Affinity of uterine luminal proteins for rat blastocysts*

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Summary. The binding of ^{125}I -labelled rat uterine luminal proteins from Day-5 pregnant rats showed higher binding affinity to blastocysts than did the binding of proteins in uterine fluid from pro-oestrous rats (Day 0), rat serum albumin (RSA) or bovine serum albumin (BSA). Apparently little uptake of proteins into cells by phagocytosis or entry into the blastocoelic cavity occurred since similar results were obtained in the presence of sodium azide or cytochalasin B. Autoradiographic studies showed that the proteins were localized on the outer surface of the blastocyst. The binding was Ca^{2+} -dependent. Denaturation of Day-5 uterine proteins at 80°C reduced the counts to the values obtained with undenatured RSA and Day-0 fluids; this residual binding was considered as non-specific. The binding of labelled Day-5 uterine proteins was substantially reduced in the presence of unlabelled Day-5 proteins but to a lesser extent in the presence of RSA or rat serum. The dissociation of the bound labelled Day-5 uterine proteins occurred most rapidly in the presence of unlabelled Day-5 proteins. However, dissociation occurred within 2 h in the presence of other macromolecules, suggesting that the binding was not strong.

Introduction

Studies on several species show that blastocyst implantation occurs when particular groups of proteins are detected in the uterine lumen (Daniel, 1971; Renfree, 1972; Beier, 1974; Aitken, 1974; Beato, 1977; Pratt, 1977; Surani, 1977a; Fishel, 1979). Treatment with oestrogen and progesterone sensitizes the uterus for blastocyst implantation in the rat and the mouse (Psychoyos, 1973) and once the embryos have entered the uterine lumen, a period of at least 1 day intervenes before implantation commences. Considerable changes are detected in the uterine luminal proteins during the oestrous cycle and early pregnancy as a result of de-novo synthesis and secretion of uterine proteins and selective transudation of serum macromolecules into the lumen (Beier, 1974; Surani, 1977a, b; Edwards & Surani, 1978). Little is known about the role of luminal proteins or indeed the nature of the association of the proteins with blastocysts before implantation.

Implantation does not proceed during pro-oestrus or in ovariectomized rats and mice experiencing delayed implantation (Surani, 1977a; Pratt, 1977; Fishel, 1979) when blastocysts remain in a quiescent state (McLaren, 1973; Weitlauf, 1974); implantation resumes when the spayed animals are injected with ovarian steroids and after the appearance of characteristic macromolecules in the lumen (Surani, 1977a). In the rabbit, the appearance of the appropriate luminal proteins can be delayed by injecting oestradiol soon after mating, and implantation is similarly delayed until the appearance of the macromolecules characteristic for the time of

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implantation (Beier, 1974). Hence, sensitization of uterus by oestradiol and progesterone for implantation and the metabolic activity of blastocysts are apparently closely related to the occurrence of characteristic groups of macromolecules in the lumen. In this study, therefore, we investigated the nature and the extent of the association between uterine luminal proteins and blastocysts in rats.

Materials and Methods

Animals

Adult virgin female Wistar rats (Olac, Bicester, Oxon.) of 130–150 g body weight were kept under standard conditions and vaginal smears were taken daily to detect rats at pro-oestrus. For mating, females were housed 2 per cage with a male. The day on which spermatozoa were detected in the vaginal smear was taken as Day 1 of pregnancy. Some rats were killed at 12:00 h on Day 5 *post coitum* to obtain uterine luminal proteins while others were killed at 14:00–15:00 h on Day 5 of pregnancy to provide blastocysts.

Chemicals

Iodine-125 (sp. act. 100 mCi/ml) was obtained from the Radiochemical Centre, Amersham, Bucks, U.K. The amino acids for embryo culture medium, rat serum albumin, bovine serum albumin and cytochalasin B were obtained from Sigma, London. Fetal calf serum, vitamins and antibiotics were from Gibco Biocult (Paisley, Scotland), Sephadex-G25 from Pharmacia (Uppsala, Sweden) and the salts and other reagents were of Analar grade from the British Drug House (Poole, Dorset).

