Patterns of lactic dehydrogenase isozymes in mouse embryos over the implantation period in vivo and in vitro

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SUMMARY

Following blastocyst implantation, or outgrowth in vitro, the LDH isozyme pattern changes from that of the maternally inherited B subunit isozyme form (LDH-1) to a pattern dominated by A subunits (Auerbach & Brinster, 1967, 1968). In preimplantation embryos we have also observed additional isozyme bands, as yet unidentified. An analysis of the pattern of newly synthesized LDH isozymes and specific activity of LDH in different regions of early postimplantation embryos suggests that there is a sequential activation of A and B subunits, and that activity first appears in ICM-(inner cell mass) derived tissues and then in trophoblast-derived tissues. In vitro, in the absence of ICM cells, the transition of LDH-isozyme pattern does not occur in outgrowing trophoblast giant cells. This suggests a possible inductive interaction between ICM and trophoblast.

INTRODUCTION

Lactic dehydrogenase (LDH, E.C. 1.1.1.27) is a tetramer composed of combinations of two subunits, A and B, coded for by separate genes (Shaw & Barto, 1963). There are five isozymic forms; LDH-1 (4B), LDH-2 (1A3B), LDH-3 (2A2B), LDH-4 (3A1B), and LDH-5 (4A), which vary in electrophoretic mobility. The relative proportion of these forms varies from tissue to tissue in the adult animal presumably because of the differences in the rates of expression of the two genes (Markert & Möller, 1959). A sixth LDH isozyme, termed LDH-X, is synthesized by primary spermatocytes (Hawtrey & Goldberg, 1968). It is a tetramer of C subunits, C being a third type of subunit under independent genetic control (Zinkham, 1968).

In mouse embryos, the distribution of LDH amongst the five isozymic forms, LDH-1 to LDH-5, varies with stage of development. The level of LDH in the mouse egg is high (Brinster, 1965; Epstein, Wagienka & Smith, 1969) and is thought to be exclusively in the B subunit form, or LDH-1 (Auerbach & Brinster, 1967; Rapola & Koskimies, 1967). This maternally inherited LDH-1

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is progressively degraded during preimplantation development (Brinster, 1965; Spielmann, Erickson & Epstein, 1974) and there is evidence that newly synthesized enzyme does not appear before implantation (Epstein et al. 1969; Erickson et al. 1975). At about the time of implantation (between 4 and 6 days gestation) the pattern changes, presumably due to the appearance of LDH coded for by the embryonic genome, LDH-5 (the A subunit form) predominating (Auerbach & Brinster, 1967; Wolf & Engel, 1972; Engel & Petzoldt, 1973). Diapause blastocysts, where implantation has been prevented by ovariectomy on the 3rd day of pregnancy, fail to show this transition, suggesting that the implantation event itself is required for activation of embryo-coded LDH expression (Auerbach & Brinster, 1967). Outgrowths obtained after 4 days culture of blastocysts in vitro also show a transition of LDH isozyme activity to that of LDH-5 (Auerbach & Brinster, 1968).

The possibility that the appearance of LDH-5 is causally related to implantation suggests its use as a marker in the investigation of processes regulating postimplantation development. In this paper we investigate postimplantation isozyme patterns in different regions of the early embryo in vivo and in vitro.

**MATERIALS AND METHODS**

Mouse embryos were obtained from the randomly breeding stocks, CD1 (Charles River & Co.), MF1 (Olac, Bicester) or ‘Q’, bred in our laboratory. Medium used for collection of embryos was Dulbecco’s phosphate buffered saline (PBS), or PBS supplemented with bovine serum albumin, sodium pyruvate, glucose, penicillin and phenol red (PB1, Whittingham & Wales, 1969). On the day of the copulation plug, unfertilized eggs were teased from oviducts of females mated with vasectomized males. Cumulus cells were removed by treatment for 5 min with hyaluronidase (Sigma, 300 units/ml in PB1). Eight-cell eggs were obtained by flushing oviducts dissected from females on the 3rd day of pregnancy, and blastocysts by flushing uteri of females on the 4th day of pregnancy. Postimplantation embryos were dissected out of the uterus into PB1. Samples for electrophoresis were washed through several changes of PB1 or PBS, frozen and thawed twice, clarified by centrifugation, and the supernatants stored in liquid nitrogen. Eight-cell embryos or blastocysts for culture were transferred to droplets of culture medium under oil.

Culture medium for 8-cell embryos was medium 16 (Whittingham, 1971). Trophoblastic vesicles were prepared by the addition of tritiated thymidine at a final concentration of 0.05 μCi/ml to the medium for 24 h (Snow, 1973). Outgrowths from trophoblastic vesicles or control blastocysts (produced by culture from the 8-cell stage without tritiated thymidine) were harvested after culture in medium 16 supplemented with 5% foetal calf serum for a further 5 days. In some experiments blastocysts were cultured in Eagle’s minimum essential medium (MEM, Flow Laboratories) supplemented with sodium
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bicarbonate (2 g/l), glutamine (2 mm), penicillin (100 units/ml), streptomycin (50 μg/ml) and 20 % foetal calf serum (Flow Laboratories) or human cord serum (kindly supplied by University College Hospital). The media were filtered and equilibrated to 37 °C and 10 % CO₂ in air atmosphere before the embryos were introduced.

