Phenotypic modulation in sub-populations of human articular chondrocytes

in vitro

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Summary

Human articular cartilage has been separated into surface (approx. 15% of tissue depth) and deep zones (remaining tissue) and the constituent chondrocytes released by enzymic digestion. Subsequent culture either as a low density monolayer or as a suspension over agarose revealed distinct morphological and synthetic behaviour in the two populations. Whilst in monolayer these morphological differences disappeared with time in culture, over agarose they remained. Surface zone cells formed two types of cell cluster; one that was highly cellular with little extracellular matrix, and the other less frequent, which formed copious amounts of fibrillar matrix. Both types of cluster were surrounded by a layer of flattened chondrocytes. In contrast, deep cells formed a single cluster type that lacked a surrounding cell layer, but formed large amounts of sparse cartilage-like matrix and comprised morphologically typical chondrocytes. In monolayer, both populations gradually ceased to synthesise cartilage matrix components with the exception of link protein. In suspension, whilst the chondrogenic phenotype per se was preserved, there was, nevertheless, a loss in qualitative synthetic heterogeneity, which exists between surface and deep cells, that was not accompanied by changes in the differential rate of \textsuperscript{35}S incorporation into proteoglycan. Under these conditions, surface cells that normally do not synthesise keratan sulphate initiated de novo synthesis of this glycosaminoglycan. Consequently, it appears that the observed modulation in synthetic ability of the cell sub-populations is independent of the cluster morphology, which, once established, remains constant throughout the culture period.

Key words: chondrocyte, human cartilage, culture.

Introduction

Articular cartilage is an avascular tissue whose chief structural characteristic is a high matrix to cell volume ratio. The matrix comprises predominantly type II collagen, which forms a three-dimensional network encompassing cartilage-specific proteoglycan. The tissue has biochemical heterogeneity that seems to be paralleled by morphologically distinct types of chondrocytes (Royce and Lowther, 1979). The most obvious differences are apparent when one compares surface-located cells with those found in deeper regions of the tissue. Surface cells tend to be discoid and they synthesise less proteoglycan than more deeply located cells, which are rounded. Significant quantitative and qualitative differences in proteoglycans also exist through the depth of the tissue (Bayliess et al. 1983). More recently, Zanetti et al. (1985) have shown that a correlation exists between the paucity of the glycosaminoglycan keratan sulphate in the surface layer of articular cartilage, and the lack of keratan sulphate synthesis by the surface-located cells.

It is now established that cell shape plays an important role in the control of the chondrogenic phenotype in vitro. Many authors have stressed the importance of maintaining a rounded cell configuration in order that chondrocytes continue to synthesise type II collagen and/or cartilage-aggregating proteoglycan (von der Mark et al. 1977; Benya and Shaffer, 1982; Glowacki et al. 1983; Watt and Dudhia, 1988). It would also appear that a rounded cell shape maintains the quantitative differences in synthetic rate between surface zone and deep zone cells grown in culture and, to a certain extent, the qualitative differences (Aydellote et al. 1988). In contrast, cell flattening or, more precisely, the acquisition of a fibroblast-like morphology, is usually associated with the loss of the cartilage phenotype (Benya and Shaffer, 1982; Watt and Dudhia, 1988). More recent work has also related chondrocyte modulation to the degree of polymerization of the actin cytoskeleton (Benya et al. 1988).

Whilst there is a large literature on chondrocyte biology, very few workers have utilized adult human chondrocytes. The main reasons for this are difficulty in obtaining a reliable source of normal human articular cartilage and the variable number of cells obtained from specimens. Nevertheless, in order that we can relate knowledge gained from animal species to the human condition, and...
ultimately to the study of degenerative joint disease, the characteristics of human chondrocytes need to be determined.

In this study, we have separated adult human articular cartilage into surface and deep zones and cultured the isolated chondrocytes either as a monolayer or as a suspension over agarose. Using morphological, biochemical and immunocytochemical techniques we have monitored phenotypic modulation in both cell populations grown under the respective culture conditions. In particular, we have analysed the stability of the heterogeneity that exists between the separated cell populations.

