ANIMAL EXPERIMENTATION

Synthesis of Early Pregnancy Factor Using Red Deer (*Cervus elaphus*) as a Delayed Implantation Model

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Submitted: July 3, 1996

Accepted: September 9, 1996

Purpose: This study measured serum early pregnancy factor (EPF) in pregnant red deer (*Cervus elaphus*) and ascertained whether EPF synthesis is associated with implantation.

Methods: Serial serum samples were taken from mated hinds up to 42 days postconception and analyzed for EPF activity using the rosette inhibition test. EPF activity was then correlated with calving records and stages of preimplantation development.

Results: EPF was detected in all pregnant animals, with a twin pregnancy giving increased EPF activity. Three animals gave an EPF response following fertilization but failed to continue beyond the preimplantation embryo stage. The increase in EPF synthesis previously associated with implantation in other mammals occurred at the blastocyst stage in red deer.

Conclusions: EPF synthesis in red deer (*Cervus elaphus*) is consistent with the preimplantation period, as occurs in other mammals. However, the second phase of the biphasic increase in early pregnancy factor production is associated with blastocyst formation, not implantation.

KEY WORDS: blastocysts; deer; early pregnancy factor; implantation.

INTRODUCTION

Early pregnancy factor (EPF) is one of the first detectable indicators of pregnancy. In all mammals investi-

gated including mice (1), humans (2), and several domestic and exotic species (3–5), EPF has appeared at a time corresponding to the pronuclear stage of the embryo and is generally present for at least the first two-thirds of gestation (1). No functional role for EPF has been identified, although it has been proposed to have roles in pregnancy immunoregulation (6) and as a possible growth factor (7).

Little is known about the control of EPF synthesis, however, it has been proposed that EPF is synthesized by the ovary in response to a signal from the zygote and that, during the periimplantation period, synthesis moves to the site of implantation (8). This change in EPF synthesis can be detected by a biphasic response using the rosette inhibition test, which indicates an increase in EPF synthesis (8–10). However, as blastocyst development in most mammals is closely associated with implantation, it has been difficult to identify whether the proposed switch in EPF synthesis from the ovary is associated with blastocyst development, implantation, or both events.

Recent research investigating preimplantation embryo development in the red deer (*Cervus elaphus*) demonstrated that blastocyst development was completed by day 8 following conception and that the blastocysts were located in the uterotubal junction or as free uterine blastocysts (11). Much earlier research demonstrated that implantation does not take place in deer until approximately 42 days postconception (12), which was recently confirmed for red deer (13).

The present study utilizes the differences in deer preimplantation embryo development to investigate the reported increase in EPF synthesis associated with implantation in other mammals including humans.

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MATERIALS AND METHODS

Animals and Management

Farmed adult red deer (*Cervus elaphus*) hinds ($n = 28$; mean live weight, 98.4 ± 1.3 kg) were run on Invermay Agricultural Research Centre (latitude $45^\circ 53'S$) and were used for estrous synchronization investigations (14). The animals had received one of four regimes designed to induce a range of ovulation rates and then $750 \mu\text{g}$ cloprostenol (14), to induce luteolysis 11 or 15 days after the initial estrous synchronization (14). The herds were run in one of two single-sire mating groups, each with an adult stag.

Blood Sampling

Blood samples were taken via jugular venepuncture from 28 hinds at 12 time points while the deer were restrained in a pneumatic deer crush, at 3- to 4-day intervals from 11 or 15 days after cloprostenol administration (i.e., 24 or 26 days after the initial estrous synchronization). The blood was allowed to clot, and the serum removed and frozen at -20°C until required for analysis.

Pregnancy and Calving

Pregnancy was diagnosed by rectal ultrasonography 25 or 31 and 45 or 51 days after cloprostenol treatment (39 and 59 days after the initial estrous synchronization). The date of calving was determined by daily observation, and the date of conception calculated on a gestation period of 233 days (15).

Mice

Mature C57BL/6J $\times$ BALB/cJ or BALB/cJ $\times$ C57BL/6J F_1 hybrid males were used as spleen donors for lymphocytes in the rosette inhibition test. All animals were fed with reliance stock food pellets (Reliance, Dunedin, New Zealand) and were on a 12L:12D cycle, lights on at 0730.

Early Pregnancy Factor Analysis

EPF was assayed with the rosette inhibition test (1, 16) using murine lymphocytes, a rabbit anti-mouse antilymphocyte serum, guinea pig complement, and fresh human red blood cells, collected each day into sterile Hanks balanced salt solution (HBSS). Lymphocytes were isolated from the spleens of the F_1 hybrid

male mice using a previously described technique (16). Rabbit anti-mouse antilymphocyte serum was raised using splenic lymphocytes from the F_1 hybrid male mice as previously described (17). Guinea pig complement was purchased from Serotec Ltd. (Oxford, England).

The rosette inhibition titer was defined as the highest dilution of antilymphocyte serum in which the number of rosettes formed was less than 75% of the number formed in the controls (16) and was calculated as $\log_2$ (reciprocal concentration of the antilymphocyte dilution $\times 10^{-4}$).

Previous investigations (18) indicated that EPF has a characteristic dose-response curve over a range of dilutions for a given species; therefore the range of dilutions over which the pregnant deer were positive had to be defined. This investigation used serum dilutions from 10^{-1} to 10^{-6} . The results are expressed as the reciprocal of the largest dilution to give a positive EPF response.

