Viability and proliferation of epithelia and the initiation of osteogenesis within mandibular ectomesenchyme in the embryonic chick

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SUMMARY

Ectomesenchyme, a derivative of the embryonic neural crest, forms the membrane bones of the mandibular skeleton, but will only do so after undergoing an inductive interaction with mandibular epithelium. Previously, non-mandibular epithelia have been shown to act as effective substitutes for the mandibular epithelium in this interaction. The role of epithelial viability was examined by enzymatically separating the mandibular epithelium from its ectomesenchyme, killing the epithelium, recombining the epithelium with vital ectomesenchyme and either organ culturing the recombinant or grafting it to the chorioallantoic membrane of a host embryo. Epithelia killed by distilled water, air drying, 80% ethanol, freeze-thawing or with 2500 rad of gamma irradiation did not elicit osteogenesis from the ectomesenchyme while vital epithelia did. Exposure of epithelia to gamma irradiation at doses between zero and 2000 rad resulted in a progressive reduction in the incidence of osteogenesis in ectomesenchyme. However, the incidence of osteogenesis progressively increased after irradiation of the mandibular epithelium with 3000 to 5000 rad, only to decrease again after 10000 rad. [3H]thymidine autoradiography was used to show that this pattern of induction of bone by irradiated epithelia could be correlated with the proliferative activity of the epithelia. A similar pattern of induced osteogenesis and epithelial proliferation was seen after epithelia were treated with colchicine. It was concluded that the ability of the mandibular epithelium to permit osteogenesis within mandibular ectomesenchyme was correlated with some property of epithelial proliferation. Several possibilities are discussed and related to other instances of induction of heterotopic bone by epithelia.

INTRODUCTION

It has recently been shown that osteogenesis for the membrane bones of the cranial and mandibular skeletons of the embryonic chick is only initiated after the preskeletal ectomesenchyme has undergone an inductive interaction with epithelia at the site where the bones will form. In the case of the quadratojugal of the maxillary skeleton the interaction requires that cells leave the neural crest and migrate to the maxillary process where they interact with the maxillary epithelium (Tyler, 1978; Hall, 1978a). In the case of the six membrane bones

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of the mandibular skeleton, neural-crest cells migrate into the developing mandibular process where they undergo an interaction with the mandibular epithelium which is a prerequisite for the initiation of mandibular osteogenesis (Tyler & Hall, 1977). It has further been shown that the interaction between the mandibular ectomesenchyme and the mandibular epithelium is not highly specific with respect to the epithelial requirement—epithelium from wing or leg buds, as well as back or abdominal epithelium, will substitute for mandibular epithelium and initiate osteogenesis when combined with mandibular ectomesenchyme and the recombinants maintained either in vitro or as grafts on the chorioallantoic membrane (Hall, 1978b).

Although mandibular, wing- and leg-bud epithelia all permitted initiation of osteogenesis, the epithelia only possessed that ability at some ages during their development and these ages differed for the different epithelia. Mandibular epithelia possessed this inductive ability between three and four days of incubation (Hamburger & Hamilton (1951) stages H.H. 18 to 23). H.H. stage 23 also marked the latest developmental stage when the mandibular ectomesenchyme required interaction with the epithelium. Thus duration of the requirement for epithelial interaction by the ectomesenchyme and loss of the ability of the epithelium to provide the interaction were coincident (Hall, 1978b). Wing-bud epithelium possessed the ability to initiate osteogenesis within ectomesenchyme only after four and a half days of incubation (after H.H. stage 24), while leg-bud epithelium possessed this ability from H.H. stage 18 onwards.

