Intercellular relationships during cavitation of aggregates of extraembryonic endoderm cells from gastrulating chick embryos

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SUMMARY

Extraembryonic endoderm cells from gastrulating chick embryos undergo epiboly and change from a multilayered cell group to a single cell layer surrounding the yolk. Single cell suspensions from this cell layer can aggregate in vitro to form aggregates that cavitate. To study the stages of cavitation aggregates were harvested after different times in culture, and fixed and processed for light and electron microscopy. In aggregates harvested at 75 min of culture cell contact consisted of areas of parallel and close membrane apposition and interdigitation. Desmosomes were occasionally observed. Aggregates in the early stages of cavitation (24 h) contained numerous intercellular spaces bordered by irregularly shaped cells which appeared to be digesting their yolk and releasing material extracellularly. Long cytoplasmic projections were extended into these spaces. In addition to regions of parallel membrane apposition and interdigitation, desmosomes and adherens junctions were observed. Cells closer to the periphery of the aggregates displayed fewer cell projections and also showed signs of release of material extracellularly. After 48 h of culture, a single smooth-walled central cavity was present and cells still exhibited signs of extracellular release of material. These same cell shapes and intercellular junctions were also observed when area opaca tissue dissected from gastrulating embryos was examined. Aggregates of different sizes were created and cultured. The results suggest that a critical tissue mass may be important for cavitation.

INTRODUCTION

The extraembryonic area opaca of the gastrulating avian embryo spreads down over the surface of the yolk and encompasses it throughout the first few days of development. During this process, known as epiboly, its innermost multilayered population of endoderm cells is gradually transformed into the single cell layered epithelium of the yolk sac. This epithelium will absorb and process the yolk components before passing them to the overlying blood vessels for transport to the developing embryo (Bellairs, 1963, 1964; Mobbs & McMillan, 1979, 1981; Romanoff, 1960).

Previously published work has suggested that the rearrangement of extra-

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embryonic endoderm cells during epiboly as well as their reorganization into a coherent epithelial cell layer is dependent upon the presence, in the area opaca, of the mesoderm which migrates peripherally from the area pellucida (Bennett, 1973; Bremer, 1960). We questioned this after observing the development of cellular aggregates obtained when pure single cell suspensions of extraembryonic endoderm cells were allowed to aggregate in rotating flasks. Initially the aggregates were solid but after being cultured 24 to 48 h they cavitated and developed into hollow vesicles of cells (Milos, Zalik & Phillips, 1979). In some instances these vesicles became very thin walled, as little as a single cell layer in thickness (Milos et al. 1979). The observation that endoderm cells in ectoderm and mesoderm-free aggregates become relocated to eventually surround a central cavity suggested to us that aggregate vesiculation and yolk sac formation could be a manifestation of developmental information already present in the endoderm cells and independent of the presence of the other germ layers. Thus, developing extraembryonic endoderm cell aggregates may provide a relevant in vitro model system for studies on the reorganization of these cells during yolk sac formation in vivo.

To learn more about how extraembryonic endoderm cells participate in cavity formation, studies on the changes in cellular morphology and intercellular relationships are required. It is also important to determine if the kinds of intercellular junctions present in aggregates resemble those occurring in the extraembryonic endoderm in situ.

We have now studied the intercellular relationships in solid, cavitating and cavitated aggregates in more detail using light and electron microscopy. We have also examined these relationships in the area opaca of chick embryos incubated for 24 h. Our results show that similar intercellular adhesive junctions and cell-shape changes occur in cavitating aggregates in vitro and in the spreading endoderm cell population in vivo. We have also created aggregates of different sizes and determined the relationship between aggregate size and cavitation. Our results suggest that a critical tissue mass may be important for the formation of a cavity. Some of these results have previously been presented in abstract form (Milos, Zalik, Sanders & Ledsham, 1982).

