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In vivo patterns of expression of urokinase and its inhibitor PAI-1 suggest a concerted role in regulating physiological angiogenesis

(plasminogen activator/ovary/*in situ* hybridization)

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ABSTRACT **To evaluate the role of plasminogen activators (PAs) in physiological angiogenesis, we have investigated the *in vivo* patterns of expression of urokinase-type PA (uPA) and PA-inhibitor type 1 (PAI-1) during neovascularization of ovarian follicles, the corpus luteum, and the maternal decidua. Using *in situ* hybridization, we detected uPA mRNA in the ovary along the route of capillary extension, originating at the existing ovarian vasculature, extending toward growing follicles, and terminating at the newly formed capillary sheaths surrounding each growing follicle. Following ovulation, uPA mRNA was expressed in capillary sprouts within the developing corpus luteum. During the process of decidual neovascularization, uPA expression was detected in endothelial cell cords traversing the maternal decidua in the direction of the newly implanted embryo. uPA mRNA was not detected in endothelial cells upon completion of neovascularization, suggesting that uPA expression is a part of the angiogenic response. During *in vitro* "angiogenesis" of cultured aortic explants, uPA was expressed in capillary sprouts but not in underlying endothelial cell sheets, suggesting that the expression of uPA depends on the histological context of the endothelial cell. Interestingly, during corpus luteum development and decidual neovascularization, and in aortic explants, PAI-1 expression was preferentially activated in cells in the vicinity of uPA-expressing capillary-like structures. These findings suggest a functional interplay between uPA- and PAI-1-expressing cells and support the idea that natural PA inhibitors function during angiogenesis to protect neovascularized tissues from excessive proteolysis.**

For neovascularization to occur, endothelial cells must be freed from the constraints of their enveloping basement membranes. Local dissolution of the basement membrane is achieved by targeted proteolysis and is thought to affect the organization and orientation of endothelial cells in a fashion that promotes their migration and proliferation (1, 2). Subsequent migration of endothelial cells and capillary extension through solid tissues also require active proteolysis.

Plasminogen activators (PAs) have been implicated as activators of proteolysis associated with angiogenic responses. The cellular localization of the various components involved in plasmin production (plasminogen, PAs, and their respective receptors) outlines a remarkable pathway of cell surface-mediated proteolysis (3). This feature, in conjunction with the observations that urokinase-type PA (uPA) receptor may polarize expression of the protease to cell–cell and cell–substratum contacts (4, 5) or to the leading edge of migrating cells (6), has prompted suggestions that the system operates under circumstances that require restricted and targeted proteolysis. PA activity has indeed been correlated with cell migration (7) and in a number of invasive biological processes, including invasiveness of tumor cells (8, 9) and embryonic trophoblast cells (10).

Production of uPA by cultured endothelial cells is enhanced by treatment with angiogenic factors (11, 12) and the production of matrix-degrading proteolytic enzymes plays an important role in capillary morphogenesis (13). However, the production of uPA *in vivo*, in the context of natural angiogenic processes, has not been shown. Angiogenesis is mostly repressed in the fully developed healthy organism and is activated predominantly under various pathological settings. Yet extensive angiogenesis naturally takes place in the female reproductive system.

The requirement for mechanisms that will locally protect certain cells and matrices from adverse proteolysis has prompted the suggestion that specific protease inhibitors may be locally activated to restrain excessive proteolysis in particular microenvironments. Imbalanced proteolysis during angiogenesis leads to subversion of the normal morphogenetic program of endothelial cells (13, 14), and a cartilage-derived inhibitor of angiogenesis possesses a collagenase-inhibitor activity (15). PA inhibitor type 1 (PAI-1), by virtue of its ability to bind, and bring about internalization of active uPA complexes (16) and its localization on the cell surface or deposition in the extracellular matrix (17, 18), is a good candidate to serve as a local attenuator of uPA-mediated proteolysis. To examine this possibility we determined the spatial relationship between uPA- and PAI-1-expressing cells in the course of physiological angiogenesis.

MATERIALS AND METHODS

Animals, Embryos, and Organs. Eight-week-old C57BL/6 female mice or, when indicated, prepubertal 25-day-old rats were used. Hyperstimulation of ovaries and derivation of embryonic implantation sites were performed as described (19).

