Ovarian angiogenesis in rabbits: endotheliotrophic chemoattractant activity from isolated follicles and dispersed granulosa cells

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To test the hypothesis that angiogenesis is an important variable in ovarian folliculogenesis, we measured endothelial cell migration (chemotaxis) in media conditioned by rabbit ovarian cells. Endothelial cell migration, a reliable predictor of angiogenesis *in vivo*, was stimulated by media conditioned by isolated intact follicles (0.4–2.2 mm in diameter) from either unstimulated or hCG-stimulated (pseudopregnant) rabbits. In separate experiments, endothelial cell migration was also stimulated by granulosa cell-conditioned media. Follicular chemoattractant activity was associated with a molecular weight greater than 30 000 but was not correlated with follicular size or steroid concentrations in the media, although there was no evidence to suggest that the biological activity detected in media conditioned by either intact follicles or dispersed granulosa cells was the same. Demonstration of nonsteroidal chemoattractant activity in media conditioned by intact follicles or by dispersed granulosa cells provides evidence that follicles secrete a vascular chemotactic factor, and is consistent with a role for angiogenesis in follicle growth.

Introduction

Ovarian follicular growth is accompanied by an increase in thecal blood and lymphoid vessels which remain outside the basal lamina until ovulation. Follicular growth is sequential; follicles continue to grow until they either become atretic or ovulate. Differences in local ovarian follicular vasculature may influence follicular growth: some follicles thrive in response to extraovarian factors (i.e. gonadotrophins) provided by an increased blood supply, whereas others may suffer from inadequate support and hence become atretic (Jones, 1978; Moor and Seamark, 1986).

Since differential access to blood-borne extraovarian factors may explain why some follicles continue to grow and mature while others become atretic, growing follicles may secrete a factor(s) that induces local angiogenesis (neovascularization). To date, potential ovarian angiogenic factors include fibroblast growth factor (Gospodarowicz *et al.*, 1985; Neufeld *et al.*, 1987), epidermal growth factor (Skinner *et al.*, 1987) and a heparin-binding vascular endothelial growth factor (Phillips *et al.*, 1990).

To provide data relevant to the participation of an ovarian angiogenic factor in the regulation of follicular growth, we examined the effect of media conditioned by either intact rabbit follicles or dispersed granulosa cells on endothelial cell migration. Vascular endothelial cell migration is an integral part of new blood vessel formation and cell migration *in vitro*

(chemotaxis) correlates closely with angiogenesis *in vivo* (Folkman, 1984). The purpose of this study was therefore to determine (i) whether rabbit follicles produce potential angiogenic factors; (ii) whether follicular production of angiogenic factor(s) is related to follicular size, health or steroidogenesis and (iii) whether follicular production of angiogenic factor(s) is influenced by gonadotrophins.

Materials and Methods

Animals

New Zealand White virgin female rabbits (3–5 kg) were obtained from local suppliers, and housed in air conditioned quarters with a 12 h lighting schedule. Food and water were available *ad libitum*. Rabbits were caged individually at least three weeks before any experiment. Both unstimulated and hCG-stimulated (pseudopregnant) rabbits were used. Pseudopregnancy was induced by i.v. injection of hCG (62.5 iu per doe) (Sigma, St Louis, MO); day of injection is day 0 of pseudopregnancy (which lasts approximately 18 days in the rabbit). In hCG-stimulated (pseudopregnant) rabbits, ovaries were removed on day 3, day 5 or day 7 of pseudopregnancy. Animals were killed by decapitation in a guillotine or by rapid i.v. injection of a lethal dose of sodium pentobarbitone.

