THE EFFECT OF FIBRONECTIN AND
SUBSTRATUM-ATTACHED MATERIAL ON THE
SPREADING OF CHICK EMBRYO MESODERM
CELLS IN VITRO

E. J. SANDERS
Department of Physiology, University of Alberta, Edmonton, Alberta, Canada, T6G 2E1

SUMMARY
Endoblast and hypoblast tissue, dissected from early chick embryos, was explanted and cultured on glass or plastic substrata. These tissues grew rapidly to form epithelial sheets. Under the same conditions, mesoderm, dissected without the aid of dissociating agents, grew poorly. After 24 h in culture, the mesoderm explants consisted of a sparse outgrowth of fibroblast-like cells. When pieces of mesoderm were seeded onto the dorsal surface of the epithelia, however, the cells penetrated the sheet and rapidly spread on the substratum within 4 h. If the epithelial sheet was detached from the substratum and the mesoderm then seeded onto areas of substratum previously occupied by epithelium, similar rapid spreading occurred. This effect could be produced in the absence of serum. The method used to remove the epithelium (EDTA, detergent or manual dissection) did not influence the result. When the substratum-attached material (SAM) was examined by scanning electron microscopy, 2 types of material were seen. One type appeared to be the remains of detached filopodia and cytoplasmic lamellae, while the other appeared to be of extracellular origin. Both these types reacted positively by immunofluorescence using anti-fibronectin serum. SAM derived from mesoderm reacted negatively. When mesoderm was cultured in the presence of plasma fibronectin on unmodified plastic or glass, spreading was complete in 4-5 h and thus was similar to mesoderm seeded onto SAM. The morphology of mesoderm explants on SAM or in the presence of plasma fibronectin was more epithelial than on untreated substratum in normal medium. Hypoblast and endoblast cultured in the presence of anti-fibronectin serum failed to spread normally, apparently being unable to attach to the substratum. Mesoderm did not spread rapidly on SAM in the presence of this antiserum. Cycloheximide reversibly inhibited the spreading of hypoblast and endoblast, and this effect could be eliminated, at least for hypoblast, by the addition of plasma fibronectin. Covering attachment sites on the substratum with bovine serum albumin, thereby preventing the attachment of SAM or fibronectin, also inhibited spreading. It is proposed that mesoderm cells have low levels of surface fibronectin in comparison with endoblast and hypoblast, and that this results in a comparatively low adhesiveness, which is important for its morphogenetic activity within the embryo.

INTRODUCTION
During the process of gastrulation in the chick embryo, cells of the upper layer (the epiblast) migrate through the primitive streak and undergo a morphological conversion from an epithelial to a fibroblastic cell type as they form mesoderm. The mesoderm, initially a very loosely packed tissue of individually moving cells, spreads outward from the primitive streak between the epiblast and the lower layer (the endoblast), eventually to organize into segmental plate mesoderm and lateral plate mesoderm (Bellairs, 1971, 1979; Bellairs, Sanders & Portch, 1980). The migration of
these mesoderm cells in this tissue space has engendered interest in recent years as
an example of a situation in which extracellular materials may control and direct
morphogenesis (Bancroft & Bellairs, 1975; Ebendal, 1976; Solursh, 1976; England & Walkey, 1977; Walkey & England, 1977; Solursh & Revel, 1978; Sanders, 1979a; Sanders & Anderson, 1979). As a result of these studies, we have a picture of the
mesoderm at this stage of development as a tissue that migrates on glycosaminoglycan-
rich extracellular matrix in the space between the epiblast and endoblast, and also on
the surface of these 2 cell layers, the former of which is lined by a basal lamina.

The endoblast, which at the primitive streak stage comprises the lower layer of
the 3-layered embryo, is, like the mesoderm, derived from the epiblast which in-
vaginates through the streak (Vakaet, 1970; Rosenquist, 1972). As the endoblast
emerges from the primitive streak, it displaces the original lower layer, the hypoblast,
probably by a process of invasion (Sanders, Bellairs & Portch, 1978), so that the
hypoblast takes up a peripheral position. The endoblast then differentiates into
embryonic endoderm and also provides a substratum for mesoderm migration, while
the hypoblast forms the extraembryonic endoderm. These relationships have been
discussed in detail elsewhere (Sanders et al. 1978).