What is the nature of the interactions between these epithelia and the ectomesenchyme? The fact that the responding ectomesenchymal cells lie deep within the mandibular processes and are not in contact with the epithelium would seem to rule out direct cell to cell interaction, or interaction between ectomesenchymal cells and the basement membranes of the epithelia (see Saxén, 1977a, b, and Hay & Meier, 1978 for good recent discussions of the role of such contacts in inductive interactions). That there is a developmental stage-dependent ability of these epithelia to allow ectomesenchyme to form bone suggested that some time-related property of the epithelia might be important. Among possible temporal components would be synthesis of a morphogen, attainment of a specific state of differentiation of the epithelia, rate or amount of cell division and/or aspects of the cell cycle. As a first step directed toward understanding the basis of the epithelial–ectomesenchymal interaction which lead to the initiation of osteogenesis within the mandible, mandibular epithelia were isolated from mandibular processes, killed by a variety of treatments, recombinated with vital mandibular ectomesenchyme and either maintained in organ culture or as grafts to the chorioallantoic membranes of host embryos. As a second step to determine whether the ability to induce osteogenesis was related to epithelial proliferative activity, epithelia were treated either with one of a graded series of doses of gamma irradiation or with colchicine, recombinated and cultured or grafted. In all cases the incidence of bone produced was
compared with the incidence of bone in companion cultures or grafts in which the epithelia had been treated with the vehicle used for the experimental epithelia.

**MATERIALS AND METHODS**

**Incubation procedure**

Fertile eggs of the domestic fowl, *Gallus domesticus*, White Leghorn, shaver starcross 288 strain, were obtained from Lone Pine Farm, Berwick, Nova Scotia, and from Cook's Hatchery, Truro, Nova Scotia. Eggs were incubated within 24 h of their arrival without rotation in a forced-draft, Humidaire Incubator (Model 350, Humidaire Incubator Co., New Madison, Ohio, U.S.A.) maintained at 37 ± 0.5 °C and 57 ± 4 % relative humidity.

**Preparation of tissue**

Eggs which had been incubated for 96 h were opened under sterile conditions and the embryos removed and placed into Petri dishes containing sterile saline (0.85 % NaCl). These embryos were staged according to the morphological series of H.H. stages described by Hamburger & Hamilton (1951). Only embryos of H.H. stage 22 were used in this study. The mandibular processes were dissected from these H.H. stage-22 embryos and placed into a sterile solution of trypsin and pancreatin in calcium and magnesium-free Tyrode solution (257 mg beef pancreas trypsin and 43 mg pig pancreas pancreatin/10 ml, both obtained from BDH chemicals, Toronto, Ontario, Canada). Sixty minutes of enzyme treatment at 4 °C was sufficient to loosen the mandibular epithelium from the mandibular mesenchyme and ectomesenchyme. The mandibles were then transferred to a mixture of the complex culture medium BGJb and horse serum (1:1, v/v) to slow down the action of the enzymes. The epithelia were then separated from the mesenchyme and ectomesenchyme by microdissection using watchmaker forceps and sharpened hypodermic needles. The isolated mesenchymal cores of the mandibles remained in BGJb horse serum during the subsequent treatment of the epithelia.

**Treatment of epithelia**

The isolated epithelia were treated in one of the following ways:

1. Epithelia were soaked in sterile distilled water for 30 min at room temperature.
2. Epithelia were allowed to dry in air for 20 min at room temperature.
3. Epithelia were exposed to 80 % ethanol for 30 min at room temperature.
4. Epithelia were exposed to three alternating periods of freezing and thawing. Each freeze/thaw cycle lasted for 60 or 120 min.
Table 1. The incidence of cartilage and bone found in mandibular ectomesenchyme after recombination with killed mandibular epithelia and maintenance for 7 days either in organ culture or as grafts to the chorioallantoic membrane

<table>
<thead>
<tr>
<th>Treatment of epithelia*</th>
<th>Cartilage†</th>
<th>Bone†</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Control†</td>
<td>84/90</td>
<td>60/90</td>
</tr>
<tr>
<td>(2) Distilled water</td>
<td>9/9</td>
<td>0/9</td>
</tr>
<tr>
<td>(3) Air drying</td>
<td>10/10</td>
<td>1/10†</td>
</tr>
<tr>
<td>(4) 80% ethanol</td>
<td>23/23</td>
<td>0/23</td>
</tr>
<tr>
<td>(5) Freeze thaw</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) 3 x 60 min</td>
<td>10/10</td>
<td>0/10</td>
</tr>
<tr>
<td>(b) 3 x 120 min</td>
<td>20/20</td>
<td>1/20</td>
</tr>
<tr>
<td>(6) 2500 rad</td>
<td>10/10</td>
<td>0/10</td>
</tr>
</tbody>
</table>

* See Materials and Methods for details of the treatments.
† The control epithelia were maintained at room temperature in BGJb : Horse serum (1:1) for the time needed to treat the epithelia from 2–6 (20–120 min). As all time periods gave similar results the data have been pooled.
‡ The epithelium was alive in this one specimen.
§ As the results did not vary significantly between cultures and grafts the data have been combined.

