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Pattern of interleukin 6 gene expression *in vivo* suggests a role for this cytokine in angiogenesis

(vascularization/ovarian follicles/embryo implantation/decidua/*in situ* hybridization)

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ABSTRACT **Interleukin 6 (IL-6) is a cytokine that acts on various cell types. Here we show that IL-6 mRNA is produced *in vivo* in two self-limiting physiologic angiogenic processes: (*i*) the formation of the vascular system accompanying development of ovarian follicles and (*ii*) the formation of a capillary network in the maternal decidua following embryonic implantation. *In situ* and RNA blot hybridization analyses detected transient expression of IL-6 mRNA in gonadotropin-primed hyperstimulated ovaries, with maximal mRNA levels coinciding with the period of formation of a capillary network around follicles. Expression of IL-6 mRNA was detected in the vasculature extending from the ovarian medulla to the forming capillary sheath in the thecal layer of individual growing follicles. No expression was detected in more-developed preovulatory follicles once angiogenesis had been completed. IL-6 mRNA was also detected in the uterus of pregnant mice 9.5 days postcoitum, and there was no appreciable IL-6 mRNA at later stages of embryonic development. Expression in the uterus was confined to cords of endothelial cells in the process of formation of an anastomosing network that traversed the maternal decidua towards the developing embryo. The expression of IL-6 mRNA in two independent physiological angiogenic processes and the transient nature of its expression in endothelial cells suggest a role for IL-6 in angiogenesis.**

Interleukin 6 (IL-6) is a cytokine that is produced by different cell types and can act on various cells including B cells, T cells, myeloid cells that produce macrophages, granulocytes, or megakaryocytes, hepatocytes, plasmacytomas, and hybridomas (1–9), as well as epithelial cells (10), endothelial cells (11), keratinocytes (12), and mesangial cells (13). In order to study IL-6 gene expression in the context of interactions between different cell types in a natural setting, we have used *in situ* hybridization to identify cells in which IL-6 mRNA is produced during defined physiological processes *in vivo*. Here we report that IL-6 mRNA is produced during angiogenesis.

Angiogenesis, the process of generating new blood vessels by an outgrowth of specific cell types, is regulated by a variety of factors including acidic and basic fibroblast growth factor, transforming growth factors α and β, heparin, and certain prostaglandins and steroids (14–17). In the healthy mature animal, vascular turnover is low and angiogenesis rarely occurs. One exception is the female reproductive system, where follicular growth (18), formation of the corpus luteum (19), and the cyclic changes in the uterine endometrium (20) are all accompanied by vascularization (21). Vascularization is also an important event in the maternal response to embryonic implantation and during early embryonic development (22). Here we show that IL-6 mRNA is transiently expressed during angiogenesis that accompanies two natural processes, folliculogenesis and formation of the maternal decidua during early postimplantation development. This suggests a role for IL-6 in angiogenesis.

MATERIALS AND METHODS

Animals, Embryos, and Organs. Eight-week-old C57BL/6 female mice were used. Hyperstimulation of ovaries was induced by injecting 5 international units of pregnant mare's serum gonadotropin (PMSG) (G-4877, Sigma). Five international units of human chorionic gonadotropin (hCG) (GG-2, Sigma) was injected 44 hr later. Matings were with C57BL/6 males. Embryos were staged as 0.5 day postcoitum at noon of the day on which the vaginal plug was observed. Ovaries and uteri from nonpregnant or postimplantation pregnant mice were collected into 4% paraformaldehyde in phosphate-buffered saline (PBS) and fixed overnight at 4°C (excluding organs used for RNA extraction, which were collected into PBS). Fixed organs were incubated overnight in 0.5 M sucrose in PBS before embedding in Tissue-tek O.C.T. medium (Miles Scientific) and *in situ* hybridization. Spleens were stimulated with bacterial lipopolysaccharide (LPS) by injecting 200 μg of LPS per animal intraperitoneally and collecting the spleens 2 hr later.