The present work originated from experiments in which the interactions between
these tissues were examined in vitro by the use of confronted cultures (Sanders et al.
1978) and by seeding tissue onto the surface of an established culture of another
tissue (Bellairs, Ireland, Stern & Sanders 1980). In the latter study, an effort was
made to duplicate the in vivo situation by seeding pieces of mesoderm onto epithelial
sheets of endoblast or hypoblast cells. The present investigation shows that these
epithelial sheets were able to modify the surface of the culture vessel in a way that
had a drastic effect on the morphology and rate of spreading of the mesoderm cells,
which penetrated the sheet and rapidly attached to the substratum. The substratum-
attracted material (SAM) derived from the epithelia was then investigated to determine
the nature of the component responsible for the enhancement of mesoderm spreading.

Substratum-attached materials, or ‘microexudates’, often in the form of ‘condi-
tioned’ medium, have been known to affect cell attachment for some time (reviewed
by Grinnell, 1978), and a number of investigations have been concerned with the
morphology and composition of the material left behind on the substratum after cell
detachment (see for example Revel, Hoch & Ho, 1974; Rosen & Culp, 1977; Badley
et al. 1978). It is becoming clear that both the conditioned medium and the cell
surface-derived substratum-attached materials frequently contain the glycoprotein
fibronectin (Yamada & Olden, 1978), and that this is active in the enhancement of
cell adhesion to the substratum (Culp, 1976; Hynes, Destree, Mautner & Ali, 1977;
Chen et al. 1978; Hedman, Vaheri & Wartiovaara, 1978; Furcht, Mosher & Wendel-
Schafer-Crabb, 1978; Grinnell & Feld, 1979; Thom, Powell & Rees, 1979; Hughes
Pena, Clark & Dourmashkin, 1979). Of the possible general function of such materials
during embryogenesis, little is known (Wartiovaara, Stenman & Vaheri, 1976; Wartiovaara, Leivo, Virtanen & Vaheri, 1978), although fibronectin has recently
been shown to be present in the early chick embryo (Critchley, England, Wakely &
Hynes, 1979).
Chick embryo mesoderm cells in culture

In this study, the influence of SAM from hypoblast and endoblast on the morphology and spreading of mesoderm cells is examined and shown to be similar to the effects of fibronectin. Other experiments are described which also suggest the involvement of fibronectin. The results are discussed in terms of the possible roles of these substances in the early morphogenesis of the chick embryo. Some of the results described here have been published previously in abstract form (Sanders, 1979b).

MATERIALS AND METHODS

Hypoblast was obtained from embryos incubated to Stage XII or XIII of Eyal-Giladi & Kochav (1976), while endoblast and mesoderm were dissected from embryos at Stage 5 of Hamburger & Hamilton (1951). The hypoblast and endoblast were taken from most regions of the area pellucida, while mesoderm was taken only from regions lateral to the primitive streak for reasons of ease of dissection. The embryos were manipulated in sterile Tyrode’s solution and the tissues required were dissected out using tungsten needles without the use of ethylenediaminetetraacetic acid (EDTA) or enzymes. Under such conditions, pure samples of each tissue could routinely be obtained. The tissues were cultured in a medium consisting of medium 199 (with modified Earle’s salts and L-glutamine), foetal bovine serum and penicillin-streptomycin mixture (5000 units/ml) in the proportions 18:2:1. Incubation at 37 °C was carried out either in Falcon 3001 plastic Petri dishes or on glass coverslips (Corning Glass Works) waxed onto a cavity slide.

Seeding of one tissue onto another was performed by culturing hypoblast or endoblast for 24 h and then placing small pieces of mesoderm on top of the cell sheet in fresh medium (Bellairs et al. 1980). The culture chamber was then returned to the incubator or to the microscope for further observation. Substratum-attached material was prepared by culturing hypoblast or endoblast for 24 h and then removing the cell sheet by one of 3 methods. Firstly, the cultures were washed in calcium- and magnesium-free (CMF) Tyrode’s solution, followed by 20 min incubation at 37 °C in 0.002 M EDTA in CMF Tyrode’s solution. Secondly, cultures were rinsed in CMF Tyrode’s solution and then incubated in 1% nonidet P40 detergent (NP40, BDH Chemicals Ltd.) for 20 min, followed by several saline rinses (Chen et al. 1978). Thirdly, cultures were removed manually from the substratum by dissection with a tungsten needle. This technique takes advantage of the fact that epithelial cell sheets are principally attached to the substratum at their margins (DiPasquale, 1975) so that once the marginal cells were lifted from the plastic or glass, the remainder of the sheet peeled off with relative ease. Before removal, the area covered by the explant was accurately marked using a wax pencil on the reverse side of the dish or coverslip. In all 3 cases the final removal of the cells was effected by directing jets of saline from a finely drawn out pipette over the cultures. All preparations of SAM were rinsed and incubated with culture medium before use. Pieces of mesoderm tissue were then placed on the substratum previously occupied by the epithelial sheet and the culture returned to the microscope for observation.

