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Induction of bone by epithelial cell products

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SUMMARY

The bones of the head and face of vertebrate embryos only form after their progenitor cells have undergone an inductive interaction with embryonic epithelia. We have investigated whether epithelial cell products can substitute for epithelia in allowing mandibular ectomesenchyme to form bone. Mandibular epithelia from embryonic chicks were cultured on Millipore filters for 28 days to allow them to deposit an extracellular matrix, shown by electron microscopy to be a basal lamina-like material. Mandibular ectomesenchymal cells formed bone when placed on to these epithelial extracellular products and grafted to chorioallantoic membranes of host embryos. Treatment of epithelial cultures with trypsin or L-azetidine-carboxylic acid removed both the extracellular products and their ability to induce bone formation. Hyaluronidase treatment did neither. We concluded that a proteinaceous component of epithelial basal lamina provides a sufficient inductive stimulus to initiate differentiation of bone within mandibular ectomesenchyme.

INTRODUCTION

As in the induction of most, if not all, tissues of vertebrate embryos, epigenetic factors are required before membrane bones of the embryonic craniofacial skeleton can differentiate.

Bones of the avian mandible will not form unless mandibular mesenchyme (a neural crest derivative) interacts with the inductively active mandibular epithelium until four days of incubation (Tyler & Hall, 1977). Similar inductive interactions have been shown to be required for osteogenesis to begin in the maxillary, palatine, scleral and cranial skeletons of the embryonic chick (Schowing, 1968; Hall, 1978*a*, 1981*a*; Tyler, 1980, 1981; Tyler & McCobb, 1980), and in the mandible and calvarium of the foetal mouse (Hall, 1980*b*, *c*). Reviews and discussions of these data have recently been published or are in press (Hall, 1980*b*, *c*, 1981*b*, *c*, 1982*a*, *b*).

The use of recombinations between mandibular mesenchyme and epithelia from mandible, maxilla, sclera or limb bud, have shown that 'foreign' epithelia can allow mandibular mesenchyme to form bone (Hall, 1978*a*, *b*, 1981*a*; Tyler, 1980, 1981; Tyler & McCobb, 1980).

In an effort to identify the mode of action of these osteogenically inductive

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epithelia we have shown that their inductive ability is correlated with viability and mitotic activity (Hall, 1980*a*) and that pharmacological suppression of epithelial collagen and/or glycosaminoglycans renders them inductively inactive (Bradamante & Hall, 1980).

To further analyse these interactions, we have carried out two sets of experiments – one utilizing transfilter tissue recombination and the other, reported here, to determine whether the extracellular products of cultured epithelial cells contain inductive activity, which they do. Ultrastructural evidence localizes this inductive activity within basal lamina-like material deposited by epithelia *in vitro*.

MATERIALS AND METHODS

Incubation of eggs

Fertilized eggs were obtained from Cook's Hatchery, Truro, Nova Scotia and incubated for four days in a forced-draught incubator at 37 ± 0·5 °C and 55 ± 4 % R.H.

Isolation and culture of epithelia

Embryos were removed from eggs under sterile conditions and staged according to the morphological series of stages of Hamburger & Hamilton (1950). Embryos of H.H. stages 21 and 22 were used.

Mandibles were dissected from these embryos and separated into their epithelial and ectomesenchymal components using the trypsin–pancreatin digestion procedure described earlier (Hall, 1980*a*). Epithelia were placed on to squares of sterile, black Millipore filters (0·45 μm porosity, 125 μm thick, Millipore Filter Corp., Montreal, Quebec) and cultured on stainless steel mesh grids in 30 mm diameter Falcon plastic petri dishes. Each dish contained 1·5 ml of the synthetic culture medium BGJ_b, supplemented with 15 % horse serum (both from GIBCO, Grand Islands, N.Y.) and ascorbic acid (150 μg/ml). These cultures were maintained in a Forma Scientific CO_2 incubator in a humidified atmosphere of 5 % CO_2 in air at a temperature of 37 °C. The culture medium was completely changed every three days and the cultures maintained for up to 28 days. Empty Millipore filters were cultured as a control for the accumulation of components from the medium.

Association of ectomesenchyme with cultured epithelia

After various times *in vitro*, epithelial cells, or the products which they had deposited into the Millipore filters, were tested for their osteogenic inductive activity by associating them with mandibular ectomesenchyme freshly isolated from H.H. stage-21 and -22 embryos. Some epithelial cultures were used without further treatment. These provided a substrate of intact epithelial cells on to which ectomesenchyme was placed. Other epithelial cultures were treated with distilled water for one hour at 37 °C to lyse the epithelial cells. These filters were

rinsed in culture medium and used as a substrate of epithelial extracellular material on to which ectomesenchyme was placed.

