Detection of Maternal Esterase in Mouse Embryonic Tissues
(trophoblast/yolk sac/placental barrier)

MICHAEL I. SHERMAN* AND NANCY J. CHEW*

Department of Zoology, University of Oxford, England; and Department of Cell Biology, Roche Institute of Molecular Biology, Nutley, New Jersey 07110

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ABSTRACT A genetic variant of esterase in mice is used to demonstrate for the first time that an enzyme of maternal origin is taken up intracellularly by two mid-gestation tissues derived from the embryo: trophoblast and yolk sac (visceral endoderm). Although other alternatives are still possible, it is likely that the esterase is transported from the mother to the trophoblast via the serum. If so, the phenomenon is selective, since at least one other prominent serum esterase is not found in trophoblast or yolk sac cells. The enzyme has not been detected in the embryo proper, even at very late stages of pregnancy.

Trophoblast cells developing from blastocysts implanted under the kidney capsule of a male mouse also appear to have the ability to take up host esterase. Efforts to demonstrate uptake of the enzyme by trophoblast cells in blastocyst cultures have been unsuccessful.

A mammalian embryo develops in the reproductive tract, and as such is constantly exposed to, and protected by, the maternal environment. Preimplantation embryos removed from the reproductive tract and placed in ectopic sites can implant in the foreign tissue and proceed to develop normally for several days (1–4). Blastocysts maintained in culture medium are able to give rise to some cell types that are at least partially differentiated: trophoblast (5–7), yolk sac (7, 8), and part of the embryo proper (8). It would thus appear that signals for early differentiation need not come from the mother. There is, however, a great advantage to be gained from development in the reproductive tract, namely the selective passage of molecules and macromolecules from mother to fetus. By this phenomenon, for example, the fetus is provided with maternal antibodies that protect it after birth until its own immune system is functional (9). Studies on the passage in mice of injected serum proteins from the maternal bloodstream into embryonic tissues late in pregnancy have revealed that some fractions (e.g., human IgG) can cross the placental barrier more easily than others (e.g., human albumin) (10, 11). The studies described here are the first to illustrate that an enzyme naturally present in the maternal blood penetrates the embryonic tissues of the placenta in the first half of gestation.

METHODS AND MATERIALS

The following strains of mice were used in this study: Swiss PO, C57Bl/6, SWR/J, SJL/J, C57Bl/6J, and C57Bl/6J x DBA/2J hybrids. The latter four strains were obtained from the Jackson Laboratory, Bar Harbor, Me. Mice were randomly mated when postimplantation embryos were required. Preimplantation blastocysts were collected from females superovulated (12) before mating. The day on which vaginal plugs, indicating successful matings, were detected is referred to as the first day of pregnancy. The following tissues were dissected (6) on the 10th or 11th days of gestation: the outer or trophoblast layer of the fetal placenta, containing mainly trophoblast cells, but also Reichert's membrane, parietal endoderm, and, at later stages, part of the allantois; the visceral endoderm layer of the placenta, referred to here as the yolk sac; the embryo proper, surrounded by the amnion; and the decidua, or the maternal moiety of the placenta, which surrounds the trophoblast. The tissues were dissected in phosphate-buffered saline (pH 7.0; GIBCO, New York). Pooled tissues were frozen in PBS at −70°. After thawing, they were washed twice in PBS by centrifugation, resuspended in PBS, homogenized with a Teflon pestle homogenizer, and centrifuged at 2,000 rpm (1120 × g) for 5 min to remove debris. When tissues from individual conceptuses were being analyzed, they were washed twice with PBS, resuspended in water, and then disrupted by two cycles of freeze-thawing (13). Unless otherwise indicated, these preparations were mixed with ethylene glycol and bromphenol blue (7), and subjected directly to electrophoresis.