**Materials and Methods**

White Leghorn eggs obtained from the University of Alberta farm or from Woodman’s Hatcheries, Edmonton were incubated for 24 h at 39 °C. Embryos were dissected into Pannett and Compton saline (Pannett & Compton, 1924) (PCS) and single cell suspensions of extraembryonic endoderm cells were obtained from stage-4 embryos (Hamburger & Hamilton, 1951) by a differential dissociation method described in detail elsewhere (Milos et al. 1979). Briefly, areae opacae are pipetted with a wide-bore pipette in PCS (pH 7-5) and strained through Nitex mesh (44 µm). This traps sheets of ectoderm and mesoderm with some adhering endoderm on the mesh while endoderm cell clumps pass through it. The
endoderm cell clumps collected in the filtrate are resuspended in Ca$^{2+}$-Mg$^{2+}$-free PCS (pH 7-8). After incubation at 0°C for 10 min the suspension is pipetted three times. The cells are then washed twice in Ca$^{2+}$-Mg$^{2+}$-free PCS. This procedure yields a cell suspension consisting of approximately 80% single cells, 10% pairs and 10% small cell clumps with slight or no contamination by ectoderm or mesoderm (Milos et al. 1979).

For production of cell aggregates, 5 x 10$^6$ single cells in 3 ml of medium were placed in 10 ml flasks and allowed to aggregate at 80 r.p.m. in a gyratory shaker (New Brunswick Scientific Co.) at 37°C. The culture medium consisted of L-15 medium containing glucose (900 mg/l) instead of galactose, and gentamycin sulphate (Sigma, 20 μg/ml). In some experiments aggregates were harvested after 75 min of rotation. For aggregates cultured for longer time intervals, the shaker speed was increased to 120 r.p.m. Small aggregates were created by increasing the shaker speed to 120 r.p.m. after 30 min of rotation. Aggregates were cultured for 24 or 48 h.

Blastoderms were fixed by two methods. In the first the egg was opened into a beaker and the albumin was removed. Then, cold 3% glutaraldehyde in 0.1 M-phosphate buffer (pH 7.35) was added to the beaker to cover the yolk. The beaker was then placed at 5°C overnight. In the second method cold fixative was pipetted over the surface of the blastoderm and yolk, as well as injected through the vitelline membrane beneath the blastoderm. The beaker was then filled with fixative and stored at 5°C overnight. The following day the glutaraldehyde was replaced with 0.1 M-phosphate buffer and the blastoderms were removed from the yolk. Then, pieces of tissue from different areas of the blastoderm were dissected and processed further. The areas removed included (a) the outer area pellucida and adjacent area opaca and (b) the intermediate and outer area opaca.

Tissues were postfixed in 1% osmium tetroxide in 0.1 M-phosphate buffer, dehydrated and embedded in araldite. Thick and thin sections were cut on a Sorvall MT2 ultramicrotome. Thick sections were stained with Richardson's stain. Thin sections were stained with uranyl acetate and lead citrate and examined using a Phillips 300 electron microscope. Aggregates were processed as above. To preserve orientation whole aggregates were sectioned so that the location of cells within different regions of the aggregate would be known for each section.

RESULTS

A. Extraembryonic endoderm cell associations in vitro

1. Early aggregates*

Large irregularly shaped aggregates form rapidly when suspensions of single cells are cultured in gyratory shakers. These early aggregates consist of a number

*We have used the descriptions of intercellular junctions in Fawcett (1981) to identify the junctions in the present study. Junctions resembling the zonula adherens have been designated as adherens junctions while those resembling the macula adherens are called desmosomes.
Fig. 1. Early aggregate (1-25 h).

(A) Cross section, light microscope (LM). Numerous small irregularly shaped clusters of yolk-laden cells have formed. These small groups are loosely adherent to one another and form a branching network of clusters separated by large spaces. ×210.

(B–D) Similar intercellular junctions are observed at all levels of the aggregates. (B) Electron microscope (EM). An area of localized parallel membrane apposition (arrow). ×38,700. (C) EM. Localized membrane apposition with increased local membrane and extracellular density (arrow). ×50,800. (D) EM. Morphologically mature desmosome. Desmosomes are observed occasionally. ×50,150.

(E) EM. Cells have begun to spread over one another's surfaces and are changing shape from the round morphology of freshly dissociated cells to more irregular forms. Long cytoplasmic projections have been extended, some of which interdigitate (*). Cell-to-cell contact is sporadic and limited to localized areas of opposing cell surfaces (arrows). Type A yolk (lipid plus lipoprotein) (A), Type B yolk (mainly lipid) (B), and complex yolk (protein plus lipoprotein plus lipid) (C), (Bellairs, 1963), are present in the cytoplasm. n: nucleus; g: Golgi apparatus; m: mitochondria. ×8,540.
Fig. 2. 24 and 48 h aggregates.

