Expansion of chondrocytes for tissue engineering in alginate beads enhances chondrocytic phenotype compared to conventional monolayer techniques

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Submitted 01-08-31. Accepted 02-04-01

ABSTRACT – Chondrocytes are known to dedifferentiate when cultured in monolayer culture, which may compromise the efficacy of cartilage repair systems in which cells are expanded by repeat passage in monolayer prior to implantation. We tested the hypothesis that repeat passage in alginate beads can provide sufficient expansion of cells, while producing cells with enhanced chondrocytic phenotype. Bovine articular chondrocytes were seeded in 2% alginate beads or in monolayer. 4 passages at 7-day intervals were performed. Values of 9.1 days for monolayer expansion and 12.5 days for alginate expansion were estimated for a 10-fold increase in cell number. For assessment of chondrocytic and fibroblastic phenotype, expanded cells were seeded in alginate beads or on glass coverslips and cultured for 7 days. On subsequent seeding in alginate, cells which had previously been subcultured in alginate showed higher levels of both DNA and GAG synthesis than cells passaged in monolayer. Furthermore, the alginate-passaged cells retained a chondrocytic phenotype, indicated by synthesis of type II collagen and chondroitin-6-sulphate, while cells passaged in monolayer synthesised type I collagen, indicating a fibroblastic phenotype. In conclusion, expansion of cells for autologous cartilage repair systems, using subculture within alginate beads, provides a potentially attractive alternative to monolayer expansion.

There is growing interest in the development of cartilage repair systems which involve the implantation of autologous chondrocytes into defect sites for the treatment of localised full or partial thickness lesions in young adults (Bentley and Greer, 1971, Grande et al. 1989, Vacanti et al. 1991, Brittberg et al. 1994, Romaniuk et al. 1995, Paige et al. 1996, Kawamura et al. 1998). These cells are typically isolated from a small tissue biopsy, removed from a low load-bearing site at the periphery of the cartilaginous joint surface (Brittberg et al. 1994). Using a small biopsy minimises potentially harmful effects at the donor site, but limits the number of available cells. The isolated cells must, therefore, be cultured in vitro to increase the cell number. Typically, a 10-fold expansion of cells is required in approximately 4 weeks to ensure the practicality and financial viability of the technique (Brittberg et al. 1994).

Most repair systems use monolayer culture techniques to expand the chondrocytes. While these methods are effective in inducing cell proliferation, chondrocytes are known to dedifferentiate and adopt a fibroblastic phenotype when cultured in monolayer and this process is only slowly reversible (Mayne et al. 1976, Benya et al. 1978, Benya and Shaffer 1982, deHaart et al. 1999). A system which allows sufficient expansion of cells, but which maintains chondrocytic phenotype is, therefore, preferable.

The dedifferentiation process is believed to be mediated by the formation of actin stress fibres which occurs when the cells spread on an adhesion-permitting substrate (Mayne et al. 1976, Benya et al. 1978). Culture systems which maintain the chondrocyte in a rounded morphology

prevent the formation of stress fibres (Takigawa et al. 1984, Benya et al. 1988, Brown and Benya 1988, Newman and Watt 1988, Loty et al. 1995). One such system involves the culture of articular chondrocytes encapsulated within calcium alginate beads (Guo et al. 1989, Hauselmann et al. 1992, 1994, Bonaventure et al. 1994, Yaeger et al. 1997, Binette et al. 1998, Lemare et al. 1998, deHaart et al. 1999). During culture the chondrocytes divide to form cell clusters and also synthesise cartilage-specific matrix components which form a halo around the cells (Almquvist et al. 2001, Kikuchi et al. 2001). While cell numbers increase during initial culture, a plateau is reached at a later time indicating an inhibition of cell proliferation, which may involve processes similar to contact inhibition in monolayer cultures (Guo et al. 1989, Beekman et al. 1997, Enobakhare 1998, Enobakhare et al. 2001). Individual cells can be recovered by dissolving the alginate, using calcium chelating agents followed by enzymatic digestion to remove elaborated matrix, allowing repeat passage to be performed.

We tested the hypothesis that repeat passage in alginate beads can provide sufficient expansion of cells for autologous chondrocyte transplantation while producing cells with enhanced chondrocytic phenotype as compared to monolayer expansion.