Rat Aortic Ring Explant Cultures. Rat aortic ring explant cultures, grown in tissue culture chamber slides (Lab-Tek), were prepared essentially as described by Diglio *et al.* (20). Cultures showing spontaneous generation of an array of vascular-like channels were fixed and processed for *in situ* hybridization.

***In Situ* Hybridization.** This procedure was performed as described (21), with some modifications (19).

Hybridization Probes. The following DNA fragments were cloned onto the polylinker of a pBS vector (Stratagene) to serve as templates for synthesis of specific cRNAs: a 1.1-kilobase (kb) *Eco*RI–*Bgl* II fragment derived from the coding and 3′ noncoding regions of the mouse uPA cDNA (22), a 2.4-kb *Xho* I–*Eco*RI fragment derived from the coding and 3′ noncoding regions of the mouse PAI-1 cDNA clone *mrl* (23), and an 8.8-kb *Eco*RI–*Sal* I fragment derived from a human

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Abbreviations: PA, plasminogen activator; uPA, urokinase-type PA; PAI-1, PA inhibitor 1; PMSG, pregnant mare's serum gonadotropin; hCG, human chorionic gonadotropin; vWF, von Willebrand factor.

von Willebrand factor (vWF) cDNA clone (24). Constructs in pBS vector were linearized by digestion with the appropriate restriction endonuclease to allow synthesis of a ^{35}S-labeled complementary RNA (cRNA) in either the antisense or the sense orientation (by using T3 or T7 RNA polymerase). RNA probes were fragmented by a mild alkaline treatment prior to use for *in situ* hybridization.

RESULTS

uPA Is Transiently Expressed During Neovascularization of Ovarian Follicles. Noninduced and small preantral follicles have no vascular supply of their own. However, further development of follicles is accompanied by their neovascularization, and accordingly, angiogenesis is "switched on" shortly after the onset of follicular growth. To follow uPA expression during development of ovarian follicles, we analyzed follicular development induced by follicle-stimulating hormone. In this experimental system a larger than usual cohort of follicles is induced to develop in a roughly synchronous manner (25), imposing a demand for rapid generation of a very extensive vascular network.

RNA was extracted from pooled ovaries at various times after injection of pregnant mare's serum gonadotropin (PMSG) and subjected to RNA blot hybridization analysis with a uPA-specific probe. Steady-state levels of uPA mRNA increased shortly after PMSG administration, reached maximal levels about 5 hr after the onset of follicular growth, and declined rapidly thereafter (data not shown). Thus, the wave of PMSG-induced uPA expression coincided with the period of follicular neovascularization (26). To correlate the PMSG-induced wave of uPA expression with a particular process, we determined the identity of cells that express uPA by *in situ* hybridization with a ^{35}S-labeled uPA-specific cRNA probe. Fig. 1 shows a representative ovary, withdrawn 5 hr after treatment with PMSG. In the medullar region, uPA mRNA was abundantly expressed in the lining endothelium of large blood vessels, as well as in cells of the surrounding interstitial tissue (Fig. 1 *A* and *B*).

Capillaries are known to extend through the interstitial tissue toward cortical follicles and to form two concentric capillary sheaths in the theca externa and theca interna of each individual growing follicle (reviewed in ref. 27). uPA mRNA was detected in the interstitial stroma along the pathway of capillary extension toward the follicles, and in cells embedded within the theca layers of growing follicles (Fig. 1 *C* and *D*). Due to insufficient resolution, however, it was not possible to unambiguously identify expressing cells embedded within the theca as endothelial cells forming the single-layered capillary plexus. Consistent with the suggestion that the thecal expression of uPA is associated with a transient developmental process, we could barely detect hybridization signals in the steroidogenic theca of follicles that had passed stage 8 [using the staging system of Peters (28)] (data not shown). We assume that the complex pattern of preovulatory uPA expression reflects a role of uPA in additional ovarian remodeling processes.

