 0.0002 (7 days). For the 7-day cultures, the Tukey–Kramer multiple comparisons test gave the following P values: <0.1% vs 5, P<0.01; <0.1% vs 10%, P<0.001; <0.1% vs 20%, P<0.01; 10% vs 20%, P<0.05. All other pairs of oxygen tensions gave P>0.05.

cultures of bovine articular cartilage explants: lactate production decreased under hypoxic culture conditions concomitant with a decrease in the uptake of external glucose.[30] This indicates that chondrocytes are metabolically less active when cultured under <0.1% oxygen, in contrast to many other cell types which increase anaerobic metabolism under near-anoxic conditions to meet the cell's energy requirements. The pH dropped by similar amounts in all other cultures (5–20% oxygen), suggesting that the rate of conversion of glucose to lactate is the same regardless of oxygen tension. Lane *et al.* showed previously that chondrocytes use anaerobic respiration even under aerobic conditions.[31]

The maximum cell pellet volume was achieved by chondrocytes maintained for 7 days in 10% pO_2. This oxygen tension is probably close to that which is present at the surface of articular cartilage. The cell/matrix pellet was visibly smaller when cells were cultured under <0.1% oxygen compared to 5–20% oxygen. Pellet volume peaked in 10% oxygen and pellets from <0.1% oxygen cultures were ~50% smaller than those from 10% oxygen cultures. Differences in cell pellet volume were not due to different numbers of cells. The reduction in cell pellet volume is probably due to decreased expression of type II collagen at both the mRNA and protein levels (data not shown; manuscript submitted). As a major extracellular matrix protein this would predictably lead to a decrease in matrix volume. Integrity of the ECM may also be affected, allowing smaller proteins normally entrapped in the collagen network to diffuse away from the pericellular matrix. Additionally we cannot exclude the possibility that enhanced degradation of pericellular matrix occurs in low oxygen tension, decreasing the volume of the cell pellet.

The highest RNA levels were present in chondrocytes cultured in 5–10% oxygen. This oxygen tension is probably representative of that found in the upper half of articular cartilage. The rRNA yield from cultures maintained for seven days in <0.1% oxygen was decreased 60–90% compared to 10% oxygen cultures. This decrease in rRNA may be related to the decrease in matrix production under anoxic culture conditions. Much of a chondrocyte's protein production will be geared towards matrix synthesis. Thus decreased levels of collagen transcription would lower the demand for ribosomes for translation. However, it has been suggested that decreased protein synthesis by lattice-cultured fibroblasts compared to monolayer cultured cells is partly regulated at the ribosomal level[32,33] and this could also be the case for chondrocytes cultured under very low

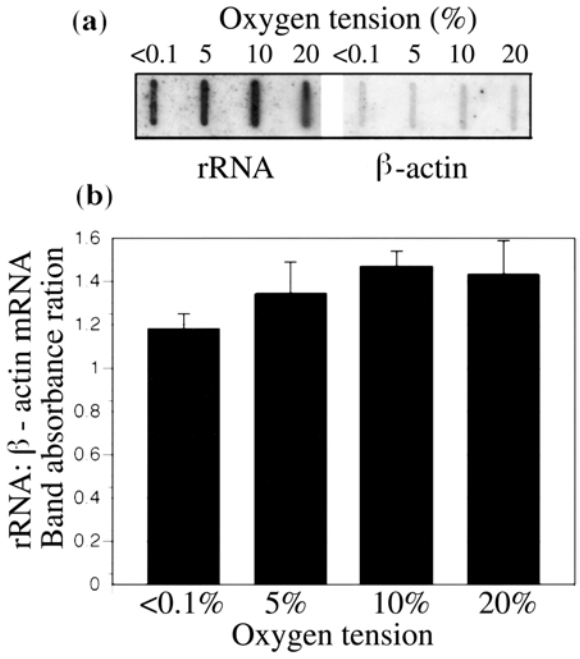

Fig. 4. (a) Autoradiograph of a slot blot using total RNA from chondrocytes cultured for 7 days under different oxygen tensions. The slot blots were probed for rRNA or β-actin. (b) Quantitation of rRNA:β-actin mRNA ratio. Results are the mean±S.E.M. (N=3). Repeated measures ANOVA shows P>0.05.

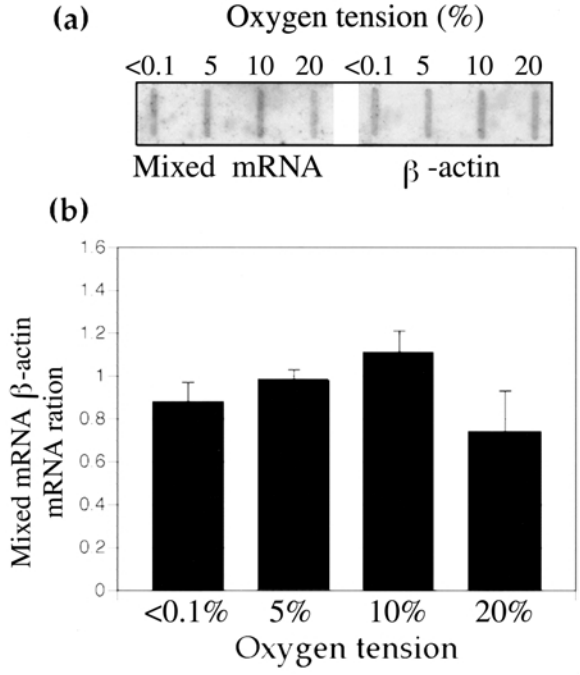

Fig. 5. (a) Autoradiograph of a slot blot using total RNA from chondrocytes cultured for 7 days under different oxygen tensions. The slot blots were probed for mixed transcripts and β-actin. (b) Quantitation of mixed transcripts mRNA: β-actin mRNA ratio under different oxygen tensions. Figures are mean±S.E.M. (N=6). Repeated measures ANOVA shows P>0.05.

oxygen tensions. It is also noteworthy that rRNA levels are decreased by heat-shock,[34] which is similar in its effects in many cell types to hypoxic stress. HeLa cells incubated at 42°C decrease cytoplasmic levels of 18S and 28S rRNA by as much as 95% as well as decreasing the mRNA abundance of non-heat-shock proteins. However, there was little effect on transcription.

Even though the 18S and 28S rRNA levels are dramatically decreased after 7 days' culture under <0.1% oxygen compared to 10% oxygen, the rRNA/β-actin or mRNA/β-actin mRNA ratio does not change significantly. This indicates that as well as decreasing the abundance of the rRNA species, chondrocytes cultured under <0.1% oxygen decrease the abundance of almost *all* mRNA species. However, the overall decline of the mRNA pool does not imply that specific mRNA species cannot be up-regulated. Overall the data from this study highlight the complications of normalizing mRNA data according to the total RNA, rRNA or the expressed mRNA pool when comparing expression under different oxygen tensions.

In summary, articular chondrocytes cultured in near-anoxic conditions survive yet show less matrix production and produce less metabolic acid than cells cultured at higher oxygen tensions. Comparison of rRNA:mRNA ratios suggests that the abundance of almost *all* mRNAs is down-regulated under near-anoxic conditions. Together, these observations suggest that the chondrocytes enter a state of 'metabolic dormancy' under very low oxygen tension. Whilst chondrocytes are capable of withstanding anoxic conditions for much longer than most other cell types, some oxygen is required for the cells to fulfil their primary function—production of matrix components. If the effects reported here were mimicked *in vivo* through low oxygen conditions in rheumatoid arthritis, this would be likely to compromise articular cartilage function.

Acknowledgments

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