Material and methods

Chondrocyte isolation

Full depth slices of cartilage were removed from the metacar palphalangeal joints of 18-month-old steers. For each experiment, cartilage was removed from the joints of at least 6 animals. The cartilage slices were diced finely, and incubated at 37 °C for 1 hr in Dulbecco's minimal essential medium supplemented with 20% (v/v) foetal calf serum, 2 mM L-glutamine, 20 mM HEPES, 100 unit.mL^{-1} penicillin, 100 μg.mL-1 streptomycin and 150 μg.mL^{-1} L-ascorbic acid (DMEM + 20% FCS, all Gibco, Paisley, UK) + 700 unit.mL-$^{-1}$ pronase (BDH Ltd., Poole, UK) and for 16 hr at 37 °C in DMEM + 20% FCS + 100 unit.mL^{-1} collagenase type IX (Sigma, Poole, UK). The supernatant containing released chondrocytes was passed through a 70 μm pore size sieve (Falcon, Oxford, UK), washed twice in DMEM + 20% FCS and finally resuspended in DMEM + 20% FCS.

Preparation and passage of monolayer and alginate bead cultures

For monolayer cultures, the cell suspension was adjusted to 8.5 × 10^4 cells.mL^{-1} and 35 mL was added to tissue culture flasks (175 cm^2 growth area, Falcon, Oxford, UK). On initial seeding, each flask contained approximately 3 × 10^6 cells at an initial seeding density of 1 × 10^4 cells.cm^{-2}. The cells were maintained at 37 °C/5% CO_2 and culture media were changed every 2 days. For alginate bead culture the cell suspension was adjusted to 2 × 10^7 cells.mL^{-1} and added to an equal volume of 4% (w/v) alginate (Keltone LV, Kelco Nutrasweet, Poole, UK) in Earle's Balanced Salt Solution (Gibco, Paisley, UK) to give a final concentration of 1 × 10^7 cells.mL^{-1} in 2% (w/v) alginate. The cell/alginate suspension was slowly expressed through a 23-gauge needle into a solution containing 100 mM $CaCl_2$ in DMEM + 20% FCS. The resultant beads, each of a volume of approximately 11 μL and containing approximately 1.1 × 10^5 cells, were incubated in the $CaCl_2$ supplemented media for 10 min at room temperature to induce crosslinking of the alginate gel. The beads were subsequently washed in 4 changes of DMEM + 20% FCS. The beads were cultured in 35 mL DMEM + 20% FCS in petri dishes (140 mm diameter, Falcon, Oxford, UK) at 37 °C / 5% CO_2. 27 beads, containing approximately 3 × 10^6 cells were cultured in each dish. Culture media were replaced every 2 days.

After 7 days in culture, chondrocytes in monolayer culture were recovered by incubation for 15 min in 0.25% (w/v) trypsin (Sigma, Poole, UK) in phosphate buffered saline, pH 7.4 (Sigma, Poole, UK). Chondrocytes within alginate beads were recovered by incubation for 15 min in 50 mM sodium citrate, 150 mM sodium chloride, pH 7.4. Single cell suspensions were produced by incubation in DMEM + 20% FCS + 700 unit.mL^{-1} pronase + 100 unit.mL^{-1} collagenase for 2 hr. Isolated cells were seeded in alginate beads or monolayer as above. At each passage, the number of cells recovered and cell viability were determined, using a haemacytometer and the trypan blue exclusion test. Expansion of cells was calculated, using the following equation:

