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ALKALINE PHOSPHATASE ACTIVITY IN THE PREIMPLANTATION MOUSE EMBRYO

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SUMMARY

Alkaline phosphatase (AP) activity has been assayed in frozen sections of preimplantation mouse embryos by an azo dye cytochemical method. The results indicate that during preimplantation mouse development AP activity is first expressed between the 8- and 16-cell stages and develops in all cells by the late morula stage. During blastocyst formation AP activity is lost or greatly reduced in trophoblast cells while activity is maintained in the inner cell mass.

Introduction

Mouse embryos develop from a single cell zygote into a blastocyst ready to implant in the uterine wall during a preimplantation period of 4-5 days. Blastocyst formation occurs between the 32- and 64-cell stages and involves the differentiation of the outer embryonic cells into a single layer of cells (trophoblast) which encloses the fluid-filled blastocoel and an inner cell mass (ICM). These two cell populations demonstrate different developmental pathways the trophoblast cells forming the ectoplacental cone and giant cells, and the ICM forming the embryo proper subsequent to implantation. Differences in cell surface morphology [1], intercellular junctions [2] and protein composition [3] between ICM and trophoblast have been described. However, with the exception of the acid and alkaline phosphatases [4, 5], little information is available concerning possible biochemical differences between these 2 cell populations at the blastocyst stage.

Since the initial report of alkaline phosphatase (AP) activity in the preimplantation mammalian embryo [4], the point in early development at which this enzyme activity first appears and its intraembryonic localization have been the subjects of some controversy. Mulnard [4], assaying whole-mounts of rat and mouse embryos by the Gomori cytochemical technique [6], reported the earliest appearance of AP activity in the central cells of 16-cell embryos. At the blastocyst stage, cells of the ICM were reported to be positive for AP activity while those of the trophoblast were negative [4]. Izquierdo [7] and Ortiz et al. [8] have described similar results with Gomori-assayed AP activity appearing in the central region of whole mouse embryos as early as the 12- to 16-cell stage and in the ICM but not the trophoblast of blastocysts. A more

recent study by Izquierdo and Ortiz [9] confirmed the first appearance of AP activity assayed by the Gomori technique in 17-cell stage mouse embryos and demonstrated similar AP activity in embryos developed *in vivo* or *in vitro*.

Rodé et al. [10] and Solter et al. [11], utilizing both Gomori and azo dye [12] techniques, have described the appearance of AP activity in late 4- to 8-cell embryos and increasing activity through the blastocyst stage. However, in contrast to previous results these investigators detected no differences in the intraembryonic distribution of reaction product between inner and outer cells of any stage of rat or mouse preimplantation development. Izquierdo and Marticorena [13] were also unable to identify regional differences of activity within preimplantation embryos using an azo dye technique and suggested that differences in the phosphatases identified by the cytochemical techniques of Gomori and Burstone could be responsible for the varying results. These authors feel that the experimental observations might be reconciled by assuming that the technique of Burstone demonstrates all phosphatases while the Gomori technique identifies only phosphatase activity attached to certain cytomembranes absent from the trophoblast and that a decrease in such membrane-bound activity may be involved in the gradual differentiation of trophoblast cells.

Biochemical assay of whole mouse embryos developed *in vivo* [13] has detected initial AP activity at the 8-cell stage, an increase to a maximum at 8- to 16-cell stages and a gradual decline subsequently. Sherman [14] has reported that *in vivo* - developed mouse blastocysts do not produce any sign of AP activity on electrophoretic gels, while blastocysts cultured in outgrowth medium for 4 or 7 days have an electrophoretic band of AP activity with a mobility similar to that of

postimplantation trophoblast. Electrophoretic assay of trophoblast on the tenth day of gestation demonstrated 2 distinct forms of AP activity while embryo homogenates contained only 1, the slower moving form [14].

Previous cytochemical investigations of the distribution of AP activity within preimplantation mouse embryos have been conducted on whole-mount embryos. It has been argued [10, 11] that the higher activity levels reported for inner cells may be due to cell overlap in the center of whole-mounts and/or other artifacts. In order to circumvent these difficulties this study utilizes frozen sections of mouse embryos to examine the development of AP activity during the preimplantation period.

Materials and Methods

Preimplantation mouse embryos (ICR strain) were flushed from the oviducts of superovulated pregnant females at the 2-cell stage and cultured in a modified embryo culture medium [15]. Embryos of 2-cell, 8-cell, early morula (compacted, 8 - 16 cells), late morulae (16 - 32 cells), and blastocyst stages were assayed for AP activity by an azo dye technique [12]. Primary oocytes obtained by puncturing ovarian follicles, unfertilized eggs from superovulated unmated females and in vivo-developed late morulae and blastocysts were similarly assayed. An in vitro blastocyst outgrowth and an ICM isolated by immunosurgery of a mouse blastocyst [16] were also assayed for AP activity.

Samples of 20 to 30 embryos of a given developmental stage were placed within an ant pupa case, fixed with 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.1) for 10 minutes and briefly rinsed in phosphate buffered saline (pH 7.1 - 7.2). In addition, a group of blastocysts was fixed for 1 hour to analyze the effects of increased fixation. The pupa

case was frozen by a 5 - 15 second immersion in liquid nitrogen-cooled 2-methyl butane (Matheson, Coleman and Bell) and subsequent immersion in liquid nitrogen for 1 - 3 minutes. Sections of approximately 8 μ were cut on an International cryostat and mounted on coverslips previously subbed with a solution of 0.5% gelatin and 0.05% chromium potassium sulfate [17].

