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Ectodermal inhibition of cartilage differentiation in micromass culture of chick limb bud mesenchyme in relation to gene expression and cell shape

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Summary

Ectoderm inhibits the formation of cartilage by chick wing bud mesenchyme in micromass culture. This suggests that the pattern of cartilage formation in the limb bud may result from a restriction of cartilage cell differentiation to the limb bud core as cells leave the progress zone. We have used *in situ* hybridization to investigate whether ectodermal inhibition in micromass culture occurs at the level of gene transcription. We found that ectoderm completely inhibited the accumulation of cartilage-specific type II collagen transcripts in the mesenchyme cells, whilst the level of type I collagen transcripts was unaffected. Morphometric analysis of electron micrographs revealed that inhibition of chondrogenesis in micromass culture was not preceded by cell flattening. In fact, a rounded cell shape was found not to be a prerequisite for cartilage cell differentiation in micromass.

Key words: chick limb bud, ectoderm, cell shape, cartilage.

Introduction

As the chick wing bud grows out, cartilaginous elements develop within the mesenchyme. They are laid down in a proximodistal sequence; first the humerus, then the radius and ulna, and finally the wrist and hand. This patterning of the cartilaginous elements has been discussed in terms of mechanisms based on both prepattern and positional information (Wolpert & Stein, 1984; Wolpert & Hornbruch, 1987).

One aspect of such mechanisms is the role played by the dorsal and ventral ectoderm in inhibiting cartilage formation; a role which has been suggested by experiments carried out *in vitro*. High-density cultures of limb bud mesenchyme develop cartilaginous nodules (Ahrens *et al.* 1977), but if the mesenchyme cells are taken from the distal end of the limb underlying the apical ectodermal ridge (i.e. from the progress zone), a sheet of cartilage develops (Cottrill *et al.* 1987). When ectoderm is placed on the surface of the micromass, cartilage formation is inhibited in the region both below and adjacent to the ectoderm (Solursh *et al.* 1981).

Solursh (1984) has suggested that this inhibition provides a means of confining the cartilage to the core of the limb bud. However, Martin & Lewis (1986) have removed up to 70% of the dorsal ectoderm by u.v. irradiation, leaving the apical ectodermal ridge intact, and yet the resulting cartilaginous pattern was normal. A possible explanation for this apparent contradiction might be found in a prepattern model for cartilage specification based on a reaction – diffusion mechanism (Wolpert & Murray, unpublished). The model requires the inhibitory effect of the ectoderm, but the interactions are such that removal of the ectoderm from one side still permits a normal pattern to develop.

The inhibition of cartilage by ectoderm in micromass cultures allows us to investigate the role of cell shape in regulating cartilage differentiation. It has been proposed that a rounded cell shape may be a trigger for cartilage differentiation in limb bud mesenchyme (Archer *et al.* 1982; Solursh *et al.* 1982). In addition, Zanetti & Solursh (1986) have suggested that the ectoderm exerts its inhibitory effect by causing cells to flatten. Studies with mature chondrocytes suggest that flattened cells do not synthesize cartilage-specific type II collagen (Benya & Shaffer, 1982). We have investigated the relationship between cartilage differentiation and cell shape and in particular whether cell shape change precedes or is a consequence of the ectodermal inhibition of cartilage formation.

It is important to know how ectoderm inhibits the development of cartilage. It may act by altering cartilage-specific gene expression, or by affecting the assembly or stability of the cartilage matrix. Using specific antisense RNA probes we have recently studied the spatial distribution of type I(α2) and type II(α1) collagen transcripts during chick limb bud development by *in situ* hybridization (Devlin *et al.* 1988). High levels of type II collagen transcripts map closely with the development of cartilage, suggesting that type II col-

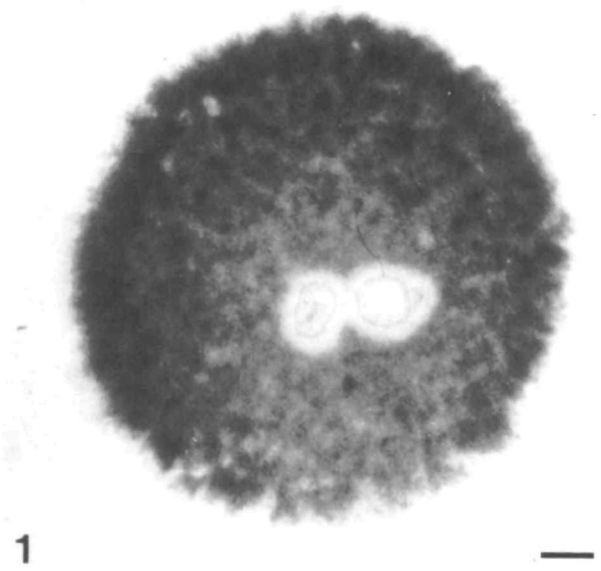

Fig. 1. Wholemount of micromass culture with 2 pieces of ectoderm grafted on day 1. Culture stained with Alcian blue on day 4. Bar, 500 μm.

lagen synthesis is controlled principally at the level of transcription or mRNA accumulation. Type I collagen synthesis seems by contrast to be controlled at the level of translation or of RNA processing. We have now used these probes to study the inhibition of cartilage formation by ectoderm in micromass.

