Journal of Cell Science 108, 1991-1999 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995

Expression of cartilage-specific molecules is retained on long-term culture of human articular chondrocytes

Evangelos Kolettas[1], Lakjaya Buluwela[1], Michael T. Bayliss[2] and Helen I. Muir[1,*]

[1]Department of Biochemistry, Charing Cross and Westminster Medical School, University of London, Fulham Palace Road, London W6 8RF, UK
[2]Kennedy Institute of Rheumatology, 6 Bute Gardens, London W6 7DW, UK

*Author for correspondence

SUMMARY

Normal human adult articular chondrocytes were used to determine how the chondrocyte phenotype is modulated by culture conditions following long-term culture. We report here for the first time that human articular chondrocytes have a lifespan in the range of 34-37 population doublings. While chondrocytes cultured as monolayers displayed a fibroblastoid morphology and grew faster, those cultured as suspensions over agarose adopted a round morphology and formed clusters of cells reminiscent of chondrocyte differentiation in intact cartilage, with little or no DNA synthesis. These morphologies were independent of the age of the culture. Despite, these morphological differences, however, chondrocytes expressed markers at mRNA and protein levels characteristic of cartilage: namely, types II and IX collagens and the large aggregating proteoglycans, aggrecan, versican and link protein, but not syndecan, under both culture conditions. However, they also expressed type I collagen α1(I) and α2(I) chains. It has been suggested that expression of collagen α1(I) by chondrocytes cultured as monolayers is a marker of the loss of the chondrocyte phenotype. However, we show here, using reverse transcriptase/polymerase chain reaction, that normal fresh intact human articular cartilage expresses collagen α1(I). The data show that following long-term culture human articular chondrocytes retain their differentiated characteristics and that cell shape does not correlate with the expression of the chondrocyte phenotype. It is proposed that loss of the chondrocyte phenotype is marked by the loss of one or more cartilage-specific molecules rather than by the appearance of non-cartilage-specific molecules.

Key words: chondrocyte differentiation, lifespan, gene expression

INTRODUCTION

Articular chondrocytes are specialised cells of mesenchymal origin found exclusively in cartilage, an avascular tissue whose physical properties depend on the extracellular matrix produced by these cells. The matrix is composed of several macromolecules including cartilage proteoglycan or aggrecan, hyaluronan and link protein, embedded within a framework of collagen fibrils consisting predominantly of type II collagen and smaller amounts of type IX and XI collagens (Mendler et al., 1989; Upholt and Olsen, 1991). Since these components occur only in cartilage, they are markers of chondrocyte differentiation. During endochondral ossification, however, chondrocytes in cartilage undergo a process of maturation leading to cellular hypertrophy, which is characterised by the onset of expression of type X collagen (Upholt and Olsen, 1991; Reichenberger et al., 1991; Kirsch et al., 1992; Stephens et al., 1993).

Studies with human articular chondrocytes have been hampered because of the availability and quality of human articular cartilage, and the difficulty in isolating and maintaining human articular chondrocytes in culture. Several culture systems have been used to maintain chondrocytes in vitro including monolayer cultures, suspension cultures such as over agarose gels, within agarose gels, on alginate beads or spinner cultures (Aulthouse et al., 1989; Archer et al., 1990; Häuselmann et al., 1994; Bonaventure et al., 1994). It has been shown that when human chondrocytes are grown in monolayer cultures, they proliferate but they cease to express the specialised proteins of cartilage and become fibroblastic in appearance (Aulthouse et al., 1989; Archer et al., 1990; Bonaventure et al., 1994), suggesting that under these conditions the chondrocyte phenotype is unstable. However, this can be reversed using suspension cultures which promote the re-expression of the chondrocyte phenotype (Bassleer et al., 1986; Delbruck et al., 1986; Aulthouse et al., 1989; Archer et al., 1990; Bonaventure et al., 1994). Under these conditions chondrocytes form clusters of rounded cells, cell proliferation ceases and cartilage-specific molecules are resynthesised, suggesting that cell shape plays an important role in the control of the chondrocyte phenotype in vitro (Benya et al., 1978; Benya and Shaffer, 1982; Glowacki et al., 1983; Watt and Dudhia, 1988; Aulthouse et al., 1989; Archer et al., 1990; Häuselmann et al., 1994; Bonaventure et al., 1994).

The expression of the human chondrocyte phenotype has been investigated with either freshly isolated cells or cells that were passaged for as few as six times in vitro (Aulthouse et al., 1989; Archer et al., 1990; Bonaventure et al., 1994); however, nothing is known about the lifespan of human articular chondrocytes (HAC) or the expression of the chondrogenic phenotype following long-term subculturing of the cells. In this report, adult human articular chondrocytes isolated from cartilage were cultured either as monolayers or as suspensions over agarose, and passaged extensively in order to determine their lifespan. Using biochemical and molecular biological techniques, evidence is presented for the first time of how the chondrogenic phenotype, the growth behaviour of the cells and the expression of cartilage-specific molecules are modulated by long-term culture and different culture conditions.

MATERIALS AND METHODS

Source of cells and culture conditions

Human articular cartilage was obtained from the knees of three female patients aged 11, 21 and 33 years old, undergoing amputation for osteosarcoma distant from the joint. Sliced cartilage was maintained overnight at 37°C, 5% CO_2 as submerged explant in Hepes-buffered DMEM containing 10% foetal calf serum (FCS), 1.4 mM L-glutamine, 200 units/ml penicillin, 200 μg/ml streptomycin and 50 μg/ml ascorbic acid to ensure sterility of the specimens prior to enzymic digestions. The cartilage preparations were washed in phosphate-buffered saline (PBS) containing antibiotics and diced finely. The tissue was then incubated in the above growth medium containing 700 i.u./ml Pronase at 37°C on a roller for 30 minutes. Supernatants were discarded and the cartilage further digested in growth medium containing 300 i.u./ml collagenase for 3 hours on a roller at 37°C. The digested tissue was then allowed to settle and the supernatant containing the cells was removed and centrifuged at 200 ***g*** for 10 minutes at 4°C. The remaining tissue was further subjected to collagenase digestion as above. The cells were washed three times in PBS containing 10% FCS and antibiotics. The cells were resuspended in Hepes-buffered DMEM supplemented with 10% FCS, 1.4 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml ascorbic acid, seeded into a 80 cm^2 tissue culture flask and incubated at 37°C, 5% CO_2 until confluent. The medium was changed every 3-4 days.

Confluent monolayers were subcultured at 1:2 and 1:4 split ratios using 1 ml of 1× trypsin-EDTA solution. The cell suspension was diluted with PBS and the cells were seeded onto tissue culture flasks. Human articular chondrocytes were also grown over 1% agarose (Sigma type V) prepared in phosphate buffered saline (PBS) (Archer et al., 1990).