(5) Epithelia were exposed to gamma irradiation using an Atomic Energy of Canada Ltd cobalt 60 gamma cell irradiator (model 220). The dose rate was 2.5 rad/sec, with total irradiation ranging from 500 to 10000 rad.

(6) Epithelia were exposed to colchicine at concentrations of either 0.1 or 1.0 μg/ml for 4 h at 37 °C.

(7) As a control epithelia were exposed to BGJb : horse serum both at room temperature and at 37 °C for times similar to those listed under 1–6. The mesenchymal cores were maintained under similar conditions.

At the end of each of the above seven treatments the epithelia and mesenchymes were rinsed in the culture medium BGJb.

Procedures for organ culturing.

The treated epithelia were placed onto squares of sterile, black Millipore filter (0.45 μm porosity; Millipore Filter Corporation, Montreal, Quebec, Canada) and secured to them by gentle pressure at the corners. The isolated mesenchymes were then placed directly onto the epithelia, one on one. The recombined tissues were placed onto stainless steel grids in 35 x 10 mm Falcon plastic Petri dishes containing 1.5 ml of BGJb supplemented with 15% horse serum (both from GIBCO Laboratories, Grand Island, N.Y., U.S.A.) and 150 μg ascorbic acid/ml (Matheson, Coleman and Bell, Norwood, Ohio, U.S.A.). The cultures were maintained at 37 °C in a humidified CO₂ incubator (Forma Scientific Model 3156) in an atmosphere of 5% CO₂ in air for 7 days. The medium was completely changed every third day. For the number of cultures examined see Table 1.
Procedure for chorioallantoic grafting

Epithelia and mesenchymes were recombined and placed onto Millipore filters as described above. Embryos which had been incubated for 8 days were used as hosts. A window in the shell was made to expose the highly vascular chorioallantoic membrane. The recombined tissues on the filters were placed onto the chorioallantoic membrane so that the tissue was in direct contact with the chorioallantoic membrane. The shell window was replaced, sealed with tape and the host and graft incubated at 37 °C for 7 days. Further details of the grafting procedure may be found in Hall (1978c). The number of grafts analyzed in this study is shown in Table 1.

Histological procedures

After 7 days of in vitro cultivation or as grafts, the tissues were fixed on the filters by irrigation with neutral buffered formal saline, dehydrated, cleared and embedded in 54 °C melting point paraffin. Serial sections were cut at 5–6 μm and stained with haematoxylin, alcian blue and chlorantine fast red, a procedure which renders cartilage a bright blue and bone a brilliant red. Sections were examined for the presence or absence of bone and cartilage and for the status of the epithelia.

Isotopic labelling

As a further check on their viability and proliferative activity some epithelia treated with 80 % ethanol, gamma-irradiation or colchicine, along with untreated epithelia were also exposed to [³H]thymidine (specific activity 46·8 Ci/m mole, New England Nuclear, Lachine, Quebec, Canada) and processed for autoradiography.

One set of epithelia was exposed to 80 % ethanol at room temperature for 30 min, rinsed in BGJb and then exposed to 1 ml BGJb containing 10 μCi [³H]thymidine for 3 h at 37 °C. After a further rinse in cold BGJb these epithelia were recombined with mandibular mesenchyme, cultured for 7 days and processed autoradiographically.

A second set of isolated epithelia was exposed to 3000 rad of gamma-irradiation. Half was then exposed to 10 μCi [³H]thymidine in BGJb for 2 h and processed immediately for autoradiography. The other half was recombined with mesenchyme and grafted to the chorioallantoic membrane for 7 days. These grafts were recovered under sterile conditions, exposed to 10 μCi [³H]thymidine for 2 h and processed autoradiographically.