Discontinuous polyacrylamide slab gel electrophoresis was carried out using the solutions of Davis (1964) with a 2.5 % acrylamide stacking gel pH 6.7, 7 % separating gel pH 8.9, and a Tris-glycine tank buffer pH 8.3. Approximately 10 μl of sample was mixed with an equal volume of 40 % sucrose containing bromophenol blue (0.01 %). Electrophoresis was carried out at 4 °C for approximately 3 h at 100 V or until the bromophenol blue marker reached the bottom of the gel. The gels were stained at 37 °C for LDH activity with the staining solution of Engel & Kreutz (1973). When staining had reached the desired intensity gels were fixed in 7 % acetic acid. Densitometer tracings were made with a Joyce Loebl Densitometer.

LDH activity was assayed by the method of Brinster (1965) and protein determinations were made by the Lowry technique using bovine serum albumin as the standard (Lowry, Rosebrough, Farr & Randall, 1951).

RESULTS

In vivo development

Fig. 1 shows the distribution of LDH isozyme activities in an extract of unfertilized eggs (Slot 4) as well as in extracts of adult skeletal muscle and testis (Slots 1 and 2). The eggs, obtained from the oviducts of females mated with vasectomized males, contain predominantly LDH-1. In addition there are two faint bands, one running just behind LDH-1, and the other running slightly more slowly than LDH-2. We have observed these additional bands in all preimplantation embryos examined (1-cell, 8-cell, early and late blastocysts) as well as in unfertilized eggs, whether spontaneously ovulated or hormonally induced. They are present in all of the three strains of mice examined (Q, CD1 and MF1). We have not identified these additional bands. However, the band running behind the position of LDH-2 could be a tetramer of 3B subunits and 1C subunit since it runs midway between that of a 4B tetramer (LDH-1) and that of a 2B2C tetramer artificially created by dissociating and reassociating the subunits of a whole testis extract (Slot 3).

Fig. 2 shows isozyme patterns in blastocysts and in postimplantation embryos dissected from the uterus early and late on the 7th day of gestation. Blastocysts contain LDH-1 together with the additional bands (Slot 1). Postimplantation embryos (Slot 2) show the transition of LDH isozyme pattern to one dominated by A subunits, LDH-5 (4A) and LDH-4 (3A1B). A faint residual band of maternally inherited LDH-1 is visible. With further development, LDH-3 activity appears (Slot 3) owing to an increasing contribution to the pattern
Fig. 1. LDH isozyme patterns in unfertilized eggs (Slot 4), and in adult mouse testis before (Slot 2), and after (Slot 3), treatment to dissociate and reassociate LDH subunits. Under the conditions of enzyme extraction and running of the gel there is no dissociation and reassociation of subunits between independently synthesized forms of the tetrameric enzyme. However, such dissociation and reassociation can be artificially engineered by freezing and thawing mixtures of isozymes in one molar sodium chloride in sodium phosphate buffer at pH 7 (Markert, 1963). Adult mouse skeletal muscle (Slot 1) is shown to give the position of LDH-5 and LDH-4. An extract of a total of 48 unfertilized eggs, obtained from oviducts of CD1 mice approximately 10 h after mating with vasectomized males, was applied to the gel. The direction of migration is shown. Further details are provided in Materials and Methods.

of de novo production of B subunits in the embryo. Measurements of specific activity of total LDH in the extracts, have shown a progressive increase in embryos from mice on the 7th, 8th and 9th day of pregnancy.

Fig. 3 shows the pattern of LDH isozymes, in different regions of embryos, obtained on the 8th (primitive streak stage) and 9th days of gestation. Slots 1, 2 and 3 (Fig. 3) show LDH isozymes in extracts of ectoplacental cone, and extra-embryonic and embryonic regions of the egg cylinder of 8th day embryos.
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Fig. 2. LDH isozyme patterns before and after implantation. Polyacrylamide slab gel electrophoresis of extracts of 33 CD1 blastocysts (Slot 1) and of approximately ten CD1 postimplantation embryos (Slots 2 and 3) dissected from the uterus early and late on the 7th day of pregnancy. Adult mouse heart (Slot 4) and skeletal (Slot 5) muscle serve to indicate the positions of the five isozyme bands.