Blastocysts used for embryo transfers (22), ectopic pregnancies (2, 3), and cultures were collected from superovulated females on the fourth day of gestation. They were flushed from uterine horns in PBS, placed in Eagle's medium (14) supplemented with 10% heat-inactivated fetal-calf serum (60° for 15 min, GIBCO, New York), and incubated at 37° in an atmosphere of 5% CO₂ in air until transfer. Foster mothers were mated with sterile males 4 days before blastocyst transfer. 10 Blastocysts in a small drop of culture medium were injected into each uterine horn of these females midway through the third day of pseudopregnancy. 8 Days later, successful transfers were removed, dissected, washed, and disrupted by freeze-thawing according to the protocol described above. Blastocysts were implanted in pairs under kidney capsules of adult males. After 8 days in situ, successful ectopic growths were apparent as large, often very hemorrhagic, masses of tissue. These were carefully dissected free of kidney tissue and were individually washed with PBS and disrupted by freeze-thawing. For culture experiments, blastocysts were placed in small petri dishes containing Eagle's medium plus 10% heat-inactivated fetal-calf serum (7). Where indicated, the medium was also made 10% with respect to mouse serum. Mouse serum was obtained by centrifugation of whole blood collected from the inferior vena cava. Cultures were washed.

Abbreviation: PBS, phosphate-buffered saline, pH 7.0.

* Present address: Roche Institute of Molecular Biology, Nutley, N.J.
Fig. 1. Esterase electrophoretic profiles in midgestation decidua. Crude homogenates of decidual tissue from a pregnant Swiss PO (slot 1) or C57Bl/6 (slot 3) mouse were electrophoresed through a 4.5% polyacrylamide spacer gel and an 8% separating gel in Tris-borate buffer (pH 7.2), and stained for acetyl esterase activity (7). In slot 2, the homogenates were mixed and electrophoresed together. The different regions of esterase activity are identified alphabetically (7) from the cathode (top) to the anode (bottom). The drawing beside the photo is a representation of the slow and fast bands in the F region of the gel.

twice with PBS, dislodged from the dish with a rubber policeman, and washed again by centrifugation. The cells were then resuspended in water and disrupted by freeze–thawing.

Slab acrylamide gel electrophoresis and staining of the gels for acetyl esterase activity were as described (7).

RESULTS

Typical esterase profiles from homogenates of decidual tissue obtained late on the 11th day of pregnancy are illustrated in Fig. 1. Slot 1 shows the profile obtained from a Swiss PO female, while the slot 3 profile is characteristic of the C57Bl/6 strain. The profiles are the same except in the F region; the C57Bl/6 isozyme (F_{fast}) is anodal to the PO species (F_{slow}). A mixture of the two homogenates (Fig. 1, slot 2) shows that the F isozymes can be separated by this method. Deciduas from SWR/J, SJL/J, and DBA/2J mice all possessed the F_{slow} species. Insofar as the kidney esterase isozyme denoted E_3 by Popp and Popp (15) and Ruddle et al. (16) migrates identically with the F isozyme (7), and shows the same strain-specific variation (Sherman, M. I., unpublished observations), it is assumed that the F isozyme is in fact E_3. The F_{fast} and F_{slow} bands would then be equivalent to E_{1a} and E_{1b}, respectively. Mapping studies are required to definitively establish the identity of E_3 and F isozyme. F isozyme has been detected in trophoblast on the 8th–12th days of gestation, and in late 10th–12th-day yolk sac. The concentration in the yolk sac appears to be substantially less than that in trophoblast or decidua (7).

Because the decidua is formed by division of endometrial cells, it would be expected to possess only maternal esterases. Therefore, if a Swiss female were mated with a C57Bl/6 male, the F-region esterase in the decidua would be expected to be only F_{slow}. Conversely, in a C57Bl/6 female x Swiss male cross, the decidua should contain F_{fast} esterase. Only if the female were hybrid would one predict the presence of both F isozymes. Fig. 2 illustrates that these expectations are realized. Intermediate hybrid bands are not seen with F isozyme, in agreement with observations with E_3 (15, 16).

Because the trophoblast layer of the placenta is of embryonic origin, the above hybrid matings would be expected to produce a mixed profile of F isozymes in that tissue. This is not observed; Fig. 3 illustrates that the resultant profiles of the F region are identical to those obtained with decidual homogenates, i.e., only the maternal F esterase is observed, regardless of the cross. There could be several explanations for this observation. The following three were considered most likely:

(a) the trophoblast layer has been incompletely dissected from the decidua, and the esterase activity observed in the F region arises from contaminating decidual cells;
(b) the paternal F isozyme is not synthesized in trophoblast tissue at the gestation stages under observation; or
(c) the maternal enzyme is passing from the mother to the fetal placenta.