Assays of cell growth properties

The growth rates of human articular chondrocytes (HAC) grown either as monolayers or as suspensions over agarose were measured by seeding cells at a density of 5×10^4 into 60 mm dishes. About 24 hours after seeding and every 24 hours thereafter for 12 to 15 days, cells from two plates were harvested and counted with a Coulter counter (model ZB1, Coulter Electronics Ltd, Harpenden, UK). The saturation density, that is the number of cells per unit culture surface area, was determined from cultures of HAC that had reached the stationary phase of growth.

Plating efficiencies (PEs) were determined by harvesting exponentially growing cells and replating them, in triplicate, at a density of 1,000 and 3,000 cells per 60 mm dish in growth medium supplemented with 10% FCS. The cells were fed every 3 to 4 days and stained 3 to 4 weeks later with 0.5 mg/ml Crystal Violet. The colonies were counted and PEs were expressed as the percentage of the total number of cells seeded.

To determine the lifespan of the cells, confluent monolayers were subcultured at 1:2 or 1:4 split ratios every 3 to 5 days. To retain equivalence of population doublings (p.d.l.s) with passages, a 1:2 split was scored as 1 p.d.l. and 1:4 split as 2 p.d.l.

RNA extraction and northern blot analysis

Total cellular RNA was prepared from cultured cells by the NP-40 lysis method (Sambrook et al., 1989). Briefly, cell pellets were lysed in 400 μl of NP-40 lysis buffer for 5 minutes on ice and cell lysates were collected by centrifugation at 13,400 ***g*** for 10 minutes at 4°C. The clear supernatant was transferred to a clean tube and extracted twice with phenol/chloroform/isoamyl alcohol (25/24/1, by vol.). The RNA was precipitated with ethanol overnight at –20°C, washed with 70% ethanol and dissolved in sterile distilled water. A total of 30 μg RNA was electrophoresed in a 1% agarose/2.2 M formaldehyde gel and transferred to Genescreen according to standard procedures (Sambrook et al., 1989). Hybridisation was performed in 5× SSC, 1× Denhardt's solution, 1% sodium dodecyl sulphate (SDS), 20 mM sodium orthophosphate, pH 6.8, 50% formamide and 250 μg/ml denatured salmon sperm DNA at 42°C using ^{32}P-labelled probes (Feinberg and Vogelstein, 1983). Filters were washed in 0.1×SSC, 0.5% SDS at 65°C for 30 to 45 minutes and exposed to autoradiography.

The probes used were: a 1.65 kb *Eco*RI human procollagen α1(I) cDNA fragment from HF-677 (Chu et al., 1982), a 2.0 kb *Eco*RI human procollagen α2(I) cDNA fragment from HF-1131 (Bernard et al., 1983), a 0.96 kb *Eco*RI-*Pst*I fragment carrying the 3′ end of the human procollagen type II gene from pSCg119KS (Cheah et al., 1985; Lovell-Badge et al., 1987), a 0.75 kb *Eco*RI rat collagen type α1(IX) cDNA fragment from pKS.KT(RS) (Kimura et al., 1989), a 0.7 kb *Hin*dIII cDNA fragment encoding most of the C-terminal non-collagenous domain and part of 3′ untranslated region of the human collagen type X cDNA from pUChColX (Thomas et al., 1991), a 2.1 kb *Eco*RI human aggrecan cDNA fragment from pKS.H4, a 1.3 kb human versican cDNA fragment from PG-350 encompassing the 3′ region of human versican (Krusius et al., 1987; obtained from Bioquote Limited, UK), a 1.1 kb *Bam*HI human syndecan cDNA fragment from pUC19-hsyn4 (Mali et al., 1990), a 1.8 kb *Eco*RI human link protein cDNA fragment from pKS8.1D3 (Perkins et al., 1989; Dudhia and Hardingham, 1990) and a 1.9 kb *Sal*I-*Bam*HI human β-actin cDNA fragment from PIβ-A1 (pHF-β-A1; Gunning et al., 1983).

Reverse transcriptase/polymerase chain reaction (RT-PCR)

Total RNA isolated from human articular cartilage, human articular chondrocytes (HAC) cultured as monolayers or as suspensions over agarose, Syrian hamster embryonic chondrocytes and human foetal lung diploid fibroblasts was reverse-transcribed using 'Super RT' grade reverse transcriptase (HT Biotechnology, Cambridge, UK). The reaction was performed in 20 μl volume containing 2 μg of total RNA, with the exception of early-passage HAC (100 ng) and human articular cartilage (50 ng), 10 mM DTT, 1 mM dNTPs (Pharmacia), 10 ng random primers, $pd(N)_6$ in 1× RT reaction buffer (50 mM Tris-HCl, pH 8.3, 40 mM KCl, 6 mM $MgCl_2$) and 10 units of reverse transcriptase. The tubes were incubated on a Hybaid Combi (TR2) thermal reactor at 37°C for 1 hour. Following a 5 minute heat-inactivation at 95°C, the cDNAs were amplified in a 100 μl reaction volume. The reaction contained 2 μl of cDNA with the exception of early-passage HAC and human cartilage (10 μl), 1 mM $MgCl_2$, 200 μM dNTPs, 0.5 μl of *Taq* Polymerase (Promega UK), 1 μM of forward (5′-AACGGCAAGGTGTTGTGCGATG-3′) and 1 μM of reverse (5′-AGCTGGGGAGCAAAGTTTCCTC-3′) primers to human type I collagen in 1× reaction buffer. PCR reactions were

carried out on a Hybaid Combi (TR2) thermal reactor as follows: one cycle of 95°C (2 minutes) and 72°C (2 minutes), 35 cycles of 95°C (1 minute), 55°C (1 minute) and 72°C (1.5 minutes) followed by one cycle of 72°C for 5 minutes. The reaction products were analysed on 1.2% agarose gel stained with ethidium bromide.

Isolation of proteoglycans and link protein

Proteoglycans and link protein were isolated from the culture medium of chondrocytes at P18 (passage 18) cultured as monolayers for 3-4 days or as suspensions over agarose for 1-2 weeks. Culture media were mixed with proteinase inhibitors and dialysed against 50 mM sodium acetate, pH 6.8, in the presence of proteinase inhibitors for 48 hours at 4°C. Following dialysis, the media were concentrated by freeze-drying at 4°C and stored at −20°C until further use.

Isolation of collagens

Culture media were collected from chondrocytes cultured under both conditions as described above, mixed with proteinase inhibitors and dialysed against 0.5 M acetic acid at 4°C for 24 hours. Solubilised collagens were freeze-dried and stored at −20°C (Marriott et al., 1991; Gibson et al., 1991).

Analysis of proteoglycans

Proteoglycans were resuspended in 8 M urea and equilibrated overnight at 4°C. Samples were separated on a polyacrylamide-agarose composite gel as previously described (Carney et al., 1986) under dissociative conditions. The gel was blotted onto Immobilon for 2 hours at room temperature and the filter was incubated for 1 hour at room temperature with 0.05 unit/ml of ABC chondroitinase. The blot was washed with PBS, blocked for at least 3 hours with low fat milk (Marvel) and then incubated with an anti-human hyaluronic acid binding region (anti-HABR; 1:1000)-specific antibody. Visualisation of the proteoglycans was performed using an ECL western blot detection kit (Amersham International, UK).

