**In vitro** studies on the morphogenesis and differentiation of the mesoderm subjacent to the apical ectodermal ridge of the embryonic chick limb-bud

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**SUMMARY**

It has been suggested that one of the major functions of the apical ectodermal ridge (AER) of the embryonic chick limb-bud is to maintain mesenchymal cells directly subjacent to it (i.e. cells extending 0.4-0.5 mm from the AER) in a labile, undifferentiated condition. We have attempted to directly test this hypothesis by subjecting the undifferentiated subridge mesoderm of stage-25 embryonic chick wing-buds to organ culture in the presence and absence of the AER and the ectoderm that normally surrounds the mesoderm dorsally and ventrally. During the period of culture, control explants comprised of the subridge mesoderm capped by the AER and surrounded by the dorsal/ventral ectoderm undergo progressive morphogenesis characterized by polarized proximal to distal outgrowth and changes in the contour of the developing explant, and ultimately form a structure grossly resembling a normal distal wing-bud tip. In contrast, explants from which the AER and dorsal/ventral ectoderm have been removed (minus ectoderm explants) or from which just the AER has been removed (minus AER explants) form compact, rounded masses exhibiting no signs of morphogenesis. During the polarized proximal to distal outgrowth control explants undergo during the first 3 days of culture, as cells of the explant become located greater than 0.4-0.5 mm from the AER, they concomitantly undergo a sequence of changes indicative of their differentiation into cartilage. However, those cells which remain 0.4-0.5 mm from the AER during this period retain the characteristics of non-specialized mesenchymal cells. In marked contrast to control explants, virtually all of the cells of minus ectoderm explants initiate chondrogenic differentiation during the first day of culture. Cells comprising the central core of minus AER explants also initiate chondrogenic differentiation during the first day of culture, but in contrast to minus ectoderm explants, non-chondrogenic tissue types form along the periphery of the explants subjacent to the dorsal/ventral ectoderm. These results indicate that the AER maintains cells directly subjacent to it in a labile, undifferentiated condition, and that when mesenchymal cells are freed from the AER's influence either artificially or as a result of normal polarized outgrowth, they are freed to commence cytodifferentiation. The results further suggest that the dorsal/ventral ectoderm may have an influence on the differentiation of the mesenchymal cells directly subjacent to it, once the cells have been removed from the influence of the AER.

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INTRODUCTION

The embryonic chick limb-bud following its initial formation during the third day of development essentially consists of a bulge or core of mesodermal cells surrounded by a thin rim of ectoderm. The ectoderm extending around the distal periphery of the limb-bud is a thickened cap of pseudostratified columnar epithelium called the apical ectodermal ridge (AER). Since the classical experiments of Saunders (1948), it has been known that the AER has a profound effect upon the development of the mesodermal cells of the limb-bud. For example, surgical removal of the AER results in the formation of limbs possessing severe distal deficiencies, the severity of the distal deficiencies produced depending upon the stage of limb development at which the AER is removed (Saunders, 1948; see also Summerbell, 1974). The influence of the AER has also been demonstrated in a positive manner, since grafting an extra AER onto the mesodermal cells of the limb-bud results in the formation of limbs possessing distal duplications (Saunders & Gasseling, 1968; Zwilling, 1956; Saunders, Gasseling & Errick, 1976). On the basis of the above observations it appears that the AER is required for the outgrowth and formation of distal limb structures by the mesodermal cells. The mechanism by which the AER exerts this profound effect on the mesodermal cells has, however, remained obscure.

The mesodermal cells that constitute the bulk of the limb-bud during the earliest stages of its development (stages 16–22; Hamburger & Hamilton, 1951) appear to be a homogeneous population of unspecialized mesenchymal cells that are virtually identical to one another ultrastructurally (Searls, Hilfer & Mirow, 1972; Thorogood & Hinchliffe, 1975), and biochemically (Searls, 1965a, b; Linsenmayer, Toole & Trelstad, 1973). Furthermore, experimental studies suggest that the mesenchymal cells in various regions of the limb at these earliest stages are identical in their developmental potential, i.e. their ability to subsequently form either cartilage or muscle components of the limb (Zwilling, 1966; Searls, 1967; Zwilling, 1968; Searls & Janners, 1969), although several recent studies suggest that during normal development the limb musculature and its associated connective tissue may be derived from somitic or somatic plate mesoderm (Christ, Jacob & Jacob, 1977; Chevallier, Kieny & Mauger, 1977).

