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Cartilage morphogenesis *in vitro*

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SUMMARY

The morphogenetic capacity of prechondrogenic mesenchyme from two developmentally distinct sources was investigated in high density micromass cultures. We confirmed an earlier report (Weiss & Moscona, 1958) that scleral mesenchyme formed cartilage sheets whilst limb bud mesenchyme formed distinct cartilage nodules. It was thus suggested by these authors that this morphogenesis was tissue type specific. However, by varying cell density at inoculation (which controls cell configuration) and by varying the relative amount of prechondrogenic mesenchyme present in cultures we found that dramatic changes in morphogenesis could be brought about. Viewed in these terms we suggest that cartilage morphogenesis *in vitro* is dependent on cell configuration and the presence of non-chondrogenic cell types and hence is not necessarily a function of an intrinsic morphogenetic potential of the constituent cells.

INTRODUCTION

The occurrences of cartilage in a developing vertebrate embryo take numerous forms: these range from the sheets of the sclera through the rod-like form of Meckels cartilage to the rudiments of the future long bones of the limb and the complex three-dimensional structures of the vertebrae prior to ossification.

In the past 15 years there has been an immense amount of work published concerning factors which influence the commitment and differentiation of mesenchyme cells particularly in relation to the expression of the chondrogenic phenotype. Surprisingly, very little of this information has been correlated with early cartilage morphogenesis even though earlier investigators, Weiss & Amprino (1940) and Weiss & Moscona (1958) reported findings that suggested intrinsic differences in prechondrogenic mesenchyme which led to 'tissue-specific' morphogenesis even after dissociation and reaggregation *in vitro*. These authors took dissociated chick embryo mesenchyme from two separate sources which, *in vivo*, give rise to cartilage of very different morphologies, and cultured them at

Key words: cartilage, scleral mesenchyme, tissue type, cell density, chick embryo.

high density *in vitro*. Periocular mesenchyme gave rise to flat sheets of cartilage similar to the sheet of scleral cartilage which surrounds the retina *in vivo*. In contrast, limb mesenchyme gave rise to whorled nodules of cartilage *in vitro* which they considered analogous to the initial cartilage formation in long bone rudiments.

Further indirect evidence for 'tissue-specific' morphogenesis derives from work using isolated mature chondrocytes from different sources which also have different embryological origins and therefore different developmental histories. Levenson (1969, 1970) has shown that isolated epiphyseal chondrocytes (mesodermal origin) formed aggregates *in vitro* in a totally different fashion to isolated chondrocytes from Meckels cartilage (neuroectodermal origin). Moreover, pieces of cartilage of similar developmental origin will invariably fuse when placed in association, a phenomenon that has been shown to work across species (Silberzahn, 1968), but when the cartilages were of different embryological origin, fusion occurs much less frequently or not at all (Chiakulus, 1957; Silberzahn, 1968; Foret, 1970; Fyfe & Hall, 1977). Clearly, a degree of heterogeneity exists but whether this is cell- or matrix-mediated remains unknown.

It has been appreciated for over half a century that the morphogenesis of skeletal tissue involves an interaction of intrinsic (genetic) and extrinsic (environmental) factors (Murray & Huxley, 1925; Murray, 1936). The question is to what extent these respective factors contribute to the overall morphogenetic process at any specific time in a given system.

We have carried out micromass cultures of prechondrogenic and non-chondrogenic mesenchyme (Ahrens, Solursh & Reiter, 1977) and varied such parameters as cell density and cell type. Unlike Weiss & Moscona (1958) we have interpreted our results in terms of cell-density-dependent morphogenesis at the time of plating, and not necessarily as a 'tissue-specific' morphogenesis. In addition, the cartilage form was found to be highly dependent on the presence of non-chondrogenic cells.

MATERIALS AND METHODS

Culture

Fertilized White Leghorn eggs from a local breeder were incubated at 38+1°C and windowed on the fourth day of development. Quail eggs were obtained from our own stocks and incubated as above until required. The embryos were staged according to Hamburger & Hamilton (1951). Limb mesenchyme was obtained from the wing and leg buds of stage-23 or -24 embryos. Limb buds were dissociated by trypsin digestion (0·1% in PBS for 10 min at 37°C) with frequent agitation. Cell clumps that remained were broken up by gentle aspiration with a pipette.

Periocular mesenchyme was obtained from the developing eyes of 6- to 6½-day embryos as described by Weiss & Moscona (1958). Briefly, the eyes were removed to sterile phosphate-buffered saline (PBS), dissected free of adhering soft tissue, punctured and left for approximately 15 min. This latter procedure facilitated the separation of the periocular mesenchyme which was then dissected free with forceps and dissociated as described above. Generally, periocular mesenchyme needed longer trypsinization (approximately 20 min) than the younger limb bud mesenchyme.

Chick heart cells from 7-day embryos were also harvested. Hearts were shredded into small pieces using fine forceps and trypsinized for 15 min with frequent agitation. Again, gentle aspiration with a pipette dispersed any remaining cell clumps.

Epiphyseal and scleral chondrocytes were obtained from 8- and 9-day embryos respectively. Cells were isolated from chopped explants after prolonged trypsinization and collagenase treatment (0·2 % for 1–2 h at 37 °C).

Cells were inoculated at medium and high density in a manner similar to the 'micromass' techniques described by Ahrens *et al.* (1977). Briefly, medium density cultures comprised a suspension of 5×10^6 cells ml^{-1} which were inoculated as 10 μl drops (5×10^4 cells) in 35 mm Petri dishes (Nunc, U.K.). Normally, three or four cultures occupied each dish. Cells were allowed 1 h to attach to the substratum before the Petri dish was gently flooded with nutrient medium.