Materials and methods

Preparation of micromass cultures

Fertilized White Leghorn × Sykes Tinted eggs obtained from Poyndon Farm, Waltham Cross, Herts were incubated at 38 ± 1 °C until stages 22–23 (Hamburger & Hamilton, 1951). Wing buds were dissected in calcium-/magnesium-free saline (CMF) with 10 % newborn calf serum (Gibco). Using an eyepiece graticule, 300 μm was measured from the wing tip along the proximodistal axis. The distal tip (i.e. the progress zone) was excised with a single cut. Limb pieces were placed in 2 % trypsin in CMF at 4 °C for 15 min, washed and the ectoderm removed using tungsten needles. The mesenchyme cells were gently dissociated in 1–2 ml of medium to produce a single-cell suspension. Cell density was determined using a haemocytometer and cells were suspended at 2×10^7 cells per ml in a mixture of 60 % culture medium (Nutrient Mixture F-12 Ham with 10 % fetal calf serum, 1·4 mM-L-glutamime, 1 % antibiotic–antimycotic [all from Gibco] and 200 μg ml^{-1} ascorbic acid [BDH]) with 40 % CMF containing 10 % newborn calf serum. A single 10 μl drop of this suspension was plated on to each 35 mm tissue culture dish (Sterilin) and incubated at 37 °C in an atmosphere of 5 % CO_2/95 % air in a humidified incubator (Forma Scientific USA). Cells were allowed to attach for 50 min and then flooded with 1–2 ml of culture medium. Cultures received fresh medium daily.

Preparation and grafting of ectoderm on to micromass cultures

Anterior, posterior and distal edges of the donor limb buds were cut off using a cataract knife. The remaining central limb pieces, with only dorsal and ventral ectoderm, were treated with 2 % trypsin in CMF at 4 °C for 15 min. After washing in culture medium, each limb piece was put into a culture dish which had been previously cooled to 4 °C. The ectoderm was peeled off cleanly using tungsten needles and positioned on the micromass, taking care to keep it unfolded. The culture medium was removed and the cultures returned to the incubator for 4–6 h before reflooding with medium.

Histology for wholemount, light and electron microscopy

Cultures for wholemount were fixed in 1/2-strength Karnovsky's fixative (Karnovsky, 1965) at 4 °C for 2 h and then stained with Alcian blue pH 1 (Luna, 1968), dehydrated and cleared in glycerol.

Micromasses for light microscopy were fixed in 1/2 strength Karnovsky's as above. They were then dehydrated and embedded in Araldite. Sections of 1 μm were cut, either perpendicular or parallel to the culture dish, and stained with toluidine blue.

Micromasses for electron microscopy were prepared according to a modification of the procedure described by Hunziker *et al.* (1982). Prior to fixation, some cultures were detached from the culture dish using a piece of silastic to ensure even penetration of fixative. Micromasses were fixed first in 2 % (v/v) glutaraldehyde and 0·7 % (w/v) ruthenium hexammine trichloride (Johnson Matthey Chemicals) in 0·05 M-sodium cacodylate (pH 7·4) for 30 min, followed by three washes in 0·05 M-sodium cacodylate (pH 7·4). They were then fixed in 1 % (w/v) osmium tetroxide and 0·7 % (w/v) ruthenium hexammine trichloride in 0·1 M-sodium cacodylate (pH 7·4) for 30 min and finally washed three times in 0·1 M-sodium cacodylate (pH 7·4). All fixative and washing solutions had an osmolarity of 330 mosmol and were used at room temperature. Fixative solutions were made up fresh and were left to stand for 1 h to allow the pH to stabilize to 6·75. After fixing, micromasses were dehydrated and embedded in Araldite. Perpendicular sections for electron microscopy were cut at 80–90 nm. Grids were stained with either 4 % aqueous uranyl acetate followed by lead citrate, or a saturated solution of uranyl acetate in absolute ethanol followed by lead citrate. Sections were viewed using a Philips EM 300 at 60 kV.

Cell shape determination

Cell shape was determined from electron micrographs by measuring the maximum horizontal and vertical cell lengths of nucleated cells, and is expressed as:

$$\text{index of asymmetry} = \frac{\text{maximum horizontal cell length}}{\text{maximum vertical cell length}}$$

Statistical analysis was done using a Student's *t*-test.

Probe preparation for in situ *hybridization*

^{32}P-labelled antisense RNA probes, which we have previously shown to be specific for chicken type I collagen or type II collagen transcripts, were prepared as described by Devlin *et al.* (1988). ^{32}P-labelled sense RNA probes for use as negative controls were synthesized using the same templates.

In situ *hybridization, tissue preparation and detection*

Micromasses were fixed in fresh PBS containing 4 % (w/v) paraformaldehyde and were wax embedded. Perpendicular sections were cut at 7 μm. Each slide contained sections of 10-day embryo leg as a hybridization control, as well as micromass sections. *In situ* hybridization was performed as described by Devlin *et al.* (1988).