In a number of experiments, substances were added to the culture medium before use. Chicken plasma fibronectin (a gift from Dr R. Rajaraman) and human plasma fibronectin (Collaborative Research Inc., Waltham, Mass.) were used at final concentrations of between 10 and 100 μg/ml. It was necessary to pretreat glass, but not plastic, with plasma fibronectin in order to get spreading activity. Plasma fibronectin (50 μg/ml) in serum-free medium was placed on the glass for 45 min at room temperature and then drained off before the substratum was used. Anti-human plasma fibronectin serum raised in rabbit (a gift from Dr R. Rajaraman) was used at a final dilution of 1:40. This antiserum gave a single band in immunodiffusion against human and chicken plasma as well as fibronectin from these sources. The results show that the presence of rabbit plasma fibronectin in this serum did not appear to influence the activity of the preparation at the dilution used. Controls were run using normal rabbit serum at the same dilution. Cycloheximide (Sigma Chemical Co.) was used alone at a concentration of 5 μg/ml and in combination with plasma fibronectin. In one series of experiments, medium
containing 1% bovine serum albumin (fraction V, Sigma Chemical Co.) was added to cultures of hypoblast or endoblast after these tissues had been allowed to attach to the substratum for 4 h. Albumin-coated coverslips were prepared according to the method of Grinnell & Feld (1979) by incubating dishes for 10 min at room temperature in 1% bovine serum albumin (BSA) followed by thorough rinsing in culture medium.

For indirect immunofluorescence studies, SAM was prepared as above and fixed overnight in 95% ethanol with 1 change. After 3 rinses in phosphate-buffered saline (PBS) for 1 min each, coverslips were incubated with anti-fibronectin serum (1:40 dilution in PBS) for 30 min at 37°C. Control samples were incubated in PBS. After further rinsing in PBS (3 x 5 min), the samples, including the controls, were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (Miles Laboratories, Inc., Elkhart, Ind.), diluted 1:10 with PBS for 30 min at 37°C. The coverslips were rinsed in PBS (3 x 5 min), allowed to air-dry, and mounted in Fluormount (Edward Gurr, Ltd., London). Observations were made on a Zeiss Photomicroscope II or a Leitz Orthoplan equipped with fluorescence optics.

Light microscopy was performed using a Nikon model M inverted microscope with phase-contrast or dark-field optics and an incubation chamber. Time-lapse films were made using a Bolex 16-mm movie camera driven by a Nikon CFMA controller and Kodak Plus-X negative film 7231. Stained cultures were examined after preparation with Harris' haematoxylin.

For scanning electron microscopy (SEM), samples were rinsed in Tyrode's solution and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 4 h at room temperature. Specimens were washed in buffer and postfixed with 1% buffered osmium tetroxide for 1 h at room temperature. Dehydration was performed using a graded acetone or ethanol series and critical-point drying from carbon dioxide. After coating with gold, samples were examined with a Cambridge Stereoscan 150 microscope.

For transmission electron microscopy (TEM), cultures were grown in Falcon dishes, fixed as for SEM and dehydrated with a graded series of ethanol solutions. Araldite was poured into the dish and after polymerization was sectioned with the plastic dish still in place. Sections, stained with uranyl acetate and lead citrate, were examined with a Philips 300 microscope. For ruthenium red staining, the specimens were fixed in a freshly prepared solution of 0.05% ruthenium red (BDH Chemicals Ltd.) in 2.5% glutaraldehyde buffered with 0.1 M cacodylate, pH 7.4, for 4 h at room temperature. After a buffer rinse, postfixation was carried out for 1 h at room temperature in 0.05% ruthenium red in 1% buffered osmium tetroxide (Cohn, Banerjee & Bernfield, 1977). Tannic acid staining was carried out as described previously (Sanders, 1979a) by dissolving 1% tannic acid (Fisher Scientific Co.) in the glutaraldehyde fixative and continuing the preparation as above.

RESULTS

Morphology of the tissues in culture

The morphology of epithelial sheets of hypoblast and endoblast has been described previously (Sanders et al., 1978; Sanders & Prasad, 1979). Briefly, the hypoblast (Fig. 1) spread out as an epithelium of large cells, each having yolk droplets aggregated around the nucleus and a thin, wide area of lamellar cytoplasm. Endoblast cells (Fig. 2) were readily distinguishable from hypoblast, being smaller, with less yolk and slower to attach to the substratum. Hypoblast explants were well spread after only 2 h in culture, while endoblast required 4–6 h for a similar degree of spreading (see Table 1). By 24 h, both tissues had usually spread completely, leaving no residual explant mass. The nature of the substratum, either glass or plastic, made little or no difference to these spreading characteristics. Cells within sheets of both types were coherent, although transient gaps appeared between them. Hypoblast was able to grow almost as well in medium lacking serum as in whole medium, the cells being not
Fig. 1. Phase-contrast micrograph of a hypoblast culture grown on glass for 24 h, showing yolk droplets surrounding each nucleus and broad cytoplasmic lamellae. × 150.