**Materials and methods**

**Cell isolation and culture**

Human articular cartilage was obtained from knees of patients undergoing amputation or massive joint replacement for osteosarcoma or chondrosarcoma distant from the joint. Patients were in their second or third decade of life. Surface cartilage was separated from underlying cartilage with the aid of a scalpel, and transferred to a Petri dish (Fig. 1). This portion represented approximately the uppermost 15% of the entire tissue depth. The remaining cartilage was removed in a similar fashion, and transferred to a separate Petri dish. Sliced cartilages were maintained overnight as submerged explants in Ham’s F12 containing 10% fetal calf serum (FCS), 100 μg/ml ascorbic acid and 2% antibiotic/antimycotic to ensure sterility of the specimens prior to enzymic digestion. The cartilage preparations were washed in phosphate-buffered saline (PBS) and diced finely. Both sets of tissue were then incubated in Hepes-buffered Ham’s F12 medium (GIBCO) supplemented with Pronase (700 i.u. ml⁻¹) and collagenase (Sigma, type 1A) for 3 h at 37°C to allow viable cells to attach. Supernatants were discarded, and the cartilage further digested in Hepes-buffered Ham’s F12 supplemented with 10% FCS, 2% antiantimicrobial/antimycotic and collagenase (Sigma, type 1A) for 3 h at 37°C on a roller. Surface zone and deep zone cartilage was digested in 300 i.u. and 900 i.u. collagenase ml⁻¹ medium, respectively. The digested tissue was then allowed to settle and the supernatant removed and centrifuged at 1000 g. The cells were resuspended in an equal volume of 10% FCS in calcium- and magnesium-free PBS, re-centrifuged and the cells plated in 9 cm Petri dishes (~5 × 10⁵ cells per dish) into Ham’s F12 medium supplemented with 10% FCS, 1.4 mM L-glutamine, 100 μg/ml ascorbic acid, 2% antibiotic/antimycotic (standard growth medium). Viability was determined by Trypan Blue exclusion. Cultures were incubated overnight to allow viable cells to attach. These normally accounted for between 70 and 90% of the original total cell count. The attached cells were then harvested using 0.1% trypsin (1500 BAEE units mg⁻¹) and 0.05% EDTA in Ca²⁺ and Mg²⁺-free PBS, centrifuged and resuspended in growth medium. Cells were then plated into 35 mm Petri dishes (2.5 × 10⁶/dish) either as a monolayer or as a suspension over 1 ml of pre-set high-melting-point agarose (Sigma, type V). The growth medium was as described above except that human serum (prepared in our laboratory; see below) was substituted for FCS. Cultures were fed twice weekly.

**Human serum preparation**

Serum was prepared by bleeding two people from the laboratory (M.S. and C.W.A.). This was done during the morning in order to reduce the fat content of the serum. The blood was left at 37°C for 30 min to allow clotting and the remaining serum was then decanted off, centrifuged to precipitate erythrocytes and the supernatant removed to a sterile Universal tube and stored at −20°C.

**Morphological studies**

Cultures were examined and photographed routinely using an Olympus inverted microscope and viewed under phase-contrast optics. Cell diameters of the subpopulations immediately after plating over agarose were also calculated with the aid of these photographs. In addition, some cultures were fixed in 1.5% glutaraldehyde and stained as wholemounts with 0.5% Alcian Blue at pH 1.9 to visualise sulphated glycosaminoglycans.

**Electron microscopy**

At various time intervals (0–20 days) cultures were fixed in 1.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in a graded series of ethanol, and embedded in Spurr resin. Semi-thin 1 μm sections were cut on a diamond knife, stained with Toluidine Blue, examined and photographed on a Zeiss Photomicroscope III. Thin sections (80–100 nm) were also cut and viewed in a Philips CM12 transmission electron microscope.

**Immunofluorescence studies**

Again, at various time intervals during culture, cells were fixed for antibody labelling. Monolayer cultures were rinsed in PBS, fixed in 95% ethanol (4°C, 10 min), air-dried and stored at −20°C until required. Suspension cultures were centrifuged (1000 g for 5 min), rinsed in PBS, re-centrifuged, and fixed in 95% ethanol (4°C, 10 min). The dispersed pellet was plated on to glass slides, air-dried and stored at −20°C. For intracellular labelling, monoclonal antibodies were added to the culture medium (final concn 5 μg/ml) for 4 h prior to fixation. Ethanol fixation was then followed by incubation in absolute methanol (4°C, 10 min). This often provided additional resolution when counting numbers of positive cells.