Analysis of link protein

Freeze-dried samples were resuspended in 35 µl of 2× SDS sample loading buffer, boiled for 5 minutes, centrifuged and resolved on a 8% (w/v) SDS-polyacrylamide gel (Laemmli, 1970). Following immunoblotting overnight at 4°C, the filters were processed as above and then incubated overnight at 4°C with a mouse monoclonal antibody, 8-A-4, raised against rat chondrosarcoma link protein (Caterson et al., 1985). Link protein was visualised using an ECL western blot detection kit (Amersham International, UK).

Analysis of collagens

Collagens were dissolved in 0.5 M acetic acid and digested with 200 µg/ml pepsin in 0.5 M acetic acid overnight at 4°C. Samples were freeze-dried, resuspended in 2× SDS loading buffer and resolved by SDS-PAGE on a 8% (w/v) separating gel and a 4.5% (w/v) stacking gel (Laemmli, 1970). The gel was blotted onto nitrocellulose membrane overnight at 4°C and the filter was blocked with a low fat milk for at least 3 hours at room temperature. Collagen type II was detected with a mouse monoclonal anti-human collagen type II antibody at 1:250 dilution (Chemicon International, UK). A similar immunoblot was probed with a rabbit anti-human collagen type X at 1:10 dilution (a kind gift from Dr K. von der Mark) or a monoclonal anti-chicken type X collagen (Stephens et al., 1992). Collagens were visualised using an ECL western blot detection kit (Amersham International, UK).

RESULTS

Morphology of cultured human articular chondrocytes

Freshly isolated human adult articular chondrocytes were plated onto 80 cm^2 tissue culture flasks. After two to three days the cells formed confluent monolayers and appeared flat, exhibiting a polygonal to elongate-spindle morphology (Fig. 1). In contrast, when chondrocytes were cultured as suspensions over agarose, they aggregated and formed clusters after about two days, reminiscent of chondrocyte differentiation in vivo (Fig. 1). The size of the clusters increased over a period of one week and remained constant over the next ten days. The phenotype of the chondrocytes cultured as monolayers or as suspensions over agarose remained stable following long-term subculturing of the cells.

Growth characteristics of cultured human articular chondrocytes

Several cultural parameters of the human chondrocytes such as

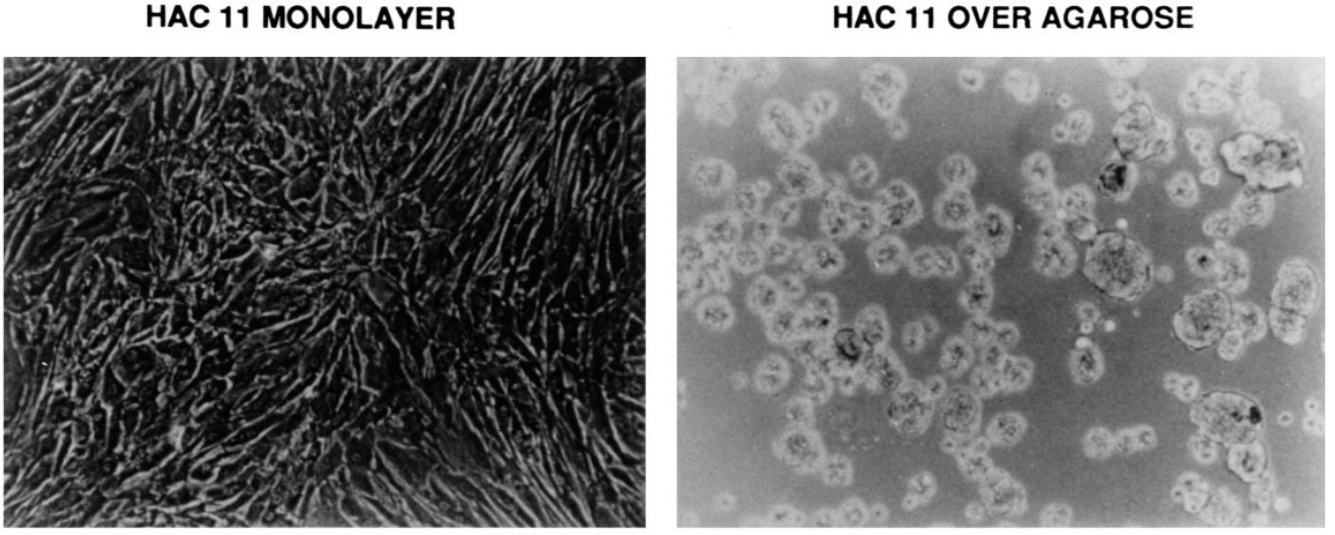

Fig. 1. Morphology of human articular chondrocytes isolated from an 11 year old female (HAC 11), grown as monolayers on plastic and as suspensions over 1% agarose. ×10.

Table 1. Growth properties of human articular chondrocytes

Cell type	Morphology		Life span (p.d.l.)	Growth rate (days)		Saturation density (cells/cm^2)	PE (%)
	Monolayer	O/A		Monolayer	O/A		
HAC11	F	Round, Cluster	34	3.2	14.8	1.10×10^4	0.2
HAC 21	F	Round, Cluster	35	3.4	NT	1.26×10^4	0.2
HAC33	F	Round, Cluster	37	4.3	16.0	0.92×10^4	0.2
MRC-5	Foetal fibroblasts	–	60-70	1.08	–	5.70×10^4	12
149BR	Adult fibroblasts	–	45-50	1.25	–	4.60×10^4	6

F, fibroblastoid morphology; O/A, over agarose; p.d.l., population doubling; PE, plating efficiency; NT, not tested.

growth rates, saturation densities and plating efficiencies were determined and compared with those of human fibroblasts (Table 1).

When grown as suspensions over agarose, HAC grew slowly for about one week and then cell proliferation slowed down and eventually ceased after about two weeks in culture (Fig. 2). The growth rate of the cells was about 14.8-16 days (Table 1). This was further supported by cellular DNA synthesis assays (not shown). The cells in the clusters underwent very little DNA synthesis and cell division eventually ceased. In contrast, cells cultured on plastic as monolayers grew much faster with an average growth rate of about 3-4 days (Fig. 2 and Table 1). As expected, the rate of cell division was also higher than those grown as suspensions over agarose, as determined by thymidine incorporation. Although the cells displayed a fibroblastic-like morphology, they did not grow as fast as human foetal fibroblasts or adult dermal fibroblasts, whose growth rates were 26 hours and 30 hours, respectively. Moreover, with in vitro propagation the growth rate of HAC gradually decreased to about 6-7 days (determined at population doubling 15) and at much later passages (over 28 population doublings) to about two weeks, and remained constant thereafter.

