Angiogenic activity of placental tissues of cows

L. P. Reynolds, D. S. Millaway, J. D. Kirsch, J. E. Infeld and D. A. Redmer

*Department of Animal and Range Sciences, North Dakota State University, Fargo,
North Dakota 58105, U.S.A.*

Summary. In Exp. 1, maternal (caruncle) and fetal (cotyledon) portions of the placenta as well as uterine endometrium were obtained from cows at mid-gestation and evaluated for angiogenic activity by placing tissue samples on chick chorioallantoic membranes (CAM). Only caruncular tissues exhibited angiogenic activity in the CAM assay. In Exp. 2, lyophilized homogenates of caruncular tissues obtained from cows at mid-gestation were evaluated for angiogenic activity on CAM and for their ability to stimulate mitosis of bovine aortic endothelial cells *in vitro*. Homogenates of caruncular tissues again were angiogenic on the CAM and also were mitogenic for endothelial cells. In Exp. 3, maternal (caruncle and endometrium) and fetal (cotyledon and fetal membrane) portions of the placenta were obtained from cows at mid-gestation and fine minces (explants) of each were cultured for 24 h. Explant-conditioned media were then tested for angiogenic activity by their abilities to stimulate mitosis and migration of bovine aortic endothelial cells *in vitro*. Conditioned media from caruncular explants, but not from explants of other tissues, exhibited both mitogenic and migration-stimulating activities. When pools of caruncular explant-conditioned media were fractionated by ultrafiltration, mitogenic activity was not present in fractions of M_r < 10 000, < 30 000 and < 100 000, but was retained in fractions of M_r > 10 000, > 30 000 and > 100 000. Mitogenic activity was not observed in any fractions subjected to heat treatment. On the basis of these data, we conclude that, during mid-gestation, angiogenic activity of bovine placenta is associated primarily with the maternal caruncular portion of the placenta which, therefore, may direct growth of the placental microvasculature. This angiogenic activity seems to have a molecular weight of > 100 000 and be heat labile.

Introduction

Low birth weights are associated with increased morbidity and mortality of newborns in several mammalian species, including man (Record *et al.*, 1952; Anderson *et al.*, 1978; Fahmy *et al.*, 1978; Huffman *et al.*, 1985). Birth weight depends primarily on rate of fetal growth, which is highly correlated with placental weight (Ibsen, 1928; Warwick, 1928; McKeown & Record, 1953; Alexander, 1964). The role of the placenta in determining rates of fetal growth and development probably depends, to a large extent, on development of an adequate placental blood supply (Barcroft, 1946; Barcroft & Barron, 1946; Rosenfeld *et al.*, 1974; Reynolds *et al.*, 1986). Growth of placental vascular beds continues throughout gestation even though placental weight in ruminants is maximal by about midgestation (Barcroft, 1946; Hutchinson, 1962; Teasdale, 1976). Associated with growth of placental vascular beds throughout gestation is a corresponding increase in rates of uterine and umbilical blood flows as well as fetal oxygen and nutrient uptakes (Rosenfeld *et al.*, 1974; Reynolds *et al.*, 1986). In spite of the importance of angiogenesis to placental function, little is known about how development of the placental blood supply is directed. There is some indication, however, that the stimulus for growth of placental vascular beds may derive from the placenta itself, since vasoactive factors (e.g. prostaglandins, glycosaminoglycans) produced by the placenta

have been shown to stimulate growth of microvasculature in other systems (Rankin, 1976; Form & Auerbach, 1983; Greiss & Wagner, 1983; West *et al.*, 1985).

Ruminant placentas are classified by shape as cotyledonary because intimate contact between maternal and fetal tissues occurs only in discrete structures, termed placentomes, which comprise the most vascular portion of the placenta (Ramsey, 1982). This arrangement provides a unique opportunity to study differences not only between maternal and fetal portions but also between functionally specialized portions of the placenta. The purposes of the present study were to determine whether bovine placental tissues contain angiogenic activity and, if so, from which tissue(s) this activity derives.

