Changes in secreted uterine proteins associated with embryo implantation in the mouse

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Summary. A dual-label ratio method was used in conjunction with two-dimensional polyacrylamide gel electrophoresis to measure the relative changes in rates of production of individual secreted proteins by mouse uteri at the start of the process of decidualization. A characteristic pattern of differential changes in the rates of synthesis and secretion of the proteins was found to be associated with development of a positive Pontamine Blue reaction at the site of embryo implantation. These changes were compared with those associated with development of experimentally induced deciduomata and although the patterns were similar, presumably reflecting common processes in transformation of the endometrium, there was preferential enhancement of a subset of small (M_r 14 000–20 000) acidic proteins in the authentic implantation sites. It is suggested that this embryo-dependent modification of constitutive changes associated with decidualization reflects a form of embryo–maternal signal–response mechanism that may be important for the process of implantation in mice.

Keywords: implantation; uterine proteins; embryonic signalling; decidual reaction; mouse

Introduction

There are several physiological adjustments in the mammalian female associated with the recognition of pregnancy (see Heap *et al.*, 1979). Because these changes depend on the presence of a viable embryo in the uterus and may occur before the time of implantation, it is generally accepted that some form of communication involving diffusible signals must exist between mother and embryo, and various substances including steroids, prostaglandins, histamines and proteins have been proposed as possible messages (see Heap *et al.*, 1979; Sauer, 1979; Kennedy, 1983, for reviews). Although the actual mechanism is not clearly understood, the hypothesis that it involves changes in secreted uterine proteins (Gore-Langton & Surani, 1976; Pratt, 1977; Aitken, 1977a, b, 1979; Fishel & Surani, 1978; Fishel, 1979, 1980) is particularly appealing. In the first place, it can be imagined that protein regulators of blastocyst metabolism or growth could be highly specific and might, therefore, be ideal for co-ordinating interactions between an embryo and the uterus as pregnancy is becoming established. Second, quantitative and qualitative changes in uterine proteins have been observed at the time of implantation in a variety of animals and hence there are candidates for the roles of diffusible signal factors that can be tested experimentally.

To evaluate this hypothesis, it is essential to determine whether cause and effect relationships exist between changes in specific proteins and some variable aspect of blastocyst development or the process of implantation itself. Although several investigators working with mice have attempted to develop evidence along these lines (Gore-Langton & Surani, 1976; Pratt, 1977; Aitken, 1977a, b, c; Fishel, 1979, 1980), there were technical problems associated with separating individual protein species from the complex mixture of uterine secretions as well as with quantitating changes in the small amounts of material available. Hence, little progress has been made

towards rigorously defining mechanisms involving protein signal factors that might be important for the process of implantation in the mouse.

However, with the introduction of high-resolution two-dimensional polyacrylamide gel electrophoresis (O'Farrell, 1975), problems associated with separating the proteins have been solved and, using that method, Nieder *et al.* (1987) and Nieder & Macon (1987) observed that there are qualitative changes in the electrophoretic pattern of secreted uterine proteins associated with embryo implantation in mice. In the present work, high-resolution two-dimensional-PAGE has been coupled with a dual-label ratio method for quantitating relative changes in rates of production of individual uterine secreted proteins in order to evaluate their potential to act as part of a signal-response mechanism at implantation.

Materials and Methods

A dual-label ratio method was used in conjunction with two-dimensional-PAGE to compare rates of production of individual secreted proteins by Pontamine Blue-positive and -negative segments of mouse uteri. The procedure was adapted from those described by Brinster *et al.* (1979) and Chen *et al.* (1980). Briefly, the method involves incubating tissue from Pontamine Blue-stained regions with [³H]methionine and that from non-reactive regions with [³⁵S]methionine *in vitro* to allow synthesis and secretion of labelled proteins. Aliquants of conditioned medium containing the differentially labelled proteins are mixed together and electrophoresed simultaneously, and radioactivity in the individual proteins is measured by scintillation spectroscopy. Statistical comparisons of the ratios of isotopes are used to determine which proteins have greatest differences in rates of production between the experimental and reference conditions.