The saturation densities of the cells grown as monolayers were also determined and found to be 0.9 to 1.2×10^4 cells/cm^2 as compared with 5.7×10^4 cells/cm^2 and 4.6×10^4 cells/cm^2 for human foetal and adult dermal diploid fibroblasts, respectively, suggesting that chondrocytes have a larger volume than other mesenchymal cells such as fibroblasts.

To determine the lifespan of the human articular chondrocytes, the cells were grown and passaged as monolayer cultures until they stopped dividing or failed to form confluent monolayers after about one month with frequent changes of the growth medium, probably due to the exhaustion of their growth potential. Under these conditions, the lifespan of cultured human articular chondrocytes was found to be in the range of 34 to 37 population doublings (p.d.l.). There was no significant difference in the lifespan of chondrocytes isolated from patients of different ages (Table 1).

Plating efficiencies were determined as there were a number of cells floating in the medium (Table 1). Human chondrocytes at p.d.l. 6 and 18 had PEs of 0.5% to 0.2%, respectively, and this decreased further to about 0.05% with in vitro propagation as determined at p.d.l. 27. These plating efficiencies were very low compared with those for human foetal and adult dermal fibroblasts, which were about 12% and 6%, respectively (Table 1).

Expression of cartilage collagens

To determine whether human articular chondrocytes retain the expression of cartilage-collagens following long-term subculture, total RNA was isolated from HAC 11 at p.d.l. 18 and analysed by northern blot hybridisation using ^{32}P-labelled human type B collagen-specific cDNA probes (lacking exon 2) (Fig. 3A). Human articular chondrocytes cultured as monolayers expressed a 5.3 kb collagen type II mRNA (Fig. 3A, lane

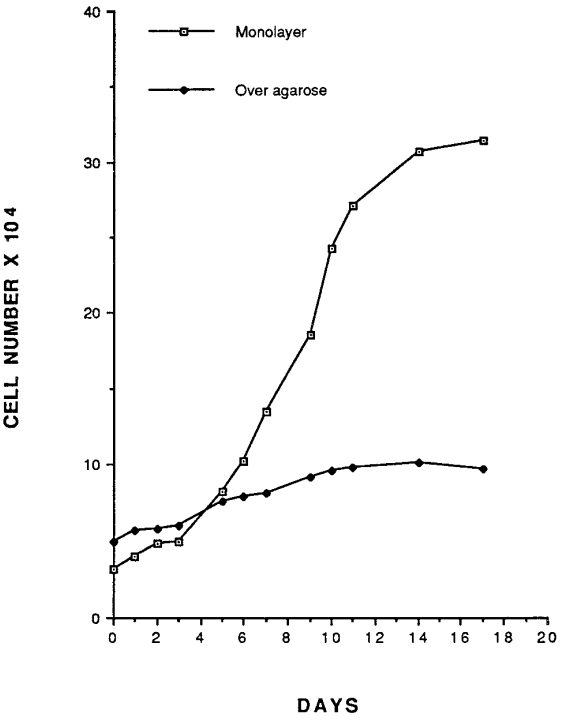

Fig. 2. Growth curves of HAC11 grown as monolayers on plastic and as suspensions over 1% agarose; 5×10^4 cells were seeded onto 60 mm culture dishes and three plates were harvested; the cell number was determined using a Coulter Counter at the time points indicated.

1) and a 4.2 kb collagen type IX mRNA (Fig. 3A, lane 2). In addition, the cells expressed procollagen α1(I) and α2(I) mRNAs (Fig. 3A, lanes 3 and 4, respectively). There was no hybridisation to human collagen type X collagen in cells cultured either as monolayers on plastic or as suspensions over agarose. The relative expression of type II collagen mRNA did not decrease between passage 18 and passage 25.

It has been reported in the literature that expression of type I procollagen by cultured articular chondrocytes is associated with the loss of chondrocyte differentiation (Aulthouse et al., 1989; Archer et al., 1990; Bonaventure et al., 1994; Häuselmann et al., 1994), hence it was important to investigate whether intact freshly isolated human articular cartilage expressed collagen type I mRNA. Total RNA extracted from intact human articular cartilage from a 15-year-old individual, human adult articular chondrocytes cultured as monolayers, embryonic Syrian hamster chondrocytes or human diploid fibroblasts was subjected to RT-PCR using specific primers to collagen α1(I) (Fig. 3B). A RT-PCR product of 296 bp was detected in all the lanes. Collagen type I was expressed by human chondrocytes in vivo (Fig. 3B, human cartilage) and in vitro (Fig. 3B, HAC 11) and by embryonic Syrian hamster chondrocytes (SHEC), cultured as monolayers. Total RNA isolated from cultured human diploid fibroblasts, MRC-5, was used as positive control for the expression of collagen α1(I).

To determine whether HAC 11 synthesised and secreted type II collagen, collagens isolated from the culture media of cells cultured as monolayers or as suspensions over agarose, as described under Materials and Methods, were separated by SDS-PAGE, western blotted and the blot reacted with an anti-human collagen type II mouse monoclonal antibody (Fig. 3C). Collagen type II was detected in both culture media but it was present at higher levels in cells grown as suspensions over agarose. Purified chicken and human collagen type II were used as controls (Fig. 3C).

Expression of cartilage proteoglycans

To determine whether the expression of aggrecan, the main cartilage proteoglycan, is affected by long-term culture of human articular chondrocytes, total RNA was isolated from chondrocytes (HAC 11) at p.d.l. 18 cultured as monolayers on plastic and analysed by northern blot hybridisation using a ^{32}P-labelled human aggrecan-specific cDNA probe (Fig. 4A). HAC 11 cells cultured as monolayers expressed an 8.5 kb aggrecan mRNA at high levels (Fig. 4A, lane 1) as previously reported (Doege et al., 1991). Versican mRNA has been detected in human articular chondrocytes (Grover and Roughley, 1993), hence the expression of versican at mRNA and protein levels was examined. Northern blot hybridisation analysis using a ^{32}P-labelled human versican-specific cDNA fragment as probe showed that human chondrocytes expressed a 8.0 kb versican mRNA (Fig. 4A, lane 2). Comparison of aggrecan and versican mRNA levels to β-actin showed that chondrocytes produced 5-fold less versican message than aggrecan message.

The synthesis and secretion of large aggregating proteoglycans were followed by western blotting. Proteoglycans isolated from the medium of cells cultured as monolayers or as suspensions over agarose were electrophoresed on dissociative agarose-polyacrylamide composite gels, western blotted and probed with a rabbit polyclonal antibody raised against the hyaluronic acid binding region (HABR) of human aggrecan.

