Ectoderm-mesoderm interactions in relation to limb-bud chondrogenesis in the chick embryo: transfilter cultures and ultrastructural studies

By MADELEINE GUMPEL-PINOT

From the Institut d'Embryologie du CNRS Nogent-sur-Marne

SUMMARY

Limb ectoderm induces cartilage differentiation in mesoderm from chick embryo limb buds. Transfilter cultures have shown that this interaction requires 'contact' conditions and cannot take place at a distance. In vivo, a basement membrane is always present between ectoderm and mesoderm.

The present paper demonstrates that the relationship between ectoderm and mesoderm is similar in vivo and in transfilter cultures. In culture conditions, the filter appears to be infiltrated by mesodermal cell outgrowths which form a continuous mesodermal cover on the filter. A basement membrane is always present between the mat of mesodermal cell processes and the ectoderm.

Mesodermal cell processes are able to cross the Nuclepore filters (pore size 0.6–0.8 μm) within 15 min. After 2 h in culture, the surface of the filter opposite to the mesodermal explant is completely covered with mesodermal outgrowths.

The extracellular material accumulating at the ectoderm-mesoderm interface appears to be mainly of mesodermal origin.

INTRODUCTION

The differentiation of chondrocytes in the wing bud of the chick embryo is controlled by the ectoderm (Gumpel-Pinot, 1972, 1973, 1980). Transfilter cultures have shown that the inductive process cannot take place at a distance and requires conditions of 'contact' between the two tissues (Gumpel-Pinot, 1980). However a basement membrane is always found between ectoderm and mesoderm of the limb in vivo (Jurand, 1965; Berczy, 1966, Ede, Bellairs & Bancroft, 1974; Smith, Searls & Hilfer, 1975; Kaprio, 1977; Gumpel-Pinot, unpublished observations) so that a true contact between the cell membranes of the two tissues is never achieved. This suggests a possible role for the basement membrane in the interaction. This hypothesis is supported by work on the mouse limb bud by Milaire & Mulnard (1968) pointing out that disappearance or profound alteration of the basement membrane suppressed or at least reduced the inductive properties of the epiblast on cartilage differentiation of the mouse mesoblast.

1 Author's address: Institut d'Embryologie du CNRS et du College de France, 49 bis Avenue de la Belle-Gabrielle. 94130 Nogent-sur-Marne, France.
In transfilter cultures, it has been shown (Gumpel-Pinot, 1980) that 'contact' can be established across a Nuclepore filter with at least 0.4-1 μm pore size by means of long filopodia issuing from mesodermal cells and spreading on the filter surface opposite to the culture. The ectoderm itself is not able to cross the filter.

The nature of ectomesodermal interaction established across the filter between ectodermal cells and mesodermal processes was therefore examined. It is important to establish, for instance, whether a basement membrane is present between the two tissues in such conditions. In order to answer this question, we have studied by transmission electron microscopy sections of explants formed by ectoderm and mesoderm placed on each side of Nuclepore filters. Another point concerns the time factor involved in the interaction. If we observe on one hand a control explant formed by ectoderm and mesoderm (stages 14–16 Hamburger & Hamilton (1951), when mesoderm is unable to differentiate into cartilage without ectoderm) cultured on the same side of the filter and, on the other hand, an experimental explant in which ectoderm and mesoderm of the same age are cultured separated by the filter (0.6-0.8 μm pore size), the first signs of differentiation of cartilage are detectable at the same time in the two types of explants (unpublished observations). This underlines the importance of timing establishment of contact between ectoderm and mesoderm across the filter. We have thus carried out a series of cultures with fixation after various time intervals to find out how long it takes for the mesodermal processes to cross the filter and to establish contact. This study has been made by scanning observation of the surface of the filter opposite to the mesodermal culture.

In addition these experimental observations throw some light on the question of the origin of the extracellular material normally found in the space between ectoderm and mesoderm.

**MATERIAL AND METHODS**

The experiments used White Leghorn chick embryos. The stage reached at the time of excision of the wing primordia was specified by the number of somites and by Hamburger & Hamilton (1951) stages.

(i) **Dissociation of the wing bud**

The wing primordia were excized from stage 14 to early 17 (22- to 30-somite embryos). Ectoderm was separated from mesoderm by means of enzymatic digestion. Trypsin or collagenase were used as dissociating agents. The procedure for trypsin treatment has been described in detail in previous papers (Gumpel-Pinot, 1972, 1973). For collagenase treatment, the limb buds were placed in cold enzymatic solution (0.5 mg/ml collagenase (CLS II Worthington Biochemical Corporation) in culture medium without serum) for 20 min. They
Ectoderm-mesoderm interactions in chick embryo

were then transferred to culture medium at room temperature where ectoderm and mesoderm were mechanically separated. Both components were then washed three times in culture medium supplemented with serum.

