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Ovarian Angiogenesis

Phenotypic Characterization of Endothelial Cells in a Physiological Model of Blood Vessel Growth and Regression

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Angiogenesis occurs during embryogenesis and is a down-regulated process in the healthy adult that is almost exclusively linked to pathological conditions such as tumor growth, wound healing, and inflammation. Physiological angiogenic processes in the adult are restricted to the female reproductive system where they occur cyclically during the ovarian and uterine cycle as well as during pregnancy. By systematically analyzing the phenotypic changes of endothelial cells during bovine corpus luteum (CL) formation and regression, we have established a physiological model of blood vessel growth and regression. Quantitation of vessel density, percentage of vessels with lumen, and ratio of* Bandeiraea simplicifolia*-I to von Willebrand Factor-positive endothelial cells were established as parameters of angiogenesis. Sprouting endothelial cells invade the growing CL and continue to grow throughout the first third of the ovarian cycle. Thereafter the mature CL is characterized by a dense network of vessels with gradually decreasing vessel density. During luteolysis and for several weeks thereafter (regressing and residual CL) all newly formed vessels regress, which is accompanied by gradual foreshortening and rounding of endothelial cells and subsequent detachment. Based on histochemical detection of nucleosomal fragmentation products physiological blood vessel regression in the cyclic CL does not appear to involve endothelial cell apoptosis. Lectin histochemical analysis revealed a distinct alteration of endothelial cell glycoconjugate expression during ovarian angiogenesis comparable with the distinct pattern of hyperglycosylation of cultured migrating endothelial cells (up-regulation of binding sites for* Lycopersicon esculentum *lectin, wheat germ agglutinin, neuraminidase-treated peanut agglutinin, and* Ricinus communis *agglutinin-I on sprouting ECs). Northern blot analysis of glycosyltransferases during the different stages of angiogenesis revealed an up-regulation of β-galactoside α2,6-sialyltransferase and α1,3-galactosyltransferase mRNA expression during the angiogenic stages of CL formation. These data establish the ovarian angiogenesis model as a suitable experimental system to study the functional and phenotypic properties of endothelial cells in sprouting and regressing blood vessels and provide additional evidence for the importance of endothelial cell surface glycoconjugates during angiogenesis. (Am J Pathol 1995, 147:339–351)

Angiogenesis, the sprouting of new vessels from pre-existing blood vessels, is a developmental process that occurs during embryogenesis and is down-regulated in the healthy adult.[1] In adults, it is almost exclusively linked to pathological conditions such as tumor growth, wound healing, inflammatory processes (eg, rheumatoid arthritis), retinopathies, and hemangiomas.[2,3] Physiological

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angiogenesis is restricted to the female reproductive system where it occurs cyclically in the ovary and the uterus as well as during pregnancy. It has long been recognized that angiogenesis is a requirement for ovarian and uterine function,[4,5] yet few investigators have taken advantage of the physiological angiogenic processes in the female reproductive system to study mechanisms that underlie the induction of angiogenesis.[6–12]

The ovary was among the first organs in which a growth factor with angiogenic activity was detected.[6] This activity was later identified as basic fibroblast growth factor, which exerts potent angiogenic activity in a variety of experimental systems.[7,8] More recently, some other heparin-binding growth factor activity was detected in the corpus luteum.[9] The endothelial cell specific growth factor VEGF has also been implicated in ovarian angiogenesis. VEGF could be shown to be dynamically regulated during the ovarian cycle being expressed at highest levels during the angiogenic phases of corpus luteum (CL) and follicle development.[10–12]

Experimental models of angiogenesis *in vivo* include the rabbit corneal angiogenesis assay, the hamster cheek pouch angiogenesis assay, and the chicken chorioallantois model as well as laboratory animal models of tumor growth and metastasis including transgenic models of angiogenesis induction.[13,14] It is common to all of these models that they are unidirectional; ie, angiogenesis is induced experimentally and proceeds throughout the course of the experiment. This pattern of blood vessel growth is reminiscent of the unidirectional growth of tumors and their vasculature but might not be representative of all phases of blood vessel growth during physiological angiogenesis, for example, during wound healing. In contrast to tumor angiogenesis, physiological angiogenesis follows a controlled program that includes a period of vessel maturation and tissue remodeling after the active angiogenic phase has come to an end and will eventually lead to the regression of the newly formed blood vessels during scar formation. It is for this difference that tumors have also been designated as wounds that do not heal.[15] Clearly, although it is easily conceivable that the induction of angiogenesis ceases after removal of the angiogenic stimulus, it is evident that blood vessel maturation and particularly regression must follow specific off pathways that are different from the simple absence of the on signal.

Here we have systematically analyzed cyclic angiogenic processes in the bovine CL. Studying a monovulatory species, this experimental system is very similar to the cyclic angiogenic processes in the human ovary. In contrast to small laboratory animals with a less well defined ovarian cycle, the different stages of CL development can easily be identified. The degree of angiogenic activity in the developing CL can be appreciated considering that the mature CL weighing approximately 5 to 6 g forms in less than 10 days from the approximately 200 mg of ruptured ovulatory follicle tissue.[5] Morphological and quantitative histochemical analysis with endothelial cell markers could establish the time course of blood vessel growth and regression in the cyclic CL. Furthermore, the lectin binding pattern of endothelial cells in the cyclic CL was analyzed based on the characterized pattern of hyperglycosylation of cultured migrating endothelial cells.[16] Semiquantitative analysis of the lectin binding intensities of endothelial cells in the cyclic CL revealed distinct changes of cell surface glycoconjugate expression of angiogenic endothelial cells. These data support the concept that cell surface carbohydrate moieties may play a critical role in determining the angiogenic phenotype of endothelial cells possibly mediating the adhesive interactions of sprouting endothelial cells with their microenvironment.

Materials and Methods

Tissue Samples

Tissue specimen of bovine ovaries were collected at the local abattoir within 20 minutes of slaughter. Samples for morphological and histochemical analysis were transported on ice and further processed in the laboratory. Samples to be used for high power light microscopy, ultrastructural analysis, and *in situ* Apop Tag detection were immediately trimmed and fixed. Likewise, samples for RNA analysis were immediately frozen in liquid nitrogen and stored at −70°C until further processing.

Staging of Ovaries

Ovarian CL were staged independently by two different investigators. Both ovaries were inspected visually considering the size of the follicles, ovulation and subsequent hemorrhage, size, color, and tissue consistency of the developing CL, and signs of CL regression including appearance of connective tissue in the residual CL (corpus albicans). Following these criteria, CL were grouped into early corpus rubrum (corpus hemorrhagicum), late corpus rubrum, midstage CL, regressing CL, and residual CL. Only those tissue samples were

used for the subsequent establishment of a stage-defined bank of cyclic CL that were macroscopically and microscopically evaluated by the investigators. Given the well characterized endocrine functions of the 21-day ovarian cycle, cyclic CL can reliably be staged within 2 to 3 days by morphological criteria.

