Relationships between cellular condensation, preosteoblast formation and epithelial-mesenchymal interactions in initiation of osteogenesis

LANA-LEE T. DUNLOP and BRIAN K. HALL*

Department of Biology, Dalhousie University, Halifax, Nova Scotia, Canada

ABSTRACT Initiation of osteogenesis or bone formation is dependent on cell and tissue interactions. We investigated the events between 4 and 7 days of incubation that translate epithelial-mesenchymal signalling into overt differentiation of osteoblasts and deposition of bone in the mandibles of chick embryos. Condensation of mandibular mesenchyme (the membranous skeleton), visualized with PNA-lectin, occurred at H.H. mid-26 (5.75 days), lasted 12 h and preceded osteoblast differentiation by 1.5 days. As determined from 3D-reconstruction all mandibular membrane bones arose from a single condensation closely associated with the stomodeal epithelium. The finding that the osteogenic condensation in the mandibular arch is a major branch of a common condensation that provides osteogenic mesenchyme to both maxillary and mandibular arches establishes a closer link between mechanisms controlling development of the skeleton in these two arches than previously suspected. Preosteoblasts (alkaline phosphatase-positive cells) form in the mandible at H.H. early 25, which is before condensation but after the epithelial-mesenchymal interaction upon which preosteoblast formation and condensation depend — neither form in isolated mesenchyme, whereas both form after recombination of mesenchyme and epithelium. Tenascin was present in the mandibular epithelium only at H.H. stage 19 but not in the mesenchyme at any age. Therefore, the epithelial-mesenchymal interaction controls initiation of osteogenesis at the preosteoblast stage. Preosteoblasts then condense, transform into osteoblasts and deposit bone matrix. Differentiation of preosteoblasts precedes condensation which amplifies their number. This is in contrast with chondrogenesis where condensation triggers prechondroblast differentiation.

KEY WORDS: condensation, osteogenesis, epithelial-mesenchymal interaction

Introduction

Initiation of cell proliferation and formation of a condensation — the "membranous skeleton" of Gruneberg (1963) — is the earliest detectable cellular stage of chondrogenesis (see Hall and Miyake, 1992 for a review). Condensation brings cells of a similar differentiation potential into proximity, enabling increased cell-to-cell communication, as evidenced by a dramatic increase in the numbers of gap junctions (Coelho and Kosher, 1991; Hall and Miyake, 1992).

Condensation marks initiation of selective gene expression during skeletogenesis. Many mutant genes or teratogenic agents alter skeletal development by exerting their primary action at this stage (Merker et al., 1975; Johnson, 1986; Hall and Miyake, 1992). Talpid in the chick and brachypodism and congenital hydrocephalus in the mouse, exert their primary action at the condensation stage by altering cell surface properties that affect cellular aggregation and/or cell-cell interactions (Johnson, 1986), both of which greatly influence condensation development (Hall and Miyake, 1992).

The importance of the "membranous skeleton" was further emphasized by Atchley and Hall (1991) in a model of the development and evolution of complex skeletal elements in which condensations are identified as the basic morphogenetic units from which skeletal elements are constructed. A "simple, single" bone like the mammalian dentary was considered to be constructed from 6 separate condensations, each representing a different cell lineage. Confirmation of this model comes from such studies as the recent finding that the homeobox gene Msx1 affects only the condensation associated with the incisor and molar teeth and associated alveolar bone, which are absent or severely reduced in Msx1-deficient mice (Satokata and Maas, 1994).

The generality of the condensation phase is evident from the

Abbreviations used in this paper: AP, alkaline phosphatase; CAMs, chorioallantoic membranes; DMSO, dimethyl sulfoxide; E/P, epithelial/mesenchymal; HBQ, Hall-Brunt Quadruple stain; PBS, phosphate-buffered saline; PNA, peanut agglutinin lectin; TFG-β, transforming growth factor-beta.
appearance of condensations during development of almost all tissues and organs including lung, nose, eye, ear, vibrissae, hair follicles, kidney, muscle, cartilage, and endochondral bone. In skeletal development the condensation phase may play one or more of 3 fundamental roles: (1) provide the appropriate quantity of cells (tissue mass) from which morphology can be constructed; (2) turn on specific genes (selective gene activation) as demonstrated in prechondrogenic condensations (Kosher et al., 1986a,b); and (3) provide the high cell density required for initiation of cytodifferentiation (Aulthouse and Solursh, 1987; Browder et al., 1991). Any of these 3 events could be regulated by epithelial-mesenchymal interactions. Stein et al.'s (1990) analysis and model of the relationship between proliferation and differentiation during rat osteoblast development, illustrates the fact that cell proliferation and formation of a condensation are required for the initiation of differentiation, but that cell proliferation must cease before differentiation can be initiated. Very recently this group has demonstrated that TGF-β at concentrations as low as 0.1 ng/ml blocks the transition between proliferation and differentiation (Breen et al., 1994).

Three models of the temporal relationship between condensation and differentiation in the initiation of mandibular bones in avian embryos are summarized in Fig. 1. Model 1 assumes that condensation precedes cytodifferentiation as in chondrogenesis and as proposed in the differentiation of rat osteoblasts (Stein et al., 1990). Model 2 assumes the reverse — initiation of cytodifferentiation precedes condensation formation. If differentiation is defined as the appearance of a cell-specific product, then this model is consistent with Kosher et al. (1986b), who showed that a cartilage-specific product — mRNA for type II collagen — appears before condensation during chick limb bud development. Model 3 assumes that condensation and cytodifferentiation are simultaneous, and is consistent with Kosher et al. (1986a) who showed that mRNA for cartilage core protein appeared with condensation.