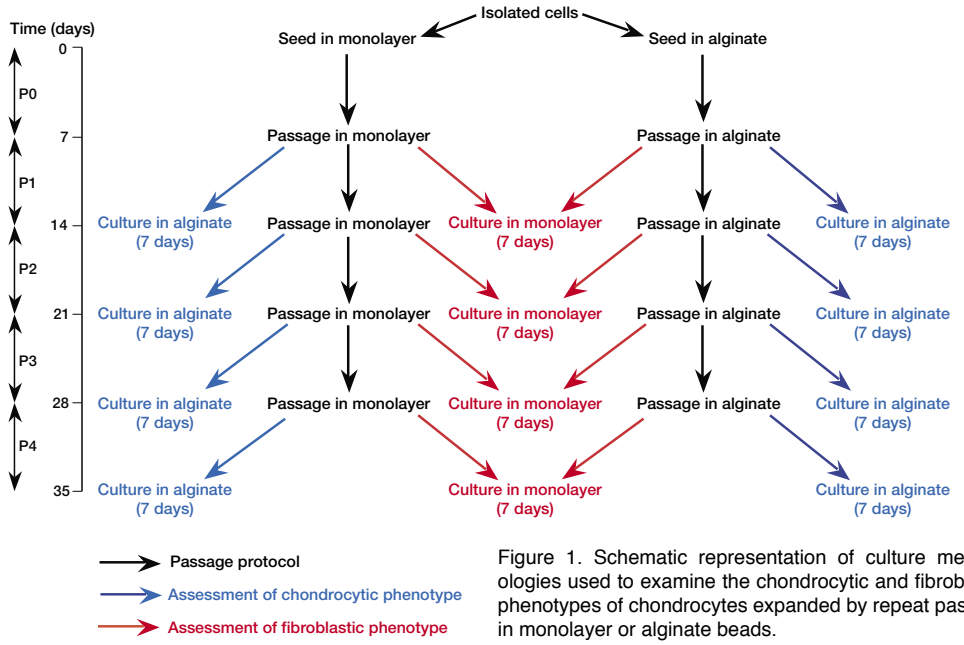

Figure 1. Schematic representation of culture methodologies used to examine the chondrocytic and fibroblastic phenotypes of chondrocytes expanded by repeat passage in monolayer or alginate beads.

$$\text{Cell expansion}_{(p_n - p_{n+1})} = \frac{\text{Viable cell number recovered}_{(p_{n+1})}}{\text{Cell number seeded}_{(p_n)}}$$

p_n = passage number

4 passages, at 7-day intervals were performed. The initial 7 day culture period was called passage 0 and subsequent 7-day culture periods were called passages 1–4. At various stages during culture, cell morphology in monolayer or within alginate beads was monitored using phase contrast microscopy. Details of the culture protocols are given in Figure 1.

Assessment of chondrocytic and fibroblastic phenotypic potential

To assess chondrocytic potential, the passages of cells in monolayer or within alginate bead culture were subsequently seeded in alginate beads at 1 × 10^7 cells.mL^{-1} and cultured for 7 days in 2.75 mL of DMEM + 20% FCS in 12 well plates (4 cm^2 cell growth area, Falcon, Oxford, UK). 1 bead was added to each well. To assess fibroblastic potential, the passages of cells using each system were subsequently seeded on glass coverslips (13 mm diameter, Agar Scientific, Stansted, UK) at 2 ×10^4 cells.cm^{-2} and cultured for 7 days in 1 mL DMEM + 20% FCS in 24 well plates (2 cm^2 cell growth area, Falcon, Oxford, UK). Culture media were replaced every 2 days.

At time 0 and at the end of the culture period, representative alginate bead and glass coverslip samples were removed from culture and solubilised by incubation in 50 mM sodium citrate, 150 mM sodium chloride, pH 7.4, supplemented with 10 mM cysteine HCl, 10 mM EDTA and 5 μL.mL^{-1} papain suspension (391 unit.mL^{-1}, Sigma, Poole, UK) for 24 hr at 60 °C. The digests were assayed for total DNA, using the Hoechst 33258 method (Rao and Otto 1992) and for glycosaminoglycan, using the method of Enobakhare et al. (1996).

At the end of the culture period, more alginate bead and monolayer samples were fixed by incubation in 1 mL of 4% paraformaldehyde in 0.1M sodium cacodylate buffer + 10 mM $CaCl_2$, pH 7.4 for 4 hr at room temperature. All samples were washed exhaustively in 0.1M sodium cacodylate buffer + 10 mM $CaCl_2$, pH 7.4 and stored in 0.1M sodium cacodylate buffer + 50 mM $BaCl_2$, pH 7.4 at 4 °C before immunolocalisation. The alginate bead samples were dehydrated and then embedded in paraffin wax. Sections, approximately 9 μm in thickness, were cut from the specimens and rehydrated before immunolocalisation.

The streptavidin biotin-immunoperoxidase method (Woods and Warnke, 1981) was used for immunolocalisation with the primary antibodies

raised against collagen type I (COL-1, Sigma, Poole, UK, at 1:100), collagen type II (CIICI, DSHB, Iowa, USA, at 1:2) and chondroitin-6-sulphate (3-B-3, ICN, Basingstoke, UK, at 1:2). Sections and coverslips were incubated for 15 min in a solution containing hydrogen peroxide and methanol in a 50:1 ratio to block endogenous peroxidase activity. Enzymatic pretreatment with 0.1% (w/v) trypsin for 10 min at 37 °C or 0.13 unit.mL^{-1} chondroitinase ABC for 1 hour at 37 °C was used to unmask the antigenic sites for collagen and chondroitin-6-sulphate respectively. The samples were incubated with primary antibody, diluted in 0.05M TRIS, 0.15M NaCl, pH 7.6, for 30 min at room temperature, washed in 0.05M TRIS, 0.15M NaCl, pH 7.6 and incubated at room temperature for 30 min with biotinylated rabbit anti-mouse Ig (Dako, Ely, UK), diluted 1:200 in TBS. The samples were washed in 0.05M TRIS, 0.15M NaCl, pH 7.6 and incubated with Streptavidin-Biotin complex (Dako, Ely, UK) for 30 min, followed by 3',3'-diaminobenzidine tetrahydrochloride liquid substrate solution (Sigma, Poole, UK). The samples were washed in tap water and counterstained with Harris's haematoxylin, dehydrated and mounted. Staining was ranked using a scale from –, indicating minimal staining to +++ indicating intense staining. Control samples were incubated in 0.05M TRIS, 0.15M NaCl, pH 7.6, without adding the primary antibodies.