Expression of uPA and PAI-1 During Development of the Corpus Luteum Vasculature. To more clearly show that expression of uPA is intrinsic to angiogenesis, we examined the pattern of uPA expression in the process of capillary invasion into the collapsed postovulatory follicle, accompanying the development of the corpus luteum. Rat ovaries were used in these experiments because, in contrast to the mouse system, uPA is not expressed in rat granulosa cells (29). This allowed us to better visualize expression of uPA in capillary sprouts that had invaded the membrana granulosa (i.e., the matrix separating theca and granulosa layers). Ovaries were withdrawn after the presumed timing of ovulation [>12–14 hr after administration of human chorionic gonadotropin (hCG)] and processed for *in situ* hybridization analysis. Fig. 2 shows representative stages in the development of the corpus luteum: a preovalutory follicle (Fig. 2*A*), intermediate stages where the antrum of the collapsed follicle has been partially filled with granulosa and differentiated lutein cells (Fig. 2 *A* and *B*), and a fully developed corpus luteum, distinguished by a lutein cell mass filling the entire space of the former follicular antrum (Fig. 2*F*). It is clear that uPA mRNA was transiently expressed in the developing corpus luteum, with no expression detectable in the granulosa compartment either before ovulation or once the development of the corpus luteum had been completed. uPA expression within the developing corpus luteum was mostly restricted to endothelial cells organized in cords (Fig. 2*E*), presumably representing extending capillary sprouts. No expression was detected in the extensive, functional vascular network of the mature corpus luteum (Fig. 2*F*).

To determine whether the uPA inhibitor PAI-1 plays a role in regulating angiogenesis of the corpus luteum, we determined its patterns of expression in adjacent serial sections. During formation of the corpus luteum, PAI-1 mRNA was expressed inside the collapsed follicle in a subpopulation of cells localized at the innermost layers of the proliferating granulosa (Fig. 2*D*). PAI-1 expression remained restricted to the inner cell layers throughout the progressive expansion of the granulosa/lutein cell mass and until the closure of the follicular cavity. Thus, a physical proximity was observed between the populations of uPA- and PAI-1-expressing cells.

Identity of Cells Expressing uPA and PAI-1 During Neovascularization of the Decidua in Early Postimplantation Development. To analyze an independent angiogenic process, we monitored uPA and PAI-1 expression during neovascularization of the maternal decidua following embryonic implantation. Timed pregnancies were set up, uterine segments that contained implantation sites were collected into a fixative at daily intervals from 8.5 to 11.5 days postcoitum, and speci-

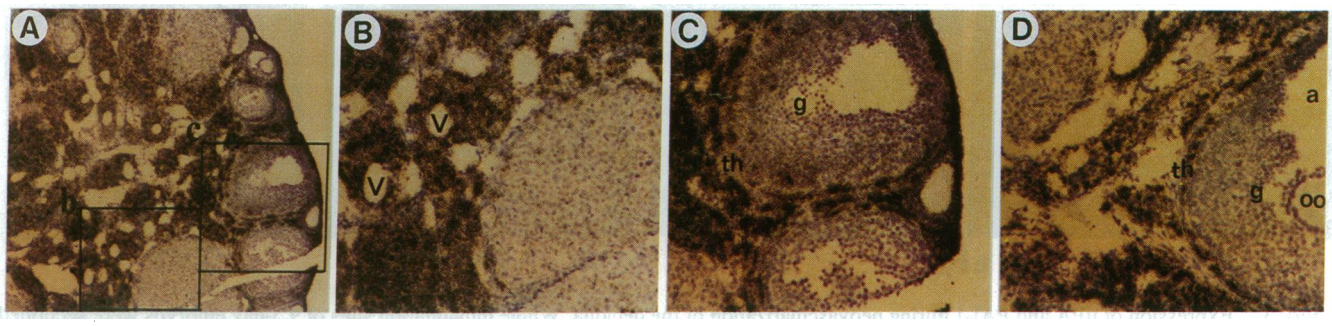

FIG. 1. Identification of cells expressing uPA during development of ovarian follicles. A representative section of an ovary, withdrawn 5 hr after injection of PMSG, was hybridized with a uPA-specific probe. Different regions of the same section are shown. v, Blood vessel; th, theca; g, granulosa; oo, oocyte; a, antrum. (*A*) Low magnification. (×45.) (*B* and *C*) Higher magnification of the two boxed regions (b and c, respectively) shown in *A*. (×112.) (*D*) A different region of the same section. (×112.)

