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Distribution of osteo- and chondrogenic neural crest-derived cells and of osteogenically inductive epithelia in mandibular arches of embryonic chicks

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SUMMARY

Mandibular arches of H.H. stage-22 embryonic chicks were divided into halves (lateral/medial; cephalad/caudal; or proximal/distal) and grafted to the chorioallantoic membranes of host embryos. All six halves formed both cartilage and membrane bone. Epithelial–mesenchymal recombinations performed between half mandibles showed (*a*), that the cephalad half of the mandible contained more chondrogenic cells than did the caudal half, (*b*), that the proximocaudal quarter of the mandible contained more osteogenic cells than the remainder of the mandible, and, (*c*), that the epithelium of the caudal half was more osteogenically inductive than was the cephalad epithelium. Differential distribution of mesenchymal cells and differentially inductively active epithelia are both components of this epithelial–mesenchymal interaction.

INTRODUCTION

The mandibular arch first appears in the embryonic chick at H.H. (Hamburger & Hamilton, 1951) stage 14, by which stage neural crest-derived cells have left the mesencephalic region of the neural tube and begun to accumulate under the future mandibular epithelium. This population of neural crest-derived cells differentiates into a mass of ectomesenchyme, destined to form the future cartilage, bone, fibrous connective tissue, endothelial smooth muscle and dermal portion of the feathers of the mandibular arch (LeLievre, 1978; Noden, 1978). We have been investigating the epigenetic events required for this ectomesenchyme to form both Meckel's cartilage and the membrane bones of the lower jaw.

Ectomesenchymal cells have to undergo a permissive inductive interaction with the mandibular epithelium until H.H. stage 24 ($4\frac{1}{2}$ days) in order to begin to form bone at H.H. stage 33 ($7\frac{1}{2}$–8 days, Tyler & Hall, 1977). Various parameters of this interaction have now been established, including (*a*), that other epithelia can substitute for mandibular epithelium (Hall, 1978*a*, 1981*a*, *b*), (*b*) that inductive activity is correlated with epithelial viability and proliferative

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activity (Hall, 1980); (*c*) that epithelially derived collagen and glycosaminoglycans are related to this inductive activity (Bradamante & Hall, 1980); and (*d*) that epithelially deposited extracellular products akin to basal lamina contain inductive activity (Hall & Van Exan, 1982).

Ectomesenchymal cells form cartilage in the absence of the mandibular epithelium (Tyler & Hall, 1977). In fact neural crest cells dissected from neural tubes *before* or *during* migration can form cartilage (Hall & Tremaine, 1979), apparently because of having interacted with the cranial ectoderm (Bee & Thorogood, 1980).

Having identified the major tissue interactions responsible for initiating chondrogenesis and osteogenesis we wanted to begin to explore the events which occur between the end of the inductive period and the onset of overt cytodifferentiation. We know that the end result of these interactions is the formation of specific cartilages and bones but we know nothing about the initial response of the neural crest-derived or ectomesenchymal cells to the epithelia. Because we would have to study mandibular arches before cytodifferentiation, and because we wished to examine proliferative activity in cells destined to form cartilage, bone and fibrous tissue – tissues which form in close proximity to one another – we started by determining whether cells with these differentiative potentials were evenly or unevenly distributed throughout the mandibular arch. Jacobson & Fell (1941) described the localization of chondro-, osteo- and myogenic centres on the basis of organ culturing various fragments of the mandibular arches of 3- to 7-day-old embryos. However they removed the epithelium from their mandibles after they had been in culture for two days. Given the timing of the osteogenic induction with the epithelium, their system may have prevented osteogenesis within mandibles from the younger embryos.

In the present study, mandibular arches were halved in one of three planes (lateral/medial; proximal/distal; cephalad/caudal) and the halves grafted to chorioallantoic membranes of host embryos. To control for possible differences in inductive ability of epithelia from various sites on the mandibular arch, half mandibles were separated into their epithelial and ectomesenchymal portions, recombined in various combinations, e.g. lateral ectomesenchyme with medial epithelium, and grafted.

MATERIALS AND METHODS

Incubation of Eggs

Eggs of the domestic fowl were obtained from Cook's Hatchery, Truro, Nova Scotia and incubated in a forced-draft, Humidaire Incubator (Model 350, Humidaire Incubator Co., New Madison, Ohio, U.S.A.) at $37 \pm 0{\cdot}5$ °C and 59 ± 4 % relative humidity.