A third set of epithelia was exposed to colchicine at 1 μg/ml for 4 h and then to 10 μCi [³H]thymidine for 1 h. Half the epithelia was fixed immediately while the other half was recombined with unlabelled mesenchyme, grafted to the chorioallantoic membrane for 7 days and then processed for autoradiography.
 Autoradiography

The tissues which had been exposed to [³H]thymidine were fixed, processed and sectioned as outlined above. After staining with haematoxylin and alcian blue and exposure to a phosphomolybdic acid mordant the slides were roller coated with Kodak NTB3 emulsion (1:1 with distilled water), dried for 30 min and exposed in a light-tight box containing silica gel for 6 days. The autoradiographs were developed for 2 min in Kodak D-19 developer, fixed for 5 min, washed for 20 min and stained with 0-5 % chlorantaine fast red. The emulsion was destained in acid alcohol (3 % HCl in 70 % ethanol), and the slides placed in two changes of ethylene glycolmonoethylether, dehydrated and mounted. These autoradiographs were examined for the presence and distribution of labelled cells.

RESULTS

Chondrogenesis

As shown in previous studies (Tyler & Hall, 1977; Hall, 1978b) H.H. stage-22 mandibular ectomesenchyme can be recombined with mandibular or non-mandibular epithelium to form cartilage and bone and maintain epithelial differentiation when subsequently organ cultured or grafted to the chorio-allantoic membrane. Cartilage formed from isolated ectomesenchyme but osteogenesis required that the epithelium be present until H.H. stage 23. These data provided the baseline for the present study for they indicated that the procedures of tissue dissection, enzymatic separation of epithelium from ectomesenchyme and recombination of the two components did not prejudice the differentiative ability of either component. The present paper utilized the same experimental methods to determine whether the epithelial cells had to be vital and/or proliferating in order to be able to exert their influence on the mandibular ectomesenchyme.

As chondrogenesis within the ectomesenchyme does not require the presence of an epithelium, the appearance of cartilage in the present cultures or grafts was taken as an indication that the ectomesenchymal cells were healthy and undergoing normal differentiation. The data on the incidence of cartilage given in Table 1 indicate that chondrogenesis was initiated in virtually every culture and graft. Furthermore the incidence of cartilage was independent of any and all treatments of the epithelia (Figs 1, 3, 7). This was taken to indicate that recombination of ectomesenchyme with treated epithelium did not lead to inhibitory effects of the epithelia on the mesenchymal and ectomesenchymal cells.

Osteogenesis

The data in Table 1 also indicate that osteogenesis was initiated in two thirds of the control cultures and grafts. These were recombinations where both epithelium and mesenchyme had been placed in BGJb : Horse serum at room
Epithelia and osteogenesis

Fig. 1. A low power photomicrograph of a recombination between mandibular ectomesenchyme and epithelium exposed to BGJb: Horse serum for 30 min before being enzymatically separated, recombined and grafted to the chorioallantoic membrane for 7 days. Membrane bone (b) and rods of cartilage (c) have differentiated. m, Millipore filter substrate; e, mandibular epithelium. Haematoxylin, alcian blue and chlorantine fast red, × 52.

Fig. 2. Membrane bone (b) and highly differentiated mandibular epithelium (e) in a recombinant between components exposed to BGJb: Horse serum for 120 min before being separated, recombined and grafted. Note the close contact between the mandibular epithelium and the adjacent ectomesenchyme and mesenchyme. Haematoxylin, alcian blue and chlorantine fast red, × 338.

temperature for the same length of time (20–120 min) that companion epithelia were exposed to one of the experimental treatments (Figs. 1, 2). Because time of exposure to BGJb: Horse serum and whether organ cultures or grafted did not influence the incidence of bone formation, the controls from each experimental treatment were grouped together.

All of the treatments which were used to kill the epithelia suppressed its ability to initiate osteogenesis from mandibular ectomesenchyme (Table 1). This suppression was either complete or virtually complete. In the one instance in which bone was found in a recombinant of air-dried epithelium and ectomesenchyme, the epithelium was found to be mitotically active and evidently vital.
Fig. 3. [3H]thymidine and alcohol-treated epithelia (e), recombined with untreated ectomesenchyme and cultured for 7 days do not take up any isotope (cf. Fig. 4). c, cartilage; m, millipore filter. Haematoxylin, alcian blue and chlorantine fast red, × 201.

