Cell membrane regions in preimplantation mouse embryos

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SUMMARY

Cell membrane regions characterized by alkaline phosphatase activity are described in cleaving mouse embryos and early blastocysts. Enzyme activity is demonstrated by light and electron microscopy, from the late 4-cell stage onwards, on the cell surfaces between blastomeres but not on the outer surface of the embryo. Experiments with dissociated morulae show that this is probably not an artifact due to the retention of the enzyme reaction product between the blastomeres. With the electron microscope the activity is also demonstrated in crystalloid bodies within the cytoplasm. The localization and growth during cleavage of cell membrane regions with enzyme activity is interpreted as the result of new cell membrane formation and/or as a relation of the crystalloid bodies with the cell membrane through the cortical system of microtubules and filaments.

INTRODUCTION

Since the first demonstration by Mulnard (1955) of non-specific alkaline phosphatase activity (APA) in early mammalian embryos, several research groups have been attracted to the subject because the onset of the enzyme activity and its localization suggest a pattern of cell differentiation and morphogenesis (Dalcq, 1957; Sherman, 1972; Solter, Damjanov & Skreb, 1973; Mulnard, 1974; Izquierdo & Marticorena, 1975; Izquierdo & Ortiz, 1975; Izquierdo, 1977; Vorsbrodt, Konwinski, Solter & Kopolowski, 1977; Johnson, Calcarco & Siebert, 1977; Mulnard & Huygens, 1978).

Regarding the onset of APA, the information was controversial until a direct biochemical assay showed the sudden appearance of the enzyme activity in 8-cell mouse embryos. For further precision, individual embryos were tested by means of a cytochemical method and the enzyme activity was demonstrated in some late 4-cell embryos (Izquierdo & Marticorena, 1975). Observations on mouse, rat and hamster embryos, as well as on mouse embryos cultured in vitro, confirmed and extended our previous cytochemical results (Ishiyama & Izquierdo, 1977).

As far as the localization of APA in morulae and blastocysts is concerned

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reports are at variance on whether inner and outer cells stain differentially. These contradictory observations have been ascribed to artifacts caused either by unreliable cytochemical methods or by the unequal thickenss of the embryos, when examined in whole mounts (Wilson, Bolton & Cuttler, 1972; Solter et al. 1973). However, recent reports on APA localization are largely in agreement. Electron microscope observations of mouse morulae and blastocyst stained by various modifications of the Gomori–Takamatsu method show that APA is confined to the cell surface between blastomeres and to crystalloid bodies found in the cytoplasm, and is detected neither at the outer surface of the embryo nor at the lining of the blastocoel (Izquierdo, 1977; Mulnard & Huygens, 1978). Light microscope observations on mouse embryos stained by an azo-dye cytochemical method show a somewhat similar distribution of APA (Johnson et al. 1977). The disparate results of Vorbrodt et al. (1977) have been attributed to unspecific staining produced by long incubation at a relatively low pH (Mulnard & Huygens, 1978).

In this paper we deal with the possibility of an artifact due to the retention of the enzyme reaction product between the blastomeres, then we describe the regionalization of the cell membrane as revealed by APA and finally we discuss the mechanisms that might be involved. Our results support the idea that during

Figures 1–8

Fig. 1. Whole mount of an early 4-cell stage (42 h of development). No APA is recognized.

Fig. 2. Whole mount of an 8-cell stage (57 h of development). APA is observed between blastomeres.

Fig. 3. Whole mount of two morulae of about 10 cells (60 h of development). Embryo at the left side reveals APA, embryo at the right side is a control incubated without substrate.

Fig. 4. Whole mount of a pair of blastomeres isolated from a morula of about 10 cells (60 h of development) and immediately processed for APA demonstration. APA patches are observed between blastomeres and on the surfaces exposed by dissociation (arrows).

Figs. 5 and 6. A whole mount of one pair of blastomeres isolated from a morula of about 12 blastomeres (61 h of development) photographed in two positions. After their isolation from the morula the pair was cultured in vitro for 17 h before fixation and APA demonstration. An APA patch is shown between the blastomeres, but no other patches are observed on the surface.

Fig. 7. Whole mount of a morula of about 16 cells (64 h of development), halved in two parts and cultured in vitro for 15 h before fixation and APA demonstration. The part represented shows APA between the compacted blastomeres and a patch on the surface exposed by halving (arrows).