Materials and Methods

Materials. For cell and tissue cultures, Dulbecco's Modified Eagle medium (DMEM), Eagle Minimal Essential medium (EMEM), MEM non-essential amino acids, calf serum, fetal bovine serum (FBS), trypsin, Trypan Blue stain (0.4%) and penicillin-streptomycin (10 000 U penicillin G sodium/ml and 10 000 µg streptomycin sulphate/ml) were purchased from Gibco (Grand Island, NY). Dextrose, bovine serum albumin (BSA, fraction V), bovine pancreatic insulin, antibiotic-antimycotic solution, and ethylenediaminetetraacetate (EDTA) were purchased from Sigma (St Louis, MO). Plasma-derived horse serum used for culture of endothelial cells was from HyClone (Logan, UT). Fluorescent low density lipoprotein (acetylated LDL labelled with 1,1'-dioctadecyl-1-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate; Biomedical Technologies, Cambridge, MA), antisera for Factor VIII-related antigen (Accurate Chemical and Scientific, Westbury, NY; Calbiochem-Behring, San Diego, CA), and radioassay kit for angiotensin-converting enzyme (Ventrex, Portland, ME) were used for the identification of endothelial cells.

Isolation of endothelial cells. Bovine aortic endothelial cells (BAEC) were obtained from thoracic aortas by using procedures similar to those of Redmer *et al.* (1985), except that cells were initially cultured in DMEM containing 20% plasma-derived horse serum and 1% penicillin-streptomycin. At the third and subsequent subcultures, BAEC were cultured in DMEM containing 10% calf serum and 10% FBS. At the sixth subculture, BAEC were frozen and then stored in liquid nitrogen until used for bioassays. The BAEC were identified as being endothelial cells by the following criteria (Shepro & D'Amore, 1984): strict maintenance of a monolayer at confluence and cobblestone appearance, rapid uptake of fluorescent LDL, specific immunofluorescent staining for Factor VIII-related antigen (with both antisera), and expression of substantial angiotensin-converting enzyme activity (240 U/10⁶ cells).

Experimental procedures. Three experiments were conducted to evaluate angiogenic activity of bovine placental tissues. In Exp. 1, samples (≈ 2 g) of maternal (caruncle) and fetal (cotyledon) portions of the placentome, intercaruncular uterine endometrium and fetal semitendinosus muscle (control tissue) were obtained from 14 cows at slaughter on Day 133 $\pm$ 7 of gestation. Samples of corpus luteum, which previously have been shown to be highly angiogenic (Gospodarowicz & Thakral, 1978; Redmer *et al.*, 1985), were obtained at slaughter from 13 non-pregnant cows to serve as positive control tissue. All tissues were handled in a sterile fashion and were placed into siliconized glass Petri dishes containing DMEM. Tissue samples were minced and then transferred to fresh DMEM. Angiogenic activity of these samples was evaluated by measuring neovascularization ('spoke-wheel' response) using the chicken chorioallantoic membrane (CAM) bioassay. The CAM bioassay was similar to the false air sac technique described by Phillips & Kumar (1979) except that 'windows' (1 cm square) were cut through the shell and shell membrane of each egg on Day 7 of incubation. On Day 8 of incubation of eggs, a single sample (≈ 5 mg) of each tissue was placed on each of 5 CAM (within 2 h after tissue collection). On Day 13 of incubation (5 days after placement of tissues samples) each CAM was evaluated *in situ* by using stereomicroscopy. Grading was done by 3–5 trained individuals without prior knowledge of the treatment to which each egg was assigned. The angiogenic response was graded 0 (no response), 1 (questionable response), 2 (spoke-wheel response), 3 (exceptional spoke-wheel response) or 4 (exceptional spoke-wheel response with hyperaemia) as described previously (Vu *et al.*, 1985).