Recovery of uterine luminal proteins

The detailed procedure for the recovery of uterine luminal proteins has been described elsewhere (Surani & Webb, 1977). About 30 µg protein were obtained from each pregnant rat at 12:00 h on Day 5 of pregnancy. The samples were pooled from 4–8 rats, centrifuged at 12 000 g for 1 h and total protein was estimated using the method of Lowry, Rosebrough, Farr & Randall (1951). The sample volumes were adjusted to give 0.3 mg protein/ml in 0.25 M- PO_4^{3-} , pH 7.3. Uterine luminal fluid was similarly obtained from 4 pro-oestrous rats and combined to give a final concentration of 0.3 mg protein/ml. The samples were collected on the day the iodination procedure was carried out and kept at 0°C for 2–3 h during this time.

Blood samples were obtained by cardiac puncture just before the animals were killed, and centrifuged at room temperature for 1 h at 12 000 g and stored at –20°C.

Iodinations

Iodination of uterine luminal proteins and albumins was carried out using the chloramine-T method (Greenwood, Hunter & Glover, 1963). The stock solutions required for iodination of proteins were prepared as follows: 5.0 mg chloramine-T/ml 0.05 M-sodium phosphate buffer, pH 7.5, 12 mg sodium metabisulphite/ml 0.05 M-sodium phosphate buffer, pH 7.5, and 100 mg potassium iodine/ml phosphate buffer. Small amounts (1.0 mCi) of Na^{125}I solution were dispensed into 4 ml polystyrene tubes followed by 17 µg protein in 0.25 M- PO_4^{3-} , pH 7.5 and 20 µg chloramine-T. The tube was agitated by hand for 30 sec and 48 µg sodium metabisulphite were added followed by 0.5 mg potassium iodide. The total volume of the reaction mixture was usually 80–85 µl.

The ^{125}I -labelled proteins were separated from free iodine by elution through a Sephadex G-25 column prepared before the iodination procedure. The 0.5×7 cm column was presaturated by passing 10 mg rat albumin in $100\ \mu\text{l}$ $0.25\ \text{M-PO}_4^{3-}$, pH 7.3, through the column followed by extensive washing with the protein-free embryo culture medium (Spindle & Pedersen, 1973) to prevent adsorption of the small amount of iodinated proteins to the gel (Greenwood *et al.*, 1963). Fractions, each of $40\ \mu\text{l}$, were collected from the column when the iodinated sample was eluted through the column with the protein-free embryo culture medium. Thirty fractions were collected and $1\ \mu\text{l}$ aliquots were counted in a gamma counter (Nuclear Enterprises) for 10 min to determine the radioactivity of the individual fractions. The iodinated protein fractions were found in a total of $80\text{--}150\ \mu\text{l}$ culture medium and between 45 and 65% of ^{125}I was usually bound to the proteins in all cases. The specific activities of the preparations were $25\text{--}35\ \mu\text{Ci}/\mu\text{g}$ uterine luminal proteins and $30\text{--}40\ \mu\text{Ci}/\mu\text{g}$ albumins. Samples of the iodinated proteins were treated with trichloroacetic acid at 4°C to precipitate the labelled proteins (Fishel & Surani, 1978). Between 90 and 97% of the radioactivity in the fresh samples was precipitable with TCA illustrating that the samples had been essentially rendered free of unbound ^{125}I .

In the initial experiments the iodinated samples were used in binding assays without the addition of rat albumin. In later experiments it was established that the addition of 4 mg albumin/ml reduced the counts obtained after binding assays with embryos by only 10–13%. Therefore 4 mg rat serum albumin/ml were added to all the samples immediately after iodinations and before 5–10 μl fractions of the samples were stored at -20°C . Addition of albumin to the samples improved the stability of the iodinated proteins. Samples were stored for a maximum of 2 weeks, after which fresh preparations of the iodinated proteins were obtained.

Culture of blastocysts

Zonae pellucidae were removed from the blastocysts by incubating the embryos for 3–5 min at 37°C in phosphate-buffered saline containing 0.5% pronase (Calbiochem, Bishops Cleeve, Herts) and 1% polyvinylpyrrolidone (May and Baker, Dagenham, Essex). The embryos were then washed 6 times and cultured overnight in the culture medium with 10% fetal calf serum. The embryos were washed twice in the medium without protein and 6 times in medium with 10 mg polyvinylpyrrolidone/ml (PVP) and cultured in this medium for 1 h. To test whether embryos rendered free of the zona pellucida by pronase *in vitro* behaved similarly to the zona pellucida-free embryos from the uterus, the latter were flushed from uteri at 20:00 h on Day 5 of pregnancy. In these pilot experiments, the counts obtained after binding assay with Day-5 uterine luminal proteins were similar in both cases (approximately 260 c.p.m./embryo), and therefore embryos denuded of the zona pellucida *in vitro* were used in all the subsequent experiments.