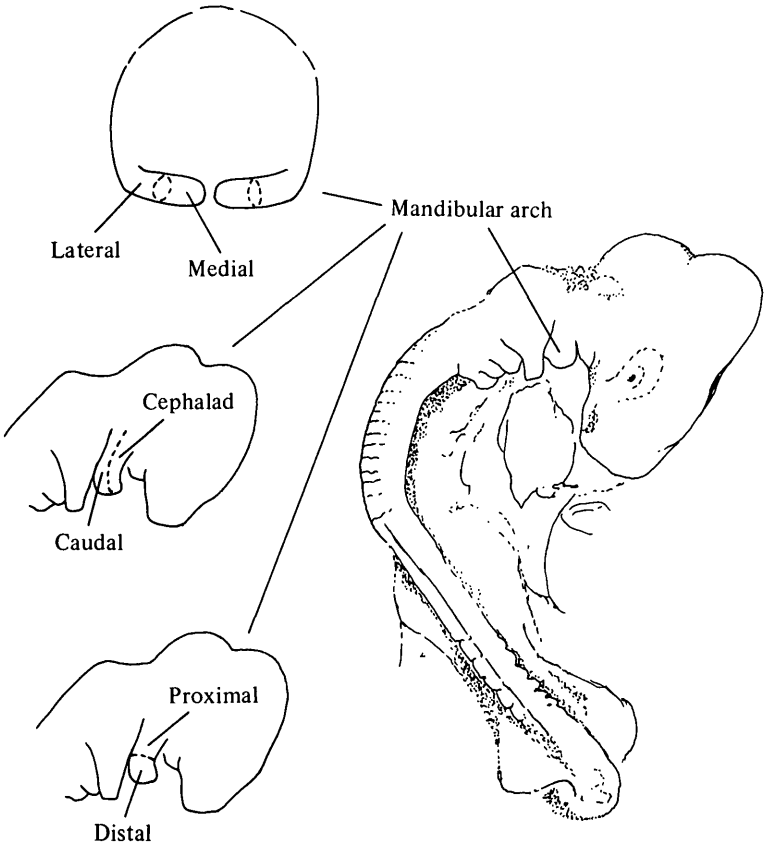

Fig. 1. The three planes used to obtain lateral/medial, cephalad/caudal, or proximal/distal half mandibles are shown diagrammatically. Lateral/medial halves are shown in frontal view, the remainder in side view.

Tissue preparation

Eggs which have been incubated for 3½ days were opened and the embryos removed. Only embryos of H.H. stage 22, as described by Hamburger & Hamilton (1951) were used. Mandibular arches were dissected from these embryos under sterile conditions and halved so as to produce (*a*), proximal and distal halves, (*b*), lateral and medial halves, or (*c*), cephalad and caudal halves. The three planes of dissection are illustrated in Figure 1. Normally one half was fixed immediately as a time zero control and the other grafted. The controls served as a check on the accuracy of the planes of dissection.

Tissue separation

Some of the halves mandibles were separated into their epithelial and ecto-mesenchymal components by microdissection following treatment with trypsin

and pancreatin as previously described (Tyler & Hall, 1977; Hall 1978*a*). These components were recombined in various combination and grafted (see Table 2 for details).

Chorioallantoic grafting

Intact half mandibles or recombined half mandibles were grafted to the chorioallantoic membranes of 8-day-old host embryos as previously described (Hall, 1978*b*).

Recovery of tissues and histology

After 8–10 days as grafts, the mandibles were dissected away from the hosts' chorioallantoic membranes, and, as the time zero controls had been, were fixed in neutral buffered formal saline, dehydrated, cleared, paraffin embedded, serially sectioned, stained with haematoxylin, alcian blue and chlorantine fast red (modified from Lison, 1954), and examined for the presence or absence of cartilage and bone.

RESULTS

Whole mandibles

In a variety of previous studies (see Introduction) we have shown that whole mandibles from H.H. stage-22 embryos virtually always form cartilage and bone when grafted to the chorioallantoic membranes of host embryos. The aim of the present study was to delineate the distribution of osteo- and chondrogenic cells within mandibular arches of H.H. stage-22 embryos.

Controls

For each graft a mandible was divided into two halves. Visual examination of halved mandibles was used to ensure that the halves were of comparable size. Those that were not were discarded. To further check on the uniformity of both the size and the shape of the halved mandibles, one half was fixed immediately as a time zero control and the other half was grafted. Grafts were only used to provide data for the results when their control halves had been dissected at right angles to their long axes. These two controls ensured that half mandibles were of comparable size and included comparable areas of the mandible.