(A) 24 h aggregate – cross section, LM. The aggregate has become regular in outline. Initially it was solid but between 12 to 24 h of culture numerous irregular cavities began to form in the interior. The largest cavities have formed in the centre. ×156.

(B) Enlargement of the area in the box.

I. Central area. Some cells bordering the small internal cavities contain very little yolk (I). Many cells have long cytoplasmic projections (black arrows) and are elongate in shape. Because of the large size of the cells, the nuclei (n) are not abundant in the sections.

II. In the intermediate area, a loose, lacy type of intercellular arrangement is observed.

III. At the periphery, the most compact cell arrangement is observed.

At all three levels of the aggregate, the yolk platelets are undergoing structural changes indicative of digestion. In some areas, yolk platelets have coalesced and become very large (*). Type B yolk is being extruded from some cells into the extracellular spaces (white arrows). ×580.
Cavitation in endoderm cell aggregates

of loosely joined smaller aggregates each comprising its own loosely attached cell group (Fig. 1A). Within these groups, cellular outlines are beginning to change from the round shape of freshly dissociated cells (Milos et al. 1979) to more irregular and lobose forms (Fig. 1E). The cells are beginning to spread over one

(C) 48 h aggregate – cross section, LM. The irregular central cavities have coalesced to form a large smooth-walled single central cavity. ×118.

(D–E) Enlargements of regions I and II. (D) Inner face. The cells are closely packed and numerous droplets of Type B yolk are being extruded into the central cavity (arrows) and between some cells (white arrows). n: nucleus. ×770. (E) Outer face. The outer face of the aggregate is smoother than the inner face. Type B yolk is also being extruded into the medium from the outer face (arrow). ×770.
another's surfaces and areas of parallel membrane apposition are frequently observed (Fig. 1B). Many cells have extended long cytoplasmic projections into the extracellular spaces some of which run parallel and close to the opposing cell surface. In some areas these processes interdigitate (Fig. 1E). In areas where surfaces of opposing cells are in close contact, some patches of increased extracellular density with associated cell surface density are observed (Fig. 1C). These may represent early stages of desmosome formation. Desmosomes that appear more mature in morphology are observed sporadically (Fig. 1D). Adherens junctions are not observed at this stage.

Numerous yolk inclusions including Type A (lipid plus lipoprotein), Type B (mainly lipid) and complex yolk (protein, lipoprotein and lipid) (Bellairs, 1963, 1964) are present in the cytoplasm. The cells also contain an eccentrically placed nucleus, a Golgi apparatus and mitochondria (Fig. 1E).

2. Cavitating and cavitated aggregates

During the next 24 h of culture the aggregates undergo striking morphological changes. They become smooth and round in outline (Milos et al. 1979) and in the interior, the large spaces observed at early stages of culture (Fig. 1A) disappear due to gradual coalescence of small aggregates. This phase of aggregate development lasts from approximately 12 to 24 h of culture. It is followed by the reappearance of numerous irregular cavities in the interior (Fig. 2A,B). In general, the largest cavities form near the centre of the aggregate (Fig. 2A,B, area I). These cavities are bordered by irregularly shaped or extremely elongated cells connected to one another by long cellular projections. Some of these cells contain very little yolk. In cells that contain yolk, the yolk inclusions appear to have coalesced and undergone structural changes indicative of digestion (Fig. 2B) (Bellairs, 1963, 1964).
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Closer to the periphery of the aggregate (Fig. 2A,B, area II), the large central cavities are replaced by a lacy, loose type of cellular association (Fig. 2B). In this area cells containing both small and large amounts of yolk are observed and more Type B yolk is present than in early aggregates. In the most peripheral regions of the aggregate (Fig. 2A,B, area III) the cells are packed most closely although intercellular spaces still are present (Fig. 2B). In this area most of the cells are...
lobose and contain a great deal of yolk. Extrusion of Type B yolk is observed into extracellular spaces within the aggregates at this time (Fig. 2B). We do not believe that fixation induces release of the granule contents because we have never observed this process in early aggregates where Type B yolk is also often found close to the cell membrane (Fig. 1).

By 48 h of culture all large aggregates contain a large central cavity (Fig. 2C). In cross section the interior and exterior surfaces bordering the central cavity and the periphery, respectively, have a smooth outline when examined with the light microscope at low magnification (Fig. 2C). Upon examination at higher magnifications, however, it is observed that the outer wall is smoother than the inner wall (Fig. 2D,E).

