

# Fibronectin and Cell Shape In Vivo: Studies on the Endometrium during Pregnancy

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**ABSTRACT** The rat endometrium during pregnancy was used as a model system to study fibronectin in vivo. Fibronectin distribution on stromal fibroblasts, as determined by indirect immunofluorescence staining, was studied in relationship to cell shape during decidual transformation. Fibroblasts of the estrus endometrial stroma were elongated cells with a fibrillar pattern of fibronectin on their surfaces. During days 1-6 of pregnancy, as these elongated cells acquired a round morphology, fibronectin changed first to a patched distribution on the cells' surfaces and then disappeared. The change in fibronectin was specific for the fibroblasts since over the same time period there was no decrease in fibronectin found associated with blood vessels or in the epithelial-stromal basement membrane. These results support the proposed relationship between cell surface fibronectin and cell shape that has been inferred from in vitro experiments. After implantation, fibronectin distribution was studied in relationship to the position of the conceptus. In the stroma proximal to the implanting conceptus, fibronectin was absent except around blood vessels, which may help explain how decidual tissue could act as a barrier to trophoblast invasion. Finally, fibronectin distribution was studied in the uterus after parturition. Debris in the uterine lumen was coated with fibronectin, which may be important in the rapid removal of this material by phagocytic cells. Also, fibronectin associated with the epithelial-stromal basement membrane was reorganized after reepithelialization had occurred.

Because fibronectin has been shown to play a role in cell adhesion in vitro (9, 18, 39), studies on the physiological relevance of fibronectin have become of particular interest. In wound healing research, for instance, a combination of biochemical and morphological studies, carried out both in vitro and in vivo, have demonstrated that fibronectin becomes covalently linked to fibrin during blood clotting and provides an essential substratum for fibroblast adhesion and migration during formation of granulation tissue (7, 11, 12, 22, 23). In other in vitro and in vivo studies, fibronectin has been implicated in phagocytosis of tissue debris and in systemic function of the reticuloendothelial system (13, 27, 32).

Much of the original interest in fibronectin occurred because transformed fibroblasts were found to have decreased levels of surface fibronectin (3, 15, 35). The absence of fibronectin has been suggested as a possible cause of the more rounded morphology of transformed cells when compared to their normal counterparts. The addition of cellular fibronectin to transformed cells induced at least partial reversion of the transformed phenotype to a more normal morphological appearance (2, 40). In related studies, cells that entered into mitosis and rounded up were found to lose their cell surface fibronectin

(16, 31), and treatment of normal fibroblasts with antifibronectin caused redistribution of cell surface fibronectin and partial cell rounding (38). Finally, a temperature sensitive Chinese hamster ovary (CHO) mutant cell line has been described that undergoes reversible rounding and loss of cell surface fibronectin at the nonpermissive temperature (20).

The above studies have demonstrated a positive correlation between cell shape and the presence of cell surface fibronectin in vitro. Also, a number of in vivo studies have shown that fibronectin present in mesenchymal tissue disappears during differentiation of epithelial components of these tissues (reviewed in reference 36). Nevertheless, a critical analysis of individual cell shape and fibronectin distribution in vivo has not yet been reported. The uterine endometrial stroma during decidualization represents a suitable tissue in which to study this problem. During the time period just before blastocyst implantation in the endometrium, the elongated stromal fibroblasts transform into decidual cells that have an epithelioid shape (5, 37). Therefore, it might be predicted that the fibronectin distribution on these fibroblasts would change during this transformation.

In addition to decidualization, other events occur in the

endometrium during pregnancy that are of interest in terms of fibronectin distribution and cell behavior. For instance, trophoblast invasion of the stroma may be controlled by the decidual tissue (4, 17). Studies on cell invasion during embryogenesis have implicated fibronectin as an important component of some cell migratory pathways (14, 19, 21, 23). Therefore, the organization of fibronectin in the decidual tissue might provide some insight into how trophoblast invasion is controlled. Also, after parturition, collagenous material in the uterine lumen is quickly removed by the activity of phagocytic cells (26). This removal might involve opsonization of collagen by fibronectin.

To study the various possibilities described above, fibronectin distribution in the rat endometrium was studied by indirect immunofluorescence staining during decidualization, trophoblast invasion, and after parturition. Herein, the results of these studies are reported.

## MATERIALS AND METHODS

### Animals

Adult female (Fischer  $\times$  DA) $F_1$  rats were maintained on a 12-h light:12-h dark cycle in the local animal colony. Animals in the estrus phase were selected by examining vaginal smears for the presence of cornified epithelial cells and the absence of leukocytes (1). To obtain pregnant animals, females were caged with males overnight and vaginal smears were examined the following morning for the presence of sperm. The day sperm were found in the vaginal smear was designated day 1 of pregnancy.

A total of 21 animals were used in these studies. There was at least one representative of every day from estrus to day 10 of pregnancy and postpartum, days 1-3 (day 1 postpartum being the day after delivery, which occurred during the night).

### Biopsy of Specimens

Experimental animals under ether anesthesia were subjected to laparotomy, allowing the two horns of the uterus to be exposed and excised. For animals at days 1-5 of pregnancy, at least one horn was flushed with Hank's balanced salt solution (HBSS), and the flushings were observed microscopically to insure the presence of fertilized ova. At day 6, implantation sites were identified by Pontamine Blue staining (25). At days 7-10, implantation sites could be observed by gross visual inspection. One uterine horn from each animal was fixed (see below) and used for histological analysis, and the other was frozen (see below) and used for indirect immunofluorescence analysis.

Uterine horns used for histological analysis were prevented from contracting by pinning on a wax surface and fixed by immersion in 2% glutaraldehyde, 1% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 24 h at 4°C. Uterine horns to be used for indirect immunofluorescence analysis were sliced into transverse sections ~0.5 cm in length and placed in BEEM capsules filled with embedding medium (O.C.T. Compound) for frozen tissue specimens and immersed in liquid nitrogen. For animals in estrus, at days 1-5 of pregnancy, and postpartum, the specimens were taken at random. For animals at days 6-10 of pregnancy, the specimens were taken at the blastocyst implantation sites.