Statistics

Where appropriate, two-tailed Student's unpaired t-tests were used to compare results with chondrocytes expanded, using the monolayer and alginate passage techniques.

Results

Chondrocyte passage in monolayer and alginate bead culture

On initial seeding in monolayer (P0), the chondrocytes rapidly became attached to the growth surface, but typically retained a rounded morphology for approximately 3 days. Between days 3 and 7, the cells began to spread on the growth surface. After the first passage in monolayer, however, the cells attached and spread more rapidly, such that after

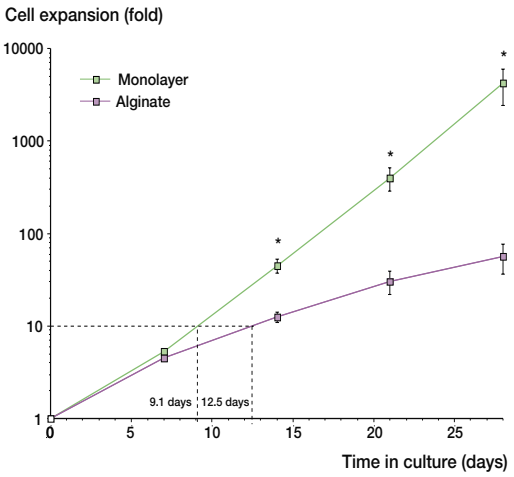

Figure 2. Cumulative cell expansion for chondrocytes cultured in monolayer or alginate for up to 28 days and passaged at 7-day intervals. Each point represents the mean ± standard deviation of 6 measurements from two separate experiments. Unpaired Student's t-test results indicate differences between monolayer and alginate values * = $p < 0.05$.

2 days in culture the majority of cells had adopted a fibroblastic morphology with a small proportion retaining a rounded morphology. They proliferated rapidly and had formed a confluent monolayer by day 7. A similar pattern was observed following subsequent passages in monolayer. Cells seeded within alginate beads at P0 were evenly dispersed within the alginate and had a rounded morphology. After 7 days in alginate culture, clusters of up to 16 cells were observed within the alginate beads. A similar pattern was seen following subsequent passages in alginate beads.

Cell expansion, assessed as an increase in the number of cells, during passage 0 was similar for cells cultured in either monolayer or alginate beads (Figure 2). During subsequent passage periods, expansion in monolayer was significantly greater than in alginate. Cumulative expansion suggested that during the entire 28-day culture period, a 4000-fold expansion of cells in monolayer was possible, while a 56-fold expansion of cells in alginate was attainable (Figure 2). Due to the similarity in expansion during the initial 7-day passage period, however, the time required for a 10-fold expansion of cells did not differ markedly for either passage methods with estimated values of 9.1 days and 12.5 days for monolayer and alginate, respectively.

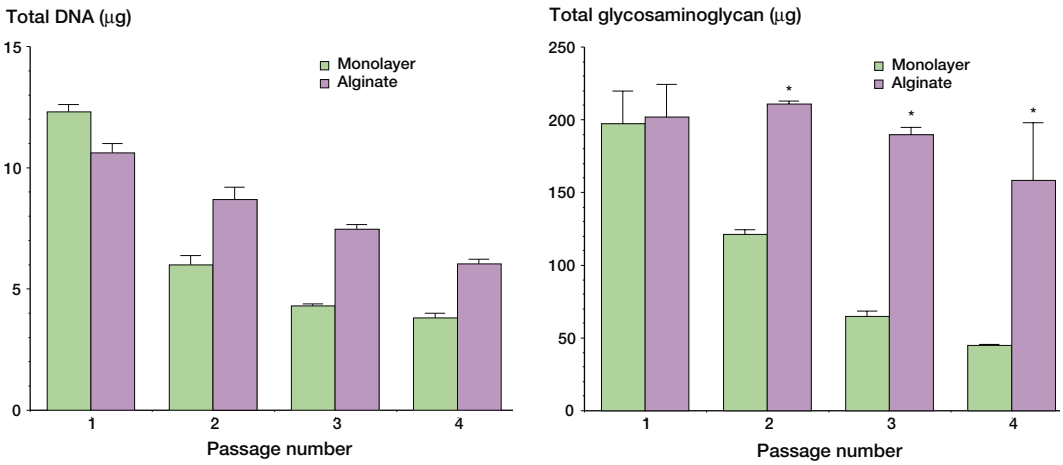

Figure 3. Total DNA (A) and glycosaminoglycan (B) content of alginate bead cultures prepared from chondrocytes passaged between 1 and 4 times in monolayer or alginate and subsequently cultured in alginate beads for 7 days. Each value represents the mean and standard deviation of 3 replicates. Unpaired Student's t-test results indicate differences between monolayer and alginate values * = $p < 0.05$.