Immunohistochemical Analysis

Samples to be processed for microscopic and histochemical analysis were fixed overnight in 4% buffered formaldehyde and embedded in paraffin. Aliquots of the samples were also shock frozen and stored at −70°C for cryostat sectioning. Serial sections of 4 μm were cut, deparaffinized, rehydrated, and either stained with hematoxylin and eosin or used for histochemical studies. Histochemical analysis of endothelial cells of the cyclic CL was performed by standard von Willebrand factor (vWF) histochemistry.[17] In brief, deparaffinized and rehydrated tissue sections were incubated with 0.3% H_2O_2 in methanol to inhibit endogenous peroxidase. Tissue sections were trypsinized (0.1% trypsin, 0.1% $CaCl_2$ for 45 minutes at 37°C), incubated for 10 minutes with blocking solution (10% normal goat serum), and subsequently incubated with polyclonal rabbit anti-human antibody against von Willebrand Factor (Dako, Hamburg, Germany) in a humid chamber at 37°C for 45 minutes. Sections were incubated with second antibody (biotinylated goat anti-rabbit immunoglobulin antibody; Zymed, South San Francisco, CA) and exposed to streptavidin peroxidase. Peroxidase activity was detected with chromogen with either diaminobenzidine (DAB) or 3-amino-9-ethylcarbazole (AEC) as substrate. Sections were weakly counterstained with hematoxylin or methyl green and embedded with either xylene soluble mounting medium (DAB) or water-soluble mounting medium (AEC).

Lectin Histochemistry

Lectin histochemistry was performed to analyze the glycoconjugate expression pattern of endothelial cells in different stages of CL development. Serial sections were deparaffinized and endogenous peroxidase was blocked by incubation with 0.3% H_2O_2 in methanol. Rehydrated sections were incubated with biotinylated lectins in a humid chamber for 8 hours at 4°C. Lectin binding was visualized by incubating with streptavidin peroxidase that was developed with either DAB or AEC as substrate. To determine the specificity of lectin binding, staining of negative controls was carried out by incubating serial sections with lectins that were preincubated with the appropriate inhibiting sugar (20 minutes at 0.2 mol/L).

The following lectins (acronym, concentration, binding specificity, blocking sugar) were used in this study: *Bandeiraea simplicifolia-I* (BS-I, 10 μg/ml, α-*N*-acetylgalactosamine and α-galactose, lactose), *Ricinus communis* agglutinin-I (RCA-I, 4 μg/ml; galactose > *N*-acetylgalactosamine, lactose), concanavalin A (Con A, 4 μg/ml, α-mannose, α-D-mannose), peanut agglutinin (PNA, 4 μg/ml, galactosyl-β1→3-*N*-acetylgalactosamine, galactose), *Lycopersicon esculentum* lectin (LEL, 4 μg/ml, oligomers of *N*-acetylglucosamine, *N,N′,N′′*-triacetylchitotriose), wheat germ agglutinin (WGA, 20 μg/ml, *N*-acetylglucosamine and *N*-acetylneuraminic acid (sialic acid), mixture of *N*-acetylglucosamine and sialic acid), and soybean agglutinin (SBA, 4 μg/ml, *N*-acetylgalactosamine > galactose, *N*-acetylgalactosamine). Additionally, sections to be stained with WGA, PNA, and SBA were treated with neuraminidase (type V; Sigma Chemical Co., St. Louis, MO); at 0.5 U/ml for 30 minutes at pH 6.6 before incubation with the lectin (N-WGA, N-PNA, or N-SBA).

Lectin binding intensities of arteries, arterioles, capillaries, venules, and veins in the cyclic CL were graded independently by two investigators on an arbitrary semiquantitative scale ranging from 0 = negative to 5 = strong. Endothelial cell lectin binding intensities of the cyclic CL were compared with the binding intensities of endothelial cells in the adjacent connective tissue present on the same slide. Binding intensities of at least eight different ovaries of each stage of CL formation were analyzed to determine the average lectin binding intensity of the respective endothelial cell population.

Histochemical Detection of Apoptotic Cells

Apoptotic cells were visualized by histochemical detection of nucleosomal fragmentation products applying the Apop Tag *in situ* detection kit (Oncor, Gaithersburg, MD) following the manufacturer's instructions. In brief, nucleosomal fragmentation products in deparaffinized and proteinase K digested tissue sections were 3′ end labeled with digoxigenin-dUTP with terminal deoxynucleotidyl transferase. Labeling was visualized with a peroxidase-labeled anti-digoxigenin antibody with DAB as substrate and sections were counterstained with methyl green.

Quantitation of Vessel Densities

Vessel densities were quantitated as described.[17] Sections stained either for binding of antibody to vWF or the lectin BS-I were inspected at low power (×100) to assess uniformity of vessel staining and average vessel density. Individual vessels were counted on a ×400 field (ie, ×40 objective lens and ×10 ocular lens) discriminating between immunoreactive spots with lumen and those without a lumen. At least 10 randomly selected fields were counted per section to quantitate average vessel density and to assess the heterogeneity of vessel densities within the CL.

RNA Isolation and Northern Blot Analysis

Total cellular RNA was isolated from 500 mg of whole tissue as well as 3×10^7 cultured bovine aortic endothelial cells. Frozen tissue samples were homogenized and pulverized with a mechanical dismembranator. Bovine aortic endothelial cells were isolated, cultured as either quiescent resting or activated migrating cells, and harvested for RNA isolation as described.[18] RNA from pulverized whole tissue and cultured bovine aortic endothelial cells was isolated by the single-step guanidin thiocyanate-phenol-chloroform extraction procedure.[18] Twenty micrograms of RNA from different stages of CL development were separated by electrophoresis, capillary transferred to nylon membranes (Hybond Nylon Membrane, 0.45-μm pore size; Amersham, Braunschweig, Germany), and hybridized with the following cDNA probes: bovine α1,3-galactosyltransferase cDNA (α1,3-GT; 800 bp),[19] rat α-galactosyl-β1,4-*N*-acetylglucosamine-α2,6-sialyltransferase (α2,6-ST; 1434 bp),[20] rat galactosyl-β1,3(4)-*N*-acetylglucosamine-α2,3-sialyltransferase (889 bp),[21] and human galactosyl-β1,3/1,4-*N*-acetylglucosamine-α2,3-sialyltransferase (822 bp).[22] For 18 S rRNA probing, a 30-mer oligonucleotide of an 18 S rRNA region conserved between *Xenopus laevis*, rat, and rabbit was synthesized: 5′ CGG CAT GTA TTA GCT CTA GAA TTA CCA CAG 3′.[18] The 30-mer oligonucleotide was labeled with [^{32}P]dATP (Amersham) by 3′ end labeling with deoxynucleotide terminal transferase at 37°C for 4 hours. Labeled cDNA and labeled 30-mer oligonucleotide were separated from unincorporated label by Sephadex G-50 gel filtration. For hybridization, 10^6 cpm of ^{32}P-labeled cDNA or oligonucleotide (specific activities, 10^8 to 10^9 cpm/μg) were added per milliliter of hybridization solution and incubated at 42°C overnight. Stringency washes were performed at 65°C in 0.1 × standard saline citrate, 0.1 sodium dodecyl sulfate for 20 minutes. X-ray films were exposed for 2 to 5 days.