As a further check on the effect of size of half mandibles on the incidence of cartilage and bone formation, half mandibles were grafted either alone, or in contact with a second half mandible of the same type, e.g. two lateral halves in contact. Incidence of cartilage and bone formation was similar in the two groups so that size of the explant at the outset was not a factor influencing the results.

Lateral and medial half mandibles

In the first experiment, mandibular arches were subdivided into their lateral and medial halves as illustrated in Fig. 1. Medial and lateral halves from left

Table 1. *Percentage(N) of grafts of intact half mandibles which formed cartilage and bone*

	Cartilage	Bone
(1) Lateral	100 (16)	56 (9/16)
(2) Medial	92 (12/13)	46 (6/13)
(3) Cephalad	100* (29)	34 (10/29)
(4) Caudal	87 (26/30)	47† (14/30)
(5) Distal	100 (24)	33 (8/24)
(6) Proximal	96 (24/25)	68 (17/25)

* These cartilages were much larger than those formed from caudal halves.
† These bones were much larger than those formed from cephalad halves.

and right mandibles were grafted separately but as there was no difference between them they are reported together.

There were no differences either in the percentages of grafts which formed cartilage and bone or in the sizes of the cartilages and bones which formed when lateral and medial half mandibles were compared (Table 1, lines 1 and 2). Because of these similarities, no tissue recombinations were performed.

Cephalad and caudal half mandibles

In this second set of grafts, mandibular arches were divided into their cephalad and caudal halves as illustrated in Fig. 1. There was no difference in the percentage of grafts which formed cartilage, either when comparing intact half mandibles (Table 1, lines 3 and 4) or the various recombinations between cephalad and caudal epithelia and ectomesenchyme (Table 2, lines 1–6). However the rods of cartilage which formed from cephalad halves were much larger than those which formed from caudal halves. This size difference was presumably due to cephalad halves containing more chondrogenic cells than caudal halves, because the mandibular epithelium has no effect on chondrogenesis (see Introduction).

Somewhat more caudal half mandibles formed bone than did cephalad halves (47 *vs.* 34% Table 1) and they also formed much larger amounts of bone than did cephalad halves. Given that the mandibular epithelium is required for the initiation of mandibular osteogenesis, these differences could have been due either (*a*), to differential distribution of osteogenic ectomesenchyme within the mandibles, and/or (*b*), to differential distribution of inductively active epithelium. Tissue recombinations were performed in an attempt to discriminate between these two alternatives.

The incidence of bone from caudal ectomesenchyme was decreased in the presence of cephalad epithelium (from 47 to 29%; cf; Table 1, line 4 with Table 2, line 1), but percentage of cephalad ectomesenchyme forming bone was the

Table 2. *Percentage (N) of grafts of recombined half mandibles which formed cartilage and bone*

Epithelium	Ectomesenchyme	Cartilage	Bone
	(*a*) Cephalad/caudal		
(1) Cephalad	Caudal	100 (17)	29 (5/17)
(2) Caudal	Cephalad	96 (23/24)	33 (8/24)
(3) Cephalad + caudal	Caudal	100 (17)	59 (10/17)
(4) Cephalad + caudal	Cephalad	94 (17/18)	33 (6/18)
(5) Caudal	Cephalad + caudal	87 (7/8)	62 (5/8)
(6) Cephalad	Cephalad + causal	100 (8)	37 (3/8)
	(*b*) Proximal/distal		
(7) Distal	Proximal	100 (13)	69 (9/13)
(8) Proximal	Distal	77 (10/13)	31 (4/13)
(9) Distal + proximal	Proximal	100 (25)	76 (19/25)
(10) Distal + proximal	Distal	83 (20/24)	29 (7/24)
(11) Proximal	Distal + proximal	100 (13)	46 (6/13)
(12) Distal	Distal + proximal	100 (14)	43 (6/14)

same in the presence of caudal epithelium (33%; Table 2, line 2) as in the presence of cephalad epithelium (34%, Table 1, line 3). This pair of results was consistent with cephalad epithelium being less inductively active and with cephalad ectomesenchyme containing fewer osteogenic cells than caudal ectomesenchyme. Caudal epithelium is inductively active for the incidence of bone from caudal ectomesenchyme rises to 59% when grafted in contact with caudal + cephalad epithelium (Table 2, line 3). That the incidence of bone formation from cephalad ectomesenchyme was unchanged under similar conditions (Table 2, line 4) was consistent with cephalad ectomesenchyme containing fewer osteogenic cells than caudal ectomesenchyme. Recombinations of caudal or cephalad epithelia with the combined ectomesenchymes from both halves (Table 2, lines 5 and 6) was also consistent with the interpretation that cephalad epithelium was less inductively active than caudal, and that caudal ectomesenchyme contained more osteogenic cells than did cephalad ectomesenchyme.