***In Situ* Hybridization.** This was performed essentially as described (23). In brief, 10-μm-thick frozen sections were collected on poly(L-lysine)-coated glass slides, refixed in 4% paraformaldehyde, and dehydrated in graded ethanol solutions. Before hybridization, sections were pretreated successively with 0.2 M HCl, 2× SSC (1× SSC is 0.15 M NaCl/0.015 M sodium citrate), Pronase (0.125 mg/ml), 4% paraformaldehyde, and acetic anhydride in triethanolamine buffer. Hybridization was carried out at 50°C overnight in 50% (vol/vol) formamide/0.3 M NaCl containing 10% (wt/vol) dextran sulfate, 1× Denhardt's solution (0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin), carrier tRNA (1 mg/ml), 10 mM dithiothreitol, 5 mM EDTA, and ^{35}S-labeled RNA probe (2×10^8 cpm/ml). Washing was performed under stringent conditions that included an incubation at 50°C for >14 hr in 50% formamide/0.3 M NaCl and a 30-min incubation at 37°C with RNase A (20 μg/ml). Autoradiography was performed using Kodak NTB-2 nuclear track emulsion and autoradiographic exposure was for 4–5 days. All experiments were repeated with three separate animals or embryos for each time point and >10 sections per specimen. Control hybridizations with "sense" RNA probes were carried out in all experiments.

Isolation and Blot Analysis of RNA. Total RNA was extracted from tissues homogenized in LiCl/urea solution (procedure C in ref. 24). RNA was denatured in formaldehyde and electrophoresed through a 1.3% agarose/formaldehyde gel in

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Abbreviations: IL, interleukin; M-CSF, macrophage colony-stimulating factor; PMSG, pregnant mare's serum gonadotropin; hCG, human chorionic gonadotropin; LPS, lipopolysaccharide.

Mops buffer. RNAs were transferred onto a nylon-based membrane (GeneScreen, NEN) and hybridized with the indicated probes as described (25). The experiments were repeated three times with different RNA preparations. For each time point RNA was extracted from ovaries pooled from four animals.

Hybridization Probes. A mouse IL-6 cDNA clone was provided by S. C. Clark (3) in a pXM plasmid vector. The ≈1-kilobase-long insert was excised by *Pst* I digestion and recloned in a pBS vector (Stratagene). This cDNA clone is missing sequences upstream of the third amino acid codon but is otherwise complete. The mouse IL-1α cDNA clone pIL-1 1301 was provided by P. T. Lomedico (26). A 430-base-pair *Pst* I–*Pvu* II fragment (corresponding to amino acids 101–239) was subcloned in the pBS vector. A mouse macrophage colony-stimulating factor (M-CSF) cDNA clone was provided by S. C. Clark. An ≈800-base-pair fragment was subcloned in the pBS vector. Constructs in pBS vector were linearized by digestion with the appropriate restriction endonuclease to allow synthesis of an ^{35}S-labeled complementary RNA in either the antisense or the sense orientation (by using T3 or T7 RNA polymerase, respectively). RNA probes were fragmented by a mild alkaline treatment prior to use for *in situ* hybridizations. For RNA blot analysis the same IL-6 cDNA fragment was labeled with ^{32}P by randomly primed DNA synthesis.

RESULTS

In situ hybridization enabled us to study IL-6 mRNA production under natural settings that preserve the contacts and interactions between different cell types in a given tissue, and to detect IL-6 mRNA at its site of synthesis. As a source of IL-6-specific sequences we used a cDNA clone of mouse IL-6 that includes almost the complete coding region of the gene. ^{35}S-labeled cRNA in an antisense orientation was synthesized *in vitro* and used as a hybridization probe. Frozen sections (10 μm thick) were prepared from the appropriate organs, processed, and hybridized *in situ* under stringent conditions. mRNA/antisense RNA hybrids were subsequently visualized by autoradiography. Preliminary screening of a number of candidate developmental processes suggested an association between IL-6 expression and angiogenesis. To determine whether IL-6 mRNA is specifically expressed during angiogenesis, we selected for further study two processes in which vascularization occurs in a readily accessible tissue over a relatively short period of time and in a well characterized schedule.