Statistical Analysis

Quantitative data were analyzed for statistical significance by Student's *t*-test when appropriate.

Results

Morphological Analysis of Angiogenic Processes in the Cyclic Corpus Luteum

Tissue remodeling during CL formation and regression is associated with distinct phases of angiogenesis and blood vessel regression that can be appreciated by visual macroscopic inspection of CL at different stages of the ovarian cycle. After ovulation, a massive burst of angiogenesis precedes actual CL formation. The very early CL is characterized by hemorrhage into the ovulatory cavity (corpus hemorrhagicum; Figure 1A), which is accompanied by intense vascular sprouting throughout the first third of the ovarian cycle. Individual sprouting vessels can be identified within 1 to 2 days after ovulation (Figure 2A). In monovulatory species, the developing CL continues to grow for approximately one-third of the duration of the ovarian cycle until it reaches its maximal size (Figure 1B). Its macroscopic appearance throughout this period is characterized by prominent red coloration (corpus rubrum, ie, red body), which is an expression of the intense ongoing tissue vascularization

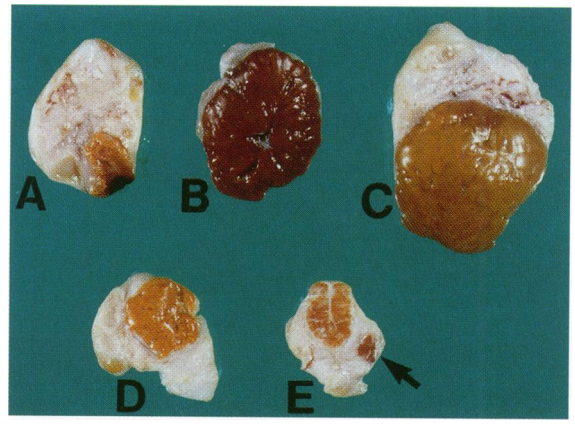

Figure 1. *Cross sections of bovine CL at different stages of the ovarian cycle.* **A:** *After ovulation, the blood-filled ovulatory cavity forms the corpus hemorrhagicum.* **B:** *Intense red coloration of the growing CL indicates angiogenesis in the corpus rubrum.* **C:** *The midcycle CL is characterized by its intense yellow coloration.* **D:** *Luteolysis leads to rapid regression of the CL.* **E:** *Tissue regression and connective tissue growth dominate in the residual CL. Remnants of an even older residual CL (corpus albicans) can also be seen (arrow).*

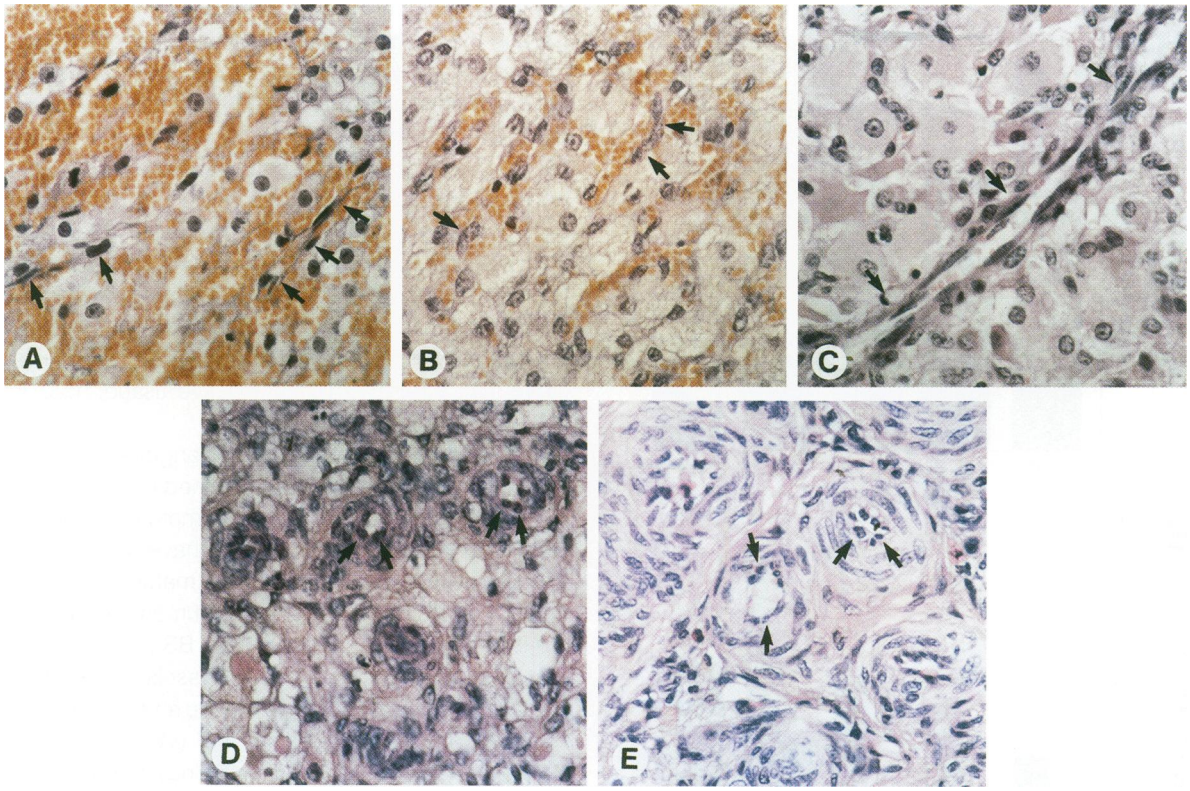

Figure 2. *Characteristics of blood vessel growth and regression in the cyclic CL.* **A:** *The early corpus rubrum is characterized by massive hemorrhage. Directional growth of numerous sprouting blood vessels can be identified (arrows).* **B:** *At later stages of CL growth, cuboidal endothelial cells line blood-filled caverns.* **C:** *The midcycle CL is characterized by mature blood vessels of varying calibers (arrows) and large fully mature lutein cells, most of which are lined by two or more capillaries.* **D:** *Luteolysis leads to rapid tissue dissociation. Endothelial cells show signs of rounding and condensation.* **E:** *Onions-like arrangement of surrounding myofibroblastic cells in regressing blood vessels with rounded, condensed endothelial cells dominates in the residual CL.*