### Histological Analysis

Fixed specimens were cut into transverse sections ~0.5 cm long. The specimens were selected as described for the frozen samples, above. The specimens were dehydrated through a graded series of ethanol solutions and finally infiltrated with JB-4 methacrylate (activated with benzoyl peroxide according to the manufacturer's instructions) in 1:1 ethanol. After overnight incubation at room temperature, the tissue was further infiltrated for two 1-h time periods with fresh, activated JB-4 solution in a vacuum oven at 28-lb/in<sup>2</sup> pressure and room temperature. The infiltrated specimens were then placed in BEEM capsules filled with activated JB-4 solution mixed with catalyst (20:1) and allowed to harden at room temperature. 3- $\mu$ m sections were cut with a glass knife on a Porter-Blum microtome (Sorvall MT-2; DuPont Instruments-Sorvall Biomedical Div., DuPont Co., Newtown, CT). The sections were stained with hematoxylin-eosin (8).

### Indirect Immunofluorescence Staining

Frozen specimens were placed in a Tissue Tek II cryostat (Miles Laboratories Inc., Research Products Div., Elkhart, IN) and the BEEM capsules were cut

away. 5- $\mu$ m sections were cut and placed on 22-mm<sup>2</sup> glass cover slips that previously had been subbed with a solution of 0.25% gelatin and 0.05% chrome alum in H<sub>2</sub>O for 10 min and then air-dried. The sections on the cover slips then were placed in 30-mm Falcon tissue culture dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA) for subsequent handling. All incubations were carried out in 1-ml solutions at room temperature. The sections were rinsed for 10 min with phosphate saline (0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2) containing 1% bovine serum albumin (BSA), and then for 10 min with 1% BSA, 2% goat serum in phosphate saline. They then were treated for 30 min with phosphate saline containing specific anti-rat fibronectin antiserum or preimmune serum at a final dilution of 1:400. The sections then were rinsed 3  $\times$  10 min with 1% BSA in phosphate saline. Next, the cover slips were incubated for 30 min with fluorescein-conjugated goat anti-rabbit antiserum (1:50). Finally, the sections were rinsed 3  $\times$  10 min with 1% BSA in phosphate saline and the cover slips were inverted and mounted on glass slides using 0.1 M Tris, 90% glycerol, pH 9.4. The sections were examined and photographed with a Zeiss Photomicroscope III equipped with epifluorescence.

### Preparation of Antifibronectin Antiserum

Specific antiserum to rat fibronectin was prepared by a method similar to that previously used to prepare specific antisera to human plasma fibronectin (12) and guinea pig plasma fibronectin (11). Briefly, rat blood was obtained by aortic cannulation and allowed to clot at 37°C. Fibronectin was purified from the serum by affinity chromatography on gelatin-Sepharose and anti-rat fibronectin antiserum was produced in rabbits. To obtain specific anti-rat fibronectin antiserum (i.e., remove antibodies to nonfibronectin serum proteins) the crude antiserum was incubated for 60 min at 37°C in a 1:1 mixture with rat serum from which plasma fibronectin had been removed by two passages through the gelatin-sepharose column. The precipitate that formed was removed by centrifugation and the resulting antiserum was tested against purified rat fibronectin and against whole serum. One precipitate band was observed both by Ouchterlony analysis and by immunoelectrophoresis.

### Materials

Pontamine Sky Blue 5BX was obtained from Biomedical Specialties Co. HBSS and goat serum were obtained from GIBCO Laboratories (Grand Island Biological Co., Grand Island, NY). JB-4 methacrylate embedding kit was obtained from Polysciences, Inc. (Warrington, PA). O.C.T. compound was obtained from Lab-Tek Div. (Miles Laboratories, Naperville, IL). Fluorescein-conjugated goat anti-rabbit antiserum was obtained from Meloy Laboratories Inc. (Springfield, VA).

## RESULTS

### Cell Shape Change during Decidual Transformation

The typical changes in cell shape that occur during decidualization are shown in H&E stained sections in Fig. 1 (29). In estrus, the stromal fibroblasts of the endometrium were elongated with long processes (Fig. 1A). By day 2 of pregnancy, the fibroblasts had enlarged (Fig. 1B). A rounded morphology was observed by day 5 (Fig. 1C); shortly thereafter (by day 6) the rounded decidual cells had aggregated into a closely packed layer at the antimesometrial end of the uterus, where blastocyst implantation occurs (Fig. 1D). The largest decidual cells were those found in the stroma proximal to the implanted blastocyst, while smaller decidual cells were observed in the stroma distal to the implantation site (5, 37).

### Fibronectin Distribution during Decidual Transformation

Unfixed, frozen thick sections of uterine specimens were examined for fibronectin distribution by indirect immunofluorescence staining. During estrus, fibronectin was prominent in the endometrial stroma in what appeared to be a fibrillar pattern on the surfaces of fibroblasts (Fig. 2A, circled) and around blood vessels (Fig. 2A, arrows). Fibronectin was also present in the basement membrane region of the stromal-