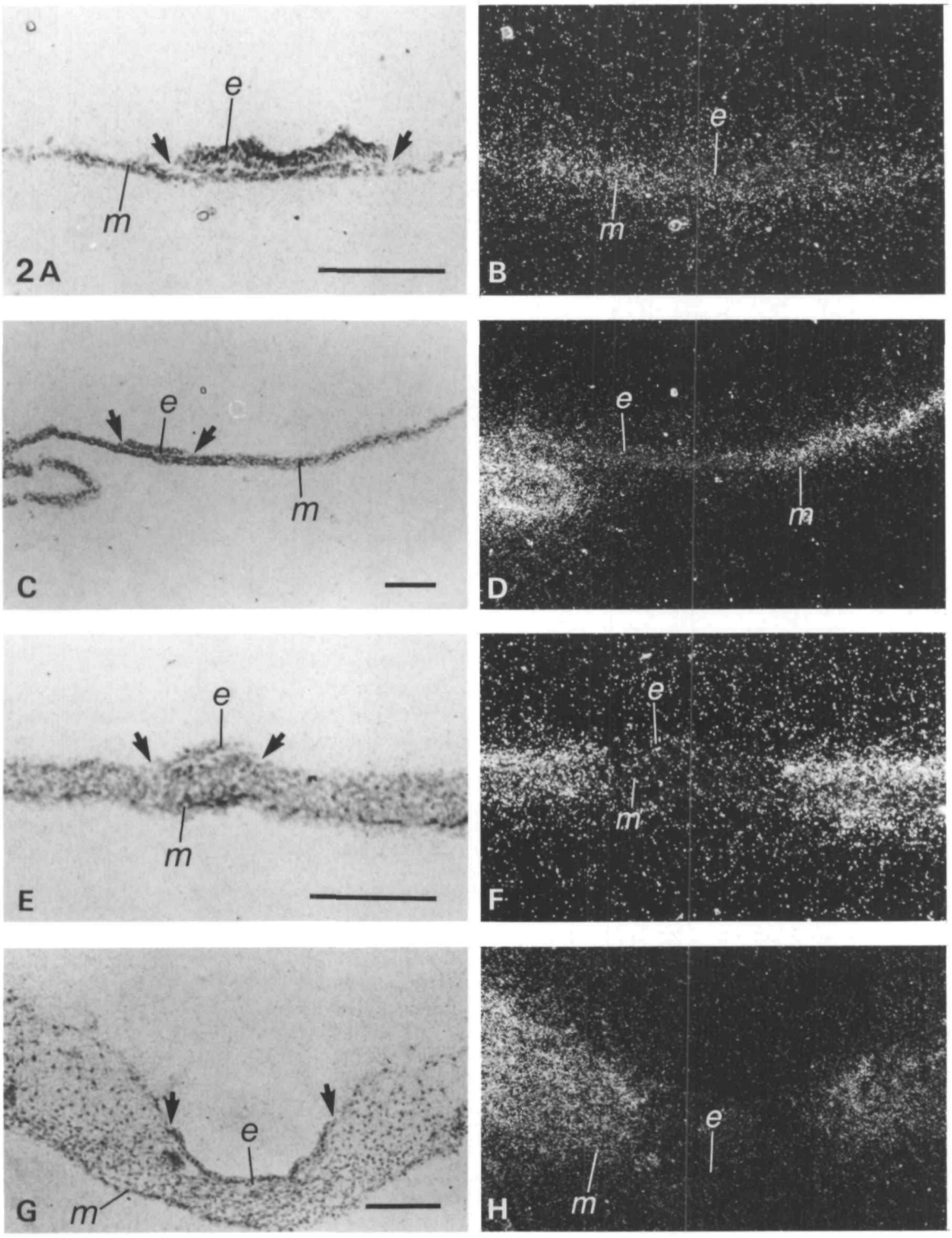

Fig. 2. Localization of type II collagen transcripts in micromass cultures with ectoderm grafts, using a type II collagen-specific antisense RNA probe. (A), (C), (E) and (G) are bright-field photographs of perpendicular sections; (B), (D), (F) and (H) are dark-field photographs of the same sections. Sections are of 1-day micromass cultures, grafted with ectoderm and sectioned 3 hr (A,B), 1 day (C,D) and 2 days (E,F) later, and of a 3-day micromass culture grafted with ectoderm and sectioned 4 days later (G,H). m, micromass; e, ectoderm graft; arrows indicate edges of ectoderm graft. Bar, 100 μm.

Table 1. *Localization of type II collagen transcripts in micromass cultures with ectoderm grafts, using a type II collagen-specific antisense RNA probe and a control sense probe*

Age of micromass (no. cultures; no. sections)	Time since ectoderm grafting	Probe	Grains per cell ± 1 S.D.		
			Under ectoderm	Adjacent to ectoderm[a] (Pa)	Distant from ectoderm[b] (Pb)
1 day (2; 10)	3 hr	Antisense	3·28 ± 1·09	4·08 ± 0·87 ($P < 0·002$)	4·43 ± 0·96 ($P < 0·05$)
2 days (2; 12)	1 day	Antisense	2·90 ± 0·82	3·52 ± 1·17 ($P > 0·05$)	9·34 ± 2·85 ($P < 0·001$)
2 days (2; 10)	1 day	Sense	0·34 ± 0·32	0·29 ± 0·33 ($P > 0·1$)	0·29 ± 0·34 ($P > 0·1$)
4 days (2; 12)	3 days	Antisense	3·79 ± 2·99	4·38 ± 2·31 ($P < 0·1$)	10·85 ± 5·31 ($P > 0·001$)
7 days (2; 12)	4 days	Antisense	3·26 ± 2·18	8·66 ± 3·56 ($P < 0·001$)	10·00 ± 5·03 ($P > 0·1$)
7 days (2; 10)	4 days	Sense	0·17 ± 0·14	0·14 ± 0·13 ($P > 0·1$)	0·13 ± 0·09 ($P > 0·1$)

[a] 2–3 cell diameters from the edge of the ectoderm graft.
[b] >20 cell diameters from the edge of the ectoderm graft.
Pa paired sample t-test: grains per cell adjacent to[a] edge of ectoderm graft significantly different from grains per cell under ectoderm graft.
Pb paired sample t-test: grains per cell distant from[b] edge of ectoderm graft significantly different from grains per cell adjacent to[a] ectoderm graft.