Fig. 8. Whole mount of a 2-cell stage (38 h of development) fused to a morula of about 16 cells (62 h of development) and cultured in vitro for 5 h before fixation and APA demonstration. No APA is observed in the 2-cell stage nor in its contact with the morula. The morula shows the typical localization of APA at this stage.
cleavage an intrinsic spatial pattern gradually unfolds, providing cells with information about their position in the embryo (Izquierdo, 1977).

MATERIALS AND METHODS

Preimplantation embryos were obtained from natural matings of Swiss-Rockefeller mice. Their stage is indicated in number of cells or in hours of development, reckoned since the beginning of the day when the vaginal plug is found.

The embryos recovered from the oviduct were either immediately processed for APA demonstration or they were first treated so as to dissociate blastomeres or associate embryos. To this end, eggs were denuded of the zona pellucida by pipetting them in 5% Pronase (Calbiochem) in Biggers's culture medium (Biggers, Whitten & Whittingham, 1971) devoid of albumin, for 4 min at room temperature; the embryos were then rinsed in Biggers's medium and manipulated under mineral oil with fluid jets produced by a pipette and with the help of gentle touches. Thereafter they were either fixed, or cultured in vitro before fixation. Fixation lasted for 60 min in cold 3% glutaraldehyde in 0.05 M cacodylate buffer at pH 7.4. After fixation, embryos were rinsed in buffer for 30 min and incubated for 90 min at room temperature in an alkaline lead citrate medium at pH 9.5 (Mayahara, Hirano, Saito & Ogawa, 1967). Best results were obtained when the medium was buffered with tris-maleic acid and stabilized with 8% glucose (Sundström & Mörnstadt, 1975).

For light microscopy, after incubation the embryos were rinsed in buffer and the enzyme reaction product was developed with ammonium sulphide. The embryos were then immersed in glycerol and whole mounts observed under oblique illumination so as to cast shadows (Hlinka & Sanders, 1970) which clearly reveal the contours of the nuclei and blastomeres, though this illumination somewhat hinders an accurate recognition of APA localization on black and white photographs.

For electron microscopy, embryos were rinsed in buffer (pH 7.4) and post-fixed for 60 min in 1% OsO₄ in cacodylate buffer; then they were dehydrated in acetone and embedded in low-viscosity resin (Spurr, Polyscience). Thin sections

FIGURES 9–14

Figs. 9–11. Morulae of 8 cells (60 h of development). The reaction product accumulates on the outer surface of the plasma membrane at the edge of the cleavage furrows.

Figs. 12 and 13. Morula of 8 cells (60 h of development). Discrete patches of APA on the surface of adjoining cells. Fig. 12 shows that the perivitelline space (pvs) is continuous with the interblastomeric cleft.

Fig. 14. Morula of about 16 cells (65 h of development). Extended APA patches on the cell membrane between blastomeres. APA is also detected in crystalloid bodies (cb) within the cytoplasm.
were stained with uranyl acetate and observed in a Philips EM 300 electron microscope.

Controls were performed on embryos whose enzyme was heat-denatured after fixation and on embryos which were incubated in a substrate-free medium, keeping all other steps of the procedure unchanged. Out of a total number of approximately 300 embryos studied, 45 were observed with the electron microscope.

RESULTS

Light microscopy of whole embryos and electron microscopy of thin sections confirm previous observations in that no APA is detected prior to the late 4-cell stage (see Introduction). The results concerning the localization of APA in later stages of development will be summarized in this order: light microscope observations on intact embryos and on manipulated embryos, and observations with the electron microscope.

**Light microscopy.** From the late 4-cell stage onwards (Fig. 1) while APA is neatly observed on the cell surface between blastomeres, no activity is detected on the outer surface of the embryos nor is it possible to affirm that there is activity on cytoplasmic structure (Figs. 2, 3). If embryos are heated after fixation or incubated in a substrate-free medium, no traces of APA are detected (Fig. 3).

When pairs of blastomeres are dissociated from a morula, processed for APA demonstration and observed in whole mounts, interblastomeric patches are evident and APA patches can also be recognized on the cell surface that has become outer surface by virtue of the dissociation procedure (Fig. 4). If the dissociated pairs are cultured overnight before APA demonstration, the outer patches are not always evident (Figs. 5, 6); actually, they are more clearly seen if morulae were dissociated into portions with several blastomeres each (Fig. 7). The variability of these results and the limited number of observations do not allow a systematic description, but they support the reality of outer APA patches on the cell surface exposed by dissociation.