Expression of IL-6 mRNA During Folliculogenesis. Small pre-antral follicles have no special vascular supply of their own. Concomitant with their further growth, however, follicles acquire individual vascular sheaths (18, 27). In order to follow IL-6 expression during follicular development, we used PMSG-primed female mice. In this experimental system ovaries are hyperstimulated by follicle-stimulating hormone in response to injection of PMSG. As a result, a larger than usual cohort of follicles is induced to develop in a roughly synchronous manner (28). RNA was extracted from pooled ovaries at various times after PMSG injection and subjected to RNA blot hybridization analysis with an IL-6-specific probe. IL-6 was found to be transiently expressed in PMSG-stimulated ovaries: the mRNA was detected at 5 hr after PMSG administration, was barely detectable at 20 hr, and was undetectable at 44 hr, after the onset of follicular growth (Fig. 1). Down-regulation of IL-6 mRNA thus preceded the progression of follicles to preovulatory stages. Maximal IL-6 mRNA expression coincided with the period of rapid growth and differentiation of follicles, the period in which the vascular sheath surrounding the follicle is established (29). On a quantitative basis, the apparent maximal level of IL-6 mRNA

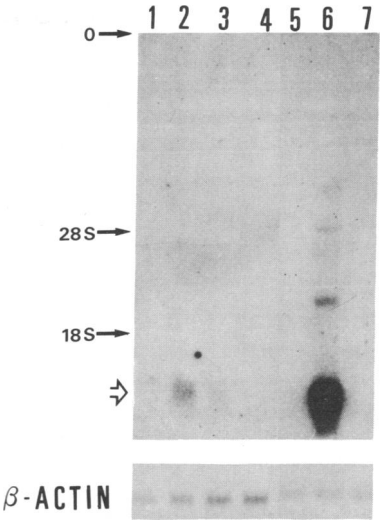

FIG. 1. Detection of IL-6 mRNA in mouse ovaries. Animals were injected with PMSG and sacrificed at the indicated times afterwards. Ovaries were removed and total ovarian RNA was extracted and subjected to RNA blot hybridization analysis with an IL-6-specific probe. Eight micrograms of total RNA was loaded in each lane. Filters were rehybridized with a β-actin probe (36) in order to serve as internal standards for the amount of RNA loaded and RNA quality. Lanes: 1, randomly cycling ovaries; 2, 5 hr after PMSG; 3, 20 hr after PMSG; 4, 44 hr after PMSG; 5, RNA from control spleens; 6, RNA from LPS-treated spleens; 7, RNA from spleens of PMSG-treated animals (5 hr after PMSG). Top of gel (o, origin) and positions of 28S and 18S rRNA are indicated.

in ovaries is lower than in LPS-stimulated spleen cells (Fig. 1). However, whereas IL-6 is expressed in a large fraction of cells in the stimulated spleen, only a minor fraction of ovarian cells express IL-6 (see below). The amount of IL-6 mRNA in producer ovarian cells may, therefore, be comparable to the amount in activated spleen cells.

The identity of ovarian cells that express IL-6 was established by *in situ* hybridization (Figs. 2 and 3). To allow direct comparison of relative IL-6 mRNA levels, ovaries removed at various times after PMSG injection were cosectioned and subsequently processed and hybridized all on the same slide. In agreement with the RNA blot hybridization data (Fig. 1), hybridization signals were detected only in ovaries collected earlier than 24 hr after administration of PMSG (Fig. 2). Both the RNA blots and *in situ* hybridizations showed similar kinetics for the IL-6 signal. No hybridization signals were detected with an IL-6 RNA probe in the sense orientation. Higher magnification views of an ovary undergoing extensive follicular growth (ovary no. 2 in Fig. 2) are shown in Fig. 3 to allow a clearer identification of the IL-6 mRNA-producing cells. IL-6 mRNA within the ovary was detected in endothelial cells constituting the ovarian vasculature. The ovarian vasculature consists of a medullary vasculature and a capillary network extending towards cortical follicles. The latter form two concentric networks of vessels in the theca interna and the theca externa, respectively, of each growing follicle. A typical growing cortical follicle, staged at maturation stage 6 (30), was boxed (A) in Fig. 2 and is shown at high magnification in Fig. 3*A*. Hybridization signals in the cortical region were found in cells embedded within the thecal layers of the growing follicle (Fig. 3*A*). Although it is difficult to attribute the hybridization signals to the small single-layered capillary plexus embedded within thecal layers (arrow in Fig. 3*A*), the distribution pattern of expressing cells is consistent with this assignment. In addition, the lack of detectable hybridization in granulosa cells is consistent with the observation that capillaries do not penetrate the follicular mem-