Cells were incubated in M215 (a monoclonal antibody recognising keratan sulphate, Zanetti et al., 1985), CIIC1 (a monoclonal antibody recognising collagen type II, Holmdahl et al., 1986), M38 (a monoclonal antibody recognising type I procollagen, McDonald et al., 1986) a rabbit anti-human hyaluronic acid binding region (diluted 1:25) (Ratcliffe and Hardingham, 1983; Ratcliffe et al., 1984) and a rabbit anti-swine link protein (diluted 1:20) (Ratcliffe and Hardingham, 1983; Ratcliffe et al., 1984) for 45 min at room temperature, washed in Tris-buffered saline (TBS, pH 7.2) and incubated in rhodamine-conjugated rabbit anti-mouse IgG (Dako, Denmark) or fluorescein-conjugated swine anti-rabbit IgG (Dako, Denmark).
Denmark), washed in TBS and mounted in glycerol/water (9:1) containing 1,4-diazobicyclo [2.2.2.] octane (DABCO, 25 mg ml\(^{-1}\)) (Sigma) to retard fading. Cells were examined on a Zeiss Photomicroscope III under epifluorescence. Controls comprised second antibody incubation alone or pre-absorption of the primary antibody to its relevant antigen. In these cases, minimal or no fluorescence was observed. In some cases, particularly when staining for hyaluronic acid binding region, staining intensity was increased by predigestion of the cells in chondroitinase ABC (0.25 i.u. ml\(^{-1}\) in PBS for 45 min at 37°C). By combined viewing of epifluorescence and phase-contrast microscopy, the percentage of positive cells could be determined. This percentage was determined from a total of 500 cells.

**Measurements of Incorporation of \(^{35}\)SO\(_4\) into proteoglycan**

Cultures were labelled for 18 h with 20 \(\mu\)Ci ml\(^{-1}\) of \(^{35}\)SO\(_4\) (Amerham, England) at various time intervals. Medium was stored immediately, whilst cell layers were rinsed in PBS and then stored at −20°C. Cell pellets and monolayers were digested in papain (Royce and Lowther, 1979) at 60°C overnight. Label incorporated into extracellular matrix was separated from unbound label by passing 100 \(\mu\)l of digest down a Sepharose G50 column (Pharmacia, Sweden). Similarly, \(^{35}\)SO\(_4\)-labelled proteoglycan released into the medium was also separated from free isotope in the same way. In both cases, 0.5 ml fractions were collected directly into 4 ml of Scintillator 299 (Packard) and counted on a Minaxi Tricarb 4000 (Packard) scintillation counter.

Determination of DNA in the cell digests was by the fluorometric ethidium bromide binding assay described by Royce and Lowther (1978).

**Qualitative analysis of newly synthesised proteoglycans by gel chromatography**

Cell layers and their accompanying media were extracted overnight with 1 ml of 8 M guanidine hydrochloride buffered with 0.05 M sodium acetate (pH7.0) containing protease inhibitors (Oegema et al. 1975). The crude extracts were applied directly to a column of Sepharose CL-2B (0.6 cm×150 cm) equilibrated with 2 M guanidine hydrochloride in 0.05 M sodium acetate buffer (pH7) at a flow rate of 4 ml h\(^{-1}\). Aggregation of proteoglycan was determined by adding 1 mg of swine laryngeal cartilage proteoglycan monomer as a carrier and 2 % (w/w) hyaluronic acid to dissociative extracts. Guanidine was removed by dialysis and the proportion of \(^{35}\)SO\(_4\)-labelled proteoglycan that could aggregate was measured by gel chromatography on Sepharose CL-2B equilibrated with 0.5 M sodium acetate (pH 7). The \(V_o\) and the \(V_t\) of the column was determined using \(^{35}\)S-labelled proteoglycan aggregate and \(^{3}H\)-labelled water, respectively.

**Results**

Fig. 2A depicts a transverse section through normal human articular cartilage. The lightly staining surface tissue comprising 10–15 % of the total tissue depth represents the surface zone whilst the remaining tissue comprises the deep zone. In addition, representative electron micrographs of surface and deep zone cells in situ are included to demonstrate their distinctive morphologies (Fig. 2B and C, respectively). Whilst in this plane of section surface cells appear elongate, they are in fact discoid.

**Cell isolation**

Samples generally showed a viability of greater than 95 % by Trypan Blue exclusion. However, this was not indicative of whether or not cells would attach to the sub-stratum. The vast majority of non-adherent cells died within 3 days. It was subsequently found that human chondrocytes were extremely sensitive to batch variations in commercial bacterial collagenase. Therefore, for good cell recovery, batch testing of collagenase is essential. There did not appear to be a similar sensitivity to batch variations in Pronase.