During the fourth–fifth day of development (stages 22–25) the mesenchymal cells in the proximal central core of the limb undergo a variety of changes indicative of their differentiation into cartilage. At this time, these cells which were previously separated from one another by rather extensive intercellular spaces undergo condensation, i.e. become closely packed and exhibit large areas of close surface contact (Fell & Canti, 1934; Gould, Day & Wolpert, 1972; Thorogood & Hinchliffe, 1975), and concomitantly undergo amplified production of sulfated glycosaminoglycans, one of the major constituents of
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cartilage matrix (Searls, 1965a). Shortly thereafter, the cells become separated from one another by a histochemically and ultrastructurally detectable cartilage matrix (Searls et al. 1972; Thorogood & Hinchliffe, 1975) and initiate synthesis of cartilage-characteristic Type II collagen (Linsenmayer et al. 1973). As the mesenchymal cells in the central proximal core (the so-called chondrogenic area) are differentiating into cartilage, peripheral mesenchymal cells in the dorsal and ventral proximal regions of the limb (the so-called myogenic areas) undergo a depressed rate of sulfated glycosaminoglycan production (Searls, 1965a) and acquire the ultrastructural characteristics of muscle-forming and connective tissue cells (Hilfer, Searls & Fonte, 1973). However, as the mesenchymal cells in the proximal chondrogenic and myogenic areas of the limb initiate differentiation at stages 22–23, those mesenchymal cells directly subjacent to the AER, i.e. cells extending 0.4–0.5 mm from the AER, retain the characteristics of unspecialized mesenchymal cells (Searls, 1965; Searls et al. 1972), and exhibit no signs of differentiation. During subsequent stages of development as the limb undergoes polarized outgrowth in a proximal to distal direction, the size of the non-differentiating subridge region remains constant (Searls, 1965a, 1973; Stark & Searls, 1973; Summerbell, 1976), although the cells in the subridge region double in number every 11–13 h (Cairns, 1966; Janners & Searls, 1970; Lewis, 1975; Cairns, 1977). Apparently, therefore, when polarized limb growth and division in the subridge region causes cells to become located greater than 0.4–0.5 mm from the AER, the cells become part of the proximal chondrogenic and myogenic areas and initiate differentiation (Searls, 1965a, 1973; Stark & Searls, 1973).

On the basis of the above observations, it has been suggested that one of the major functions of the AER may be to maintain mesenchymal cells in the subridge region in a labile, undifferentiated condition, and that when, as a result of polarized limb outgrowth, cells become located greater than 0.4–0.5 mm from the AER, they are then freed from the AER’s ‘negative’ influence and thus commence differentiative changes (i.e. chondrogenesis and myogenesis) (Stark & Searls, 1973). Other investigators have similarly suggested that the function of the AER is to maintain the mesenchymal cells directly subjacent to it in a state of developmental lability (Summerbell, Lewis & Wolpert, 1973). In the present investigation we have attempted to directly test the above hypotheses by subjecting the undifferentiated subridge mesoderm of embryonic chick wing-buds to organ culture in the presence and absence of the AER and the ectoderm that normally surrounds the mesoderm dorsally and ventrally.

MATERIALS AND METHODS

Preparation of cultures. Forelimb buds were removed from stage-25 (Hamburger & Hamilton, 1951) embryos of White Leghorn chicks and placed into Simms’ balanced salt solution (SBSS) (Simms & Sanders, 1942). Distal wing-bud
Fig. 1. A freshly excised distal wing-bud tip (subridge region) which has been cut away from a stage-25 embryonic chick forelimb bud. The size of the excised tissue is 0.4-0.5 mm from the distal apex to the proximal cut edge. Note the apical ectodermal ridge (AER) extending around the distal periphery of the tissue. × 70.

tips (the subridge region, see Introduction) comprised of the subridge mesoderm capped by the AER and surrounded dorsally and ventrally by epithelium (see Fig. 1) were cut away from the limb-buds. The size of the excised distal wing-bud tip was 0.4-0.5 mm from the distal apex of the tissue to the proximal cut edge (see Fig. 1 and Introduction). Three types of explants were prepared for organ culture. The first type of explant utilized was the intact distal wing-bud tip described above and comprised of the subridge mesoderm capped by the AER and surrounded by the dorsal/ventral ectoderm. Such explants will hereafter be referred to as control explants. The second type of explant utilized was the distal wing-bud tip from which both the AER and dorsal/ventral ectoderm have been removed following brief (15-20 min) treatment at room temperature with 1.25% trypsin in SBSS. Thus, these second type of explants are composed of the subridge mesoderm in the complete absence of ectoderm and will hereafter be referred to as minus ectoderm explants. The third type of explant utilized was the distal wing-bud tip from which the AER was surgically removed, but the dorsal/ventral ectoderm left intact. Such explants will hereafter be referred to as minus AER explants.