Fig. 2. A culture of endoblast cells grown on glass for 24 h. The cells are less yolky than hypoblast. × 150.

Fig. 3. Hypoblast cultured for 24 h in the absence of serum. The cells attach and spread, although some multilayering is evident. × 150.

Fig. 4. Endoblast cultured for 24 h in the absence of serum. The tissue fails to attach to the substratum. × 150.

Fig. 5. Mesoderm explant cultured on glass for 24 h. An unspread mass of tissue remains and few cells have grown out. × 150.

Fig. 6. Scanning electron micrograph (SEM) of a 24-h mesoderm culture on glass, emphasizing the fibroblastic morphology of the out-wandering cells. × 230.
Table 1. Attachment and spreading of chick embryo tissues under various conditions

<table>
<thead>
<tr>
<th>Substratum</th>
<th>Time, h</th>
<th>Glass or plastic</th>
<th>Glass + FN or plastic + FN</th>
<th>Seeded onto epithelium or SAM on glass or plastic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypoblast</td>
<td>1</td>
<td>+1</td>
<td>+1</td>
<td>.</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>+2</td>
<td>+2 → +3</td>
<td>.</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>+3 (21)</td>
<td>+3</td>
<td>.</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>+3 (1)</td>
<td>+3</td>
<td>.</td>
</tr>
<tr>
<td>Endoblast</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>.</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0 → +1</td>
<td>0 → +1</td>
<td>.</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>+1 → +2</td>
<td>+2</td>
<td>.</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>+3 (2)</td>
<td>+3</td>
<td>.</td>
</tr>
<tr>
<td>Mesoderm</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>.</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0</td>
<td>+1</td>
<td>+1</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0</td>
<td>+2 (20)</td>
<td>+2 (13)</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>+2 (5, 6)</td>
<td>+3</td>
<td>+3 (14)</td>
</tr>
<tr>
<td>Morphology</td>
<td>at 24 h</td>
<td>fibroblastic</td>
<td>epithelial</td>
<td>epithelial</td>
</tr>
</tbody>
</table>

Symbols used are as follows: o = no attachment; +1 = attachment and initial spreading; +2 = advanced spreading with some explant mass remaining in the culture; +3 = complete spreading, no explant mass remaining; FN = plasma fibronectin (50 μg/ml); SAM = substratum-attached material.

Figures in brackets refer to Fig. numbers in this paper.

as greatly spread (Fig. 3). Endoblast, by contrast (Fig. 4), would not attach or spread in the absence of serum.

Mesoderm grew with an entirely different morphology from that described above. The explant required 16–24 h to attach and begin to spread (Table 1), and after this

Fig. 7. Transmission electron micrograph (TEM) of section through the ventral surface of an endoblast cell grown on plastic. A ruthenium red-stained deposit is present between the cell and the substratum. x 55650.

Fig. 8. TEM showing tannic acid-stained material between the ventral surface of a mesoderm cell and the plastic substratum. x 55650.

Fig. 9. TEM of a section through an endoblast culture which was fixed in the presence of tannic acid. Note that there is an electron-dense deposit on the ventral surface of the cells but not on the dorsal surface. x 5100.

Fig. 10. Frames from a time-lapse movie using dark-field optics. Two pieces of mesoderm have been placed on the glass substratum and several pieces placed on top of a sheet of endoblast cells. The times at each frame A, B, C are 0, 4, and 8 h, respectively. The tissue on the glass does not spread, while that on the endoblast does. x 28.

Fig. 11. Frames from a time-lapse movie using dark-field optics. Pieces of mesoderm have been explanted onto SAM derived from endoblast. The times at each frame A, B, C are 0, 2, and 4 h, respectively. Compare the spreading with the mesoderm in Fig. 10. x 28.
Chick embryo mesoderm cells in culture
time a central mass of residual tissue always remained (Fig. 5). This timing was the same for glass and plastic substrata. The outwandering cells were fibroblastic in appearance (Figs. 5, 6) and resembled those from unsegmented mesoderm at a later stage of development (Bellairs et al. 1980). This tissue would not grow in the absence of serum, the cells failing to attach and frequently dissociating from one another.