High density cultures were made in a similar fashion to medium density cultures but using a final suspension of 2×10^7 cells ml^{-1} (2×10^5 cells 10 μl^{-1} drop).

Culture combinations

All cells were chick embryo derived unless otherwise stated:

High density scleral mesenchyme
Medium density scleral mesenchyme
High density limb mesenchyme
Medium density limb mesenchyme
High density limb mesenchyme/chick heart cells (3:1)
High density scleral mesenchyme/chick heart cells (3:1)
High density limb mesenchyme/quail scleral mesenchyme (1:1)
High density epiphyseal chondrocytes
Medium density epiphyseal chondrocytes
High density scleral chondrocytes
Medium density scleral chondrocytes
High density epiphyseal chondrocytes/chick heart cells (3:1)
High density scleral chondrocytes/chick heart cells (3:1)

In all cases a minimum of 15 cultures were analysed for each experimental combination after 4 or 5 days culture.

Culture medium

Hams F12 nutrient medium (Gibco) supplemented with foetal calf serum (10 %), L-glutamine (1·4 mmol), L-ascorbic acid (150 $\mu g\,ml^{-1}$) and 1 % antibiotic/antimycotic solution (Gibco) was used in all experiments. Cultures inoculated into 35 mm Petri dishes were flooded with 1·5 ml culture medium which was changed every other day.

Microscopy and histology

Cultures were examined daily and photographed using an inverted microscope (Zeiss, W. Germany). Samples were fixed in 10 % buffered formol saline (10 min), rinsed briefly with distilled water and stained with 0·1 % alcian blue at pH 1 (30 min) (Luna, 1968) for sulphated glycosaminoglycans and/or with 0·2 % toluidine blue (2 min) (Lillie, 1929) for metachromasia.

Thick Araldite sections were prepared from cultures fixed in half-strength Karnovsky (Karnovsky, 1965), postfixed in 1 % OsO_4 dissolved in cacodylate buffer, dehydrated in a graded series of ethanols and embedded in Araldite. Two micron sections were cut with a Cambridge MK11 microtome (Cambridge Instruments) and stained with 0·1 % toluidine blue in borax (Trump, Smuckler & Benditt, 1961). Sections were photographed using a Zeiss photomicroscope 11.

Immunofluorescence

Cartilage-specific type II collagen was localized in cell cultures by indirect immunofluorescence (von der Mark, von der Mark & Gay, 1976). Affinity-purified rabbit antiserum to

chick type II collagen was kindly donated by Dr G. Shellswell, ARC Meat Research Institute, Long Ashton, Bristol and Dr Klaus von der Mark, Max-Planck-Institut fur Biochemie, Munich.

Cultures were rinsed once in PBS and fixed in 95 % ethanol at 4°C for a minimum of 2 h. At this stage they could be gently scraped off the substratum and the floating cultures were then dehydrated in absolute ethanol (×3, 1 h each), cleaned in xylene (×3, 1 h each) and embedded in wax (×3, 30 min each) at just melting point. All dehydrating and clearing procedures were carried out at 4°C. Serial sections (7 μm thick) were cut on a rotary microtome. Mounted sections were dewaxed and hydrated in precooled xylene, alcohols and rinsed in PBS (×3 at 4°C). Prior to the addition of antibodies, sections were pretreated with hyaluronidase (1 % at 37°C for 30 min) and washed in PBS. Sections were incubated with antiserum (1:10 dilution) at room temperature (30 min), washed three times in PBS and overlaid with a 1:10 dilution fluoresceine isothiocyanate (FITC)-conjugated goat anti-rabbit gamma globulin (Wellcome Labs, Beckenham, England) (30 min at room temperature in a moist dark atmosphere). After further washing (×2 in PBS) the cultures were mounted in glycerol/PBS (1:9) and viewed with a Zeiss photomicroscope II equipped with a u.v. light source for fluorescence microscopy.

RESULTS

Scleral mesenchyme

At high density (2×10^5 cells/culture) scleral mesenchyme formed a rather homogenous sheet of polygonally shaped cells many of which showed evidence of a refractile extracellular matrix after 2 days in culture (Fig. 1). Histologically, it was apparent that most of the cells were typically polymorphic and surrounded by a metachromatic matrix (Fig. 2). Both the cells and the extracellular matrix in these cultures stained with antibody to type II collagen (Fig. 3).

Medium density cultures (5×10^4 cells) behaved, initially, in a similar manner to high density cultures. However, discrete islands or nodules of cartilage separated by fibroblastic cells were apparent after 5 days (Fig. 4). Unlike the cartilage nodules, the fibroblastic regions showed no evidence of intra or extracellular fluorescence when stained with collagen type II antibody (not shown).

Limb mesenchyme

The behaviour of high density limb mesenchyme cultures has been extensively documented by Solursh and his colleagues (see review by Solursh, 1984 and Ede, 1981). Initially (day 1 or 2), cells formed concentric aggregates (Fig. 5), the centre of most aggregates acting as a focus for cartilage formation which was detectable by alcian blue and toluidine blue staining at day 3 (Figs 5, 6). Immunofluorescent labelling to type II collagen was found predominantly within the nodular regions (Fig. 7) and is consistent with the findings of Solursh *et al.* (1982*b*).