(ii) Transfilter cultures

The culture medium was GIBCO H 21 medium supplemented with 12% heat-inactivated foetal calf serum. Nuclepore filters (pore size 0.6 and 0.8 µm, thickness 10 µm. Nuclepore Corporation, Pheasanton, USA) were sterilized in 50% ethanol, washed three times in Tyrode’s solution and left in the culture medium supplemented with 12% serum in the CO₂ incubator.

Ectoderm was attached to the lower surface of the filter with agar (1/4 agar in Gey solution, 1/4 culture medium). Mesoderm was placed on the opposite side (see Gumpel-Pinot, 1980). In some cases, mesoderm was cultured alone on the upper surface of the filter. In both type of cultures each explant consisted of four to eight mesodermal components.

The cultures were kept at 38 °C in a humidified incubator in an atmosphere of 5% CO₂ in air.

(iii) Time-lapse cultures

In the case of time-lapse experiments, the explants consisted of (1) mesoderm (six to eight components), (2) mesoderm + ectoderm treated by collagenase but cultured together, (3) ectoderm alone. In the three types of cultures, the explant was placed on the upper surface of the filter. The culture was prepared in such a way that the temperature of the medium and the explant was 38 °C at the start of explantation. When the explant was laid on the filter, the surface of the filter was at the same level as that of the medium, so that the pores of the filter were completely filled with medium.

(iv) Cytological techniques

(a) Transmission electron microscopy

After 2 days, the transfilter cultures were fixed for 45 min in 2.5% glutaraldehyde in 0.1 m cacodylate buffer (pH 7.3) at room temperature. They were washed in three changes of 0.2 m cacodylate buffer and then post-fixed for 1 h in 2% osmic acid in the same buffer (0.1 m) and washed in three changes of 0.2 m buffer. Serial dehydration in ethanol was followed by embedding in Epon. A Reichert microtome was used for sectioning. Thick sections (1 µm) were stained with toluidine blue and thin sections were treated with uranyl acetate and lead citrate. Thin sections were examined in an Hitachi HS 9 electron microscope.

(b) Scanning electron microscopy

After 15 min to 72 h in culture, explants formed by mesoderm, mesoderm and ectoderm or ectoderm cultured on the upper surface of the filter were fixed and washed in buffer as described above. The specimens were then briefly rinsed in
distilled water, dehydrated through a graded series of ethanol followed by an ethanol isoamylacetate series. They were then stored in pure isoamylacetate for a period varying from a few hours to a few days. Critical-point drying was done in a Balzers apparatus using liquid CO$_2$ according to Anderson (1951). Dried specimens were mounted on stubs so that the surface of the filter opposite to the culture was visible, coated with gold in an Epi 500 Polaron and observed in a Jeol scanning electron microscope operated at 25 kV.

RESULTS

(1) T.E.M. studies

(1) Transfilter cultures of ectoderm and mesoderm

The results described in this chapter have been obtained by studying 19 transfilter explants of stage-15 to -16 ectoderm and mesoderm fixed after 48 h in culture.

The mesoderm was healthy with rounded nuclei. In the centre of the culture, the relations between the cells appeared to be similar to those observed in vivo:
cells contacted each other by a network of long filopodia. Near to the filter, the cells became more densely packed although forming numerous cell processes orientated towards the filter (Fig. 1). In the layer of mesodermal cells in contact with the filter the nuclei were generally ovoid, the smaller axis being parallel to the filter. The Golgi apparatus, numerous mitochondria and rough endoplasmic reticulum were most often localized on the side of the cell which contacts the filter. It should be emphasized that there is no extracellular material accumulation that can be revealed by TEM either near to the filter, or in the intercellular spaces between the cells in the mesodermal tissue.

Many outgrowths penetrated into the pores of the filter (Fig. 1, Fig. 2a, b, c). Some of them completely filled the pores (0.6-0.8 μm) but others had a diameter which did not exceed 0.2-0.3 μm. In such cases several cell processes could be present in the same pore. The cell processes were composed of light cytoplasm in which mitochondria and vacuolar structures could be distinguished. They were mainly characterized by the presence of microtubules (Fig. 2c) and microfilaments or intermediate (filaments Fig. 2b) the latter being parallel to the axis or forming a network.