Reagents

Powdered media and supplements, fetal bovine serum (FBS), and calf serum were obtained from Sigma or Gibco (Grand

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Island, NY). Media and supplements were reconstituted in deionized water (Nanopure, Barnstead, Rochester, NY), and sterile filtered (0.2 μm Sterivex filters, Millipore Corp., Bedford, MA) before use. Collagenase type CLS (168 U mg^{-1}) was purchased from Cooper Biomedical (Malvern, PA); bovine serum albumin (BSA, Fraction V) was purchased from Sigma. Ovine luteinizing hormone (NIADDK-oLH-25) and ovine follicle stimulating hormone (NIH-oFSH-13) were obtained from the National Hormone and Pituitary Program, Baltimore, MD. Stock solutions of ovine LH (200 $\mu\text{g ml}^{-1}$) and ovine FSH (100 $\mu\text{g ml}^{-1}$) were kept frozen (-20°C) in sterile aliquots in Dulbecco's Modified Eagle Medium (DME) plus 0.1% BSA until used.

Follicular culture

Each pair of ovaries was removed, using aseptic technique, placed in DME (supplemented with 25 mmol Hepes l^{-1} and 100 iu penicillin ml^{-1} and 100 μg streptomycin ml^{-1}) and trimmed of extraneous tissue. Follicles were mechanically dissected using no. 5 watchmaker forceps under a stereomicroscope (at $\times 10$). Follicular diameter was measured via calibrated ocular reticle, and follicles were transferred to holding dishes containing similarly sized follicles. When the dissection of each ovary was completed, individual follicles were transferred to separate wells of 24 or 96 well plates (Falcon, Becton Dickinson and Co., Lincoln Park, NJ) containing 0.5 or 0.2 ml of incubation media per well, respectively, and cultured at 37°C in a CO_2 incubator. Conditioned media were collected after 24 h and stored in 12 $\times$ 75 mm glass culture tubes (VWR Scientific, Bridgeport, NJ) at -20°C until assayed; aliquots of identical unconditioned control media were stored in the same way.

Incubation media were either Ham's F12 plus insulin (5 $\mu\text{g ml}^{-1}$), transferrin (5 $\mu\text{g ml}^{-1}$), and selenium (5 ng ml^{-1}) (ITS; Collaborative Research or Sigma) or Ham's F12 plus 0.5% FBS plus ITS. Ascorbic acid (50 $\mu\text{g ml}^{-1}$) and glutamine (2 mmol l^{-1}) were routinely added to all incubation media. Oestradiol, testosterone and progesterone concentrations in unextracted follicular-conditioned media (diluted 1:10–1:100) were measured by established radioimmunoassay (RIA) procedures (Goodman and Hodgen, 1982), previously validated for culture media (Goodman, 1984).

Granulosa cell culture

Granulosa cell-conditioned media were obtained by trimming fat from ovaries (from ten rabbits in four separate experiments) and rinsing in DME. Under a stereomicroscope, three to eight follicles per ovary of surface diameter 0.3–0.9 mm were punctured with a sterile 30-gauge needle held on a 1 ml tuberculin syringe, which was also used to dislodge mural granulosa cells (Goodman, 1984). Media containing cells were pooled, centrifuged at 100 g for 10 min and the cells were resuspended in DME. An aliquot was removed to estimate cell concentration (haemocytometer) and cell viability (trypan blue exclusion). Initial viability was 20–25%. Granulosa cells were plated in 10% FBS DME; after 24 h, media were replaced with 0.5% FBS DME with and without hormones. In two experiments, granulosa cells were plated at 50 000 viable cells cm^{-2} ; media contained

either no hormone, 100 ng LH ml^{-1} , 100 ng FSH ml^{-1} , or 100 ng LH ml^{-1} plus 100 ng FSH ml^{-1} . In two additional experiments, granulosa cells were plated at varying densities from 2500 to 40 000 viable cells cm^{-2} and media contained either 0, 10, 30, 90 ng LH ml^{-1} , or 0 or 90 ng FSH ml^{-1} at 40 000 viable cells cm^{-2} . Granulosa cell-conditioned media and identical unconditioned media were collected after 24, 48 and 72 h in culture at 37°C in a CO_2 incubator, centrifuged at 1500 g for 20 min to remove cellular debris and particulate matter, then stored at -20°C until bioassayed.

