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Binding of Proteins to Mouse Blastocysts after the Attachment Stage of Implantation

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ABSTRACT

Experimentally delayed mouse blastocysts were activated to implant by exogenous estradiol and after varying duration of estrogen influence, the blastocysts were flushed out from the uterine horns. Then they were incubated in the cold with different, ^{125}I-conjugated proteins and the amount of protein bound to the blastocysts was determined by radioassay. Three radiolabelled proteins: human serum albumin (^{125}I-HSA), human serum transferrin (^{125}I-HST) and normal rabbit IgG (^{125}I-RIgG) were tested and it was found that the uptake of each protein markedly increased between 14 and 24 hours of estrogen-activation. It was possible to partially block the binding of ^{125}I-HSA and ^{125}I-RIgG with the respective unlabelled protein. Unlabelled RIgG could also partially block the uptake of ^{125}I-HSA whereas HSA did not impede the binding of ^{125}I-RIgG. During delay of implantation the protein binding was low and approximately the same as after 14 hours of activation. However, at 18 and 24 hours of activation protein uptake increased gradually. Raising the incubation temperature from 0 to 37°C did not significantly influence the protein binding capacity of the 24-hour-activated blastocysts. Whole blastocyst autoradiography indicated that the labelled protein was heavily bound in patches, preferentially located in the abembryonic half of the postattachment blastocysts.

It is assumed that the binding of protein to abembryonic trophoblast cells of the implanting blastocyst can be attributed to the presence of protein receptors on the surface of these cells.

INTRODUCTION

During studies on the antigenicity of mouse blastocysts before and during implantation it was observed that blastocysts flushed from the uterus after the attachment stage of implantation exhibited a marked tendency to bind protein. It could be demonstrated that gammaglobulin was bound to these blastocysts and that the binding was independent of the specificity of the gammaglobulin (Håkansson & Sundqvist, unpublished). Since the protein binding occurred at 0°C, it was considered unlikely that it was due to an active metabolic process. Rather it appeared that the uptake reflected the presence of protein binding sites on the trophectoderm of the attaching blastocyst.

The purpose of the present investigation is to test this hypothesis by experiments designed to characterize the protein binding capacity of the postattachment blastocyst.

MATERIALS AND METHODS

Mice of the CBA strain were used throughout the investigation. The animals were obtained from an inbred line maintained at this laboratory (Biomedical Centre, Uppsala, Sweden). Nulliparous females, 8 to 12 weeks of age, were caged overnight with fertile males. The presence of a vaginal plug on the following morning indicated day 1 of pregnancy. Experimental delay of implantation was induced by bilateral ovariectomy on day 3 of pregnancy, followed by daily s.c. administration of 1.0 mg progesterone (Ikapharm, Ramat Gan, Israel) in 0.04 ml peanut oil. Activation of delayed blastocysts was achieved by a s.c. injection of 0.1 μg estradiol-17-β (AB Leo, Helsingborg, Sweden) in 0.1 ml propylene glycol. Blastocysts were flushed from the excised uterine horns with ice-cold phosphate buffered saline (Dulbecco's PBS, pH 7.2) supplemented with 1% heat-decomplemented fetal calf serum and recovered in a watchglass on ice. All manipulation was then, unless otherwise stated, performed in a cold room (+4°C). The blastocysts used in this study were from days 9, 10 or 11 of pregnancy.

Radioiodination of protein proceeded as follows: Human serum albumin, HSA (Kabi Ltd., Stockholm, Sweden), human serum transferrin, HST (Kabi Ltd.) and normal rabbit IgG, RIgG (obtained by ammonium sulphate precipitation and ion exchange chromatography on DEAE-Sephadex A-50) were conjugated with ^{125}I (100 mCi/ml, Radiochemical Centre, Amersham, U.K.) by the lactoperoxidase method (9). The A_{412}/A_{280} ratio of the lactoperoxidase preparation was 0.74. Unbound iodide was removed on a Sephadex G-25 column. The protein contents of the conjugates were 10 mg/ml and the specific activity was in each case 4 mCi/mg.