Numerous cell processes crossed the filter and spread on it, some emerging between the first processes and spreading on them (Fig. 1, 3) The processes joined together, thus forming a mesodermal cytoplasmic cover 5-12 μm thick in the ectodermal side of the association. The extremities of the cell outgrowths contained mitochondria, microfilaments or intermediate filaments and microtubules. The cell membranes of the filopodia were often in close contact but specialized junctions have never been observed. A very abundant extracellular material (collagen fibers and granules) was present among the mesodermal cell outgrowths and between the ectodermal cells and the mesodermal filopodia (Fig. 1, Fig. 2d).

The ectodermal mass was formed by several ectodermal layers packed together on the filter. The ectoderm thus forms a dense multilayered tissue (Fig. 3). The nuclei are generally rounded, sometimes irregular. The cells themselves are rounded and massive compared to in situ ectoderm. Desmosomes were observed (Fig. 3, arrowed). Numerous ectodermal cells are characterized by a well-developed rough endoplasmic reticulum. Ectoderm is repulsed by mesodermal cell outgrowths (Fig. 1, 3). It contacted the filter only in the rare places where pores were empty or incompletely filled with mesodermal material (Fig. 1). The ectodermal cells in contact with the filter never possessed a recognizable basal lamina. By contrast, the sheet of ectodermal cells in front of mesodermal processes had a clear basal lamina (Fig. 1, 3). The cell membrane lining the basal lamina often showed empty depressions as described in vivo in normal conditions (Kaprio, 1977). The basal lamina might be interrupted in some places but was always seen at the contact points with mesodermal processes. Therefore, direct contacts between the cell membranes of a mesodermal process and an ectodermal cell have never been observed.
Fig. 2. (a) Stage-16 mesoderm cultured 2 days alone on a Nuclepore filter. Mesoderm cell process passing through the filter to the opposite side. (b) Higher magnification of the cell process represented in (a). (c) Microtubules in a fragment of mesodermal cell outgrowth inside the filter. (d) Extracellular material accumulated in transfilter culture in the interspace between mesodermal cell processes and ectoderm. mf, Micro- or intermediate filaments.
Fig. 3. Two days transfilter culture of stage-15 to -16 ectoderm and mesoderm. The ectodermal side of the filter. A sheet of cytoplasmic mesodermal cell outgrowths (M) have repulsed the ectoderm (E). A basal lamina is present between the two tissues (→). The different sheets of ectoderm accumulated on the filter form a very dense tissue. Desmosomes are observed (→). The ectodermal mass delimits an empty vesicle, far away from mesoderm, lined by a continuous basal lamina (→). F = filter.
Fragments of basal lamina were also found inside the mass of ectoderm between two sheets of ectodermal cells. In one of the explants, the ectoderm contained an empty vesicle entirely lined by a continuous basal lamina (Fig. 3). Except for the basal lamina itself and a very light granular or fine fibrillar material along it, no extracellular material was observed in the centre of the ectodermal mass.

(2) Cultures of mesoderm alone:

Four explants of this type have been studied. The results are comparable to those described above as far as the mesoderm and the cell outgrowths inside and outside the filter are concerned (Fig. 2a).

(II) S.E.M. studies of the surface of nuclepore filters after culture on the opposite surface

The pore size of the Nuclepore filter was 0.6 and 0.8 μm. No significant difference could be observed in the results according to the pore size; thus, the two series are described together.

The cultures were fixed after 15 min to 72 h and the filters were prepared for scanning observation. The wing primordia used for these series of experiments have been excised at stage 14–17 (Hamburger & Hamilton, 1951).

(1) Cultures of mesoderm:

Each explant comprised four to eight mesodermal cores the ectoderm of which had been removed by collagenase treatment. Seventy-six explants of this type have been observed: eight after 15 min, thirteen after 30 min, fifteen after 1–2 h, six after 4 h, twenty after 6–12 h, eleven after 48 h and three after 72 h.

(a) After 15 min, mesodermal cell processes appeared on the surface of the filter opposite to the culture (Fig. 4a). Some were visible within the pores at the level of the surface but some other processes were already projecting on to the filter. Figure 4(a) represents the most frequent situation. In one case (pore 0.8 μm) the processes were more numerous and larger; in two cases, they appeared just at the level of the surface (pores 0.6 and 0.8 μm).

(b) After 30 minutes culture (Fig. 4b) the cell processes became more numerous, bulged on the surface of the filter and sent out long filopodia which generally made a network on the filter surface. It is noteworthy that these sometimes very thin filopodia (no more than 0.1 or 0.2 μm in diameter) appeared to be able to jump the pores (0.6–0.8 μm) as if they were completely rigid (arrowed).