Transmission electron microscopy using ruthenium red or tannic acid in the fixative to enhance the contrast of extracellular material showed that material was detectable adhering to the substratum and to the ventral surface of the cells (Figs. 7, 8). Fig. 9 shows that whereas large amounts of extracellular material were present on the ventral surface of the cells, little or none was detectable on the dorsal surface by these techniques. There was little difference between the various cell types in this regard, although it appeared that the mesoderm laid down somewhat less of this material than the others.

**Seeding experiments**

When pieces of mesoderm were seeded on top of an established sheet of hypoblast or endoblast, the mesoderm rapidly penetrated the sheet, gained access to the substratum and spread out. Fig. 10 shows time-lapse frames of the spreading event. In this particular experiment, pieces of mesoderm were placed both on the endoblast sheet and on the glass beside the sheet. The rapid spreading of the former in comparison with the latter is readily apparent. The mesoderm on glass did not spread until 18–24 h, which was beyond the duration of this film. The rapidity of this process, in comparison with spreading of mesoderm on unmodified substratum, can also be seen from Table 1. Spreading in the seeded configuration was virtually complete within 4 h. By 24 h there was no trace of the explant mass, all its cells having spread—a situation which never occurred on unmodified substratum. That the mesoderm was not spreading on top of the epithelial sheet was shown by scanning electron microscopy (Fig. 12). Further confirmation that the mesoderm was migrating on the substratum and not on an underlying cell layer was obtained by phase-contrast microscopy, cinemicrography and TEM.

In order to determine whether or not the presence of the epithelial cells themselves

---

Fig. 12. SEM of mesoderm (m) seeded onto endoblast (e). The mesoderm cells can be seen to have penetrated the endoblast sheet and have moved underneath the endoblast cells. \( \times 2500 \).

Fig. 13. Phase-contrast micrograph of mesoderm cultured for 5 h on SAM derived from endoblast. Considerable spreading has occurred in this time. \( \times 150 \).

Fig. 14. Fixed and stained explant of mesoderm cultured for 24 h on SAM derived from endoblast. No mass of unspread tissue remains and the culture has an epithelial appearance, compare with Fig. 5. \( \times 110 \).

Fig. 15. SEM of a culture similar to that in Fig. 14. The cells are flattened and coherent and are outlined by ruffling edges. \( \times 1700 \).

Fig. 16. SEM of SAM derived from hypoblast. The remains of lamellae and retraction fibres are seen. \( \times 3800 \).

Fig. 17. As Fig. 15, showing filamentous deposits. \( \times 3800 \).
Chick embryo mesoderm cells in culture
was required for the rapid spreading of the mesoderm, the epithelium was removed and the mesoderm was seeded onto substratum previously occupied by the hypoblast or endoblast. Three techniques were used for removal of the epithelium (detailed in the Methods section): EDTA, detergent and manual dissection. The result was the same whichever technique was used: the mesoderm spread rapidly and with the same time course as when seeded onto the intact sheets (Table 1 and Fig. 11). In Fig. 11, frames from a time-lapse movie are shown to indicate the time course of the spreading process. SAM derived from both hypoblast and endoblast had the same effect. In addition, the morphology of the spread culture was clearly different from that of cultures on unmodified substratum. The appearance of the explant on SAM at 5 and 24 h (Figs. 13, 14) was more epithelial, with no individual cells breaking away from the periphery of the outgrowth (compare with Fig. 5). SEM (Fig. 15) showed the epithelial nature of the culture, which was cohesive, the cells having large ruffles and microvilli delineating their borders.

A similar experiment to that described above was performed in the absence of serum. Thus, a hypoblast sheet was grown for 24 h with no serum. This was detached from the substratum and mesoderm was then seeded in the absence of serum. Spreading occurred as rapidly, and with the same morphology, as in the presence of serum, although the mesoderm outgrowth could not be maintained for longer than 7 or 8 h under these conditions.

**Morphology of SAM**

When examined using the SEM, the morphology of SAM derived from hypoblast and endoblast was similar, and of 2 distinct types seen with approximately equal frequency (Figs. 16, 17). One type was clearly the result of the amputation of fine cytoplasmic processes or lamellae, leaving membrane-enclosed sacs and cell fragments (Fig. 16). The other type (Fig. 17) could be considered to be extracellular matrix material. SAM derived from mesoderm cultures was less abundant but similar to that described above.

---

Fig. 18. Indirect immunofluorescence of SAM from endoblast with antifibronectin serum. The staining is in patches. Control immunofluorescence preparations were non-reactive. \( \times 680 \).

Fig. 19. As Fig. 18, showing strongly reactive filaments attached to the substratum. \( \times 680 \).