Results

Description of wholemounts of micromass cultures grafted with ectoderm

After 1 day of culture, phase-contrast microscopy of living progress zone micromass cultures revealed a uniform pavement of polygonal cells with a narrow rim of flattened cells. After 2 days the cells were closely packed and optically dense extracellular matrix containing type II collagen (Cottrill *et al.* 1987) was present throughout the culture. The micromass stained uniformly with Alcian blue on days 3 and 4.

Ectoderm from stage-24 limbs was placed on to 1-day micromass cultures, which were then fixed at 3 h, 1 day, 2 days or 3 days after ectoderm grafting. 1 day after grafting, phase-contrast microscopy of wholemounts revealed no obvious difference in cell morphology or matrix deposition between cells in the vicinity of the ectoderm and cells in the main culture. 2 days after grafting, the mesenchyme cells directly underneath and within 100–200 μm of the ectoderm edge were clearly different from those in the main culture. They had lost their polygonal morphology and those around the edge of the ectoderm appeared to be whorled. Alcian blue staining showed that there was no cartilage matrix underneath and surrounding the ectoderm. 3 days after ectoderm grafting, Alcian blue staining showed a sharply defined region of cartilage inhibition (Fig. 1), which did not change with further culture. These observations confirm those of Solursh *et al.* (1981).

When stage-24 ectoderm was placed on to 2-day micromass cultures, the inhibition of cartilage was essentially as described above. However, when stage-24 ectoderm was placed on to 3-day cultures, there was no change in matrix 1 day later, as judged by Alcian blue staining. After 4 days there was some reduction directly beneath the ectoderm but the peripheral inhibition and cell whorling were completely absent.

Localization of type I and type II collagen transcripts by in situ *hybridization*

After 1 day of culture, type II collagen transcripts were easily detectable in the micromass using the type II collagen-specific antisense probe (Fig. 2A,B; Table 1). There was a slight but significant depression of the levels of type II collagen transcripts directly beneath the ectoderm, which was grafted 3 h prior to fixation (Fig. 2A,B; Table 1). No hybridization was seen with the type II collagen control sense probe at this time (data not shown), or in older cultures (Fig. 3A,B; Table 1). After 2 days of culture (i.e. 1 day after ectoderm grafting), there were high levels of type II collagen transcripts in the main culture, but underneath and immediately surrounding the ectoderm the levels were significantly reduced (Fig. 2C,D; Table 1). 2 days and 3 days after ectoderm grafting, hybridization to cells underneath and immediately surrounding the ectoderm graft was at background levels (Fig. 2E,F; Table 1).

When ectoderm was grafted on to a 3-day culture, a less-well-defined inhibition of type II collagen transcripts was detected on day 7. Cells right up to the edge of the ectoderm contained levels of type II collagen transcripts similar to those of the main culture. However, directly underneath the ectoderm the level of type II collagen transcripts was reduced (Fig. 2G,H; Table 1).

Very low levels of type I collagen transcripts were detected in micromasses cultured for 2, 3 and 4 days, using the type I collagen-specific antisense probe (Fig. 3C,D and data not shown), although these were above the background levels seen in these cultures with