The viability of cells recovered following passage was similar with the monolayer and alginate techniques and ranged from 92% to 97%.

Assessment of chondrogenic phenotypic potential (Figures 3 and 4, Table 1)

An inverse relationship between passage number and DNA value was observed, with both passage techniques. There was no significant difference between the normalised DNA values for cells passaged once in either monolayer or alginate. By contrast, cells passaged between 2 and 4 times in alginate showed significantly higher normalised DNA values, than those passaged an equivalent number of times in monolayer.

Alginate beads containing cells passaged between 2 and 4 times in alginate ha significantly higher glycosaminoglycan levels than those containing cells passaged for an equivalent number of times in monolayer. Indeed, there was a clear inverse relationship between passage number and glycosaminoglycan content within beads containing cells passaged in monolayer. By contrast, the glycosaminoglycan content of alginate beads containing cells passaged in alginate did not vary markedly with the number of passages.

Morphological assessment of immunolocalisation micrographs indicated that alginate beads prepared using cells passaged 4 times in monolayer contained small cell clusters with up to 4 cells, after 7 days in culture. By contrast, beads prepared from cells passaged 4 times in alginate contained much larger cell clusters. Immunolocalisation indicated a degree of staining for collagen I within alginate beads prepared using cells passaged in monolayer, with a greater staining intensity detected in beads prepared from cells which had undergone 3 or 4 passages in monolayer. By contrast, minimal staining for type I collagen was detected in beads prepared from cells passaged in alginate. Cells passaged in monolayer did not stain for collagen type II. Intense staining for collagen type II was, however, observed in beads containing cells passaged in alginate, the staining being primarily associated with the pericellular region surrounding cell clusters. Some degree of staining for

Table 1. Intensity of immunostaining within alginate bead cultures prepared from chondrocytes passaged between 1 and 4 times in monolayer (Mon) or alginate (Alg) and subsequently cultured in alginate beads for 7 days

	Passage 1		Passage 2		Passage 3		Passage 4	
	Mon	Alg	Mon	Alg	Mon	Alg	Mon	Alg
Coll I	+	–	+	–	++	–	++	–
Coll II	–	+++	–	+++	–	+++	–	+++
C-6-S	++	+++	++	+++	+	+++	+	+++

Scale ranges from –, indicating minimal staining to +++ indicating intense staining

