Tissue interactions and the initiation of osteogenesis and chondrogenesis in the neural crest-derived mandibular skeleton of the embryonic mouse as seen in isolated murine tissues and in recombinations of murine and avian tissues

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SUMMARY

Mandibular processes from 9- to 13-day-old embryonic mice formed both bone and cartilage when grafted to the chorioallantoic membranes of host embryonic chicks. Isolated ectomesenchyme, taken from 9-day-old embryos did not form bone or cartilage, while older ectomesenchyme formed both. Recombination of the epithelial and ectomesenchymal components confirmed that the presence of the epithelium was a sufficient stimulus for the initiation of both chondro- and osteogenesis. Recombinations between components of mouse and chick mandibular processes showed that 9-day-old mouse ectomesenchyme could respond to chick epithelium but that, although older murine epithelia could initiate osteogenesis from the avian ectomesenchyme, epithelia from 9-day-old embryos could not. These results indicated that an epithelial-ectomesenchymal interaction was responsible for the initiation of both osteo- and chondrogenesis within the mandibular arch of the mouse; that the interaction began at 10 days of gestation; that the ectomesenchyme was capable of responding at 9 days, but that the epithelium did not acquire its ability to act on the ectomesenchyme until 10 days of gestation.

INTRODUCTION

Both the cartilaginous and the bony elements in the mandibular skeletons of vertebrate embryos form from ectomesenchymal cells derived from the embryonic neural crest. These cells have their origin within the crests of the neural folds. During neurulation these neural crest cells migrate away from the neural folds. Those cells which originate in the cranial neural crest become preferentially localized within the primordia of the cranio-facial skeleton. The ability of these cells to form the cartilages and bones of the craniofacial skeleton depends upon

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interactions which they undergo, either during or after, their migration. Thus, in the embryo of the lamprey, *Lampetra fluviatilis* L., contact with the endoderm is required before chondrogenesis can be initiated (Damas, 1944; Newth, 1951, 1956). While it is known that cranio-facial skeletal elements are of neural crest origin in the fishes (Jollie, 1971; Berkovitz & Moore, 1974; Schaeffler, 1977), the role of tissue interactions in their genesis has not been studied. Interaction of neural crest cells with pharyngeal endoderm is a prerequisite for the initiation of chondrogenesis in the visceral skeleton of the urodele amphibians (Wagner, 1949; Wilde, 1955; Cusimano-Carlo, 1963, 1972; Drews, Kocher-Becker & Drews, 1972; Epperlein, 1974; Corsin, 1975). However, although neural crest cells are known to form bone in the urodeles (de Beer, 1947; Cassin & Capuron, 1977), tissue interactions have not been studied. Nor is there any information for the anuran amphibians (see Horstadius, 1950 and Hall, 1978a for a discussion).

The initiation of intramembranous osteogenesis within the scleral, maxillary and mandibular skeletons of the domestic fowl results from inductive interactions between scleral, maxillary, or mandibular epithelia and post-migratory ectomesenchymal cells from the neural crest (Murray, 1943; Tyler & Hall, 1977; Tyler, 1978; Hall, 1978a, b, 1980; Bradamante & Hall, 1980; Tyler & McCobb, 1980). Pre-migratory neural crest cells must interact, (a) with cranial epithelial ectoderm if they are to be able to form Meckel’s cartilage later in development (Hall & Tremaine, 1979; Bee & Thorogood, 1980), or (b) with the pigmented retinal epithelium if they are to form scleral cartilage (Reinbold, 1968; Newsome, 1972, 1976; Hall, 1978a).

Although the details vary, epithelial-mesenchymal interactions emerge as important prerequisites for the initiation of osteogenesis and chondrogenesis in the sub-mammalian vertebrates. The possible existence of similar interactions in the mammalian embryo has not been explored. Indeed, even the origin of the mammalian cranio-facial skeleton from neural crest cells has not been substantiated experimentally. There is histological evidence for spurs of ectodermal cells leading into the visceral arches (Bartelmez, 1952; Mulnard, 1955; O’Rahilly, 1965; Mayer, 1973); histochemical visualization of presumed neural crest cells (Milaire, 1959; 1974); indications that high doses of vitamin A both arrest migration of cranial neural crest cells (Morriss & Thorogood, 1978) and produce first arch defects such as Treacher Collins syndrome (Poswillo, 1974), and the evidence that ectoderm or teratocarcinomas isolated from rat embryos form both cartilage and bone (Levak-Švajger & Švajger, 1971; Damjanov, Solter & Serman, 1973) – all of which provide circumstantial evidence for contributions to the facial skeleton from the neural crest.