A simultaneous coupling azo dye technique [12] was utilized for the cytochemical demonstration of AP activity because of its precise localization of final reaction product and applicability to frozen sections [18]. The AP reaction medium consisted of 0.08% Fast Blue RR and 0.02% Na-α naphthyl acid phosphate in 0.2M Tris-HCl buffer (pH 9.4). Control medium contained no substrate. Following Millipore filtration (.22μm) drops of experimental or control medium were placed over tissue sections and incubated at 37°C for 15 minutes. To determine the effects of extended incubation times, blastocysts and late morulae were also incubated in reaction medium for periods of 30 minutes, 1, 2, and 4 hours. Coverslips were then rinsed in phosphate buffered saline, mounted on microscope slides with a glycerol-water (1:1) solution, observed under bright-field microscopy and photographed.

Results

All preimplantation stages prior to the early morula, including primary oocytes and unfertilized eggs, were negative for AP activity. Reaction product, which appeared as a finely granular, grey-black deposit, was first identified in some cells of early morulae (Fig. 1a). At the late morula stage reaction product was apparent in most cells and was more dense than that in early morulae (Figs. 1b, 1c). Central cells did not show a more positive AP reaction than peripheral cells in either

of the 2 morula stages assayed. In blastocysts, however, there developed a marked difference in AP activity between the ICM and the trophoblast. Cells of the ICM and polar trophoblast (those overlying the ICM) were strongly positive in their reaction while cells of the mural trophoblast (those surrounding the blastocoel) appeared negative or at least much less positive (Figs. 1d, 1e). Distribution of reaction product in late morulae and blastocysts developing *in vivo* was similar to that observed in the *in vitro* samples. In all cases, reaction product was more dense over the cell periphery than over the cytoplasm. This is consistent with the observation that in most cell types the majority of AP activity is localized on the plasma membrane with areas of lesser activity existing at intracellular sites such as the nucleus and the Golgi complex [19]. The embryonic portion of the blastocyst outgrowth and the isolated ICM also demonstrated a positive reaction for AP activity. Control sections consistently showed no evidence of reaction product (Fig. 1f).

Extended incubation times in AP reaction medium (up to 4 hours) produced a slight increase in density of reaction product but no differences in its intraembryonic distribution in late morula and blastocyst stages. Blastocysts undergoing extended fixation (1 hour) also displayed an increased density of reaction product throughout the embryo but a marked difference in intensity between ICM and trophoblast was still readily apparent.

Discussion

This study demonstrates by an azo dye technique a higher level of alkaline phosphatase (AP) activity in the ICM than in the trophoblast of sectioned preimplantation mouse embryos whether they have developed *in*

vivo or in vitro. This observation supports previous reports [4, 7, 8] of such an intraembryonic distribution of AP activity as assayed in blastocyst whole-mounts by the Gomori cytochemical technique. Since this study utilizes sectioned embryos it is unlikely that cell overlap is responsible for the more intense staining of ICM than trophoblast as has been suggested for those studies utilizing whole-mounts [10, 11]. However, the demonstration of ICM-trophoblast differences in AP activity by an azo dye technique is in conflict with the results of previous investigations utilizing similar techniques in which no such differences were evident [10, 11, 13]. The basic for this conflict in results is unclear.

The results of this study do not, however, reveal intraembryonic differences in the distribution of AP activity at the morula stage as reported previously [4, 10, 11]. Rather, the present study supports the contentions of Solter et al. [11] that AP activity is equally distributed in all cells of morulae, and that observations of more pronounced activity in inner cells of whole-mounts may in fact be due to cell overlap. In early morulae it is clear that not all blastomeres are positive for AP activity (Fig. 1a) but no regular aggregations of positively-reacting cells in the central region of morulae are apparent. Since almost all cells in the late morulae are positive for AP activity (Figs. 1b, 1c) it is possible that negatively-reacting cells of early morulae have not yet initiated synthesis or activation of AP activity.

During the process of blastocyst formation, when the morphological differentiation of ICM and trophoblast takes place, AP activity is apparently lost or greatly reduced specifically in the cells of the mural trophoblast (Figs. 1d, 1e). Izquierdo & Marticorena [13] have suggested that such a decrease may be indicative of trophoblast differentiation.

It has been a common observation that elevated levels of AP activity present in all 3 primary germ layers are reduced during the process of differentiation [20]. During the growth of teratocarcinomas, a system with some analogies to mouse development, the differentiation of pluripotent stem cells (embryonal carcinoma cells) also appears to be accompanied by a decline in AP activity [21, 22, 23]. However, the observations of relatively high levels of AP activity in trophoblast derivatives of early postimplantation embryos [10, 14] suggest that the observed decrease in activity is only temporary. Alternatively, the reduction in alkaline phosphatase activity in trophoblast might be explained by the expression of a less reactive form of the enzyme due to _de novo_ synthesis or activation of a preexisting protein. Sherman [13] has described the existence on electrophoretic gels of a region of AP activity which is present in trophoblast but not embryonic tissues as early as day 10 of gestation in the mouse. A change in the molecular form of AP has been reported to occur at the time of gastrulation in sea urchin embryos [24] and changes in isozymic forms of AP have also been observed during the development of embryonic chick limb [25] and in the functional development of postnatal mouse intestinal epithelium [26].

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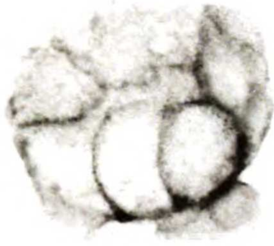
Figure Captions

Figure 1. Unstained 8μ frozen sections of preimplantation mouse embryos with the cytochemical identification of alkaline phosphatase activity appearing as a dark precipitate. (a) early morula; (b) and (c) late morulae; (d) and (e) blastocysts; (f) control blastocyst. (All figures X450.)



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