In contrast, medium density limb mesenchyme cultures rarely became chondrogenic. By day 2, numerous fibroblasts were evident and these quickly overgrew the entire culture (Fig. 8). Regions in which the cells remained polygonal rarely established a refractile extracellular matrix. Invariably, the whole culture was fibroblastic after 5 days *in vitro* and there was no marked fluorescence above background levels after antibody labelling for type II collagen (not shown). In addition, these cultures showed a complete absence of metachromasia after staining with toluidine blue and reacted negatively with alcian blue at pH 1.

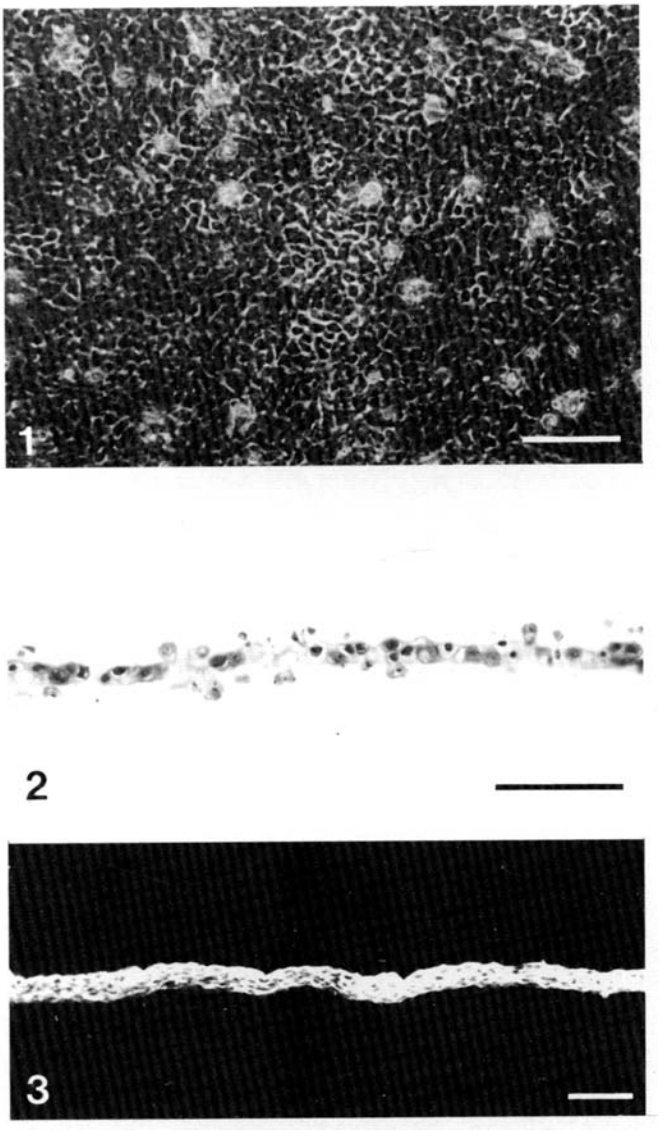


Fig. 1. Phase-contrast image of the centre region of a high density scleral mesenchyme culture after 2 days. Note the homogenous nature of the cell layer and also the refractile intercellular partitions indicative of matrix secretion. Scale bar equals 100 μm.

Fig. 2. Thick Araldite section through a 4-day high density scleral mesenchyme culture. Note that most cells are surrounded by a metachromatic matrix. Toluidine blue. Scale bar equals 50 μm.

Fig. 3. Wax section through a 4-day high density scleral mesenchyme culture and stained with antibody to type II collagen. The entire culture stains positively. Scale bar equals 50 μm.

Chondrocytes

High density scleral and high density epiphyseal chondrocytes both gave rise to flat sheets of cartilage which were very similar in appearance (Figs 9, 10). Similarly, when plated at medium densities, both types of chondrocytes produced similar results, namely discrete nodules of cartilage separated by non-chondrogenic fibroblast-like cells (Figs 11, 12).

Mixed cell cultures

In both high density scleral mesenchyme/chick heart cell and high density limb mesenchyme/chick heart cell cultures chondrogenesis was drastically reduced compared with non-mixed cultures (Figs 13, 14). In the former case small areas containing polygonal cells surrounded by refractile matrix were occasionally observed (Fig. 13). Although in the limb mesenchyme culture (Fig. 14), the characteristic concentric aggregation of cells was not abolished by the addition of heart cells, there was little accumulation of matrix.

Equal mixtures of chick limb mesenchyme with quail scleral mesenchyme resulted in substantial chondrogenesis (Fig. 15). In histological section it was seen that like the addition of non-chondrogenic cell types (e.g. chick heart cells), scleral mesenchyme did not entirely abolish the concentric cellular arrangement characteristic of limb mesenchyme (Fig. 16). However, unlike chick heart cells, scleral mesenchyme did not impair chondrogenesis. On the contrary, chondrogenesis appeared to be enhanced with large areas of cartilage interspersed with whorled aggregates which were also metachromatic. However, it was unclear from our Feulgen-stained sections whether cell sorting was taking place.