In Exp. 2, 1-g samples of caruncular tissue were obtained from 7 additional cows at slaughter on Day 133 $\pm$ 5 of gestation. In addition, 1-g samples of corpus luteum were obtained from 7 non-pregnant cows at slaughter. Samples were minced, homogenized on ice in 2 ml distilled water by using a Polytron (Brinkmann, Westbury, NY) and centrifuged at 1500 *g* for 30 min at 4°C. The resulting supernatants then were lyophilized. By the use of previously described methods (Langer & Folkman, 1976; Form & Auerbach, 1983), one of the lyophilized samples of each tissue was incorporated into 1 g ethylene-vinyl acetate copolymer (Elvax, E.I. Dupont de Nemours, Wilmington, DE). Five discs (≈ 1.5 mm diam. and 0.3 mm thick) were cut from each Elvax preparation, as well as from 3 control Elvax preparations to which no lyophilized homogenate had been added. Each disc was dialysed in sterile 0.15 M-NaCl overnight before being utilized in the CAM bioassay as described in Exp. 1. Prepared in this way, Elvax discs have been shown to achieve sustained release of lipids, proteins and other macromolecules when placed onto the CAM (Langer & Folkman, 1976; Form & Auerbach, 1983). For some of the cows, a duplicate lyophilized homogenate of caruncle or corpus luteum was redissolved in 2 ml distilled water and evaluated for angiogenic activity *in vitro* by its ability to stimulate mitogenesis of BAEC in culture. The BAEC were preincubated (37°C; 5% CO₂, 95% air) for 24 h in 24-well plates (35 $\times$ 10³ cells/well) in DMEM containing 10% calf serum, 10% FBS and 1% penicillin-streptomycin.

After preincubation, medium was changed to a maintenance medium (DMEM with 5% FBS and 1% penicillin-streptomycin; 5 wells) or maintenance medium containing 20% (v/v) redissolved homogenate (3 wells per tissue). After 72 h incubation, wells were rinsed with a 0.02% EDTA solution, lightly trypsinized (0.05% trypsin, 0.02% EDTA), and number of BAEC per well determined by using a Coulter counter (model ZF, Coulter, Hialeah, FL). To ensure accuracy of counts obtained with the Coulter counter, aliquants were removed from some wells after trypsinization and cell counts were determined by using a haemocytometer. Cell counts determined by haemocytometer or Coulter counter were always within 5% of each other. In addition, aliquants from some wells were stained with Trypan Blue (0.2%), and viability of BAEC was always at or near 100%. Protein concentrations of redissolved homogenates were determined by the method of Lowry *et al.* (1951) with BSA used as the standard.

In Exp. 3, samples (≈ 2 g) of caruncle, cotyledon, intercaruncular endometrium and intercotyledonary fetal membrane were obtained from 6 cows at slaughter on Day 180 of gestation. All tissue samples were handled in a sterile fashion and were placed into siliconized glass Petri dishes containing EMEM. Explants (193 ± 12 mg) of each tissue were placed into fresh EMEM, finely minced, blotted on sterile gauze and transferred to siliconized 50-ml glass culture tubes containing 10 ml explant medium. Explant medium was similar to that described by Basha *et al.* (1979) and consisted of EMEM supplemented with dextrose (5 g/l), MEM non-essential amino acids (10 ml/l), bovine pancreatic insulin (300 U/l) and antibiotic-antimycotic solution (20 ml/l), pH 7.3. Explant incubations were conducted under 5% CO₂, 95% air in a Dubnoff shaking water bath (30 cycles/min, 37°C) for 24 h. After incubation, explant-conditioned medium was collected and stored at -80°C until assayed for mitogenic and migration-stimulating activities. Ability of conditioned media to stimulate mitosis of BAEC was evaluated as described above except that after 24 h preincubation in 24-well plates (35×10^3 cells/well), medium was changed to DMEM with 5% FBS and 1% penicillin-streptomycin (control medium, 6 wells) or control medium containing 20% (v/v) conditioned media (3 wells for each tissue from each cow). The number of BAEC per well was determined 72 h later by using a Coulter counter. Ability of conditioned media to stimulate migration of BAEC was evaluated as described by Redmer *et al.* (1985). Microchemotaxis chambers (48-well; Neuro Probe, Bethesda, MD) with polycarbonate membranes (8 μm pores; Nucleopore, Pleasanton, CA) were used. The bottom portion of each well contained unconditioned explant medium (control, 5 wells) or unconditioned explant medium containing 30% (v/v) conditioned media (3 wells for each tissue from each cow). The top portion of each well contained 58×10^3 BAEC in unconditioned explant medium. Microchemotaxis chambers were incubated (37°C, 5% CO₂, 95% air) for 5 h. After incubation, non-migrated BAEC were wiped from the top of the membrane, and migrated BAEC (bottom of membrane) were fixed and stained (Diff-Quik, American Scientific Products, McGaw Park, IL). After the membrane was mounted on a glass slide, the number of BAEC that had migrated through the pores in the membrane was determined for each well as described by Redmer *et al.* (1985).