(c) Between 1 and 4 h, the surface of the filter became rapidly covered with the
cell processes (Fig. 4c, d, e, f). The maximum invasion of the surface by cell processes seemed to be achieved between the second and the fourth hour of culture.

(d) Extracellular material. The first signs of accumulation of extracellular material were visible after 30 min culture (6 out of 13 explants). Between the cell processes it was possible to distinguish filaments or very thin amorphous matrix (probably collagen) and isolated granules (probably proteoglycans) on the filaments, on the filter and on the amorphous matrix (Fig. 5a). This ECM accumulation became progressively more marked (14 out of 15 explants between 1 and 2 h culture). After 48 and 72 h of culture, it completely covered the surface and most of the cell processes seemed to have retracted or to have been embedded in it (Fig. 5b, c).

As concerns the invasion of cell processes or the accumulation of ECM, no significant differences could be detected according to the stage of the mesoderm cultured. Stage-17 mesoderm, which is able to chondrify without ectoderm had the same behaviour as stage-14 to-15 mesoderm for which chondrogenesis requires the presence of ectoderm.

(2) Cultures of mesoderm and ectoderm

The wing buds were treated by collagenase and the dissociation of ectoderm from mesoderm was achieved or not. In any case, ectoderm and mesoderm were cultured together. Each explant was composed of four to six wing buds. Nineteen cases of this type have been observed and fixed after 30 min to 2 h (11 cases) or after 72 h of culture (8 cases). The invasion of the filter by cell processes was significantly lower for all periods of cultures (Fig. 5e). After \( \frac{1}{2} \) to 2 h very few cell processes crossed the filter. The accumulation of ECM was very marked (Fig. 5d), at least as much as when mesoderm is cultured alone. Very few processes were seen and what was observed seemed to be almost pure ECM.

(3) Cultures of ectoderm

Each explant was composed of 10 to 12 ectodermal components (stages 14–17). Eleven cultures of this type were fixed after 72 h. No cell processes were observed. No ECM accumulated on the surface of the filter opposite to the culture (Fig. 5f).

Fig. 5. (a) 30 min culture of stage-15 to -16 mesoderm. First sign of accumulation of ECM; (b) 72 h culture of stage-17 mesoderm (capable of autonomous differentiation of cartilage) showing ECM accumulation; (c) 72 h of stage-14 to-15 mesoderm (incapable of chondrogenesis) showing ECM accumulation; (d) 72 h culture of stage-16 ectoderm and mesoderm. Cartilage differentiates. Accumulation of ECM which looks like collagen II and proteoglycans lattice (Lash & Vasan, 1977); (e) 2 h culture of stage-15 to -16 ectoderm and mesoderm. Compare with Fig. 4(d): the density of cell processes is much lower; (f) 72 h culture of ectoderm alone. No cell processes. No accumulation of ECM.
CONCLUSIONS AND DISCUSSION

The basement membrane

In a number of systems, the basement membrane has been described as having an important role during morphogenesis, for instance in skin, salivary gland and lung (Grobstein, 1956; Wessels, 1967; Bernfield, Cohn & Banerjee 1973). In the case of the development of the tooth, the role of the basement membrane has been particularly studied (Karcher-Djuciric et al. 1978), and Thesleff, Lehtonen & Saxen (1978) suggested that ‘differentiation of odontoblasts is triggered via contact of the mesenchymal cells with the basement membrane’.

The present work shows that in limb mesoderm-ectoderm interaction, even through a filter, contact between ectodermal and mesodermal cells is mediated by a basement membrane as in vivo conditions. This result supports the hypothesis of a significant role played by the basement membrane in ectodermal-mesenchymal interaction involved in cartilage differentiation in the limb bud.

As to the restoration of the basement membrane after the action of the dissociating agents, some differences seem to exist between the various systems. There is increasing evidence that in many organs the basement membrane is predominantly derived from epithelium (Hay & Dodson, 1973; Banerjee, Cohn & Bernfield, 1977). In the case of salivary epithelium, Banerjee et al. (1977) showed that the basement membrane is restored within 2 h in culture in the absence of mesenchyme. In the case of dental epithelium, however, while epithelial cells presumably participate in the formation of the basement membrane since they are able to synthesize type-IV collagen, present in the basement membrane and not synthesized by mesenchymal cells (Trelstad & Slavkin, 1974), it seems that a close association between the epithelial and mesenchymal cells is required for the restoration of the basement membrane (Thesleff et al. 1978). By contrast, while the basement membrane of the limb bud seems not to be restored normally when the epithelial cells are in direct contact with the filter, it does become restored in the absence of mesoderm when ectoderm is cultured alone in the vitelline membrane (Gumpel-Pinot, 1981) or along an empty vesicle in the centre of cultured ectoderm, far away from mesoderm.