**Culture morphology**

Surface and deep zone cells were photographed immediately after plating over agarose. By measuring the diameter of at least 50 cells from each zone from five patients, it was found that deep zone cells were a mean 28 % larger in volume than surface cells (Table 1) \((P<0.05)\). Although statistically significant, this result is likely to represent a trend rather than a firm rule, since the differences be-
Table 1. Surface zone and deep zone cells were photographed immediately after plating over agarose, and the resulting enlarged micrographs analysed.

<table>
<thead>
<tr>
<th></th>
<th>Surface zone cells</th>
<th>Deep zone cells</th>
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<tbody>
<tr>
<td>Diameter (µm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume (µm³)</td>
<td>10.75</td>
<td>13.68</td>
</tr>
<tr>
<td></td>
<td>11.49</td>
<td>11.58</td>
</tr>
<tr>
<td></td>
<td>11.19</td>
<td>12.23</td>
</tr>
<tr>
<td></td>
<td>10.73</td>
<td>10.53</td>
</tr>
<tr>
<td></td>
<td>11.23</td>
<td>12.74</td>
</tr>
<tr>
<td></td>
<td>11.07</td>
<td>12.22</td>
</tr>
</tbody>
</table>

Cell diameters were measured for five individual patients incorporating at least 50 cells from each patient. Cell volumes were calculated from diameters using the formula 4/3πr³, where r is radius. Means are expressed in bold-type at the base of each column.

Data between the cells in two of the five patients were small. Because of the small sample size, possible age-related differences were not examined. In one experiment cells were measured after isolation and after 1 day in suspension culture and it was found that surface cells expanded to the size of deep zone cells after that time (data not shown).

After 1 day in culture, cells plated over agarose began to form cell clusters. Initially, there were no obvious differences between surface and deeply located chondrocytes, and both elaborated an Alcian Blue-staining matrix (Fig. 3). After 5 days, certain differences became apparent and remained throughout the culture period. Surface zone clusters possessed smooth outlines. Most of these clusters appeared highly cellular but were interspersed with less frequent larger clusters that appeared ‘amorphous’ (Fig. 4A). Deep zone clusters, however, appeared as a single type that was lobulate in outline (Fig. 4B). Surface zone cells plated onto plastic typically spread in a bipolar fashion (Fig. 4C), a morphology that was retained for a few days. Whilst these types of cells were also apparent in deep zone cells in monolayer, most displayed polygonal morphology with few filopodia. These cells also tended to spread more slowly than surface cells (Fig. 4D). With time in culture, both cell populations tended to adopt a more fibroblastic morphology, and by 14 days were often indistinguishable from each other (Fig. 4E and F).

Histological and electron-microscopical examination of the clusters confirmed our phase-contrast observations. Surface clusters comprised an outer whorl of flattened cells enclosing central polygonal cells with little extracellular matrix. These clusters were interspersed with the occasional larger ‘amorphous’ clusters that displayed copious amounts of extracellular matrix (Fig. 5A). Deep zone cells, in contrast, formed clusters of polygonal cells interspersed with a faintly staining cartilaginous-like extracellular matrix (ECM). Unlike the surface zone, there was no enveloping layer of cells around the deep zone clusters (Fig. 5B).

At the electron-microscopical level, morphological differences were apparent between the surface and deep zone cells immediately after isolation. Surface zone cells often possessed fine cell processes and significant amounts of rough endoplasmic reticulum and lysosomes (Fig. 6A), which were more abundant when compared with deep zone cells. In contrast, deep zone chondrocytes displayed few cell processes, fewer lysosomes but more Golgi vesicles than corresponding surface cells (Fig. 6B).

After several days in culture, the whorled surface clusters were striking in their lack of elaborated extracellular matrix (Fig. 7A), contrasting with the large interterritorial spaces of deep zone clusters (Fig. 7B). Interestingly, by 5 days in vitro, most deep zone cells displayed numerous fine processes (Fig. 7B). In the larger amorphous surface zone clusters that did elaborate quantities of ECM (see Fig. 5A), the highly fibrillar nature of this matrix (Fig. 7C) contrasted markedly with the matrix of deep zone clusters, which showed copious amounts of proteoglycan granules (Fig. 7D).