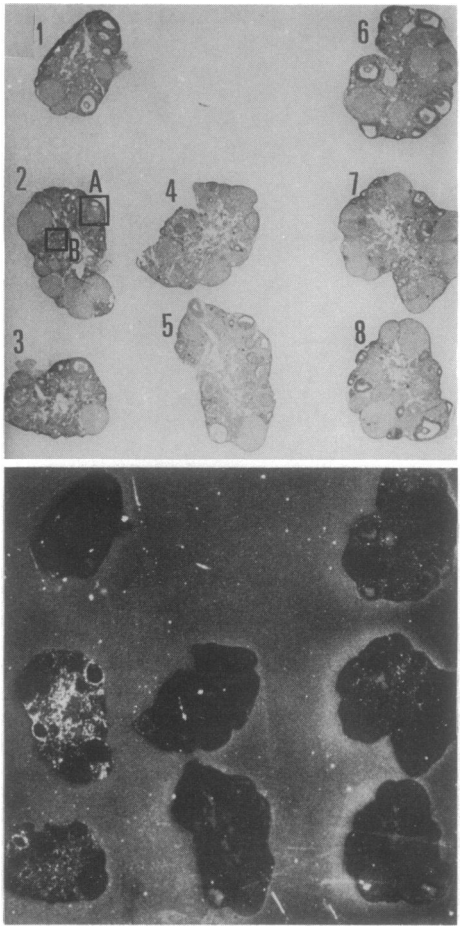

FIG. 2. *In situ* hybridization for IL-6 mRNA in cycling ovaries. PMSG treatment and removal of ovaries were as described for Fig. 1. Ovaries were collected directly to paraformaldehyde for immediate fixation. All ovaries were embedded in Tissue-tek O.C.T. embedding medium in a single cryomold, to allow cosectioning and coprocessing of all samples on a single slide. Ovary 1 was from a randomly cycling mouse; ovaries 2 and 3 were collected 5 hr after PMSG injection (from different animals); ovaries 4 and 5 were collected 24 hr post-PMSG; ovaries 6 and 7 were collected 44 hr post-PMSG (zero time for hCG injection); ovary 8 was collected 5 hr post-hCG. Ovaries were photographed under bright-field (*Upper*) and dark-field (*Lower*) illumination. (×6.)

brana granulosa prior to ovulation (21, 27). Localization of IL-6 mRNA to blood vessels was particularly clear in the medullary region, where numerous transverse sections through the highly coiled vessels were present in the fields that were screened (box B in Fig. 2, magnified in Fig. 3*B*). Occasionally, sections displaying early stages of folliculogenesis were also obtained from ovaries derived from randomly cycling animals. In these cases a pattern of hybridization similar to the one obtained with PMSG-primed ovaries was observed, indicating that IL-6 was not activated by some nonphysiological action of the injected PMSG. Furthermore, RNA extracted from the spleens of the PMSG-primed animals contained no detectable IL-6 mRNA, as also found in control spleens (Fig. 1), indicating that activation of IL-6 in the developing follicles was not due to another stimulating factor contaminating the PMSG preparation.

IL-6 mRNA could not be detected in follicles that had progressed in their pre-ovulatory development for >24 hr after injection of PMSG. Several examples of follicles at stages 8 and onwards with no detectable IL-6 mRNA are shown in ovaries 4–8 in Fig. 2. The undetectability of IL-6 mRNA in the vasculature of follicles with an apparent complete capillary plexus suggests that IL-6 expression is associated with the angiogenic process *per se*, rather than reflecting expression of IL-6 in vascular endothelium in general.

Expression of IL-6 mRNA in Maternal Decidua During Early Postimplantation Development. To determine whether our findings regarding the expression of IL-6 mRNA could be extended to another angiogenic process, we monitored IL-6 expression during vascularization in the maternal decidua following embryonic implantation. Timed pregnancies were set up, uterine segments that contained both interimplantation regions and multiple implantation sites were collected into a fixative at daily intervals from 8.5–11.5 days postcoitum, and organs were sectioned and processed for *in situ* hybridization. Fig. 4*A* shows a general view of an implantation site 9.5 days postcoitum where maximal expression of IL-6 was observed. Although the embryo proper is not included in the sectioning plane of this particular section, the yolk sac cavity, some extraembryonic tissues, and the large mass of maternal decidual cells are visible. The relatively homogeneous mass of large decidual cells is traversed by strands of endothelial cells. The latter are better seen at higher magnification (Fig. 5 *A–C*). The *in situ* hybridizations show that IL-6 mRNA is localized to these cords of endothelial cells. A control hybridization with a sense RNA probe (identical in sequence to IL-6 mRNA) gave no signal under identical experimental conditions (Fig. 4*B*). The observed arrangement of IL-6 mRNA-expressing cells was that of individual cords of endothelial cells of variable lengths (Fig.