Cell migration assays

Endothelial cells were isolated from rabbit thoracic aorta or rabbit abdominal fat pads and characterized on the basis of morphological and immunocytological (presence of angiotensin-converting enzyme) criteria (Goodman and Rone, 1987; Rone and Goodman, 1987). Endothelial cells were used in the chemotaxis bioassay between the third and ninth passage.

Cell migration assays were conducted in 48-microwell modified Boyden chamber assemblies (Neuroprobe, Cabin John, MD) using 8 μm pore polycarbonate membranes (Neuroprobe; PVP-free) as previously described (Goodman and Rone, 1987; Rone and Goodman, 1990). The number of migrated cells mm^{-2} in 2 h was determined for each well; each test and control medium (conditioned versus unconditioned medium) was assayed in triplicate on the same membrane.

Statistical analysis

Statistical significance of differences between test and control media was estimated by one-way analysis of variance and by Student's *t* tests, using the mean square error term from the analysis of variance (Goodman and Rone, 1987); comparisons of several treatments with one control were performed according to Dunnett's procedure (Dunnett, 1964). Conditioned media were considered to contain chemoattractant activity when it produced a statistically significant increase in cell migration ($P < 0.05$) versus otherwise identical unconditioned media. The coefficient of variation of triplicate determinations ranged from 4 to 20%; because the number of migrated cells mm^{-2} membrane is Poisson distributed, percentage error was lower at higher migration rates (Goodman and Rone, 1987).

Ultrafiltration

To begin characterization of the angiogenic factor, conditioned media containing significant chemoattractant activity were combined for ultrafiltration and reversed-phase chromatography. Rabbit follicular-conditioned media, rabbit granulosa cell-conditioned media, and identical unconditioned media were fractionated and concentrated using 10 and 30 kDa cutoff (MWCO) ultrafilters (Centricon, Amicon Corp., Danvers, MA) according to manufacturer's instructions. Retentates were diluted in Dulbecco's phosphate-buffered saline containing Ca^{2+} and Mg^{2+} to an even multiple or fraction of the starting volume of unfractionated media before assay (Goodman and Rone, 1987). Both filtrate and retentate were bioassayed for chemoattractant activity.

Table 1. Steroid accumulation and chemoattractant activity in media conditioned by isolated follicles from three rabbits in oestrus

Follicle diameter (mm)	Oestradiol (pg ml ⁻¹ in 24 h)	Progesterone (ng ml ⁻¹ in 24 h)	Testosterone (ng ml ⁻¹ in 24 h)	Chemotaxis ^a
1.14	488	1.79	0.85	+
1.23	3331	2.51	2.52	+
1.42	2625	< 1.7	4.31	+
1.14	2279	< 1.7	3.46	+
1.33	2329	2.10	1.17	+
1.14	212	< 1.7	3.29	+
1.14	58	3.87	1.67	+
0.95	< 20	< 1.7	0.94	—
1.33	4462	< 1.7	3.01	—
0.95	54	< 1.7	0.65	—
0.95	115	< 1.7	0.77	—

^a+ indicates significant ($P < 0.05$) stimulation of endothelial cell migration compared with migration in identical unconditioned media.

Reversed-phase chromatography (steroid extraction)

Chemoattractant activity was determined in unfractionated follicular-conditioned media, in the 30 kDa retentate of follicular-conditioned media and in identically treated unconditioned media (Ham's F12 plus 0.5% serum plus ITS) after reversed-phase chromatography (extraction) on Baker C₁₈ minicolumns according to manufacturer's instructions (J.T. Baker, Phillipsburg, NJ).