Electron-microscopical examination of surface and deep zone chondrocytes grown on plastic showed few if any morphological differences between the two sub-populations, beyond those described at the light-microscopical level. Little ECM was elaborated.

Immunofluorescence studies

Examples of labelled monolayer and agarose cultures are shown in Fig. 8. Table 2 shows the percentages of cells that stained positively during culture over agarose and as a low density monolayer. Certain patterns are evident. Irrespective of culture condition, initially, a much smaller percentage of cells synthesised keratan sulphate in the surface zone when compared with the deep zone. This is consistent with findings using swine chondrocytes (Zanetti et al.)
In monolayer culture, the percentage of immunopositive cells in both the surface and deep zones decreased markedly for antibodies raised against keratan sulphate, hyaluronic acid binding region and type II collagen, with time in culture. In contrast, in both surface and deep zone cultures, there was an increase in the percentage of cells immunopositive for link protein and, more predictably, type I collagen.

In agarose suspension culture, the percentage of immunopositive cells for keratan sulphate, hyaluronic

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Fig. 5. (A) Semi-thin section of cell clusters formed by surface cells after 10 days in culture over agarose. A large 'amorphous' cluster is surrounded by numerous smaller 'whorled' clusters. Note the lack of elaborated matrix in the smaller clusters, contrasting with the copious amount of fibrillar matrix in the rather sparsely populated large cluster. Clusters developed from surface zone cells were always surrounded by a flattened cellular sheath. (B) Semi-thin section of a corresponding deep cell cluster. Most cells are polygonal and are interspersed with significant quantities of faintly staining intercellular matrix. Bar, 75 μm.

Table 2. Percentages of cells immuno-positive for various matrix components in monolayer and agarose suspension culture

A. Agarose culture

<table>
<thead>
<tr>
<th>Days in culture</th>
<th>Surface zone</th>
<th>Deep zone</th>
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<tbody>
<tr>
<td></td>
<td>KS</td>
<td>BR</td>
</tr>
<tr>
<td>1-2</td>
<td>60 (16)</td>
<td>ND</td>
</tr>
<tr>
<td>10-11</td>
<td>92 (7.8)</td>
<td>100</td>
</tr>
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B. Monolayer culture

<table>
<thead>
<tr>
<th>Days in culture</th>
<th>Surface zone</th>
<th>Deep zone</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>KS</td>
<td>BR</td>
</tr>
<tr>
<td>1-2</td>
<td>60 (16)</td>
<td>ND</td>
</tr>
<tr>
<td>10-11</td>
<td>92 (7.8)</td>
<td>100</td>
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</table>

KS, keratan sulphate antibody (ab); BR, hyaluronic acid binding region ab; LP, link protein ab; CI, procollagen type I ab; CII, collagen type II ab. Percentages were calculated from 500 cells for each time point. ND, value not determined. The values in parenthesis represent standard deviations.

Acid binding region, link protein and collagen type II all increased with time and few cells initiated type I collagen synthesis.

Analysis of 35SO4 incorporation and newly synthesised proteoglycans

The rate of incorporation of radioactive sulphate into sulphated glycosaminoglycans with time in culture is shown in Table 3. It can be seen that cells grown over agarose synthesised more sulphated proteoglycan than cells grown as a monolayer. Furthermore, under both culture conditions deep zone cells synthesised more sulphated proteoglycan than surface zone cells. In addition, under both culture conditions there is a significant decrease in the rate of incorporation between day 5 and the remainder of the culture period.

Table 3. Incorporation of 35S into proteoglycans by chondrocytes grown as a monolayer or suspension over agarose

<table>
<thead>
<tr>
<th>Days in culture</th>
<th>Agarose culture</th>
<th>Monolayer culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Surface</td>
<td>Deep</td>
</tr>
<tr>
<td>4-5</td>
<td>31.1 (2.4)</td>
<td>43.6 (35)</td>
</tr>
<tr>
<td>10-12</td>
<td>13.4 (3.3)</td>
<td>16.1 (9.2)</td>
</tr>
<tr>
<td>14-16</td>
<td>9.8 (4.3)</td>
<td>16.7 (4.8)</td>
</tr>
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Values in parenthesis represent standard deviations. Data for suspension cultures taken from three separate experiments. Monolayer data taken from one experiment.
Fig. 6. (A) Electron micrograph of a freshly isolated surface zone chondrocyte. Note the numerous cell processes, lysosomes (arrowheads) and quantities of rough endoplasmic reticulum. (B) Electron micrograph of a deep zone cell corresponding to that shown in A. In contrast to the surface cells, deep zone cells possessed few cell processes, fewer lysosomes and more abundant quantities of Golgi vesicles (asterisk). Bar, 4 \mu m.