Preparation of animals. Virgin white Swiss mice (6–8 weeks of age) at random stages of the oestrous cycle were used. Animals being used to provide early authentic implantation sites were induced to ovulate with gonadotrophins (Fowler & Edwards, 1957) and placed with fertile males; the presence of a vaginal plug on the following morning confirmed mating (designated as Day 1 of pregnancy). The animals were ovariectomized bilaterally on the morning of Day 3 and injected with progesterone (2.0 mg/day s.c. in 0.1 ml sesame seed oil) on Days 4, 5, 6, 7, 8 and 9. In addition they received oestradiol-17 β (25 ng s.c. on Day 9). Animals required to provide experimental deciduomata were not mated; the virgin females were ovariectomized bilaterally, allowed to recover for 1 week and divided into two sub-groups: those in (a) were given hormones as described above but had a sterile surgical thread (3.0 braided silk) inserted in the uterine lumen at the time of the last injection; those in (b) were given oestradiol (0.1 μ g s.c.) for 3 days followed by 2 days without treatment and 3 days of oestradiol combined with progesterone (0.0125 μ g and 1.0 mg respectively) followed by instillation of 10 μ l arachis oil into one uterine horn (Finn & Martin, 1970). Animals in all groups were given Pontamine Blue dye (0.1 ml 1% in 0.154 M-NaCl, i.v.) 24 h after the last hormone treatment and killed 15 min later.

Preparation of uterine explants. In preliminary experiments (Weitlauf, 1988), embryos were flushed from the excised uterine horns of pregnant animals with a stream of tissue culture medium (BMOC₃; Brinster, 1971; with 100 μ g bovine serum albumin/ml) from a 1-ml syringe fitted with a blunt needle. Cross-sections of uterus (3–6 mg wet weight) were cut from fertile horns and opened longitudinally to expose the endometrial surface. These explants were placed in 50 μ l BMOC₃ (with 100 μ g bovine serum albumin/ml) to which [³⁵S]methionine was added (sp. act. 1100 Ci/mmol; New England Nuclear, Boston, MA; final concentration 200 μ Ci/ml). The incorporation of labelled methionine into TCA-precipitable secreted proteins was linear from 2 to 6 h (c.p.m. = 31 000 [h] – 43 400; r^2 = 0.98) and 3 h was selected as a standard incubation period for the subsequent experiments.

Tissue from authentic implantation sites (i.e. blue areas) or interimplantation regions (i.e. clear areas) was taken only after flushing the uteri and accounting for all embryos (i.e. the number of blastocysts recovered equalled the number of blue bands in the horn) and the segments were opened longitudinally for incubation as described above. Tissue explanted from the implantation sites was placed in 50 μ l medium (BMOC₃ with 100 μ g bovine serum albumin/ml) to which [³H]methionine (sp. act. 85 Ci/mmol; Amersham; Arlington Heights, IL) was added (final concentration 400 μ Ci/ml); tissue from interimplantation sites was placed in medium to which [³⁵S]methionine was added (as above). After incubation the explanted uterine strips were removed and the conditioned medium was lyophilized, reconstituted in 35 μ l of an electrophoresis lysis buffer consisting of 8 M-urea, 4% Nonidet P-40, and 2% ampholytes pH 3.5–9.5 (LKB, Gaithersburg, MD) and stored at –70°C. Uteri with deciduomata resulting from placement of an intraluminal thread or instillation of oil were treated similarly, that is Pontamine Blue regions were incubated with [³H]methionine and clear areas incubated with [³⁵S]methionine. In some experiments, tissue was washed and dissolved (1 N-NaOH at 60°C for 0.5 h) after incubation and protein content was determined by the method of Lowry *et al.* (1951).