Follicular morphology

Intact follicles, dissected from ovaries at oestrus or day 7 pseudopregnant rabbits, were cultured fully submerged (37°C in a CO₂ incubator) in Ham's F12 for 1 day. After culture, follicles were fixed overnight in Bouin's fluid and processed for standard paraffin embedding; conditioned media were assayed for endotheliotrophic chemoattractant activity. Serial sections, 7 µm, stained with haematoxylin and eosin were examined by an observer unaware of follicular-derived chemoattractant activity.

Results

Chemoattractant activity in intact follicular-conditioned media

Media conditioned by intact individual follicles (0.4–2.2 mm in diameter) were collected from 14 unstimulated rabbits and 11 pseudopregnant rabbits and examined in the chemotaxis bioassay. Endotheliotrophic (chemoattractant) activity was detected in media conditioned by 45% of the follicles ($n = 530$) recovered from unstimulated rabbits and in 21% of the follicles ($n = 146$) recovered from pseudopregnant rabbits relative to identical unconditioned media bioassayed on the same membrane. Sixty per cent ($n = 319$) of all follicles examined from unstimulated rabbits ranged in size from 0.7 to 1.0 mm; of these, endotheliotrophic activity was detected in 144 (27%) of the follicles bioassayed.

Intact individual follicles were collected from nine day 7 pseudopregnant rabbits, and from one day 3 and one day 5 pseudopregnant rabbit. Thirty-nine (27%) of the total follicles collected from pseudopregnant rabbits were 0.8–1.0 mm in diameter; endotheliotrophic activity was detected in media conditioned by three of those follicles. Twenty per cent ($n = 29$) of all follicles bioassayed from pseudopregnant rabbits were 1.4 mm in diameter; of these, 11 (7.5%) of the follicles bioassayed demonstrated endotheliotrophic activity.

Chemoattractant activity in media conditioned by intact follicles from either unstimulated or pseudopregnant rabbits was not related to follicular size or steroid concentration (Table 1). To determine whether follicular-derived chemoattractant activity was steroidal, conditioned media were assayed after fractionation by reversed-phase C₁₈ minicolumns (which removes > 95% of steroids; Redmer *et al.*, 1985a). Follicular-conditioned media were compared to identical unconditioned media subjected to the same treatment in parallel, as discussed in the Methods section.

Endotheliotrophic activity was not removed by reversed-phase chromatography (123 ± 15 , follicular-conditioned media before extraction versus 61 ± 15 cells mm⁻² in 2 h, identical unconditioned media before extraction; 168 ± 7 , follicular-conditioned media after extraction versus 98 ± 6 cells mm⁻² in 2 h, identical unconditioned media after extraction, $P < 0.01$). Chemoattractant activity in follicular-conditioned media was retained by 30 000 MWCO ultrafilters and by ultrafiltration after extraction (Table 2). Chemoattractant activity was detected after ultrafiltration and extraction, but there was not enough material for serial dilutions of either ultrafiltered or extracted samples.

Chemoattractant activity in granulosa cell conditioned media

Endothelial cell migration was significantly stimulated by granulosa cell-conditioned media (Fig. 1). Endotheliotrophic activity varied directly with the number of granulosa cells per well; however, addition of gonadotrophins to the culture media

Table 2. Chemoattractant activity after fractionation of rabbit follicular conditioned media by reversed phase chromatography and ultrafiltration

Treatment	Rabbit follicular	
	Conditioned media ^a	Unconditioned media ^a
None	81 ± 3	23 ± 3*
Reversed phase chromatography	93 ± 9	40 ± 10*
Centricon 30 retentate	79 ± 7	31 ± 1*
Centricon 30 filtrate	41 ± 2	74 ± 10
Centricon 30 after chromatography retentate	88 ± 13	36 ± 8*
Centricon 30 after chromatography filtrate	78 ± 10	72 ± 8

^aValues are expressed as the number of migrated aortic cells mm⁻² in 2 h.

*P < 0.01, conditioned versus unconditioned media.

between the conditioned and unconditioned filtrate (132 ± 14 versus 112 ± 7 cells mm⁻² in 2 h).