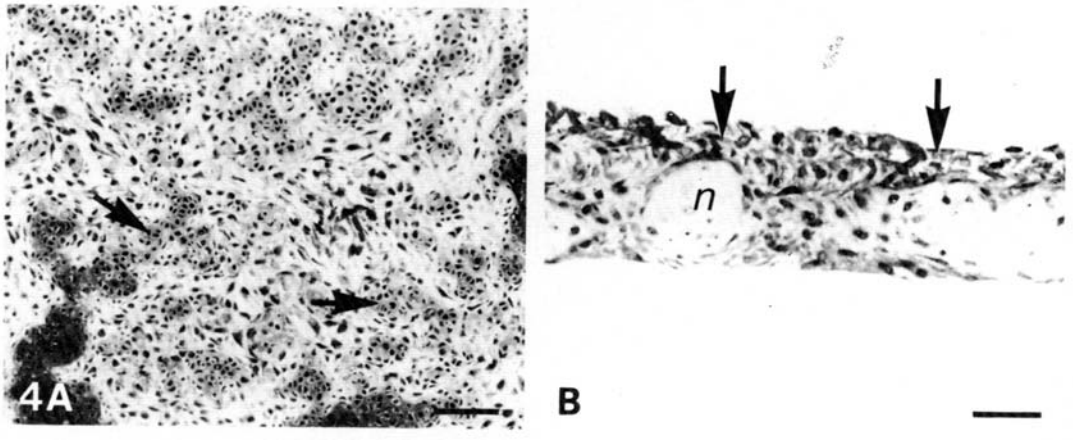
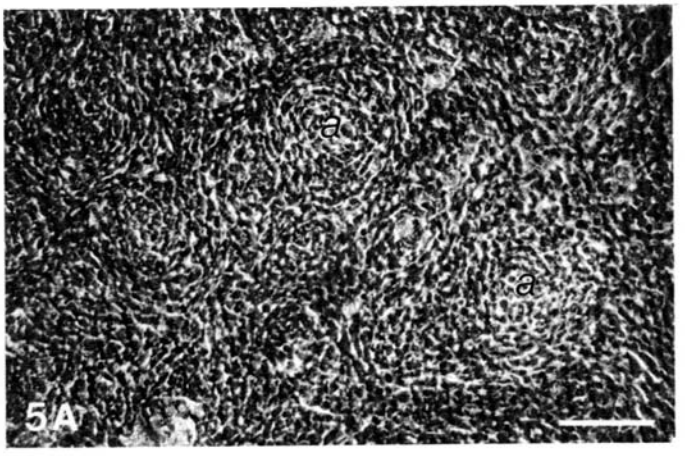
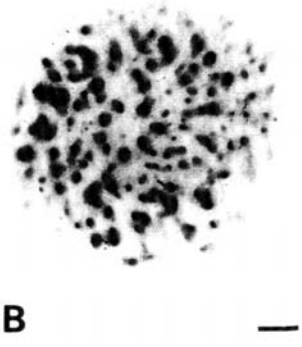
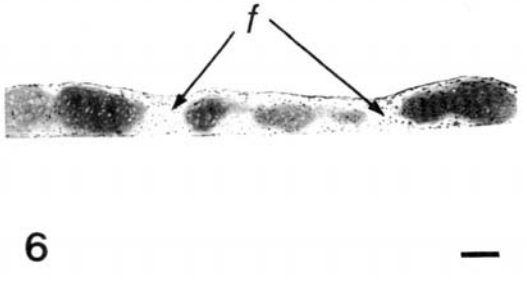


Fig. 4. (A) Low-power planar section of a medium density scleral mesenchyme culture after 5 days. Numerous chondrogenic islands (arrowed) are present interspersed by fibroblastic-like cells. Scale bar equals 100 μm. (B) Transverse section through a similar culture showing detail of the chondrogenic nodules (*n*) and the large number of fibroblastic-like cells (arrowed) on the culture surface. Scale bar equals 50 μm.







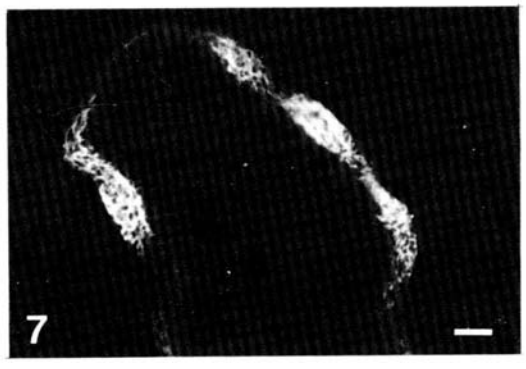


Fig. 5. (A) Phase-contrast image of the centre region of a high density limb mesenchyme culture after 2 days. Distinct aggregates (*a*) can be seen with the surrounding cells forming characteristic whorls. Scale bar equals 100 μm. (B) Whole mount micrograph showing the distribution of alcian blue staining foci within a single culture after 4 days. Scale bar equals 1 mm.

Fig. 6. Thick Araldite section cut at right angles to the planar surface of a 5-day limb mesenchyme culture showing the chondrogenic nodules interspersed by non-metachromatic 'fibrous' regions (*f*). Toluidine blue. Scale bar equals 100 μm.

Fig. 7. A transverse wax section through a high density limb mesenchyme culture after 4 days and stained with antibody to type II collagen. Unlike scleral mesenchyme, only the nodular regions of the culture stains positively. Scale bar equals 100 μm.

In contrast, an equal combination of epiphyseal and scleral chondrocytes produced a homogenous sheet of cartilage (Fig. 17). Combinations of epiphyseal and scleral chondrocytes with chick heart fibroblasts resulted in indistinguishable patterns of cell behaviour, both sets of cultures exhibiting substantial areas of chondrogenesis separated by fibroblastic tracts (Figs 18, 19).