Electrophoresis. Two-dimensional electrophoresis on polyacrylamide gels was carried out on the conditioned media according to the procedure of O'Farrell (1975) incorporating the modifications of Anderson & Anderson (1978a, b). Samples of secreted proteins labelled with each isotope were mixed together (i.e. a total of ~100 000 c.p.m.

of ^3H and ^{35}S [1:1]). The isoelectric dimension was run in 1.5×105 mm tube gels with an ampholyte range of 3.5–9.5 at 1000 V for 11–15 h. The second dimension was a 10–18% linear polyacrylamide gradient (sodium dodecyl sulphate) slab gel run at a constant 3.5 W per slab. Molecular weight standards (^{14}C -mixture: Amersham, Arlington Heights, IL) were run in the second dimension to provide estimates of the sizes of proteins in the samples. Slab gels were fixed overnight in water:methanol:acetic acid (63:30:7 by vol.) and were saturated with fluor (Amplify: Amersham) before being dried onto filter paper. Gels were exposed on Kodak XAR-5 X-ray film for 7 days at -70°C .

In pilot experiments it was found that two-dimensional PAGE arrays of secreted proteins from authentic implantation sites were not obviously different from those secreted by non-reactive areas (compare Figs 1a and 1b) and the 15 most prominent spots were selected for analysis with the dual-label method; the numbering scheme is consistent with that used in previous work (Nieder *et al.*, 1987; Weitlauf, 1988). The fluorographs were used to locate radioactive areas that corresponded to these 15 spots, the gels were marked and the appropriate areas were cut out with scissors. The gel fragments were placed in scintillation vials, rehydrated with water (400 μl), and incubated with tissue solubilizer (Protosol, New England Nuclear; 1.0 ml at room temperature for 24 h). Acetic acid (200 μl) and scintillation cocktail (Ready-Solv HP, Beckman, Fullerton, CA; 10 ml) were added and the piece of gel was fragmented with a tissue homogenizer (10 sec with 12 mm Polytron probe generator operated at full speed: Brinkmann Instruments, Westbury, NY). The amounts of ^3H and ^{35}S in the individual proteins were determined simultaneously by scintillation spectroscopy using a dual-label d.p.m. programme with automatic quench control and correction for crossover on a Beckman LS 6800 scintillation counter. The ratio of ^3H to ^{35}S for each of the 15 individual proteins on the gel was determined (R_p), as was the overall average ratio for all proteins on the gel (R_i) and a normalized value for each protein was then calculated [R_p/R_i]. The advantage of this approach is that, with the exception of the factors representing absolute rates of protein synthesis and secretion, those variables that influence the amount of each isotope in the protein spots on a given gel are the same in R_p and R_i and so are eliminated mathematically. This can be shown as follows: the amount of radioactivity (d.p.m.) detected in a specific protein (A) under a given condition (i) will be a function of several factors: (1) the absolute rate of synthesis of A (R_{Ai}); (2) the number of copies of the tracer amino acid (e.g. methionine) that are contained in A (β_A); (3) several factors including the mass of the tissue, length of incubation and the specific activity of the intracellular pool of the tracer amino acid etc (ζ_i) for a specific experimental cycle (e.g. ω); (4) several other factors related to efficiency of loading the gel for any given electrophoretic run (δ); (5) the efficiency of detecting radioactivity for the isotope involved (e.g. $^3\text{H}_i$); and some constant (κ). Thus, the amount of radioactivity detected in protein A from decidualized uterine tissue (ii) in incubation (χ) and electrophoresis run (δ) can be expressed as follows: d.p.m. [^3H]A $_{ii}$ = $\kappa_1[R_{Aii}][\beta_A][\zeta_{ii}][\delta][^3\text{H}_i]$. Similarly, the amount of radioactivity detected in protein A from the non-reactive tissue (i) in incubation (ω) and some electrophoresis run (δ) is given by: d.p.m. [^{35}S]A $_i$ = $\kappa_2[R_{Ai}][\beta_A][\zeta_{\omega i}][\delta][^{35}\text{S}_i]$. The amount of radioactivity detected in total protein (T) for (ii) and (i) above is given by: d.p.m. [^3H]T $_{ii}$ = $\kappa_3[R_{Tii}][\beta_T][\zeta_{ii}][\delta][^3\text{H}_i]$ and d.p.m. [^{35}S]T $_i$ = $\kappa_4[R_{Ti}][\beta_T][\zeta_{\omega i}][\delta][^{35}\text{S}_i]$ respectively, where (R_T) is the average rate of protein synthesis and (β_e) reflects the amino acid (e.g. methionine) content of the average protein. Hence, R_p is:

$$\frac{\text{d.p.m. } [^3\text{H}]A_{ii}}{\text{d.p.m. } [^{35}\text{S}]A_i} = \frac{\kappa_1[R_{Aii}][\beta_A][\zeta_{ii}][\delta][^3\text{H}_i]}{\kappa_2[R_{Ai}][\beta_A][\zeta_{\omega i}][\delta][^{35}\text{S}_i]}$$

and R_i is:

$$\frac{\text{d.p.m. } [^3\text{H}]T_{ii}}{\text{d.p.m. } [^{35}\text{S}]T_i} = \frac{\kappa_3[R_{Tii}][\beta_T][\zeta_{ii}][\delta][^3\text{H}_i]}{\kappa_4[R_{Ti}][\beta_T][\zeta_{\omega i}][\delta][^{35}\text{S}_i]}$$

and therefore:

$$\begin{aligned} \frac{\text{d.p.m. } [^3\text{H}]A_{ii}}{\text{d.p.m. } [^{35}\text{S}]A_i} &= \frac{\kappa_1[R_{Aii}][\beta_A][\zeta_{ii}][\delta][^3\text{H}_i]}{\kappa_2[R_{Ai}][\beta_A][\zeta_{\omega i}][\delta][^{35}\text{S}_i]} & \frac{[R_{Aii}]}{[R_{Ai}]} \\ [R_p/R_i] &= \frac{\text{d.p.m. } [^3\text{H}]T_{ii}}{\text{d.p.m. } [^{35}\text{S}]T_i} = \frac{\kappa_3[R_{Tii}][\beta_T][\zeta_{ii}][\delta][^3\text{H}_i]}{\kappa_4[R_{Ti}][\beta_T][\zeta_{\omega i}][\delta][^{35}\text{S}_i]} & \frac{[R_{Tii}]}{[R_{Ti}]} \end{aligned}$$

The value of [R_p/R_i] is therefore proportional to the ratio of absolute rates of production of the individual protein of interest under the two conditions being studied relative to the overall average ratio of rates of protein production under those conditions. Because the values are normalized to a constant R_i for each gel, the data from different gels can be pooled and the results analysed statistically. In the experiments reported here the samples to be run on each gel were derived as follows: duplicate paired samples (e.g. 2 explants from implantation sites, and 2 explants from interimplantation sites) were removed from a single animal and incubated individually as described in the text (i.e. 2 with ^3H and 2 with ^{35}S). Paired samples were mixed (i.e. 1 from an implantation site incubated with ^3H and 1 from an interimplantation site incubated with ^{35}S) and the resulting mixture was run on a single gel. The data presented are from 19–24 gels for each experiment. In general then, for each experiment, tissue was collected from 9–12 animals; this resulted in 18–24 incubations with ^3H and a like number with ^{35}S ; between 19 and 24 gels were prepared from this material depending on the total number of samples actually collected and successfully processed in any given experiment. Because the dual-label ratios method controls for internal tissue and incubation variations, the gels

Fig. 1. Fluorographs of [^{35}S]methionine-labelled proteins synthesized and released by explants from (a) a Pontamine Blue-positive implantation site (b) a Pontamine Blue-negative inter-implantation region of the same horn, and (c) a Pontamine Blue-positive region of a deciduomata induced by an intraluminal thread. The samples contained 36 000 c.p.m. TCA-precipitable material and the fluorographs were exposed for 7 days. See text for details.