Although a condensation phase has been clearly and repeatedly observed during organogenesis of other tissues, there is no direct evidence that a condensation phase exists during differentiation of intramembranous bones. Indirect evidence for increased proliferation preceding cytodifferentiation dates to the pioneering studies of Fell (1925) and Jacobson and Fell (1941) using light microscopic histology, and to the 3H-thymidine-labeling studies of Fyfe and Hall (1983), but proliferation need not equal condensation. Condensations may arise by prevention of migration away from a center (Gould and Wolpert, 1972; Gould et al., 1974) and/or by aggregation of cells toward a center (Epplerlein and Lehmann, 1975), neither of which need involved increased proliferation.

Therefore, we sought to determine (1) whether osteogenic condensations exist; (2) if they do, when they form, and (3) their general morphology. Tissue separation and recombination experiments were used to determine whether the epithelial-mesenchymal interactions known to be required for osteogenesis (Hall et al., 1983; Van Exan and Hall, 1984; Hall, 1988, 1994) control osteogenesis at the condensation stage. Condensations were visualized with peanut agglutinin lectin (PNA), using a method developed by Zimmermann and Thies (1984) and applied widely to detect prechondrogenic condensations, most notably by Milella (1991). Aulthouse and Solursh (1987) and Bagnall and Sanders (1989). PNA binds the Galβ1-3GalβNAc residues on condensing cells, thereby binding preferentially to cellular aggregates (ibid.). The only previous reference to specificity of this lectin for osteogenic cells is that PNA preferentially binds to osteoblast cell membrane domains (Watson et al., 1989).

Whether condensation coincides with initiation of osteogenic differentiation was determined by whether alkaline phosphatase-positive cells appeared coincident with, before, or after, condensation. Alkaline phosphatase activity, which is enhanced during very early stages of bone cytodifferentiation, is a well-recognized marker for osteoblast differentiation (see Weinreb et al., 1990 for a recent analysis). Alkaline phosphatase rather than another molecule(s) was chosen for 2 reasons: (1) type I collagen is not specific for bone (Rodan and Noda, 1991); and (2) bone-specific proteins such as osteonectin, osteocalcin or osteopontin, only appear during late stages of osteogenesis. Often coincident with mineralization and hence too late to be used as markers for osteoblast differentiation (Bronckers et al., 1987; Weinreb et al., 1990; Rodan and Noda, 1991).

In addition, we examined a possible role for tenascin, a novel extracellular matrix molecule, in the initiation of osteogenesis, condensation formation, and/or the epithelial-mesenchymal interaction. Tenascin, characterized by a 6-armed oligomeric structure (Chiquet, 1989) is deposited in condensing mesenchyme as a result of epithelial-mesenchymal interactions during organogenesis of vibrissae, kidney, gut, mammary glands, teeth and lungs.

Results

Do osteogenic condensations exist?

Whole heads between 3 and 7 days of incubation were collected and fixed for staining with PNA or Hall-Brunt Quadruple stain (HBO; Hall, 1986). Three days was the earliest age examined because it precedes the completion of the osteogenic epithelial-mesenchymal interaction, which lasts until H.H. stage 24, approximately 4 days of incubation (Hall, 1978a). Seven days (H.H. stage 31) was the
latest age examined because it is when osteogenic cells become secretory osteoblasts and lay down matrix (Tyler and Hall, 1977).

Careful observation of serial sections using blue-filtered light to enhance contrast, revealed osteogenic condensations in the mesenchyme of mandibular arches between H.H. stage mid-26 (approximately 5.5 days of incubation; Table 1) and late-28 (approximately 6.25 days incubation). PNA was very faint at mid-stage 26, strongest at early stage 28 (Fig. 2A) and extremely faint by late stage 28. Duration of the condensation phase therefore corresponded to an incubation time of 12 h. Secretory osteoblasts, identified by early deposition of bone matrix with HBQ, coupled with negative PNA binding were first detected at mid-stage 30, approximately 7 days incubation. That the condensations were osteogenic was based upon several criteria.

1) A process of elimination. By the 5th day of incubation, muscles, nerves and Meckel’s cartilage have all begun to differentiate. Muscle and nerve can be readily distinguished. Meckel’s cartilage and the mandibular bones lie close to one another. As perichondria are PNA-positive (Sasano et al., 1992, and present study; Fig. 2B and C), Meckel’s cartilage was carefully analyzed to ensure that the condensation was not perichondral, which it was not.

Fig. 2. PNA-positive condensations. (A) A typical oblique section through the mandibular (md) and maxillary (mx) arches of an H.H. stage early-28 chick embryo to show the PNA-positive condensation (*). (B and C) Representative oblique sections of 5 and 7 day old chick embryos (B and C respectively) to show PNA-positive staining (arrows) in the perichondrium of Meckel’s cartilage (c). (Top is dorsal). Scale bar: 1 cm = 4 mm.
2) Consistency of timing. Condensation of prechondrogenic mesenchyme for Meckel's cartilage occurs approximately 24 h prior to differentiation (Hall, 1978a). The putative osteogenic condensation also formed approximately 24 h prior to bone matrix deposition.

3) Location. The condensed mesenchyme was closely associated with the stomodeal epithelium laterally and with Meckel's cartilage medially (Fig. 3). This localization conforms to the classic description by Jacobson and Fell (1941). The condensation was also in close proximity to the mandibular nerve and mandibular blood vessels; osteogenesis requires vascularization (Hall, 1988) and is initiated in association with nerves (Kjaer, 1990). Location of the condensation was confirmed with 3D-reconstruction (see below).

**Morphology of the osteogenic condensation: 3-D reconstruction**

The osteogenic condensation was larger and more prominent dorsolaterally where it was closely associated with the facial ectoderm (Fig. 3A and B). It progressively thinned out to occupy a narrow space between the stomodeal epithelium and Meckel's cartilage (Fig. 3C).