Explants of each of the above types were cultured on 1.2% nutrient agar containing Ham’s F12X medium (Marzullo & Lash, 1970; Gordon & Lash, 1974; Kosher & Lash, 1975) supplemented with 10% fetal calf serum (FCS; Grand Island Biological Co., GIBCO) and 1% Bovine Albumin Fraction V (BSA; Miles Laboratories). Ham’s F12X medium is Ham’s (1965) F12 medium containing twice the normal concentration of amino acids and pyruvate. Explants were fed with liquid nutrient feeding medium comprising F12X supplemented with 10% FCS and 1% BSA. For the biochemical studies described below the nutrient agar and feeding medium contained 5 μCi/ml of H$_2$SO$_4$ (carrier free; New England Nuclear). Cultures were maintained in a humid atmosphere of 95% air–5% CO$_2$ for periods up to 3 weeks depending upon the experimental design.
**Histological and histochemical procedures.** At various time intervals following the initiation of culture, explants were fixed in 4% formalin containing 0.5% cetylpyridinium chloride (Kvist & Finnegan, 1970), dehydrated, embedded in paraffin, sectioned at 5 or 10 μm, and routinely stained with hematoxylin and eosin. In addition, in order to histochemically examine the accumulation of sulfated glycosaminoglycans by the explants, sections were stained as described by Yamada (1970) with 0.5% Alcian blue 8GX, pH 1.0.

**Electron microscopy.** Explants were fixed for 30 min in 1.0% paraformaldehyde and 1.0% glutaraldehyde in 0.1 M cacodylate buffer, rinsed in cacodylate buffer, and postfixed for 30 min at room temperature in 1% osmium tetroxide in the same buffer. Following a brief rinse in buffer, the explants were then stained **en bloc** for 5 min with 0.5% aqueous uranyl acetate, rapidly dehydrated, and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate.

**Biochemical analyses.** Glycosaminoglycans were extracted from explants continuously exposed to [35S]sulfate by procedures previously described (Kosher, 1976). DNA was determined by a micromodification of the procedures of Abraham, Scaletta & Vaughan (1972) and Richards (1974).

**RESULTS**

**Comparison of the gross in vitro morphogenesis of control, minus ectoderm, and minus AER explants.** Figure 2 demonstrates photographs of living explants of each of the three types described in Materials and Methods during the first 5 days in culture. A photograph of a freshly excised control explant just prior to its being placed in organ culture is shown in Fig. 1.

During the first day of culture, explants from which the AER and dorsal/ventral ectoderm have been removed (minus ectoderm explants, Fig. 2F–J), or from which just the AER has been removed (minus AER explants, Fig. 2K–O), form essentially compact, rounded masses, and remain as compact masses exhibiting no signs of morphogenesis throughout the 5-day culture period. There do, however, appear to be some grossly detectable differences between the living minus AER explants and minus ectoderm explants. Whereas the minus ectoderm explants (Fig. 2G–J) give the appearance of being a completely homogeneous mass, cells in the center of the minus AER explants differ somewhat in their translucency from cells along the periphery of the same explant (see particularly Fig. 2M). In contrast to minus ectoderm and minus AER explants, during the first day of culture, control explants composed of the subridge mesoderm capped by the AER and surrounded by the dorsal/ventral ectoderm begin to undergo morphogenesis and polarized outgrowth in a proximal to distal direction (compare Fig. 2A with Fig. 1). During the subsequent days of culture (Fig. 2B–E), control explants continue undergoing quite striking morphogenesis characterized primarily by polarized proximal to
Fig. 2. For legend see opposite.
In vitro morphogenesis of mesoderm in chick limb-bud distal outgrowth and changes in the contour of the developing explant. The gross morphogenesis that control explants undergo in vitro as shown in Fig. 2A–E, is quite similar to the sequence of morphogenesis distal wing-bud tips undergo in vivo during stages 25–32. For example, our 4-day control explants in vitro bear a close resemblance to stage-30 distal wing tips in vivo, and the appearance of successively younger explants bears a close resemblance to successively younger distal wing-bud tips in vivo (see, for example, Hamburger & Hamilton, 1951 and Saunders, Gasseling & Saunders, 1962, fig. 6). Ultimately by the fifth day of culture, control explants have formed a structure grossly resembling a normal wing-bud tip comprised proximally of a large, rounded cartilaginous structure resembling the carpometacarpus, and distally of a digit-like cartilaginous anlage resembling the 3rd metacarpal (see Figs. 3D, E, 4D, E, and 8 which will be described in more detail later).

Late on the third or early on the fourth day of culture, the AER of control explants grossly undergoes a change in appearance. It loses its previous smooth contour, flattens, acquires a jagged appearance, and terminal wispy structures (compare the AER in Fig. 2A, B with the AER in Fig. 2D, E). These grossly detectable changes in the AER occur when the control explants grossly resemble stages 29–30 in vivo distal wing-bud tips.

**Differentiation of control explants.** Histological sections stained with hematoxylin and eosin of control explants during the first 5 days of culture are shown

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**Figure 2**

(A) A living control explant (comprising the subridge mesoderm capped by the AER and surrounded by the dorsal/ventral ectoderm) during the first day of culture. The AER is indicated by arrows. Compare with Fig. 1, and note the polarized proximal to distal outgrowth and changes in the contour of the explant that have occurred. × 70.

(B), (C) Living control explants during the second (B) and third (C) days of culture. The AER is indicated by arrows. Note the progressive polarized proximal to distal outgrowth and contour changes that occur during this period. × 70.