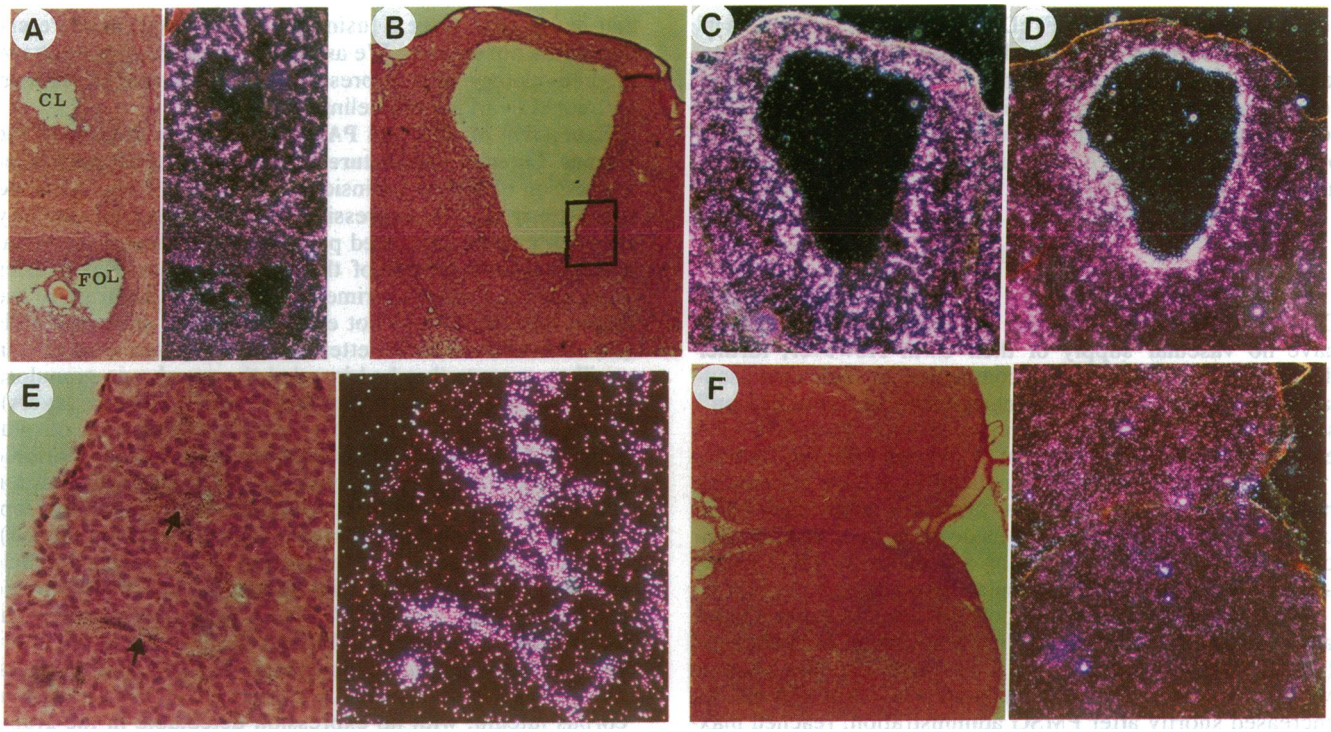

FIG. 2. Expression of uPA and PAI-1 during development of the corpus luteum. Ovulation in prepubertal rats was induced by injecting PMSG and hCG. Ovaries were withdrawn at the indicated times post-hCG, embedded in the same mold, cosectioned, and hybridized *in situ*. All uPA-specific hybridizations shown (*A*, *B*, *C*, *E*, and *F*) were photographed from the same hybridized slide. An adjacent serial section was hybridized with a PAI-1-specific probe (*D*). (*A*) Twenty-six hours post-hCG; bright- and darkfield photographs. CL, corpus luteum; FOL, preovulatory follicle. (*B* and *C*) Thirty-two hours post-hCG; bright- and darkfield photographs, respectively. (*D*) PAI-1 hybridization of an adjacent serial section; same CL is shown. (*E*) Higher magnification of the boxed area in *B*; bright- and darkfield photographs. (*F*) Fifty-six hours post-hCG; bright- and darkfield photographs. Sections were counterstained with hematoxylin/eosin. (*A*, ×23; *B–D* and *F*, ×45; *E*, ×225.)