Binding of protein. The binding of radioiodinated HSA,

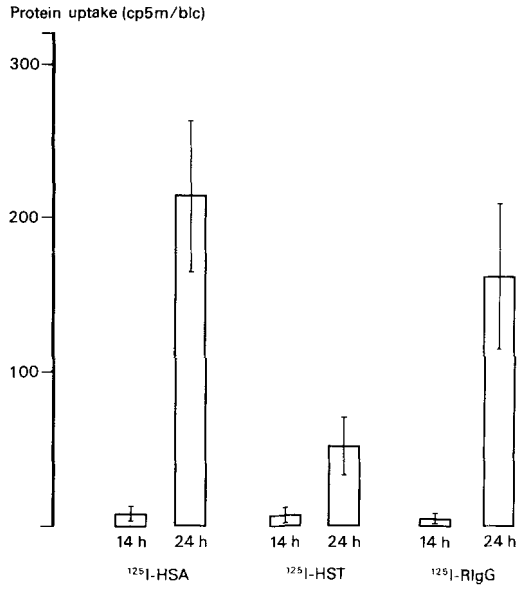

Fig. 1. Diagram illustrating uptake of the three different radiolabelled proteins by 14- and 24-hour-activated blastocysts. Each category of blastocysts in figures 1 to 4 is depicted by indication of mean ±estimated standard deviation of observations.

HST and RIgG to blastocysts which were flushed from the uterus 14 and 24 hours after estradiol-activation was compared. The blastocysts were incubated for 30 minutes in PBS containing 2 mg/ml of labelled protein. Then they were washed by transfer to watchglasses with fresh flushing medium until the radioactivity in the last change of medium was equal to background levels. This was generally achieved after 4 or 5 washings, but routinely 6 changes of medium were performed. After washing, 3 blastocysts were placed on a small, punched out piece of filter paper and the added radioactivity from the blastocysts was determined in a dual channel Wallac Gamma Sample Counter (GTL 300–500). Each group of blastocysts was counted for 5 minutes and the average blastocyst activity was calculated by dividing the total count by a factor of 3. Thus, protein binding was measured in units of counts/5 min/blastocyst (cp5m/blc). The total number of blastocysts in each of the six different groups was 24 (^{125}I-HSA, 14 and 24 h), 35 (^{125}I-HST, 14 and 24 h), 27 (^{125}I-RIgG, 14 h) and 30 (^{125}I-RIgG, 24 h).

These criteria concerning incubation, washing and measurement of radioactivity were followed for each different category of blastocysts in the experiments described below.

Blocking of protein binding. To determine whether the protein uptake of the post-attachment blastocysts could be blocked, 24-hour-activated blastocysts were pre-incubated with unlabelled HSA or RIgG (10 mg/ml) for 2 hours. Following 3 washes, the blastocysts were incubated with the respective ^{125}I-conjugated protein (2 mg/ml, 30 min).

The reciprocal pre-incubation procedure, i.e. RIgG followed by ^{125}I-HSA and HSA followed by ^{125}I-RIgG, was employed to investigate whether the binding of one protein could influence the binding of a second, different protein. The four different groups comprised the following number of blastocysts: HSA+^{125}I-HSA=30 blastocysts, RIgG+^{125}I-HSA=27, RIgG+^{125}I-RIgG=30 and RIgG+^{125}I-HSA=36 blastocysts.

Influence of blastocyst activation on protein binding. The effect of the degree of blastocyst activation on protein binding was studied by measuring the uptake of ^{125}I-HSA (2 mg/ml, 30 min) by experimentally delayed (24 blastocysts), 14-hour-activated (data previously obtained were used), 18-hour-activated (27 blastocysts) and 24-hour-activated (data previously obtained were used) blastocysts.

Influence of temperature on protein binding. The effect of incubation temperature on blastocyst protein binding was investigated by determining the uptake of ^{125}I-HSA (2 mg/ml) by 24-hour-activated blastocysts at 0 (data previously obtained were used) and 37°C (24 blastocysts).

Distribution of bound protein. To visualize the binding of protein to blastocysts, autoradiography of whole blastocysts was performed. After incubation in ^{125}I-HSA or ^{125}I-RIgG (2 mg/ml, 30 min) and washing, the blastocysts were individually transferred to a gelatin-coated glass-slide with the aid of a micro-capillary connected to a micrometer syringe. The blastocysts were oriented in a minute drop of rinsing medium with the long axis parallel to the plane of the glass. The surrounding fluid was carefully sucked away, the blastocysts becoming flattened against the gelatin surface as originally oriented. The in-