(D), (E) Living control explants during the fourth (D) and fifth days of culture. Note that during this period the AER (arrows) has undergone a gross change in appearance in that it has lost its previous smooth contour, flattened, acquired a jagged appearance and terminal wispy structures. × 70.

(F)–(J) Living minus ectoderm explants (comprising the subridge mesoderm from which both the AER and dorsal/ventral ectoderm have been removed) during the first (F), second (G), third (H), fourth (I) and fifth (J) days of culture. Note that the explants form a compact, rounded mass, and remain as a compact mass exhibiting no signs of morphogenesis throughout the 5-day culture period. The explants give the appearance of being a completely homogeneous mass. × 70.

(K)–(O) Living minus AER explants (from which the AER has been removed, but the dorsal/ventral ectoderm left intact) during the first (K), second (L), third (M), fourth (N) and fifth (O) days of culture. The explants form compact, rounded masses and remain as such throughout the culture period. Note particularly in Fig. 2M that cells in the center of the minus AER explants differ somewhat in their translucency from cells along the periphery of the same explant. × 70.
(A) A hematoxylin and eosin (H and E) stained section through a 1-day control explant. The cells retain the appearance of non-specialized mesenchymal cells separated from one another by fairly extensive extracellular spaces. × 125.

(B) An H and E stained oblique section through a 2-day control explant. The proximal cells of the explant (small arrow) are condensing (becoming closely packed and apposed to one another) while the cells directly subjacent to the AER (arrow head) retain the appearance of non-specialized mesenchymal cells. In the photograph the size of the mesenchymal subridge region appears shorter than its actual length (0.4–0.5 mm), because of the obliqueness of the section. × 125.

(C) An H and E stained section through an early 3-day control explant. The most proximal cells of the explant (arrow head) have become separated from one another by an extracellular matrix; more distal cells (arrow) have undergone condensation;
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in Fig. 3A–E. Fig. 3A is a section of a 1-day control explant that has just initiated polarized outgrowth and morphogenesis (see Fig. 2A). During this early period of in vitro morphogenesis the vast majority of the cells of the explant are still within 0.4–0.5 mm of the AER, and the cells retain the histological appearance of non-specialized mesenchymal cells separated from one another by fairly extensive intercellular spaces and exhibiting no indications of differentiation (Fig. 3A). A similar section of a 1-day control explant stained with Alcian blue 8GX, pH 1.0, to histochemically examine sulfated glycosaminoglycan accumulation demonstrates a complete absence of Alcian blue-positive material throughout the explant (Fig. 4A).

During the second and third days of culture, as control explants continue undergoing polarized proximal to distal outgrowth (Fig. 2B–C), those cells which remain 0.4–0.5 mm from the AER retain the histological characteristics of non-specialized mesenchymal cells (Fig. 3B, C), and exhibit a complete absence of Alcian blue-positive staining (Fig. 4B, C). In contrast, those cells and, the most distal cells (those which are still located 0.4–0.5 mm from the AER) remain mesenchymal (region surrounding the X).

(D) An H and E stained section through a 4-day control explant. The proximal cells of the explant have formed a large, rounded cartilaginous structure resembling the carpometacarpus and the subridge cells have condensed and formed a digit-like cartilaginous anlage resembling the third metacarpal. This section further demonstrates that occasionally a second digit-like cartilaginous structure (which probably represents the fourth metacarpal) forms adjacent to the larger third metacarpal. × 125.

(E) An H and E stained section through a 5-day control explant demonstrating the proximal, rounded, cartilaginous carpometacarpus, and the distal digit-like third metacarpal. Note also in this section (and in Fig. 3D), the non-chondrogenic tissue subjacent to the ectoderm throughout the explant. × 125.

(F) An H and E stained section through a 1-day minus ectoderm explant. Virtually all of the cells of the explant have undergone condensation, i.e. become closely packed and apposed to one another. × 125.

(G)–(J) H and E stained sections through second (G), third (H), fourth (I) and fifth (J) day minus ectoderm explants. On the second and subsequent days of culture the previously condensed cells have become separated from one another by an extracellular matrix. Note also the small circumferential layer of flattened, elongated cells resembling a perichondrium along the extreme periphery of the explants. × 125.

(K) An H and E stained section through a 1-day minus AER explant. Cells in the central core of the explant have undergone condensation, while those along the periphery have not. × 125.

(L), (M) H and E stained sections through second (L) and third (M) day minus AER explants. Previously condensed cells in the central core of the explants have become separated from one another by an extracellular cartilaginous matrix. However, cells along the periphery of the explants subjacent to the ectoderm do not differentiate into cartilage. × 125.

(N), (O) H and E stained sections through fourth (N) and fifth (O) day minus AER explants demonstrating the cartilaginous central core, and peripheral non-chondrogenic tissue subjacent to the ectoderm. Note particularly in Fig. 3 (O) the strap of fibrous-appearing cells in the non-chondrogenic peripheral region of the explant (arrow). × 125.
FIGURE 4

(A) A section through a 1-day control explant stained with Alcian blue, pH 1.0. There is a complete absence of Alcian blue-positive material throughout the explant. $\times 125$.