After finding that conditioned media from caruncle exhibited angiogenic activity, a pool was made of caruncular explant-conditioned media from each of the 6 cows. Two additional pools of caruncular explant-conditioned media were obtained, one from 4 cows slaughtered on Day 122 and another from 3 cows slaughtered on Day 142 of gestation. Samples (2 ml) of each of these pools were fractionated by using a pressurized ultrafiltration cell with low-adsorption (YM series) membranes (Amicon, Lexington, MA) to obtain filtrates with molecular weight ranges of $<10\,000$, $<30\,000$ and $<100\,000$. In addition, retentates from each aliquant were resuspended in 2 ml unconditioned explant medium to obtain fractions of $M_r >10\,000$, $>30\,000$ and $>100\,000$. One-half of each fraction was placed into a water bath at 80°C for 30 min to determine whether angiogenic activity was heat-labile. The untreated and heat-treated molecular weight range fractions then were utilized in the BAEC mitogenesis bioassay (3 wells per fraction) as described above, except that only 20×10^3 BAEC were initially plated into each well.

Statistical methods. Data were analysed statistically by using analysis of variance for a completely randomized design, and when the F-test was significant, differences between specific means were evaluated by using Bonferroni's *t* test (Kirk, 1968). Data are reported as means $\pm$ s.e.m.

Results

The results of Exp. 1 are shown in Table 1. The angiogenic response of CAM to caruncular tissues was greater ($P < 0.05$) than the responses to tissues from corpora lutea, both of which stimulated neovascularization more ($P < 0.05$) than did other tissues. Responses to intercaruncular endometrium, cotyledon and fetal muscle were similar. In Exp. 2, lyophilized homogenates of caruncle and corpus luteum incorporated into Elvax discs stimulated ($P < 0.01$) angiogenesis on the CAM compared with control Elvax discs (Table 2). Homogenates of caruncle and corpus luteum also contained potent mitogenic activity. Fourfold increases ($P < 0.05$) in numbers of BAEC after 72 h of incubation were observed with homogenates of caruncle and corpus luteum compared with medium alone (Table 3). In Exp. 3, conditioned media from explant incubations of caruncular tissue were mitogenic ($P < 0.01$) for BAEC compared with unconditioned medium (Fig. 1a). In

Table 1. Angiogenic responses of chick chorioallantoic membranes to various bovine tissues*

	Caruncle	Corpus luteum	Intercaruncular endometrium	Cotyledon	Fetal muscle
Response	2.4 ± 0.2 ^a	1.8 ± 0.2 ^b	1.2 ± 0.2 ^c	0.7 ± 0.1 ^c	0.7 ± 0.2 ^c
No. of cows	14	13	12	10	12

*A single sample (≈ 5 mg) of each tissue was placed on each of 5 chorioallantoic membranes. Values are mean $\pm$ s.e.m.

Values with different superscripts differ ($P < 0.05$).