Fig. 4. Epithelia (e) exposed to [3H]thymidine, recombined with unlabelled ectomesenchyme and organ cultured for 7 days show incorporation of isotope into proliferating cells (arrows). C, cartilage. Haematoxylin, alcian blue and chlorantine fast red. × 367.

The one case of osteogenesis after recombination of ectomesenchyme with freeze-thawed epithelium remains an exception to the pattern exhibited by the other recombinants.

**Viability of epithelia**

Several lines of evidence were used to determine that the epithelia had in fact been killed by the various treatments. Comparison between the epithelia in recombinations of treated epithelia with ectomesenchyme and untreated epithelia with ectomesenchyme showed that in the former the epithelium was thin and the nuclei often pyknotic while in the latter the epithelia were multilayered, mitotically active and maintaining stratified squamous epithelial differentiation (except where the epithelia were physically separated from the ectomesenchyme in which areas the epithelium had keratinized).
Fig. 5. The percentage of grafts containing membrane bone in grafts of mandibular ectomesenchyme recombined with irradiated epithelia is shown as a function of the dose of gamma irradiation (0–10000 rad). Number of samples – see Tables 1 and 2.

Treated and untreated epithelia were cultured in isolation from ectomesenchyme. Untreated epithelia spread over the millipore filters as the cells continued to proliferate. Treated epithelia showed no attachment to the filters, their cells did not divide and their nuclei appeared pyknotic.

When epithelia which had been treated for 30 min with 80 % ethanol and then exposed to $[^3]$H]thymidine for 3 h were cultured, no uptake of label could be found.

Nor was isotopic labelling observed when such epithelia were recombined with ectomesenchyme and cultured (Fig. 3). This contrasted with control epithelia which contained labelled cells after 7 days of having been organ cultured either alone or after recombination with mandibular ectomesenchyme (Fig. 4). A similar lack of $[^3]$H]thymidine-labelled cells was observed in epithelia exposed to 3000 rad of gamma irradiation.

In summary, common features of the various treatments were that they prevented uptake of $[^3]$H]thymidine by the epithelia, killed the epithelial cells and eliminated their ability to initiate osteogenesis from mandibular ectomesenchyme.

**Proliferation of epithelia**

Was the inability of the epithelia to initiate osteogenesis within ectomesenchyme a function of the loss of epithelial proliferative activity or was it a function of the lack of viability of the epithelia? To answer these questions epithelia were exposed to one of a series of doses of gamma irradiation between 0 and 10000 rad.
The epithelia were then labelled with [³H]thymidine and either autoradiographed immediately or recombined with unlabelled, untreated mesenchyme, grafted to the chorioallantoic membranes of host embryos for 7 days and then examined autoradiographically. It was reasoned that increasingly higher doses of irradiation would eliminate progressively more cells from the epithelia, with mitotically active cells being more sensitive to irradiation-induced removal than non-mitotic cells. [³H]thymidine-labelling was used to monitor the number of cells engaged in DNA synthesis within the epithelia. The data obtained on the incidence of cartilage and bone in these grafts are shown in Table 2 and in Fig. 5. The percentage of grafts forming bone decreased with increasing doses of gamma irradiation up to 2500 rad (Fig. 6). Surprisingly the incidence of bone increased with doses of gamma irradiation between 2500 and 5000 rad (Fig. 7). A replicate of this experiment confirmed the reproducibility of these results (Table 2). Irradiation of epithelia with 10000 rad inhibited bone formation to
Table 2. The incidence of cartilage and bone found in mandibular ectomesenchyme after recombination with irradiated mandibular epithelia and maintenance for 7 days as grafts to the chorioallantoic membrane