**Figures 15-19**

Fig. 15. Morula of about 16 cells (65 h of development). Discrete APA patches observed on the cell surface between three blastomeres.

Fig. 16. Morula of about 16 cells (65 h of development). Typical crystalloid bodies in the cytoplasm showing APA.

Fig. 17. Advanced morula (80 h of development). A wide interblastomeric space shows APA on both cell surfaces.

Fig. 18. Advanced morula (80 h of development). The nucleolus (nu) and cytoplasmic organelles do not show APA. The activity is found on the cell surfaces.

Fig. 19. Advanced morula (80 h of development). In a wide interblastomeric space (arrow) no APA is shown on the cell membranes facing each other.
Figs. 20 and 21. Advanced morulae (80 h of development). APA is detected on the surface of blastomeres and in crystalloid bodies (cb). No APA is detected in the nucleolus (nu) nor in mitochondria nor other cytoplasmic organelles, except crystalloid bodies.
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By fusing synchronous or asynchronous morulae in pairs we tried to convert a part of their outer surface, which never reveals APA, into interblastomeric surface, which always does. After 5 h in culture the fused embryos were processed for APA demonstration and observed in whole mounts. Under these conditions the artificial interblastomeric surfaces do not reveal any APA (Fig. 8).

Electron microscopy. In early morulae, APA is localized on the cell membrane both at the advancing edge of the cleavage furrows (Figs. 9-11) and in discrete patches on adjoining blastomers (Figs. 12, 13). In late morulae and blastocysts, APA patches have spread out, and comprise most of the cell membrane bordering the interblastomeric clefts (Figs. 18, 20-25).

Although the cytochemical procedure interferes with a clear definition of the cell membrane, the enzyme reaction product appears to be lumped on the outer surface while the inner surface shows a smooth profile. Narrow interblastomeric spaces usually appear full of lead phosphate, but when the spaces are sufficiently wide, the reaction product may be detected on both cell membranes or on neither of them, as if cell membranes facing each other were symmetrical regarding their APA localization (Figs. 17-19).

In all developmental stages studied, the cell membranes around the embryo appear to be devoid of APA. The transition from the outer inactive region to the active region between blastomeres is quite abrupt, even in early morulae when apical tight junctions are still absent (Fig. 12). The cell surface bordering the blastocoelic cavity lacks APA, and as a consequence APA patches are exhibited exclusively between trophectoderm cells (Fig. 23). Lead phosphate grains are usually found scattered in the blastocoel associated with cellular debris which do not reveal clear-cut membrane structures.

The membrane of the many diverse cytoplasmic organelles does not show APA, and this is also the case for non-membranous structures except for crystalloid bodies, which are known to arise during cleavage in mammalian embryos of several species (see Calarco & Brown, 1969; Van Blerkom, Manes & Daniel, 1973). In the mouse embryo, crystalloid bodies appear as structures of approximately 0.5 μm in diameter that reveal a substructure periodicity of 120-130 nm in the tightly packed crystalline form. These structures may also be recognized in diverse transitional forms, from typical crystalloid bodies to a network of loosely associated cross-striated microtubules or filaments.

Crystalloid bodies may show a strong APA (Figs. 14, 16, 21) or no activity at all (Fig. 23), even at the same stage of development; the less structured forms do not show APA. We have not observed an association of crystalloid bodies with the APA patches nor with any other part of the cell membrane.
DISCUSSION

Mulnard & Huygens (1978) analysed several possible causes of false localization of APA in preimplantation embryos but they did not consider an eventual artifact caused by the retention of the enzyme reaction product in the narrow clefts between blastomeres while it freely diffuses out from the surface around the embryo and from the surface bordering the blastocoel. In fact, lead phosphate is insoluble in our experimental conditions; however, this artifact would closely mimic the APA distribution we describe in intact embryos. The presence of APA patches on the new outer surface produced by dissociating morulae and the absence of APA patches on the new interblastomeric surface produced by fusing embryos is inconsistent with an artifact due to differential retention of lead phosphate, though further investigation may be required to dismiss it entirely. On these bases we submit that the pattern of APA distribution in preimplantation embryos reveals a true regionalization of the cell membrane that deserves to be discussed.