Table 2. Angiogenic responses of chick chorioallantoic membranes (CAM) to lyophilized homogenates of bovine caruncular and luteal tissues

	Caruncle	Corpus luteum	Control
Response	2.6 ± 0.2 ^a	2.2 ± 0.3 ^a	0.1 ± 0.1 ^b
No. of cows or control samples	7	7	3
No. of CAM evaluated*	35	35	15

Values are mean $\pm$ s.e.m.

Values with different superscripts differ ($P < 0.01$).

*For each tissue (1 sample/cow) or control sample, a single Elvax disc was placed on each of 5 CAM.

Table 3. Stimulation of mitogenesis of bovine aortic endothelial cells by homogenates of bovine caruncular and luteal tissues

	Control	Medium + caruncular tissue	Medium + CL
Cells ($\times 10^3$) per well*	31 ± 0.8 ^b	117 ± 34.8 ^c	138 ± 23.7 ^c
No. of cows or control samples	5	5	6

*Number of cells per well determined after 72-h incubation with test sample.

Values are mean $\pm$ s.e.m.

Values with different superscripts differ ($P < 0.05$).

contrast, mitosis of BAEC was not affected by exposure to conditioned media from intercotyledonary fetal membrane, cotyledon or intercaruncular uterine endometrium for 72 h (Fig. 1a). Migration of BAEC also was stimulated ($P < 0.05$) by caruncular explant-conditioned media compared with unconditioned medium (Fig. 1b). Conditioned media from intercotyledonary and intercaruncular tissues had no significant effect on migration of BAEC. Cotyledonary explant-conditioned media, however, inhibited ($P < 0.01$) migration of BAEC compared with unconditioned medium (Fig. 1b).

Fig. 1. Effects of explant-conditioned media from intercotyledonary fetal membrane (ICOT), cotyledon (COT), intercaruncular endometrium (ICAR) and caruncle (CAR) on (a) mitosis and (b) migration of endothelial cells.

Table 4. Effects of various fractions of caruncular explant-conditioned media pools on mitosis of bovine aortic endothelial cells*

	Unfractionated	<i>M_r</i> values					
		<10 000	>10 000	<30 000	>30 000	<100 000	>100 000
Untreated	136.5 ± 3.3	90.3 ± 4.4	128.2 ± 4.2	88.1 ± 6.5	124.0 ± 6.5	100.3 ± 8.1	126.3 ± 4.7
Heat-treated (80°C, 30 min)	99.5 ± 3.4	98.5 ± 6.4	82.0 ± 3.5	96.1 ± 3.4	84.8 ± 0.8	91.4 ± 9.1	92.9 ± 4.4

*Numbers of cells per well expressed as % ($\pm$ s.e.m.) of control wells. Control wells contained $47.0 \pm 3.0 \times 10^3$ cells (untreated; $n = 19$) and $53.7 \pm 9.1 \times 10^3$ cells (heat-treated; $n = 8$) after 72 h incubation. Data obtained from 3 pools of media, each obtained from 3–6 cows at Days 122–180 of gestation.

The pools of caruncular explant-conditioned media also exhibited mitogenic activity and stimulated a 36% increase ($P < 0.05$) in numbers of BAEC at 72 h compared with unconditioned (control) medium (Table 4). When pools of caruncular explant-conditioned media were subjected to ultrafiltration, activity was not apparent in filtrates of $M_r < 10\,000$, $< 30\,000$ and $< 100\,000$ but was retained ($P < 0.05$) in fractions of $M_r > 10\,000$, $> 30\,000$ and $> 100\,000$ (Table 4). When the unfractionated pools or molecular weight fractions were heat-treated, mitogenic activity was no longer observed (Table 4).