Fig. 20. Phase-contrast micrograph of a mesoderm explant cultured for 5 h in the presence of fibronectin on plastic. The spreading is similar in degree and character to mesoderm spreading on SAM (Fig. 13). \( \times 150 \).

Fig. 21. Hypoblast cultured on glass for 5 h. The explant is well spread after this time. \( \times 150 \).

Fig. 22. Hypoblast cultured on glass for 5 h in the presence of antifibronectin serum. Cells are poorly attached to the substratum and fail to form a coherent sheet. \( \times 150 \).

Fig. 23. Mesoderm explanted onto SAM derived from endoblast for 5 h in the presence of anti-fibronectin serum. The tissue does not attach or spread (compare with Fig. 20). \( \times 150 \).
Chick embryo mesoderm cells in culture
Immunofluorescence studies of the SAM using anti-fibronectin serum also showed the 2 distinct types. The first type appeared as patches of fluorescence (Fig. 18), while the second appeared as a strongly reactive fibrillar material (Fig. 19). When SAM from mesoderm cultures was subjected to this technique, little or no fluorescence was detectable. In all cases, control coverglasses bearing SAM incubated in PBS were negative.

**Explants cultured with plasma fibronectin**

In view of the above immunofluorescence results, the tissues were grown on glass and plastic in the presence of human or chicken plasma fibronectin (Table 1). When mesoderm was explanted with plasma fibronectin (50 µg/ml) on untreated plastic or glass pretreated with plasma fibronectin, the time course and morphology of spreading were the same as for mesoderm seeded onto an epithelium or onto SAM (Fig. 20). The progress of spreading was followed using time-lapse cinemicrography. After incubation for 24 h, these cultures remained fully spread, with no remaining tissue mass in the centre, often with a more epithelial appearance than in normal medium. TEM of mesoderm cultures in the presence of plasma fibronectin did not demonstrate any particular change in the contact relationship between the ventral surface of the cells and the plastic substratum. When mesoderm was explanted in the presence of plasma fibronectin on glass which had not been pretreated, no enhancement of spreading was observed.

When hypoblast and endoblast were explanted on glass and plastic in the presence of plasma fibronectin (50 µg/ml), the initial rate of outgrowth of hypoblast may have been slightly accelerated but was not consistently so. The final size of the cultures was not different from controls in normal medium.

**Explants cultured with anti-fibronectin serum**

The above experiments indicated that hypoblast and endoblast might be laying down fibronectin during outgrowth. These tissues were therefore cultured in the presence of anti-fibronectin serum which, it was reasoned, would bind to fibronectin on the substratum and on the cell surface, and thus interfere with spreading. Both of the epithelia behaved similarly. In comparison with normal spreading after 5 h (Fig. 21), the presence of the antiserum prevented sheet formation. This is illustrated by Fig. 22, which shows spindleshaped cells indicative of an impaired ability of the cells to attach and spread. This situation is still apparent at 18–24 h after explanting, and time-lapse studies confirmed that the cells emerging from the explant were not able to grip the substratum. Lamellae were generated but failed to obtain an adhesion along a broad front (as in Fig. 21), but instead were pulled back, forming long thin retraction fibres.

In a similar experiment, mesoderm was seeded onto SAM in the presence of anti-fibronectin antibody (Fig. 23). In this situation, spreading was always inhibited (compare Figs. 13 and 23). In all cases, control cultures were grown in the presence of an equivalent concentration of normal rabbit serum. All tissues grew normally under these conditions.
Chick embryo mesoderm cells in culture

Explants cultured with cycloheximide

In order to inhibit the cellular production of fibronectin during outgrowth, explants were cultured in the presence of 5 μg/ml cycloheximide. Both endoblast and hypoblast (Fig. 24) failed to spread under these conditions. The hypoblast was still viable after 24 h in cycloheximide, since replacement of the medium containing cycloheximide at this time with normal medium resulted in complete recovery and spreading.

Fig. 24. Hypoblast cultured on glass for 24 h in the presence of cycloheximide. Spreading is poor and a large explant mass remains. ×150.
Fig. 25. Hypoblast cultured on glass for 24 h in the presence of both cycloheximide and fibronectin. Spreading is extensive in comparison with spreading in the presence of cycloheximide alone, indicating that fibronectin can attach these cells to the substratum. ×150.
Fig. 26. Mesoderm explanted in the presence of cycloheximide. Dissociation occurs and the cells do not spread. The presence of fibronectin does not affect this result. ×150.
Fig. 27. Mesoderm explanted for 5 h in the presence of fibronectin on BSA-treated plastic. The tissue does not attach and spread. ×150.
Endoblast was recoverable after at least 10 h cycloheximide treatment. When hypoblast was cultured with cycloheximide but with plasma fibronectin (50 μg/ml) also added, spreading was essentially normal (Fig. 25), although some multilayering of the cells was evident and the culture size was reduced somewhat in comparison with controls in normal medium. Endoblast did not respond as well to the addition of plasma fibronectin, the cultures remaining small and fragmented, although some spreading was clearly evident.