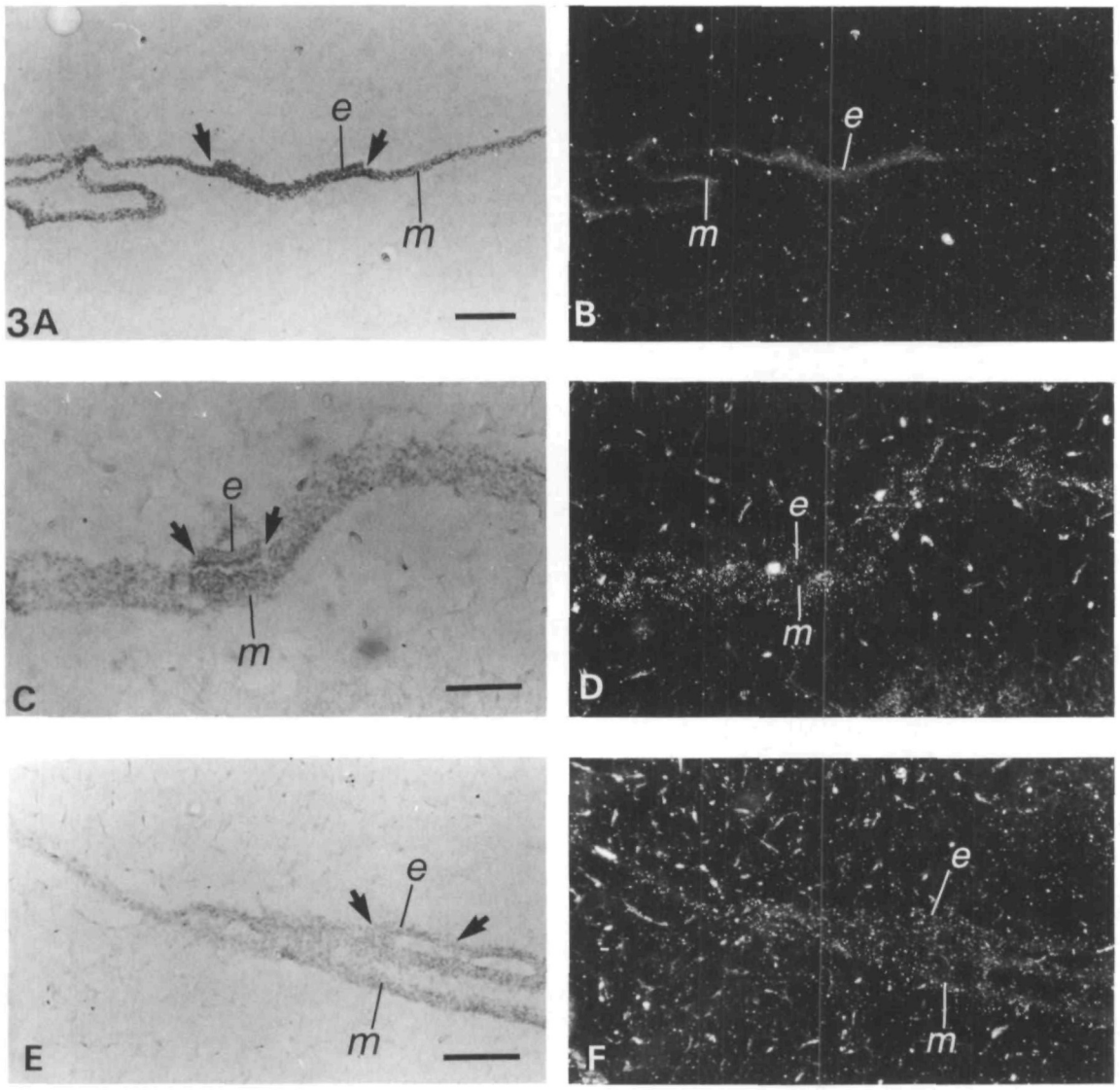

Fig. 3. Control sections of micromass cultures with ectoderm grafts. (A), (C) and (E) are bright-field photographs of perpendicular sections; (B), (D) and (F) are dark-field photographs of the same sections. Sections are of 1-day micromass cultures, grafted with ectoderm and sectioned 1 day (A,B,E,F) or 2 days (C,D) later. Sections have been hybridized with a type II collagen sense probe (A,B), a type I collagen-specific antisense probe (C,D) and a type I collagen sense probe (E,F). m, micromass; e, ectoderm graft; arrows indicate edges of ectoderm graft. Bar, 100 μm.

the type I collagen control sense probe (Fig. 3E,F and data not shown). Ectoderm grafted on to 1-day cultures did not affect the levels of type I collagen transcripts in micromass cells in its vicinity after 1, 2 or 3 days of further culture (Fig. 3C,D and data not shown).

The specificity of the probes for type I or type II collagen was checked by the inclusion on every slide of transverse sections of 10-day chick embryo limb. The type II collagen-specific probe hybridized only to cartilage, whilst the type I collagen-specific probe hybridized only to the perichondrium and to tendons, as we have described previously (Devlin *et al.* 1988). Neither sense probe hybridized above background, as we have shown previously (Devlin *et al.* 1988).

Histology of micromass cultures

Electron microscopy of sections stained with ruthenium red showed that as early as day 1 of culture a significant amount of matrix had been secreted by the micromass cells. Each cell had a pericellular coat rich in proteoglycans (data not shown). By the second day of culture the amount of extracellular matrix had increased. Sections through the micromass showed that matrix was most abundant in the middle level of the culture (Fig. 4A). Collagen fibrils were present, orientated in all planes. Some cells, particularly those in the top two layers of the micromass, were tightly apposed, separated only by their pericellular coats. All of the cells looked flat and this was confirmed by measurements of the maximum