mens were sectioned and processed for *in situ* hybridization. Fig. 3*A* shows a low-magnification view of an implantation site 9.5 days postcoitum, including some embryonic, extraembryonic, and maternal tissues. The relatively homogeneous mass of decidual cells is traversed by strands of endothelial cells in the process of forming a dense network of vessels converging near the embryo. The network is better seen at a higher magnification (Fig. 3 *B–D*) and was unambiguously shown to be composed of endothelial cells on the basis of its specific hybridization with the endothelial cell-specific vWF probe (Fig. 3*D*). *In situ* hybridization analysis revealed that uPA mRNA was localized to cords of endothelial cells that were in the process of joining each other or anastomosing (Fig. 3 *A* and *B*). Sections of implantation sites at later stages of embryonic development (>10.5 days postcoitum) failed to show appreciable decidual hybridization (data not shown). As with neovascularization of ovarian follicles and corpus luteum, these observations emphasize

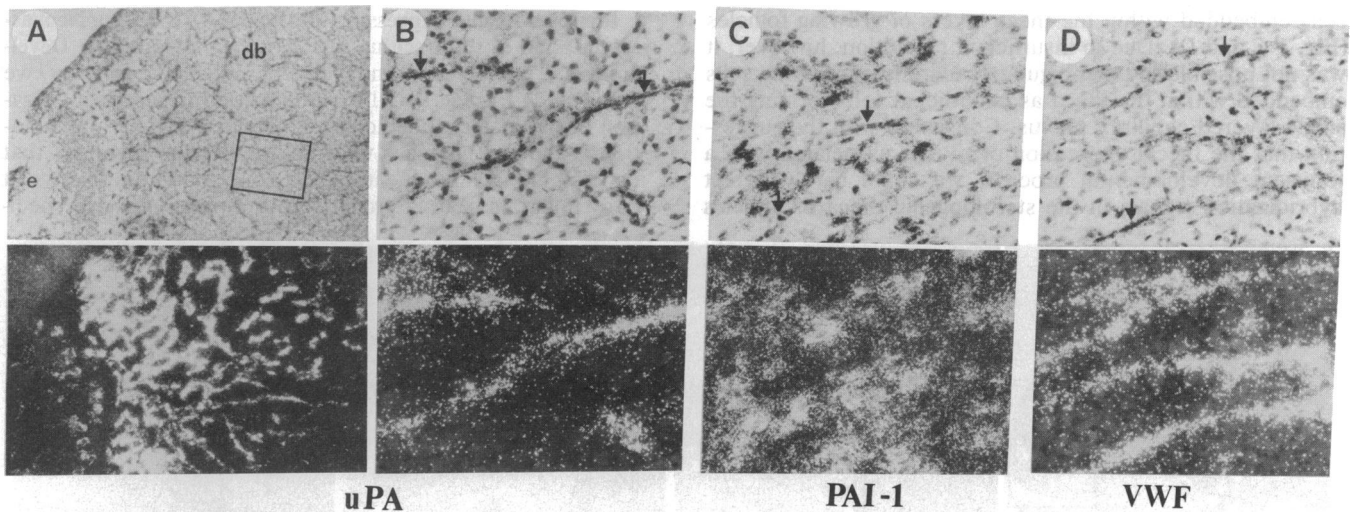

FIG. 3. Expression of uPA and PAI-1 during neovascularization of the decidua. Whole implantation sites of 9.5-day embryos were sectioned and processed for *in situ* hybridization. Both brightfield (*Upper*) and darkfield (*Lower*) photographs are shown. (*A*) A small part of the embryo (e), some extraembryonic tissues, and a relatively large mass of the maternally derived decidua basalis (db); hybridization with uPA probe. (*B*) Enlargement of the boxed area in *A*. Cords of endothelial cells (indicated by arrows) are clearly distinguishable from decidual cells. (*C* and *D*) Serial sections from the same implantation site; hybridization with PAI-1-specific probe and vWF-specific probe, respectively. (*A*, ×23; *B–D*, ×112.)

the transient nature of uPA expression during the angiogenic process.

PAI-1 RNA was not expressed in the endothelial cell cords (i.e., in cells expressing uPA). Interestingly, however, PAI-1 mRNA was expressed in the mass of decidual cells through which the endothelial cell cords migrate and organize (Fig. 3*C*). The spatial proximity of PAI-1-expressing decidual cells to endothelial cells that express uPA would allow, in principle, for functional interaction between the two proteins.