(B), (C) Alcian blue-stained sections through second (B) and third (C) day control explants. The proximal cells of the explant have acquired an Alcian blue-positive extracellular matrix, while the distal cells of the explant that are still within 0.4–0.5 mm of the AER continue to exhibit a complete absence of Alcian blue staining. $\times 125$.

(D), (E) Alcian blue-stained sections through fourth (D) and fifth (E) day control explants demonstrating the proximal Alcian blue-positive carpometacarpus and distal digit-like third metacarpal. Note the Alcian blue-negative tissue subjacent to the ectoderm throughout the explants. $\times 125$.

(F) Alcian blue-stained section through a late 1-day minus ectoderm explant.
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Fig. 5. Accumulation of $[^{35}S]$sulfate-labelled glycosaminoglycans by the proximal and distal segments of control explants during the first 3 days of culture.

of the explant which gradually become located greater than 0.4-0.5 mm from the AER undergo a sequence of changes indicative of their differentiation into cartilage. These cells initially undergo condensation, i.e. become closely packed and apposed to one another (Fig. 3B, C), and subsequently become separated from one another by extracellular spaces (Fig. 3C, D) that stain intensely with Alcian blue (Fig. 4B, C).

To confirm and quantify the above histological and histochemical observations, chondrogenesis in the distal and proximal portions of control explants was studied biochemically by examining the accumulation of sulfated glycosaminoglycans (GAG), one of the major constituents of cartilage matrix, by each portion. In this experiment, control explants were cultured in the presence of $[^{35}S]$sulfate, and at the end of each period of culture, the explants were cut

Faint traces of Alcian blue-positive material are detectable throughout the explant. × 125. (G)-(J) Alcian blue-stained sections through second (G), third (H), fourth (I) and fifth (J) day minus ectoderm explants. More or less uniform Alcian blue-positive staining is seen throughout the explants from the second day of culture onward. × 125.

(K) An Alcian blue-stained section through a late 1-day minus AER explant. Faint traces of Alcian blue-positive material are detectable in the central core of the explant. However, the peripheral cells of the explant remain Alcian blue-negative. × 125.

(L)-(O) Alcian blue-stained sections through second (L), third (M), fourth (N) and fifth (O) day minus AER explants demonstrating the Alcian blue-positive central cartilaginous core surrounded by Alcian blue-negative tissue subjacent to the ectoderm. × 125.
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(A) An electron micrograph of a 1-day minus ectoderm explant the cells of which are histologically undergoing condensation (Fig. 3F). Note the large areas of intimate surface contact between adjacent cells and the virtual lack of intercellular space. ×7341.

(B) An electron micrograph of a minus ectoderm explant whose cells are histologically becoming separated from one another by Alcian blue-positive extracellular spaces. Note the numerous Golgi-derived cytoplasmic vacuoles containing amorphous, electron-dense material, and the extensive extracellular spaces containing numerous 25–30 nm in diameter densely staining granules and thin unbanded or faintly banded fibrils (arrows). ×7341.

(C) A higher magnification electron micrograph of a minus ectoderm explant demonstrating the densely staining granules and thin unbanded or faintly banded fibrils seen throughout the extracellular spaces of the explants from the second day of culture onward. ×91770.

into distal and proximal segments and sulfated GAG accumulation in each segment was determined. As demonstrated in Fig. 5, during the first day of culture there is little difference in sulfated GAG accumulation by distal and proximal segments of control explants, but by the second and third days, sulfated GAG accumulation by the proximal cells which are located greater than 0.4–0.5 mm from the AER is two- to three-fold greater than accumulation by distal cells that are still under the ridge’s influence.

One further observation provides support for the indication that when cells are freed from the AER’s influence, they initiate differentiation. As previously
described during the fourth day of culture, the AER of control explants undergoes a gross change in appearance in that it loses its previous smooth contour, flattens, and acquires a jagged appearance (Fig. 2D, E). These changes in the AER occur when control explants grossly resemble stages 29–30 in vivo distal wing-bud tips (Fig. 2D). It is of particular interest to note that it is at stages 29–30 that the AER has been demonstrated to lose its influence on limb-bud mesodermal cells, i.e. lose its capacity to induce the outgrowth and formation of distal limb structures by the mesodermal cells (Rubin & Saunders, 1972). With this in mind, note, as demonstrated in Figs. 3D–E and 4D–E, that during the fourth day of culture the mesenchymal cells in the region 0.4–0.5 mm from the AER condense (Fig. 3D), then become separated from one another by an Alcian blue-positive extracellular matrix (Figs. 3E and 4D–E), and form a digit-like cartilaginous structure that resembles the third metacarpal (Figs. 3E and 4E). In other words, mesenchymal cells within 0.4–0.5 mm of the AER undergo differentiation at precisely the time the AER undergoes gross changes in its appearance and the control explant resembles a stage-29 to stage-30 distal limb tip, i.e. at a time corresponding to the stage in vivo that the AER loses its influence on limb mesodermal cells (Rubin & Saunders, 1972).