Binding assay

Groups of embryos (6–20) were transferred to 5–10 μl droplets of iodinated proteins eluted in the protein-free culture medium kept at 37°C under paraffin oil in 5% CO_2 in air. After the completion of incubation the embryos were washed 12 times in droplets of medium with 10 mg PVP/ml and counted in groups of 2–6 in a gamma counter for 10–20 min. The counts of the last three drops of the washing medium were found to be near the level of background.

Autoradiography

Groups of embryos (2–4) were fixed in Bouin's fixative, after the binding assay, for 2 h in an embryological dish and then washed 4 times in 70% alcohol. They were then placed on glass slides and coated with the Ilford photographic emulsion (Ilford, Essex) at 50°C . The slides were kept at 4°C in a light-tight container for a maximum of 1 week before developing and fixing the slides. The embryos were stained with haematoxylin and observed under Wild M20 microscope.

Results

Changes in the binding of the iodinated proteins with time were investigated at 37°C (Text-fig. 1). The association of ^{125}I -labelled uterine luminal proteins from pregnant rats with blastocysts was higher than that obtained with rat serum albumin or proteins in uterine luminal fluid from pro-oestrous females. The reaction reached a plateau after 2–3 h of incubation. Similar results were obtained at 0°C although all the counts were about 4 times lower. In subsequent experiments 4 mg unlabelled RSA/ml were added to all the iodinated samples. The results were similar to those shown in Text-fig. 1, apart from a reduction in all the counts by 10–13%. In all subsequent experiments, unlabelled RSA was added to the iodinated samples to reduce the non-specific binding.

Text-fig. 1. Time-dependent changes in the binding of iodinated (^{125}I) proteins (O = Day-5 pregnant rat uterine luminal proteins; Δ = pro-oestrous rat uterine luminal proteins; $\bullet$ = rat serum albumin) to blastocysts at 37°C. Each point represents mean values of c.p.m./blastocyst $\pm$ s.e.m. for 15–35 blastocysts from 4–6 experiments.

Attempts were made to obtain precise values for any non-specific binding using high concentrations of unlabelled uterine luminal proteins, which is the procedure usually adopted for binding assays (Cuatrecasas, 1971). However, it proved difficult to concentrate uterine luminal proteins beyond 0.6–0.8 μg protein/ml since the proteins formed aggregates. Therefore, the effects of heat denaturation of the samples and their binding to the blastocysts were estimated. The results in Text-fig. 2 clearly demonstrate a substantial reduction in the association of ^{125}I -labelled Day-5 proteins with blastocysts. The counts obtained after heat denaturation of the Day-5 proteins at 80°C were considered as representing non-specific binding of uterine luminal proteins and were therefore subtracted from the total counts to obtain values for specific binding. The non-specific binding counts were similar to those obtained with ^{125}I -labelled RSA proteins from Day 0 rats in the presence or absence of 4 mg unlabelled RSA/ml. Trichloroacetic acid (TCA) protein precipitation of heat-denatured Day-5 proteins showed that 85% of the counts were in the precipitable fraction compared to 90–95% in fresh samples. The reduction in the counts after heat denaturation is therefore not due to the reduction in the bound iodine *per se*. Moreover, samples that were stored for 4 weeks or more gave 70% counts in the precipitable fraction and the counts per blastocyst after binding assays gave about 50% lower counts, presumably because of denaturation of the high specific activity proteins in the samples.

Various drugs were used to assess if substantial uptake of proteins into the cells by phagocytosis or entry into the blastocoelic cavity occurred. In the presence of 1 mM-sodium azide, which is an inhibitor of energy metabolism and prevents phagocytic activities of cells (Hollenburg & Cuatrecasas, 1975), the counts were similar to those obtained in the controls (Table 1). When blastocysts were incubated in 5–10 μg cytochalasin B/ml, an inhibitor of microfilaments (Wessells *et al.*, 1971), the embryos collapsed and the blastocoelic cavity was obliterated.

Text-fig. 2. Effect of heat denaturation for 30 min at 55°C, 65°C and 80°C on the binding to blastocysts of ^{125}I -labelled Day-5 pregnant rat uterine luminal proteins (●) and ^{125}I -labelled rat serum albumin (O).