In order to establish the morphology of the osteogenic condensation(s), serial sections from whole heads of H.H. stage early-28 embryos were captured, printed and processed to obtain a 3-D image (Fig. 4).

Surprisingly, the 3-D reconstruction revealed that a single osteogenic condensation contributed to both the mandibular (md) and maxillary (mx) arches (Figs. 3C and 4). At H.H. stage early-28, the maxillary portion of the condensation has already migrated to its...
Fig. 4. A 3-D reconstruction of osteogenic condensations taken from the mandible of a PNA-stained, serially sectioned early-stage 28 embryo. (A) Frontal view showing the common osteogenic condensation contributing to both the mandibular (Md) and maxillary (Mx) arches. At this stage, the condensation has migrated deep within the maxillary arch. In the mandibular arch, much of the condensation remains in the rostral position from which the cells must migrate into the arch. (Blue, osteogenic condensation; green, buccal cavity; green and marked with an arrow, notochord; red, maxillary vein; top is dorsal). (B) A more ventral orientation to show the extent of the mandibular condensation, the connection between the maxillary and mandibular portions of the condensation, and the close association of maxillary vein and condensation. (Red, osteogenic condensation; green, buccal cavity; green and marked with an arrow, notochord; yellow, maxillary vein; top is dorsal).

final position deep within the first branchial (maxillary) arch. In the mandibular arch, however, the condensation is still primarily localized to the original rostral position — the migration of the cells into the second branchial (mandibular) arch has not been completed. The close association between the condensation and stomodeal epithelium was very evident. Thus, a common condensation located laterally against the facial ectoderm bifurcated to contribute to both mandibular and maxillary arches. The skeletons of the lower and upper jaws therefore arise from a common condensation.
Establishing a relationship between the osteogenic epithelial-mesenchymal interaction and the condensation

Grafts of isolated mandibular mesenchyme and direct recombinations of mesenchyme and epithelium were used to investigate any relationship between the osteogenic epithelial-mesenchymal interaction and condensation formation. A comment on terminology is required because the age of grafted tissues can be expressed in 3 different ways. Donor age refers to the incubation time of the donor embryo at the time the mandibular arches were removed and grafted. Graft time refers to the duration of the incubation period during which grafted tissues were maintained on the CAMs of host embryos. The day of grafting was designated day 0. Combined age is the sum of donor age and graft time.

Establishing the timing of onset of bone matrix deposition in grafted tissues

Because it takes a little time for a CAM graft to become vascularized, development proceeds at a different rate in grafts than in ovo. To facilitate determining the timing of osteogenic condensation formation in grafted tissues we first determined the time of onset of bone matrix deposition in grafted tissues (Table 2). Osteogenic condensations were most prominent in ovo 24 h before bone matrix deposition (6 and 7 days respectively). We therefore expected osteogenic condensations to appear approximately 24 h prior to bone matrix deposition in grafted tissues.

Mandibular arches were dissected from embryos of H.H. stage 23 (4 days donor age) and grafted to host CAMs for 2 to 4 days (graft time). Three days as a graft (7 days combined age) would coincide with the time of bone matrix deposition in ovo if grafts developed at the same rate.

The results of 35 grafts, summarized in Table 2, show that onset of bone matrix deposition was between 7 and 8 days combined age — 3 and 4 days graft time. At 7 days combined age, osteogenic cells were just becoming secretory osteoblasts. However, by 8 days combined age, considerable bone matrix had been laid down. Taking 7.5 days combined age as an age when osteogenesis was well advanced, grafting delayed the timing of developmental events by approximately 12 h, and so condensations were expected at 6.5 days combined age. The delay of 12 h is comparable to delays found when other elements are grafted (Hall, 1978b) and reflects the time required to revascularize the graft.

Establishing the timing and duration of osteogenic condensations in grafted tissues

Grafts of 7 and 8 days combined age (above) were negative when stained with PNA. Binding specificity decreases as overt differentiation of bone commences. Using this timing, a series of grafting experiments were performed to establish the timing and duration of osteogenic condensations in grafted tissues (Table 2). Mandibular arches were removed from H.H. stage 23 embryos and grafted to the CAMs of host embryos for 2 or 3 days.

Osteogenic condensations as visualized with PNA were not present at 6.25 or 7 days combined age, but were present at 6.5 days combined age (5.25 days on CAM). They persisted until 6.75 days combined age (5.75 days on CAM) (Fig. 5A and B). Therefore the duration of the condensation in these grafts was a minimum of 6 and a maximum of 12 h, comparable to in ovo.

Do osteogenic condensations form in the absence of the epithelium?

Using the timing of condensation formation determined above, we then investigated whether condensation formation depended on the epithelial-mesenchymal interaction. Mandibular mesenchyme from H.H. stage 23 embryos, grafted for 4.5-5.5 days served as controls for the efficacy of tissue separation and isolation. No bone formed in grafts of isolated mesenchyme even when maintained for up to 5.5 days (5.5 days combined age; Table 3) and no epithelium was present. The appearance of Meckel's cartilage in the grafts served as an internal control to monitor the health and vitality of the tissues.