A further set of epithelia was cultured for 28 days and then exposed to: (*a*) 26 mg trypsin/ml of medium for 1 h at 4 °C; (*b*) 0·5 or 1·0 mg bovine testicular hyaluronidase or 10 T.R.U. *Streptomyces* hyaluronidase/ml of medium for 2 h at 37 °C, or (*c*) 300 μg L-azetidine–2-carboxylic acid (LACA)/ml of medium either overnight or for 2½ days. After rinsing in culture medium, these treated epithelial cells were also used as substrates for mandibular ectomesenchyme. Controls consisted of epithelial cells maintained in distilled water for times and at temperatures as in (*a–c*) above.

Ectomesenchyme was obtained from mandibles of H.H. stage-21 and -22 embryos using the enzymic procedures referred to above. Ectomesenchyme from the mandible of a single embryo was placed: (*a*) on to the epithelial cells; (*b*) on to filters from which epithelial cells had been removed by lysis; (*c*) on to filters which had been cultured without epithelial cells, or (*d*) on to filters whose epithelial cells had been treated with trypsin, hyaluronidase, LACA or distilled water. These tissue assemblies were grafted on to the chorioallantoic membranes of 8-day-old host embryonic chicks as previously described (Hall, 1978*c*).

Tissue recovery and histology

After 8 days as chorioallantoic grafts, the tissues were recovered, fixed in neutral buffered formol saline, processed for histology, serially sectioned at 6 μm, stained with haematoxylin, alcian blue and chlorantine fast red and examined for the presence of bone and cartilage.

Electron microscopy

Mandibles from H.H. stage-22 embryos and mandibular epithelial cells cultured for 28 days were examined using transmission electron microscopy. Mandibles were fixed in half-strength Karnovsky's fixative (Karnovsky, 1965) for 3 h and postfixed in 1 % osmium tetroxide in 0·1 M cacodylate buffer for 2 h. Cultured epithelial cells were fixed for 2 h and postfixed for 1 h. Sections of both were stained with lead citrate and uranyl acetate after Hardy, Sweeny & Bellows (1978) and examined in a Philips 201 electron microscope.

RESULTS

Induction of bone by epithelial cells and their products

In the first experiment, mandibular epithelia were isolated, placed on to Millipore filter substrates and maintained in organ culture for up to 28 days. Mandibular ectomesenchyme from H.H. stage-21 and -22 embryos (i.e. young enough not to have been induced to form bone, Tyler & Hall, 1977) was placed either (*a*) on to the cultured epithelial cells, or (*b*) on to the Millipore filters after the cells had been removed by distilled water lysis. These recombinations

Table 1. *Induction of bone in grafts of mandibular ectomesenchyme previously exposed to cultured epithelial cells or to epithelial cell products*

Duration of epithelial culture (days)	Substrate	
	epithelial cells	epithelial cell products
1	0/6	0/7
2	0/4	0/5
3	0/3	0/4
14	0/9	0/8
21	0/10	0/24
28	12/25	18/24
28 (controls)*	0/7	0/7

* Controls consisted of ectomesenchyme grafted on to filters previously cultured without epithelial cells.

of ectomesenchyme with epithelial cells or their products were then grown as grafts to the chorioallantoic membranes of host embryos.

Table 1 shows the results from this experiment as assessed by histological examination of serial sections of all grafts. Bone was identified as such only when present as an ossicle containing osteocytes, with surfaces lined by osteoblasts. The first series (column 2, Table 1) was established to determine whether epithelial cells retained their osteogenic inductive activity after various periods of *in vitro* cultivation. Ectomesenchyme only formed bone when placed on to epithelial cells cultured for a minimum of 28 days (column 2, Table 1). Lack of osteogenesis at earlier times was not because the ectomesenchyme failed to survive, for all grafts formed nodules or rods of Meckel's cartilage. (Chondrogenesis from mandibular ectomesenchyme does not require induction from mandibular epithelium, Tyler & Hall, 1977.) No bone formed from ectomesenchyme maintained on Millipore filters previously cultured without epithelial cells (Table 1, 28-day control).