The first of these possibilities cannot completely explain the observed phenomenon, since the esterase activity in the F region of the yolk sac follows the same maternal pattern as trophoblast and decidua (Fig. 4). The yolk sac is easily separated from other tissues, and, at the gestation stages under study, is prevented from direct contact with maternal tissues by the contiguous layer of trophoblast. Furthermore, experiments reported elsewhere have demonstrated that contamination of the trophoblast preparation with decidual cells is minimal (7, 13).

To distinguish between possibilities (b) and (c), blastocysts were flushed from the reproductive tract of a female on the fourth day of gestation and transferred to a foster mother that differed genetically at the F esterase locus. Fig. 5 demonstrates that when a C57Bl/6 blastocyst is transferred to a Swiss foster mother, the F isozyme is of the foster mother's
yolk sac. In the reciprocal experiment (not shown), the maternal phenotype was again observed. Possibility (b) is, therefore, eliminated. It appears that in the first half of embryonic development, F esterase in the embryonic layers of the placenta is maternal in origin. The enzyme is prevented, however, from reaching the embryo proper (Fig. 5), even at later stages in gestation (7).

To determine whether the F esterase penetrates trophoblast and yolk sac cells, or whether it has an extracellular location, dissected tissues were washed several times with PBS. After each wash, the tissue was pelleted, and a fraction of it was homogenized and analyzed electrophoretically (Fig. 6). Although some F esterase activity is lost with each wash, the band in the F region can still be seen after five washes (Fig. 6, slots 1-5). After a sixth wash, the remaining tissue was homogenized and mitochondria, nuclei, and cell debris were pelleted by centrifugation. The bulk of activity is in the soluble fraction after this fractionation (Fig. 6, slots 6 and 7); therefore, the enzyme is not being adsorbed to the outside of the cell. Similar observations were made when yolk sac and decidua were treated in the same way.

Experiments were done to determine whether trophoblast cultured outside the uterus possessed the ability to take up maternal esterase. Fourth-day blastocysts were placed under a kidney capsule of an adult male that differed from the transplant in its F esterase profile. After 8 days of incubation, the hemorrhagic ectopic growths, containing giant trophoblast-like cells, were removed and freed of adhering kidney tissue. Those growths containing relatively low levels of contamination with blood were analyzed electrophoretically (Fig. 7). As was observed with growths under the testis capsule (7), all have activity in the F region, although one of the growths (Fig. 7, slot 4) showed much less activity than the others. Furthermore, the F isozyme in all four cases is that of the host, rather than the implant. None of the implants exhibits more than a trace of activity anodal to the F esterase, as do the kidney samples (Fig. 7, slots 2 and 3), although in one...

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estimates were incubated for 8 days beneath kidney capsules, and the resultant growths were analyzed electrophoretically as in Fig. 1. Slot 5: trophoblast tissue from (C57Bl/6J x DBA/2J)F2 hybrids, showing the position of the $F_{slow}$ and $F_{fast}$ phenotypes; slots 3 and 4: individual growths resulting from (SWR/J x SJL/J)F1 blastocysts implanted beneath the kidney capsule of C57Bl/6J males; slots 6 and 7: C57Bl/6J blastocysts implanted beneath the kidney capsule of SJL/J males; slots 1 and 9: serum from C57Bl/6J and SJL/J males, respectively; slots 8 and 6: kidney tissue from C57Bl/6J and SJL/J males, respectively. The kidney preparations show an unusually weak staining reaction in the $G$ region. In most gels, esterase activity in the $G$ region greatly exceeds that found in the $F$ region. The drawing beside the photo is a representation of the $F$ and $G$ regions of the gel. $\ast$, major band of activity; $\ast\ast$, minor band of activity; $\ast\ast\ast$, trace of activity.