Within the tissue mass the cells are generally closely packed and cell shapes tend to be lobose. The long cellular projections observed during cavitation are absent. Most of the yolk drops remaining in the cells have been converted to Type B drops which are still being extruded into the cavity as well as into the culture medium from the external face of the aggregate (Fig. 2D,E). Release of Type B yolk into the extracellular spaces is also occurring to a limited extent between cells inside the aggregate at this time (Fig. 2D).

Similar intercellular junctions are present at all levels of cavitating and cavitated aggregates (Fig. 3). Cells are joined to one another by adherens junctions (Fig. 3A,B,C,F) and desmosomes (Fig. 3A,B). Areas of focal close and parallel membrane apposition and interdigitation are also present (Fig. 3C,D,F). Structures that we interpret as desmosomes in the process of splitting have also been observed in the interior of the aggregates at these times (Fig. 3E).

By 48 h of culture many interior and exterior cells also show well-developed microvillar projections, coated pits, coated vesicles, membrane-bound vacuoles and smooth-surfaced canaliculi, (Fig. 3D,F) all of these indicative of the absorptive stage of yolk sac development (Mobbs & McMillan, 1979, 1981). Glycogen granules are also present, (Fig. 3F) possibly reflecting one of the other suggested functions of the yolk sac, that of a transitory liver (Bellairs, 1964).

B. Extraembryonic endoderm cell associations in vivo

It was of interest to determine whether the cell associations present during cavitation resemble those present in the extraembryonic endoderm in situ. Although Bellairs (1963) has presented observations of the area opaca at this stage, new features are noted here. For orientation purposes, a sketch of a cross section of a blastoderm is shown in Fig. 4A. The numbers in the sketch correspond to the regions of the blastoderm examined in this study and can be used for reference in Fig. 4.

1. Before colonization by the mesoderm

At this stage of development the endoderm cells are arranged in a loose array from one to four cells deep, separated by intercellular spaces of varying sizes and
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yolk spheres (Fig. 4C,D). In general, the intercellular spaces and yolk platelets are larger and more numerous closer to the periphery of the area opaca while in more medial areas the cells are packed more closely. In fact, in the outer regions, some cells appear to have no contact at all with neighbouring cells. Cell shapes are lobose or more elongate and where cell-to-cell contact does occur it is by areas of parallel membrane apposition. Areas of membrane interdigitation and desmosomes are observed only very occasionally; adherens junctions have never been observed. Cells resting directly on the underlying yolk have extended long cytoplasmic projections ventrally (Fig. 4C,D). In some areas these projections appear to rest on extracellular deposits of material (Fig. 4C) possibly corresponding to the material described as yolk membranes by Bellairs (1963). The cells contain all three types of yolk platelets and Type B yolk is being extruded from some cells at this time.

2. During colonization by the mesoderm

Changes occur in the arrangements and morphologies of extraembryonic endoderm cells at the time that the area opaca is being colonized by mesoderm. The cells become more lobose in shape, more closely packed and form a layer from four to seven cells deep (Fig. 4B). Extracellular yolk is less abundant. Villous projections are present at the ventral surface of the cell group and among these cells contact occurs via parallel membrane apposition and interdigitation (Fig. 4E). Adherens junctions are also frequent (Fig. 4E). More dorsally, cells adhere mostly via parallel membrane apposition and interdigitation. However, some adherens junctions and desmosomes are observed.

Type B yolk is also being extruded from some cells in this area (Fig. 4F). In this figure it can be seen that the partially extruded yolk maintains its overall round shape. The dense outer layer appears as a light amorphous covering in regions that have been extruded.

Influence of cell number on aggregate cavitation

Cavitation could be regarded as one of the manifestations of cell commitment. However, a minimal critical number of cells may still be necessary for this to occur as has been found for tissue differentiation in other systems (Deuchar, 1970; Grobstein, 1952; Grobstein & Zwilling, 1953). To determine if a similar situation occurs in the extraembryonic endoderm cell population, aggregates of different sizes were created, cultured for 24 or 48 h, and examined for the presence of cavities. Table 1 shows that after 24 h of culture the only aggregates to cavitate were those containing more than 16,000 cells. However after 48 h, a single central cavity was also present in aggregates consisting of 7000 cells or more. At this time numerous irregular internal cavities were observed in aggregates containing between approximately 3000 to 7000 cells. Thus, cavitation is progressively inhibited as cell number decreases suggesting that a critical tissue mass is important for this process to occur.
Fig. 4. *Area opaca* cell associations *in vivo*.