Morphology

To relate follicular structure and angiogenic activity individual follicles (0.8–1.0 mm) were fixed and stained after 1 day in culture. Angiogenically active follicles (chemotaxis bioassay) had a well developed thecal layer, which was preserved in culture (Fig. 3). In contrast, granulosa cells were largely necrotic and there was no evidence of luteinization. Media conditioned by comparably sized wedges of ovarian cortex, containing structurally well preserved admixtures of germinal epithelium, stroma, primordial and preantral follicles and interstitial cell patches, were inactive in the angiogenesis bioassay.

Discussion

Conditioned media from intact individual follicles and media conditioned by dispersed granulosa cells contained an activity that stimulated endothelial cell migration; stimulation of endothelial cell migration *in vitro* is considered a reliable predictor of angiogenic activity *in vivo* (Folkman, 1984). These data support the hypotheses that follicles secrete angiogenic factors and that differential access to blood-borne factors contributes to follicular selection, further growth and development of ovarian follicles or to both (Jones, 1978; Moor and Seamark, 1986). Differential access may be regulated by follicular production of angiogenic factors as well as by other paracrine factors. Differences in local ovarian angiogenesis might account for differences in follicular growth: some follicles thrive in response to an increased blood supply and others suffer from inadequate support and hence become atretic. Preovulatory follicles may emerge because of increased vascular supply; increased vascularization of the dominant follicle of monkeys results in preferential uptake of gonadotrophins (Zelevnik *et al.*, 1981).

In the study reported here, chemoattractant activity was unequivocally detected in media conditioned by some isolated, intact, individual follicles from both unstimulated and hCG-stimulated rabbits. In addition, endotheliotropic activity was detected in media conditioned by dispersed granulosa cells from unstimulated rabbits; this activity was dependent upon the density of granulosa cells in culture. However, we have no evidence to indicate that the biochemical factor(s) producing chemoattractant activity in media conditioned by intact follicles is (are) the same as the factor(s) responsible for chemoattractant activity in media conditioned by dispersed granulosa cells. Chemoattractant activity in granulosa cell-conditioned media may be the result of luteinization; there is considerable evidence demonstrating luteal-derived angiogenic activity (Gospodarowicz *et al.*, 1985; Redmer *et al.*, 1985a, 1988; Phillips *et al.*, 1990; Rone and Goodman, 1990).

In the study reported here, chemoattractant activity in ovarian-conditioned media was associated with a nonsteroidal fraction of molecular mass > 30 kDa. A nonsteroidal angiogenic activity (45–60 kDa) has also been detected in human follicular fluid (Frederick *et al.*, 1985). In addition, our laboratory has reported nonsteroidal chemoattractant activity derived

Fig. 1. Comparison of migration rates of rabbit aortic-derived endothelial cells in response to rabbit granulosa cell-conditioned media containing 0, 10, 30 or 90 ng LH ml⁻¹. Granulosa cells from three rabbits were pooled and plated at 0 (□), 2500 (■), 10 000 (▨) or 40 000 (▩) viable cells cm⁻² in Dulbecco's Modified Eagle Medium (DME) containing 10% fetal bovine serum (FBS); after 24 h, media were replaced with 0.5% FBS in DME with or without LH and collected after an additional 48 h. Each point represents the mean ± SEM of triplicate determinations in the chemotaxis bioassay.

did not significantly affect chemoattractant activity (Fig. 1; FSH data are not shown).