DISCUSSION

The results in this paper confirm the distinct patterns of chondrogenesis by mesenchyme from different locations in the embryo. The 'whorled' cartilage

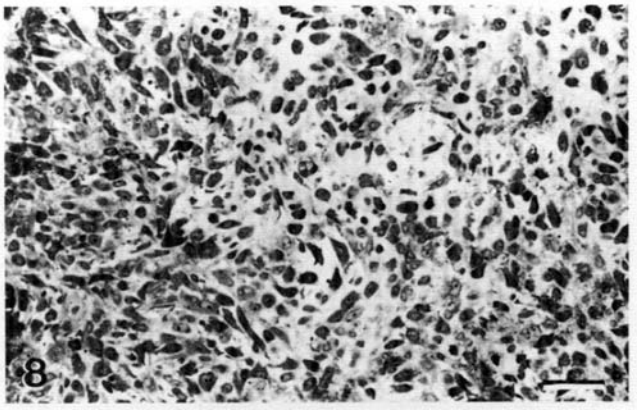


Fig. 8. Planar Araldite section of a medium density limb mesenchyme culture after 4 days. Very few cells remain polymorphic, the majority showing fibroblastic-like morphology. Scale bar equals 50 μm.

nodules observed in high density limb bud mesenchyme cultures are undoubtedly similar to those described by Weiss & Moscona (1958) as are the cartilage sheets formed from high density scleral periocular mesenchyme. More interestingly, the types of chondrogenesis mentioned above seem to reflect the behavioural patterns of the mesenchyme prior to matrix secretion, that is, limb bud mesenchyme forms numerous concentric aggregates in which the foci normally become chondrogenic (Ahrens *et al.* 1977). In contrast, at high density scleral mesenchyme remains as a sheet of rounded/polygonal cells from plating to the onset of overt chondrogenesis.

A central question is whether these cellular arrangements represent a tissue morphogenetic response, that is, an intrinsic property of the cells which has been shown to operate for other cell types *in vitro* (e.g. keratinocytes, Green & Thomas, 1978). For example, can scleral mesenchyme form whorled aggregates or limb mesenchyme a sheet of polygonal cells which gives to a flat sheet of cartilage – the reverse of the normal *in vitro* behaviour? In order to answer these questions, we

Fig. 9. Thick Araldite section through a 5-day high density scleral chondrocyte culture. Apart from the boundary cells, the entire culture is clearly chondrogenic. Toluidine blue. Scale bar equals 100 μm.

Fig. 10. Thick Araldite section through a 5-day high density epiphyseal chondrocyte culture. The morphology is almost identical to that of scleral chondrocytes (Fig. 9). Toluidine blue. Scale bar equals 100 μm.

Fig. 11. Medium density scleral chondrocyte culture after 4 days. Note the distinct nodules of cartilage (*n*) interspersed by fibroblastic-like cells. Toluidine blue. Scale bar equals 100 μm.

Fig. 12. (A) Medium density epiphyseal chondrocyte culture after 4 days. Again the appearance is very similar to that shown for scleral chondrocytes, namely, rounded nodules of cartilage (*n*) interspersed by fibroblastic-like cells. Phase contrast. Scale bar equals 100 μm. (B) Representative section of a medium density epiphyseal chondrocyte culture after 6 days. The nodules of cartilage are interspersed by non-chondrogenic fibrous-like cells. An identical morphology was obtained using scleral chondrocytes. Phase contrast. Scale bar equals 100 μm.

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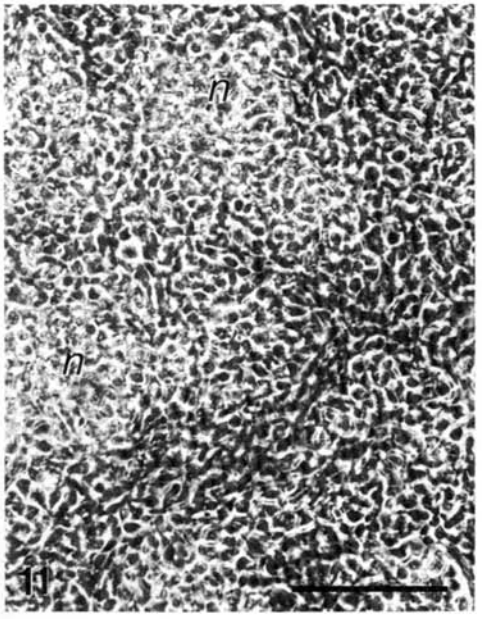

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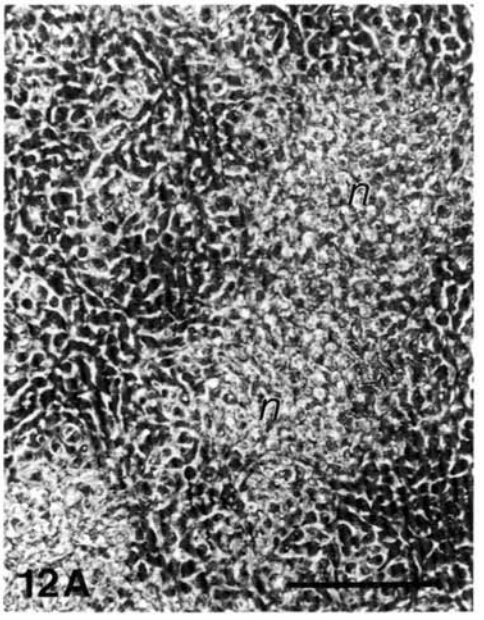

12A

12B

must first try and understand the cell interactions which occur prior to phenotype expression.