Mesenchyme was grafted for sufficient time to allow condensations to form (7.75 days combined age) and for an additional 24 h (8.75 days combined age) in case condensation formation was further delayed. No osteogenic condensations formed in isolated mesenchyme from H.H. stage 23 embryos grafted for 3.75 or 4.75
Fig. 5. Timing and duration of osteogenic condensations in grafted tissues. (A-B) Photomicrographs of typical 5μm sections of chick mandibular arches grafted at H.H. stage 23 and maintained for a combined age of 6.5 days. (A) Toluidine blue-stained section to show cartilage (purple). Scale bar: 1 cm = 16 mm. (B) PNA-stained section showing osteogenic condensation (J) in close association with Meckel's cartilage (c) and the stomodeal epithelium (arrows). Scale bar: 1 cm = 16 mm. (C) Mandibular arch recombined and grafted at H.H. stage 23 and maintained for a combined age of 11 days, produces bone (red) and cartilage (blue) (Stain, HBO). (D and E) Mandibular arches recombined and grafted at H.H. stage 20 and maintained for a combined age of 6.75 days. (D) Toluidine blue-stained section to show cartilage (purple). Scale bar: 1 cm = 9 mm. (E) PNA-stained section showing osteogenic condensation (J) in close association with the stomodeal epithelium (arrows). Scale bar: 1 cm = 4 mm.
were left on CAMs for the appropriate length of time. Tissues which were from Chlorantline donors embryos formed when the epithelial-mesenchymal interaction was reinitiated. Tissue recombinations were performed to determine whether osteogenic condensations nor bone matrix deposition were observed. Days, nor in H.H. stage 23 mesenchyme grafted for 2.75 or 3.75 days (Table 3). These grafts of mandibular mesenchyme were all negative for both PNA and I-1BO, i.e. neither osteogenic condensations nor bone matrix deposition were observed.

Do osteogenic condensations form in mesenchyme recombined with epithelium?

After establishing the fact that osteogenic condensations do not form in the absence of the epithelium, recombination experiments were performed to determine whether osteogenic condensations formed when the epithelial-mesenchymal interaction was reinitiated.

Mesenchyme and epithelium from H.H. stage 23 embryos were recombined and grafted for 7 days (11 days combined age) as a control for the recombination and grafting techniques. The results clearly revealed (Table 3) that the techniques were suitable; overt differentiation of bone and deposition of substantial matrix occurred within all grafted tissues maintained for 7 days (Fig. 5C). Recombined epithelium and mesenchyme was then grafted for shorter periods.

Osteogenic condensations formed in recombinations of mesenchyme and epithelium from H.H. stage 20 and H.H. stage 23 embryos, grafted to the CAM for 3.75 and 2.75 days, respectively (Table 3; Fig. 5D and E). As in ovo, the condensation lay in very close proximity to the epithelium. Therefore, formation of the osteogenic condensation is dependent on the epithelial-mesenchymal interaction and the interaction acts at least as early in osteogenic differentiation as the condensation stage.

The relationship between condensation and transition to preosteoblasts during osteogenic differentiation

Does transition of mesenchymal cells to preosteoblasts occur before, after or simultaneous with condensation (Models 1-3, Fig. 1)? Alkaline phosphatase (AP) was used as the marker to determine the timing of formation of preosteoblasts, initially in vivo and then in grafts.

Whole heads of precondensation (H.H. stages 20-25) and condensation (H.H. stage early-23) embryos, corresponding to incubation times of 4-6 days, were processed for AP histochemistry (Table 4). AP-positive cells were present at H.H. stage early-25, which is before condensation but after completion of the epithelial-mesenchymal interaction (H.H. stage 24). The number of preosteoblasts increased at the condensation stage, i.e., condensation enhanced preosteoblast formation, either through increased proliferation of existing preosteoblasts or recruitment of new preosteoblasts from surrounding mesenchyme (see Discussion).

Two areas of AP activity were observed at the precondensation stage (H.H. stage early and mid-25; Fig. 6A) and increased in number (3 areas) as well as size and intensity by the condensation stage (H.H. stage early-28; Fig. 6B). AP activity was also present in H.H. stage mid-20 and late-21 embryos. However, using other criteria these AP areas were demonstrated not to be areas of preosteoblasts.

In order to localize preosteoblasts, serial sections from whole heads at precondensation and condensation stages were examined. The importance of establishing the general morphology and localization of preosteoblast areas was two-fold: (1) to identify which of the mandibular membrane bones were initiated at these stages; and (2) to determine whether preosteoblasts could be traced back to a common origin.

Two areas of preosteoblasts were observed at H.H. stage early and mid-25 and early-28 (Fig. 6A and B). Both were proximal, one in close proximity to the retroarticular process of Meckel's cartilage (Area I), the other more ventrally and medially (Area II). At the condensation stage (H.H. stage early-28) both areas were larger and stronger in staining intensity. Furthermore, an additional area (Area III) was present distally on the lateral side of Meckel's cartilage, opposite preoosteoblast Area II (Fig. 6B). By following serial sections below Meckel's cartilage the preosteoblastic areas at both stages could be traced to a common origin in a larger area of preosteoblasts.

### Table 3

<table>
<thead>
<tr>
<th>H.H. stage of donor embryos</th>
<th>Graft time (days)</th>
<th>% of specimens with osteogenic condensations*</th>
<th>% of specimens with bone matrix deposition**</th>
<th>Total number of specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated mesenchyme</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>3.75</td>
<td>0</td>
<td>N/A</td>
<td>5</td>
</tr>
<tr>
<td>23</td>
<td>3.75</td>
<td>0</td>
<td>N/A</td>
<td>5</td>
</tr>
<tr>
<td>Tissue recombinations</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>3.75</td>
<td>100</td>
<td>N/A</td>
<td>2</td>
</tr>
<tr>
<td>20</td>
<td>3.75</td>
<td>100</td>
<td>N/A</td>
<td>2</td>
</tr>
</tbody>
</table>

* Determined from PNA-positive staining in serial sections. ** Determined from Chlorantline Fast Red staining in serial sections. N/A refers to grafted tissues which were not expected to have any bone matrix deposition as they were not left on the CAMs for long enough (a minimum of 8 days is required for graft tissues to lay down bone matrix). 0 refers to grafted tissues which were negative for bone matrix deposition even though they were left on CAMs for the appropriate length of time. ** These specimens served as the controls to test the suitability of the tissue recombination techniques.