(or replicates) were considered to be the source of variance for statistical purposes. Kruskal-Wallis non-parametric analysis of variance was used to determine whether the ratios for any individual protein differed; when appropriate the a-posteriori Least Significant Difference Test (LSD; Kirk, 1968) was used to identify those with ratios significantly different from $[R_p/R_i] = 1.00$. The analyses were performed with an IBM 4341 mainframe computer using SAS (Statistical Analysis System; Keppel, 1982). When the values for $[R_p/R_i]$ are not different from 1.00, the difference in rates of production of the protein of interest in the two situations is not different from the overall average; with an $[R_p/R_i] < 1.00$ the difference is less than the average; and with an $[R_p/R_i] > 1.00$ it is greater than average. It is to be expected, therefore, that identical tissue samples incubated in $[^3H]$ methionine and $[^{35}S]$ methionine and compared in this way will have values of $[R_p/R_i]$ for each protein that are not different from 1.00. That premise was tested in pilot studies for the present experiments using tissue samples explanted from the same Pontamine Blue-positive early implantation sites; because the rates of production of each protein in the two samples were the same, there were no values of $[R_p/R_i]$ different from 1.00 (Fig. 2). Similar results were obtained in comparisons of tissue from artificial deciduomata explanted from animals with and without injections of Pontamine Blue dye, indicating that the dye does not have differential effects on rates of production of the individual proteins (data not shown).

Fig. 2. Evaluation of changes in rates of synthesis and secretion of individual uterine proteins by means of the dual-label ratio method. The comparison shown is from experiments in which two pieces of tissue were explanted from the same Pontamine Blue-positive early implantation site; because the rates of production of each protein in the two samples are identical, there are no values of $[R_p/R_i]$ different from 1.00. See text for details.

In some experiments duplicate samples were incubated with both isotopes and a variation of the dual-label ratio method introduced by Vallet *et al.* (1987) was used to compare rates of production of individual proteins by authentic implantation sites and deciduomata. The explants were prepared and incubated, and the conditioned medium with 3H -labelled proteins from authentic implantation sites (i.e. condition ii) was mixed with medium containing ^{35}S methionine-labelled proteins from deciduomata (i.e. condition i). The samples were electrophoresed, and the radioactivity determined as described above; the reciprocal mixtures were also made and treated similarly (i.e. 3H -labelled proteins from deciduomata and ^{35}S -labelled proteins from implantation sites). The 3H to ^{35}S ratios for each protein (e.g. A) from both sets of incubations were determined (i.e. R_{p1} and R_{p2} respectively). R_{p1} is given by:

$$\frac{\text{d.p.m. } [^3H]A_{ii}}{\text{d.p.m. } [^{35}S]A_i} = \frac{\kappa_i [R_{Aii}] [\beta_A] [\zeta^3H_i] [\delta_i] [^3H_i]}{\kappa_2 [R_{Ai}] [\beta_A] [\zeta^{35}S_o] [\delta_o] [^{35}S_o]}$$

where [i and ii], $[R_{Ai}]$ and $[R_{Aii}]$, $[\beta_A]$, and the factors depicting counting efficiency are as described above and $[\zeta^3H_i]$ and $[\zeta^{35}S_o]$ represent the first set of incubations and $[\delta_o]$ that electrophoretic run. R_{p2} then is given by:

$$\frac{\text{d.p.m. } [^3H]A_i}{\text{d.p.m. } [^{35}S]A_{ii}} = \frac{\kappa_1 [R_{Ai}] [\beta_A] [\zeta^3H_i] [\delta_i] [^3H_i]}{\kappa_2 [R_{Aii}] [\beta_A] [\zeta^{35}S_o] [\delta_o] [^{35}S_o]}$$

where $[\zeta^3H_i]$ and $[\zeta^{35}S_o]$ and $[\delta_i]$ represent the second set of incubations and electrophoretic run. Therefore, $[R_{p1}/R_{p2}]$ is given by:

$$\frac{\text{d.p.m. } [^3H]A_{ii}}{\text{d.p.m. } [^{35}S]A_i} = \frac{\kappa_1 [R_{Aii}] [\beta_A] [\zeta^3H_i] [\delta_i] [^3H_i]}{\kappa_2 [R_{Ai}] [\beta_A] [\zeta^{35}S_o] [\delta_o] [^{35}S_o]}$$

$$\frac{\text{d.p.m. } [^3H]A_i}{\text{d.p.m. } [^{35}S]A_{ii}} = \frac{\kappa_2 [R_{Ai}] [\beta_A] [\zeta^3H_i] [\delta_i] [^3H_i]}{\kappa_1 [R_{Aii}] [\beta_A] [\zeta^{35}S_o] [\delta_o] [^{35}S_o]}$$