Chemoattractant activity was detected in both the 10 000 and 30 000 MWCO retentates of granulosa cell-conditioned media. Furthermore, serially diluted Centricon 10 retentates produced dose-dependent decreases in endothelial cell migration rates (Fig. 2). Even though there appears to be activity in the 10 000 MWCO filtrate, compared with identically treated unconditioned media, there was no difference ($P > 0.05$)

Fig. 2. Comparison of migration rates of rabbit adipose-derived endothelial cells in response to unfractionated (neat) and to serially diluted retentates of rabbit granulosa cell-conditioned media (○) and to identical unconditioned media (●), using Centricon 10 ultrafilters. Retentate ($\log_2$) is a relative concentration, which is the inverse of the dilution. Each point represents the mean $\pm$ SEM of triplicate determinations in the chemotaxis bioassay. Granulosa cells were obtained from two rabbits and plated at 50 000 viable cells cm^{-2} . Rabbit granulosa cell-conditioned media (10% FBS DME; 2 ml) were pooled for assay; the conditioned media were taken from dispersed granulosa cells after 24 and 48 h in culture.

Fig. 3. Light microscopy of an intact rabbit follicle cultured for 24 h, then fixed and stained in haematoxylin and eosin; follicle conditioned media was active in chemotaxis bioassay. T: theca; G: granulosa. Bar: 50 μm .

from rat corpora lutea (nominal molecular mass 20–30 kDa) that is distinct from fibroblast growth factor (Rone and Goodman, 1990). Rat luteal-derived chemoattractant activity does not depend on the addition of gonadotrophins to the culture media, which is in agreement with our present findings in rabbits. Follicular chemoattractant activity was not associated with steroid secretion, unlike epidermal growth factor and transforming growth factor (Caubo *et al.*, 1989), nor is detection of chemoattractant activity affected by the addition of steroids to unconditioned media (Goodman and Rone, 1987). Although follicular size and steroid secretion are both considered indices of follicular health, we found no evidence to link angiogenesis and steroidogenesis directly in rabbits.

Well-developed theca, as assessed histologically in our study, coincided with significant chemoattractant activity from the intact rabbit follicle. The establishment of the vascular sheath within the theca coincides with the period of rapid growth and differentiation of the follicle (Burr *et al.*, 1951; Richards, 1980). Isolated theca from monkeys stimulate chemotaxis (Redmer *et al.*, 1985b); isolated theca from ovine (Taraska *et al.*, 1989), bovine (Redmer *et al.*, 1991) and porcine (Makris *et al.*, 1984) follicles stimulate mitogenesis. Endothelial cell migration in response to a stimulus (chemotaxis) and mitogenesis of endothelial cells behind the front of migrating cells are two of the principal components of neovascularization (Folkman, 1984). Expression of chemoattractant activity may precede endothelial cell mitogenesis and vascular growth of a differentiating follicle.

Because the rabbit is a reflex ovulator, it does not have a spontaneous ovarian cycle with distinct follicular and luteal phases; follicles grow and are steroidogenically active even after

ovulation (Osteen and Mills, 1980). Continuous development of follicles in the rabbit could account for the detection of follicular chemoattractant activity from both the unstimulated and hCG-stimulated (pseudopregnant) rabbit. Fewer follicles from pseudopregnant animals had detectable chemoattractant activity compared with unstimulated does, perhaps because we were unable to obtain as many follicles from these animals during early pseudopregnancy. The rabbit ovary is temporarily depleted of the largest follicles following ovulation (Asami, 1920; Osteen and Mills, 1980; Kranzfelder *et al.*, 1984; Setty and Mills, 1987). Alternatively, because the attraction and capture of a blood supply is an early event in follicular development, it is possible that follicles may not continuously secrete a chemotactic substance.

Stimulation of endothelial cell migration by rabbit granulosa cell-conditioned media did not depend on the presence of LH or FSH in the media. Similar findings have been reported in rats (Koos, 1986) and women (Katz *et al.*, 1992). In addition, granulosa cell-conditioned media from rats treated with pregnant mares' serum gonadotrophin has a mitogenic effect on bovine granulosa cells (Koos and LeMaire, 1983) that was independent of gonadotrophin treatment. If angiogenesis is analogous to the blood coagulation cascade, i.e. one step leading to the next through the action of specific mediators (Findlay, 1986), it is not surprising that chemoattractant activity is not directly dependent upon gonadotrophin treatment. More than one mediator may be responsible for the release of angiogenic activities from the follicle, that is the mitogenic activity of epidermal growth factor is potentiated by ovarian glycosaminoglycans (Sato *et al.*, 1991). There may be additional activities of lower molecular mass (i.e. epidermal growth factor, leukotrienes) released by growing follicles, which cannot be detected in our bioassay.