Apposition of uPA- and PAI-1-Expressing Cells During "Angiogenesis" in Aortic Ring Explant Cultures. The observations that uPA and PAI-1 are expressed in different, yet neighboring, cells during physiological angiogenesis, prompted us to examine another system of capillary sprouting, "*in vitro* angiogenesis" of aortic explants. Rat aortic rings were maintained in culture as explants for up to 3 weeks. Most rings showed initial outgrowth of endothelial cells (often accompanied by spreading of smooth-muscle cell populations), followed by spontaneous development of extensive sprouts of new vascular channels radiating on and through the established cell sheet (20). To correlate patterns of gene expression with the organizational status of endothelial cells, cultures were fixed and processed for *in situ* analysis. uPA was abundantly expressed in endothelial cells that were rearranged into tube-like structures, but it could not be detected in endothelial cells comprising the underlying cell sheet (Fig. 4 *A* and *B*).

Analysis of PAI-1 expression revealed a strikingly different pattern. PAI-1 mRNA was undetectable in capillary-like structures but was abundantly expressed in cells adjacent to capillaries (Fig. 4 *C* and *D*). Thus, uPA and PAI-1 mRNAs are expressed in neighboring, but distinct, cell populations. Both uPA- and PAI-1-expressing cells were identified as endothelial cells on the basis of positive *in situ* immunostaining with anti-vWF antibodies (data not shown). These results indicate that the expression program of endothelial cells (i.e., whether expressing uPA, PAI-1, or neither) is likely to be determined by the cellular organization and/or cellular contacts. Close examination of the aortic cultures revealed that PAI-1 expression was not correlated with cell density. Rather, the levels of PAI-1 expression seemed to be positively correlated with the proximity of the expressing cell to the capillaries, with the highest levels of PAI-1 mRNA detected in cells juxtaposed to uPA-expressing capillary cells. These findings suggest that PAI-1 expression is locally induced by nearby uPA-producing capillaries.

DISCUSSION

We have shown that expression of uPA is transiently activated during *in vivo* angiogenesis in a "process-specific" manner. We analyzed four different angiogenic processes. Three of them—neovascularization of ovarian follicles, the corpus luteum, and the maternal decidua—represent physiological angiogenesis. Since these processes are self-limiting, they also provide suitable systems to explore the nature of the built-in mechanisms that down-regulate the angiogenic response. The fourth system, capillary sprouting from aorta explants, does not represent natural angiogenesis but has been suggested to resemble wound healing-associated angiogenesis (20). In all four processes uPA was shown to be expressed in endothelial cells (in the ovary and embryonic implantation sites, uPA was found to be expressed also in nonvascular cells), and in all three physiological processes uPA expression was transient. These observations, when viewed in conjunction with the failure to detect uPA transcription in fully developed blood vessels in any mouse tissue investigated (ref. 30; confirmed by our unpublished observations), support the thesis that a transient uPA expression is built into the angiogenic program. In this respect, our *in vivo* study provides support to the widely assumed role of uPA in controlling angiogenesis-associated proteolysis that has been proposed on the basis of numerous *in vitro* studies.

In the natural processes examined, uPA expression was only transiently activated. This finding implies that both the "on" and "off" switches for uPA transcription are built into self-limiting angiogenic processes. It should be emphasized, however, that the experiments reported here measured steady-state levels of the respective mRNAs. It is possible that posttranscriptional controls, factors effecting the conversion of the proenzyme into an active enzyme, and availability of uPA receptors may further restrict uPA activity to a fraction of the RNA-expressing cell populations defined by our *in situ* hybridization analysis.