Distal and proximal half mandibles

The incidence and amount of chondrogenesis was similar from both intact distal and proximal halves (Table 1, lines 5 and 6). The incidence of chondrogenesis was reduced when distal ectomesenchyme was recombined with proximal epithelium, either in the presence or absence of distal epithelium (Table 2, lines 8 and 10).

An even more pronounced difference in the incidence of osteogenesis was seen between distal and proximal halves than has already been described between cephalad and caudal halves. Proximal halves (68%) formed large rods of bone

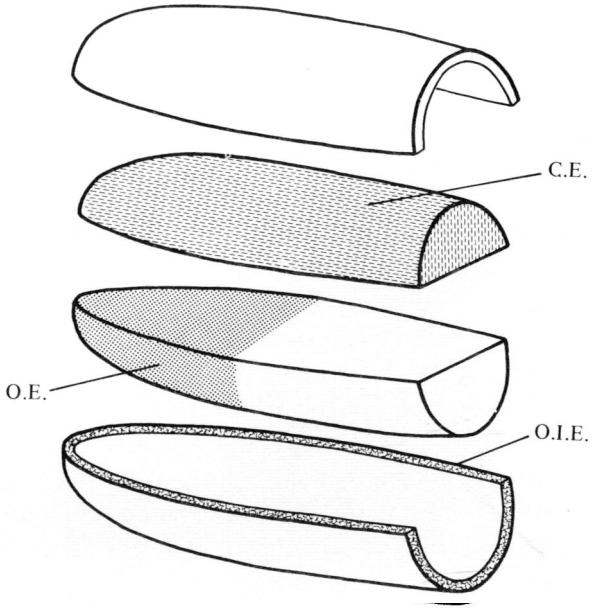

Fig. 2. A diagrammatic representation of a separated mandibular arch with epithelium at the top and bottom, ectomesenchyme in between and proximal to the left. At the top is the cephalad epithelium. Next is the cephalad ectomesenchyme (C.E.) which contains the most chondrogenic ectomesenchyme. Next is the ectomesenchyme from the caudal half of the mandible. Its proximal region contains the most osteogenic ectomesenchyme (O.E.) of the mandible. At the bottom is the osteogenically inductive epithelium (O.I.E.) of the caudal half of the mandible.

in comparison with 33 % of distal halves which formed much smaller nodules of bone (Table 1, lines 5 and 6). Tissue recombinations were then performed on these distal and proximal half mandibles.

The incidence of bone was unchanged when epithelia were exchanged between the half mandibles – proximal ectomesenchyme still formed bone twice as often as did distal ectomesenchyme (cf. Table 1, lines 5 and 6 with Table 2, lines 7 and 8). A similar finding when epithelium from both halves was recombined with either distal or proximal ectomesenchyme (Table 2, lines 9 and 10) indicated that proximal and distal epithelia were equally inductively active but that distal ectomesenchyme contained fewer osteogenic cells than did proximal ectomesenchyme. This conclusion was further supported by the last set of recombinations where combined distal and proximal ectomesenchyme was recombined with either distal or proximal epithelia (Table 2, lines 11 and 12). The incidence of bone was equal in both sets (46 *vs.* 43 %) and equalled the average incidence obtained from the three sets containing ectomesenchyme from only one half, *viz.* 50, 50 and 51 % from Table 1, lines 5 and 6 and Table 2, lines 7–10.

DISCUSSION

Several general conclusions emerged from this study. The first is that lateral and medial half mandibular arches from H.H. stage-22 embryos are equivalent with respect both to ectomesenchyme and to the distribution of inductively active epithelium. The second is that all sections of the mandibular arch contain ectomesenchyme capable of forming cartilage with the cephalad half containing more chondrogenic ectomesenchyme than the remainder of the mandible. There was also some indication that the distal half of the mandible contained somewhat less chondrogenic ectomesenchyme than the proximal half (Table 2). The third general conclusion is that all parts of the mandible contain osteogenic ectomesenchyme with the caudal and proximal halves containing more than the rest. The fourth conclusion is that the caudal epithelium is more osteogenically inductively active than the cephalic epithelium but that proximal and distal epithelia are equally active.