Scleral mesenchyme

The presumptive fate of neural-crest-derived periocular mesenchyme is to produce a cup or capsule of supporting cartilage around the eye (Smith &

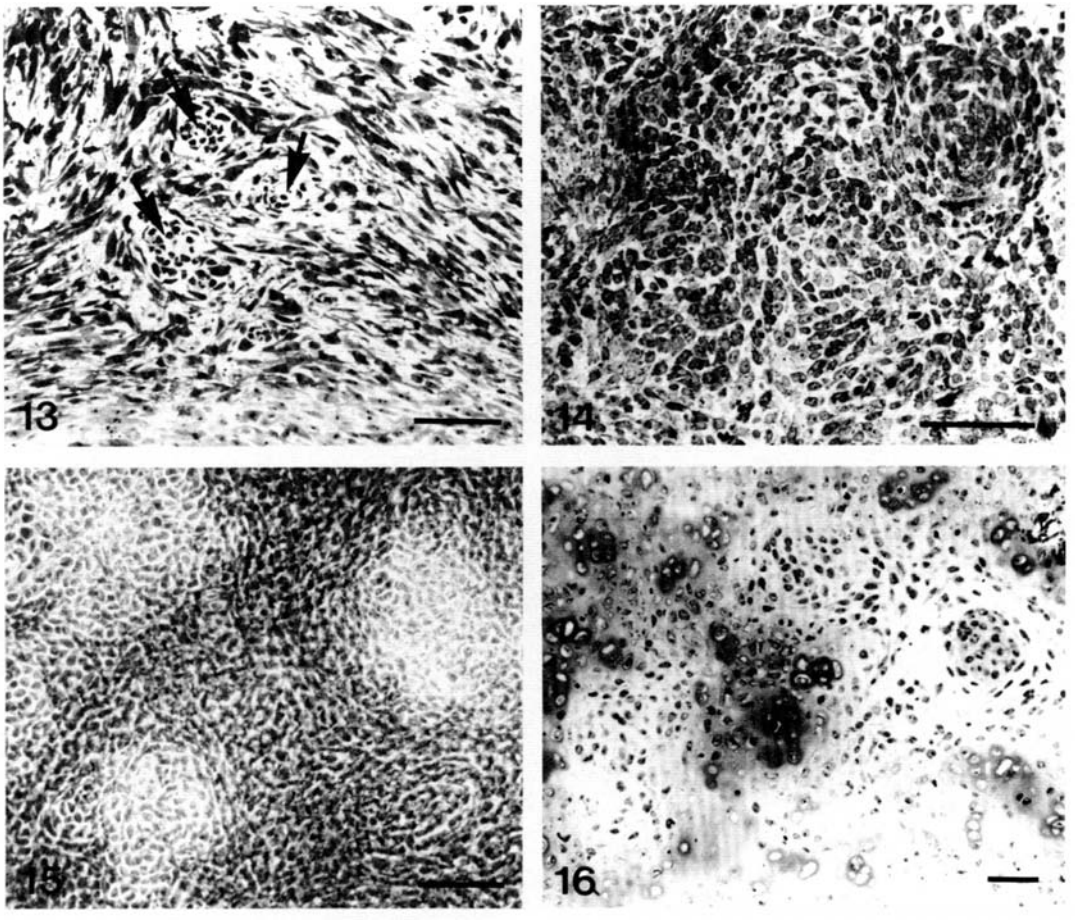

Fig. 13. Planar section through a scleral mesenchyme chick heart fibroblast culture after 4 days. The largely fibroblastic culture is interspersed with small chondrogenic islands (arrowed). Toluidine blue. Scale bar equals 100 μm.

Fig. 14. Planar section through a chick heart fibroblast/limb mesenchyme culture after 4 days. Whilst whorling is clearly obvious, there is very little evidence of matrix secretion, and whole mounts stained with alcian blue revealed very little positive staining. Toluidine blue. Scale bar equals 100 μm.

Fig. 15. Phase-contrast image of a 4-day scleral mesenchyme/limb mesenchyme (1.1) combination. Note substantial matrix secretion. Scale bar equals 100 μm.

Fig. 16. Planar Araldite section through a 4-day scleral/limb mesenchyme culture. Large areas of chondrogenesis appear interspersed by whorled aggregates which also stain faintly metachromatic. Toluidine blue. Scale bar equals 50 μm.