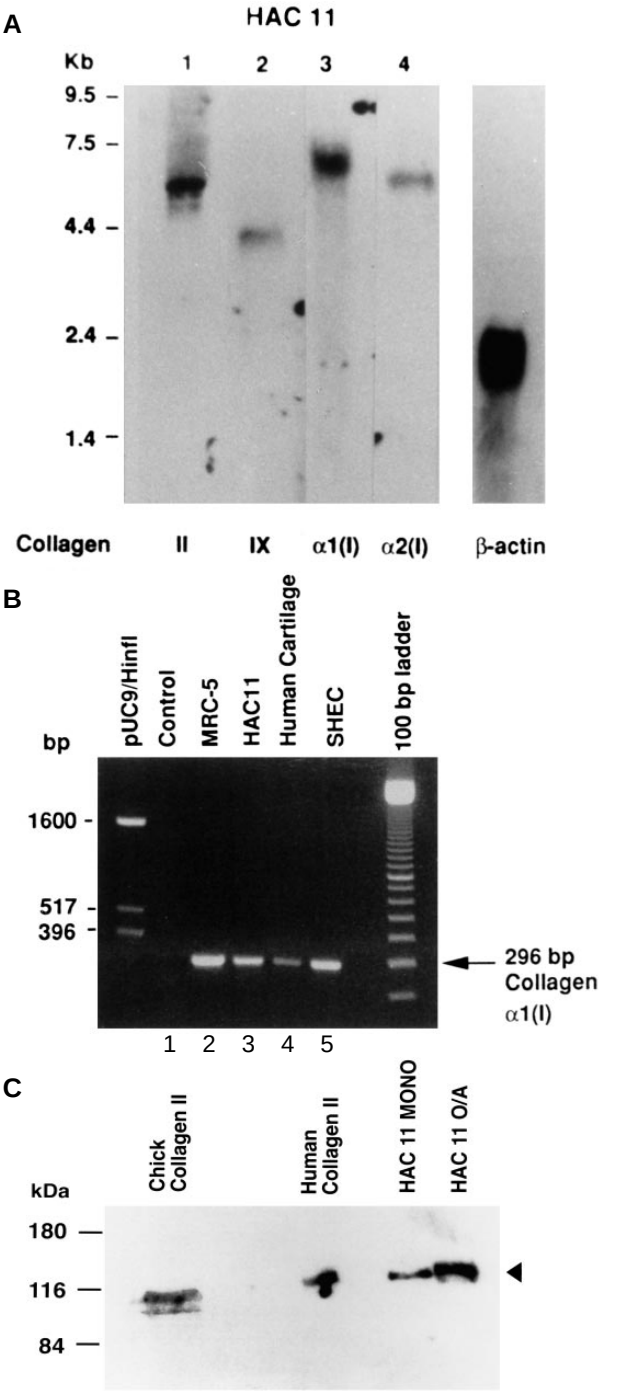

Fig. 3. Expression of collagens. (A) Northern blot analysis of total RNA extracted from HAC11 grown as monolayers on plastic. A 25 μg sample of RNA was fractionated on 1% denaturing agarose gel, blotted onto GeneScreen and hybridised with ^{32}P-labelled collagen-specific cDNA probes as indicated below the lanes. (B) RT-PCR analysis of total RNA extracted from human diploid fibroblasts, MRC-5 (lane 2), cultured human articular chondrocytes, HAC 11 (lane 3), human articular cartilage (lane 4) and embryonic Syrian hamster chondrocytes, SHEC (lane 5) for the expression of collagen α1(I). (C) Western blot analysis of collagen type II isolated from culture media of HAC 11 grown as monolayers on plastic (MONO) or as suspensions over agarose (O/A). Purified chicken and human collagen type II were used as positive controls. The position of collagen type II is indicated by an arrowhead.

Anti-HABR detected a large proteoglycan species in the media of cells from both types of culture (Fig. 4B, lanes 1 and 2). There was no increase in the level of the aggregating proteoglycan with prolonged incubation of the cells cultured as suspensions over agarose (Fig. 4B, lane 3). However, the HABR region is also present in versican, suggesting that the protein band detected could be a mixture of aggrecan and versican. Dot blots were therefore prepared from proteoglycans isolated from the medium of chondrocytes from donors of two different ages, 11 and 33 years old, cultured under both conditions. Purified human aggrecan from the cartilage of the 21 year old female and bovine versican were used as controls. The blots were probed with a rabbit polyclonal antibody raised against bovine versican or anti-HABR (Fig. 4C). Anti-HABR reacted with proteoglycan(s) found in the media of cells cultured under both conditions. There was a very slight cross-reactivity of the antibody with purified versican (Fig. 4C). Similar protein dot blots reacted with anti-versican showed that chondrocytes synthesise and secrete versican under both conditions (Fig. 4C). The antibody did not react with purified aggrecan. The data suggest that the large aggregating proteoglycan detected by western blot shown in Fig. 3B was mainly aggrecan.

To determine whether cultured chondrocytes express cell surface proteoglycans, northern blot hybridisation analysis was performed using a human syndecan cDNA fragment as probe. There was no expression of syndecan in chondrocytes cultured as monolayers or as suspensions over agarose.

Expression of link protein

The expression of link protein (LP) at mRNA and protein levels was followed by northern and western blotting, respectively. Total RNA was isolated from monolayer cultures of HAC 11 at p.d.l. 18, and analysed by northern blot hybridisation using a ^{32}P-labelled human link protein cDNA as probe (Fig. 5A). The cells expressed two major RNA transcripts of 2.7 kb and 3.0 kb, appearing as a diffuse band and two minor mRNA species of 1.7 kb and 4.8 kb (Fig. 5A), as previously reported (Caterson et al., 1985; Doege et al., 1986; Mallein-Gerin and Olsen, 1993).