The only study known to the author which explores the role of tissue interactions in the development of the mammalian mandibular skeleton is that of Švajger and Levak-Švajger (1976). These workers separated first branchial arch mesenchyme of 13-day rat embryos from their epithelia, organ cultured the mesenchyme, and observed the subsequent development of bone and cartilage.
Skeletogenesis in the murine mandible

If an epithelial–mesenchymal interaction was involved, it must have taken place before thirteen days of gestation.

The aim of the present study was to determine whether an epithelial–mesenchymal (ectomesenchymal) interaction was involved in the initiation of chondrogenesis and/or osteogenesis within the mandibular skeleton of the embryonic mice. To this end, whole mandibular processes, and mandibular ectomesenchyme, separated from the mandibular epithelium by treatment with proteolytic enzymes, were grafted to the chorioallantoic membranes of host embryonic chicks. (This vascularized site is preferred over organ culture when studying osteogenesis.) The separated components were also either recombined with one another or combined with components isolated from the mandibular processes of embryonic chicks and grafted to chorioallantoic membranes. Histological analysis was used to document the presence or absence of cartilage and bone in the recovered grafts.

MATERIALS AND METHODS

Animals

ICR Swiss albino mice obtained from Bio-breeding Laboratories of Canada, Ottawa, Ontario, were used as the experimental animal. Mice were weaned at 4 weeks of age, housed with three or four of the same sex per cage, and fed Purina lab. chow and water ad libitum. Matings were carried out using animals which were at least 8 weeks old. Each male was placed with from one to three females overnight (16.00 to 08.00 hours), 08.00 hours was designated as the commencement of day zero of the pregnancy. Pregnancy was determined by presence of a vaginal plug and by weight gain, as measured on alternate days.

Embryos

Pregnant females were killed by an overdose of ether, the embryos removed by dissection under sterile conditions and aged using the morphological series of stages of Grüneberg (1943). Embryos of 9–13 gestational days were used in this study. The 9-day-old embryos had well developed mandibular processes, lacked posterior limb buds, and had unsegmented posterior somitic mesoderm. By 13 days of gestation the embryos were recognizably murine, with five rows of whiskers containing hair follicles, footplates on the limbs and marked segmentation to the distal tips of the tails.

Mandibular processes

Mandibular processes were dissected from these embryos and either grafted intact, or separated into their epithelial and ectomesenchymal components and then grafted. Separation was achieved by placing the processes into a solution of trypsin and pancreatin in calcium- and magnesium-free Tyrode's solution (257 mg bovine pancreatic trypsin + 43 mg porcine pancreatic pancreatin/10
ml. both obtained from BDH Chemicals, Toronto, Ontario) for 2 h at 4°C. The mandibular processes were then placed into a solution of the synthetic culture medium BGJb (GIBCO, Grand Islands, N.Y.) and Horse Serum (1:1) to stop the enzymic digestion and the epithelia separated from the ectomesenchyme by microdissection using sharpened hypodermic needles. A similar separation was performed on mandibular processes obtained from embryonic chicks which had been incubated for 4 days and attained Hamburger & Hamilton (1951) stage 22.

Grafting

Whole mandibular processes, recombined components from the mouse, or components combined with epithelia or ectomesenchyme from mandibular processes of the chick, were grafted to the chorio-allantoic membranes of 8- or 9-day-old embryonic chicks following the procedure outlined by Hall (1978c). Briefly, the mandibular process or epithelium was placed onto a square of sterile, black, Millipore filter (0.45 μm porosity, 125–150 μm thick, Millipore Filter Corp., Montreal, Quebec), mandibular ectomesenchyme was placed in direct contact with the epithelium and the intact, or recombined processes, grafted for 7 days.

