Estrogen-induced surface coat and enzyme changes in the implanting mouse blastocyst

By P. V. HOLMES1 and A. D. DICKSON2

From the Division of Morphological Science, Faculty of Medicine, The University of Calgary

SUMMARY

Blastocysts from estrogen-stimulated mice tend to be very sticky, a characteristic which can be correlated with estrogen-induced enzyme activation and changes in the surface coat of the trophoblast cells. The blastocyst surface coat was investigated using colloidal iron-Prussian blue stain. In addition, the activity of tyrosine aminotransferase was assayed histochemically in preimplantation blastocysts. The surface and enzyme changes were correlated with the presence of estrogen by investigations in normal blastocysts from intact mice before and after the endogenous estrogen surge and in delayed-implantation blastocysts from ovariectomized mice preceding and following estrogen treatment. Colloidal iron stains the surface coat of the blastocyst after, but not before, the endogenous estrogen surge and the exogenous estrogen treatment. Tyrosine aminotransferase activity also is increased considerably after estrogen induction.

INTRODUCTION

The preimplantation stickiness of the zona pellucida-free mouse blastocyst has been referred to previously (Böving, 1966). Whether this adhesiveness contributes to the attachment phase of blastocyst implantation is the concern in this work.

In 1961, working with dissociated embryonic cells, Moscona found that rotation of the cultures caused the cells to reaggregate. Subsequently, Moscona & Moscona (1963) were able to inhibit the adhesiveness and aggregation of dissociated embryonic cells by blocking RNA and protein synthesis. They suggested that actinomycin D and puromycin may interfere with formation of extracellular materials having specific cell-binding roles. Lin & Florence (1971) dissociated the cells of four- to eight-cell mouse ova and observed reaggregation, some aggregates developing to the blastocyst stage. Sheffield (1970) studied the morphological characteristics of cell adhesion in embryonic cell reaggregation and postulated that an adhesive material may be released on the cell surface to

1 Author’s address: Institute of Human Anatomy, University of Uppsala, Uppsala, Sweden.
2 Author’s address: Division of Morphological Science, Faculty of Medicine, The University of Calgary, Calgary 44, Alberta, Canada.
provide a means of cell–cell recognition and attachment for histogenetic aggre-
gation. This possibility could apply to the attachment phase of mouse blasto-
cysts, for Jones & Kemp (1969) observed, on day 6 of gestation, deposition of
a fibrinoid material containing mucoproteins, sialic acid and hyaluronic acid
on the outer surface of the trophoblast cells. A similar surface mucoprotein
layer was observed and histochemically characterized on human trophoblast
by Bradbury, Billington, Kirby & Williams (1969, 1970). These investigators
demonstrated the presence of the surface coat using colloidal-iron stain for light
and electron microscopy.

Blocking the synthesis of new RNA and protein species induced by estrogen
(Dass, Mohla & Prasad, 1969) in the blastocyst could prevent the attachment
phase of implantation. Unger & Dickson (1971) have shown that actinomycin D
and cycloheximide, administered to mice at the preimplantation stage, stops both
the estrogen-induced trophoblastic giant cell transformation (Dickson, 1963,
1967) and implantation from occurring.

There is a possibility that the enzyme neuraminidase may influence the
adhesion of blastocysts to the uterine luminal epithelium. Gasic & Gasic (1970)
administered neuraminidase intravenously to post-coitum female mice and noted
a total suppression of pregnancy, an effect reversible by progesterone treatment.

Thompson, Tomkins & Curran (1966) and Granner, Hayashi, Thompson
& Tomkins (1968) treated cell cultures, respectively, with glucocorticoids and
dexamethazone phosphate and were able to induce increases in tyrosine
aminotransferase (TAT) activity. Correlating this work with that of Moscona
& Moscona (1963), Ballard & Tomkins (1969) linked both the synthesis of
a specific cell surface adhesive factor and the increase in intracellular TAT
activity with a number of administered steroid-hormone inducers. They postu-
lated that tyrosine aminotransferase may be on the pathway of steroid-induced
increases in cell adhesion.