### Table 4

<table>
<thead>
<tr>
<th>H.H. stage of embryos</th>
<th>Phase</th>
<th>% of specimens with AP activity (n)</th>
<th>Number of areas of preosteoblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>mid-20</td>
<td>precondensation</td>
<td>100(3)</td>
<td>0</td>
</tr>
<tr>
<td>late-21</td>
<td></td>
<td>50(4)</td>
<td>0</td>
</tr>
<tr>
<td>late-23</td>
<td></td>
<td>0(4)</td>
<td>0</td>
</tr>
<tr>
<td>mid-24</td>
<td></td>
<td>0(4)</td>
<td>0</td>
</tr>
<tr>
<td>early-25</td>
<td></td>
<td>100(5)</td>
<td>2</td>
</tr>
<tr>
<td>mid-25</td>
<td></td>
<td>100(5)</td>
<td>2</td>
</tr>
<tr>
<td>early-28</td>
<td>condensation</td>
<td>100(5)</td>
<td>3</td>
</tr>
</tbody>
</table>

* Established on the basis of the first appearance of alkaline phosphatase-positive cells in relation to condensation and precondensation as determined by PNA binding. ** Total number of specimens sampled at each stage= 5.
Fig. 6. Condensation and preosteoblast formation. (A and B) Photomicrographs of representative 5 μm oblique sections through whole mandibles. (A) H.H. stage mid-25 (precondensation) to show two areas of preosteoblasts. Scale bar: 1 cm = 5 mm. (B) H.H. stage early-28 (condensation) to show three areas of preosteoblasts. Scale bar: 1 cm = 4 mm. (C and D) Mandibular arches recombined and grafted at H.H. stage 20 and maintained for a combined age of 6.75 days. (C) Low magnification showing location of alkaline phosphatase activity. Scale bar: 1 cm = 22 mm. (D) High magnification of alkaline phosphatase in (C). Scale bar: 1 cm = 4 mm. I, preosteoblast area 1; II, preosteoblast area 2; III, preosteoblast area 3; c, Meckel's cartilage; arrows, areas of alkaline phosphatase activity; top is dorsal.
Establishing a role for the epithelium in the differentiation of preosteoblasts

Having shown that initiation of differentiation — formation of preosteoblasts — precedes condensation formation and that condensation formation requires epithelial-mesenchymal interaction, we then asked: "does the epithelial-mesenchymal interaction control osteogenesis at the preosteoblast stage?" Mandibular mesenchyme from H.H. stages 20 and 23 was grafted for 3.75-4.75 and 2.75-3.75 days, respectively to obtain a combined age of 6.75-7.75 days. Preosteoblasts did not form in the absence of the epithelium (Table 5). Mesenchyme was negative for AP activity, implicating epithelial control in preosteoblast formation.

Recombination experiments were then performed using tissues from H.H. stage 20 and 23 embryos grafted to the CAMs for 3.75 and 2.75 days (Table 5). Re-establishment of the epithelial-mesenchymal interaction was sufficient to reinitiate osteogenic differentiation. Areas of AP activity (preosteoblasts) were present in all recombinants and lay in close association with the epithelium (Fig. 6C and D).

Tenascin

After the sequence and timing of major developmental events associated with the early stages of osteogenic differentiation were established, our attention turned to establishing a possible role for tenascin in the epithelial-mesenchymal interaction, transition of mesenchymal cells to preosteoblasts, and/or condensation. Whole mandibles from H.H. stages 19-28 were collected and fixed for tenascin immunohistochemistry (Table 6). H.H. stages 19 and 21 were chosen as they preceded the completion of the epithelial-mesenchymal interaction (H.H. stage 24; Hall, 1978a). H.H. stage 25 was chosen as it is the stage at which the mesenchymal cells differentiate into preosteoblasts. H.H. stage 28 was chosen as it is during the osteogenic condensation phase and has the highest intensity of PNA-binding (Table 1).

Careful observation of serial sections, using blue-filtered light microscopy to enhance contrast, revealed the presence of tenascin in the mandibular epithelium of H.H. stage 19 embryos (trypsinized and non-trypsinized; Fig. 7). No tenascin was present in any of the other stages examined (trypsinized and non-trypsinized) even when the concentration of the tenascin monoclonal antibody was increased to a dilution of 1:200.

Discussion

The stage of osteogenesis regulated by the epithelial-mesenchymal interaction

Mesenchymal cells within the mandibular arches of chick embryos were shown to differentiate into alkaline phosphatase-positive preosteoblasts at H.H. stage early-25, immediately following the completion of the epithelial-mesenchymal interaction. Preosteoblast differentiation requires epithelial-mesenchymal interaction and epithelial-mesenchymal interaction controls osteogenesis at the preosteoblastic stage.

Furthermore, from the localization of different areas of preosteoblasts it was evident that the epithelial-mesenchymal interaction initiates a large number of proximally located mesenchymal cells which subsequently (or simultaneously) subdivided into individual membrane bones. The proximo-caudal localization of the preosteoblasts is consistent with previous mapping of osteogenic mesenchyme at later stages. Hall (1982) examined the distribution of osteogenic mesenchyme and osteogenically inducive epithelium by dividing mandibular arches of H.H. stage 22 embryos into 6 halves (lateral/medial; cephalad/caudal; or proximal/distal) and grafting them to the CAMs of host embryos. Halved mandibles were also separated and recombined in various combinations (example: lateral mesenchyme with medial epithelia, and so on). Proximo-caudal epithelium and mesenchyme were shown to be the most osteogenically active and to contain the most osteogenic precursors, corresponding with the AP histochemistry reported herein.