Histology

Grafts were recovered, dissected away from the chorioallantoic membranes, fixed in neutral buffered formal saline, processed histologically, serially sectioned and stained with haematoxylin, alcian blue and chlorantine fast red (modified from Lison, 1954).

RESULTS

Intact mandibular processes

The chorioallantoic membrane (CAM) has previously been shown to be a graft site which supports the initiation and maintenance of chondrogenesis and osteogenesis from avian mandibular processes (Tyler & Hall, 1977; Hall, 1978b, 1980). To determine whether the CAM would support cartilage and bone formation from murine tissue, and to determine whether the initiation of either tissue was dependent upon the age of the donor embryo, whole mandibular processes from mouse embryos of 9–13 gestational days were grafted to the CAMs of host embryonic chicks. Osteogenesis and chondrogenesis were initiated in at least 75% of the grafts, irrespective of the ages of the mouse embryos (column I, Table 1). Typically, the cartilage consisted of one or two rods of hypertrophic cartilage, with scant amounts of extracellular matrix and an ill-defined perichondrium. The bone typically consisted of one or two ossicles of trabecular, membrane bone, usually separated from the cartilage by intervening connective tissue and often resting on the Millipore Filter substrate.
Table 1. Number of grafts forming cartilage and/or membrane bone

<table>
<thead>
<tr>
<th>Age embryo (days)</th>
<th>I Intact mandibular processes</th>
<th>II Isolated ectomesenchyme</th>
<th>III Recombined epithelium and ectomesenchyme</th>
<th>IV† Mouse ectomesenchyme + chick epithelium</th>
<th>V† Chick ectomesenchyme + mouse epithelium</th>
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<td>Cartilage</td>
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* n, number of grafts analysed.
† The chick tissues were obtained from embryos of H. H. stage 22 (4 days of incubation). When chick mandibular ectomesenchyme was recombined with chick epithelium and grafted, all grafts formed both cartilage and bone.
Both the large percentage of grafts forming skeleton tissues and the advanced state of differentiation attained by the tissues, indicated that the chorioallantoic membrane was a suitable graft site for these mouse cells. The mandibular epithelium also continued to differentiate and formed a multi-layered squamous epithelia, with numerous buds extending into the underlying ectomesenchyme.

**Isolated mandibular ectomesenchyme**

Mandibular ectomesenchyme which had been dissected away from the mandibular epithelium after treatment of the intact mandibular process with trypsin and pancreatin was then grafted. Provided that the embryo was at least 10 days old, cartilage and membrane bone formed in a similar percentage of grafts to that seen when intact mandibular processes were grafted, viz. at least 75% (see column II, Table 1, Fig. 1). The lack of murine epithelial cells in these grafts attested to the adequacy of the tissue separations. Although the amount of cartilage and bone present was not quantified, it was not noticeably less than that obtained from intact mandibular processes.

The ectomesenchyme isolated from the 9-day-old embryos behaved quite differently. Chondrogenesis was not initiated in any graft, and osteogenesis only in one out of the eight grafts. The grafts consisted of a loose meshwork of ectomesenchymal cells (Fig. 6).

Thus, mandibular ectomesenchyme would only form bone and cartilage if the epithelium was present until 10 days of gestation.
Recombined epithelia and ectomesenchyme

To confirm that the inability of ectomesenchyme from 9-day-old embryos to form cartilage and bone was a consequence of its isolation from mandibular epithelium, these two components were recombined and grafted to the CAMs of host embryonic chicks. Recombinations of components from 10- to 13-day-old embryos served as a control for the procedure. In these specimens chondrogenesis and osteogenesis were initiated at the same, on a higher rate, than when intact mandibular processes or isolated ectomesenchyme were grafted (column III, Table 1, Fig. 2).

Having confirmed that the procedure of recombination did not inhibit skeletogenesis in these older tissues, epithelia and ectomesenchyme from mandibular processes of 9-day-old embryos were recombined and grafted. Both osteogenesis and chondrogenesis were initiated in such grafts (column III, Table 1), indicating that the lack of cartilage and bone in the isolated ectomesenchymes was attributable to the absence of the mandibular epithelia.

Heterospecific recombinations

Mandibular processes from embryonic mice and from H. H. (Hamburger & Hamilton, 1951) stage-22 embryonic chicks were separated and recombined heterospecifically.