A similar temporal sequence of duration of epithelial culture and initiation of osteogenesis was seen in mandibular ectomesenchyme exposed to epithelial cultures lysed with distilled water. Provided that the period of epithelial culture was prolonged (28 days), the ectomesenchyme responded to the filters impregnated with epithelial extracellular products by forming bone (column 3, Table 1). No bone formed in the controls.

Two approaches were then taken to determine the nature of the products deposited by epithelial cells on to the filters; (*a*) enzymic removal of material(s) from Millipore filters on which epithelial cells had been cultured for 28 days and (*b*) transmission electron microscopy of the products in the filters.

Table 2. *Formation of bone and cartilage by mandibular ectomesenchyme grafted in contact with enzymically treated epithelial cell products*

Enzyme	Dose (μg/ml)	Duration (h)	Temperature (°C)	N	Cartilage	Bone	T/C*
1. Testicular hyaluronidase	0·5	2	37	8	8	3	0·56
	1·0	2	37	9	9	1	0·16
Control	—	2	37	6	6	4	
2. *Streptomyces* hyaluronidase	1 OT.R.U.	2	37	12	12	2	0·37
Control	—	2	37	9	9	4	
3. Trypsin	26	1	4	20	20	0	0·00
Control	—	1	4	19	18	12	
4. *a.* LACA	300	60	37	9	9	0	0·00
Control	—	60	37	7	7	4	
b. LACA	300	12	37	14	14	0	0·00
Control	—	12	37	10	10	6	

* Incidence of bone formation in treated (T) versus control (C) grafts.

Enzymic removal of epithelial inductive activity

The results obtained after maintaining ectomesenchyme on epithelial cells previously treated with one of the three enzymes are summarized in Table 2. In each case the results are reported in comparison with controls which consisted of epithelial cell cultures treated with distilled water for the same times and at the same temperatures as their partners were being exposed to enzymes. Ectomesenchyme was placed on to these cultures and they too were grafted to host embryos.

Some, but not all, grafts in each control group formed bone (Table 2). Under ideal conditions 100 % of the controls should form bone. However, the combination of long-term culture of the epithelia, possible misplacement of ectomesenchyme on the filters, possible poor vascularization of the grafts, etc., means that controls never attained 100 %. That half to two-thirds of the controls formed bone was acceptable, given the limitations outlined above.

Initiation of osteogenesis was reduced to between one-sixth to one-half of control values in ectomesenchyme grafted in contact with hyaluronidase-treated epithelia (Table 2). Under none of the concentrations of either testicular or *Streptomyces* hyaluronidase used was osteogenesis completely inhibited. On the other hand no ectomesenchyme formed bone when trypsin or LACA-inhibited epithelial cells were used as the inductive substrate (Table 2). Cartilage formed in virtually all grafts, confirming the continued viability of at least the chondrogenic, and presumably the osteogenic, ectomesenchyme.