<table>
<thead>
<tr>
<th>Dose of gamma irradiation (rad)</th>
<th>Cartilage</th>
<th>Bone</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>50/57</td>
<td>32/57</td>
</tr>
<tr>
<td>500*</td>
<td>5/6</td>
<td>4/6</td>
</tr>
<tr>
<td>1000</td>
<td>13/13</td>
<td>6/13</td>
</tr>
<tr>
<td>1500</td>
<td>7/7</td>
<td>3/7</td>
</tr>
<tr>
<td>2000</td>
<td>13/13</td>
<td>3/13</td>
</tr>
<tr>
<td>2500†</td>
<td>10/10</td>
<td>0/10</td>
</tr>
<tr>
<td>3000*</td>
<td>11/11</td>
<td>1/11</td>
</tr>
<tr>
<td>4000</td>
<td>8/8</td>
<td>1/8</td>
</tr>
<tr>
<td>5000*</td>
<td>15/16</td>
<td>4/16</td>
</tr>
<tr>
<td>10000</td>
<td>12/12</td>
<td>7/14</td>
</tr>
<tr>
<td></td>
<td>10/10</td>
<td>4/10</td>
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<tr>
<td></td>
<td>15/15</td>
<td>3/15</td>
</tr>
</tbody>
</table>

* A second experiment was performed with each of these doses to check the initial results.
† These are the grafts listed in Table 1.

the same extent as did 3000 rads. Chondrogenesis within the ectomesenchyme was not affected by irradiation of the epithelia (Table 2).

The subset of recombinants which had been exposed to [³H]thymidine were examined and it was found that epithelial cells were labelled even after 10000 rad of gamma irradiation (Fig. 8). Evidently the epithelia were not being killed even with the largest dose of irradiation used.

In order to determine whether the pattern of incidence of osteogenesis observed after various doses of gamma irradiation could be correlated with proliferative activity of the epithelia, a series of epithelia were exposed to 0, 500, 2000, 3000, 5000 or 10000 rad and examined immediately. These epithelia provided an indication of the proliferative activity at the time that other similarly irradiated epithelia were recombined with ectomesenchyme. As shown in Fig. 9 the per cent labelling index varied with the level of irradiation in a manner which paralleled the incidence of osteogenesis in the recombinants. The per cent labelling index was lowest after 2500 and 10000 rad and highest after 5000 rad. As Fig. 10 indicates there was a close linear relationship between [³H]thymidine labelling of the epithelia and the incidence of osteogenesis ($r = 0.765$, $N = 6$, $P = 0.05$). If the 5000 rad dose is removed the correlation is even closer ($r = 0.900$, $N = 5$, $P = 0.05$).

As a further check that the initiation of osteogenesis was related to mitotic activity of the epithelia, a group of mandibular epithelia was exposed to colchicine (0.1 or 1.0 $\mu$g/ml) at 37 °C for 4 h, labelled with [³H]thymidine and
Fig. 8. Mandibular epithelium exposed to 10000 rad of gamma irradiation and labelled with [3H]thymidine shows uptake of label into cells synthesizing DNA. Haematoxylin, alcian blue and chlorantine fast red, ×1100.

![Image of mandibular epithelium](image)

Fig. 9. The % labelling index of uncultured mandibular epithelia irradiated with gamma irradiation (0–10000 rad) was calculated from autoradiographs prepared after exposure of the epithelia to [3H]thymidine. Based on six specimens per dose.

![Graph showing % labelling index](graph)

either processed immediately for autoradiography or recombined with ectomesenchyme, grafted and subsequently autoradiographed.

Epithelia exposed to medium without colchicine for 4 h at 37 °C remained mitotically active and initiated osteogenesis in two thirds of the ectomesenchymes with which they were recombined (9/14). However, in colchicine-treated epithelia the number of labelled cells was reduced below that seen in noncolchicine-treated epithelia and the incidence of osteogenesis initiated in
Fig. 10. The linear relationship between the % labelling index of irradiated epithelia and the percentage of grafts forming bone in recombinants between mandibular ectomesenchyme and irradiated epithelium is a very close one ($r = 0.765$, $P, 0.1-0.05$). The regression line was calculated by the least squares method and has the form, $Y = 0.773x + 6.253$.

Fig. 11. A plate of trabecular bone (b) formed in this one graft of mandibular ectomesenchyme which had been recombined with epithelium (e) exposed to 0.1 μg/ml colchicine for 4 h before being grafted to the chorioallantoic membrane for 7 days. Haematoxylin, alcian blue and chlorantine fast red, × 276.

mandibular ectomesenchyme was 2/9 after 0.1 μg colchicine/ml and 1/23 after 1.0 μg colchicine ml (Fig. 11). There was a linear relationship between concentration of colchicine and incidence of bone (Fig. 12) and, as with the irradiation
Fig. 12. The percentage of grafts forming bone and the % labelling index of the epithelium in recombinants between mandibular ectomesenchyme and colchicine-treated epithelium both decline as a function of the dose of colchicine (µg/ml culture medium) to which the epithelia were exposed.