RESULTS

EPF Response

No EPF response was identified in the eight hinds that failed to calve. Seventeen of the animals that calved gave a positive EPF response over the serum dilution range of 10^{-3} to 10^{-5} . Three of the animals (one of which was known to twin) were positive for EPF over an increased dilution range of 10^{-5} to 10^{-7} (Fig. 1). The approximate gestation times for all ani-

Fig. 1. Changes in the dose-response curve between nonpregnant red deer hinds and red deer with single or multiple pregnancies. A sample is positive if it has a rosette inhibition titer >16 .

mals tested was different but ranged from 13 to 36 days postconception (pc).

Twins

The hind known to have twinned and the two other hinds with identical serum EPF profiles were further investigated using serum samples from day 10 to day 15 pc, over a dilution range of 10^{-1} to 10^{-8} . All samples from the three hinds were positive over the dilution range 10^{-3} to 10^{-4} , with an increase in EPF response occurring between day 15 and day 17 pc giving a positive response of 10^{-5} to 10^{-7} . Serum samples from all three hinds mimicked those of a single pregnancy at <9 days pc (dilution range, 10^{-3} to 10^{-4}), although the three animals were at day 10 to day 15 pc. The EPF response from two of the animals returned to that of a singleton pregnancy between day 24 and day 28 pc for one hind and between day 27 and day 31 pc for the second hind. The third hind, which twinned, continued to have an increased EPF response.

Noncalving Animals

These animals ($n = 8$) were tested for an EPF response at the earliest serum sample point available (putative gestation not known since gestation times were determined on calving dates). Three of the eight animals had a positive EPF response (dilution range, 10^{-3} to 10^{-5}), which was taken as an indication that fertilization had occurred. However, a second serum sample taken 7 days later was negative over the dilution range 10^{-1} to 10^{-6} . None of these animals were pregnant by ultrasonography.

Establishment of Biphasic Response

Serum samples from known pregnant hinds were analyzed for EPF response in two groups, days 6 to 10 pc (day 6, $n = 2$; day 7, $n = 1$; day 8, $n = 1$; day 9, $n = 2$; day 10, $n = 2$) and days 40 to 41 pc ($n = 8$). The biphasic EPF response, i.e., the increase in production occurred between day 8 and day 9 pc (10^{-3} to 10^{-5} ; Fig. 2). This corresponds to the time of blastocyst formation. No further change in EPF response was detected to days 40 to 41 pc, corresponding with implantation. This change in EPF response was further confirmed by analysis of serum samples at 5-day intervals from days 5 to 6 pc up to days 40 to 41 pc, with no further change identified following the EPF activity increase at days 8 to 9 pc.

Fig. 2. Changes in the dose-response curve during blastocyst formation in red deer at approximately day 8 pc.

DISCUSSION

We have recently reported that a transient increase in EPF synthesis occurs in nonpregnant mice when superovulated (19). In the present series the blood sampling times were well beyond the period where a transient EPF response was detected. All animals which gave a persistent EPF response had successful pregnancies, however, the results from the three animals with an increased EPF response warrants comment. We observed that the animal which gave birth to twins had a greater EPF response than those giving birth to singleton pregnancies. However, two other animals had identical responses at a corresponding early stage of pregnancy (day 17 pc). Currently we are unsure whether two embryos developed separately but did not reach the blastocyst stage until after day 15 pc, or there was a period of embryonic diapause, or a single embryo developed slowly and then split. None of these speculations can be answered until further embryological investigations are carried out in association with EPF synthesis.

Previous research (18) has determined that serum samples for EPF analysis must be analyzed over a range of dilutions to avoid false-negative results and each dose-response curve must be defined for the species being investigated. In this retrospective analysis we have determined that EPF is synthesized in the red deer (*Cervus elaphus*) and is consistent with reports from other species (1-5). However, during our investigations, it became apparent that the biphasic response of increased EPF previously associated with the peri-implantation period in other mammals (8-10) was associated with blastocyst formation and not implantation. As there is a well-defined period between blasto-

cyst formation and implantation in the red deer (11–13), it was possible to determine clearly that the biphasic switch, which increases EPF synthesis, was blastocyst mediated and did not occur during implantation. Investigations into EPF synthesis, to date, have used animal models where the time of implantation is closely associated with blastocyst formation; therefore the nature of the change in EPF synthesis could not be clearly identified. In red deer (*Cervus elaphus*), however, embryo transport through the oviduct is slow compared with that in bovine and ovine blastocysts, with red deer blastocysts in the uterus at approximately day 7 pc (11). In addition, the process of implantation takes place over a longer period, with apposition occurring between day 27 and day 34 pc, adhesion between day 34 and day 41 pc, and attachment from day 41 (13). We conclude, therefore, that the red deer (*Cervus elaphus*) provides information which identified EPF synthesis being associated with the blastocyst rather than implantation. Previous research (20–23) has clearly identified protein synthesis by blastocysts from other species *in vitro*. Our findings are consistent with the concept that the blastocyst both synthesizes and secretes proteins prior to implantation.

In summary, we have used red deer (*Cervus elaphus*) as an animal model to characterize further the synthesis of EPF in mammals. Using this model we have determined for the first time that EPF synthesis is strongly associated with blastocyst formation and not implantation. In addition, we have also identified an increase in EPF synthesis in conjunction with twins and its potential for monitoring early embryo loss. However, like other investigators, we have found that the rosette inhibition test is not an efficient monitoring technique.

ACKNOWLEDGMENTS

We are extremely grateful to the staff of AgResearch, Invermay Agricultural Centre, Mosgiel, Dunedin, New Zealand, for the deer serum samples and access to the deer breeding records. We wish to acknowledge specifically the close collaboration with Lynne Meikle, Dr. Doug Lang, and Dr. Peter Fennessey. We also wish to acknowledge the technical help received from Dr. Halle Morton in establishing the rosette inhibition test. These investigations received financial support from AgResearch and the Otago Medical School Bequest Funds.

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