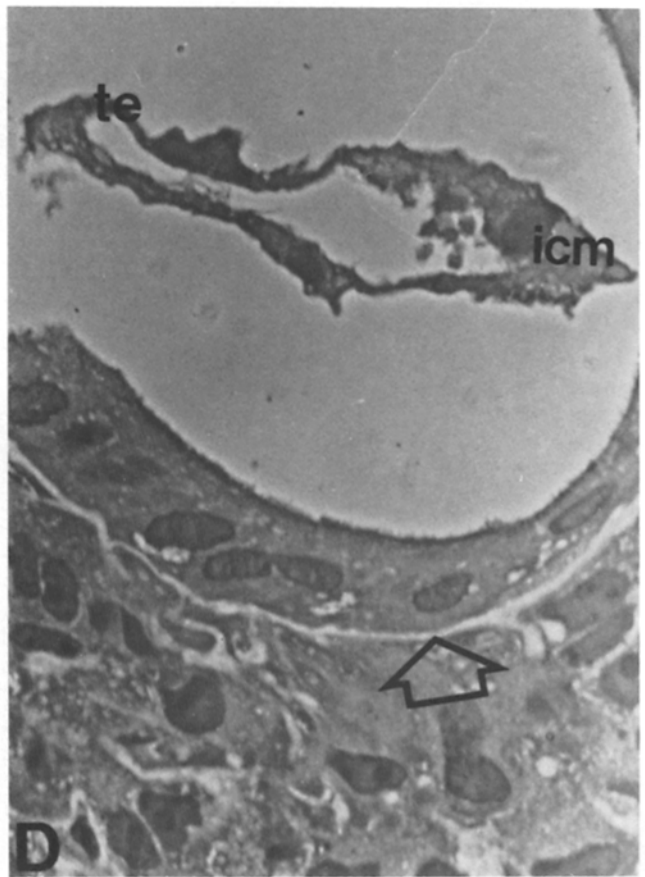
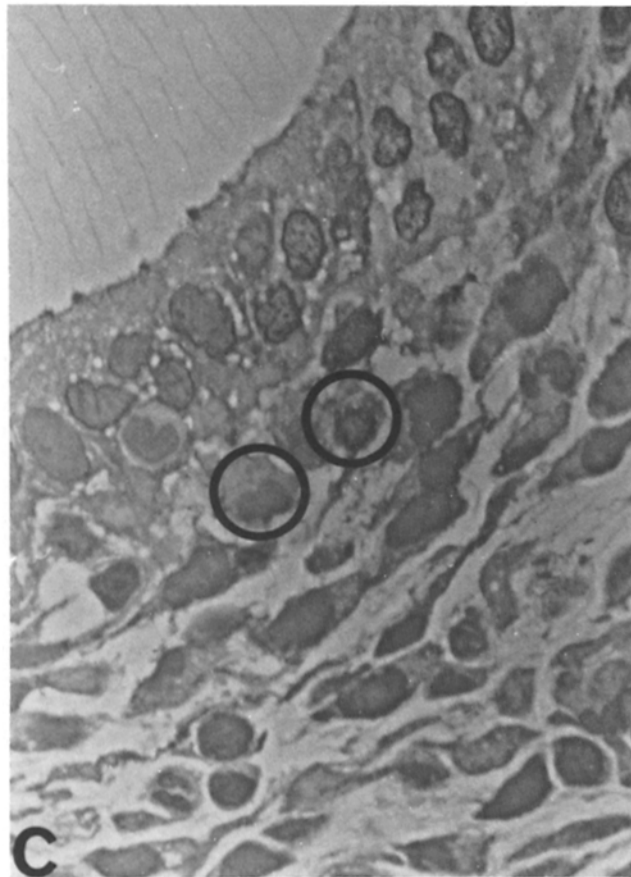
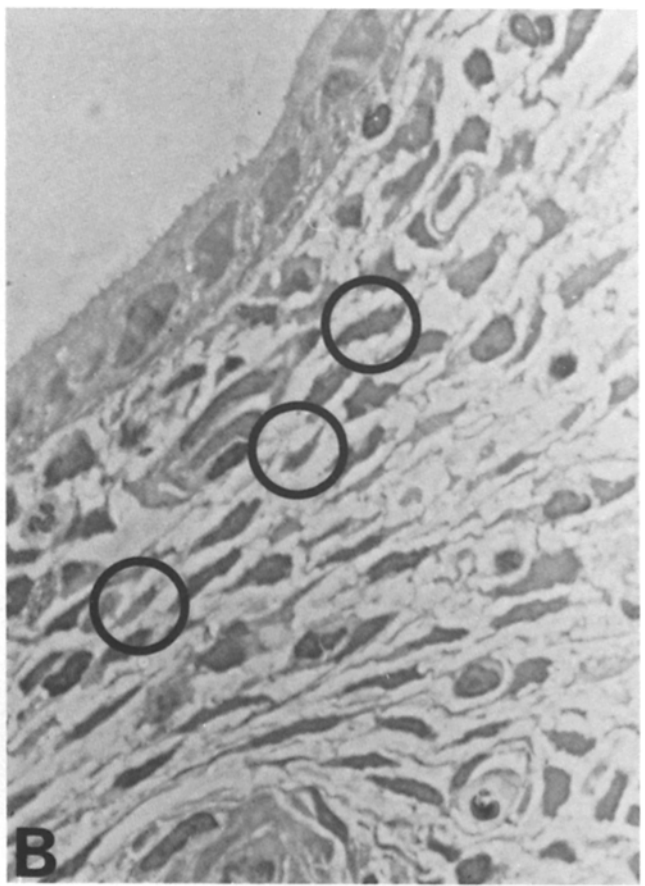
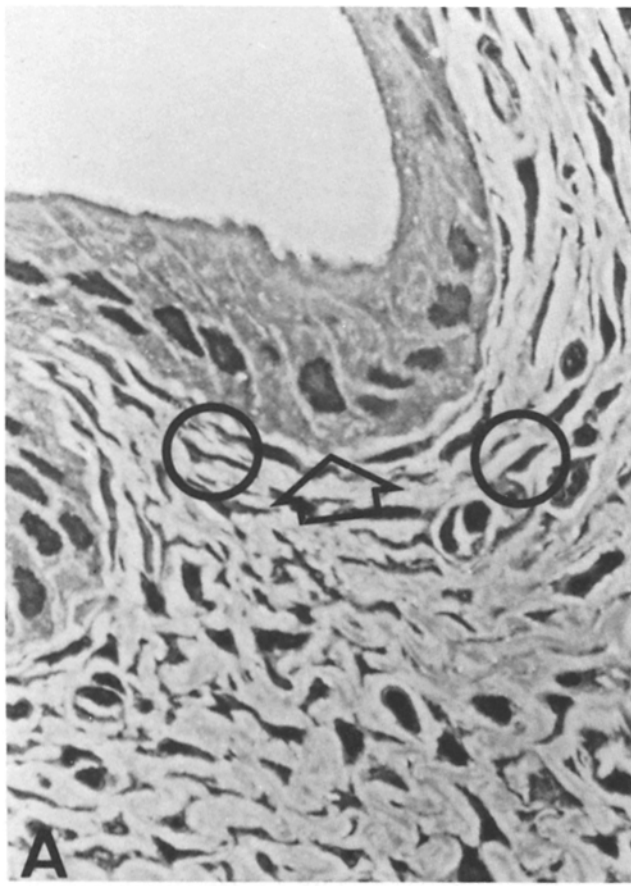


FIGURE 1 Cell shape change during decidualization. The appearance of the endometrial epithelium and subjacent stroma on the antimesometrial side of the uterine lumen at the time of mating and during early pregnancy are shown in H & E stained preparations. Stromal fibroblasts (circled) are initially very elongated (Estrus: *A*). During decidual transformation the cells become larger and round (day 2: *B*; day 5: *C*). At the time of blastocyst implantation, the decidual cells are found in a densely packed layer around the uterine lumen (day 6: *D*). The blastocyst can be seen at day 6 and the inner cell mass (*icm*) and trophoectoderm (*te*) are evident. Also at day 6, the basement membrane between the epithelium and stroma is more pronounced (arrow) than at earlier times (e.g., Estrus, arrow). Other details are in Methods and Materials.  $\times 1,000$ .