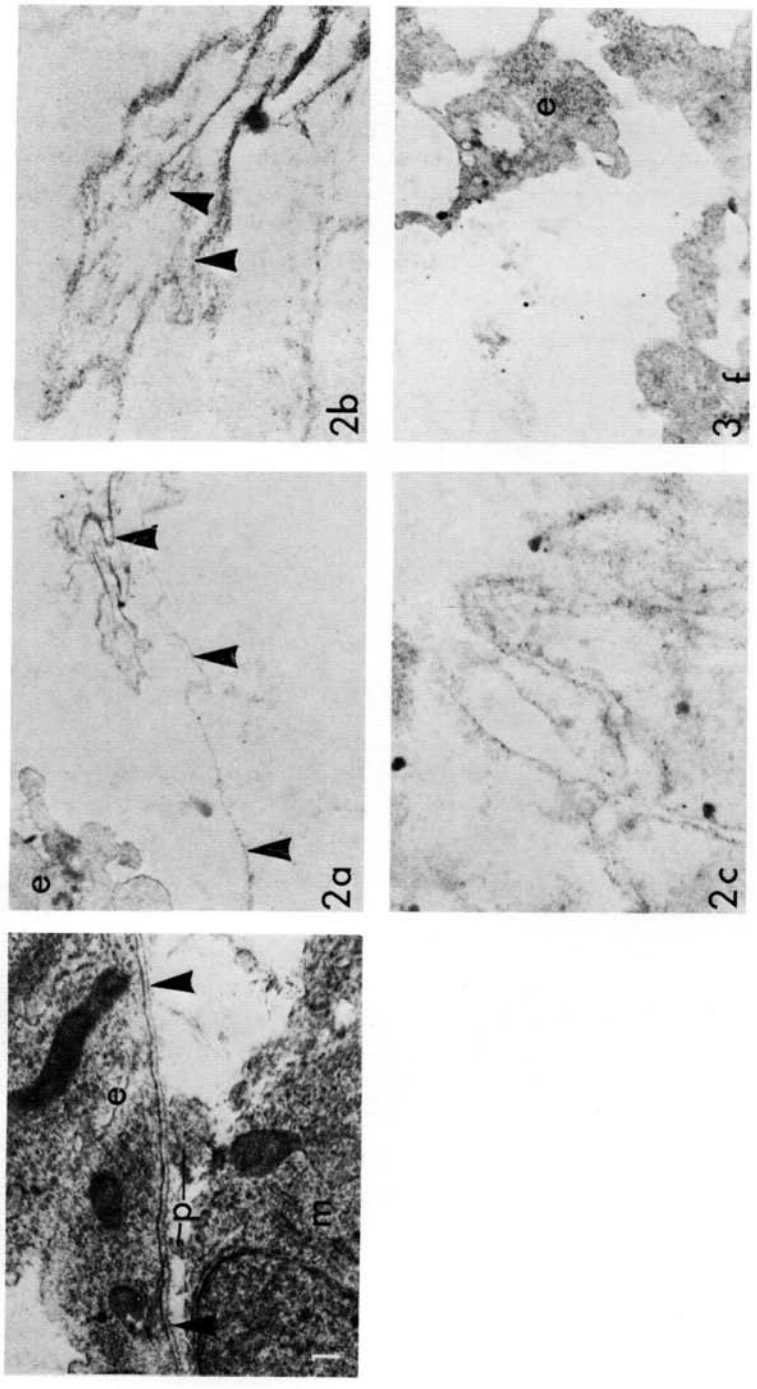

Fig. 1. Electron micrograph of the epithelial–ectomesenchymal interface in a mandible from an H.H. stage-22 embryo. The basal lamina (arrows) is continuous and separates the epithelium (*e*) from the ectomesenchyme (*m*). In numerous sections from six mandibles, ectomesenchymal cells and their processes approached but did not penetrate the basal laminae. Two such processes are shown here (*p*). ×14000.

Fig. 2. Electron micrographs from epithelial cells cultured for 28 days and immersed in distilled water for 1 h. The resulting epithelial products were inductively active (Table 1).

(*a*) This micrograph, the same magnification as Fig. 1, shows an epithelial cell remnant (*e*) overlying an epithelial cell product (arrows) which has the same size, structure and orientation relative to the epithelium as does the basal lamina shown in Fig. 1.

(*b*) A higher magnification (×51630) of a portion of the basal lamina-like material shown in (*a*), demonstrating the fine fibrous nature of these epithelial cell products (arrows).

(*c*) Frequently, portions of basal lamina-like material were convoluted, as in this example. ×41950.

Fig. 3. An electron micrograph of a treated culture in which epithelial cells were cultured for 28 days, treated overnight with LACA and then lysed with distilled water. Cultures so treated were not inductively active (Table 2). Although epithelial cell remnants (*e*) were seen close to the surface of the filter (*f*), basal lamina-like material was never seen. ×14000.

Electron microscopic localization of epithelial inductive activity in basal laminae

The interface between the inductively active epithelium and the ectomesenchyme in the normal mandible at a time when the induction is taking place consists of a continuous basal lamina with which ectomesenchymal cells or cell processes make close contact (Fig. 1). Cell processes were never seen to make direct contact with the basal surfaces of epithelial cells or to penetrate through the basal lamina. (A more detailed description of the ultrastructure of H.H. stage -20 to -26 mandibular processes is in preparation.) Any inductive interaction must either traverse or involve an intact, epithelially derived, basal lamina. This observation, coupled with the evidence that epithelially derived extracellular products are required for induction (Tables 1 and 2) led us to attempt to visualize the epithelial cell products deposited on to Millipore filters.

As visualized using light microscopy, epithelia cultured for 28 days formed a sparse sheet of cells. Cell bodies were only loosely attached to the surfaces of the Millipore filters. Epithelial cell processes did not penetrate into the pores of the Millipore filters. The majority (95 %) of these cells were removed by the distilled water lysis (the percentage removed was estimated by counting cell number on untreated filters and comparing that with the number of cells remaining on other filters treated with distilled water).

Ultrastructural visualization of 28-day epithelial cultures showed that the inductively active epithelial cells had deposited a basal lamina-like structure. This structure was *not* removed by distilled water lysis (Fig. 2*a*), a finding confirmed in all four cultures examined. Such structures were *never* seen on Millipore filters cultured without epithelial cells.