and therefore, $[R_{p1}/R_{p2}] = [R_{Aii}/R_{Ail}]^2 \cdot [\zeta^3H_i/\zeta^{35}S_o] \cdot [\zeta^{35}S_i/\zeta^3H_i]$. Assuming that conditions [i and ii] do not differentially influence the specific activities of the intercellular pools of amino acid, and that the conditions of incubation (i.e. tissue mass, time, isotope concentration etc.) for the two isotopes in each experimental cycle are identical, then $[\zeta^3H_i/\zeta^{35}S_o] \cdot [\zeta^{35}S_i/\zeta^3H_i] = 1$ and: $[R_{p1}/R_{p2}]^{1/2} = [R_{Aii}/R_{Ail}]$ which can be converted to percentage change by subtracting 1.00 and multiplying by 100.

Results

The arrays of uterine proteins that were synthesized and released by tissue from implantation sites and non-reactive areas are shown in Figs 1(a) & (b). Although there were no unique new proteins or consistently obvious differences in the species represented, the overall rate of accumulation of protein (as inferred from increasing amounts of 3H in TCA-precipitable material in the culture medium) was greater with tissue taken from developing implantation sites than with that from non-reactive interimplantation areas when both were incubated with $[^3H]$ methionine (i.e. a mean $\pm$ s.d. of 151 ± 47 vs 103 ± 30 c.p.m. acid-insoluble material/ μ g of tissue protein; $t = 2.41$, df_{14} , $P < 0.03$). This appears to reflect an increase of more than 40% in the rate of production of secretory proteins as the uterine reaction at implantation begins. The relative change in production of each of the individual proteins associated with this overall increase is shown in Fig. 3. Analysis of variance indicated that there were differences between individual ratios ($\chi^2 = 168.28$; $P < 0.0001$) and the data were subjected to the LSD test ($LSD = 0.24$; $P < 0.01$; df_{198}). Of the 15 proteins, 11 had differences in rates of production that were less than, or equal to, the overall average difference between the samples while those for the remaining proteins (Nos 25, 33, 18, and 26 in Fig. 1a) were significantly greater than the average.

Fig. 3. Comparison of rates of synthesis and secretion of individual proteins by tissue explanted from Pontamine Blue-positive authentic implantation sites and non-reactive interimplantation areas of the same uterine horn by means of dual label ratio method. * $P < 0.01$. See text for details.

Although the overall rates of synthesis and secretion of proteins by thread- and oil-induced deciduomata were higher than those for authentic implantation sites (i.e. when each type of tissue was incubated with $[^3H]$ methionine, the TCA-precipitable radioactivity was 205 ± 34 and 286 ± 63 c.p.m./ μ g tissue protein, respectively vs 151 ± 47 c.p.m.; $F_{2/25} = 16.59$; $P < 0.01$), the

arrays of proteins released were not different (Fig. 1, compare a and c). The patterns of change in production of the individual proteins associated with development of experimentally induced decidualomata are shown in Fig. 4; the impression is that overall they are quite similar to those for authentic implantation sites (compare Figs 3 and 4).

Fig. 4. Comparison of changes in rates of synthesis and secretion of individual proteins by tissue explanted from Pontamine Blue-positive decidualomata induced by an intrauterine thread (□) or intraluminal instillation of an oil droplet (■). The overall patterns of changes in production of individual proteins with development of the Pontamine Blue reaction are similar to that associated with authentic implantation sites (compare with Fig. 3).