Timing of the extension of cell processes through the filter:

The cell processes are exclusively of mesodermal origin. If the Nuclepore filter is 10 µm thick with 0.6 or 0.8 µm pore size, these processes are able to cross the filter within 15 min, virtually all the pores being filled after 2 h and the filter appearing to be almost completely covered. Thus the time for penetration of the mesenchymal processes and establishment of the contact with the inducer is short enough to explain the absence of any difference in the timing of cartilage differentiation when ectoderm and mesoderm are either in direct contact or separated by such filters.

Comparison with other systems reveals that processes from spinal cord or
Ectoderm-mesoderm interactions in chick embryo

ureter cross the same type of filter within one hour (Saxen & Lehtonen, 1978) when cultured alone. When the reactive kidney mesenchyme is on the other side, both tissues send processes into the filter and contact is established in the filter itself. The epithelial processes issued from dental epithelium cross the same type of filter within 3 days (Thesleff et al. 1978). In this case also, both the interacting tissues participate in penetration; after 7 h of transfilter culture 'extensive penetration of the epithelial as well as mesenchymal cell processes into the filter pores was seen'.

When ectoderm and mesoderm treated by collagenase are cultured together on the filter, the number of cell processes which cross the filter is significantly lower than when mesoderm is cultured alone (Fig. 4d; Fig. 5e). Thus an attractive role of ectoderm for mesodermal cells could be postulated. This hypothetical attraction is not required for the movement of mesenchymal cell processes which cross the filter without any ectodermal effect. But the ectoderm could exert an effect on the polarisation of the movement or on the direction of cell processes.

Extracellular material

Though the presence of ECM in ectoderm-mesoderm interspace in the limb bud has been frequently observed, the origin of this material is not very clear. Smith et al. (1975) note that there are differences in accumulation of ECM, particularly collagen, in the flank and limb regions between stage 10 and 18. But the authors do not give any indication of the origin of this material. Kaprio (1977) notes that 'there is a change in the number of extracellular fibrils in the interspace. The highest density is found under the AER... It may be related to a greater metabolic activity of the AER compared with adjacent ectoderm'. This implies an ectodermal origin for the fibrils found in the ectoderm-mesoderm interspace.

Our results give some insight into the origin of the fibrillar component of the interspace ECM. It is possible, to some extent, to compare the material present in the interspace in transfilter culture and the material accumulated on the filter when ectoderm or mesoderm is cultured alone. It is clear that the accumulating collagen and granular material (Fig. 2d) between the mesenchymal cell processes correspond at least partly to the material which is accumulated on the filter when mesoderm is cultured alone. The ectoderm never accumulates ECM on the opposite surface of the filter. No fibrillar material is seen in the depth of the ectodermal cultures and the basal lamina which forms far from mesoderm around the ectodermal vesicle (Fig. 3) is lined only by a very thin granule-like material.

In conclusion presumably both tissues participate in forming the ECM present in the interspace but a great part if not all of the granules and large fibres of collagen are of mesodermal origin.

What type of collagen is secreted? When ectoderm and mesoderm are cultured together and are thus capable of forming cartilage, the picture of what is
accumulated on the filter (Fig. 5d) looks very similar to the picture given by Lash & Vasan (1977) representing a lattice of collagen II and proteoglycans. This is not surprising as it is known that cartilage formation in the limb is associated with the presence of type II collagen (Miller, 1977, von der Mark & von der Mark 1977). The ECM represented in Fig. 5(b) and (c) is more difficult to explain. Stage-17 mesoderm is able to form cartilage, stage 14–15 is not. The presence of proteoglycans granules in both cases is possible since Vasan & Lash (1979) have demonstrated that proteoglycans (monomeric and aggregate forms) are present in the limb mesenchyme before the differentiation of cartilage. As far as the collagen is concerned, type I is synthesized by the limb mesenchyme before cartilage formation (von der Mark & von der Mark, 1977) and muscle cells, which are present in the explants from stage 15 onwards (Gumpel-Pinot, 1980) also produce type-I collagen (Miller, 1977). A biochemical study would be necessary to resolve this issue.

The author is most grateful for the collaboration of the Laboratoire d’Anatomie Comparée, Université de Paris VII and especially to H. Boulekbache and F. Meury.

Sincere thanks are expressed to J. R. Hinchliffe for his help during the preparation of the manuscript and to M. Bontoux and L. Boulé for their technical assistance.

REFERENCES


Ectoderm-mesoderm interactions in chick embryo


(Received 15 July 1980, revised 23 April 1981)