Thecal and granulosa cells may both secrete angiogenic activity(s), but not necessarily at the same time. The electrophoretic profiles of proteins secreted by human theca and granulosa cells of the dominant follicle in culture appear to be distinct (Lobb and Dorrington, 1987). Angiogenic activity in ovine granulosa cell-conditioned medium is detected only when endogenous LH is increased, unlike thecal-associated angiogenic activity (Taraska *et al.*, 1989). Similarly, granulosa cells of primate dominant follicles release chemoattractant activity only when cultured with LH and FSH; however, media conditioned by theca of primate dominant follicles contain angiogenic activity independent of gonadotrophin treatment (Redmer *et al.*, 1985b). Extracts of porcine theca but not of porcine granulosa cells stimulate mitogenesis (Makris *et al.*, 1984). In the rabbit follicle, granulosa and endothelial cell proliferation are closely correlated only after hCG stimulus (Kranzfelder *et al.*, 1984). Thus thecal secretion of angiogenic activity may continue throughout antral follicular growth and development, whereas granulosa cells may not express angiogenic activity until just before ovulation (Taraska *et al.*, 1989). Granulosa-derived angiogenic activity after gonadotrophin stimulus may enhance production of thecal angiogenic activity. More chemoattractant activity is released after co-culture of granulosa and theca from monkey dominant follicles than from theca cultured alone (Redmer *et al.*, 1985b). Granulosa angiogenic activity may also maintain preovulatory follicles in a nonatretic state after thecal-derived activity has contributed to early follicular selection (Taraska *et al.*, 1989).

Since many of the studies demonstrating endotheliotropic activity obtain granulosa cells from gonadotrophin-stimulated women (Katz *et al.*, 1992), rats (Koos, 1986) or mice (Sato *et al.*, 1982), there may be a species difference with regard to production of angiogenic activity and gonadotrophin dependence. In contrast to animals with cyclic gonadotrophin release, rabbits continuously produce growing follicles that will become atretic if ovulation is not stimulated by coitus; in the theca of growing and mature rabbit follicles, the number of capillaries increases linearly with increasing follicle size, with no difference between intact and less advanced atretic follicles (Kranzfelder *et al.*, 1984). However, in sheep and pigs, there is a reduction in the inner (but not the outer) wreath of thecal capillaries in atretic follicles (Cran *et al.*, 1983). In rabbits, there is continuous development of follicles irrespective of stage of sexual cycle (Asami, 1920); injection of hCG into pregnant (Adams, 1968; Mills and Gerardot, 1984) rabbits will lead to ovulation. Thus granulosa cells from rabbits may secrete angiogenic activity before gonadotrophin stimulus, whereas granulosa cells from other species may be more dependent on gonadotrophins. Alternatively, granulosa cells in culture may release endotheliotropic activity characteristic of the luteinization process, which may not be biochemically identical to chemoattractant activity associated with the growing follicle.

These novel studies in rabbits, in addition to our work with primate and rodent ovaries (Redmer *et al.*, 1985a, b; Rone and Goodman, 1990; Katz *et al.*, 1992), support the concept that angiogenesis factor(s) are involved in the paracrine regulation of ovarian cyclic structures. Demonstrations of endotheliotropic activity in ovarian-conditioned media provide evidence to support the differential access hypothesis. That is, the participation of an ovarian-derived angiogenic substance may act in a paracrine fashion on local endothelial cells and contribute to regulation of the follicle by regulating access to blood-borne factors. Production and release of ovarian factors that stimulate development of local vasculature is a complex process that requires further investigation.

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