(A) A diagrammatic cross section through the embryo is shown for reference purposes. In Fig. 4B,C,D, the numbers correspond to the numbered areas of the *area opaca* shown in A.

(B) *Area opaca* after colonization by mesoderm, LM. The extraembryonic endoderm cell group (*EEC*) is four to six cells deep and appears to be closely packed. Mesoderm (*me*) has invaded the *area opaca* from the *area pellucida* and migrated between the EEC tissue and the dorsal single cell layered epiblast (*ep*). The EEC are large, lobose in shape and laden with intracellular yolk platelets. It is from this multilayered cell group that the single cell layered endodermal epithelium will form. This section corresponds to area 1 to 2 in Fig. 4A. *n*: nucleus. ×400.

(C) Intermediate *area opaca* before colonization by mesoderm, LM. Numbers correspond to area 3 to 4 in Fig. 4A and are continuous. In the intermediate area, extraembryonic endoderm cells (*EEC*) form a layer three to four cells deep beneath the epiblast (*ep*). The cells are irregular to elongate in shape and contain intracellular yolk platelets. Long cytoplasmic projections rest on extracellular deposits of material (arrows). ×670.
(D) Peripheral area opaca, before colonization by mesoderm, EM. Numbers correspond to areas 5 to 6 in Fig. 4A and are continuous. In the peripheral area, EEC lie in a loose multilayered array from one to four cells deep. Abundant free extracellular yolk (Y) is present between the cells. Cell-to-cell spacing is large and some cells (*) appear to be loosely suspended and to have no intercellular contacts with their neighbours. The cells are irregular in shape and contain intracellular yolk platelets. Long cytoplasmic extensions are directed ventrally from some cells (arrows). ×670.

(E) Ventral area opaca after colonization by mesoderm, EM. The cells adhere by adherens junctions (arrows) and have extended many microvillous projections (mp). The cells shown here contain Type B yolk (B). ×9090.

(F) Extrusion of Type B yolk. Type B yolk was fixed in the process of extrusion from an area opaca endoderm cell in situ. The yolk retains its overall shape during extrusion and shows traces of a faint, fuzzy material peripherally (arrows). ×11500.
Table 1. Effects of cell number on aggregate cavitation

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Cavitation</th>
<th>Number of cells in tissue</th>
<th>Number of aggregates</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>absent</td>
<td>244–23148</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>complete</td>
<td>16072–77473</td>
<td>4</td>
</tr>
<tr>
<td>48</td>
<td>absent</td>
<td>579–3101</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>incomplete</td>
<td>3101–6591</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>complete</td>
<td>7406–74363</td>
<td>11</td>
</tr>
</tbody>
</table>

Aggregates of differing sizes were created by increasing the shaker speed to 120 r.p.m. at 30 or 60 min. of rotation. After processing, the aggregates were serially sectioned and the central section of each aggregate was examined. The approximate number of cells in the tissue was calculated (volume of tissue ÷ volume of one cell (~ 7349 μm³)). At 24 h a total of 20 aggregates were examined while at 48 h a total of 40 aggregates were examined.

DISCUSSION

To understand how extraembryonic endoderm cells reorganize and transform a solid into a cavitated aggregate, studies on the changes in cellular morphology and intercellular relationships are required. It is also important to determine if the kinds of intercellular junctions present in aggregates resemble those occurring in the extraembryonic endoderm in situ.

Our studies show that in the primitive-streak-stage embryo, the cells of the extraembryonic endoderm located at the periphery of the area opaca display areas of intercellular contact that are sporadic and consist of areas of parallel membrane apposition. Cellular projections appear to penetrate into the substance of the yolk and cells also appear to form a loose network with extracellular yolk spheres. These observations suggest that the cells are loosely attached to one another.

Closer to the area pellucida, as the cells increase their areas of intercellular contact and become modified to begin absorbing and processing extracellular yolk, adherens junctions and desmosomes appear. It has been shown that later in development, immediately preceding their evolution into a single cell layer, the cells of the extraembryonic endoderm located near the sinus terminalis acquire a lobose shape and are joined by the above mentioned intercellular junctions (Mobbs & McMillan, 1979).