However, the counts (224–234 c.p.m./blastocyst) were again similar to those obtained in the controls. Therefore little uptake of the proteins into the cells by phagocytosis or entry into the blastocoelic cavity occurred and the counts obtained after the assay presumably represent mainly the localization of the proteins on the surface of the blastocysts. Autoradiography of the blastocysts after binding assays also appeared to show localization of the proteins on the outer surface of the embryos. Since the binding was inhibited strongly in the presence of 1.7–2.0 mM-ethylene glycol-bis(β -aminoethyl ether)-N,N'-tetraacetic acid (EGTA, Sigma, London), which chelates Ca^{2+} , the binding appears to be dependent on extracellular Ca^{2+} .

Table 1. Mean $\pm$ s.e.m. binding of ^{125}I -labelled proteins to blastocysts after various treatments*

Labelled proteins	Treatments	No. of tests§	No. of blastocysts	Counts (c.p.m./blastocysts)		
				Total	% of maximum total	% of maximum specific
Day 5	None	27	232	236 $\pm$ 16	100 $\pm$ 6	100 $\pm$ 9
Day 5	Heat (55°C)†	2	20	231 $\pm$ 10	98 $\pm$ 4	97 $\pm$ 6
Day 5	Heat (65°C)†	2	20	118 $\pm$ 8	50 $\pm$ 2	25 $\pm$ 4
Day 5	Heat (80°C)†	3	23	78 $\pm$ 7	33 $\pm$ 3	0 $\pm$ 4
Day 5	Sodium azide (1 mM)‡	4	54	241 $\pm$ 8	102 $\pm$ 3	103 $\pm$ 4
Day 5	Cytochalasin B (5 $\mu\text{g}/\text{ml}$)‡	2	18	224 $\pm$ 14	95 $\pm$ 6	93 $\pm$ 9
Day 5	Cytochalasin B (10 $\mu\text{g}/\text{ml}$)‡	4	32	234 $\pm$ 8	99 $\pm$ 3	99 $\pm$ 4
Day 5	EGTA (1.0 mM)‡	2	18	233 $\pm$ 5	99 $\pm$ 2	99 $\pm$ 2
Day 5	EGTA (1.7 mM)‡	2	17	117 $\pm$ 7	50 $\pm$ 3	25 $\pm$ 5
Day 5	EGTA (2.0 mM)‡	2	18	76 $\pm$ 9	32 $\pm$ 4	0 $\pm$ 5
Pro-oestrous	—	2	26	68 $\pm$ 5	29 $\pm$ 2	
BSA	—	6	42	57 $\pm$ 6	24 $\pm$ 3	
RSA	—	3	23	47 $\pm$ 4	20 $\pm$ 2	
RSA	Heat (80°C)†	2	16	38 $\pm$ 5	16 $\pm$ 2	

* In all treatments 4 mg rat serum albumin/ml were added just after elution of the proteins from the Sephadex G-25 column. The blastocysts were incubated for 3 h in the droplets of ^{125}I -labelled proteins at 37°C.

† The iodinated proteins (with 4 mg rat serum albumin/ml) were immersed for 30 min in a waterbath at 55, 65 or 80°C, cooled and used for the experiments.

‡ Added to the ^{125}I -labelled proteins in $\times 40$ concentrated solutions so that only 2.5% dilution of the proteins took place.

§ Each test consisted of counts from 2 separate groups of embryos.

Competition assays were carried out to deduce the specificity of binding of Day-5 proteins to blastocysts. The results (Text-fig. 3) demonstrate that the binding was severely inhibited in the presence of unlabelled Day-5 proteins in the range of 0.3 to 0.6 mg protein/ml. A higher concentration of about 6.0 mg RSA/ml was required to give similar inhibition of binding of ^{125}I -labelled Day-5 proteins. Serum from Day-5 pregnant rats gave similar results at about 3.0–4.0 mg protein/ml.

Text-fig. 3. Competition assay to deduce the specificity of binding of ^{125}I -labelled Day-5 pregnant rat uterine luminal proteins to blastocysts. The inhibition of binding of the labelled proteins is shown in the presence of increasing amounts of unlabelled Day-5 pregnant rat uterine luminal proteins (Δ), Day-5 pregnant rat serum ($\times$) or rat serum albumin (O). In all tests, 4 mg rat serum albumin/ml were also added to reduce the non-specific binding. Each value is a mean $\pm$ s.e.m. using 20–40 blastocysts from 5 experiments.

