Synthesis of alpha-fetoprotein by the pre-implantation and post-implantation bovine embryo*

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Summary. The synthesis of alpha₁-fetoprotein (AFP) was measured by radioimmunoassay in tissues and fluids of 19 bovine embryos (14–46 days of gestation) and in tissue cultures of 4 pre-implantation embryos (17–27 days) by incorporation of radioactive methionine. AFP was first detected in Day-14 trophoblasts and secretion of AFP into allantoic fluid occurred by Day 16. Embryonic tissues and fluids in pre-implantation and post-implantation embryos contained levels of AFP that were 550 to 1 500 000 times higher than those found in maternal serum (3.9–298 000 compared with 0.07–0.25 ng/mg protein). High levels of AFP were also found in uterine fluid which suggested significant transfer of this protein from the early post-implantation conceptus. The major sites of AFP synthesis were yolk sac and fetal liver. It is concluded that the synthesis of bovine AFP is not initiated by events associated with implantation.

Introduction

Alpha-fetoprotein (AFP) is a fetus-specific protein whose origin appears to be the yolk sac and fetal liver in a variety of mammalian species (Gitlin & Boesman, 1967). This protein is not present in significant quantities in adult tissue unless associated with certain pathological conditions (Ruoslahti & Seppala, 1979). Studies of mice have demonstrated that AFP mRNA is present in yolk sac (Miura, Law, Nishi & Tamaoki, 1979; Tilghman, Kioussis, Gorin, Ruiz & Ingram, 1979) and fetal liver (Koga, O'Keefe, Iio & Tamaoki, 1974), that cultured cells of fetal liver synthesize AFP (Hannah, Simkins & Eisen, 1980), and that AFP synthesis is limited to the visceral endoderm of the post-implantation embryo (Dziadek & Adamson, 1978).

The most predominant of three fetus-specific proteins in cattle is α_1 -fetoprotein (Lai, Peters & Lorscheider, 1978), and the distribution of this protein has been reported in bovine fetal fluids from 42 days until full-term birth at 284 days (Smith, Lai, Robertson, Church & Lorscheider, 1979). Since methods are now available for the collection of pre-implantation embryos in cows (Church & Shea, 1977), the objectives of the present investigation were to establish the time of onset of AFP synthesis during early bovine embryogenesis, and specifically to determine whether implantation and placentation precede expression of the AFP gene.

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Materials and Methods

Nineteen nulliparous cows of mixed breeding were inseminated 12 h after standing heat on the day of oestrus (Day 0) (Church & Shea, 1977). Pregnant cows from Alberta Livestock Transplants (Calgary, Alberta) were slaughtered on the days indicated in Table 1 and the reproductive tract removed immediately. All gravid uterine horns were carefully opened and pinned out flat. Day 14 to 20 trophoblasts were flushed from the open horn with 2 ml phosphate buffered saline (PBS) (pH 7.1) with a large bore Pasteur pipette. Uterine fluid was obtained by flushing the exposed maternal epithelium with 5–10 ml PBS. Conceptuses of ≥ 22 days were flushed from the exposed uterine horn following careful total withdrawal of allantoic and amniotic fluids with perfusion syringes. Conceptus membranes were carefully dissected away from the embryo under a Wild M5 microscope. Conceptuses showing evidence of caruncle attachment were dissected from the gravid uterine horn in their entirety after removal of extra-embryonic fluids.