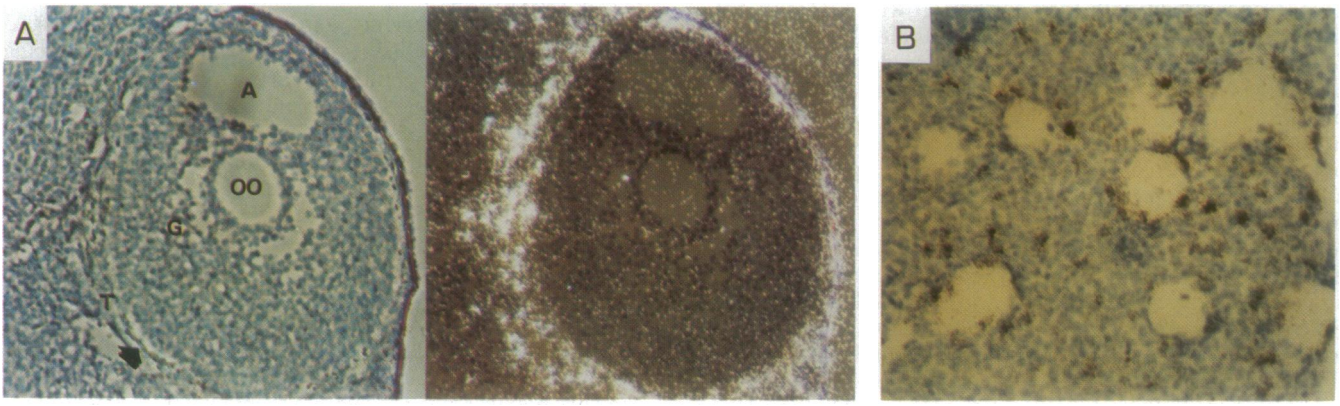

FIG. 3. Identification of IL-6 mRNA-expressing cells within the ovary. Shown is a high-magnification view of the two boxed regions (A and B of ovary no. 2) in Fig. 2. (*A*) Ovarian follicle photographed under bright-field (*Left*) and dark-field (*Right*) illumination. OO, oocyte; A, antrum; G, granulosa cells; T, thecal layer. Arrow points to a blood vessel. (×100.) (*B*) Medullary region showing sections through blood vessels. Sections were counterstained with toluidine blue (23). (×150.)

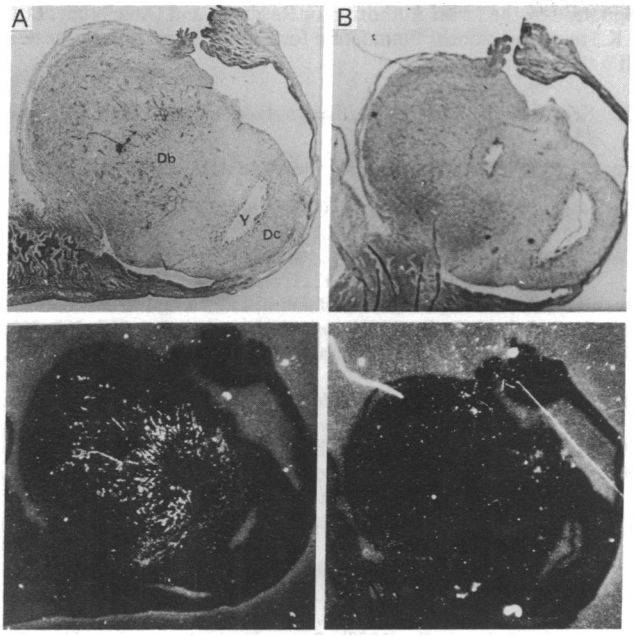

FIG. 4. Expression of IL-6 mRNA at the implantation site of a 9.5-day embryo. The embryo itself is not included in the particular sections shown. Y, yolk sac cavity; Dc, decidua capsularis; Db, decidua basalis; UT, uterus at an interimplantation region. Section was photographed under bright-field (*Upper*) and dark-field (*Lower*) illumination. (*A*) Antisense IL-6 probe. (*B*) Sense IL-6 probe. (×11.)