At higher magnification, these basal lamina-like structures were seen to include a fine network of microfibrils (Fig. 2*b*). In many areas they were thrown into folds and convolutions (Fig. 2*c*), probably formed during their deposition and not an artefact of the distilled-water lysis – similar folds were seen in cultures fixed without distilled-water treatment. These structures obviously have considerable structural integrity.

If this basal lamina-like material was causally related to epithelial induction of bone, then treatments which rendered the epithelial cells inductively inactive would have done so by removing the material. We examined LACA-treated epithelial cell cultures for evidence of basal laminae but found none (Fig. 3). Thus cultures treated with LACA neither induced ectomesenchyme to form bone (Table 2), nor deposited any basal lamina-like material.

The correlation between presence or absence of epithelial cell product and presence or absence of inductive ability strongly suggests that the induction of bone by epithelia is mediated by these extracellular epithelial cell products visualized with the electron microscope.

DISCUSSION

The data reported herein provide: (*a*) *direct* evidence that epithelial extracellular products deposited on to Millipore filters induce embryonic mandibular ectomesenchyme to form bone, and (*b*) *indirect* evidence that the inductive ability of these epithelia resides within their basal laminae. That ectomesenchymal cells can respond to these extracellular products in the absence of epithelial cells (Table 1), or without contacting epithelial cells *in ovo* (Fig. 1), provides further indirect evidence for the basal lamina as the inductive agent. That epithelial cells cultured for short periods lacked inductive ability was consistent with the possibility that epithelial extracellular products contained inductive activity and that several weeks of culture were required for such products to accumulate (Table 1). If inductive ability were a cell surface property, one would expect a more rapid recovery of inductive potency in culture. We have already shown (Hall, 1980*a*) that epithelia must be viable to be inductively active, and that fragments of lysed epithelia recombined with ectomesenchyme do not evoke osteogenesis, findings which suggest that inductive ability does not reside in the epithelial cells themselves.

The data from enzymically treated epithelial cultures, illustrated in Table 2, suggest that inductive activity does not depend upon glycosaminoglycans within the basal laminae. Testicular and *Streptomyces* hyaluronidases, which selectively degrade hyaluronate and chondroitin sulphate (Derby & Pintar, 1978) slowed, but did not prevent, initiation of bone formation. The inhibition which followed trypsin and LACA treatment implicates a protein, possibly collagen, in the induction (see also the data in Bradamante & Hall, 1980). Trypsin nonselectively breaks down protein while LACA inhibits the hydroxylation of proline, resulting in formation of underhydroxylated collagen whose release from the cell is impaired (Takeuchi & Prockop, 1969). Further tests with other analogues of collagen will be required to confirm this observation and to understand how basal lamina protein mediates this particular induction.

Although there have been previous reports of the induction of *cartilage* by extracellular products, both in the embryo (Newsome, 1976; Gumpel-Pinot, 1980) and in the adult (Urist, 1980), our is the first report of the direct induction of *membrane bone* by such products. Interestingly, in cartilage induction in the adult, a non-collagenous protein bound to collagen of demineralized bone matrix induces cartilage which is then replaced by bone. Again, protein contains the inductive activity, even though the inductive agents (bone matrix and embryonic epithelia) are quite different. Other epithelia, for example, that of the urinary bladder, are known to induce ectopic bone in soft tissues (Hall, 1978*a*, 1981*a*; Urist, 1980) but their modes of action remain unknown.

Basal laminae have been implicated in the epithelial–mesenchymal interactions involved in the initiation of several other tissues and organs, namely scleral cartilage (Newsome, 1976), limb bud cartilage (Gumpel-Pinot, 1980;

Lunt & Seegmiller, 1980), somitic cartilage (Hall, 1977) and teeth (Lesot, Osman & Ruch, 1981; Thesleff & Hurmerinta, 1981). The only one of these tissues which has been experimentally induced in response to epithelial extracellular products is scleral cartilage (Newsome, 1976), although chondrogenesis can be enhanced after culturing somitic mesenchyme in individual extracellular products, such as collagen or pro-collagen (Hall, 1977). The possibility that basal laminae are the inductively active epithelial components in these various systems should be explored.

Currently, we are using chelating agents to separate mandibular ectomesenchyme from epithelium, leaving the basal lamina on the ectomesenchyme. Bone formation from such ectomesenchyme would provide further direct evidence in support of the inductive activity of basal laminae.

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