<table>
<thead>
<tr>
<th>TABLE 5</th>
<th>ESTABLISHING A ROLE FOR THE EPITHELIAL-MESENCHYMAL INTERACTION IN THE TRANSITION OF MESENCHYME TO PREOSTEOBLASTS USING GRAFTED TISSUES OF MANDIBULAR ARCHES</th>
</tr>
</thead>
<tbody>
<tr>
<td>H.H. stage of donor embryo</td>
<td>Graft time (days)</td>
</tr>
<tr>
<td>Isolated mesenchyme</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>23</td>
</tr>
<tr>
<td>Tissue recombinations</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>23</td>
</tr>
</tbody>
</table>

* Determined from Chloroantline Fast Red staining in serial sections. N/A refers to grafted tissues which were not expected to have any bone matrix deposition as they were left on CAMs for long enough (a minimum of 8 days is required for grafted tissues to lay down bone matrix). 0 refers to grafted tissues which were negative for bone matrix deposition even though they were left on CAMs for the appropriate length of time.

<table>
<thead>
<tr>
<th>TABLE 6</th>
<th>EXPRESSION OF TENASCIN DURING THE MAJOR DEVELOPMENTAL EVENTS ASSOCIATED WITH MANDIBULAR OSTEGENESIS IN OVO</th>
</tr>
</thead>
<tbody>
<tr>
<td>H.H. stage</td>
<td>Developmental event</td>
</tr>
<tr>
<td>Non-trypsinized</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>28</td>
</tr>
</tbody>
</table>

Tissues treated with 0.1% trypsin

* Determined from immunohistochemistry using mouse anti-human tenasin monoclonal antibody (Fig. 9). In all specimens, tenasin was restricted to the mandibular epithelium. ** Determined from immunohistochemistry using mouse anti-human tenasin monoclonal antibody.
Evidence that the epithelium induces a large, common, osteogenic center which subsequently divides to form the individual bones of the mandible

A common center of preosteoblasts was visualized by detection of AP activity at both H.H. stage mid-25 (precondensation) and H.H. stage early-28 (at condensation). This area was localized in the proximal region of the mandibular arch and adjacent to the articular process of Meckel’s cartilage in H.H. stage mid-25 embryos and extended latero-medially and proximo-distally in H.H. stage early-28 (condensation stage) embryos. The individual membrane bones of the mandible therefore originate from a common center of mesenchyme which subsequently subdivides to form each membrane bone. That a common osteogenic center exists is also supported by the 3-D reconstruction of the osteogenic condensation (PNA visualization; Fig. 4).

Two areas of preosteoblasts were evident at H.H. stage mid-25. One at the proximal tip of Meckel’s cartilage near the articular process, will form the surangular. The other, located more posteriorly, will form the angular. Erdmann (1940), Jacobson and Fell (1941) and Murray (1963) all suggested that although overt differentiation of the surangular occurs anterior to the angular, its initial development is proximal to the articular process of Meckel’s cartilage. The angular was also suggested to have originated in this same area, proximal to the articular process of Meckel’s cartilage, and then either simultaneously (Jacobson and Fell, 1941) or with a temporal delay (Murray, 1963), separates from the “common area” into its final position (below the surangular and below Meckel’s cartilage). Jacobson and Fell (1941) through explantation experiments and light microscopy (hematoxylin and eosin) determined regionalization of osteogenic mesenchyme. Their results suggested that the large common osteogenic center contained the precursors for the angular, surangular, and splenial.

Both Jacobson and Fell and Murray concluded that the common condensation subdivided via a zone of cell death and/or progressively over time. Subdivision of a common osteogenic center (condensation) at different times is also supported by Erdmann (1940).

The present study supports a model of whereby individual membrane bones arise sequentially from a common center. The 2 proximal areas of preosteoblasts observed at H.H. stage mid-25 (precondensation) are identified as precursors of the surangular and angular. By H.H. stage early-28 (condensation stage) another preosteoblastic center, likely the precursor of the splenial, is present.

A common osteogenic condensation for mandibular and maxillary processes

Another surprising result was the observation that what was thought to be a ‘mandibular’ osteogenic condensation was a major branch of a common condensation also contributing cells to the maxillary arch. There is therefore a common osteogenic condensation for both arches, just as there is a common center for the several bones of the mandibular arch.

Similarities between mandibular and maxillary membrane bone development are: (1) the cells which form both mandible and maxillary arches arise from mesencephalic neural crest cells (Le Lièvre, 1974; Le Lièvre and Le Douarin, 1975); (2) both the quadratojugal of the maxillary arch and mandibular membrane bones undergo overt differentiation at the same time — 7 days of incubation H.H. stage 30-31 (Hall, 1978a); (3) both are dependent on an epithelial-mesenchymal interaction for their differentiation; and (4) the timing of these epithelial-mesenchymal interactions is the same in both arches (Tyler, 1978).

Mandibular and maxillary membrane bones arise from the same cell population, having already undergone a (common?) epithelial-mesenchymal interaction and are therefore subject to the same developmental controls during normal development or perturbations in dysmorphogenesis.

The role of condensation formation in osteogenesis

We have shown that transition of mesenchyme to preosteoblasts is controlled by the epithelial-mesenchymal interaction and that condensation occurs after this initial differentiation and increases the number of preosteoblasts. The initial phase of cytodifferentiation is amplified by condensation formation. This amplification coupled with the physical characteristics known to be associated with condensations (dramatically increasing cell-to-cell communication, increased cell-cell contact, attainment of a rounded morphology and increase in the number of gap junctions; Thorogood and Hinchliffe, 1975; Coelho and Kosher, 1991), is critical for subsequent osteogenic differentiation leading to matrix deposition and mineralization. Such an amplification of tissue specific products has been shown in cartilage (Kosher et al., 1986b) with mRNA for type II collagen being amplified 200-fold at the onset of condensation formation. The osteogenic condensation does not initiate the transition to preosteoblast but rather amplifies the number of
preosteoblasts. The sequence of events during differentiation of mandibular membrane bones may be summarized as follows:
1) An epithelial-mesenchymal interaction initiates the transition from mesenchyme to preosteoblasts.
2) Condensation amplifies the number of preosteoblasts.
3) Overt differentiation of preosteoblasts to osteoblasts and deposition of bone matrix.
4) Terminal differentiation and matrix mineralization.