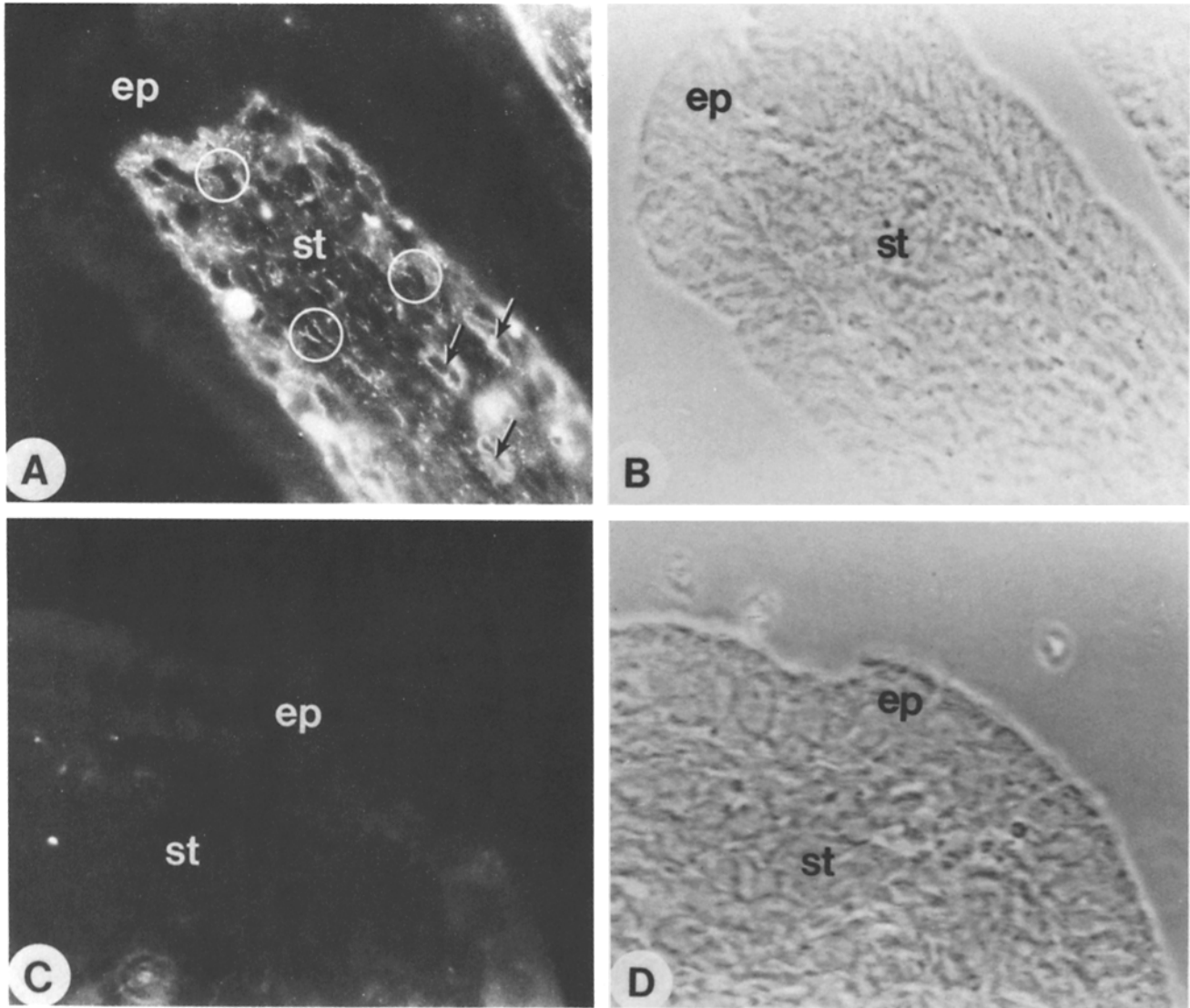


FIGURE 2 Fibronectin distribution in the endometrium of the uterus, in estrus. (A and B) Fibronectin is found in the stroma (st) but is absent from the epithelium. A fibrillar pattern of fibronectin around the fibroblasts is evident (circled) and there is also heavy staining of the blood vessels (arrows). (C and D) No fluorescent staining was observed with preimmune serum in place of immune serum. (A and C) Fluorescence. (B and D) Phase contrast. Other details are in Methods and Materials.  $\times 725$ .

epithelial junction, but absent from the epithelium (Fig. 2A and B). Control experiments revealed no staining of tissue sections when preimmune serum was used instead of immune serum (Fig. 2C and D).

During the period of decidual transformation, a dramatic change occurred in the distribution of fibronectin associated with stromal fibroblasts. At day 1 of pregnancy, fibronectin was still found in a fibrillar arrangement around the cells (Fig. 3A, circled). In contrast, by day 3, the fibrillar pattern was largely replaced by small patches of fibronectin on the cells' surfaces (Fig. 3B, circled). By day 5, the patches had disappeared and only a thin rim of fibronectin staining was present on the cells (Fig. 3C, circled). Finally, by day 6, the cells were almost devoid of fibronectin staining (Fig. 3D, circled).

The change in fibronectin associated with the decidual cells was specific since fibronectin staining of the basement membrane region (Fig. 3A–D, large arrows) and blood vessels (Fig. 3C and D, small arrows) was not decreased. In fact, the basement membrane associated fibronectin appeared to become even more prominent. This was of particular interest

since in the H & E stained material, there appeared to be an expansion of the basement membrane region in the time interval between estrus and day 6 (Fig. 1A and D, arrows). Also noteworthy was the relative depletion of fibronectin from the endometrial stroma by day 6, except where it was associated with the basement membrane or blood vessels. As will be shown below, this depletion was even more striking by day 9 during the period of trophoblast invasion.