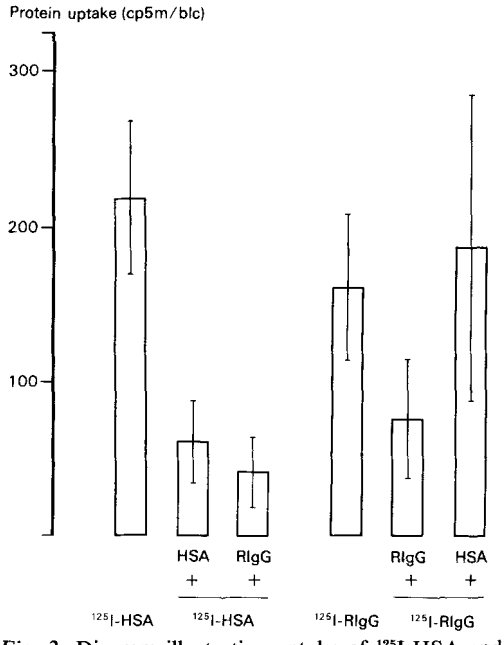

Fig. 2. Diagram illustrating uptake of ^{125}I-HSA and ^{125}I-RIgG by 24-hour-activated blastocysts after pre-incubation in HSA or RIgG. Data showing uptake of labelled protein without pre-incubation are included for comparison.

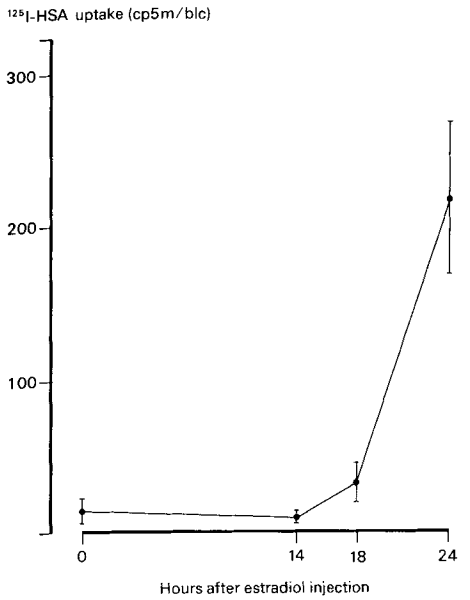

Fig. 3. Influence of blastocyst estradiol-activation on ^{125}I-HSA-uptake.

vestigation included 11 14-hour-activated and 9 24-hour-activated blastocysts incubated in ^{125}I-HSA. The number of blastocysts incubated in ^{125}I-RIgG was 15 and 11 respectively. After film coating, exposure was continued for 2 weeks. Details of procedures for film coating and development are described elsewhere (21).

For statistical analysis of data, Student's *t*-test modified for the comparison of two groups with unequal variances (15) was employed.

RESULTS

Binding of protein and blocking. All three radiolabelled proteins were more heavily bound to 24-hour-activated than to 14-hour-activated blastocysts (in each case $p<0.001$) (Fig. 1).

The binding of labelled HSA and RIgG to 24-hour-activated blastocysts could be partially blocked by pre-incubation of the blastocysts with the respective unlabelled protein. For HSA the uptake decreased from 215 (no pre-incubation) to 60 cp5m/blc ($p<0.001$). After preincubation in RIgG the ^{125}I-RIgG uptake was reduced from 162 (no pre-incubation) to 76 cp5m/blc ($p<0.01$) (Fig. 2).

Crosswise pre-incubation, i.e. RIgG followed by ^{125}I-HSA, also reduced the uptake of the labelled protein (from 215 to 41 cp5m/blc, $p<0.001$). However, the reciprocal combination, HSA followed by ^{125}I-RIgG, did not reduce the binding of the latter protein ($p>0.05$) (Fig. 2).

Influence of blastocyst activation on protein binding. Approximately the same low amount of ^{125}I-HSA was bound to experimentally delayed and 14-hour-activated blastocysts ($p>0.05$). A slight increase was seen between 14 and 18 hours of activation ($p<0.02$) and at 24 hours the uptake was very pronounced (18 versus 24 hours, $p<0.001$) (Fig. 3).

Influence of temperature on protein binding. Raising the incubation temperature from 0 to 37°C did not markedly affect the binding of ^{125}I-HSA to the 24-hour-activated blastocysts. Although a slight increase in protein uptake was observed, it was not statistically significant ($p>0.05$) (Fig. 4).

Distribution of bound protein. Whole blastocyst autoradiography revealed that the labelled protein was heavily bound in patches, probably corresponding to individual or few adjacent trophoblast cells. These patches were preferentially located in the abembryonic half of the post-attachment blastocysts (Fig. 5).

The 14-hour-activated blastocysts had only trace amounts of protein bound. Patches of intense activity were never seen, the weak radioactivity being spread diffusely over the whole blastocyst surface (Fig. 6).

The pattern of protein uptake at 14 and 24 hours of activation was identical for both ^{125}I-HSA and ^{125}I-RIgG.