with a dense network of immature, possibly leaky microvessels. Endothelial cells of these immature blood vessels have large, oval nuclei that line cavernous capillaries as well as maturating arterioles and venules (Figure 2B). Angiogenic endothelial cells are the most abundant cell population in the growing corpus rubrum accounting for as many as 50% of all cells. The plateau phase of the ovarian cycle is characterized by the typical yellow coloration of the mature CL reflecting its endocrine functions as well as the maturation of its newly formed vessels (Figure 1C). Endothelial cells acquire the characteristic elongated phenotype of mature blood vessels (Figure 2C). At the end of the ovarian cycle, luteolysis leads to rapid functional inactivation of the CL and a reduction of its size to less than one-third of its original size within 2 to 3 days (Figure 1D). Vacuolation, hyalination, and cellular condensation can be seen in all cell types of the regressing CL (Figure 2D). Dissolution of the blood vessels of the CL is associated with gradual rounding and condensation of endothelial cells of small vessels. At later stages, all lutein cells have disappeared leaving arteriole-like remnants of blood vessels with an onion-shaped arrangement of surrounding myofibroblastic cells and heavily condensed endothelial cells with dense connective tissue in the residual CL (Figure 2E). Eventually, the residual CL is completely removed and replaced by connective tissue leaving a small dark scar (corpus albicans, Figure 1E).

Quantitation of Blood Vessels during the Ovarian Cycle

The density of blood vessels in the CL was determined to characterize the temporal pattern of blood vessel growth and regression during the ovarian cycle. As expected, blood vessel counts of vWF histochemistry positive endothelial cells were significantly higher ($P < 0.01$) in the corpus rubrum and in the midstage CL compared with blood vessel counts in the regressing and residual CL as well as in the adjacent connective tissue (Figure 3A). During luteolysis, vessel densities rapidly declined by more than 50% and continued to drop in the residual CL to levels

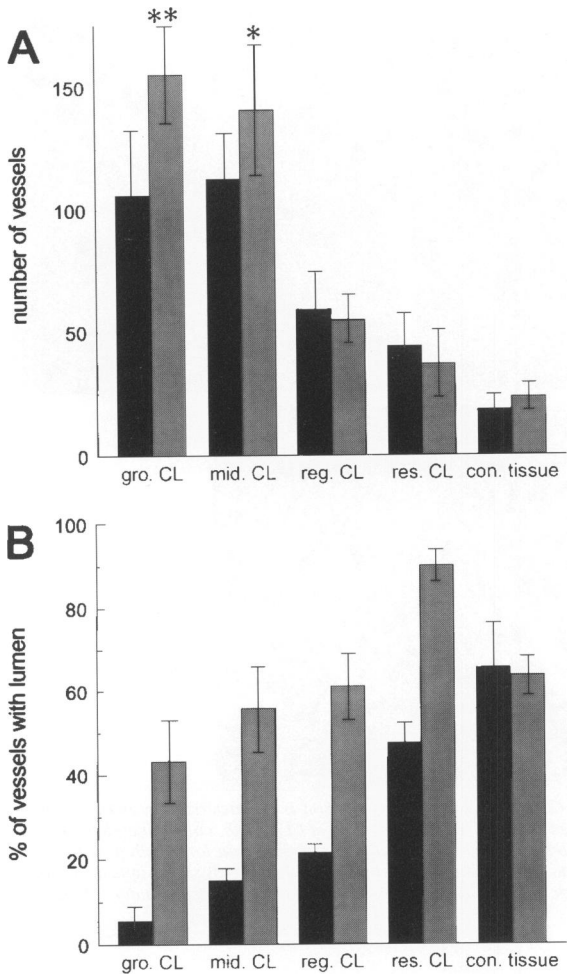

Figure 3. *Quantitation of blood vessel densities in the cyclic corpus luteum* (A) *and determination of the percentage of blood vessels with a lumen* (B) *by vWF histochemistry (black bars) and BS-I lectin histochemistry (gray bars).* A: *Highest blood vessel counts are determined in the growing corpus rubrum (gro. CL) and the midcycle CL (mid. CL). Blood vessel densities decline rapidly during CL regression (reg. CL, regressing CL; res. CL, residual CL) to levels of the adjacent connective tissue (con. tissue). BS-I staining identifies significantly more blood vessels during the angiogenic phase of CL development than vWF histochemistry (** P < 0.01; * P < 0.05).* B: *The percentage of blood vessels with a lumen increases during CL development, reflecting the gradual maturation of the newly formed vascular network.*

seen in adjacent connective tissue. Thus, it appeared that vWF histochemistry could be used to quantitatively assess angiogenic processes in the cyclic CL. Careful morphological analysis, however, indicated that vWF expression could not be detected in the most immature sprouting endothelial cells.

Histochemical analysis of several other endothelial cell markers successively led to the identification of the lectin BS-I as a more sensitive marker of angiogenic endothelial cells. Quantitative BS-I lectin histochemistry identified more blood vessels in the corpus rubrum and in the CL than vWF histochemistry (Figure 3A). In contrast, vessel density counts after BS-I and vWF staining were similar in the regressing CL and the residual CL as well as in the neighboring connective tissue (Figures 3A and 4), suggesting that the higher vessel counts after BS-I staining in the corpus rubrum and the CL reflect angiogenesis. This observation was applied by establishing the ratio of BS-I and vWF counts as an angiogenesis index indicative of the degree of ongoing angiogenesis. This index was 1.52 ± 0.15 (mean ± SEM) in the corpus rubrum ($P < 0.01$), dropped to 1.26 ± 0.10 in the CL ($P < 0.05$), and was between 0.9 and 1.2 in non-angiogenic tissues (regressing CL, 0.93 ± 0.06; residual CL, 0.92 ± 0.16; connective tissue, 1.20 ± 0.13; statistically not different).

To identify another parameter of angiogenesis, the percentage of histochemically stained blood vessels with a lumen was determined. Immature, sprouting vessels in the early CL do not yet have a lumen. As the vasculature in the growing CL matures, the percentage of blood vessels with a lumen steadily increases (Figure 3B). Staining for BS-I consistently identified a higher percentage of vessels with a lumen than staining for vWF. This difference reflects different staining qualities of BS-I and vWF (vWF staining is usually more diffuse than BS-I staining), making it important to consider the percentage of vessels with a lumen as a relative parameter. Nevertheless, both endothelial cell markers demonstrate the increase of the percentage of vessels with a lumen as a parameter of the maturation of the newly formed vasculature in the cyclic CL.