**Differentiation of minus ectoderm explants.** During the first day of culture, virtually all of the cells of explants from which both the AER and dorsal/ventral ectoderm have been removed undergo condensation (Fig. 3F). This is in marked contrast to the cells of control explants which virtually all remain mesenchymal during this period. An electron micrograph of a 1-day minus-ectoderm explant which is histologically in the condensation stage (Fig. 3F) is shown in Fig. 6A. Ultrastructurally the condensation phase is characterized by large areas of intimate surface contact between adjacent cells (Fig. 6A). Little, if any, intercellular space is detectable.

Beginning on the second day of culture (and continuing on subsequent days) the vast majority of the cells of the minus ectoderm explants leave the condensation phase and become separated from one another by extensive extracellular spaces (Fig. 3G, H) that stain with Alcian blue (Fig. 4G, H). In fact, more or less uniform Alcian blue-positive staining is seen throughout the minus ectoderm explants from the second day of culture onward (Fig. 4G–J). Essentially the only cells of the minus ectoderm explants that do not undergo the above changes are a small group of cells along the extreme periphery of the explants which become arranged in a circumferential layer of flattened, elongated cells that bear a close resemblance to a perichondrium (Fig. 3G–J). An electron micrograph of a minus ectoderm explant in which, histologically, cells are becoming separated from one another by an Alcian blue-positive extracellular matrix (Figs. 3G and 4G) is shown in Fig. 6B. The cells have undergone considerable cytological differentiation, the most striking feature of which is the presence of numerous, large Golgi-derived vacuoles containing amorphous, electron-dense material (see Godman & Porter, 1960; Goel, 1970). Furthermore,
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Fig. 7. Accumulation of $[^{35}S]$sulfate-labelled glycosaminoglycans by control (Control), minus ectoderm (– ECTO), and minus (– AER) explants during the first 3 days of culture.

the cells have become separated from one another by extensive extracellular spaces that contain predominantly numerous 25–30 nm in diameter densely staining granules and thin unbanded or faintly banded fibrils (Fig. 6B, C). The extracellular granules and fibrils (Fig. 6C) are ultrastructurally identical to the proteoglycan granules and thin, unbanded collagen (presumably Type II) fibrils that have been demonstrated by numerous other investigators to be the major ultrastructurally detectable components of hyaline cartilage matrix (see, for example, Matukas et al. 1967; Anderson & Sadjera, 1971; Searls et al. 1972; Levitt & Dorfman, 1975; Pennypacker & Goetinck, 1976).

To confirm and quantitate the above histological and histochemical observations, chondrogenesis in control and minus ectoderm explants was studied biochemically by examining the accumulation of sulfated GAG by explants of each type. During the first day of culture, sulfated GAG accumulation by minus ectoderm explants whose cells are histologically and ultrastructurally in the condensation phase is about 60% greater than accumulation by control explants whose cells are mesenchymal and still within 0.4–0.5 mm of the AER (Fig. 7). This result is consistent with the observation that concomitant with the condensation phase of chondrogenesis in vivo amplified production of sulfated GAG occurs (Searls, 1965a). During the second and third days of culture,
when the bulk of the cells of control explants remain mesenchymal and the cells of minus ectoderm explants become separated from one another by an Alcian blue-positive extracellular matrix, sulfated GAG accumulation by minus ectoderm explants is two- to three-fold greater than control explants (Fig. 7). It is of interest to note that the rate of accumulation of sulfated GAG by minus ectoderm explants increases strikingly between the first and second day of culture (Fig. 7). It appears, therefore, that biochemically, chondrogenic differentiation is biphasic, i.e. a moderately amplified production of sulfated GAG occurring during the condensation phase followed by an even greater amplified sulfated GAG production occurring as the previously condensed cells became separated from one another by a histologically and ultrastructurally detectable cartilage matrix.

**Differentiation of minus AER explants.** In contrast to control explants, during the first day of culture, cells in the central core of explants from which the AER was removed, but the dorsal/ventral ectoderm left intact undergo condensation (Fig. 3K), the first overt histological stage in chondrogenic differentiation, and during the second and subsequent days of culture the cells become separated from one another by an Alcian blue-positive extracellular matrix (Figs. 3L–M and 4L–M). However, in the minus AER explants only those cells in the central core of the explant appear to differentiate into cartilage, while those cells along the periphery of the explant form a non-chondrogenic tissue type that exhibits, for example, a complete absence of Alcian blue-positive staining (Figs. 4K–O). This is in marked contrast to minus ectoderm explants in which non-chondrogenic tissue types are not discernible.