In addition, culture media from both culture conditions were

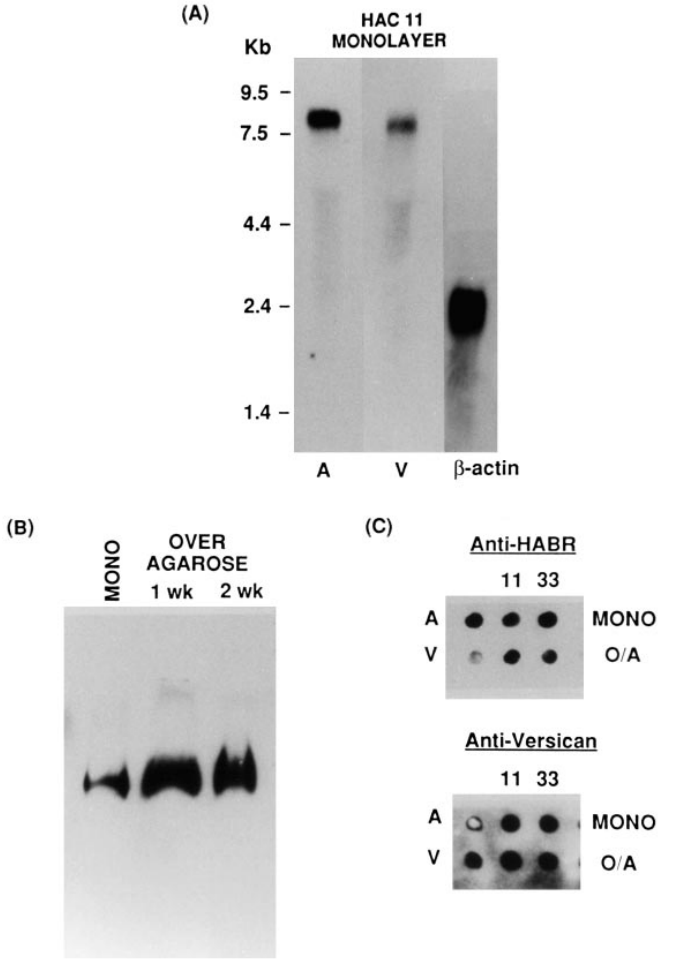

Fig. 4. Expression of large proteoglycans in HAC 11. (A) Northern blot analysis of total RNA extracted from HAC11 grown as monolayers on plastic. A 25 μg sample of RNA was fractionated on 1% denaturing agarose gel, blotted onto GeneScreen and hybridised with ^{32}P-labelled-specific cDNA probes to human aggrecan (A) or versican (V) as indicated below the lanes. (B) Western blot analysis of aggrecan isolated from the culture media of HAC11 grown as monolayers (MONO) on plastic or as suspensions over agarose for one (1 wk) or two (2 wk) weeks. (C) Western dot blot analysis of aggrecan and versican isolated from the culture media of HAC11 (11) and HAC 33 (33) grown as monolayers on plastic or as suspensions over agarose for one week using antibodies specific to hyaluronic acid binding region (Anti-HABR) or to versican (Anti-Versican). A, purified human aggrecan; V, purified bovine versican.

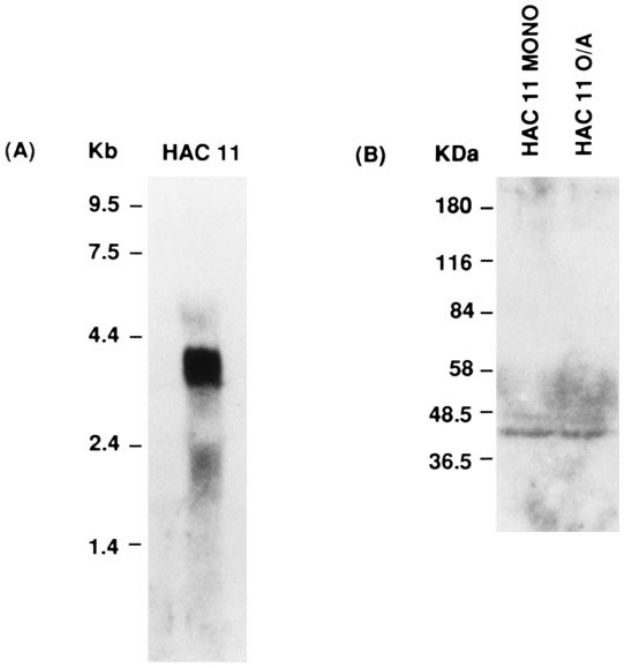

Fig. 5. Expression of link protein in HAC 11. (A) Northern blot analysis of total RNA extracted from HAC11 grown as monolayers on plastic. A 25 μg sample of RNA was fractionated on 1% denaturing agarose gel, blotted onto GeneScreen and hybridised with a ^{32}P-labelled specific cDNA probe to human Link protein. (B) Western blot analysis of link protein isolated from the culture media of HAC11 grown as monolayers on plastic (MONO) or as suspensions over agarose (O/A) for one week.

collected and processed as described under Materials and Methods. SDS-PAGE analysis of the samples followed by western blotting and immunodetection using a mouse monoclonal antibody, 8-A-4, raised against rat chondrosarcoma link protein detected a doublet of 48 kDa and 45 kDa, corresponding to LP1 and LP2, respectively, which differ in their degree of glycosylation as previously reported (Doege et al., 1986).

DISCUSSION

Here we report that human articular chondrocytes did not lose their differentiated characteristics following long-term subculture. Human articular chondrocytes from the cartilage of three female patients of different ages (11, 21 and 33 years old) were isolated and cultured as monolayers. Cultured cells were propagated extensively until they stopped dividing, despite regular changes of the growth medium, a phenomenon termed cellular senescence. Although the cells remained metabolically active, they failed to form confluent monolayers after about three months in culture. Under these conditions, the lifespan of human articular chondrocytes was found to be in the range of 34 to 37 population doublings and there was no significant difference in the lifespan of the cells isolated from patients of different ages (Table 1). During the exponential growth phase, younger cultures of human articular chondrocytes exhibited growth rates of about 3 to 4 days (Table 1). However, there was a gradual decline in the growth rate of the cells with in vitro propagation. Hence human chondrocytes can be used to study the effects of growth factors and cytokines during long-term culture and at different culture ages.

Determination and comparison of some of the cultural parameters of the cells such as growth rates, saturation densities and plating efficiencies showed that although chondrocytes grown as monolayers displayed a fibroblastic-like morphology, their growth behaviour was very different from that of embryonic lung (MRC-5) and adult dermal (149BR) human fibroblasts (Table 1). Chondrocytes grown as suspensions over agarose, showed that the cells adopted a rounded cell shape and were capable of forming clusters, a property independent of the passage number of the culture. This suggested that cultured chondrocytes retained their ability to undergo morphological changes in vitro reminiscent of chondrocyte differentiation in intact cartilage. Similar findings have been reported previously using suspension cultures (Aulthouse et al., 1989; Archer et al., 1990; Bonaventure et al., 1994). Determination of growth rates and cellular DNA synthesis assays showed that chondrocytes cultured as suspensions over agarose grow very slowly and undergo very little DNA synthesis (Fig. 2 and Table 1), consistent with earlier reports using cartilage explants.

The morphological transition from a mesenchymal to a rounded phenotype using different culture conditions prompted us to investigate whether such phenotypic modulation is linked to changes in gene expression. Chondrocytes cultured as monolayers expressed cartilage collagens such type II and type IX collagens at the mRNA level. Western blot analysis showed that chondrocytes cultured as monolayers or as suspensions over agarose expressed collagen type II and there was an apparent increase in the levels of the protein synthesised by cells cultured over agarose. The expression of collagen type II in monolayer cultures of chondrocytes is not unusual as previous studies demonstrated the expression of collagen type II in monolayer cultures of v-*myc*-immortalised rat chondrocytes, although at lower levels (Horton et al., 1988), in SV40-large T antigen-immortalised mouse chondrocytes (Mallein-Gerin and Olsen, 1993) or carcinogen-immortalised Syrian hamster embryonic chondrocytes (Cizdziel et al., 1991; unpublished data) following extensive subculturing of the cells. Recently, Bonaventure et al. (1994) also reported the expression of collagen type II in short-term monolayer cultures of human foetal chondrocytes. Monolayer and suspension cultures of chondrocytes also expressed procollagen α1(I) and α2(I) messages but at lower levels than collagen type II mRNA. It has been suggested, however, that the expression of procollagen type I in chondrocytes cultured as monolayers is associated with the loss of the differentiated chondrocyte phenotype (Aulthouse et al., 1989; Archer et al., 1990; Bonaventure et al., 1994). To resolve this, RT-PCR using collagen α1(I)-specific primers was used. The data showed that collagen type I mRNA was present in freshly dissected intact human articular cartilage. Similarly, Treilleux et al. (1992) reported the expression of type I, II and type III collagens in developing human femoral cartilage by in situ hybridisation. Expression of type I collagen mRNA, however, does not necessarily indicate synthesis of type I collagen, since mechanisms of translational control prevent type I collagen synthesis in chick vertebral chondroblasts, despite expression of α_2(I) mRNA (Bennett and Adams, 1987). On the other hand, where metabolic activity is low, as in human adult articular cartilage, expression of type I and type II collagen is not detectable by in situ hybridisation (Aigner et al., 1993).