Lectin Histochemical Analysis of Angiogenic Endothelial Cells in the Cyclic Corpus Luteum

The lectin binding pattern of endothelial cells in the cyclic CL was determined to evaluate their glycoconjugate expression pattern. Endothelial cells in the bovine ovary bind the lectins BS-I, RCA-I, WGA, and Con A as well as the lectins PNA, SBA, and WGA after pretreatment of tissue sections with neuraminidase, which cleaves terminal sialic acid (N-PNA, N-SBA, and N-WGA). The galactose- and *N*-acetylgalactosamine-specific lectin BS-I proved to be a constitutive endothelial cell marker staining endothelial cells in the different developmental stages of the CL as well as endothelial cells in vessels of different calibers with similar intensity (Figures 4 and 5). Likewise, staining of endothelial cells in mature arteries and veins with the other lectins revealed no differences in the binding intensities between endothelial cells in the different stages of CL devel-

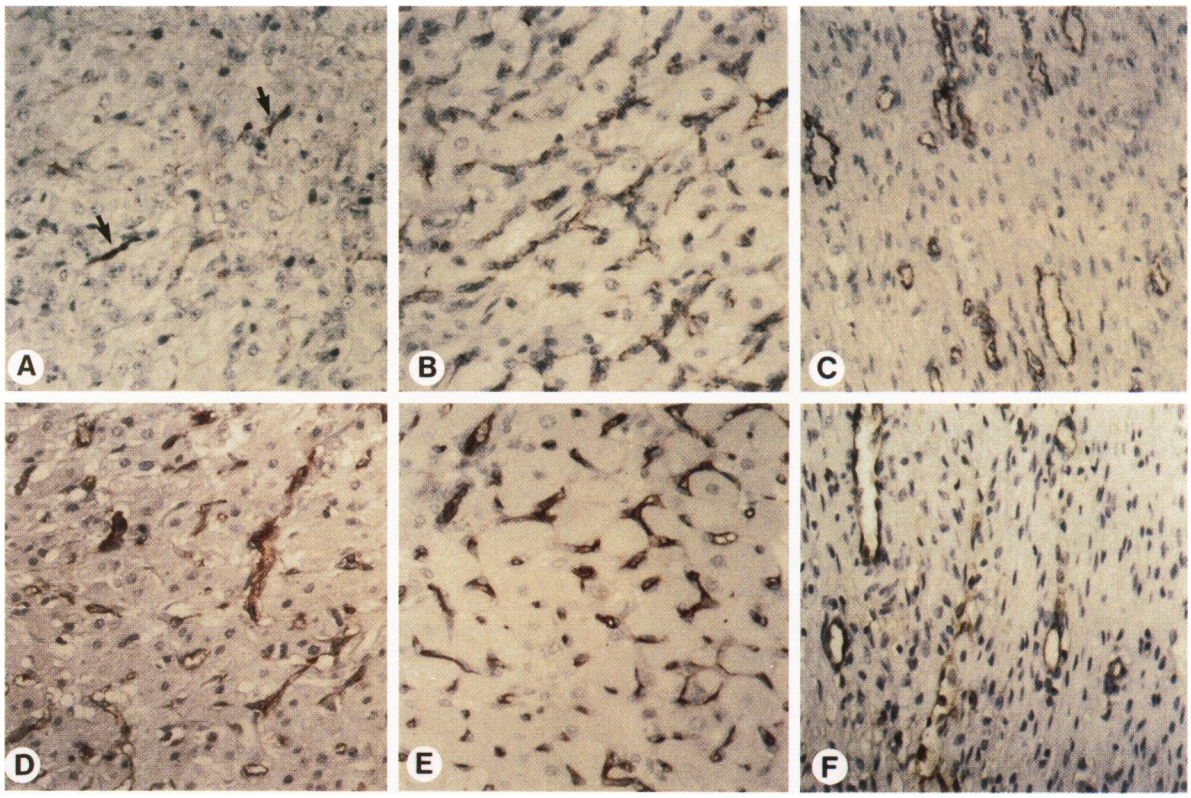

Figure 4. *Histochemical staining of endothelial cells in the cyclic CL by vWF histochemistry* (A–C) *and BS-I lectin histochemistry* (D–F). *Staining for BS-I binding identifies significantly more blood vessels during the angiogenic phase of CL development* (D) *than vWF histochemistry* (A). *In contrast, similar vessel densities were detected in the mature CL* (B *and* E) *as well as in the adjacent connective tissue* (C *and* F).

opment compared with the levels of lectin receptor expression of endothelial cells in the adjacent connective tissue. In contrast, lectin binding intensities of small vessel endothelial cells in the growing CL were up-regulated compared with the intensity of binding of resting endothelial cells in arterioles, capillaries, and venules in the adjacent connective tissue. The up-regulatory effect of lectin receptor expression of angiogenic endothelial cells was particularly prominent after staining with the lectins LEL and WGA indicating an up-regulation of *N*-acetylglucosamine and possibly sialic acid expression on cell surface glycoconjugates of angiogenic endothelial cells (Figure 6). Binding intensities of the galactose- and *N*-acetylgalactosamine-specific lectins RCA-I and PNA (PNA identifies subterminal galactose residues after neuraminidase pretreatment (N-PNA)) were weakly but consistently up-regulated on angiogenic endothelial cells in the cyclic CL compared with endothelial cells in small vessels in adjacent connective tissue. This up-regulatory effect is most likely a result of an up-regulated expression of galactose residues, as SBA staining after neuraminidase pretreatment (N-SBA, identifying subterminal *N*-acetylgalactosamine) resulted in similar staining intensities of endothelial cells in the CL and in the neighboring connective tissue (Figure 6). Although less prominent, this constitutive binding pattern confirms the constitutive binding of the lectin BS-I. Binding of the mannose-specific lectin Con A showed some up-regulation on endothelial cells in sprouting vessels. Because of the ubiquitous expression of the trimannoside core, however, this lectin exhibited a very broad expression pattern in the ovary for which reason endothelial cell Con A binding was excluded from further lectin histochemical analysis. The sialic-acid-specific lectins MAL-II and SNA exhibited a similar ubiquitous binding profile allowing only indirect evaluation of sialic acid expression on angiogenic endothelial cells. PNA and WGA detection of subterminal sialic acid masked galactose and *N*-acetylglucosamine residues after neuraminidase pretreatment (N-PNA and N-WGA) does indeed suggest that sialic acid expression on angiogenic endothelial cells is similarly up-regulated as galactose and *N*-acetylglucosamine residues. Taken together, careful semiquantitative lectin histochemical analysis of lectin binding intensities of endothelial cells in the growing CL compared with the lectin binding intensities of endothelial cells in adjacent connective tissue suggests an up-regulation of