#### *Appearance of the Stroma at the Time of Trophoblast Invasion*

By day 9, considerable development of the embryo had occurred. The endometrial epithelium was denuded, and trophoblasts were found in a thin layer adjacent to the proximal decidual tissue and in the base of the ectoplacental cone (Fig. 4A) (29). Giant trophoblast cells of the ectoplacental cone were also found invading into the decidual tissue (Fig. 4B). Distally to the embryo, small decidual cells were evident that were

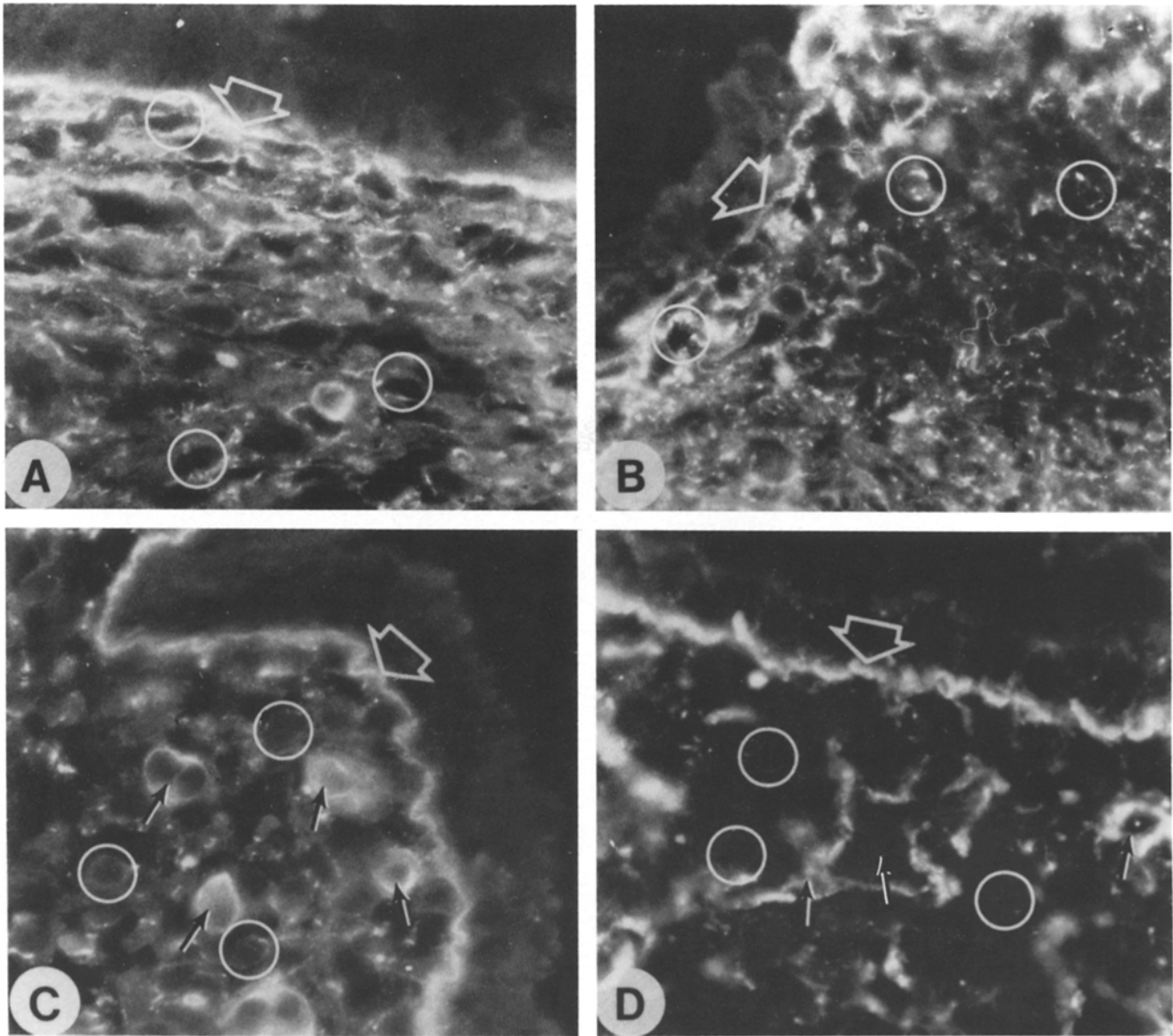


FIGURE 3 Reorganization of fibronectin during decidual transformation. The fibrillar pattern of fibronectin staining on the fibroblasts is still found at day 1 (A, circled). By day 3, fibronectin is found in patches on the cells' surfaces (B, circled). The patches disappear and only a thin rim of fibronectin staining is evident at day 5 (C, circled). Staining is further attenuated at day 6 (D, circled). In contrast to the disappearance of fibronectin on the surfaces of the fibroblasts, fibronectin in the basement membrane region persists and becomes even more prominent between days 1 and 6 (large arrows). Fibronectin also is not lost from the blood vessels (C and D, small arrows). Other details are in Methods and Materials.  $\times 825$ .

adjacent to more normal looking stromal fibroblasts and the inner smooth muscle layer (Fig. 4 C).

When examined by indirect immunofluorescence, fibronectin was found to be absent from large regions of the proximal stroma (Fig. 5 A), and at higher magnification it was found that fibronectin was generally confined to an association with blood vessels (Fig. 5 B, arrows). Fibronectin was also found in association with the mesodermal region of the embryo (closed short arrows), with Reichert's membrane (long arrows), and with regions that appeared to be remnants of the basement membrane (open short arrows) (Fig. 5 A). Distal to the embryo, fibronectin was present throughout the stroma and the inner smooth muscle layer (Fig. 5 C). At higher magnification, a patched distribution of fibronectin on the small decidual cells was observed (Fig. 5 D, circled), similarly to that found at day 3 (Fig. 3 B).