Differential of mesenchyme to preosteoblasts occurs approximately 12 h after epithelial-mesenchymal interaction is completed, i.e., 12 h after H.H. stage 24. Growth factors such as bone morphogenetic protein (BMP), a member of the transforming growth factor-β (TGFβ) family, have been given particular attention as candidate epithelial molecules modulating proliferation and/or differentiation (Hall, 1988, 1994; Hall and Ekanayake, 1991; Blessing et al., 1993). Hall (1988) localized BMP to the basement membrane of the mandibular epithelium at the right time (H.H. stage 22) to implicate BMP in a role for osteogenesis.

The role of tenascin in osteogenesis

Based on the location (mandibular epithelium) and timing of appearance (H.H. stage 19) of tenascin during mandibular osteogenesis, the only possible role(s) for this extracellular matrix molecule are one involving the epithelial-mesenchymal interaction. Tenascin may be involved in signal transduction from the epithelium to the mesenchyme during the early stages of the epithelial-mesenchymal interaction (possibly the initiation of the epithelial-mesenchymal interaction). The timing of appearance of tenascin coincides with the initiation of the epithelial-mesenchymal interaction, specifically the epithelial stimulation of mesenchymal proliferation (H.H. stage 18; Hall and Coffin-Collins, 1990). Tenascin has been widely implicated in promoting cellular proliferation as each arm of its hexabrachion structure contains a segment of epidermal growth factor repeats (Chiquet, 1989), each of which can activate growth receptors.

Time course of the major cellular events

H.H. stage 19: tenascin is expressed in the mandibular epithelium and may be playing a role in the epithelial-mesenchymal interaction — possibly in signal transduction from the epithelium to the mesenchyme.
H.H. stage 22: mesenchyme stimulates epithelial proliferation (Hall and Coffin-Collins, 1990) and may maintain the matrix-mediated epithelial signal (Hall et al., 1983; Van Exan and Hall, 1984; Hall, 1988, 1994). Increase in the basement membrane product — the osteogenic differentiation signal — may be required before interaction can occur.
H.H. stage early-25: mesenchyme known to differentiate into osteoblasts in the absence of the epithelium (Tyler and Hall, 1977). Epithelium controls the transformation of mesenchymal cells into preosteoblasts (present study). A signal within the basement membrane of the epithelium controls the initial differentiation of mesenchyme to preosteoblasts.
H.H. stage mid-26 to late-28: amplification of preosteoblasts into osteogenic condensations (this study) setting the stage for subsequent differentiation into osteoblasts and osteocytes.

Materials and Methods

Incubation of eggs

Fertilized eggs of the domestic fowl, Gallus domesticus, were obtained from Cook's Hatchery (Truro, Nova Scotia, Canada) and incubated without rotation in a forced-air Leaky incubator (Higginsville, Missouri, USA) at 36 ± 1°C.

Preparation of embryos

Eggs incubated for between 2.5 and 7 days were swabbed with 70% ethanol and opened. Embryos were placed in plastic Petri dishes containing 0.85% NaCl saline solution, staged according to Hamburger and Hamilton (1951) and mandibular arches removed by microdissection.

Tissue processing

Three fixatives and processing protocols were used.
- PNA: tissues fixed in 6% mercuric chloride, 1% sodium acetate and 0.1% glutaraldehyde (Miyake et al., 1992; personal communication) for 6 h at room temperature were transferred to 70% ethanol and kept at 4°C overnight. Tissues were then dehydrated in a graded ethanol series (30 min each), cleared for 2.5-4 h in 4 changes of 1:20 tissu volumé Histoclear and placed in regular Paraplast (melting point 56°C containing DMSO for rapid tissue infiltration, Oxford Labware, Little, MO, USA). Following 1 h at 56°C, tissues were transferred to fresh Paraplast and left for 1-4 days at 56°C to complete infiltration before being embedded in Paraplast blocks.
- AP: tissues fixed in 80% ethanol were left overnight at 4°C, changed to 95% ethanol for 2-4 h at 4°C, then to 100% ethanol at 4°C for a total of 3 h with one change at the 1.5 h interval. They were then transferred to 2 changes of Histoclear at 4°C over a 2 h period, placed in low melting point (50-54°C) Paraplast, vacuum infiltrated for 2 days at 52°C, and embedded in Paraplast blocks.
- Tenascin: tissues fixed in periodate lysine paraformaldehyde (PLP; McLean and Nakane, 1974) were left for 3 h at 4°C, and then dehydrated in an isopropanol series (50 to 100%) for 1 h each at -20°C; cleared in 2 changes of chloroform (1 h each at -20°C); vacuum infiltrated in low temperature Paraplast for 2 days at 52°C and embedded.