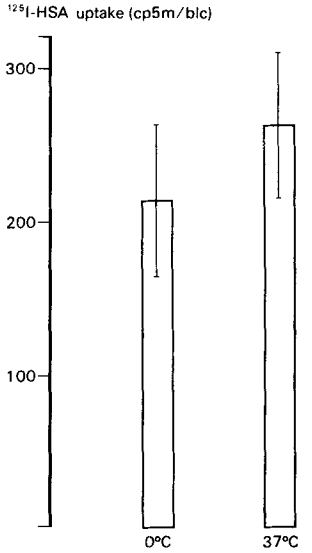

Fig. 4. Influence of temperature on ^{125}I-HSA-uptake by 24-hour-activated blastocysts.

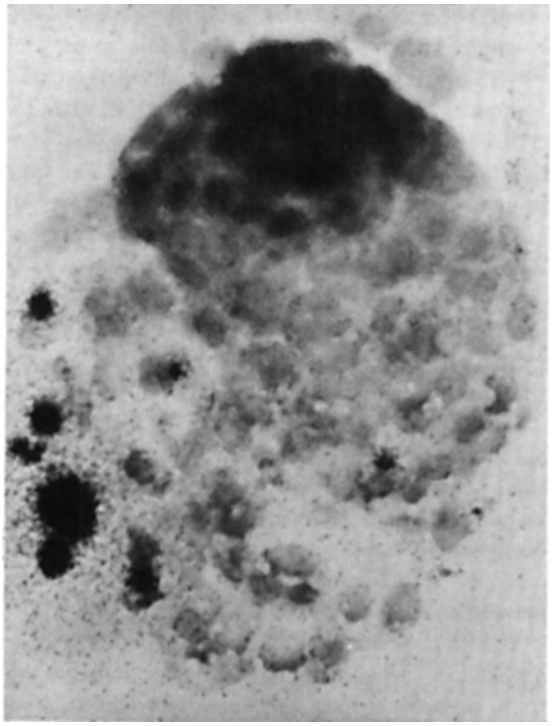

Fig. 5. A 24-hour-activated blastocyst incubated in ^{125}I-RIgG. Note heavy activity in patches localized in the abembryonic half of the blastocyst. ×350.

DISCUSSION

The results of the present investigation show that the implanting mouse blastocyst acquires the ability to bind different proteins 18 to 24 hours after estrogen-activation from experimental delay. Since it was possible to partially block the uptake of ^{125}I-HSA and ^{125}I-RIgG with the respective unlabelled protein, it is suggested that specific protein receptor sites exist on the surface of the blastocyst at this stage of implantation. This conclusion conforms to the receptor-concept created from other studies on selective protein binding to the cell surfacd, e.g. the binding of immunoglobulin to cells participating in the transfer of immunity from mother to young (2, 3) and the binding of various surface markers to B-lymphocytes (7, 8, 13). The fact that the binding of ^{125}I-HSA to blastocysts could be partially blocked by unlabelled RIgG while HSA did not impede the uptake of ^{125}I-RIgG, then may indicate that HSA and RIgG are bound to different receptor sites and that the binding of RIgG interferes with the "HSA-receptor" whereas the binding of HSA does not change the binding capacity of the "RIgG-receptor".

The degree of blastocyst activation greatly influenced the uptake of protein. During delay of implantation and at 14 hours of estradiol-activation the uptake of ^{125}I-HSA was low. After 18 hours' activation a slight increase was seen and at 24 hours the uptake was greatly enhanced. This suggests that the development of protein receptors is linked to the attachment of the blastocyst to the uterine epithelium, known to occur approximately 16 hours after estrogen-activation (Bergström & Nilsson, unpublished data). If the blastocyst is more vulnerable to mechanical trauma by flushing after than before attachment, it may be argued that protein uptake could have been due to passive diffusion of protein into damaged cells. However, considering the reduction of protein uptake by blocking, a non-specific uptake mechanism does not seem conceivable. Furthermore, preliminary data from immunoelectron microscopic studies indicate that protein uptake of 24 hour activated blastocysts is con-