When mesoderm was cultured in the presence of cycloheximide with or without plasma fibronectin, the result was that the tissue failed to attach to the substratum and considerable dissociation of cells occurred (Fig. 26).

Treatment of the substratum with BSA

Following the reasoning of Grinnell & Feld (1979), the non-adhesive protein BSA was used to occupy fibronectin adsorption sites on the substratum and thus determine the importance of these sites to the activity of the material produced by the epithelia and of plasma fibronectin. Hypoblast was cultured in the absence of serum for 4 h and then the medium was changed for medium containing 1% BSA. Further spreading of the explant was retarded but not eliminated. In the case of mesoderm, the plastic dishes were preincubated with BSA before the tissue was explanted. Pieces of mesoderm, seeded onto these dishes in medium containing plasma fibronectin, failed to spread within the first 5 h of culture (compare Figs. 27 and 20) and subsequent outgrowth was poor. Mesoderm which was explanted in pretreated dishes in the absence of plasma fibronectin also did not spread rapidly, indicating that the BSA did not influence the spreading rate. In both cases (with and without plasma fibronectin), the tissues behaved during the first 5 h as if it were seeded onto normal plastic dishes and in normal medium.

DISCUSSION

The above results show that the lower epithelial layers of the early chick embryo, the hypoblast and endoblast, laid down extracellular material on the culture substratum which accelerated the attachment and spreading of embryonic mesoderm tissue which was otherwise slow to spread. This effect could be mimicked on unmodified substrata by the addition of plasma fibronectin to the culture medium. That fibronectin was present and active in the SAM of the epithelia but not the mesoderm was shown by immunofluorescence and inhibition of the spreading activity by anti-fibronectin serum. Production of the SAM by the epithelia, and the importance of its attachment to the substratum, was confirmed by the experiments with cycloheximide and BSA.

The main point of discussion is to consider what these results can contribute to the understanding of the interactions between these tissues in vivo. Of obvious importance are previous observations which have shown the presence of fibronectin in the early (Critchley et al. 1979) and late (Linder, Vaheri, Ruoslahti & Wartiovaara, 1975) chick embryo. In the former study, fibronectin was localized on the ventral
Chick embryo mesoderm cells in culture

surface of the ectoderm (epiblast) but not on the mesoderm layer. Nothing could be said about the endoblast because it was removed. The present study did not deal with the epiblast (the presence of the basal lamina requires changes in the technique of explanting), but the results obtained here with mesoderm also suggest that it is poor in fibronectin. The present results are consistent with the proposition that if placed in order of decreasing cell surface fibronectin levels, the tissues would rank: hypoblast > endoblast > mesoderm. The placing of the hypoblast and endoblast is based on the former’s ability to spread in the absence of serum, since using Grinnell’s (1978) hypothesis, cells with high levels of fibronectin are able to deposit this on the substratum and therefore have a low serum requirement.

The morphology of mesoderm cells migrating in vitro on unmodified substratum (Figs. 5, 6) has been shown to be somewhat fibroblastic. This corresponds well with the appearance of these cells in situ, where in a 3-dimensional system they also have fibroblastic attributes (Bancroft & Bellairs, 1975; Ebendal, 1976; Wakely & England, 1977; Solursh & Revel, 1978). The acquisition of this morphology in situ seems to be the result of a gradual change from a more epithelial appearance as they migrate away from the streak (England & Wakely, 1977). In vitro, the presence of SAM or fibronectin induces the reverse morphological change from fibroblastic to epithelial.