Slots 4 and 5 (Fig. 3) show LDH isozyme activities in extracts of yolk sac and embryonic tissues dissected from 9th day embryos. Densitometer tracings of these gels are also shown in Fig. 3. The gels show that a greater degree of B subunit synthesis, as reflected by the relative amounts of LDH-4 and 3, has taken place in embryonic regions compared with extra-embryonic regions of these early postimplantation embryos. In addition the total specific activity of LDH was higher in embryonic than in extra-embryonic regions.

In vitro development

Outgrowths from blastocysts cultured for 4 days show some LDH-5 isozyme expression (Auerbach & Brinster, 1968). Fig. 4 shows further development of the LDH isozyme pattern in vitro after culture in the presence of human cord serum which favours inner cell mass (ICM) development into egg cylinder-like structures (Hensleigh, personal communication). After 6 days in culture the in vitro pattern is very similar to that of embryos dissected from the uterus on the 9th day of pregnancy.

Since the in vivo analyses suggest that expression of embryo-coded LDH occurs earlier in ICM-derived tissue than in trophoblast-derived tissue we investigated the possibility that an ICM is required for the appearance of
Fig. 3. Regional LDH isozyme patterns in postimplantation embryos. After removal from their decidua in the uterus, Q embryos were dissected into component parts as follows. From 8th day embryos ectoplacental cone and primary giant cells (epc, Slot 1) were separated from the egg cylinder, and then extra-embryonic (ee, Slot 2) and embryonic (e, Slot 3) regions were obtained by cutting the egg cylinder in half at the level of the amnion; from 9th day embryos, yolk sac (ys, Slot 4) was carefully cut away from embryonic tissue (e, Slot 5). Specific dissected regions for each stage of development were pooled, extracts prepared as described in Materials and Methods and assayed for LDH activity. Activity corresponding to approximately 26 nmol NADH oxidized per minute at 37 °C by 10⁻³ molar pyruvate at pH 7-5 was applied to each slot. Densitometer tracings were made the same day. Equivalent results were obtained using CD1 embryos.
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Fig. 4. Development of LDH isozyme pattern in vitro. LDH isoforms in 9th day embryos dissected from the uterus and in approximately 100 outgrowths from CD1 blastocysts cultured for 6 days in MEM supplemented with 20% human cord serum.

Fig. 5. LDH in outgrowths from trophoblastic vesicles and blastocysts cultured for a total of 6 days from the 8-cell stage. Extracts from approximately 50 outgrowths were applied to each slot. Trophoblastic vesicles were prepared as described in Materials and Methods. The positions for isoforms LDH-5 to LDH-1 were taken from a densitometer tracing of adult kidney extract run in the same gel.

LDH-5 in trophoblast cells. Tritiated thymidine treatment (Snow, 1973) was employed during culture from the 8-cell stage to produce trophoblastic vesicles devoid of ICM. Outgrowths from such vesicles show giant cell transformation but no proliferation of trophoblast cells (Ansell & Snow, 1975). Figure 5 shows that such outgrowths do not contain LDH-5.
DISCUSSION

Our analysis of pre- and postimplantation LDH isozyme patterns confirms the results of earlier workers that LDH-1 is the predominant form in pre-implantation embryos. Early postimplantation embryos show an initial appearance of the A subunit form, or LDH-5, followed by the appearance of other isozymes as the contribution of B subunits to the pattern increases (see also Engel & Petzoldt, 1973). This suggests a sequential activation of A and B subunit production. In addition, the pattern of appearance of these different isozymes is regulated differently in different regions of postimplantation embryos. This result, together with the observation that specific activity of LDH is higher in embryonic regions, is most easily interpreted on the hypothesis that initial embryo-coded LDH expression occurs earlier in ICM-derived, than in the trophoblast-derived tissues, extra-embryonic ectoderm and ectoplacental cone; see Gardner & Papaioannou, 1975). Alternatively, or in addition, the relative rates of production of A and B subunits could change as development proceeds, and be regulated differently in different embryonic tissues. Regional differences in esterase patterns (Sherman, 1972a) and in acid and alkaline phosphatase patterns (Solter, Damajanov & Skreb, 1973; Sherman, 1972b) also occur in postimplantation embryonic development.

We have provided evidence that ICM cells are required for the expression of new LDH activity in trophoblast cells following attachment and outgrowth. Gardner & Johnson (1972) have shown previously that the presence of an ICM is required for proliferation of neighbouring trophoblast cells to form the ectoplacental cone. However, alternative explanations of our results cannot be excluded. For instance, it is possible that giant trophoblast cells never exhibit LDH-5 either in vivo or in vitro. Our in vitro control blastocyst outgrowths include an inner cell mass; our in vivo analysis of trophoblast-derived tissues show that ectoplacental cone cells express LDH-5 but we have not tested trophoblast giant cells alone.

Further investigation of the in vitro system as a model for implantation in vivo, could provide more rigorous evidence for the supposition that implantation, or outgrowth, represents a 'trigger' event in development for the expression of embryonic LDH activity. LDH-5 would then be a convenient marker function in the analysis of the nature of this event.

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REFERENCES


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