5 *B* and *C*). Overall, cells expressing IL-6 mRNA form a pattern of anastomosing network that traverses the maternal decidua towards the embryo. This pattern is typical for an ongoing extension of blood vessels, where individual sprouts of endothelial cells join or anastomose with each other to form a contiguous vessel (21, 22). In transverse sections through decidual vessels, expression of IL-6 mRNA is seen in the lining endothelium (Fig. 5*D*). Assignment of expressing cells as parts of a vascular system was also aided by the identification, in some cases, of blood cells enclosed within the forming network of strands. Sections that included implantation sites at the later stages of embryonic development (days 10.5 and 11.5 postcoitum) did not show appreciable decidual hybridization. This emphasizes the transient nature of IL-6 expression in this angiogenic process.

Using an IL-1α-specific probe in experiments that were otherwise identical to those carried out with the IL-6 probe, we were unable to detect specific activation of IL-1α during folliculogenesis or decidua formation. As a positive control for the IL-1α probe, we readily detected hybridization signals in spleens activated with LPS. Another cytokine that we tested in a parallel study was M-CSF. Again, no expression was detected in ovaries or in the decidua of 9.5-day embryos. However, hybridization specific for M-CSF, a cytokine that is produced in embryonic implantation (31), was readily detected in the uterine epithelium in sections of pregnant females 9.5 days postcoitum, confirming previous results (32).

DISCUSSION

We have shown that IL-6 mRNA is produced in endothelial cells during the process of vascularization. It remains to be determined whether accumulation of IL-6 mRNA in these cells is due to transcriptional activation and/or to posttranscriptional stabilization of IL-6 mRNA. Transient expression of IL-6 mRNA was demonstrated in two independent angiogenic processes: the formation of a vascular sheath surrounding developing ovarian follicles, and extension of blood