Articular chondrocytes in vivo synthesise and secrete a matrix rich in proteoglycans. Aggrecan is a large aggregating proteoglycan that forms complexes with hyaluronan, stabilised by a small protein the link protein. These complexes together with the collagen fibril network determine the physical and mechanical properties of cartilage. Using biochemical and molecular biological techniques, the results show that chondrocytes cultured as monolayers or suspensions over agarose retained the expression of aggrecan and link protein, during long-term culture. Cultured chondrocytes also expressed another large aggregating proteoglycan, versican, at mRNA and protein levels, under both culture conditions. However, the expression of aggrecan was about 5-fold higher than versican as determined by densitometric analysis. These data support and extend those of Grover and Roughley (1993), who showed by RT-PCR that human chondrocytes express versican mRNA. There was no expression of syndecan, a cell surface proteoglycan, by chondrocytes cultured under both conditions. Chondrocytes cultured as monolayers also expressed high levels of link protein mRNA. The different RNA transcripts detected have been previously described (Doege et al., 1986). In addition HAC cells synthesised and secreted two forms of link protein, LP1 and LP2, which differ in their degree of glycosylation (Doege et al., 1986). There was no difference in the link protein levels in cells cultured over agarose as compared to monolayer cultures. Co-expression of aggrecan and link protein in HAC suggests that aggrecan can form aggregates with hyaluronan stabilised by link protein.

The present results show that extended propagation of adult articular chondrocytes in vitro does not result in the loss of

cartilage-specific gene expression nor does expression of type I collagen indicate loss of chondrocyte phenotype. Therefore, cell shape may be important for the cessation of cell growth, probably through cell-matrix interactions, rather than for the expression of the chondrogenic phenotype. Lack of correlation between cell shape and the expression of cartilage-specific molecules has been reported (Horton and Hassell, 1986; Mallein-Gerin et al., 1990). The transition from round to flattened shape may be necessary for proliferation and repair. Conversely, in superficial to deep layers of articular cartilage chondrocytes change in shape from flat to round cells, whose smaller volume may provide resilience to mechanical compression.

We thank Dr C. Archer for introducing E.K. to human chondrocyte cell culture; Drs R. Boot-Handford, D. Prockop, B. Olsen, T. Hardingham, J. Dudhia, F. Baralle and M. Jalkanen for cDNA probes; and Drs D. Heinegård and B. Caterson for specific antibodies. We thank Drs J. Dudhia and M. Bolton for human articular cartilage RNA. E.K. is the recipient of an Arthritis and Rheumatism Council award. This work was supported by a grant from the Arthritis and Rheumatism Council of Great Britain to H.M.

REFERENCES

Aigner, T., Bertling, W., Stross, H., Weseloh, G. and von der Mark, K. (1993). Independent expression of fibril-forming collagens I, II and III in chondrocytes of human osteoarthritic cartilage. *J. Clin. Invest.* **91**, 829-837.

Archer, C., McDowell, J., Bayliss, M., Stephens, M. and Bentley, G. (1990). Phenotypic modulation of sub-populations of human articular chondrocytes in vitro. *J. Cell Sci.* **97**, 361-371.

Aulthouse, A. L., Beck, M., Griffey, E., Sanford, J., Arden, K., Machado, M. A. and Horton, W. A. (1989). Expression of the human chondrocyte phenotype in vitro. *In Vitro Cell Dev. Biol.* **25**, 659-668.

Bassleer, C., Gysen, P., Foidart, J. M. Bassleer, R. and Frenchimont, P. (1986). Human chondrocytes in tridimensional culture. *In Vitro Cell Dev. Biol.* **22**, 113-119.

Bennett, V. D. and Adams, S. L. (1987). Characterisation of the translational control mechanism preventing synthesis of α2(I) collagen in chicken vertebral chondroblasts. *J. Biol. Chem.* **262**, 14806-148811.

Benya, P. D., Padilla, S. and Shaffer, J. D. (1978). Independent regulation of collagen types of chondrocytes during the loss of differentiated function in culture. *Cell* **15**, 1313-1321.

Benya, P. D. and Shaffer, J. D. (1982). Dedifferentiated chondrocytes re-express the differentiated collagen phenotype when cultured in agarose gels. *Cell* **30**, 215-224.

Bernard, M. P., Myers, J. C., Chu, M-L., Ramirez, F., Eikenberry, E. F. and Prockop, D. J. (1983). Structure of a cDNA for the pro α2 chain of human type I procollagen. Comparison with chick cDNA for α2(I) identifies structurally conserved features of the protein and the gene. *Biochemistry* **22**, 1139-1145.

Bonaventure, J., Kadhom, N., Cohen-Solal, L., Ng, K. H., Bourguignon, J., Lasselin, C. and Freisinger, P. (1994). Re-expression of cartilage-specific genes by dedifferentiated human articular chondrocytes cultured in alginate beads. *Exp. Cell Res.* **212**, 97-104.

Carney, S. L., Bayliss, M. T., Collier, J. M. and Muir, H. (1986). Electrophoresis of ^{35}S-labelled proteoglycans on polyacrylamide-agarose composite gels and their visualisation by fluorography. *Anal. Biochem.* **156**, 38-44

Caterson, B., Baker, J. B., Christner, J. E., Lee, Y. and Lentz, M. (1985). Monoclonal antibodies as probes for determining the microheterogeneity of the link proteins of cartilage proteoglycans. *J. Biol. Chem.* **260**, 11348-11356.

Cheah, K. S. E., Stoker, N. G., Griffin, J. R., Grosveld, F. G. and Solomon, E. (1985). Identification and characterisation of the human type II collagen gene (COL2A1). *Proc. Nat. Acad. Sci. USA* **82**, 2555-2559.

Chu, M. l., Myers, J. C., Bernard, M. P., Ding, J. F. and Ramirez, F. (1982). Cloning and characterisation of five overlapping cDNAs specific for the human pro-α1(I) collagen chain. *Nucl. Acids Res.* **10**, 5925-5934.