### Appearance of the Stroma after Parturition

Within 24 h after parturition, the epithelium of the endometrium was entirely reconstructed and large amounts of debris were found in the uterine lumen (Fig. 4 D). One day later, the material in the lumen was essentially gone and the stroma of the endometrium appeared normal including the presence of endometrial glands (Fig. 4 D).

Fibronectin appeared to be bound to the debris in the uterine lumen (Fig. 6 A, closed arrow). This staining was specific since it was not observed using preimmune serum (Fig. 6 C, arrow). The presence of fibronectin associated with the luminal debris suggests the possibility that fibronectin plays a role in the removal of this debris by phagocytic cells (26).

In the stroma of the uterus at day 1 after parturition, fibronectin was present and diffusely distributed without any

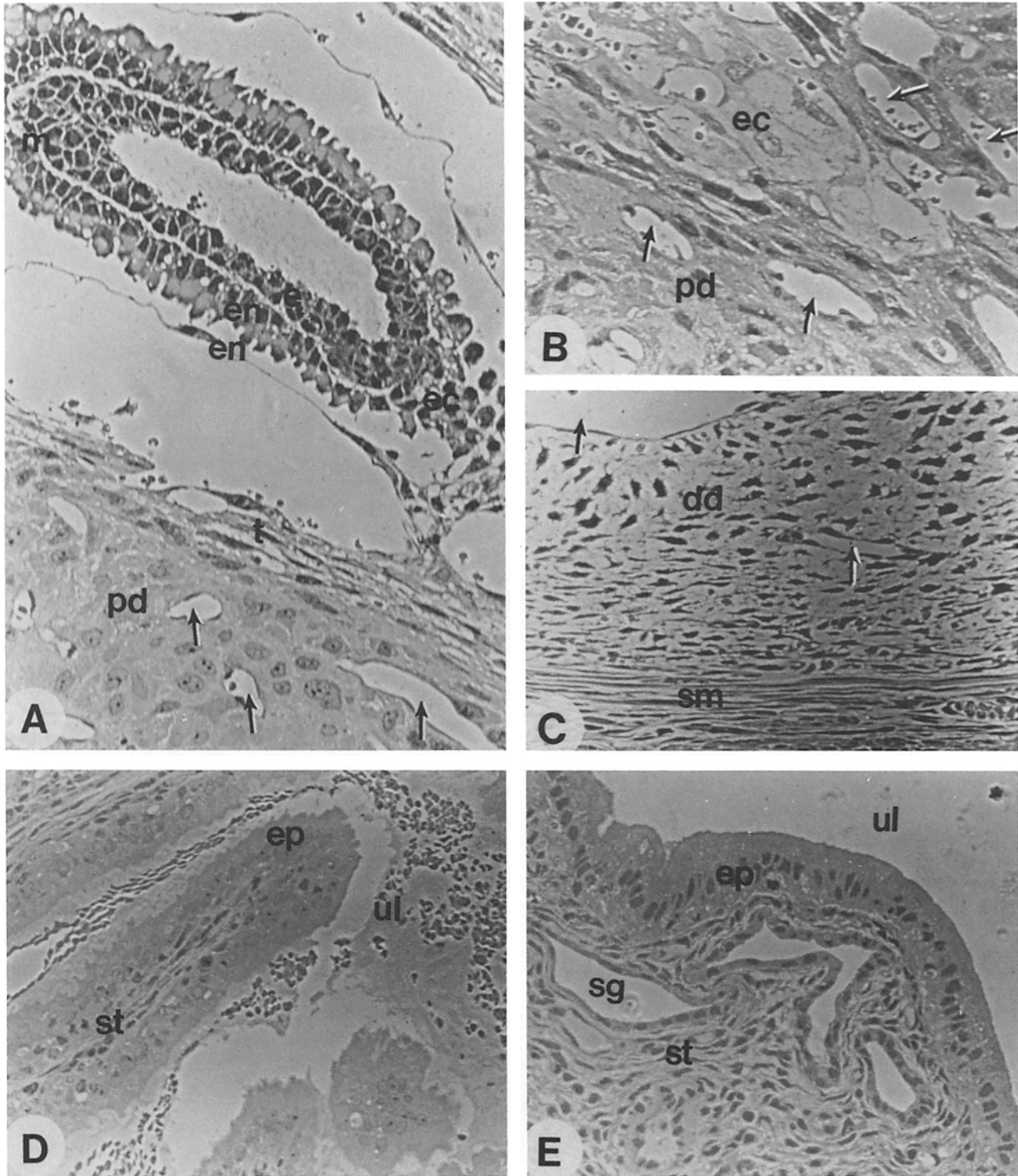


FIGURE 4 Organization of the endometrium at day 9 and postparturition. In A-C, the appearance of the embryo implantation site in the antimesometrial stroma of the uterus is shown in H & E stained preparations. (A) By day 9, embryonic development is well underway. Cells in the ectoderm (e) and proximal and distal endoderm (en) can be seen. The mesodermal region (m) is evident, but it is difficult to distinguish mesodermal cells from the others. Trophoblast cells (t) are found in contact with the proximal decidua (pd) and in the base of the ectoplacental cone (ec). The epithelium has been destroyed, and extensive vascularization of the decidua is evident (arrows). (B) The secondary trophoblast giant cells at the margin of the ectoplacental cone (ec) are seen invading into the proximal decidua that contains large decidual cells and blood vessels (arrows). (C) Distal to the conceptus, the decidual cells (dd) are much smaller and there is more extracellular space. Blood vessels are apparent (arrows). The distal decidual cells merge with more normal-looking endometrial stroma that is adjacent to the inner smooth muscle layer (sm). In D and E, the appearance of the endometrium postparturition is shown in H & E stained preparations. (D) After parturition, the endometrium is rapidly repaired. By 24 h, the stroma (st) is completely recovered with epithelium (ep). A large amount of debris containing collagen and cells is found in the uterine lumen (ul). (E) by 48 h postparturition, most of the debris in the uterine lumen (ul) is gone, the stroma (st) appears relatively normal, and endometrial stromal glands (sg) are evident. Other details are in Methods and Materials.  $\times 525$ .