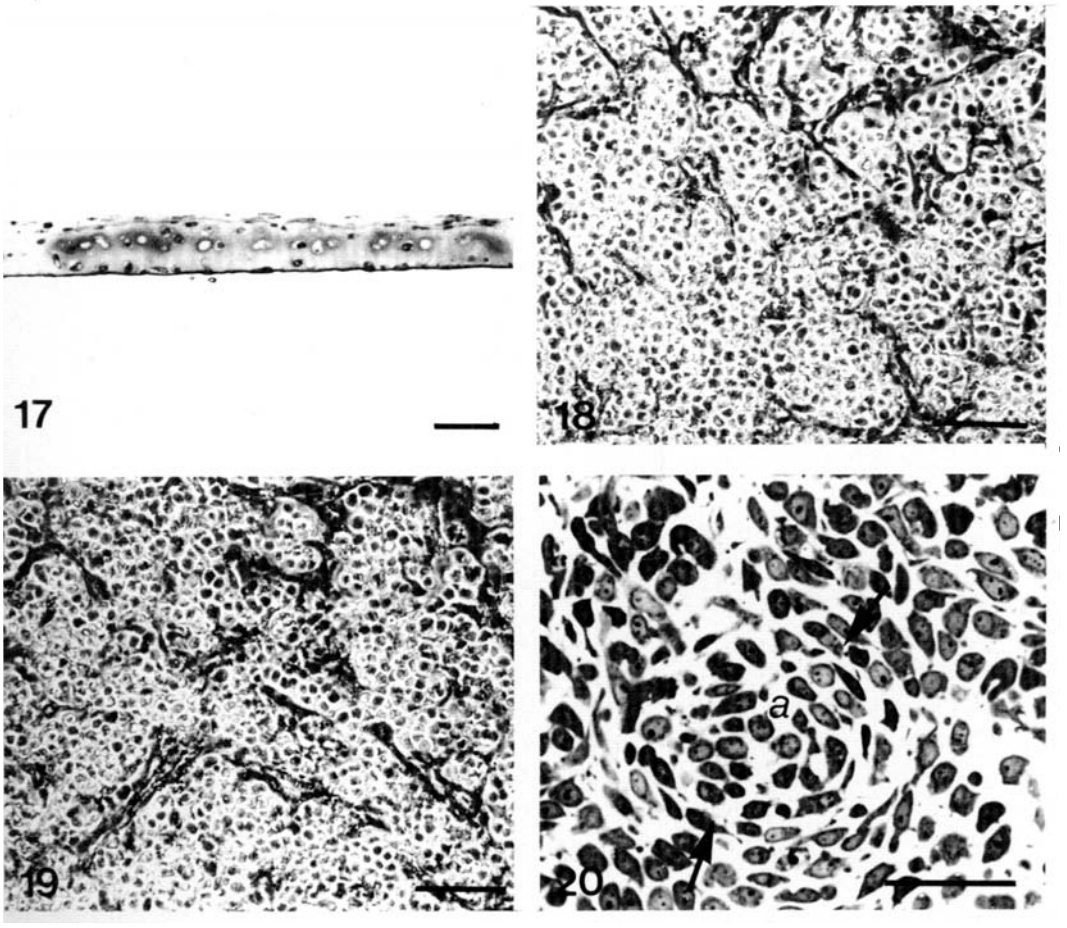

Fig. 17. Transverse section through a scleral chondrocyte/epiphyseal chondrocyte culture after 4 days. A homogenous sheet of cartilage is apparent (compare with Figs 9, 10). Toluidine blue. Scale bar equals 100 μm.

Fig. 18. Phase-contrast image of an epiphyseal chondrocyte/chick heart cell culture after 3 days. The chondrocytes are interspersed by fibroblastic-like cells. Scale bar equals 100 μm.

Fig. 19. Phase-contrast image of a scleral chondrocyte/chick heart cell culture after 3 days. The appearance is very similar for that shown by epiphyseal chondrocytes in Fig. 18. Scale bar equals 100 μm.

Fig. 20. Araldite section through a 3-day limb mesenchyme culture showing the non-oriented central cells of an aggregate (*a*) surrounded by characteristically 'whorled' aggregate cells (arrowed). Scale bar equals 50 μm.

Thorogood, 1983). At the developmental stage used in these experiments it is not difficult to separate this mesenchyme from that giving rise to the musculature of the eye and the fibrous sclera. In this respect, the periocular mesenchyme can be considered a relatively homogenous subpopulation of cells when compared to limb mesenchyme. Therefore, once induced by the apposing pigmented retinal epithelium (Newsome, 1972) plating at high density onto a surface such as tissue

culture plastic mimics the *in vivo* situation. The determined, polymorphic mesoblasts express the chondrogenic phenotype and a flat sheet of cartilage results. Our labelling experiments with antibody to cartilage-specific type II collagen show that in such cultures most or all of the cells react positively and therefore support our interpretation. It is also important to draw attention to the relationship between cell density, cell shape, and phenotypic expression. We and other workers have shown the importance for maintaining a rounded cell configuration in order that determined limb bud mesoblasts can progress to chondrogenesis (Archer, Rooney & Wolpert, 1982; Solursh, Lisenmayer & Jensen, 1982*a*). We have also suggested that the requirement for plating densities above confluence in order that limb bud mesenchyme can chondrify is directly related to the fact that cells plated at such densities remain rounded or polygonal. At lower densities, the cells can flatten, become fibroblastic and thus fail to chondrify.

We would argue that a similar process applies to scleral mesenchyme. When the plating density is dropped below confluence (5×10^4 cells ml^{-1}), some cells flatten and become fibroblastic whilst cells in localized high density areas clump and chondrify, thereby giving rise to the cartilage nodules separated by fibroblastic regions which are observed in medium density cultures. In addition, a similar effect can be obtained if non-chondrogenic cell types (chick heart cells) are added to scleral mesenchyme and plated at high density. Instead of a flat sheet of cartilage, we obtain nodules of cartilage interspersed by fibroblastic-like cells presumably of heart origin. This result implies a degree of cell sorting – a process which we have observed in micromass cultures (Cottrill & Archer, in preparation). In addition, it provides us with a result superficially similar to that shown for whole limb mesenchyme cultures, a consideration of which follows.

Limb mesenchyme

Unlike our isolated periocular mesenchyme, whole limb mesenchyme will give rise to a number of cell types (cartilage, perichondrium, tendon mesoderm and possibly bone together with somite-derived muscle). Likewise, in culture only a certain number of the cells will progress to chondrogenesis as our labelling experiments with collagen type II antibody have shown. The remaining cells are mostly non-chondrogenic and may form the whorled aggregates around the chondrogenic nodules. Indeed, Lewis (1977) has calculated that as few as 5 % of the cells in the chick limb bud will give rise to cartilage. This may explain why Dienstman & Holtzer (1975) found that only 10 % of limb bud cells gave rise to cartilage or muscle elements *in vitro*. We would like to suggest that the characteristic concentric arrangement of cells in these cultures is due to the heterogenous nature of the cells comprising the culture. For instance, it is noticeable that prior to matrix secretion the central cells of an aggregate that will become chondrogenic are not whorled, (Fig. 20 and Solursh *et al.* 1982*b*). It is possible therefore, that the whorl-forming cells are non-chondrogenic, initially at least. There is evidence for such an interpretation.