However, an additional experiment was undertaken to compare directly the patterns associated with authentic implantation sites and decidualomata induced by intrauterine threads. Tissue from the implantation sites was incubated with [3 H]methionine, and that from the decidualomata was incubated with [35 S]methionine. The conditioned medium was mixed and analysed as described above with the expectation that, if the patterns are the same, then the values of $[R_p/R_t]$ for each of the individual proteins should not be different from 1.00. Analysis of variance indicated there were differences between individual ratios ($\chi^2 = 172.15$; $P < 0.0001$) (Fig. 5) and the data were subjected to the LSD test. Whilst tissue from implantation sites produced several proteins at rates that were low relative to those for decidualomata (i.e. $[R_p/R_t] < 1.0$, Fig. 5a, proteins 4 and 6), rates for the small acidic proteins Nos 25, 33, 18 and 26 were elevated (LSD = 0.25; $P < 0.01$, df_{491}). A similar result was obtained with reciprocal double labelling and the approximation $[R_{p1}/R_{p2}]^{1/2} = [R_{Aii}/R_{Ai}]$. However, although the analysis of variance indicated that there were differences between individual ratios ($\chi^2 = 45.6$; $P < 0.0001$), the percentage change for Proteins 25, 4 and 6 did not reach statistical significance (LSD = 0.39; $P < 0.01$; df_{105} ; Fig. 5b).

Discussion

The results of the present experiments demonstrate that there are differential changes in the rates of production of secreted uterine proteins as the Pontamine Blue reaction develops at the time of embryo implantation in mice. Furthermore, it was observed that there were many similarities in the patterns of changes associated with formation of authentic implantation sites and experimental decidualomata, presumably reflecting common processes involved in transformation of the endometrium. However, the additional finding that there were characteristic differences in the reactions of specific proteins to an implanting embryo and an artificial decidualogenic stimulus indicates that the uterus is able to distinguish between them and modifies its response accordingly. It is suggested that

Fig. 5. Direct comparison of rates of synthesis and secretion of individual proteins by tissue explanted from Pontamine Blue-positive authentic implantation sites and decidualmata by means of (a) dual-label ratios and (b) reciprocal dual-label ratios. * $P < 0.01$. See text for details.

this constitutes a form of signal-response communication between the mouse pre-implantation conceptus and its mother.

It has long been believed that there are quantitative and qualitative changes in uterine proteins of mice at the time of implantation and it is generally presumed that they are important for embryo development and implantation (Gore-Langton & Surani, 1976; Pratt, 1977; Aitken, 1977a, b, 1979; Fishel & Surani, 1978; Fishel, 1979, 1980). However, there is not agreement as to what changes actually occur, or what specific function they might subserve, and disappointingly little progress has been made towards demonstrating direct links between changes in individual uterine proteins and the process of implantation. In the earliest work, fluid was recovered from the uterine lumen by lavage and proteins were quantitated after being subjected to electrophoresis in one dimension. Qualitative changes were observed at about the time of implantation but they were not different

from those occurring in pseudopregnant animals, or in ovariectomized females given appropriate hormone replacement, and it was concluded that it is principally signals from the maternal endocrine system that are responsible for alterations in protein composition of uterine fluid (Gore-Langton & Surani, 1976; Aitken, 1977b; Fishel, 1980). Failure by these workers to detect the subtle changes associated with the uterine reaction at implantation is most readily explained by their inability to resolve adequately the individual proteins by electrophoresis in only one dimension. Nieder *et al.* (1987) and Nieder & Macon (1987) have used high-resolution two-dimensional polyacrylamide gel electrophoresis to separate ^{35}S -labelled proteins synthesized and secreted by uterine tissue explanted from fertile and sterile horns of mice at about the time of implantation. On the basis of visual comparisons of fluorographic images it was concluded that there are indeed embryo-dependent changes in certain secreted proteins at the time of implantation. However, their visual evaluation of the fluorographs apparently lacked the precision necessary to quantitate all of the differences and some major changes associated with embryo implantation went unnoticed.

Technical problems with resolution of individual proteins and quantitation of changes in their rates of production were avoided in the present work by combining two-dimensional PAGE with dual-isotope labelling. The two-dimensional approach to electrophoresis utilizes separation on the basis of isoelectric properties as well as molecular size and provides an excellent method for identifying multiple protein species within complex mixtures (O'Farrell, 1975). The dual-label ratio method is a convenient way to document relative changes in rates of production of proteins that are minor components of complex mixtures and is particularly useful in situations, such as the present one, in which the population of protein species comprising the mixture of interest remains unchanged under the conditions to be compared (Brinster *et al.*, 1979; Chen *et al.*, 1980). Because the dual-label ratios for each protein are normalized to the average ratio for the sample on the gel, the results reflect differences in rates of accumulation of individual secreted proteins relative to the overall difference between the samples (i.e. $[R_p/R_i]$). With this approach then, potential problems arising from effects of isotope distribution, variations from incubation to incubation or gel to gel, and the limited range of linear responsiveness of photographic film, are eliminated and the pooled data are suitable for statistical analysis. In the reciprocal double-labelling variation of the technique (Vallet *et al.*, 1987), there is the added advantage that the result approximates the percentage change in the protein of interest.