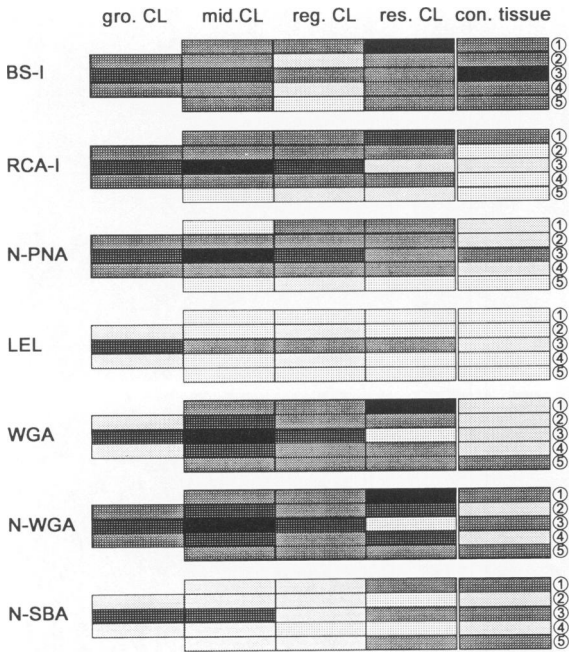

Figure 5. *Analysis of endothelial cell lectin binding intensities in the cyclic CL compared with the lectin binding intensities of adjacent connective tissue (reg., regressing; res., residual; con. tissue, connective tissue). Endothelial cell lectin binding pattern of different caliber blood vessels (①, arteries; ②, arterioles; ③, capillaries; ④, venules; and ⑤, veins) were graded on a semiquantitative scale as follows: 0, negative (□), 1 (▨), 2 (▨), 3 (▩), 4 (■), or 5, strong (■). Large vessel endothelial cells bound all lectins with similar intensity. In contrast, binding of the lectins LEL, WGA, RCA-I, and N-PNA was up-regulated on microvessel endothelial cells during the angiogenic phase of CL development. BS-I and, to a lesser degree, N-SBA were identified as constitutive markers of endothelial cells.*

cell surface glycoconjugate expression of endothelial cells in growing blood vessels. Evaluation of the corresponding lectin binding pattern suggests that particularly glycoconjugates carrying *N*-acetylglucosamine residues, sialic acid, and, to a lesser degree, galactose residues are involved in determining the angiogenic phenotype of endothelial cells.

Expression of mRNA for Glycosyltransferases in the Cyclic Corpus Luteum

To identify dynamic expression of glycosyltransferases in the cyclic CL that could account for the changes of glycoconjugate expression patterns in angiogenic endothelial cells, Northern blot analysis was performed to detect steady state mRNA levels for several glycosyltransferases in the different developmental stages of the cyclic CL. Transcript levels for the enzyme α2,6-ST were significantly up-regulated in the corpus rubrum compared with later stages of the CL as well as to the level of expression in adjacent connective tissue (Figure 7B). Expression of other sialyltransferases could not be detected in the bovine ovary because of either low levels of transcription or, more likely, lack of cross-species hybridization with the murine and human cDNA probes. Analysis of α1,3-GT expression identified moderate levels of mRNA expression of a single transcript (4.8 kb) in the corpus rubrum and in the midstage CL that was not detectable in the regressing and the residual CL (Figure 7D). Interestingly, mRNA transcripts for both α2,6-ST and α1,3-GT were also detected in cultured bovine endothelial cells. Levels of expression of both enzymes were up-regulated in subconfluent compared with confluent endothelial cells, suggesting that up-regulation of sialyltransferase and galactosyltransferase mRNA expression in the cyclic corpus rubrum could in fact represent endothelial cell expression of these enzymes (Figure 7, A and C).

Analysis of Blood Vessel Regression in the Cyclic Corpus Luteum

During luteolysis, regression of the cyclic CL is characterized by a rapid phase of tissue disintegration and involution over a few days (regressing CL) and a slow phase of tissue remodeling and connective tissue growth over several weeks (residual CL). Eventually, the CL will completely disappear leaving a small hyaline scar (corpus albicans). Blood vessel regression in the cyclic CL involves the progressive rounding and condensation of endothelial cells in small vessels that can be observed soon after luteolysis (Figure 2D). This observation prompted us to visualize apoptotic cells *in situ* by histochemical detection of nucleosomal fragmentation products applying the TdT-mediated dUTP-biotin nick and labeling technique (TUNEL).[23] Surprisingly, TUNEL-positive endothelial cells could not be identified in regressing blood vessels of the regressing CL (Figure 8A) as well as the residual CL (Figure 8B), suggesting that physiological blood vessel regression in the ovary does not involve endothelial cell apoptosis with nucleosomal fragmentation of DNA. This observation was in striking contrast to the numerous TUNEL-positive luteal cells in the regressing CL (Figure 8A). Subsequent high power light microscopic analysis of CL indicated that rounded endothelial cells do in fact detach from their basement membrane, leaving areas devoid of a covering endothelial cell monolayer, which is particularly prominent in the residual CL (Figure 8C). These findings were confirmed by ultrastructural evaluation of CL (data not shown), which also revealed a prominent basement membrane proliferation with pronounced duplication of basement membranes.