Histochemistry and immunohistochemistry

PNA: blocks were serially sectioned at 5 μm. Consecutive “strips” of 8 sections each were mounted on alternate slides coated with Haupt's (1930) adhesive and dried for 24-48 h at 37°C. Alternate strips of sections were used to visualize PNA or stained with HBB (following Hall, 1986) or toluidine blue (Graveson and Armstrong, 1987). For the PNA staining methodology see Fig. 8.
AP: blocks were serially sectioned at 5 μm and sections mounted on slides pre-coated with Haupt's (1930) adhesive. After drying for 24-48 h at 37°C, sections were deparaffinized, rehydrated, rinsed (as described in Fig. 8) and reacted with nitroblue tetrazolium (modified from Blauro et al., 1984; 0.15 M Tris buffer was used in place of veronal-acetate buffer) in the dark for 25 min. The sections were then rinsed in tap water; counterstained with 0.1% methylene green; rinsed, dehydrated, cleared and coverslipped as outlined in Fig. 8.

Tissue separation

For tissue isolation studies, mandibles were separated into their epithelial and mesenchymal components following 50 min incubation at 4°C in a...
DEPAFFINIZE sections in two changes of xylene (5 min each).

REHYDRATE tissues in graded ethanol series (100, 95, 80, 70, 50%, 1 min each).

RINSE in running tap water.

LUGOL'S SOLUTION for 10 mins.

RINSE in 0.02 M PBS pH 7.2.

5% SODIUM THIOSULFATE until bleached (max. 30 mins).

RINSE in 0.02 M PBS pH 7.2.

BLOCK ENDOGENOUS PEROXIDASE by incubating in 1% H2O2 20 mins in the dark.

RINSE in 0.02 M PBS pH 7.2.

INOCULATE with PNA conjugate (3 hours at 4°C).

RINSE in 0.02 M PBS pH 7.2.

INCUBATE in 3-3' diaminobenzidine-H2O2 substrate at pH 7.6 in the dark for 40 mins at room temperature.

RINSE in 0.03 M PBS pH 7.3.

MAYER’S HAEMATOXYLIN for 2 to 5 minutes.

RINSE in 0.02 M PBS pH 7.3.

SCOTT’S TAP WATER until tissues turn blue (2-5 minutes).

RINSE in distilled water.

Rinse with a mixture of PNA conjugate (the same mixture as above) containing 0.1 M 3,3-diaminobenzidine.

Fig. 8. PNA immunohistochemistry. PNA staining procedure modified from Miyake et al. (1989, personal communication). PNA (arachis hypogaea lectin conjugated with horseradish peroxidase) from Sigma Chemical Company, St. Louis, MO.; Lugol’s solution and Scott’s tap water from Humason (1979); 3-3’ diaminobenzidine-H2O2 substrate from Schulte and Spicer (1983).

Controls

To establish the time of appearance and duration of the putative osteogenic condensation and early bone matrix deposition in grafted tissues, mandibular arches from embryos of H.H. Stages 20-23 were removed and grafted directly onto the chorioallantoic membranes (CAMs) of host embryos for 2-7 days. These grafts formed the controls for experiments using isolated mesenchymal or mesenchyme recombined with epithelium.

Chorioallantoic grafting of mesenchyme or mesenchyme recombined with epithelium

To determine whether condensations formed in the absence of the epithelium, isolated mesenchyme from embryos at H.H. Stages 20 and 23 were grafted to the CAMs of host embryos for 2.5 to 5.5 days. To investigate a role for the epithelium in condensation formation or initiation of osteodifferentiation, mandibular mesenchyme was recombined with epithelium and grafted following the procedure outlined in Hall (1978b). Ninety five percent of the hosts were viable at the end of the graft period. Grafts were recovered and fixed for PNA or AP histochemistry.

3-D reconstruction and collection of data

The condensations from the mandible of a PNA-stained, serially-sectioned stage 29 embryo was reconstructed in three dimensions. Sections were located using a compound microscope equipped with a CoHo camera (CoHo 4610 Monochrome solid-state CCD Camera, Coho, San Diego, CA, USA) and the image 'grabbed' with a Pixelgrabber in PixelTools (TCL-Image, Perceptrics Corp., Knoxville, TN, USA) installed on a Macintosh IICl computer equipped with Ultradrive 50R (GCC Technologies, Waltham, MA, USA). 3-D reconstruction explanation of procedure. Image 1.38 software (developed by Wayne Rasband, NIH, Bethesda, MD, USA), was implemented in PixelTools. An image of each serial section was printed on a laser printer.

The condensation was reconstructed using an ICAR workstation (ISG Technologies Inc.) in two steps: 1) using a CCTV camera mounted on an enlarger stand, each section was captured to an input computer with frame grabber software (Amicus) to create an image file. Sections are automatically aligned with respect to the previous section. 2) An ICAR workstation equipped with 3-D reconstruction software (Allegro 5.0.1) was used to reconstruct a 3-D-image from images transferred from the IBM computer containing the Amicus software via ethernet. Images from the ICAR workstation were displayed on a high resolution color monitor and the outlines of the condensation, buccal cavity, notochord and mandibular vein were traced on 84 consecutive images encompassing the entire condensation. These formed the basis for the 3-D reconstruction.

Acknowledgments

Financial support was provided by the Natural Sciences and Engineering Research Council (NSERC) of Canada and by the Killam Trust of Dalhousie University. We thank Wayne Rasband for providing Image 1.38 software and Alma Cameron and Lynne Mallett-Frotten for technical advice.

Fig. 9. Tenascin immunohistochemistry. Controls for tenascin immunohistochemistry were: H.H. stage 19 chick cornea (positive control; Kaplony et al., 1991) and incubation in pre-immune serum in place of primary antibody (negative control). Pre-immune goat serum and ABC Elite Kit from Vector Laboratories Inc., Burlingame, CA; mouse anti-human tenasin from Calbiochem Corporation, La Jolla, CA.; Biotin-conjugated goat anti-mouse IgG from Gibco BRL, Grand Islands, NV.; 3-3’ diaminobenzidine from Sigma Chemical Company, St. Louis MO.
References


Accepted for publication: November 1994