The hydrodynamic size of $^{35}$S-labelled proteoglycans, released into the culture medium and cell layer at various stages of culture, was determined by gel chromatography on Sepharose CL-2B under conditions of dissociation. After 3 days, in both culture systems the major proteoglycan was of large hydrodynamic size (Fig. 9) and was capable of aggregating in the presence of exogenously added hyaluronic acid (HA) (not shown). There were also...
Fig. 7. (A) Electron micrograph of a 'whorled' cell cluster formed by surface zone cells plated over agarose and after 10 days in culture. Central polygonal cells are surrounded by enveloping flattened cells. Extracellular matrix is very sparsely distributed in this type of cluster. Note also that the surface cells still contain significant quantities of lysosomes (arrowhead). Bar, 5 μm. (B) Electron micrograph of a deep zone cell cluster after 10 days culture over agarose. Most cells are rounded, displaying 'classical' chondrocyte features. The rather sparse extracellular matrix (ECM) is just discernible. Note also that all the cells now display fine processes. Bar, 5 μm. (C) Detail of extracellular matrix elaborated by the larger 'amorphous' type of cell cluster formed by surface cells after 10 days of culture over agarose. Note the highly fibrillar nature of the matrix with little evidence of proteoglycan granules. Bar, 500 nm. (D) High-power detail of extracellular matrix elaborated by deep zone cell clusters. Numerous proteoglycan granules can be seen aligning along a fine fibrillar network, presumably collagen. Bar, 600 nm.

no significant differences in the elution profiles of cells derived from the surface or deep layers of the cartilage. After 32 days, agarose cultures continued to synthesise, predominantly, the large aggregating proteoglycan, 85% of which was still capable of aggregating with HA. In contrast, the major biosynthetic product of monolayer cultures was a proteoglycan of small hydrodynamic size that could not aggregate with HA (Fig. 9).

Discussion

The results presented in this paper show for the first time the detailed in vitro characterisation of human articular chondrocytes that, when separated on the basis of their spatial location within the tissue, represent at least two distinct sub-populations of cells that maintain morphological heterogeneity. Whilst this has been previously reported for similar chondrocytes from both steer and swine (Aydelotte and Kuettner, 1988; Aydelotte et al. 1988; Zanetti et al. 1985), the data presented here suggest that the morphological configuration adopted in culture does not reflect the qualitative changes in matrix synthesis that may be occurring. Consequently, this type of analysis (combining biochemical with immunocytochemical techniques) is essential if we wish to utilise the system to test the effects of growth factors, cytokines and other physiological modulators on sub-populations of cells derived from the whole tissue.

Whilst initially surface and deep zone cells could be distinguished in monolayer culture, both became fibroblast-like with time. However, distinct morphological differences were also apparent in cells cultured over agarose but, unlike monolayer cultures, these persisted with time. Surface cells produced two types of cluster: one sparse in extracellular matrix, the other showing copious amounts of fibrillar matrix. We do not know whether these two types of cluster represent two quite distinct sub-
Fig. 8. Immunofluorescence localisation of matrix components in monolayer and agarose cultures. (A and B) A 5-day monolayer culture of surface cells after treatment with monensin and localisation with a monoclonal antibody to type I procollagen (M38). (A) Phase-contrast; (B) same field viewed under epifluorescence illumination. Arrowheads indicate the same cell in each micrograph. Note that rounded cells are negative. (C and D) A 5-day agarose culture of deep zone cells after localisation with MZ15 antibody recognising keratan sulphate. (A) Phase-contrast; (B) same field viewed under epifluorescence illumination. Arrowheads indicate the same cell in each micrograph. Bar, 150 μm.