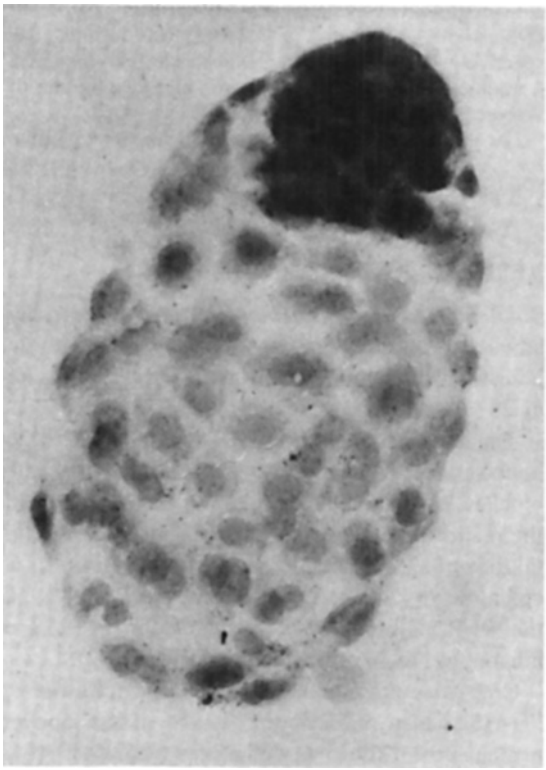

Fig. 6. A 14-hour-activated blastocyst incubated in ^{125}I-RIgG. Only trace amounts of radioactivity, distributed over the whole blastocyst surface can be detected. ×350.

fined to the trophoblast cell surface (Håkansson, unpublished data).

The mechanism of blastocyst attachment is not fully understood. It has been shown that the number of electro-negative sites on the surface of the blastocyst decreases at implantation (10, 11) and that the chemical composition of the blastocyst surface coat changes (6, 12). It is possible that these events are essential for blastocyst attachment. The development of protein binding sites on the implanting blastocyst might also contribute to attachment by establishing close contact between the trophoblast cells and the uterine epithelium.

The protein content of mouse blastocysts from normal pregnancy increases sharply immediately prior to implantation (19). This phenomenon appears to be related to an increase in the incorporation of radiolabelled amino acids (20), suggesting that the relatively high protein content of the implanting blastocyst reflects an increase in its endogenous protein synthesis. However, there exists evidence that the pre-implantation embryo also incorporates exogenous protein. In the rat, it has been demonstrated that blastocysts take up protein both *in vitro* and after injection of protein into the uterine lumen, accomplished by pinocytotic activity in the trophoblast cells (14).

The presence of protein receptors on the attaching blastocyst could presumably facilitate selective ingestion of exogenous protein. Transport of IgG across rabbit yolk membrane is preceded by specific binding and cell-membrane vesiculation (17). However, this process is probably not restricted to the transport of IgG since receptors for albumin also are present on the yolk sac membrane (16). Thus it seems, that the implanting mouse blastocyst, by carrying protein receptors and exhibiting pinocytotic activity possesses the tools required for selective utilization of an exogenous protein source.

The pinocytotic activity in the trophoblast cells of the mouse blastocyst is nil at 0°C, regardless of hormonal influence (Håkansson, unpublished data). Therefore, in the present investigation, the blastocyst incubations were maintained in the cold to avoid intracellular incorporation of protein. However, although pinocytotic activity is temperature dependent (18), raising the incubation temperature to 37°C did not to a statistically significant extent increase the uptake of ^{125}I-HSA by the post-attachment blastocysts. It appears probable though, that the technique presently used was not sufficiently sensitive to register the amounts of protein ingested by pinocytosis. The non-significant increase observed at 37°C may possibly reflect an increased pinocytotic activity in the trophoblast cells of the blastocyst.

The autoradiographic study indicated that the bound protein was not evenly distributed on the blastocyst surface, but was located in patches, probably corresponding to individual cells or cluster of juxtaposed cells preferentially located in the abembryonic half of the blastocyst. This pattern agrees with the ultrastructural appearance of the surface of the post-attachment mouse blastocyst. At this stage, proliferation of abembryonic trophoblast cells commences. However, the differentiation of these cells is asynchronous in that single or few adjacent cells show signs of close contact with the uterine epithelium whereas other cells do not seem to have established this contact (1). Thus, it may be possible that the trophoblast cells which first come in contact with the uterine epithelial lining also have developed protein receptor sites. This phenomenon, apart from its possible role in blastocyst attachment and ingestion of exogenous protein, may then also have immunological bearings. If the protein uptake masks antigenic sites on the invading trophoblast cells this could functionally resemble immunological enhancement and be of importance for the ability of the allogeneic conceptus to escape intrauterine rejection. Although it has been previously demonstrated that paternal histocompatibility antigens of the strong H-2 locus have disappeared from the surface of the mouse blastocyst shortly before attachment (5), it is possible that weaker transplantation antigens are still present (4). Obviously, the masking of any such persistent antigens could also benefit the immunologically alien fetus.

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