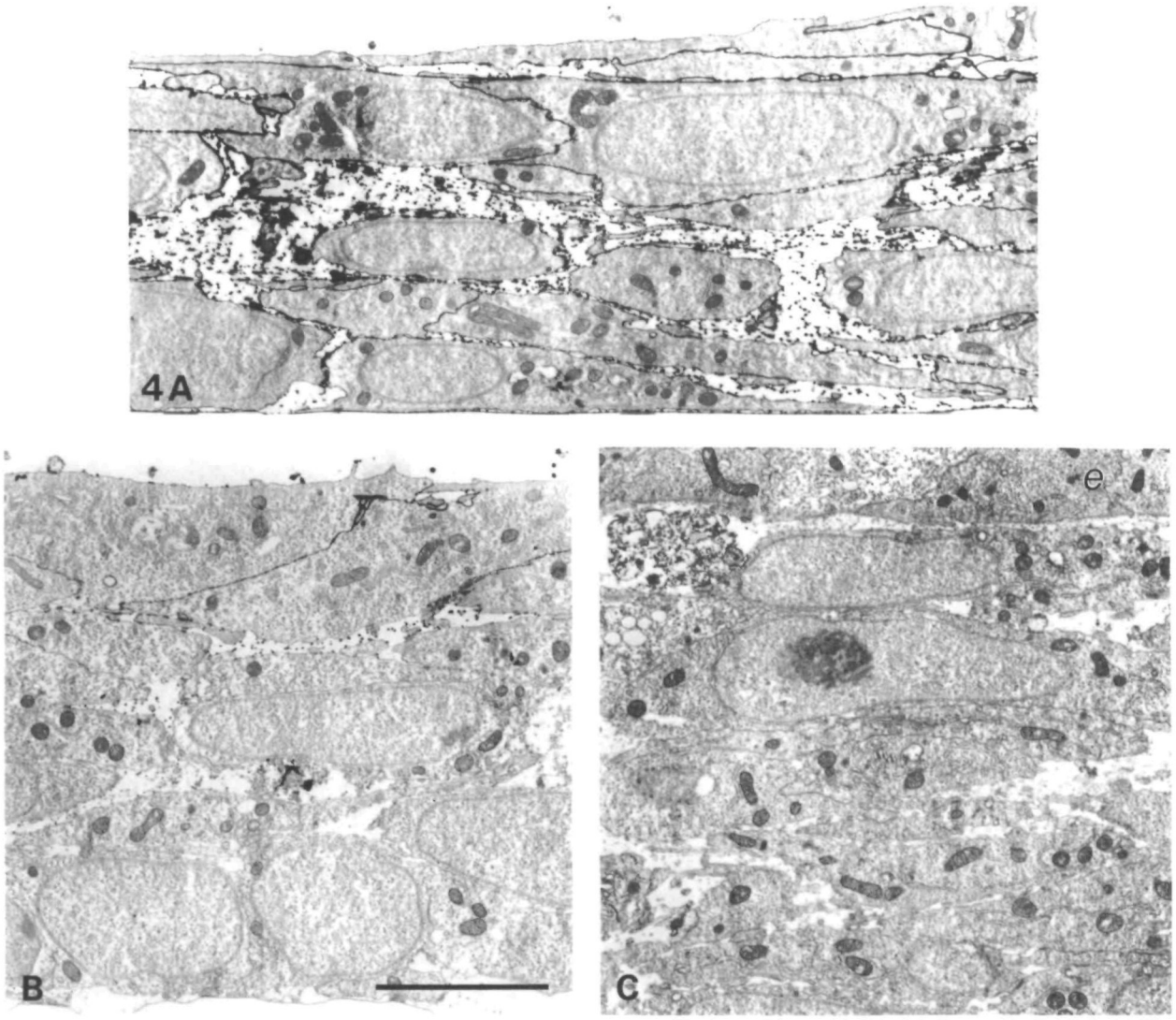

Fig. 4. Electron micrographs of ruthenium red-stained sections of 2-day micromass cultures after ectoderm graft on day 1. (A) Cells in the main culture. (B) Cells approximately 100 μm from the edge of the ectoderm. (C) Cells underneath the ectoderm (e). Bar, 5 μm.

horizontal and vertical cell dimensions which produced a mean index of asymmetry of 4·68 ± 1·8 (Table 2). Few cell processes and no specialized cell junctions were seen. After 3 days of culture the amount of extracellular matrix had increased. However, the cells were still flat and the mean index of asymmetry was not significantly different from that at 2 days (3·89 ± 1·7; Table 2). By the fourth day of culture there was extensive cartilage matrix and the cells were typical rounded chondrocytes (Fig. 5A) with a mean index of asymmetry of 1·57 ± 0·71 (Table 2). The cells had prominent Golgi and abundant rough endoplasmic reticulum. Some were vacuolated and contained lipid.

3 h after placing ectoderm on to a 1-day micromass culture, the cells underneath and adjacent to it were indistinguishable from those in the main culture. 1 day later the amount of extracellular matrix underneath the ectoderm and up to approximately 100 μm from its edge, was drastically reduced (Figs 4B and 4C). The cells lacked a proteoglycan-rich coat, and the small amount of proteoglycan present was predominantly in the extracellular space. Apart from sparsely distributed collagen fibrils most of the extracellular space appeared empty, though it presumably contained unstained hyaluronic acids. The ectoderm had a poorly defined basement membrane and was underlaid by a network of collagen fibrils, orientated parallel to the ectoderm. Directly underneath the ectoderm there was some cell death in the top two layers of the micromass. The living cells beneath the ectoderm were significantly less flattened than cells in the main micromass (Table 2). Sections cut parallel to the ectoderm showed that these cells were rounded, whereas the chondrifying cells were polygonal. They had few filopodia and there was little cell–cell contact.

2 days after ectoderm grafting a continuous basement membrane was present and there was no longer any evidence of cell death at the ectodermal–mesodermal

Table 2. *Cell shape of micromass cells, as determined by their mean index of asymmetry, 1, 2 and 3 days after an ectoderm graft on a day-1 micromass culture*

Age of micromass (no. sectioned cultures)	Time since ectoderm grafting	Mean index of asymmetry ± 1 S.D.* (no. cells measured)	
		Main micromass	Under ectoderm
2 days (3)	1 day	4·68 ± 1·8 (75)	3·29 ± 1·2† (96)
3 days (2)	2 days	3·89 ± 1·7 (44)	4·40 ± 1·6†,‡ (36)
4 days (2)	3 days	1·57 ± 0·71§ (66)	2·87 ± 1·4†,§ (93)

* Index of asymmetry = $\frac{\text{maximum horizontal cell length}}{\text{maximum vertical cell length}}$

(round cell: index of asymmetry = 1).