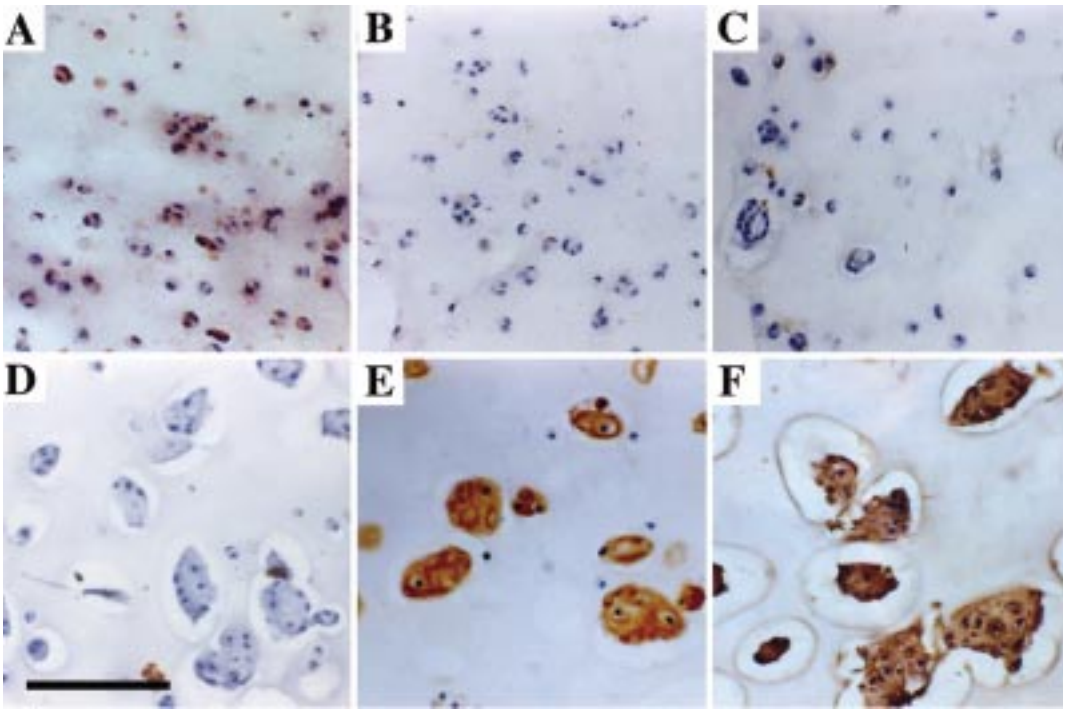

Figure 4. Micrographs representing immunolocalisation of chondrocytes passaged 4 times in monolayer (A-C) or in alginate beads (D-F). The cells were subsequently cultured in alginate beads for 7 days. Immunolocalisation for type I collagen (A, D), type II collagen (B, E) and chondroitin-6-sulphate (C, F). All micrographs are at the same magnification, scale bar = 100 μm.

chondroitin-6-sulphate was detected in the pericellular regions of cells passaged in monolayer and subsequently cultured in alginate, with a reduced staining intensity associated with passage 3 and 4 cells. Intense pericellular staining for chondroitin-6-sulphate was seen within beads prepared from cells passaged in alginate. The intensity of staining did not dependon the number of passages. Control samples, incubated in the absence of primary antibody, had no non-specific staining (results not shown).

Assessment of fibroblastic phenotypic potential (Figures 5 and 6, Table 2)

To assess fibroblastic phenotype, chondrocytes, passaged using monolayer or alginate techniques, were subsequently cultured on glass coverslips for 7 days. Between passages 2 and 4, cells passaged in monolayer had higher DNA values than those passaged in alginate. Passage 1 values were indistinguishable. There was no clear relationship between the number of passages and the normalised DNA level. The glycosaminoglycan content of glass coverslip cultures prepared using alginate-passaged cells was slightly higher than for monolayer-passaged cells, with the differences being significant at passages 2 and 4.

Staining using immunolocalisation was generally less intense for glass coverslip samples than for alginate beads. Collagen I staining was detected in coverslip cultures prepared using cells passaged in monolayer, with a greater staining intensity detected in beads prepared from cells which had

Table 2. Intensity of immunostaining for monolayer cultures prepared from chondrocytes passaged between 1 and 4 times in monolayer (Mon) or alginate (Alg) and subsequently cultured on glass coverslips for 7 days

	Passage 1		Passage 2		Passage 3		Passage 4	
	Mon	Alg	Mon	Alg	Mon	Alg	Mon	Alg
Coll I	+	–	+	+	++	+	++	++
Coll II	+	+	–	–	–	–	–	–
C-6-S	+	+	+	+	+	+	+	+

Scale ranges from –, indicating minimal staining to +++ indicating intense staining

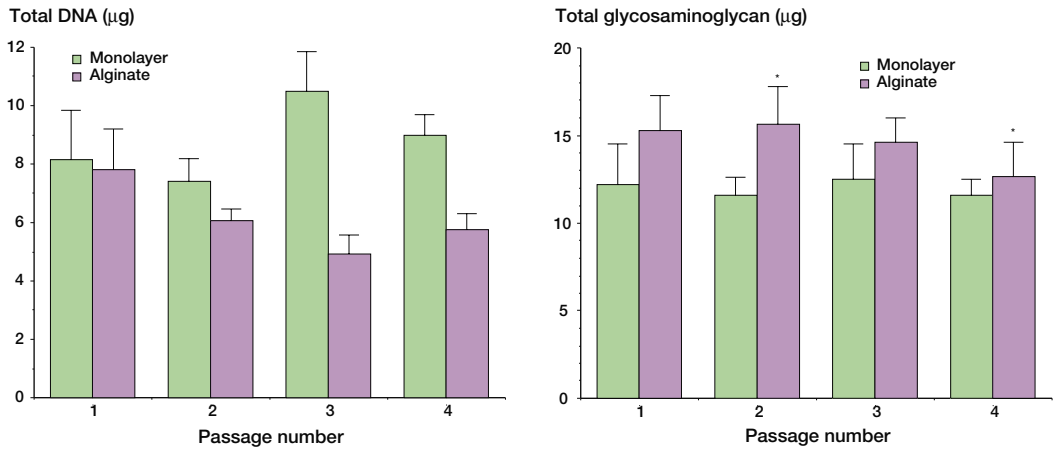

Figure 5. Total DNA (A) and glycosaminoglycan (B) content of monolayer cultures prepared from chondrocytes passaged between 1 and 4 times in monolayer or alginate and subsequently cultured on glass coverslips for 7 days. Each value represents the mean and standard deviation of 3 replicates. Unpaired Student's t-test results indicate differences between monolayer and alginate values as follows * = $p < 0.05$.