Considering first, that a large supply of cell membrane is needed during cleavage; second, that APA is detected on the cell membrane (Izquierdo, 1977; Mulnard & Huygens, 1978; this paper); third, that the activity only arises at the late 4-cell stage (Izquierdo & Marticorena, 1975; Ishiyama & Izquierdo, 1977) one would expect to recognize ‘new’ cell membrane if it is added in discrete lots to the cell surface (Izquierdo, 1977). Indeed, our observations suggest that ‘new’ membrane is formed at the leading edge of the cleavage furrow, leaving symmetrical patches between blastomeres when their division is completed. As a consequence, when development proceeds the patches of ‘new’ cell membrane cover most of the cell surface, leaving only the outer surface of the peripheral blastomeres wrapped in ‘old’ cell membrane.

This interpretation suggests that ‘new’ membrane is formed at the edge of the furrow, either by the local assembly of components or by the fusion there of preassembled components (see Doyle & Bauman, 1979). Vesicles associating...
with the cleavage furrow have been described in embryos of a species of poly-
chaete (Emanuelsson, 1974) and in the embryo of *Xenopus* (Sanders & Singal,
1975) but in our material we have not observed APA in the cytoplasm near the
place where cell membrane is supposedly formed. Therefore, in order to uphold
this interpretation we would be led to assume that alkaline phosphatase is
inactive until it reaches the outer surface of the cell membrane or that its
activity requires the kind of steric organization present at the cell membrane
and in crystalloid bodies.

The interpretation based on the formation of ‘new’ cell membrane seems to
be inconsistent with the absence of APA on the blastocoel surface. Mulnard &
Huygens (1978) suggest that the enzyme there might be lost as a result of
‘desquamation’ of the cell membrane, which would explain why they detect
APA on cellular debris in the blastocoel. Although we have also observed lead
phosphate grains in the blastocoel, we have been unable to identify membranous
debris and therefore we would regard as a possibility that the lining of the blasto-
coel derives from remnants of ‘old’ cell membranes present between blastomeres.

Up to now we have disregarded any relation between APA patches and
 crystalloid bodies, though these are the only two localizations of APA, because
they are spatially apart; however, crystalloid bodies may well be stores of
cortical material, indirectly related to the cell membrane through the system of
microtubules and filaments. This becomes a distinct possibility when one con-
siders: first, that the treatment of mouse cleaving embryos with Cytochalasin B
promotes the deposition of crystalloid bodies (Moskalewski, Sawicki, Gabara
& Koprowski, 1972; Perry & Snow, 1975); second, that crystalloid bodies in
their highly structured form look like packages of filaments or microtubules
(Daniel & Kennedy, 1978); and third, that cleaving mouse embryos synthesize
actin and tubulin at a very high rate (Abreu & Brinstner, 1978).

Now, microtubules and filaments may be involved in several ways in the
regionalization of the cell membrane. If APA patches turn out to be lots of
‘new’ membrane, microtubules and filaments would be required to prevent
alkaline phosphatase from diffusing in the plane of the fluid membrane;
moreover, the enzyme molecules on the surface might correspond to the ends of
microtubules or filaments inserted in the membrane.

However, a good morphological correlation of APA patches with cleavage
furrows and the interblastomeric surfaces does not exclude the possibility that
patches are formed by entirely different mechanisms, such as those responsible
for lymphocyte capping (Edelman, 1976). Actually, since the forces shaping the
embryo are exerted by the cortical complex of microtubules and filaments, their
local concentration at the cleavage furrows and at the inner side of blastomeres
during compaction might account for the distribution of APA patches. A
disturbance of such cortical pattern, when morulae are dissociated and cultured,
may explain the somewhat inconstant and variable distribution of APA patches
in the artificially produced outer surfaces.
The morphogenetic effect of a cell membrane regionalization during mammalian cleavage has been discussed elsewhere (Izquierdo, 1977); we should only emphasize here that a decisive morphogenetic step leading from morulae to blastocysts is the establishment of zonular tight junctions (Ducibella, Albertini, Anderson & Biggers, 1975; Izquierdo, Fernandez & Lopez, 1976), and our results suggest that these are formed around the peripheral cells of the embryo, precisely where the cell membrane region, which exhibits APA, meets the region which does not.

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REFERENCES


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