Cells taken from beneath the apical ectodermal ridge (progress zone) form a sheet of cartilage in the absence of whorling when grown in micromass culture (Archer, Cottrill & Rooney, 1984). However, whorling and cartilage nodule formation can be induced by the addition of non-chondrogenic cell types such as proximally located limb mesoderm (Archer *et al.* 1984) or mature tenocytes (Cottrill & Archer, 1984).

When the cell density of limb bud cultures is reduced to below confluence (5×10^4 cells per culture) then either the prechondrogenic cells flatten and become fibroblastic or the culture conditions select for the non-chondrogenic cell types. However, the paucity of chondrogenesis observed in cultures to which chick heart cells have been combined can only be explained in terms of the relative reduction in prechondrogenic cells present in the cultures. Conversely, the increase in chondrogenesis observed in mixed cultures of limb and scleral mesenchyme may be explained by a relative increase in prechondrogenic cells present.

The whole issue of tissue-specific morphogenesis as proposed by Weiss & Moscona (1958) revolves around the striking similarity between the concentric cell aggregates and subsequent cartilage nodules observed in limb mesenchyme cultures and the concentric cellular orientation seen in limb chondrogenesis when viewed in transverse section (Gould, Selwood, Day & Wolpert, 1974). The crucial question is, are these two events homologous or is the similarity coincidental?

Ahrens *et al.* (1977) demonstrated a stage-related capacity for cartilage differentiation in micromass cultures of limb mesenchyme. Briefly, mesenchyme from stages 19–25 (Hamburger & Hamilton, 1951) formed concentric aggregates but only those aggregates from stages 20–24 progressed to chondrogenesis, unless treated with dibutyryl cAMP (Solursh, Reiter, Ahrens & Vertel, 1981). An important point is that the electron microscope evidence of these authors shows convincingly that aggregation occurs in the absence of cartilage matrix secretion (see also Ede, 1981; Solursh *et al.* 1982*b*) although membrane-associated glycoproteins would probably be present.

The manner in which aggregation occurs is also unclear. Ede using lower density cultures has shown that there is active cell migration towards aggregation points and proposes the existence of 'founder' cells which together with transient differences in cell–cell adhesion are the major factors promoting aggregation (Ede & Agerbak, 1968; Ede, Flint, Wilby & Colquhoun, 1977; Ede, 1983). Further evidence for such a mechanism is that a similar concentric pattern is generated when certain slime moulds aggregate around a central cell (see Ede, 1981). However, it is interesting to note that in both of these cases, aggregation is occurring on what is, essentially, a flat surface. So whether similar mechanisms are operating in a three-dimensional network such as in the limb is questionable. Certainly, when condensation is first observed in the chick wing there is no clear orientation of the cells (Gould, Day & Wolpert, 1972; Thorogood & Hinchliffe, 1975) although a cellular polarity manifested in terms of the position of the Golgi apparatus with respect to the nucleus has been described (Ede *et al.* 1977; Ede & Wilby, 1981; Holmes & Trelstad, 1980).

In contrast to the active migration concept, Gould *et al.* (1972) suggested that condensation occurs through the inability of cells in the core region to move away after division. The increase in fibronectin in the core region around stage 22 (Dessau, von der Mark, von der Mark & Fischer, 1980) might lend support to this interpretation.

An important issue is whether the whorling of the condensation cells *in vivo* is brought about by cellular interactions similar to those in culture. Alternatively, the whorling orientation may be mechanically specified as a result of centrifugal forces generated by matrix secretion as suggested by Gould and his colleagues (Gould *et al.* 1974).

In contrast, Thorogood (Thorogood & Hinchliffe, 1975; Thorogood, 1983) carried out an electron microscopical study of the initiation of chondrogenesis in the stage-26 chick tibia. He found close apposition (but little contact) of oriented cells in the less mature distal region of the condensation thus favouring a cell interaction mechanism. However, a metachromatic intercellular matrix can be detected in the proximal region of a stage-26 tibia and this may easily affect the orientation of cells in the more distal levels of the condensation. Clearly, this is a contentious issue which remains to be resolved.

Differentiated chondrocytes

The formation of a cartilaginous sheet by a homogenous cell population such as scleral mesenchyme has been discussed above. We would also apply a similar explanation to high density cultures of epiphyseal (Solursh & Meier, 1974) and scleral chondrocytes, where the simultaneous secretion of cartilage matrix by almost all the cells on a flat substratum will give rise to a flat sheet. However, when the cell density at inoculation is reduced by such an extent that cells can flatten enough to become fibroblastic, then the cartilage sheet morphology is lost. Instead, groups of cells cluster and remain polygonal and therefore become cartilage nodules which are interspersed by non-chondrogenic fibroblastic-like cells. This is what we found in medium density scleral mesoderm, scleral chondrocytes and epiphyseal chondrocytes. This morphology also bears a resemblance to that found in high density limb mesenchyme where only a certain percentage of the cells will normally become chondrogenic.

In conclusion, cell shape plays a crucial role in the morphogenesis of cartilage *in vitro*. It is the inoculation density at plating, which ultimately controls cell shape, and together with the number of cells 'committed' to the chondrogenic phenotype comprise the major factors determining morphogenesis which therefore may not be necessarily determined on a tissue-specific basis.

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