Fig. 13. The linear relationship between the % labelling index of colchicine-treated epithelia and the % of grafts forming bone in recombinants between mandibular ectomesenchyme and colchicine-treated epithelium is a very close one ($r = 0.952$, $P, 0.20-0.10$). The regression line has the form, $Y = 0.519x + 12.033$.

The conclusion drawn from these experiments was that mandibular epithelium must be proliferating if it is to be able to elicit osteogenesis from mandibular ectomesenchyme.
DISCUSSION

Two general conclusions can be drawn from the results presented in this paper. First, that for the interaction between mandibular ectomesenchyme and epithelium which is a prerequisite for the initiation of intramembraneous osteogenesis, the epithelium must be alive. Epithelia killed by treatment with distilled water, air drying, 80% ethanol, freeze-thawing or by exposure to 2500 rad of gamma irradiation did not permit the initiation of osteogenesis. The second general conclusion is that there is a correlation between the proliferative activity of the mandibular epithelium (the per cent labelling index after administration of $[^3H]$thymidine) and its ability to allow ectomesenchyme to form bone. The lower the per cent labelling index the less able the epithelium is to allow osteogenesis. This conclusion is based on the parallel reduction in proliferation and in incidence of bone induced after epithelia are exposed to colchicine or to increasing doses of gamma irradiation.

The mechanism whereby irradiation reduces the per cent labelling index of the epithelium is obviously of interest, especially why the reduction in the labelling index is not linear over the dose range 0–10000 rad (Fig. 9). The highest per cent labelling index was observed after 5000 rad. As the dose range of 3000 to 5000 rad is known to lead to membrane changes in other cell types, it may be that similar membrane changes at these dose levels act to augment proliferation. The linear decrease in per cent-labelling index with increasing dosage of irradiation from 0 to 1500 rad could be explained by the known differential sensitivity of cells to irradiation as a function of the stage of the cell cycle; Cells in DNA synthesis (S phase) are the most sensitive to irradiation, cells in G2 less sensitive and cells in GI the least sensitive. If cells in the S phase are the first to be eliminated at the low doses of irradiation, and considering that such cells are augmented after 5000 rad, then the ability of the mandibular epithelium to elicit ectomesenchymal osteogenesis would be a function of a property of the epithelial cells associated with DNA synthesis. Agents such as vitamin A slow proliferation of mesenchymal cells by increasing the time spent in the S phase and in GI (Torrez-Diaz & Nanda, 1979). Subsequent studies will address the nature of the cell cycle and its control in the mandibular epithelium.

A further possibility is the occurrence of dose-related recovery of the epithelial cells from irradiation. The fact that epithelia exposed to 10000 rad contained some $[^3H]$thymidine-labelled cells (Fig. 9) is consistent with this possibility as is the recovery of cells after irradiation during amphibian limb regeneration (Desha, 1974; Wallace, Wessels & Conn, 1975; Desselle & Gontcharoff, 1978).

There are two other experimental systems in which epithelial cells induce osteogenesis. Both involve the induction of bone outside the confines of the skeleton. One is the response of connective tissue cells to the transitional epithelium of the urinary bladder (Huggins, 1931a, b; Friedenstein, 1962;
Beresford & Hancox, 1965, 1967; Friedenstein, Lalykina & Tolmacherova, 1967; Abdin & Friedenstein, 1972; Delides, 1972) or of the pelvis (Tavassoli & Crosby, 1971). The second is the induction of bone in response to cultured epithelial cells (Anderson, 1967; Wlodarski, 1969; Anderson, 1976). In the case of the transitional epithelium of the urinary bladder Huggins observed that only actively proliferating epithelium was inductively active and more recently Ioseliani (1972) has utilized [3H]thymidine labelling to show that the inductively active epithelia divide more rapidly than do other epithelia cells.