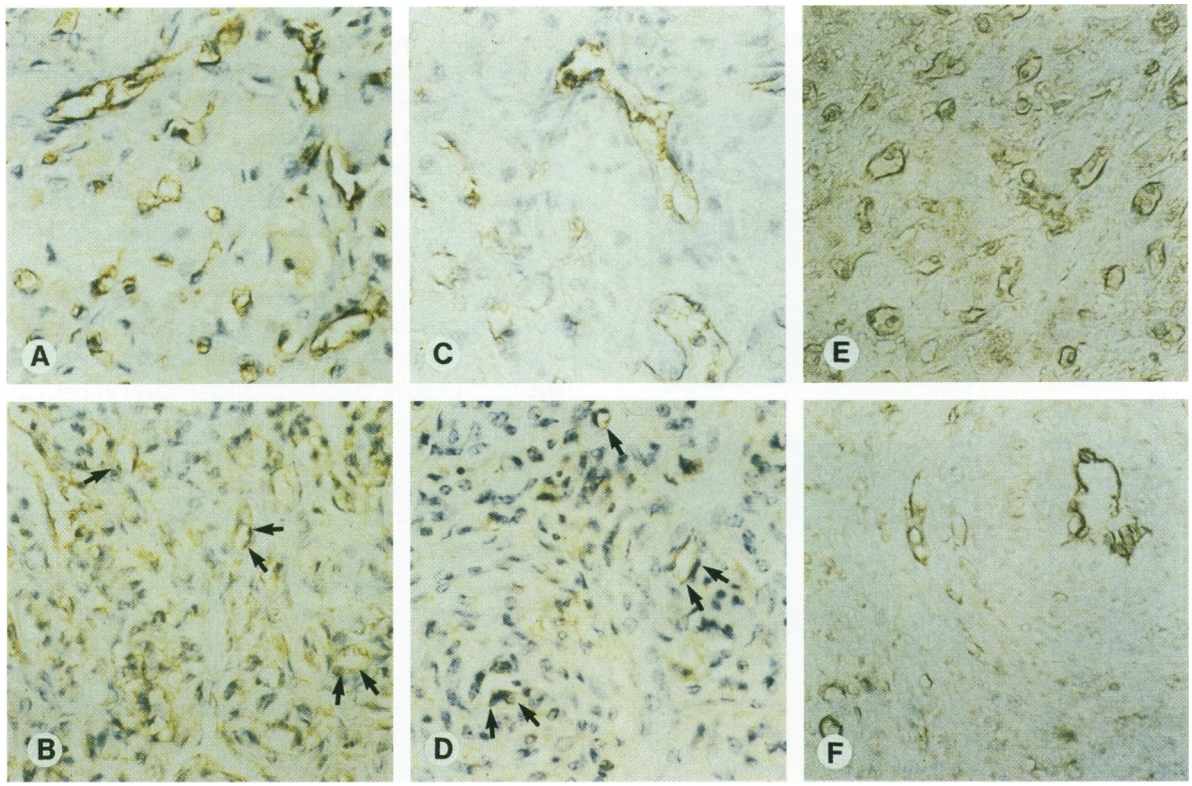

Figure 6. *Lectin histochemical analysis of endothelial cell glycoconjugate expression in the cyclic CL. Endothelial cells of small blood vessels in the growing CL express high levels of receptors for the lectins LEL* (**A**) *and WGA* (**B**). *Small vessel endothelial cells of residual CL* (**D**, *LEL*) *and endothelial cells in the adjacent connective tissue* (**E**, *WGA*) *exhibit much weaker binding of these lectins* (*arrows*). *In contrast, SBA* (*after neuraminidase pretreatment*) *binds endothelial cells in the growing CL* (**C**) *with similar intensity as in the adjacent connective tissue* (**F**).

Discussion

Angiogenesis in the ovary is a cyclically regulated process that is a necessary requirement for proper ovarian function for both follicle maturation and CL growth. In fact, considering that the endocrine functions of the ovary have been examined in detail, it is surprising that only a few studies have analyzed angiogenic processes in the ovary.[6–12] In the present study we have analyzed cyclic angiogenic processes in the bovine ovary to establish a model of physiological angiogenesis that allows the analysis of the phenotypic properties of endothelial cells during blood vessel growth and regression. Vessel density counts were employed to establish the time course of blood vessel growth and regression during the different stages of CL development. Vessel density counts have now been widely used to quantitatively assess vascularization of tumors showing that vessel densities of tumors strongly correlate with their degree of malignancy.[17,24] Similarly, we determined highest vessel counts during the proliferative phase of CL development. The very early appearance of immature sprouting vessels after ovulation does in fact suggest that, much like in tumors, angiogenesis precedes tissue growth during CL development. Vessel density counts allow the reliable quantitation of the degree of tissue vascularization, yet they provide only limited information about the level of vessel maturation. We therefore attempted to identify additional parameters that might be useful to assess the degree of ongoing angiogenesis. Staining with the lectin BS-I identified significantly more blood vessels in growing tissues than vWF histochemistry.[25,26] Thus, the BS-I/vWF ratio can be used as an angiogenesis index considering that BS-I stains a higher percentage of immature blood vessels than vWF. This angiogenesis index will be a useful parameter to assess angiogenesis in experimental animal systems. It is, however, of limited value for human endothelial cells as BS-I is not a suitable marker for human endothelial cells (unpublished observations). Additionally, determination of the percentage of vessels with a lumen could be identified as a parameter of maturation of the newly formed vasculature.

The well defined stages and the temporal pattern of angiogenesis induction, vessel maturation, and blood vessel regression in ovaries of monovulatory species allow the application of the ovarian angiogenesis model to the study of a number of steps of the

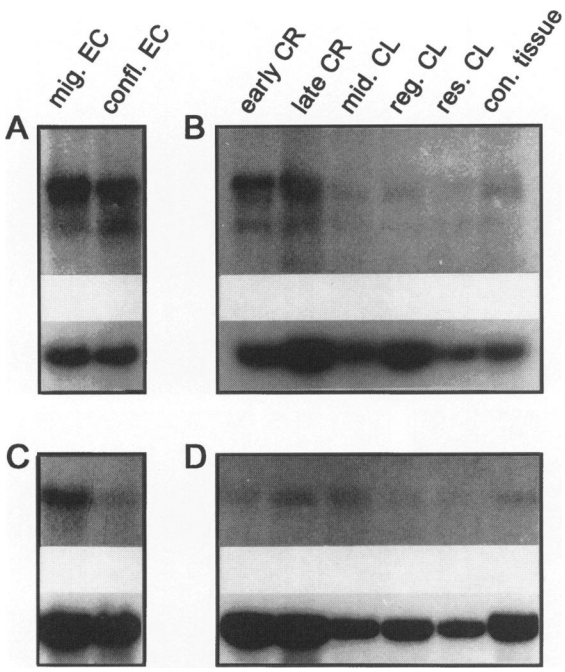

Figure 7. *Northern blot analysis of α2,6-ST* (A *and* B, upper panel) *and α1,3-GT* (C *and* D, upper panel) *expression in the cyclic CL* (B *and* C) *and cultured endothelial cells* (A *and* C). *Control hybridizations of the same membranes were performed with a 30-mer oligonucleotide to 18 S rRNA* (lower panels). *Expression of mRNA levels of α2,6-ST and α1,3-GT are up-regulated during the angiogenic phases of CL development as well as in migrating endothelial cells* (*mig. EC*). *confl. EC, confluent resting endothelial cells; CR, corpus rubrum; mid., midcycle; reg., regressing, res., residual; con. tissue, ovarian connective tissue.*

angiogenic cascade. It is not limited to the analysis of the mechanisms of angiogenesis induction but will also be suitable for the analysis of blood vessel remodeling processes during vessel stabilization and maturation as well as the mechanisms of blood vessel regression. The relevance of remodeling and regression processes is becoming increasingly evident as more compounds with anti-angiogenic activity are identified.[27,28] Recently, Brooks et al[29] reported that $\alpha_v\beta_3$ integrin antagonists promote tumor regression by inducing apoptosis of angiogenic endothelial cells. In contrast to these findings, physiological blood vessel regression in the cyclic CL does not appear to involve endothelial cell apoptosis with nucleosomal fragmentation of cellular DNA. Instead, endothelial cells in regressing blood vessels exhibit signs of rounding and detachment from the underlying basement membrane. This type of blood vessel regression has also been observed in angiostatic steroid-induced blood vessel involution in the chorioallantoic membrane.[30] These observations strongly suggest that detachment and subsequent removal from the circulation are important mechanisms of physiological blood vessel regression.