† Students *t*-test: cells in main micromass significantly different from cells under ectoderm $P < 0{\cdot}001$.

‡ Students *t*-test: cells in 2 day culture significantly diferent from cells in 4 day culture $P < 0{\cdot}001$.

§ Students *t*-test: cells in 3 day culture significantly different from cells in 4 day culture $P < 0{\cdot}001$.

interface. The micromass cells underneath and surrounding the ectoderm were very closely packed and the cells were significantly more flattened than in younger cultures (Table 2). The scant matrix in the reduced extracellular spaces consisted of collagen fibrils running parallel to the basement membrane with little associated proteoglycan. Although matrix was present throughout the culture depth there was more close to the ectoderm. Planar sections showed that directly beneath the ectoderm the cells were not stellate with long filopodia which make contact with adjacent cells. Cells surrounding the ectoderm were elongated with long cell processes and were orientated around its edge.

3 days after ectoderm grafting many of the affected cells were separated by extracellular spaces. However, there were still regions in which cells were closely packed (Fig. 5B). The amount of matrix had increased, consisting of short bundles of collagen fibrils but little proteoglycan. The structure of the ectoderm in Fig. 3B was typical of that found throughout our experiments and was different from that found in the embryo. The upper cell layer, rather than being a typical squamous periderm, consisted of cuboidal cells. There were no extracellular spaces in the lower cell layer.

Discussion

Our results show that ectoderm inhibits cartilage formation in micromass cultures when grafted after 1 or 2 days of culture. This confirms the findings of Solursh *et al.* (1981). At 1 and 2 days after the grafting of a small piece of ectoderm, there was a striking lack of matrix in the region beneath and adjacent to the ectoderm. The *in situ* hybridization experiments show that there is inhibition of cartilage-specific type II collagen transcription and/or mRNA accumulation in this region. This inhibition was detectable 3 h after grafting, and was very pronounced in older cultures. These observations show quite clearly that ectoderm exerts an effect on cartilage differentiation at the level of gene expression and not simply at the level of matrix assembly or destruction.

Ectoderm has a decreased inhibitory effect on micromass cells which have been cultured for 3 days. It produces a reduction of type II collagen transcripts only directly underneath it and does not affect the surrounding cells at all. These results suggest that the ectoderm acts upon early labile limb bud cells (even after they have initiated cartilage differentiation) in part by directly or indirectly inhibiting type II collagen transcription and/or mRNA accumulation. However, older cells which have become differentiated chondrocytes are more resistant to such inhibition. This is consistent with our unpublished observations that micromasses made from proximal regions of the limb bud are much less sensitive to inhibition by ectoderm if it is grafted later than day 1 of culture, whereas micromasses made with distal cells are still affected after 2 days of culture. The inference is that proximal cells have become irreversibly committed to cartilage differentiation by day 2.

Zanetti & Solursh (1986) have suggested that ectodermal inhibition of chondrogenesis might be mediated by a change in cell shape. However, our results show that the cell shape change occurs after a reduction in the level of type II collagen transcripts is observed. This supports the findings of Horton & Hassell (1986), who showed that retinoic acid treatment of chondrocytes in culture directly inhibits the synthesis of cartilage-specific components, with changes in cell shape being secondary. Thus, in a 2-day micromass culture on to which ectoderm was placed on day 1, the cells of the main micromass were flattened with an index of asymmetry of 4·7. Beneath the ectoderm, the cells were more rounded, with an index of asymmetry of 3·3 (Table 2). Although cells in the vicinity of the ectoderm were more flattened by the second day after ectoderm grafting their index of asymmetry was not significantly different from that of cells in the main micromass. Only at 3 days after ectoderm grafting were those cells influenced by the ectoderm significantly more flattened than the now rounded chondrocytes of the main culture. Rather than cell flattening being a signal for inhibition of cartilage differentiation our results suggest that the shape of cells affected by the ectoderm is a reflection of the amount of extracellular matrix associated with them. Cells were flattest 2 days after ectoderm grafting when there was least matrix and most rounded 3 days after ectoderm grafting when there was most matrix (Table 2).

Zanetti & Solursh (1986) found that limb bud cells cultured on collagen gels preconditioned by ectoderm did not synthesize type II collagen protein and were flat, whereas cytochalasin treatment, which caused the cells to round up, removed this inhibition. We can only assume that the effect of cytochalasin is not mediated directly by change in cell shape but acts by some other means. Brown & Benya (1988) have recently shown