From the foregoing, it may be proposed that the luteinizing hormone-induced
estrogen surge of pregnancy (Shelesnyak, Kraicer & Zeilmaker, 1963) may in-
duce increased enzyme activity in the trophoblast cells which alters their surface
mucoproteins and that this alteration is necessary for the attachment phase
of implantation.

**MATERIALS AND METHODS**

Swiss Webster albino mice were used from a random-bred colony maintained
at 22 °C with lighting controlled to provide a 10 h night centred on midnight.
Mouse chow from Tecklad Inc. of Monmouth, Illinois, and drinking water
were provided *ad libitum*. Surgical procedures were conducted under intraperi-
toneal sodium pentobarbital anesthesia and hormone injections were given
subcutaneously in the lumbar region. The mice were killed by cervical disloca-
tion and blastocysts were flushed from uterine horns with Hanks's physiological
saline and fixed for 24 h in 10% buffered neutral formalin.
Blastocysts were collected from four experimental groups of mice, namely intact females killed between 09.00 and 10.00 h on day 4 of gestation (i.e. prior to the endogenous estrogen stimulus for implantation), intact females killed between 09.00 and 10.00 h on day 5 after the endogenous estrogen stimulus, females undergoing delay of implantation, and females undergoing delay of implantation to which exogenous estrogen was administered. Delay of implantation was achieved by post-coital bilateral ovariectomy between 09.00 and 11.00 h on day 3 of gestation and administration of 1 mg of Depo Provera (medroxyprogesterone acetate suspension, Upjohn Co.) on day 3 and again on day 8 at 09.00 h. Exogenous estrogen (estradiol benzoate, 0.05 µg in corn oil) was administered to the fourth experimental group and vehicle only to the third group, both on day 8 at 09.00 h. Blastocysts undergoing delay of implantation were collected from these mice 36 h after the day-8 injections.

Trophoblast surface coat changes were visualized in the four mouse groups using the colloidal iron–Prussian blue reaction, with naphthol yellow as a counterstain and controls being treated first with α-amylase for 45 min or with neuraminidase for 60 min. After formalin fixation, blastocysts were air-dried on microscope slides before staining. The stock solution of colloidal iron was prepared by adding 12 ml of 32% w/v ferric chloride solution to 750 ml of boiling, triple-distilled water. This solution was diluted with 10 vols of glacial acetic acid immediately before use, the pH being adjusted to below 1.3, if necessary, and was applied to the tissue for 60 min. This procedure was followed by three 5 min rinses in 5% acetic acid. Colloid deposits were converted to Prussian blue by a 20 min treatment with a 1:1 mixture, prepared immediately before use, of 2% aqueous potassium ferrocyanide and 2% HCl. The effectiveness of this stain was confirmed by control staining of goblet cells in sections of small intestine and the specificity was confirmed by carrying the procedure through omitting the ferric chloride treatment.

Blastocysts from four groups of mice similar to those described above were employed for investigating tyrosine aminotransferase activity. The enzyme reaction mixture was composed of 0.05 ml of 0.33 M α-ketoglutarate, 0.2 ml of 0.025 M tyrosine, 0.005 ml of tautomerase (4.3-5.0 K units/ml), 0.005 ml of 0.02 M pyridoxalphosphate, 0.0025 ml of a suspension of 20 mg/ml of crystalline glutamate dehydrogenase in a 50% aqueous glycerol solution, 0.25 ml of iodonitrotetrazolium at 32 mg/ml, 0.1 ml of diphosphopyridine nucleotide at 10 mg/ml, and 0.05 ml of 0.4 mg/ml of phenazine methosulphate. To this mixture 0.15 M sodium phosphate solution at pH 7.9 was added to make up 1 ml. The reaction was inactivated by the addition of 7% acetic acid. Blastocysts were treated according to this technique for 30 min, formalin-fixed, washed and mounted on microscope slides as aqueous whole mounts. No counterstain was used. Specificity of the reaction was tested by enzyme inactivation and by incubation without substrate. Enzyme inactivation was accomplished by heat treatment for 60 min, at 65 °C for one group of day-5 blastocysts and at 90 °C for a second group.
All day-5 and delayed-implantation blastocysts lacked a zona pellucida when flushed from the uterus. Many of the day-4 blastocysts possessed a zona pellucida. However, those with a zona did not appear to react less to the colloid and enzyme tests than those without a zona.