We employed the ovarian angiogenesis model to analyze the phenotypic properties of angiogenic endothelial cells in the CL in comparison with resting endothelial cells in adjacent connective tissue. Endothelial cells have long been recognized as a heterogeneous multipotent population of cells,[31,32] yet the functional implications of heterogeneity mediating subpopulation-specifically-expressed endothelial cell molecules is still in its infancy.[33] Endothelial cell heterogeneity has been most extensively studied by the demonstration of specific lectin binding patterns, which exhibit species-, age-, organ-, and vascular bed-specific variations *in vivo*.[34–37] Despite gradual dedifferentiation in cell culture, some of these lectin binding differences are maintained when endothelial cells are cultured *in vitro*, supporting the study of subpopulation-specific endothelial cell properties and the identification of specific cell surface glycoconjugates.[16,38,39]

Reflecting heterogeneity and functional pluripotence, angiogenic endothelial cells express an activation-induced phenotype that is distinctly different from the phenotype of resting, quiescent endothelial cells. A number of known adhesion molecules appears to be involved in shaping the angiogenic phenotype of endothelial cells. Endothelial cell integrin $\alpha_v\beta_3$ expression, for example, was shown to be required for angiogenesis.[29,40] Expression of E-selectin by angiogenic endothelial cells might also be involved in capillary morphogenesis.[41] Similarly, angiogenic growth factor receptors, such as the receptor tyrosine kinase flk-1, have been shown to be preferentially expressed by angiogenic endothelial cells.[42] Attempts to identify other molecules that are selectively expressed on angiogenic endothelial cells have led to the production of several monoclonal antibodies that identify angiogenic endothelial cells.[43–46] Likewise, analysis of *in vitro* angiogenic endothelial cells has shown that subconfluent and migrating endothelial cells express a distinct pattern of hyperglycosylation and specific migration-associated cell surface glycoproteins.[16] Based on these findings and the well established differential lectin binding patterns of endothelial cells,[34–39] we performed a semiquantitative lectin histochemical evaluation of endothelial cell glycoconjugate expression during ovarian angiogenesis to assess the contribution of carbohydrate moieties to the angiogenic phenotype *in vivo*. Angiogenic endothelial cells *in vivo* express a very similar pattern of hyperglycosylation as migrating endothelial cells *in vitro*, which is characterized by an up-regulation of cellular glycoconjugates carrying *N*-acetylglucosamine, galactose, mannose, and possibly sialic acid moieties. The demonstration of an up-regulation of α2,6-ST and α1,3-GT

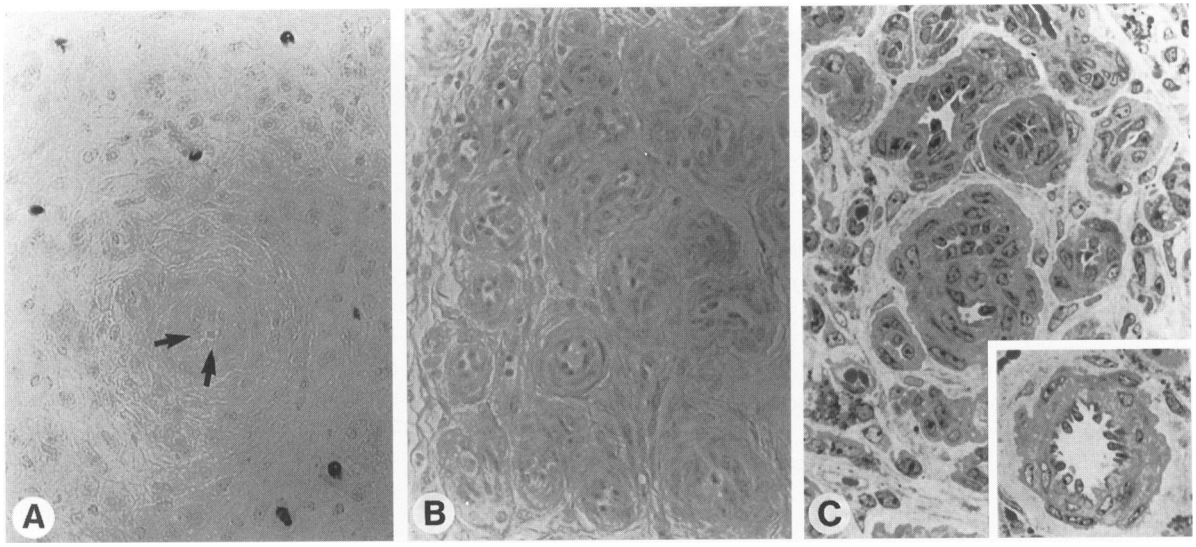

Figure 8. *Regression of blood vessels and identification of apoptotic cells in the cyclic CL.* **A** *and* **B**: *Histochemical detection of nucleosomal fragmentation products (TUNEL) identifies several apoptotic luteal cells in the regressing CL* (**A**, *dark nuclei*). *There are no TUNEL-positive cells in the residual CL* (**B**). *Endothelial cells of regressing blood vessels in the regressing CL* (**A**, *arrows*) *and in the residual CL* (**B**) *are TUNEL negative and exhibit signs of rounding and cellular condensation.* **C**: *Morphological analysis of regressing blood vessels in a 0.5-μm-thick section of a plastic embedded residual CL. Endothelial cells of capillaries and small veins lose their usually flat, elongated appearance to progressively acquire a rounded, condensed morphotype. Eventually, endothelial cells will detach, leaving denuded basement membrane that is devoid of a covering endothelial cell monolayer* (**inset**).

mRNA expression during the angiogenic phase of CL development as well as in migrating endothelial cells support these observations. Additional analysis of the regulation of the activity of different glycosyltransferases and identification of specific endothelial cell glycoconjugates will help to better understand the role of cellular glycoconjugates in morphogenetic processes such as angiogenesis. Taken together, these findings strongly support the concept that cell surface glycoconjugates are critical determinants of the angiogenic phenotype of endothelial cells with a significant functional contribution to the angiogenic cascade.

In summary, our results systematically characterize the temporal pattern of blood vessel growth and regression as well as the phenotypic properties of endothelial cells during the ovarian cycle. They support the usefulness of the ovarian angiogenesis model to study mechanisms of angiogenesis induction and vessel maturation as well as blood vessel regression. It can, for example, be used as a screening system to identify novel angiogenesis-specific endothelial cell molecules (U. Modlich et al., manuscript in preparation). Likewise, it can be used to analyze the temporal expression pattern of other known molecules that are likely involved in the angiogenic cascade. This will help to evaluate to what extent findings obtained from *in vitro* angiogenesis systems as well as experimental *in vivo* angiogenesis models (eg, rabbit cornea and animal tumor models) are applicable and/or follow the same mechanisms as *in vivo* physiological angiogenesis. Finally, cyclic angiogenic processes in the ovary offer a useful experimental approach to study mechanisms of physiological blood vessel regression.

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