In most cases (not shown), a growing containing smaller cells, as well as giant cells, did have a substantial band of activity in the $G$ region. It has been noted (7) that under these conditions of electrophoresis, the band in the $F$ region of serum preparations is much more dense than that of the trophoblast. The $F$ esterase in the ectopic growths resembles the trophoblast, rather than the serum, band (Fig., slots 1 and 9) with respect to thickness. Furthermore, there is at least one conspicuous band in the serum profile that is not present in the ectopic growth profiles.

Blastocysts cultured in vitro produce a band of activity in the $A$ region of the gel after 4 days (7). It has been proposed that this production reflects the ability of trophoblast to differentiate in vitro (7). Mouse serum was added to the culture medium to determine whether these differentiated trophoblast cells could take up $F$ esterase. Cells cultured up to 7 days show no evidence of esterase uptake. However, culture periods longer than 4 days in medium supplemented with mouse serum had a markedly adverse effect upon the cells.

**DISCUSSION**

By midgestation, maternal esterase is taken up by both the trophoblast and yolk sac layers of a fetal placenta. The proportion of enzyme that actually penetrates the cells of these tissues is difficult to determine. The amount of maternal esterase activity remaining in the trophoblast layer after the washing procedure used could be an underestimation, due to losses attributable to cell damage during repeated centrifugation. The trophoblast layer of the placenta as dissected here is not pure. However, it is likely that most of the intracellular esterase resides in trophoblast cells because Reichert's mem-

branes is acellular, the parietal endoderm represents a minute proportion of the total cell mass, and the allantois does not take up dye (17) or gamma-globulins (18) during early pregnancy in rats.

$F$ esterase activity is present in trophoblast preparations at least as early in gestation as the eighth day (7). Since there is no evidence that $F$ esterase can be synthesized by trophoblast or yolk sac, it can be assumed that maternal esterase penetrates trophoblast cells shortly after implantation. Uptake by yolk sac cells probably does not occur in significant amounts until late on the 10th day of gestation. The yolk sac must provide an effective barrier against further penetration of $F$-esterase, since the enzyme has not been detected in the amnion or fetus at any stage in gestation (7). It should be noted that the passage of maternal proteins into fertilized mouse eggs has been observed (19), and that rabbit blastocysts have the capacity to accumulate proteins from uterine fluid into their blastoocoeles (20). Preliminary studies suggest that maternal esterase may also penetrate the pre-implantation mouse embryo (unpublished observations).

The mechanism governing the uptake of maternal esterase by trophoblast cells is not clear. Although trophoblast cells are phagocytic (2, 21), engulfment of maternal cells is unlikely to account for the relatively high levels of maternal esterase present. Furthermore, blood cells, uterine cells, or, in the case of the ectopic pregnancies, kidney cells, each possess at least one esterase isozyme not found in trophoblast profiles (Fig. 7; ref 7). Since the trophoblast layer of the placenta is bathed in maternal blood, and the serum $F$ esterase level is very high (Fig. 7), it is perhaps more reasonable to assume that the enzyme is selectively taken up from the serum, as are other proteins (9–11). One other possibility, although less likely, has not been eliminated. Maternal messenger RNA for $F$ esterase, rather than the enzyme itself, may be transferred to, and translated by, trophoblast cells.

The observation that trophoblast in ectopic pregnancies can take up maternal esterase provides further evidence of the degree to which development of that cell type can take place outside of the maternal environment. Trophoblast cells developing in blastocyst cultures are unable to accumulate $F$ esterase from the culture medium. Either the individual cells have not attained that developmental capacity, or the growth as a whole loses some characteristic organizational aspect necessary for uptake of the enzyme. In the absence of $F$ esterase uptake, well-developed trophoblast and yolk sac can nevertheless arise from blastocyst cultures (7, 8). The function, if any, of this enzyme in the $in vivo$ development of trophoblast and yolk sac therefore remains to be determined.

Finally, as a result of these findings, it is suggested that studies on the appearance of new enzymes in developing extraembryonic tissues should be cautiously interpreted until it can be demonstrated that these enzymes are not of maternal origin.

**Note added in proof** Thoroughly washed leukocyte preparations have very little $F$ esterase activity, while erythrocyte preparations have none. This finding further reduces the possibility that contamination of the trophoblast and yolk sac preparations by maternal blood cells is responsible for the observed effects.
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