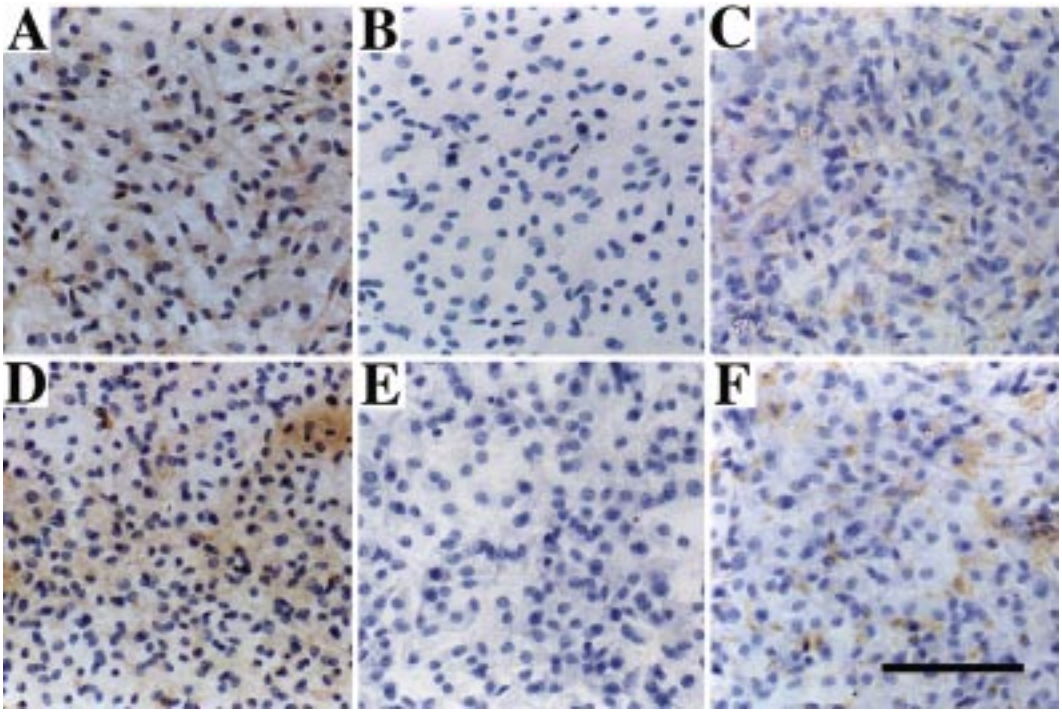

Figure 6. Micrographs representing immunolocalisation of chondrocytes passaged 4 times in monolayer (A-C) or in alginate beads (D-F). The cells were subsequently cultured on glass coverslips for 7 days. Immunolocalisation for type I collagen (A, D), type II collagen (B, E) and chondroitin-6-sulphate (C, F). All micrographs are at the same magnification, scale bar = 100 μm.

undergone 3 or 4 passages. Staining for type I collagen was also detected in alginate-passaged cells at passages 3 and 4. No staining for type II collagen was detected in any samples, while low levels of staining for chondroitin-6-sulphate staining were seen in all glass coverslip cultures.

Discussion

The aim of this study was to determine whether expansion of chondrocytes by repeat passage in alginate beads was feasible and whether this method would generate cells with an enhanced chondrogenic potential compared to cells passaged using traditional monolayer techniques. Passage is necessary for monolayer cultures to overcome contact inhibition which results in a reduction in cell proliferation once cell/cell contact is established. Previous studies have shown a similar trend among cells within three-dimensional culture systems, with a reduction in the rate of proliferation once cell clusters have formed (Guo et al. 1989, Beekman et al. 1997, Enobakhare 1998, Enobakhare et al. 2001). While techniques for the recovery of viable cells for passage are well established for monolayer systems, our findings indicate that one can recover viable chondrocytes from alginate beads, thereby confirming the efficacy of repeat passage in alginate.