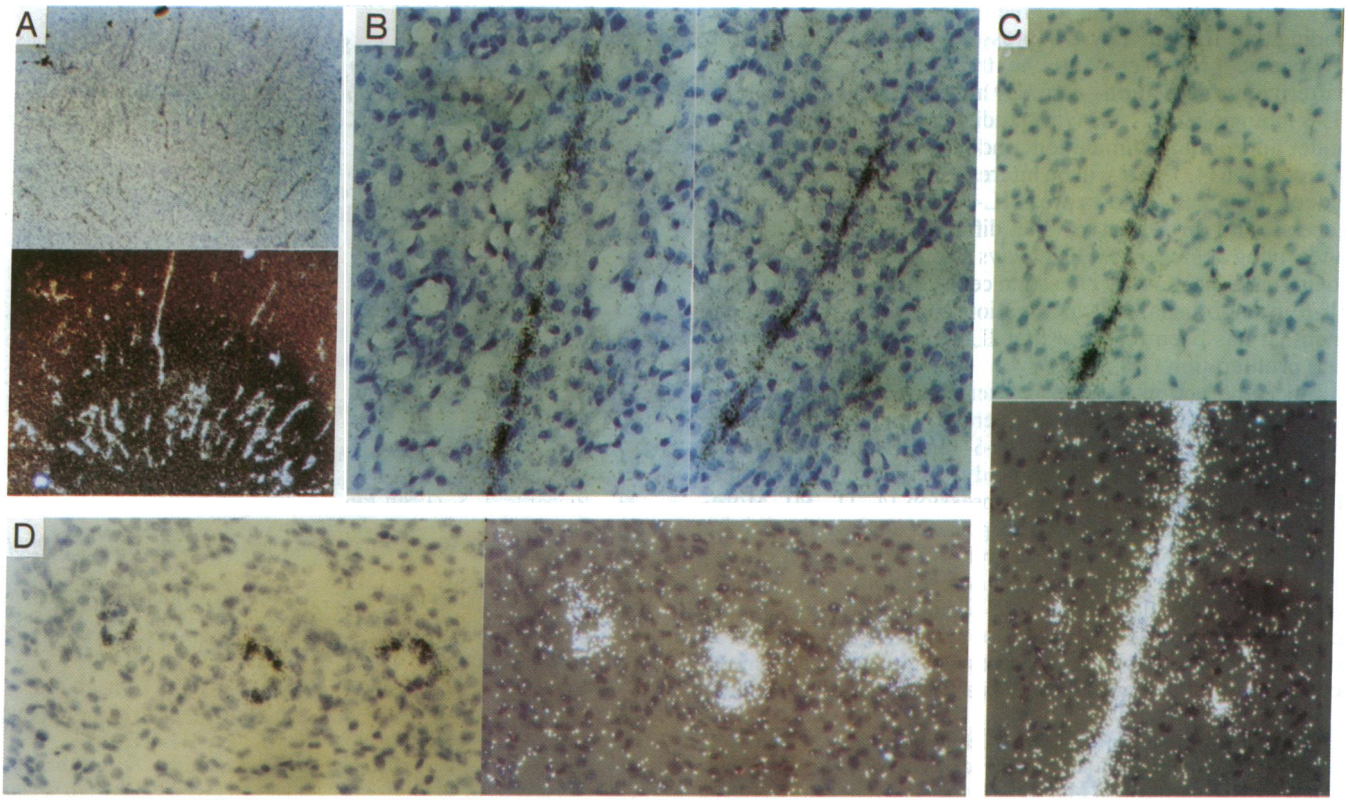

FIG. 5. IL-6 mRNA in the decidua is located in endothelial cells. Low (*A*) and high (*B–D*) magnification of different regions within the decidua basalis at an implantation site of a 9.5-day embryo show that hybridization signals are primarily over cords of endothelial cells (*A–C*) and over endothelial cells in transverse sections of vessels (*D*). Sections were counterstained with toluidine blue. *A*, *C*, and *D* were also photographed under dark-field illumination. (*A*, ×37; *B–D*, ×110.)

vessels in the maternal decidua following embryonic implantation. There are some other angiogenic processes that occur naturally in the healthy adult in reproductive tissues. One example is the formation of a capillary network within the *corpus luteum* after the rupture of mature follicles and ovulation (19). In a preliminary *in situ* hybridization analysis, we have observed expression of IL-6 mRNA in this process (data not shown). Angiogenesis also accompanies the cyclic destruction and reconstitution of the endometrium (20). It is therefore likely that the finding that freshly explanted human endometrial cells secrete IL-6 (33) reflects a role of IL-6 in renewal of endometrial blood vessels. We suggest that activation of IL-6 is a feature of all self-limiting angiogenic processes that take place in the reproductive system. It is possible that IL-6 is also activated in angiogenesis that occurs in wound healing and tumor growth.

Angiogenesis is a process involving local degradation of basement membranes, chemotaxis, cell recruitment, directed cell migration, and cell proliferation (14–16). Moreover, a negative regulation must be in operation, as vascularization is a quiescent process under normal conditions and, once initiated, is a self-limiting process. Multiple gene products participate in coordination of the different cell interactions underlying angiogenesis, and various compounds have been identified that stimulate angiogenesis in *in vivo* assay systems and that affect endothelial cell proliferation and/or locomotion *in vitro* (14–16). It has been suggested that angiogenic factors may operate either directly or indirectly when evaluated according to their targets, i.e., vascular endothelial cells or other cell types releasing active effector compounds (16). The approach taken in this study, namely, identifying genes that are activated *in vivo* in close association to a naturally occurring angiogenic process, can be considered as a complementary approach to identify candidate genes that play either a direct or an indirect role in angiogenesis. Our finding that IL-6 mRNA is expressed in several independent angiogenic processes—and the transient, process-specific pattern of its activation—strongly argues for an active role of IL-6 in angiogenesis. *In vitro* studies have also shown that, under appropriate stimulation, endothelial cells produce and secrete IL-6 (4, 11). It will be interesting to determine to what extent these cells also express IL-6 receptors during angiogenesis and in which of the different cellular reactions underlying angiogenesis IL-6 plays a role. The ability of IL-6 to increase the motility of certain cells (10) suggests a role for IL-6 in endothelial cell locomotion and their proper alignment. It has been reported that IL-6 can inhibit endothelial cell proliferation (11).

Since the action of IL-6 is exerted within a network of interacting cytokines, it is of interest to determine whether other cytokines share with IL-6 the specific pattern of expression established by this study. Of particular interest is IL-1, which can induce IL-6 expression (4, 11, 34). Moreover, IL-1 has been reported to possess angiogenic activity (cited as "unpublished data" in ref. 34) in the rabbit cornea assay (35). However, we did not detect expression of IL-1α mRNA during folliculogenesis or decidua formation. Similar experiments with IL-1β and other cytokines are required in order to establish whether expression during angiogenesis occurs with other cytokines or is a unique feature of IL-6.

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