AFP synthesis was quantitatively determined by radioimmunoassay (Lai, Smith, Church & Lorscheider, 1979) and the results were expressed in ng AFP/mg tissue protein. Total protein determinations in each sonicated tissue or fluid were performed as described by Lowry, Rosebrough, Farr & Randall (1951). AFP synthesis was also qualitatively determined by tissue culture of 4 pre-implantation embryos (17 and 27 days) in which the incorporation of radiolabelled methionine into AFP was detected by fluorography of the electrophoresed immunoprecipitates. Control experiments such as the use of non-specific antibodies or prior absorption of anti-fetal calf serum by the addition of excess AFP were performed to verify the results. Embryos or separate whole tissues were placed in 1 ml chilled 15 mM- NaPO_4 /150 mM-NaCl (pH 7.2), recovered by centrifugation (500 g) and washed twice with Eagles Minimal Essential Medium (GIBCO, Madison, Wisconsin, U.S.A.) with non-essential amino acids containing 15 mM-Hepes buffer, 100 U penicillin/ml and 100 μg streptomycin/ml at 37°C. Each tissue was cultured for 30 min at 37°C in 250 μl of the above medium in 12 $\times$ 75 mm sterile culture tubes in air. The medium was then changed to one in which methionine was replaced by 50 μCi [^{35}S]methionine/ml (sp. act. 934 Ci/mmol; New England Nuclear, Boston, Massachusetts, U.S.A.). Incubation was continued for 1 h, then sodium deoxycholate and Triton-X100 were added to a final concentration of 0.5% each and tissues were rapidly frozen and thawed 3 times. The detergent concentrations were increased to 1% each and freezing and thawing was repeated 3 more times after which the sample was centrifuged in a Beckman Microfuge at 10 000 g for 5 min at 4°C and the supernatant from the cell lysate was stored at -70°C. After thawing, 50 μl of the supernatant were incubated with 75 μl bovine amniotic fluid (48.7 μg AFP/ml) and 40 μl rabbit anti-fetal calf serum (Lai *et al.*, 1978) for 60 min at 28°C and 18 h at 4°C. Then 150 μl goat anti-rabbit γ -globulin (2 units; Calbiochem, San Diego, California, U.S.A.) and 75 μl normal rabbit serum were added and incubation was continued for 1 h at 28°C and overnight at 4°C. The precipitate was washed by re-suspending in 100 μl phosphate-buffered saline solution containing 1% sodium deoxycholate, 1% Triton-X100 and 1 mM-methionine and then centrifuging through the same solution containing 0.9 M-sucrose (Miura *et al.*, 1979). This procedure was repeated twice, after which the precipitate was dissolved in SDS buffer (Laemmli, 1970) at 90°C for 15 min and the radioactivity in a 5 μl aliquot of this solution was determined.

Equal counts of the radioactive immunoprecipitates were applied to each lane of an 8–16% gradient SDS polyacrylamide slab-gel using the Laemmli (1970) buffer. A current of 10 mA per gel was used until the tracking dye front had passed through the stacking gel, after which the current was increased to 20 mA. Bovine α_1 -fetoprotein (Lai *et al.*, 1978) was employed as the reference standard. The gel was stained with 0.05% Coomassie Brilliant Blue (G250) in methanol–water–glacial acetic acid (5:5:1 by vol.) for 2 h before destaining. Radioactive bands were detected by fluorography using Kodak XR-1 X-ray film and a 2-day exposure as described by Lasky & Mills (1975).

In one experiment the yolk sac lysate from a 27-day pre-implantation embryo was immunoprecipitated using a monospecific antiserum against bovine AFP (Lai *et al.*, 1978). The total precipitable radioactivity was electrophoresed and fluorography was performed as described above with a 3-day exposure.

Results

Table 1 lists the levels of AFP (ng/mg protein) found in tissues and fluids of the bovine embryo and maternal serum from 14 to 46 days of gestation. Embryo tissues displayed a marked increase in AFP concentration with advance of gestation but concentrations of maternal serum AFP remained similar to those found in non-pregnant cows. The embryo tissue and fluid values were approximately 550 to 1 500 000 times higher than those found in maternal serum. Concentrations were higher in fetal blood and uterine fluid than in maternal serum, especially after implantation.

Table 1. Ranges* of concentrations (ng/mg protein) of alpha-fetoprotein in tissues and fluids of the bovine embryo during early development

Specimen	Gestational age (days)				
	14-22	25-27 (pre-implantation)	25-27 (post-implantation)	31-36	40-46
Embryo (whole)	3.91-6.68† (14 days)	29 100-42 800	20 800-59 700	27 800-136 000	17 200-142 000
Allantoic fluid	220 (16 days)	18 000-60 300	24 900-33 700	16 800-62 800	17 500-41 900
Yolk sac	NA	59 700†	NA	40 800	NA
Fetal liver	NA	110-1 080†	8 660-20 900	119 000-298 000	12 700-130 000
Amniotic fluid	NA	233 000	80 000-212 000	41 200-208 000	41 400-190 000
Conceptus membranes	NA	27 300	14 600-25 900	6 130-10 800	10 600-11 700
Fetal blood	NA	NA	NA	450-74 700	NA
Uterine fluid	290-549	NA	24 300	29 200-40 200	55 700-84 700
Maternal serum‡	0.07-0.19	0.21	0.21	0.16-0.19	0.09-0.25

NA, not available.

* Ranges of values represent samples from embryos recovered from 2-4 cows, unless one value for a single animal is given.

† Supernatant of cell culture lysate (see text).

‡ Range of values for non-pregnant cow serum: 0.11-0.35 ng/mg protein.

As shown in Text-fig. 1, the major site of AFP synthesis was the yolk sac with a significant but lesser amount of synthesis being evident in fetal liver. Fetal gut tissue appeared to have a trace amount of AFP synthesis which was not detectable in brain or allantoic membrane. Various minor protein bands, which did not migrate adjacent to the immunoprecipitate of the purified bovine AFP standard, reflected precipitation of other fetal serum proteins by the rabbit anti-fetal calf serum.