At the earliest stages of aggregation examined in this study, extraembryonic endoderm cells also appear to be loosely arranged and their intercellular contacts consist mainly of areas of parallel membrane apposition and interdigitation similar to those present in situ. Although some desmosomes and incipient desmosomes are present, these junctions become more frequent with further culture; adherens junctions also appear at later times. Our dissociation method results in a cell suspension containing 80% single cells. Because of this, we
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believe that the desmosomes and adherens junctions present in situ uncouple during the cell preparation procedure or that the majority of the cell groups that remain joined by them are retained on the nitex mesh and do not form part of the cell population used in these experiments. As cavitation progresses, the cellular arrangements and intercellular junctions that develop resemble those of the extraembryonic endoderm beneath the sinus terminalis (Mobbs & McMillan, 1979). The cavities in these aggregates continue to expand and the endoderm thins out in many regions to form a single cell layer (Milos et al. 1979).

The amount of contamination by mesodermal and ectodermal cells in aggregates of extraembryonic endoderm ranges from nil to slight. This assertion is based on examination of serial sections of hundreds of aggregates over the last five years. Ectodermal and mesodermal cells are easily distinguishable histologically from those of the extraembryonic endoderm. Aggregates of extraembryonic endoderm cells thus are able to cavitate in the absence of ectoderm and mesoderm. This suggests that in vivo, the presence of these tissues may not be necessary for the formation of the single cell layered endodermal epithelium surrounding the yolk. During the expansion of the area opaca over the yolk, the epiblast can be morphologically distinguished at the rim of the area opaca under the surface of the vitelline membrane, slightly in front of the extraembryonic endoderm (Chernoff & Overton, 1979) and it has been suggested that the epiblast may play an active role in endoderm expansion. This tissue association may, however, be only coincidental and the epiblast may not necessarily be involved in causing the endoderm to undergo epiboly and differentiate. This latter tissue has no association with the ectoderm in the differentiated yolk sac (Romanoff, 1960). The influence of the epiblast (New, 1959) and mesoderm (Bennett, 1973; Bremer, 1960), may thus be secondary. Perhaps they exert a stabilizing effect on the endodermal epithelium after the completion of epiboly. In fact, Augustine (1981) has suggested that the extraembryonic endoderm itself has a role to play in the expansion of the mesoderm. It is noteworthy that Holtfreter (1944) also obtained some fluid-filled cavities in large as opposed to small aggregates of uncoated endoderm cells from the amphibian gastrula, suggesting, perhaps, that cavitation in vitro may not be restricted to the endodermal tissue studied in this paper. Nevertheless, although a certain degree of determination has occurred in the chick extraembryonic endoderm at early stages of development a certain differentiative plasticity is still retained by this cell layer (Masui, 1981).

A number of mechanisms could account for cavitation in this system in vitro.

1) Localized cell death in the centre of the aggregates followed by the removal of cell debris as has been suggested for teratocarcinoma cell aggregates (Martin, Wiley & Damjanov, 1977). However except for an occasional necrotic cell, widespread cell death has never been observed in the numerous aggregates we have examined. 2) Secretion of a temporary extracellular matrix that could act as a scaffold for cell reorganization within the aggregates. A temporary mandril
of intracellular matrix material does appear to play a role in the development of the lumen in blood capillaries (Folkman & Haudenschild, 1980). Although with the techniques used in this work we have not been able to detect an extracellular matrix between endoderm cells this possibility cannot totally be excluded.

3) Secretion of a stiff outer layer of extracellular material to which the external cells in young solid aggregates are highly adhesive. If the external cells retain their initial adhesions to this layer and change from a round to an elongate shape, small cavities would begin to form in the aggregate which could then coalesce. Although this has been suggested for sea-urchin blastulation (Wolpert & Gustafson, 1961) this possibility does not seem likely in this system since no outer layer of extracellular material has been observed at the periphery of the aggregates.

4) Sealing of the aggregate at its periphery creating an independent compartment into which fluid of defined composition is pumped from the surrounding medium. The accumulation of fluid in developing cavities could passively push cells to the periphery of the aggregate. This occurs in the mammalian blastocyst during the time of appearance of tight junctions at its periphery (Ducibella & Anderson, 1979). Although this is an attractive possibility it is ruled out because we do not have evidence of the presence of tight junctions at the perimeter of the aggregates as they undergo cavitation.