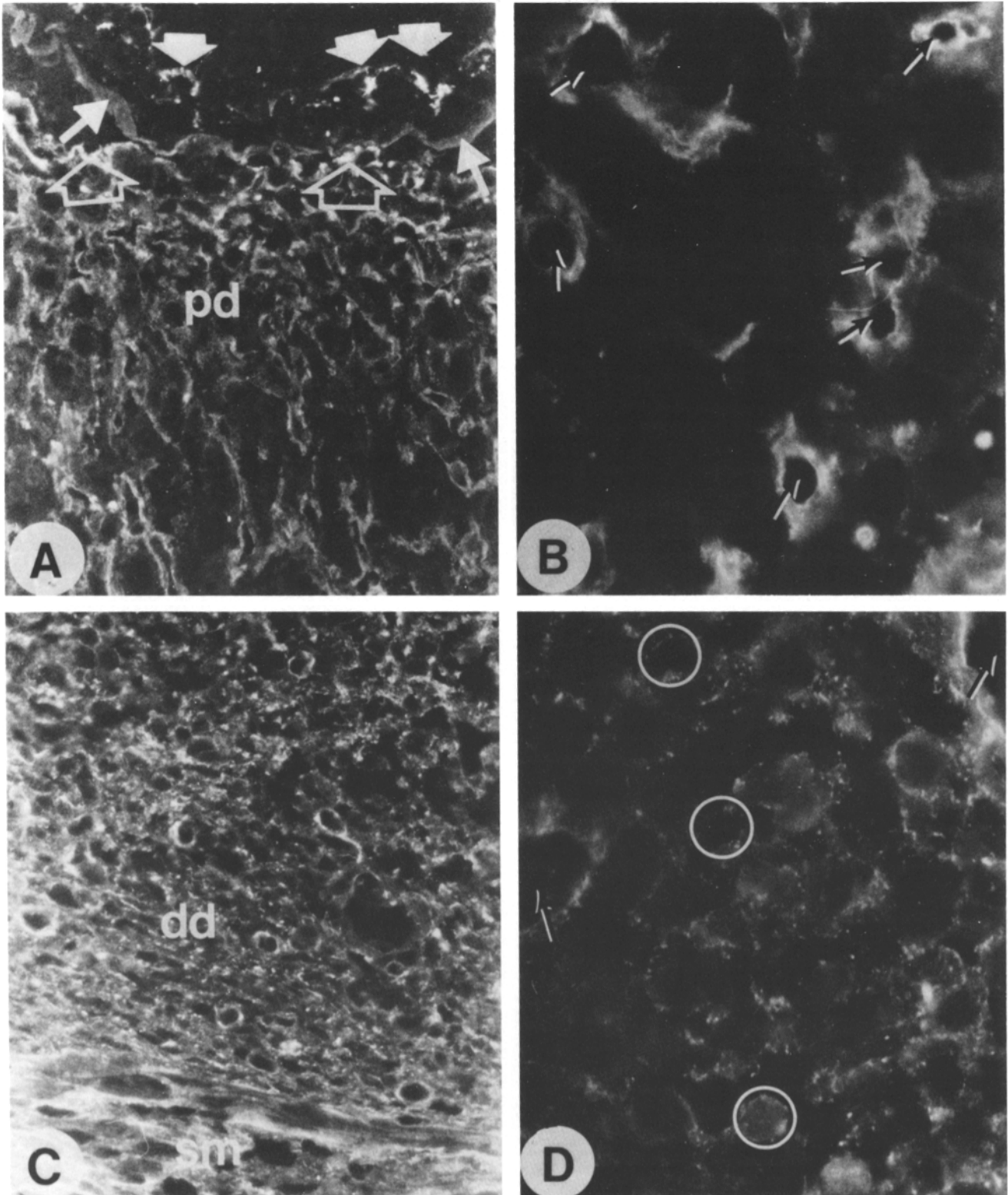


FIGURE 5 Fibronectin distribution at Day 9 in the endometrial stroma at the antimesometrial pole of the conceptus. (A) Fibronectin is observed in the mesodermal portion of the embryo (closed short arrows), in Reichert's membrane (long arrows), and in what appears to be remnants of the basement membrane (open short arrows). Large regions of the proximal decidual tissue, however, have no fibronectin present. (B) At higher magnification, it can be seen that fibronectin in the proximal decidual tissue is associated almost exclusively with blood vessels (arrows). (C) In the distal decidual tissue (*dd*), fibronectin is found in association with the decidual cells and smooth muscle (*sm*). (D) At higher magnification, the distally located small decidual cells can be seen to have patches of fibronectin on their surfaces (circled). Other details are in Methods and Materials. (A and C)  $\times 600$ . (B and D)  $\times 950$ .