Cizdziel, P. E., Hosoi, J., Montgomery, J. C., Wiseman, R. W. and Barrett, J. C. (1991). Loss of tumour suppressor gene function is correlated with downregulation of chondrocyte-specific collagen expression in Syrian hamster embryo cells. *Mol. Carcinogen.* **4**, 14-24.

Delbruck, A., Dresow, B., Gurr, E., Reale, E. and Schroder, H. (1986). In vitro culture of human chondrocytes from adult subjects. *Conn. Tiss. Res.* **15**, 115-172.

Dudhia, J. and Hardingham, T. E. (1990). The primary structure of human cartilage link protein. *Nucl. Acids Res.* **18**, 2214.

Doege, K., Hassell, J. R., Caterson, B. and Yamada, Y. (1986). Link protein cDNA sequence reveals a tandemly repeated protein structure. *Proc. Nat. Acad. Sci. USA* **83**, 3761-3765.

Doege, K., Sasaki, M., Kimura, T. and Yamada, Y. (1991). Complete coding sequence and deduced primary structure of the human cartilage large aggregating proteoglycan, Aggrecan. *J. Biol. Chem.* **266**, 894-902.

Feinberg, A. P. and Vogelstein, B. (1983). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**, 6-13.

Gibson, G. J., Francki, K. T., Hopwood, J. J. and Foster, B. K. (1991). Human and Sheep growth-plate cartilage type X collagen synthesis and the influence of tissue storage. *Biochem. J.* **277**, 513-520

Glowacki, J., Trepman, E. and Folkman, J. (1983). Cell shape and phenotypic expression in chondrocytes. *Proc. Soc. Exp. Biol. Med.* **172**, 93-98.

Grover, J. and Roughley, P. J. (1993). Versican gene expression in human articular cartilage and comparison of mRNA splicing variation with aggrecan. *Biochem. J.* **291**, 361-367.

Gunning, P., Ponte, P., Okayama, H., Engel, J., Blau, H. and Kedes, L. (1983). Isolation and characterisation of full-length cDNA clones for human α-, β- and γ-actin mRNAs: Skeletal but not cytoplasmic actins have an amino terminal cysteine that is subsequently removed. *Mol. Cell. Biol.* **3**, 787-795.

Häuselmann, H. J., Fernandes, R. J., Mok, S. S., Schmid, T. M., Block, J. A., Aydelotte, M. B., Kuettner, K. E., and Thonar, E. J. (1994). Phenotypic stability of bovine articular chondrocytes after long-term culture in alginate beads. *J. Cell Sci.* **107**, 17-27.

Horton, W. and Hassell, J. R. (1986). Independence of cell shape and loss of cartilage matrix production during retinoic acid treatment of cultured chondrocytes. *Dev. Biol.* **115**, 392-397

Horton, W. E., Cleveland, J., Rapp, U., Nemuth, G., Bolander, M., Doege, K., Yamada, Y. and Hassell, J. R. (1988). An established rat cell line expressing chondrocyte properties. *Exp. Cell Res.* **178**, 457-468.

Kimura, T., Mattei, M-G., Stevens, J. W., Goldring, M. B., Ninomiya, Y. and Olsen, B. R. (1989). Molecular cloning of rat and human type (IX) collagen cDNA and localisation of the α1(IX) gene on the human chromosome 6. *Eur. J. Biochem.* **179**, 71-78.

Kirsch, T., Swoboda, B. and von der Mark, K. (1992). Ascorbate independent differentiation of human chondrocytes in vitro: simultaneous expression of types I and X collagen and matrix mineralisation. *Differentiation* **52**, 89-100.

Krusius, T., Gehlsen, K. R. and Ruoslahti, E. (1987). A fibroblast chondroitin sulfate proteoglycan core protein contains lectin-like and growth factor-like sequences. *J. Biol. Chem.* **262**, 13120-13125.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lovell-Badge, R. H., Bygrave, A. E., Bradley, A., Robertson, E. Tilley, R. and Cheah, K. S. E. (1987). Tissue specific expression of the human type II collagen gene in mice. *Proc. Nat. Acad. Sci. USA* **84**, 2803-2807.

Mali, M., Jaakkola, P., Arvilommi, A-M. and Jalkanen, M. (1990). Sequence of human syndecan indicates a novel gene family of integral membrane proteins. *J. Biol. Chem.* **265**, 6884-6889.

Mallein-Gerin, F., Ruggiero, F. and Garrone, R. (1990). Proteoglycan core protein and type II collagen gene expression are not correlated with cell shape changes during low density chondrocyte cultures. *Differentiation* **43**, 204-211.

Mallein-Gerin, F and Olsen, B. R. (1993). Expression of simian virus 40 large T (tumour) oncogene in mouse chondrocytes induces cell proliferation without loss of the differentiated phenotype. *Proc. Nat. Acad. Sci. USA* **90**, 3289-3293.

Marriott, A., Ayad, S. and Grant, M. E. (1991). The synthesis of type X collagen by bovine and human growth-plate chondrocytes. *J. Cell Sci.* **99**, 641-649.

Mendler, M., Eich-Bender, S. G., Vaughan, L., Winterhalter, K. H. and

Bruckner, P. (1989). Cartilage contains mixed fibrils of collagen types II, IX and XI. *J. Cell. Biol.* **108**, 191-197.

Perkins, S. J., Nealis, A. S., Dudhia, J. and Hardingham, T. E. (1989). Immunoglobulin fold and tandem repeat structures in proteoglycan N-terminal domains and link protein. *J. Mol. Biol.* **206**, 737-753

Reichenberger, E., Aigner, T., von der Mark, K., Stoβ, H. and Bertling, W. (1991). In situ hybridisation studies on the expression of type X collagen in foetal human cartilage. *Dev. Biol.* **148**, 562-572.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Stephens, M., Kwan, A. P. L., Bayliss, M. T. and Archer, C. W. (1992). Human articular surface chondrocytes initiate alkaline phosphatase and type X collagen synthesis in suspension. *J. Cell Sci.* **103**, 1111-1116

Thomas, J. T., Cresswell, C. J., Rash, B., Nicolai, H., Jones, E., Solomon, E., Grant, M. E. and Boot-Handford, R. P. (1991). The human collagen X gene. Complete primary translated sequence and chromosomal localisation. *Biochem. J.* **280**, 617-623.

Treilleux, I., Mallein-Gerin, F., Guellec, D. and Herbage, D. (1992). Localisation of the expression of type I, II, III collagen and aggrecan core protein genes in developing human articular cartilage. *Matrix* **12**, 221-232.

Upholt, W. B. and Olsen, B. R. (1991). The active genes of cartilage. In *Cartilage: Molecular Aspects* (ed. B. Hall and S. Newman), pp. 43-79. CRC, Boca Raton, FL.

Watt, F. M. and Dudhia, J. (1988). Prolonged expression of differentiated phenotype by chondrocytes cultured at low density on a composite substrate of collagen and agarose that restricts cell spreading. *Differentiation* **38**, 140-147.

(Received 4 October 1994 - Accepted 24 January 1995)