5) Changes in surface topography of endoderm cells from a state in which adhesive sites are randomly distributed to a state in which these sites may be preferentially localized at particular membrane sites. Cells could then translocate within the aggregate using adhesive bonds of a temporary nature formed at distinct regions of the cell periphery using each other’s surfaces as temporary scaffolds. As a result, the inner and outer walls of cavitated aggregates might come to be composed of the non-adhesive regions of the cells’ surfaces. In the case of small aggregates the geometry of the tissue mass might be too small to permit this type of change of surface topography to occur. One of the biochemical components of extraembryonic endoderm cells that may play a role in this process is the β-galactoside-binding lectin present in these cells (Cook, Zalik, Milos & Scott, 1979). We have reported that lectin is released into the medium by extraembryonic endoderm cells and that increased levels of lectin in the medium are associated with decreased cellular adhesion (Milos & Zalik, 1982). We have suggested that controlled lectin release could render cells non-adhesive at discrete sites and promote cell rearrangement. Preliminary experiments indicate that lectin activity is also present in the fluid contained in the cavity. The lectin could be a component of the Type B yolk granules that are released by the cells during cavitation. At the present time, this latter possibility seems the most plausible. However more experiments are needed before a definite conclusion can be reached.

We did not observe a regular unit membrane surrounding the Type B yolk granules. This was the case regardless of the location of these granules inside the substance of the cytoplasm or peripherally as they were being released by the cells. Many other cellular lipid inclusions such as fat droplets of the epididimal
adipose tissue (Williamson, 1964) and triglyceride droplets of brown adipose tissue (Ahlabo & Barnard, 1974) do not appear to be surrounded by unit membranes. Type B yolk is thought to consist of a lipid core with a stabilizing protein and phospholipid cell layer (Holdsworth & Finean, 1972). This surface composition may enable this inclusion to diffuse into and through the cell membrane during its release. As mentioned previously we do not believe that the release of Type B yolk observed in the present study is a fixation artifact since it is not observed in young aggregates where this granule is located close to the cell membrane. It is possible that the frequent association of this type of yolk with the cell surface could be due to the fact that these granules once released maintain their integrity in the vesicular fluid.

The extent to which active cell migration contributes to cavitation is not known. It has previously been observed that under certain experimental conditions extraembryonic endoderm cells in vitro develop into cells with a wandering fibroblastic-like morphology, similar to the elongated shape observed in the present study (Milos & Zalik, 1981; Thomas, 1938). They also sort out in mixed aggregates formed by dissociated cells from whole primitive-streak stage embryos (Sanders & Zalik, 1976). In solid aggregates harvested at early stages of culture, adherens junctions and desmosomes are located at all levels within the aggregate. This also raises questions about the stability of these junctions in addition to questions about the mechanisms of cell translocation during cavitation. Adherens junctions are believed to be labile since they can form between dissimilar cell types during cell sorting in mixed aggregates (Armstrong, 1971; Overton, 1975). Desmosomes are generally thought to perform substantial adhesive roles, especially in tissues undergoing stretching such as certain epithelia (Bellairs, 1963; Lentz & Trinkaus, 1971; Overton, 1975; Trelstad, Hay & Revel, 1967). If desmosomes once formed, are stable, cells joined by them may translocate as groups. In fact, it has been suggested that there is a correlation between desmosome frequency and the relative positions that different cell types occupy in mixed aggregates after sorting (Overton, 1977). It is also possible that these junctions may dissociate and reform as cells change position within aggregates. In the present work desmosomes were observed in early aggregates and we have observed structures between cells that we interpret as desmosomes in the process of splitting. Borysenko & Revel (1973) have suggested that two classes of desmosomes may exist which are either ‘labile’ or ‘stable’. The internalization of both adherens junctions and desmosomes has been observed after cellular contacts have been disrupted (Kartenbeck, Schmid, Franke & Geiger, 1982; Overton, 1975). In addition, Allen & Potten (1975) have suggested that in the mammalian epidermis, entire desmosomes may be engulfed or vesicles containing hydrolytic enzymes may be released extracellularly to digest these junctions. It has also been suggested that recycling of these cell surface structures may occur as cells change locations during morphogenetic cell migrations (Kartenbeck et al. 1982).
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