Discussion

From the results of these experiments, it is concluded that (1) bovine caruncular tissues and tissue homogenates obtained during mid-gestation possess angiogenic activity, and (2) caruncular tissues secrete angiogenic activity when cultured *in vitro*. This activity was found only in conditioned media from caruncular explants and not in conditioned media from explants of intercaruncular

endometrium, cotyledon or intercotyledonary fetal membrane. In fact, cotyledonary tissue had an inhibitory effect in the endothelial cell migration bioassay. Thus, the maternal placenta may direct placental vascularization. The proposal that maternal tissues direct placental vascularization at mid-gestation does not, however, eliminate the possibility that fetal placental tissues may modulate production of angiogenic factors. In addition, the site of production of placental angiogenic factors may change with stage of gestation. Some evidence exists in support of these proposals, since angiogenic activity has been found only in the maternal portion (decidua) and not the fetal portion of the human placenta at mid-gestation (Fuchs *et al.*, 1985), whereas others have reported angiogenic activity in human fetal membranes (amnio-chorion) and placental cotyledons at term (Burgos, 1983). In addition, we have found angiogenic activity (BAEC mitogenesis bioassay) in cotyledon but not caruncle of ewes at Day 120 of gestation (Reynolds *et al.*, 1987).

Proliferation of capillary vessels is thought to consist of at least three processes: fragmentation of existing capillary basement membrane, migration of endothelial cells from existing vessels and proliferation of endothelial cells (Shepro & D'Amore, 1984). In the present study, maternal placental tissues have been shown to secrete a factor which stimulates two of these components of angiogenesis *in vitro*. The observation that bovine caruncular tissue is able to induce neovascularization of the chick chorioallantois, which represents a complete *in-vivo* angiogenic response, indicates that mitogenic and migration-stimulating activities observed in the present study indeed represent angiogenic factor(s).

Angiogenic factor from bovine maternal placenta appears to be of large molecular weight (possibly > 100 000) and also heat-labile. Angiogenic factors which are similar in molecular weight and are heat-labile have been found in other reproductive tissues, including ovarian follicles, corpus luteum and human placenta (Burgos, 1983; Buki & Seppa, 1984; Fuchs *et al.*, 1985; Koos, 1986; Redmer *et al.*, 1987). Data on molecular weight of angiogenic factors should be interpreted with caution, however, since it has been shown that angiogenic activities from several tissues are molecules of M_r 200–800 which are normally bound to a carrier of about M_r 100 000 (Weiss *et al.*, 1979; Brown *et al.*, 1980; Burgos, 1983; Kumar *et al.*, 1983; Buki & Seppa, 1984).

Placental and fetal weights are highly correlated (Eckstein *et al.*, 1955; Alexander, 1964; Knight *et al.*, 1977). Although placental growth (represented by placental size or weight) is maximal by about mid-gestation, growth of placental vascular beds continues throughout pregnancy (Barcroft, 1946; Hutchinson, 1962; Teasdale, 1976). A corresponding increase in rates of maternal placental (uterine) and fetal placental (umbilical) blood flows, as well as fetal oxygen and nutrient uptakes, is observed (Rosenfeld *et al.*, 1974; Reynolds *et al.*, 1986). Conditions associated with reduced rates of fetal growth (e.g. maternal genotype, increased number of fetuses, maternal nutrient deprivation, environmental heat stress), also are associated with reduced placental weights as well as decreased rates of uterine and umbilical blood flows and fetal oxygen and nutrient uptakes (Wallace, 1948; Alexander & Williams, 1971; Wootton *et al.*, 1977; Morriss *et al.*, 1980; Reynolds *et al.*, 1985; Ferrell & Reynolds, 1987). About 80–90% of uterine and umbilical blood flows are distributed to the maternal and fetal portions of the placenta, respectively, and have been shown to be important determinants of the quantities of oxygen and nutrients taken up by the fetus (Makowski *et al.*, 1968; Rosenfeld *et al.*, 1974; Reynolds, 1986; Reynolds *et al.*, 1986). Angiogenic factors produced by the placenta, which may be essential for development of placental vascular beds, probably therefore play a crucial role in the processes that ensure adequate supplies of oxygen and nutrients for growth of the fetus. From data presented in this paper, it can be concluded that the maternal placenta directs placental vascular growth and thereby may exert a significant influence on fetal growth and development.

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