Use of the monospecific antiserum (Text-fig. 2) revealed a major protein band which migrated adjacent to the immunoprecipitate of the bovine AFP standard, confirming the synthesis of AFP in the pre-implantation embryo.

Synthesis of AFP relative to total protein synthesis was estimated by precipitating an aliquot of the radioactive lysate supernatant with hot trichloroacetic acid (TCA) and expressing results as the ratio of radioactivity in the immunoprecipitate to that in the TCA precipitate. Such calculations revealed that in two 17-day pre-implantation embryos AFP synthesis was <0.1% of the total protein. In contrast, a 27-day pre-implantation embryo displayed AFP synthesis of 8.4% in yolk sac and 0.5% in liver when rabbit anti-fetal calf serum was used (Text-fig. 1) and 3.7% in yolk sac with a monospecific antiserum (Text-fig. 2).

Text-fig. 1. Fluorogram of radioactive immunoprecipitates from cultures of 27-day bovine pre-implantation embryo tissues. Precipitation was achieved using rabbit anti-fetal calf serum. The arrow indicates the position of migration for non-radioactive purified bovine AFP standard. Y, yolk sac; L, fetal liver; G, fetal gut; B, fetal brain; A, allantoic membrane.

Text-fig. 2. Fluorogram of radioactive immunoprecipitate from culture of the yolk sac of a 27-day bovine pre-implantation embryo. Precipitation was achieved using a monospecific antiserum to AFP. The arrow indicates the position of migration for non-radioactive purified bovine AFP standard.

Discussion

The morphology of the bovine embryo from Days 16 to 26 of gestation has been described in detail (Greenstein & Foley, 1958), and implantation occurs over 8–10 days with placentomes being present by Day 31 (Melton, Berry & Butler, 1951; King, Atkinson & Robertson, 1981). The placenta is of the epitheliochorial type (Eckstein & Kelly, 1977). In this study we classified an embryo as being post-implantation on the basis of presumptive attachment manifest by the first sign of loss of surface epithelium from the uterine caruncles in a chemotactic response to cones of trophoblastic cells on the chorio-allantoic membrane (Melton *et al.*, 1951). With this classification 2 of the embryos at 25 and 27 days gestation were designated as post-implantation, while 2 other embryos of the same gestational age were considered to be of pre-implantation status.

The results of the present investigation clearly indicate that several tissues and fluids of the pre-implantation bovine conceptus synthesize or accumulate high levels of AFP. The presence of AFP in two 14-day embryos (Table 1) reflected synthesis of this protein by the late trophoblast stage. By Day 16 AFP was evident in allantoic fluid. Pre-implantation embryos from Days 25 to 27 of gestation showed markedly elevated levels of AFP in all conceptus tissues and fluids which were assayed and the protein was still present in the same embryonic sites after implantation. Since maternal blood levels of AFP remained low, the high levels of AFP detected in uterine fluid suggest that an appreciable amount of AFP is transferred from the early conceptus, perhaps by transudation across primitive embryonic membranes.

Fluorography was first attempted on radioactive cultures from two 17-day embryos and the results indicated that AFP synthesis was <0.1% of total protein synthesis. However, cultures of 27-day-old pre-implantation embryo tissue (Text-fig. 1) and the parallel radioimmunoassay results (Table 1) clearly demonstrate that the major site of synthesis is the yolk sac with a significant but lesser contribution from the fetal liver. This latter finding is consistent with the observation that AFP mRNA levels are higher in rat yolk sac compared to fetal liver (Liao, Conn & Taylor, 1980). Since cultured cells of allantoic membrane showed no evidence of AFP synthesis (Text-fig. 1) but high levels of AFP were detected in allantoic fluid and membranes (Table 1) of the pre-implantation embryo, it seems probable that AFP is secreted by visceral endoderm cells into the allantoic fluid whence it is subsequently absorbed or taken up by membrane cells. The relatively high levels of AFP in amniotic fluid from 25 to 46 days (Table 1) may be attributed in part to a low concentration of total protein in this fluid which would, in effect, elevate the ratio of AFP to total protein.

In conclusion, AFP synthesis in the bovine embryo begins well in advance of implantation and is not restricted to the post-implantation embryo as reported for the mouse (Dziadek & Adamson, 1978). This difference may be due to technical difficulties in establishing the presence of AFP in the much smaller mouse embryo. AFP has been suggested to have an immunosuppressive role mediated by several mechanisms (reviewed by Suzuki & Tomasi, 1980). The presence of AFP in the bovine trophoblast could indicate that this fetal protein may act at the site of implantation, perhaps to enhance survival of the fetal allograft. This study demonstrates the first example of expression of a single conceptus-specific gene product that has been quantitatively monitored in a pre-implantation embryo.

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