It is known that mandibular epithelium from the H. H. stage-22 embryonic chick will allow chick mandibular ectomesenchyme to initiate osteogenesis (Tyler & Hall, 1977; Hall, 1978). Would chick mandibular epithelium substitute for murine epithelium in initiating osteogenesis? What of the initiation of chondrogenesis? Chondrogenesis in the chick mandible is not dependent on the presence of the mandibular epithelium. Would the chick epithelium allow the murine ectomesenchyme to form cartilage?

In the first recombination, mouse mandibular ectomesenchyme was recombined with chick mandibular epithelium and grafted. Ectomesenchymes from 10- to 13-day-old mice were used as controls. Both bone and cartilage formed in these recombinants (column IV, Table 1, Figs. 3, 4). Cartilage and bone also formed in four out of the nine grafts containing 9-day murine ectomesenchyme (Fig. 7). (In assessing these results all recovered grafts were counted. Given that the amount of epithelial-ectomesenchymal contact varied from graft to graft, the positive results reported represent a minimal estimate.) This recombination indicated that mandibular ectomesenchyme from 9-day-old embryonic mice could respond to chick mandibular epithelium by initiating both chondrogenesis and intramembranous osteogenesis.

In the second recombination, H. H. stage-22 chick mandibular ectomesenchyme was recombined with mouse mandibular epithelium of various ages (column V, Table 1). As noted above, the initiation of chondrogenesis in such avian ectomesenchyme is not dependent upon the presence of mandibular
epithelium. Therefore virtually all of these grafts contained nodules or rods of cartilage (Table 1, Fig. 5). Osteogenesis was also initiated in the ectomesenchyme, provided that the murine epithelium had been obtained from embryos of at least 10 days of gestation (column V, Table 1, Fig. 5). The chick ectomesenchyme also maintained the continued, normal differentiation of the mouse epithelium (Fig. 5).

Epithelium from 9-day-old embryos was not able to induce bone formation from the avian ectomesenchyme, but epithelial differentiation was maintained.

Therefore (a) the epithelial–ectomesenchymal interaction is neither species nor class-specific; (b) ectomesenchyme from all aged mice is able to respond to chick epithelia by initiating both osteogenesis and chondrogenesis, and (c) the epithelium from the mouse does not acquire the ability to induce osteogenesis.

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**Figures 2–4**

Figs. 2, 3. Cartilage (C) and bone (B) formed when mandibular epithelium and ectomesenchyme from this 12-day-old embryo were recombined (Fig. 2) and when mouse mandibular ectomesenchyme was combined with chick mandibular epithelium (Fig. 3). Note the similarity of the cartilages in the two combinations. Alcian blue, chlorantrene fast red and haematoxylin.

Fig. 4. A higher magnifications of the bone seen in Fig. 3 to show the trabeculae of bone (B), osteocytes (O), osteoblasts (ob) and flattened ectomesenchymal cells (e) presumed to be osteoprogenitor cells. Alcian blue, chlorantrene fast red, haematoxylin.
Fig. 6. Ectomesenchyme isolated from 9-day-old embryos and grafted to the chorioallantoic membrane formed a loose meshwork of cells. Neither chondrogenesis nor osteogenesis is seen. Alcian blue, chlorantine fast red and haematoxylin.

Fig. 7. Bone formed when mandibular ectomesenchyme from a 9-day-old mouse embryo was combined with mandibular epithelium from an H. H. stage-22 embryonic chick and grafted. Alcian blue, chlorantine fast red and haematoxylin.
Skeletogenesis in the murine mandible

or chondrogenesis until 10 days of gestation. The lack of bone or cartilage development from ectomesenchyme isolated from 9-day-old embryos reflected the absence of an epithelial signal rather than the inability of the ectomesenchyme to respond to such a signal.