In the present work we found that there are differential changes in rates of production of all secreted uterine proteins as the Pontamine Blue reaction develops during formation of an authentic implantation site (Fig. 3). These changes are not in response to maternal hormones because the reactive and non-reactive tissues used for comparison were taken from the same uterine horns and thus exposed to identical endocrine conditions. Although it cannot be determined from these results whether regulation of any given protein is at the step of synthesis, processing, or release, it can be anticipated from the altered rates of production that there will be corresponding changes in the composition of the secreted product. If this material is released into the uterine lumen the pattern shown in Fig. 3 will be, in essence, a recipe for relative changes in the uterine secreted proteins comprising the embryonic milieu at the time of implantation; the small acidic Proteins 25, 33, 18 and 26 will increase and Protein 5 will decrease most markedly while the others change to various degrees between these extremes.

Although the patterns associated with development of authentic implantation sites and experimentally induced deciduomata are quite similar (Figs 3 and 4), there are differences in the production of specific subsets of the secreted proteins. This was shown clearly by a direct comparison of absolute rates of production using the dual-label ratio method. The overall rate of protein production is less with tissue from an implantation site than with that from deciduomata and, as might be expected, therefore, the rates of production for most of the individual proteins are less (i.e. negative values for $[R_p/R_i] - 1$; Fig. 5a) with Proteins 4 and 6 being depressed most markedly. However, the rates of production of the small acidic Proteins 25, 33, 18 and 26 are greater in the implantation site than they are in the deciduomata (i.e. positive values for $([R_p/R_i] - 1)$, Fig. 5a; and

$[(R_{p1}/R_{p2})^{1/2} - 1]$, Fig. 5b). Therefore, although most changes in production of secreted uterine proteins that occur with the uterine reaction at implantation appear to be related to the basic process of decidual transformation, there is embryo-dependent modulation of the fundamental response. The significance of the mechanism responsible for recognizing the presence of an embryo and for selectively enhancing production of one subset of secreted proteins while reducing others is unknown.

Little is known about the processes involved in maternal recognition of pregnancy in mammals other than that a communication link, presumably utilizing some form of embryonic signal and a maternal receiver coupled to a mechanism for mounting the appropriate response, must exist. The most detailed information about such interactions has been reported for sheep in which the conceptus synthesizes and releases a complex array of stage-specific proteins in the peri-implantation period, at least one of which (oTP-1) binds to receptors in the uterine epithelium (Godkin *et al.*, 1984). As a result of specific binding to these receptors there are changes in the synthesis and secretion of specific uterine proteins (Godkin *et al.*, 1984; Salamonsen *et al.*, 1986; Vallet *et al.*, 1987). Furthermore, the local changes in the uterus and the systemic antiluteolytic effect associated with the recognition of pregnancy (Moor & Rowson, 1966a, b; Martel *et al.*, 1979) appear to be directly related to this protein because they can be duplicated in cyclic animals with various preparations of embryo-conditioned medium containing oTP-1 (see Bazer *et al.*, 1986; Vallet *et al.*, 1987, for a review of this literature).

It remains to be proven that release of stage-specific proteins by the mouse blastocyst (Nieder *et al.*, 1987) is linked to the embryo-dependent changes in uterine proteins as in sheep. However, it is tempting to speculate that the mechanisms are analogous and, even though mice and sheep are widely separated species, that they have adopted a common strategy for signalling at implantation. If true, this would suggest that communication links based on release of soluble embryonic proteins, coupled with specific uterine mechanisms for receiving the signals and responding with alterations in the luminal milieu, are a basic part of the process of embryo implantation and will be found to be conserved throughout the class Mammalia.

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