The distribution of the most active centres of osteo- and chondrogenesis and of osteogenically active epithelium are shown in Fig. 2. The localization of the more active osteogenic centre corresponds to the position of the centre described by Jacobson & Fell (1941) as occurring in the 4-day-old embryo and as forming the angular, splenial and surangular. However they found this to be the only centre of ossification in the mandible at four days. The present study indicated that the six major regions studied all contained osteogenic ectomesenchyme. That Jacobson and Fell removed the epithelia from their cultures no doubt influenced their results. They did observe a second centre distally in the 5-day-old mandible which formed the dentary and which they described as being associated with a thickened region of mouth epithelium.

It is clear from this study that prechondrogenic or preosteogenic cells cannot be localized within different regions of the H.H. stage-22 mandibular arch. Therefore other means will have to be found to separate these cell populations before overt cytodifferentiation commences. We intend to utilize clonal cell culture. The explanation for one region of the mandibular arch producing more cartilage than other regions is presumably that the cephalad half of the mandibular arch contains more chondrogenic ectomesenchyme than does the caudal half. Differential chondrogenic activity within the mandibular epithelium is not involved, for any epithelial–ectomesenchymal interactions occur before the ectomesenchymal cells reach the mandibular arch (Hall & Tremaine, 1979; Bee & Thorogood, 1980). Nor is differential innervation or vascularity involved for cartilage forms when ectomesenchyme is grown *in vitro* (Tyler & Hall, 1977). The greater amount of cartilage within the cephalad half of the mandible could result from (*a*), all regions of the mandible containing the same number of chondrogenic cells with those in the cephalad half forming more cartilage, or (*b*), from the cephalad half containing more chondrogenic cells. The latter situation could arise because of differential migration of ectomesenchymal cells

into the mandibular arch, and/or, because of differential rates of proliferation within the arch. Jacobson and Fell's data would favour differential cell proliferation but these alternatives have to be checked using [^{3}H]thymidine labelling as Searls (1967) and Fyfe (1980) have done for limb and scleral cartilage, respectively.

The pronounced osteogenesis within the caudal and proximal quadrant of the mandible could also be explained on the basis of differential migration of osteogenic ectomesenchyme into the mandibular arch or of differential proliferation once in the arch. An additional factor, not seen during chondrogenesis, is the asymmetrical distribution of osteogenic inductive activity within the mandibular epithelium. Preliminary ultrastructural evidence (Hall & Van Exan) is consistent with these possibilities for not only is caudal epithelium structurally distinct from cephalad epithelium, but more ectomesenchymal cells approximate the caudal than the cephalad epithelium. The greater osteogenic inductive activity of caudal epithelium may then either indicate that the epithelium itself is more active, and/or that more ectomesenchymal cells receive the induction than in the cephalad half of the mandible. We plan to culture different densities of ectomesenchymal cells on epithelia from different regions of the mandible to further explore these aspects.

The complexity of this system is clearly very considerable with inductive activity of epithelia, number of ectomesenchymal cells and amount of contact between epithelia and ectomesenchyme as three important variables. We also have to determine whether the same ectomesenchymal cell could form cartilage or bone depending upon its location within the mandible. Clonal cell culture will be required to determine whether these skeletogenic, neural crest-derived cells are unipotential or bipotential.

Recently Slack (1980) has studied an example of non-equivalence between anterior and posterior skin in salamander limb regeneration. Anterior skin grafted to the posterior face of the limb followed by amputation yields a normal regenerate. Posterior skin grafted to the anterior face yields double posterior duplicate regenerates. The duplications involved both redifferentiation and metaplasia of host tissues. Slack explains the morphogenetic differences between anterior and posterior skin on the basis of differential distribution of the chemical switches which control positional information. The differentiative differences may have their origin in differential distribution of inductive activity or in differential amount of contact between blastemal cells and the overlying epithelia. Skeletogenic inductive activity has been shown to reside in the basal laminae of epithelia (Gumpel-Pinot, 1980; Hall & Van Exan, 1982). The combination of differential distribution of inductively active epithelia, of competent ectomesenchyme/mesenchyme, and of varying amounts of contact between the two, would be a powerful way of controlling site-specific differentiation.

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