DISCUSSION

Although a considerable amount is known about chondrogenesis, surprisingly little information is available on the microenvironmental control of the initiation of intramembranous osteogenesis. It is known (a) that calvarial osteogenesis is only initiated after pre-osteogenetic cells have undergone an inductive interaction with the brain or spinal cord (Schoving, 1968); (b) that scleral bones only form in the avian eye after ectomesenchymal cells have interacted with epithelial scleral papillae (Coulombre, Coulombre & Mehta, 1962); (c) that the sub-periosteal bone around the shafts of developing long bones only forms after perichondrial cells have been in contact, either with hypertrophic chondrocytes or with their extracellular products (Shimomura, Yoneda & Suzuki, 1975); and (d) that membrane bones of the avian mandible and maxilla only form after ectomesenchymal cells have interacted with the appropriate epithelia (see Introduction). The dentary of the mouse may now be added to this list.

The present study demonstrates that cells of the mandibular process must interact with the mandibular epithelium if they are to initiate the sequence of differentiative events which culminate in the differentiation of osteoblasts and osteocytes, the deposition of osteoid and the mineralization of that osteoid to form bone. That this developmental interaction had not occurred by 9 days of gestation was shown by the fact that ectomesenchyme isolated from such aged embryos failed to form bone (except in one case (column II, Table 1), where epithelial cells were found to have been left on the ectomesenchyme). The re-initiation of osteogenesis in recombinants confirmed that presence of the epithelium was a sufficient osteogenetic stimulus for these ectomesenchymal cells. Therefore, the murine dentary resembles the membrane bones of the avian mandible (Tyler & Hall, 1977) in requiring a mandibular epithelial-ectomesenchymal interaction for its initiation.

Unlike Meckel's cartilage in avian embryos, initiation of the murine cartilage was dependent upon the presence of the mandibular epithelium. In the chick the ectomesenchymal cells are already able to form cartilage before they reach the mandibular arch (Tyler & Hall, 1977; Hall & Tremaine, 1979), apparently because of prior interactions with cranial epithelial ectoderm (Bee & Thorogood, 1980). In the urodele amphibians the equivalent interaction is between cells derived from the neural crest and the pharyngeal endoderm (see Introduction). Thus, these three classes of vertebrates (Amphibia, Aves, Mammalia) share epithelial-ectomesenchymal interactions as a common component for the initiation of chondrogenesis in the mandibular skeleton, differing only in the nature of the signalling epithelium. Whether these interactions are mediated via
epithelial cell products, as is the initiation of scleral chondrogenesis (Newsome, 1972, 1976), or via direct cell-to-cell contact, remains to be determined. The osteogenetic interaction in the chick requires (a) that the epithelium be vital, and proliferating (Hall, 1980), and (b) the presence of epithelially-derived collagen and glycosaminoglycans (Bradamante & Hall, 1980).

It was further shown that these interactions, like those which are responsible for the formation of epidermal structures such as hairs, feathers and teeth (Kollar, 1972; Dhouailly, 1975), were neither species nor class specific. Mouse ectomesenchyme of any age responded to chick epithelium by forming both bone and cartilage. Chick ectomesenchyme responded to mouse epithelium older than 9 days by forming bone and cartilage. From these results I concluded that mouse mandibular ectomesenchyme was capable of responding to epithelial influences at 9 days of gestation but that the murine epithelium was not active until 10 days of gestation. A qualitatively similar temporal pattern has been shown for inductively active epithelia from the chick (Hall, 1978b). In each combination the bone and cartilage formed were typical of the species providing the ectomesenchyme (cf. Fig. 4 and 5), indicating that, while the epithelium was required to initiate osteo- or chondrogenesis, it played no role in determining the type of cartilage or bone which formed.

The identification of these tissue interactions provide a new basis for the study of malformations affecting the mammalian facial skeleton. Failure of these tissues to interact, interaction with fewer than normal ectomesenchymal cells, or delayed interactions, could all alter craniofacial growth and/or morphogenesis. Mutant mice, or embryos from females given teratogens, could be used to provide epithelia or ectomesenchyme for recombination with components from wild-type or untreated embryos to further explore the role of such tissue interactions in development.

This research has been supported by the National Sciences and Engineering Research Council of Canada (Grant no. A3056). The Dalhousie University Research Development Fund in the Sciences provided funds to purchase the CO₂ Incubator. Pilot grafts of whole mandibular process and ectomesenchyme from the older embryos were performed by P. Elliott. Sharon Brunt provided expert technical assistance.

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