Biochemical evidence consistent with these histological and histochemical observations is shown in Fig. 7. Sulfated GAG accumulation by minus AER explants is greater than accumulation by control explants reflecting the precocious chondrogenic differentiation that cells in the central core of the minus AER explants have undergone (Fig. 7). However, sulfated GAG accumulation by the minus AER explants is considerably less than accumulation by minus ectoderm explants (Fig. 7). This intermediate level of sulfated GAG accumulation is precisely what would be expected, if in the minus AER explants many of the cells underwent precocious chondrogenic differentiation, while other cells in the same explant formed non-chondrogenic tissue types, and, if in the minus ectoderm explants, virtually all of the cells differentiated into cartilage.

The nature of the non-chondrogenic tissue types that surround the cartilaginous central core of the minus AER explants do not become readily discernible until the fourth and subsequent days of culture. It should be added that as would be expected the same non-chondrogenic tissue types form subjacent to the dorsal/ventral ectoderm in control explants. During the fourth and subsequent days of culture, immediately subjacent to the epidermis in control and minus AER explants is a dense, cellular tissue resembling dermal connective tissue (Figs. 3E, N, O and Fig. 8), and subjacent to this is a much
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Fig. 8. (A–G) Hematoxylin and eosin stained sections through 8- to 10-day control (A–D) and minus AER (E–G) explants demonstrating the straps of fibrous-appearing cells with elongated nuclei arranged in parallel arrays that form in the non-chondrogenic peripheral regions of the explants subjacent to the ectoderm (arrows). Note also the hypertrophying chondrocytes in the cartilaginous central core of the explants, and the considerable differentiation the ectoderm has undergone to form a layer of keratinized stratified squamous epithelium. × 230.
less cellular, looser tissue resembling subcutaneous connective tissue (Fig. 3E, N, O and Fig. 8). Finally, in control and minus AER explants, straps of fibrous appearing cells with elongated nuclei arranged in parallel arrays are invariably seen (Fig. 3E, N, and Fig. 8). Although the extent of development of these straps of fibrous appearing cells, which resemble differentiating skeletal muscle fibers is not striking, they invariably appear to originate on the distal end of the rounded cartilaginous anlage resembling the carpometacarpus and insert on the distal end of the digit-like third metacarpal.

**DISCUSSION**

The apical ectodermal ridge (AER) appears to be required for the outgrowth and formation of distal limb structures by the mesenchymal cells that comprise the core of the embryonic chick limb-bud (Saunders, 1948). On the basis of a number of observations described in detail in the Introduction, it has been suggested that one of the major functions of the AER is to maintain mesenchymal cells directly subjacent to it (i.e. cells extending 0.4–0.5 mm from the AER) in a labile, undifferentiated condition, and that, when as a result of polarized proximal to distal limb outgrowth, cells become located greater than 0.4–0.5 mm from the AER, they are freed from the AER’s influence and thus commence differentiative changes (i.e. chondrogenesis and myogenesis) (Stark & Searls, 1973). In the present investigation, direct evidence supporting the above hypothesis has been obtained by studying in an organ culture system the morphogenesis and differentiation of the unspecialized subridge mesoderm of the embryonic chick wing-bud in the presence and absence of the AER and the ectoderm that normally surrounds the mesoderm dorsally and ventrally. The evidence can be summarized as follows:

1. When the mesenchymal cells comprising the subridge mesoderm are artificially removed from the influence of the AER (minus ectoderm and minus AER explants), the cells precociously undergo the histological, histochemical, ultrastructural, and biochemical sequence of events involved in chondrogenic differentiation. When the dorsal/ventral ectoderm as well as the AER is removed, virtually all of the cells of the explant undergo these differentiative changes, whereas when just the AER is removed, the cells comprising the central core of the explant undergo chondrogenic differentiation. In contrast, in control explants which retain the AER, mesenchymal cells that remain 0.4–0.5 mm from the AER during the first 3 days of culture retain the histological, histochemical, and biochemical characteristics of unspecialized mesenchymal cells and show no indications of differentiation into cartilage or any other tissue type.

2. During the polarized proximal to distal outgrowth control explants undergo in vitro, as cells of the explant become located greater than 0.4–0.5 mm from the AER, they concomitantly undergo the sequence of events involved in chondrogenic differentiation. Thus, mesenchymal cells undergo
cartilage differentiation if they are removed from the AER’s influence either artificially or as a result of polarized outgrowth *in vitro*.

(3) Mesenchymal cells within 0.4-0.5 mm of the AER in control explants undergo chondrogenic differentiation at precisely the time the AER undergoes gross changes in its appearance and control explants resemble stage 29-30 distal wing-bud tips, i.e. at a time corresponding to the stages *in vivo* that the AER loses its influence on limb mesodermal cells (Rubin & Saunders, 1972).