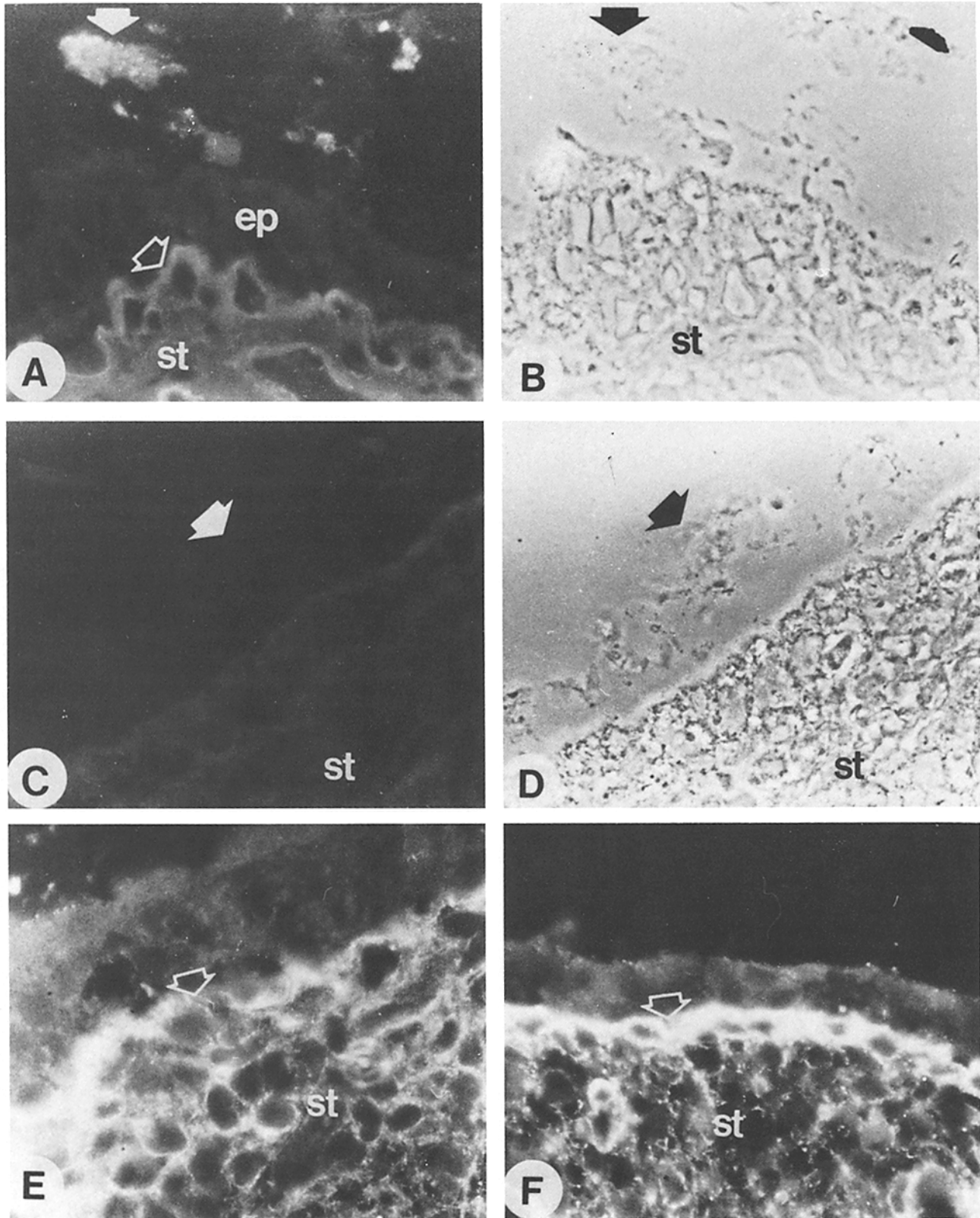


FIGURE 6 Fibronectin distribution in the endometrium, postparturition. (A and B) Fibronectin is found in the stroma (st) in a diffuse organization. Even the basement membrane region (open arrow) fails to show prominent staining. The epithelium (ep) is negative. Debris in the uterine lumen, however, is fibronectin positive (closed arrows). (C and D) No fluorescent staining of stroma or luminal material (arrows) was observed with preimmune serum in place of immune serum. (E) By day 2 postparturition, an extensive fibronectin matrix has been organized in the stroma (st) and basement membrane (arrow). (F) Further organization of fibronectin occurs by day 3. Other details are in Methods and Materials.  $\times 850$ .



marked organization in association with fibroblasts (Fig. 6A); the epithelium was fibronectin-negative. One day later a dramatic organization of fibronectin around the fibroblasts was observed (Fig. 6E), and further organization of the fibronectin matrix, especially in the epithelial-stroma basement membrane region, had occurred after another 24 h (Fig. 6F, arrow).

## DISCUSSION

The findings presented in this paper provide the first in vivo evidence for a correlation between fibroblast cell shape and fibronectin distribution. Elongated fibroblasts in the endometrial stroma of the rat uterus in estrus were found to have an apparently fibrillar pattern of fibronectin on the cell surface that looked similar to that reported for fibroblasts in in vitro studies (3, 35, 39). As the fibroblasts transformed from an elongated to round morphology during decidualization, fibronectin redistributed into a patched pattern on the cell surfaces and then largely disappeared. A sparse or patched distribution of fibronectin has been reported on fibroblasts in vitro that have a rounded morphology as a result of malignant transformation (3, 35), mitosis (31), or treatment with antifibronectin (38).

The mechanism by which fibronectin disappears from endometrial fibroblasts is unknown. There was not a uniform loss of fibronectin from the stroma such as might occur from enhanced levels of proteolytic enzymes associated with implantation (28), since fibronectin found around blood vessels was not decreased. Fibronectin in the epithelial-stromal basement membrane also stayed the same (or even increased) until after the time of trophoblast invasion when the basement membrane was destroyed, further suggesting that proteolytic enzymes were not involved.

Whether the loss of fibronectin from the surfaces of the fibroblasts undergoing decidual transformation is a cause or an effect of cell rounding cannot be ascertained at present. It should be pointed out that the uterine stroma is rich in Type III collagen (reticulin) (37), the one form of native collagen for which fibronectin has a high affinity (18). It may be, therefore, that fibronectin is involved in adhesion of stromal fibroblasts to Type III collagen, and that the loss of fibronectin results in cell rounding when the adhesive interactions are broken. This need not be the case, however, since external manipulation of cell surface fibronectin, not involved in the regions of cell-substratum adhesion, can cause cell rounding in vitro, which implicates cell surface fibronectin in modulation of cell shape (38).

Another interesting observation in our studies was the decrease in fibronectin in the proximal decidual tissue that confronts the invading trophoblast, which is consistent with a previous report on fibronectin distribution in the developing embryo (34). It has been suggested that the decidual tissue functions as a barrier to trophoblast invasion (4, 17). The absence of fibronectin from the decidual matrix might help explain why the trophoblast cannot move through this matrix. This would be consistent with the idea that a fibronectin-coated extracellular matrix is required as a cell migratory pathway during embryogenesis (14, 19, 21, 22). On the other hand the trophoblasts are epithelial cells, and fibronectin may not be important in adhesion and migration of these cells (18). Other features of the decidual tissue, such as the presence of intercellular junctions, may be important in preventing invasion of the trophoblasts (6), and the disappearance of fibronectin from the stromal matrix may be incidental.

Finally, observation of fibronectin distribution in the uterus after parturition revealed a striking reorganization of fibronectin in the endometrial stroma. It was clear that pronounced fibronectin staining in the basement membrane did not occur until after reepithelialization had already taken place. On the other hand, preliminary studies using antibodies to other basement membrane components, e.g., laminin, type IV collagen, and heparin sulfate (generously provided by Dr. George Martin, National Institute of Dental Research, National Institutes of Health) indicated that these components were organized in the basement membrane region before fibronectin.

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