How is uPA transcription up-regulated and, in turn, down-regulated during physiological angiogenesis? The stimulation of the production of protease activators and protease inhibitors is a common effect of a number of growth factors (e.g., fibroblast growth factor, transforming growth factor β, epidermal growth factor, colony-stimulating factor 1, interleukin 1) in a variety of target cells (reviewed in ref. 31). Another possibility is that, at least in the reproductive system, uPA transcription is under direct hormonal control. A number of studies have indeed shown that the uPA promoter (32–34), as

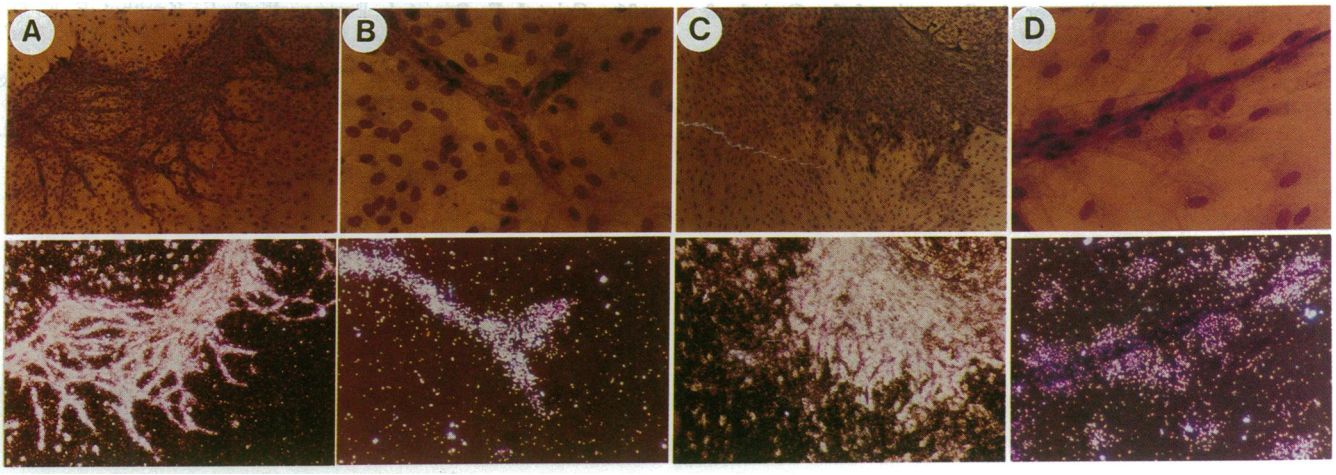

FIG. 4. uPA and PAI-1 expression during *in vitro* angiogenesis in aortic explants. Cultured rat aortic ring explants, displaying arrays of vascular-like channels, are shown 21 days after initial culture. Cultures were fixed after removal of rings and were hybridized *in situ* with the uPA-specific probe (*A* and *B*) or the PAI-1-specific probe (*C* and *D*). Both brightfield (*Upper*) and darkfield (*Lower*) photographs are shown. (*A* and *C*, ×23; *B* and *D*, ×112.)

well as the PAI-1 promoter (35) are hormone-responsive. In principle, cycles of transient uPA and PAI-1 expression within the ovary could be attained through the natural surges of gonadotropins and/or the resultant steroid outputs of steroidogenic ovarian cells.

PAI-1 is associated with the cell surface and may also be deposited in the extracellular matrix (17, 18, 36). These findings suggest that PAI-1 may protect certain cells and matrices from degradation by uPA bound to the surface of nearby cells. In fact, when uPA-producing cells and PAI-1-producing cells were cocultured, degradation of extracellular matrix was inhibited (36). Here we showed that a close proximity between uPA-expressing cells and PAI-1-expressing cells is created under the natural settings of physiological angiogenesis, and during *in vitro* "angiogenesis" (Figs. 2–4). Note, however, that PAI-1 was activated in different cell types (lutein, decidual, and endothelial or smooth muscle cells, respectively). Finally, what mechanism accounts for the close apposition of uPA- and PAI-1-expressing cells? Strikingly, PAI-1 was not uniformly expressed in the granulosa/lutein cell mass, the decidua basalis, and the endothelial cell sheets in the corpus luteum, the decidua, and the aortic explants, respectively, but was detected mostly in cells in the proximity of capillaries. It may be speculated that the apposition-dependent PAI-1 expression reflects induction of PAI-1 expression by some capillary-produced substance(s), and possibly even by uPA. Consistent with the latter suggestion are findings that synthesis of PAI-1 is induced in hepatic and endothelial cell cultures by tissue-type PA (37). A precedent for the activation of a protease inhibitor by the respective protease is the induction of the elastase inhibitor α_1-antitrypsin by elastase (38). Regardless of mechanism, the interrelationship of uPA and PAI-1 expression most likely reflects a concerted role in the spatial restriction of uPA-catalyzed proteolysis.

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