Initial cell expansion, based on the recovery of viable cells, was similar using either system. These findings appear to conflict with previous studies which have indicated that the rate of chondrocyte proliferation is greater in monolayer than in three-dimensional culture systems (Lee et al. 1993). When initially seeded in monolayer, however, chondrocytes undergo a process of attachment and spreading, associated with actin stress fibre formation and modulation to a fibroblastic phenotype (Benya et al. 1978, 1988, Newman and Watt, 1988). While typically cell attachment occurs rapidly, full spreading may take up to 5 days, thereby precluding rapid proliferation over the initial 7-day culture period. When chondrocytes have adapted to a fibroblastic morphology in monolayer, the rate of cell spreading after passage is faster. Thus a gradual increase in the proliferation rate during the subsequent passage periods was observed. The time required for a 10-fold expansion of cells, said to be necessary for autologous cell repair (Brittberg et al. 1994), was estimated at 9.1 days and 12.5 days for monolayer and alginate, respectively. Thus the rate of expansion in alginate values would be acceptable for practical use and does not differ markedly from that determined for monolayer expansion.

The success of autologous cell-based cartilage repair systems depends on the ability of the implanted cells to proliferate and synthesise hyaline cartilage matrix (Bader and Lee 2000), characterised by type II collagen and the chondroitin sulphate-rich proteoglycan aggrecan. The ability of cells, passaged in monolayer or alginate, to carry out these processes was assessed by culturing the cells within alginate beads, a system known to maintain or promote chondrogenesis (Mayne et al. 1976, Benya et al. 1978, Benya and Shaffer 1982, deHaart et al. 1999, Almquvist et al. 2001, Kikuchi et al. 2001). Indeed a recent study showed a 4-fold increase in the number of cells in human primary chondrocytes maintained in alginate over a 4-week period with maintenance of chondrocytic phenotype (Almquvist et al. 2001).

Cells expanded in alginate typically exhibited a greater rate of proliferation, assessed as an increase in total DNA, when cultured in alginate beads than monolayer-expanded cells. Examination of micrographs confirmed these findings, with larger cell clusters present in beads prepared from alginate-expanded cells. Modulation to a three-dimensional culture system may, therefore, impede cell cycle activity in monolayer-cultured cells. The synthesis of hyaline cartilage matrix was assessed quantitatively, by measuring the total glycosaminoglycan content of the samples. This technique gives a measure of the development of a proteoglycan-rich matrix, known to be a feature of hyaline cartilage. Cells passaged in alginate more than once synthesised significantly greater amounts of glycosaminoglycan, suggesting an enhanced chondrogenic ability, compared to monolayer-passaged cells. Matrix synthesis was further investigated using immunolocalisation techniques. Cells passaged in monolayer did not express the hyaline cartilage-specific marker type II collagen when cultured in alginate beads, suggesting that chondrocytic phenotype, once lost, is not rapidly re-expressed. These findings were further strengthened by the presence of type I collagen within beads prepared from monolayer-expanded cells. Type I collagen is a marker for fibrous or fibrocartilaginous tissue, but is not present in bovine articular cartilage. These data agree with previous studies which have reported limited re-expression of chondrogenic markers and maintenance of fibroblastic markers

during the first few days of culture in alginate following monolayer expansion (Binette et al. 1998, deHaart et al. 1999). By contrast, cells passaged in alginate had a chondrogenic phenotype, when cultured in alginate beads, as evidenced by intense staining for type II collagen and chondroitin-6-sulphate (Table 1). While chondroitin-6-sulphate is a non-specific marker, it is typically associated with aggrecan synthesis within alginate cultures. The semi-quantitative description of the intensity of chondroitin-6-sulphate staining correlated closely with the quantitative assessment of glycosaminoglycan for both alginate- and monolayer-expanded cells.

Comparative studies were done in which monolayer- and alginate-expanded cells were cultured on cover slips to assess fibroblastic phenotype. They indicated that monolayer-expanded cells proliferated at a higher rate than alginate-expanded ones. These differences are consistent with a lag period associated with initial culture in monolayer, as outlined above. There was some evidence that alginate-expanded cells produced greater amounts of glycosaminoglycan-rich matrix, although overall levels were generally low. Indeed, the glycosaminoglycan content of glass coverslip cultures, normalised to DNA levels, was between 8% and 12% of the levels measured within equivalent alginate beads. Immunolocalisation indicated a complete absence of collagen type II staining, while collagen type I was expressed by both monolayer- and alginate-expanded cells. These data indicate that cells, whether passaged in monolayer or alginate, rapidly adopted a fibroblastic phenotype when cultured in monolayer. Low-level staining for chondroitin-6-sulphate was detected throughout, potentially associated with versican, which is known to be upregulated within chondrocytes when cultured in anchorage-dependent conditions. Alternatively, the staining may be associated with aggrecan in the light of a previous study, which reported the isolation of aggrecan mRNA from monolayer-passaged human chondrocytes (Binette et al. 1998).

Support for this study was provided by the Engineering and Physical Sciences Research Council and the Higher Education Funding Council for England.

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