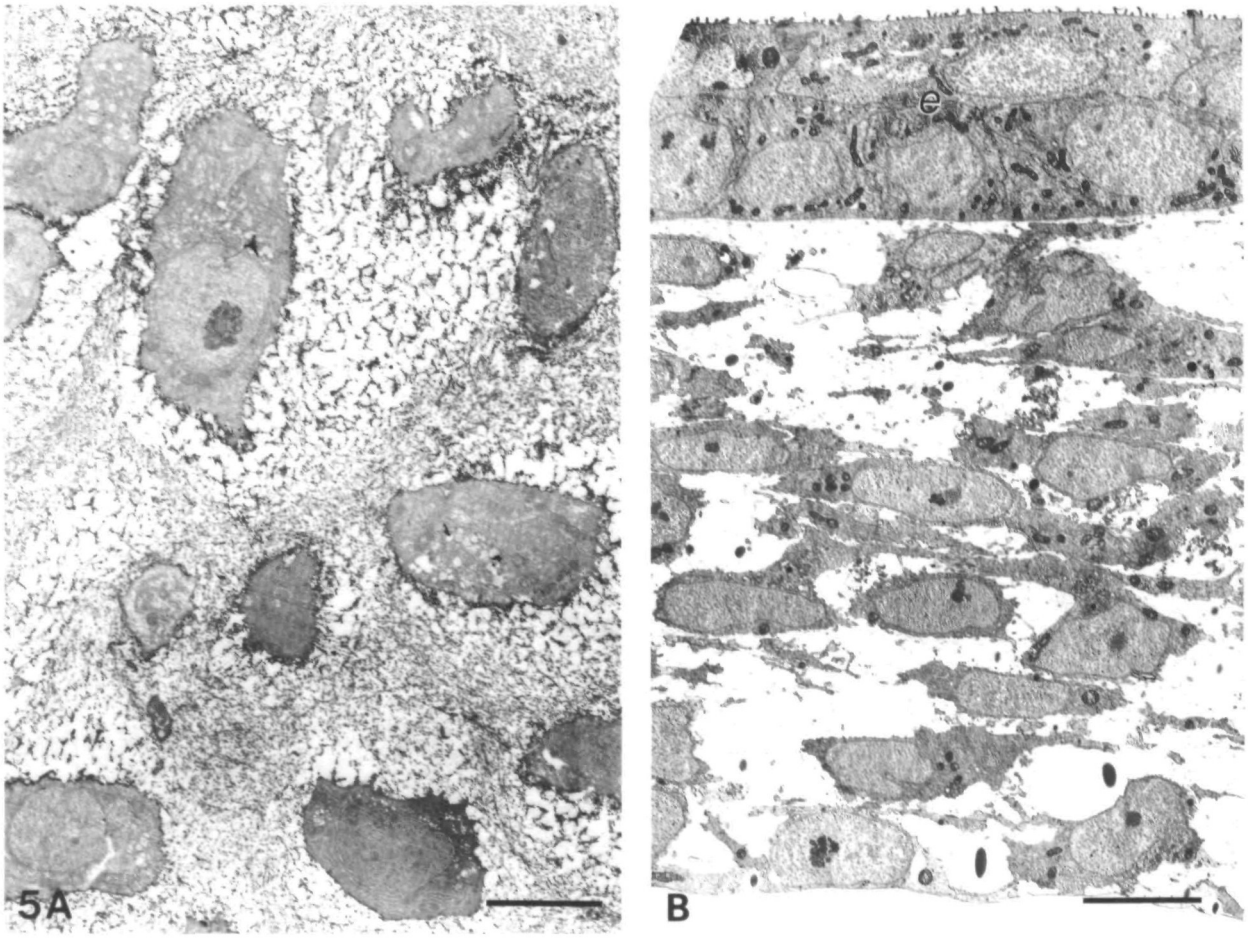

Fig. 5. Electron micrographs of Ruthenium Red-stained sections of 4-day micromass cultures after ectoderm graft on day 1. (A) Cells in the main culture. (B) Cells underneath the ectoderm (e). Bar, 5 μm.

that cytochalasin treatment produces changes in the cytoskeleton which are associated with cartilage differentiation, without a change in cell shape.

The degree of cell flattening in the chondrifying micromass was surprising, since the cells were already making matrix and contained quite high levels of type II collagen transcripts from as early as day 1 of culture. These observations are quite contrary to the idea that cells must be round in order to initiate cartilage differentiation (Archer *et al.* 1982; Solursh *et al.* 1982) and support the earlier finding of Von Der Mark *et al.* (1977) that there is no strict correlation between cell morphology and type of collagen synthesized. By 2 days of culture, micromass cells synthesize type II collagen (Cottrill *et al.* 1987), although they are still very flattened. Only after 4 days of culture, when extensive cartilage matrix has been deposited, are the cells rounded. Thus cell rounding of chondrocytes in micromass is correlated with an increasing amount of extracellular matrix.

In normal chick limb development the presumptive cartilage cells are certainly rounded at the time of condensation (Thorogood & Hinchliffe, 1975), prior to the localization of high levels of type II collagen transcripts in the limb bud core (Devlin *et al.* 1988). This is at variance with the behaviour of limb cells in micromass. However, during the development of the cartilaginous vertebral centrum the cells are not rounded prior to cartilage differentiation (Weale, 1985).

Our *in situ* hybridization experiments show that there is a uniformly low level of type I collagen transcripts throughout progress zone micromass cultures. Ectoderm does not increase the level of type I collagen transcripts in the adjacent mesenchyme cells. This is consistent with our previous finding that type I collagen transcripts are uniformly distributed throughout the chondrogenic core and the nonchondrogenic periphery of the limb (Devlin *et al.* (1988). Neither transcriptional nor translational regulation of type I collagen gene expression seems to be involved in the localization of cartilage to the limb bud core.

The nature of the ectodermal inhibitory signal remains as yet unknown. We are currently directing our attention to this.

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