Text-fig. 4. Competition assay to deduce the specificity of dissociation of bound ^{125}I -labelled Day-5 pregnant rat uterine luminal proteins. Binding of the labelled proteins was carried out at 37°C for 3 h. Dissociation of the bound proteins was monitored for up to 2 h in the presence of 10 mg polyvinylpyrrolidone/ml ($\bullet$), 4 mg rat serum albumin/ml (O), 4 mg pregnant rat serum/ml ($\times$), 0.6 mg Day-5 pregnant rat uterine luminal proteins with 3.4 mg rat serum albumin/ml (Δ), 0.6 mg pro-oestrous rat uterine luminal proteins with 3.4 mg rat serum albumin/ml ($\diamond$), or Ca^{2+} -free medium with 10 mg polyvinylpyrrolidone/ml ($\square$). Each value is a mean $\pm$ s.e.m. using 20–40 blastocysts from 5 experiments.

Study of the dissociation of the ^{125}I -labelled Day-5 proteins bound to blastocysts in the presence of various macromolecules demonstrated (Text-fig. 4) that the most rapid dissociation of the bound proteins from blastocysts occurred when the embryos were transferred to medium with 0.6 mg Day-5 proteins/ml. Dissociation of the proteins was less effective in the presence of

PVP, whereas RSA, rat serum and uterine luminal proteins from pro-oestrous rats gave intermediate results. The dissociation also occurred rapidly in medium without Ca^{2+} , which again demonstrated that the binding was Ca^{2+} -dependent. However, most of the bound proteins were dissociated after about 2 h in the presence of all the other macromolecules, which suggested that the binding of ^{125}I -labelled Day-5 proteins, even though it may be specific as judged by the two competition assays, was not especially strong.

We did not test binding to other tissues or stages of embryos since the calculations of the surface area of cells proved difficult and the nonspecific binding value of about 30% is rather high.

Discussion

This study demonstrates that the uterine luminal proteins from Day-5 pregnant females have a higher affinity for blastocysts than do uterine proteins from pro-oestrous rats or albumin. Although both the uterine samples are heterogeneous mixtures of proteins, they are qualitatively distinct (Surani, 1977a, b). The differences in the binding affinities may therefore be related to the biochemical differences in the samples. Competition assays illustrate the specific nature of the binding to blastocysts of Day-5 uterine luminal proteins, the day of blastocyst implantation in the rat. The binding is Ca^{2+} -dependent since it is inhibited in the presence of EGTA. How the binding of Day-5 uterine proteins to blastocysts affects them is not known. However, the metabolic enhancement of embryos in the presence of fetal calf serum *in vitro* has been demonstrated (McLaren, 1973; Fishel & Surani, 1978; Edwards & Surani, 1978) and is probably due to binding of some mitogenic serum macromolecules to the cell surface receptors. Perhaps some Day-5 uterine proteins may act similarly on blastocysts *in vivo*. Alternatively, the binding of luminal proteins to the embryonic surface may enhance specific adhesion at the onset of implantation since the cell surface macromolecules are important in cell adhesion (Edwards, 1978).

The finding that the bound proteins are rapidly dissociated from the blastocysts may also be significant since the embryos may remain biochemically active only so long as certain macromolecules are present in the environment. This indeed occurs *in vitro* since blastocysts in the presence of fetal calf serum macromolecules show a high rate of RNA synthesis which declines rapidly when the embryos are transferred to a medium containing only bovine serum albumin (Fishel & Surani, 1978). This response is observed perhaps because of the rapid dissociation of the bound serum proteins *in vitro*. Similar reversible response and adaptation by blastocysts *in vivo* are demonstrated by their ability to undergo diapause or implant after reciprocal transfers of embryos between females with normal implanting blastocysts and those experiencing delay of implantation (see McLaren, 1973). Significantly, the characteristic macromolecules present in the uterine lumen at the time of implantation are undetectable in females undergoing delay of implantation (Surani, 1977a). Hence the reversible binding of macromolecules to blastocysts may be relevant for synchronization of blastocyst activity and sensitization of the uterus at the time of implantation. Such reversible binding and dissociation of protein to blastocysts may account for the reversible metabolic enhancement and pleiotypic response displayed by the blastocysts (Surani, 1977a). Further tests may confirm these functions for uterine luminal proteins although the proteins and the total number of cells in embryos are highly limiting in the types of experiments that can be conducted with the rat.

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