Sixty-one blastocysts were collected from day-5 intact females and all exhibited colloidal iron–Prussian blue staining of the surface-coat material of the trophoblast cells (Fig. 1). Of the 87 collected from day-4 intact females, almost all lacked any surface-coat staining (Fig. 2). A very few of these blastocysts exhibited traces of stain on one side but it was impossible to determine whether the stain traces were consistently at the abembryonic or the embryonic pole. Fifty-four blastocysts were collected from ovariectomized, estrogen-treated mice and all exhibited light-to-heavy colloidal iron staining. From the ovariectomized, vehicle-treated group, 78 blastocysts were collected, most of which lacked any staining, with some showing trace amounts. The 30 day-5 control blastocysts untreated with ferric chloride were not stained by the colloidal iron. The same is true of the 32 day-5 blastocysts pretreated with neuraminidase and of the 28 pretreated with α-amylase.

In the tyrosine aminotransferase experiments all blastocysts exhibited at least trace amounts of enzyme activity. The lowest TAT activity observed was in the 17 substrate-control blastocysts and the 29 90 °C heat-control blastocysts. Treatment of 29 day-5 blastocysts for 60 min at 65 °C did not appear to affect their TAT activity. A comparison between 46 day-4 (Fig. 3) and 48 day-5 (Fig. 4) blastocysts from intact mice demonstrates a marked increase in TAT activity in the embryonic and trophoblast cells of the day-5 blastocysts. More advanced blastocysts collected between 12.00 and 13.00 h day 5 exhibited extremely high enzyme activity in some cells of the inner cell mass, as seen in Fig. 4, probably endoderm cells. On comparing the two groups of ovariectomized mice, the 38 blastocysts from females pretreated with estrogen were observed to have very much higher formazan deposition than the 48 blastocysts from the...
vehicle-treated females. Once again, there was very high TAT activity in the inner cell mass cells of the estrogen-stimulated blastocysts. In all cases, the formazan from the tetrazolium salt, iodonitrotetrazolium, precipitated in the form of large, reddish-brown crystals throughout the cells.

In all of the work where blastocysts were manipulated from one solution to another, it was found necessary to maintain the solutions at, or above, a physiological pH because of their extreme stickiness to siliconized glass and each other in acid solutions.

**DISCUSSION**

The marked alteration in colloidal iron staining of the trophoblast surface between days 4 and 5 of gestation in the intact mouse indicates there is probably a change in amount or functional activity of the surface glycoproteins. Since this surface change coincides temporally with a number of other known changes in the blastocyst, such as the trophoblastic giant cell transformation (Dickson, 1963, 1969), and since an increase in blastocyst stickiness was also observed at this time, it seems reasonable that the surface glycoproteins could be related to attachment of the trophoblast cells to the epithelial cells of the uterine lumen. The experiments with ovariectomized mice indicate that the colloidal iron staining or trophoblast surface changes are directly, or indirectly, the result of an estrogen stimulus.

Also at this time, between the preimplantation and implantation stages of the blastocyst, a considerable increase in tyrosine aminotransferase activity was observed in the intact mice. This coincides temporally with surface-coat and adhesiveness changes of the blastocyst and appears to be dependent on an endogenous estrogen stimulus as indicated by the work with estrogen-stimulated and non-stimulated delayed-implantation blastocysts. As mentioned previously, steroids have been shown to be responsible for increasing both the adhesiveness of cells and the intracellular activity of tyrosine aminotransferase (Ballard & Tomkins, 1969).

The substantial effect of neuraminidase on the surface coat at implantation as seen in this work compared with the lack of its effect on the zona pellucida (Bowman & McLaren, 1970) indicates that the surface coat and the zona pellucida of the blastocyst are biochemically different. In addition, intravenously administered neuraminidase was shown by Gasic & Gasic (1970) to suppress pregnancy in mice, a result possibly due to a neuraminidase-mediated change in the blastocyst surface coat, although recently, Kunii (1971) has shown that chorionic and serum extracts of human chorionic gonadotrophin are inactivated by neuraminidase.

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REFERENCES


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