Let us make the assumption that SAM in vitro to some extent corresponds with the cell surface material that can be demonstrated on tissues within the embryo (Sanders & Zalik, 1972; Sanders, 1979a). It is arguable (Critchley et al. 1979) that the mesoderm, itself lacking the ability to produce high levels of fibronectin, uses the fibronectin-rich material laid down by the epiblast and endoblast to facilitate and direct its outward migration. While this straightforward explanation may be valid to some degree, it is not entirely consistent with the present observations, which show that SAM induces in mesoderm a conversion towards the more flattened epithelial morphology. In situ, however, such flattening of mesoderm cells is not seen. Therefore, it is proposed that as the mesoderm passes through, and emerges from, the primitive streak, its ability to produce fibronectin is reduced and that a resulting overall decrease in adhesiveness is vital for its expansion and migration within the embryonic space as a fibroblastic tissue. During this conversion, the mesoderm loses the ability to form the broad areas of adhesion characteristic of epithelia. The role of the fibronectin-rich cell surface material produced by the epiblast and endoblast may be to provide the mesoderm with a substratum for the adhesion of localized attachment sites. These are in the form of the many filopodia that characterize the mesoderm cells in situ (Bancroft & Bellairs, 1975; Ebendal, 1976; Wakely & England, 1977; England & Wakely, 1977; Solursh & Revel, 1978). The SEM studies cited all show that the mesoderm cells are rounded or irregularly shaped at this stage, and not flattened onto the epithelial layers. The lack of flattening may be due to the presence of the 3-dimensional, hyaluronate-rich extracellular matrix in the tissue space, which also provides localized attachment sites, and to changes in the distribution of cytoskeletal elements. At this stage of development, collagen fibrils in the tissue space are sparse (Sanders, 1979a) and can, therefore, exert little or no effect on cell shape. From studies of the relationship between adhesiveness and cell shape (Folkman &
Moscona, 1978; Thorn et al. 1979), it has become apparent that decreased substratum adhesiveness is correlated with a loss of flattening. So the fibroblastic appearance of the mesoderm might be associated with decreased adhesiveness as a result of loss of fibronectin. It may also be significant that decreased adhesiveness has been correlated with increased cellular motility (Gail & Boone, 1972), since the mesoderm cells at this stage are presumably highly motile. At a slightly later stage of chick embryo development, the absolute adhesiveness of ectoderm and mesoderm has been compared (Bellairs, Curtis & Sanders, 1978). In this case, mesoderm was significantly less adhesive than ectoderm, in agreement with the present suggestion.

The production of substratum-attached, fibronectin-containing material by epithelia has been described previously (Chen, Maitland, Gallimore & McDougall, 1977). In the embryo, the lower layer (hypoblast or endoblast) possesses extensive cell surface material on the dorsal surface in comparison with the ventral (Sanders, 1979a). In vitro, however, these epithelia principally lay down extracellular material on their ventral surface (Figs. 7–9). Therefore, there is something to be said for the argument that the epithelium in vitro is inverted with respect to that in situ. Thus, in vitro, the mesoderm penetrates the epithelium to reach the more adhesive extracellular material in preference to the dorsal surface. In situ, however, the dorsal surface of the endoblast is provided with this material and is therefore a suitable substratum for filopodium attachment. In this connexion, it has been shown several times that the dorsal surface of cultured epithelia are not able to support the adhesion of other cells (DiPasquale & Bell, 1974; Vasiliev & Gelfand, 1978).

With regard to the morphology of the SAM revealed by SEM, 2 types were observed, the production of which was not seemingly related to the method of removal of the epithelium. The origin of these may be explained by supposing that the remnants of retraction fibrils and lamellae came mainly from marginal cells of the sheet which were firmly attached to the substratum (DiPasquale, 1975), while the extracellular deposits came from cells in the centre of the sheet that were less firmly attached and therefore removed without rupture. There is a problem in determining whether one or both of these types of SAM is involved in the enhancement of mesoderm spreading. This might be resolved by a careful study of cell removal techniques followed by correlated observations using SEM, interference-reflexion (reflexion-contrast) microscopy and immunofluorescence, such as that carried out by Badley et al. (1978).

Some observations suggest that other secreted materials in addition to fibronectin may possibly be involved in the spreading of cells studied here. For example, plasma fibronectin added to the culture medium together with cycloheximide did not abolish the cycloheximide-induced inhibition of endoblast spreading. Similarly, mesoderm did not spread in the presence of both plasma fibronectin and cycloheximide, although plasma fibronectin in the absence of cycloheximide did induce spreading.

It is interesting to note that the serum components of the normal medium, which for many cells are responsible for spreading ('spreading factors', Grinnell, 1978), are not able to spread the mesoderm rapidly. In this regard, mesoderm cells resemble malignant cells, whose poor spreading is related to a lack of cell surface fibronectin
Chick embryo mesoderm cells in culture

and is ameliorated by the addition of cell surface fibronectin to the medium (reviewed by Grinnell, 1978; Yamada & Olden, 1978).

I would like to thank the following: Mrs Sita Prasad for her skilled technical assistance; Dr R. Rajaraman for generous gifts of chicken plasma fibronectin and anti-fibronectin serum and for his suggestions concerning aspects of this work; Drs R. Bellairs, G. Ireland and C. Stern for their valuable discussion and suggestions. This work was supported by an operating grant from the Medical Research Council of Canada.

REFERENCES


E. J. Sanders


(Received 23 October 1979)