Furthermore, the results of the present investigation provide evidence indicating the ectoderm that surrounds the mesenchymal cells of the limb dorsally and ventrally may have an influence on the differentiation of the mesenchymal cells subjacent to it, once the mesenchymal cells have been removed from the influence of the AER. For example, when the AER is removed from the subridge mesodermal cells, but the dorsal/ventral ectoderm left intact (minus AER explants), only those mesenchymal cells comprising the central core of the explant precociously undergo chondrogenic differentiation, while those cells along the periphery of the same explant subjacent to the dorsal/ventral ectoderm, form non-chondrogenic tissue types which appear to be dermal and subcutaneous connective tissue and straps of skeletal muscle. In contrast, when the dorsal/ventral ectoderm as well as the AER is removed, it appears that virtually all of the cells of the explant differentiate into cartilage. These observations in themselves do not necessarily demonstrate that the dorsal/ventral ectoderm has a direct effect on the differentiation of mesenchymal cells subjacent to it. It might be argued, for example, that the subridge mesoderm is comprised of a heterogeneous group of mesenchymal cells that are restricted in their differentiative potential, in that one group might be capable of only differentiating into cartilage when removed from the AER’s influence, while another group might be capable of only differentiating into non-chondrogenic tissue types such as muscle when removed from the AER’s influence. In this regard, it is also of interest that removal of the AER or limb ectoderm may lead to cell death (Cairns, 1975). Therefore, it could then be argued that perhaps when the dorsal/ventral ectoderm as well as the AER is removed that selective death of the cells with non-chondrogenic differentiative potential occurs, and only the cells with chondrogenic differentiative potential survive. Conversely, when the dorsal/ventral ectoderm is left intact, both the prospective non-chondrogenic and chondrogenic cells might remain viable. However, several observations we have made argue against this latter possibility. If in our minus ectoderm explants selective death of a large population of mesenchymal cells was occurring, while in our minus AER explants these cells remained viable, this would undoubtedly be reflected in a reduced DNA content in the minus ectoderm explants relative to the minus AER explants (see, for example, Gordon & Lash, 1974). However, we have found that there is virtually no difference in the total DNA content of minus AER (average DNA content being 0.27 ± 0.02 μg DNA/explant) and minus ectoderm (0.28 ± 0.01 μg DNA/explant).
explants during any period of culture. Furthermore, if in the minus AER explants both prospective chondrogenic and non-chondrogenic cells remained viable, while in the minus ectoderm explants only prospective chondrogenic cells remained viable, one would expect sulfated GAG accumulation by the minus AER explants to be even greater than sulfated GAG accumulation by minus ectoderm explants. However, sulfated GAG accumulation by minus ectoderm explants is considerably and significantly greater than accumulation by minus AER explants. Thus, the most reasonable explanation for all the experimental observations is that in the presence of the dorsal/ventral ectoderm, mesenchymal cells that would have differentiated into cartilage if the dorsal/ ventral ectoderm had been absent, differentiate into non-chondrogenic tissue types. Unequivocal proof of a direct influence of the dorsal/ventral ectoderm on the differentiation of mesenchymal cells subjacent to it will, however, require further investigation. It should be added that the dorsal/ventral ectoderm may also exert some controlling influence on the dorsoventral polarity of limb parts (MacCabe, Errick & Saunders, 1974; Searls & Cioffi, 1977).

Finally, it should be added that Newman (1977; see also Newman & Mayne, 1974) has demonstrated that the subridge mesoderm of stage-25 limb-buds undergoes cartilage differentiation when grown in an organ culture system similar to that described in the present manuscript. It is not clear from the above studies, however, whether or not the morphogenesis or differentiation of the mesoderm is influenced by the AER and/or dorsal/ventral ectoderm. For example, Newman (1977) does not report whether or not the subridge mesoderm in the presence of the AER undergoes the striking morphogenesis we observe in the present study, nor does Newman report whether or not any qualitative or quantitative differences are observed in the amount of cartilage and non-chondrogenic tissues formed in the presence and absence of ectoderm. In contrast to our observations, however, Newman (1977) does not observe any indications of muscle formation, apparently even if the explants are cultured in the presence of ectoderm. In this regard, it may be noteworthy that in Newman’s studies the subridge mesoderm was subjected to organ culture for only 3 days. In the present studies, although non-chondrogenic tissues are present in our control and minus AER explants from the first day of culture onward, straps of fibrous-appearing cells which resemble skeletal muscle fibers do not become readily discernible until the fourth and subsequent days of culture.

In summary, the results of the present investigation indicate that the AER maintains limb mesenchymal cells directly subjacent to it (i.e. cells extending 0-4-0-5 mm from the AER) in a labile, undifferentiated condition, and that when mesenchymal cells are freed from the AER’s influence either artificially or as a result of normal polarized proximal to distal limb outgrowth, they are freed to commence cytodifferentiation. The results further suggest that if upon leaving the AER’s influence, the mesenchymal cells were subjected to no other influences, they would differentiate into cartilage. If the cells are, however, as
they are in normal development, subjected to the influence of the dorsal/ventral ectoderm, those peripheral cells directly subjacent to the dorsal/ventral ectoderm differentiate into non-chondrogenic tissue types including muscle, while those cells comprising the central core differentiate into cartilage. The in vitro organ culture system described in the present study should be invaluable in attempting to understand at a molecular level the mechanisms by which the AER and dorsal/ventral ectoderm exert their effects on the differentiation and morphogenesis of limb mesenchymal cells.

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