In the case of the epithelial cell lines it is the epithelial pattern of growth rather than epithelial origin which gives these cells their inductive ability (Ostrowski & Wlodarski, 1971). Thus virally-transformed fibroblasts which exhibit epithelial patterns of growth in vitro will induce bone (Wlodarski, 1969; Hall, 1978a), and the cells have to be established cell lines and not primary cultures. These inductively active cell lines are also all heteroploid and agglutinate with concanavalin A (Wlodarski, Ostrowski, Chtopkiewicz & Koziorpwska, 1974). The correlation with pattern of growth and with concanavalin A agglutination further highlights a possible role for the cell membrane in epithelial induction of bone. The proliferation and the ability of these epithelial cells to induce bone are also diminished after irradiation (Wlodarski et al. 1971). As in the present study epithelial cells were found to survive massive doses of irradiation (up to 30000 rad). These three systems in which epithelial cells act inductively on ectomesenchyme or mesenchyme have in common that the epithelia must be proliferative to be inductively active. A major difference between these systems is that the cells which produce the ectopic bone in response to transitional epithelium or to epithelial cell lines require continued contact with the epithelium for osteogenesis to be maintained while the ectomesenchymal cells only require an initial short-term interaction with their epithelia — an interaction which ends several days before the appearance of osteoblasts, osteoid or bone (Tyler & Hall, 1977). Presumably in the induction of ectopic bone from normally non-osteogenic cells, new cells have to be continually recruited to the osteogenic population by induction from the epithelium, i.e. these are instructive inductions, while in the mandible the interaction with the epithelium allows osteogenic cells to express that potential, i.e. this is a permissive induction. While this is a satisfying distinction it does beg the question of when and how these cells became osteogenic.

Also left unanswered is the question of what correlate of proliferation confers inductive ability upon these epithelia. Wlodarski et al. (1971) suggest that a proteinaceous inducer released by proliferating epithelia may be involved. Demineralized bone or dentinal matrices — well established inducers of cartilage which is then replaced by bone — contains a protein as the active inducer (Urist, Nakagawa, Nakata & Nogami, 1978; Urist, 1979) and like the above epithelia, show decreased ability to induce after irradiation (Bang & Johannessen, 1972). We are currently using a variety of pharmacological agents to explore the
possible chemical stimulation(s) which mandibular epithelium has on mandibular ectomesenchyme and *vice versa* (Bradamante & Hall, 1980).

The continued proliferation of epithelia at normal *in vivo* levels requires that they be in contact with ectomesenchyme or mesenchyme (McLoughlin, 1961; Horstadius, 1950; Holtfreter, 1968; Johnston, 1975). Epidermal growth factor, although it acts to stimulate proliferation of palatal epithelium, will only do so when the epithelium is in contact with mesenchyme (Tyler & Pratt, 1979). The inductive interaction between mandibular epithelium and ectomesenchyme which leads to osteogenesis may be akin to that which leads to odontogenesis (Slavkin, 1978) and require two-way interactions between epithelium and ectomesenchyme. The data on the interaction of non-mandibular epithelia with mandibular ectomesenchyme (Hall, 1978) are consistent with reciprocal interaction. Thus a scenario in which ectomesenchymal cells arrive in the developing mandibular process to stimulate proliferation of the epithelium thereby allowing the epithelium to elicit osteogenesis from the ectomesenchymal cells is not an unreasonable one.

One of the first steps in initiation of osteogenesis is the condensation of mesenchymal or ectomesenchymal cells (Hall, 1978). The decline of the labelling index in the maxillary process begins precisely at the time (H.H. stage 24) when the epithelial–ectomesenchymal interaction ceases (Minkoff & Kuntz, 1978; Tyler, 1978). If a similar temporal sequence is true for the mandibular process then we envisage the action of the epithelium as being related to the accumulation of ectomesenchymal cells and the cessation of the interaction as leading to lowered proliferation of ectomesenchyme and the sequence of events which initiate cytodifferentiation of osteoblasts and the deposition of bone. The demineralized bone matrix referred to above has, as its first action, a mitogenic effect on local mesenchymal cells (Rath & Reddi, 1979). The accumulation and subsequent removal of hyaluronate serve a similar function in chondrogenesis (Toole, 1972; Hall, 1978). Proliferation emerges as an important aspect of both the epithelial and the ectomesenchymal components of mandibular osteogenesis.

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Epithelia and osteogenesis


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