populations, which might arise through cell sorting, or are the same population in different stages of maturation. Both types of cluster were surrounded by a thin layer of sheath cells reminiscent of a perichondrium, a structure that was never observed in deep zone cultures. The lack of matrix elaborated by the whorled clusters of surface zone cells is consistent with their reduced incorporation of 35S when compared with deep zone cells. Whilst this is consistent with known variations in proteoglycan concentration with depth of the intact tissue (Bayliss et al. 1983) it is, nevertheless, surprising that so little extracellular matrix should have been elaborated in most surface clusters. It would also appear from ultrastructural observations that those surface clusters that do elaborate a large amount of matrix are rather sparse in proteoglycan. Clearly, we need more information as to precisely which of the surface cells are synthesising matrix and details of matrix turnover. In contrast to surface zone cells, suspension culture of deep zone chondrocytes produced a single cluster type that comprised typical rounded chondrocytes interspersed by a rather sparse but identifiable 'cartilage-like' matrix with a fine mesh of collagenous fibres and proteoglycan granules.

Analysis of the rates of incorporation of 35S supports our microscopical observations, showing that deep zone cells incorporate more label than surface cells. It is also clear that cells grown over agarose incorporate more label than those in monolayer. Again, this is consistent with other studies that have related the acquisition of a rounded cell configuration with increased synthesis of sulphated proteoglycans (Archer et al. 1982; Glowacki et al. 1983; Newman and Watt, 1988).

Qualitative analysis of newly synthesised proteoglycans by cells grown over agarose showed that both surface and deep zone cells synthesised a single large aggregating monomer for the entire culture period. This leads us to think that under such culture conditions the chondrogenic phenotype may be stable. However, a limitation of this type of analysis is that it can only reflect the overall synthetic product of an entire culture sample. Consequently, when the immunolocalisation of specific matrix components is considered, it is evident that under both culture conditions there are marked variations with time in culture in the percentage of cells synthesising individual components. Whereas, in monolayer culture, there was a decline in the percentage of cells synthesising collagen type II, keratan sulphate and hyaluronic acid binding region, the reverse pattern was found for link protein. The reduction in the percentage of cells synthesising keratan sulphate is in contrast to the findings of
Fig. 9. Elution profiles run under conditions of dissociation on Sepharose CL-2B of $^{35}$S-labelled human chondrocyte proteoglycans. (A) Monolayer cultures of surface and deep zone chondrocytes after 3 days (top panel), and 32 days (bottom panel) in culture. (B) Agarose suspension cultures of surface and deep zone chondrocytes after 3 days (top panel) and 32 days (bottom panel) in culture. Fractions 25 and 65 mark the $V_o$ and $V_r$, respectively. All axes are to the same scale. (See text for details.)

Zanetti et al. (1985), who reported an increase in the percentage of swine surface chondrocytes associated with this epitope in monolayer culture. A similar pattern of variation was obtained for cells maintained in the presence of cytosine arabinoside, an inhibitor of DNA synthesis (data not shown); thus precluding the possibility of the differential proliferation of specific cell types.

Chondrocytes maintained over agarose displayed a pattern entirely different from that of those cultured in monolayer. Whilst after a day in culture, only 25% of surface cells were positive for keratan sulphate, 70% of deep cells were positive at the same time. Again, this is consistent with data reported for swine chondrocytes (Zanetti et al. 1985). However, the percentage of positive cells from both locations increases steadily with time in culture, so that after 20 days in vitro most cells are positive. Whilst a similar pattern was obtained for type II collagen, the initial percentage differences for immunopositive cells between the two populations are likely to be related to the time required for synthetic recovery rather than innate differences between sub-populations. Other studies carried out by us indicate that, by day 3 in vitro, most cells from both locations are synthesising type II collagen. Clearly, this contrasts with the keratan sulphate data, showing that only 60% of the surface cells are positive after 10 days in culture. We do not know why some cells initiate keratan sulphate synthesis, but it may be related to cell shape changes. In the tissue, surface cells, which normally do not synthesise keratan sulphate, are discoid, whilst those cells that do synthesise keratan sulphate in the deeper regions are normally rounded. When surface cells are isolated and cultured in suspension, they assume a rounded morphology similar to that of deep zone cells. It is tempting to speculate that the basis of cellular heterogeneity within the tissue is based, in part, on cell shape differences.

Finally, it is noteworthy that whilst morphological and quantitative biochemical heterogeneity are maintained between surface and deep zone chondrocytes in suspension culture, there appears to be a convergence in terms of the matrix components synthesised. Consequently, with time in culture there are similar numbers of cells producing the matrix components analysed.

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collagen-induced arthritis, using anti-type II collagen antibodies. 


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