Laminin Production by Human Endometrial Stromal Cells Relates to the Cyclic and Pathologic State of the Endometrium

M. FABER, MD, U. M. WEWER, MD, PhD, J. G. BERTHELSEN, MD, PhD, L. A. LIOTTA, MD, PhD and R. ALBRECHTSEN, MD, PhD

From the Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland; and University Institute of Pathological Anatomy, Copenhagen, Denmark

The cyclic changes in the presence of the basement membrane glycoprotein laminin in endometrial stromal cells was studied by immunohistochemistry. The interstitial matrix around the stromal cells of the proliferative phase of the normal menstrual cycle was unreactive with antibodies to laminin. However, commencing with the secretory phase, stromal cells accumulated distinct cytoplasmic and pericellular laminin-immunoreactive material. The maximal amount of stromal cell-associated laminin was observed in predecidual cells of the late secretory phase. Thus, laminin immunostaining discriminates stromal cells of the proliferative phase (being "negative") from those in the secretory phase (being "positive"). Sixty-six cases of endometrial hyperplasia and adenocarcinomas were also stained with antibodies to laminin. Sixty-nine percent of biopsies of cystic hyperplasia and 30% of adenomatous hyperplasia contained laminin-positive stromal cells. Ultrastructural examination of stromal cells in cystic hyperplasia revealed the presence of pericellular basement membrane-like material, focally arranged into typical lamina rara and lamina densa. In contrast, stromal cells in the atypical adenomatous hyperplasia and adenocarcinomas did not react with antibody to laminin. The expression of laminin receptor in the stromal cells codistributed with laminin. Basement membranes of the surface epithelium, the glandular epithelium, and the vessels stained strongly with antibodies to laminin. In preneoplastic and neoplastic tissues, laminin immunostaining revealed discontinuous and defective basement membranes. In poorly differentiated carcinomas only sparse amounts of laminin-positive basement membrane were observed; these tumors, in contrast, exhibited cytoplasmic laminin and also significant immunoreaction with antibodies to laminin receptor. (Am J Pathol 1986, 124: 384–398)

ENDOMETRIAL STROMAL CELLS are specialized cells that express receptors for estrogen and progesterone,[1,2] produce hormones like prolactin,[3] and secrete biologically active substances like prostaglandins.[4] The morphologic features of endometrial stromal cells change during the menstrual cycle and pregnancy[5–9] During the proliferative phase of the normal menstrual cycle, stromal cells are elongated and spindle-shaped. In the late secretory phase they become predecidual cells, which are larger and attain a slightly more fusiform shape. In pregnancy the stromal cells enlarge even further and transform into polygonal epitheloid decidual cells.[6–10] The exact stimulus or set of stimuli acting on stromal cells to undergo decidualization has not been identified, although in humans, progesterone seems to play an important role.[11,12]

Recently it was shown that decidualized stromal cells of the pregnant endometrium are encircled by pericellular matrix containing laminin.[13–16] Laminin is a large (M_r = 10^6) glycoprotein present in basement membranes of normal and neoplastic tissue.[14–19] Laminin is a multifunctional protein influencing complex biological processes like cell adhesion, growth, differentiation, and migration.[20] Laminin-containing basement membranes also surround epithelial glands and specialized mesenchymal cells, such as striated and smooth muscle cells and adipocytes.

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Address reprint requests to Reidar Albrechtsen, MD, PhD, University Institute of Pathological Anatomy, Frederik V's vej 11, DK-2100 Copenhagen, Denmark.

Previous immunohistochemical studies of basement membrane alterations in various pathologic states have focused on the basement membrane derived from epithelium with little consideration of the stromal compartment.[17,21-25] After our recent observation that decidualized stromal cells of the human endometrium contain a pericellular laminin-positive basement membrane, the possibility was raised that stromal cell laminin expression might change according to the hormonal state or pathologic conditions of the endometrium. We have utilized immunohistochemical staining for laminin to compare the accumulation of this component associated with stromal cells in normal, preneoplastic, and neoplastic human endometrium. We show that the stromal cells in the proliferative phase are unreactive for laminin, whereas the stromal cells of the secretory phase express intra- and pericellularly located laminin. Furthermore, by studying endometrial hyperplasia and carcinoma, we have shown that the distribution of laminin associated with the stromal cells was altered dramatically, corresponding to the progression sequence from cystic hyperplasia to adenomatous and atypical hyperplasia and overt adenocarcinoma of the uterus.

Materials and Methods

Tissues

Specimens of human endometrial tissue were obtained by curettage or hysterectomy during routine diagnostic or therapeutic procedures conducted in Copenhagen, Denmark. The total number of cases was 114. 1) Biopsy specimens from 44 women between the ages of 17 and 53 years were investigated; 15 were in the proliferative phase (Days 4–14), 9 in the early secretory phase (Days 14–17), and 20 in the late secretory phase (Days 17–28). These endometrial biopsy specimens showed no pathologic changes. No cases with chronic inflammation were included in this study. Histologic dating was performed according to the criteria of Noyes.[7] 2) Four women (30–40 years of age) had received gestagen (Orthonett Novum, 0.5 mg norethisteron, 35 μg ethinyl estradiol, Ortho, Copenhagen, Denmark) for 2–10 years prior to curettage. 3) In 52 patients endometrial biopsies revealed hyperplastic lesions. The hyperplasia was classified according to the World Health Organization (WHO)[26] by morphologic criteria as detailed by Kurman et al[27,28] into three groups: 29 cases of cystic (proliferative, simplex) hyperplasia (age 31–66 years; mean, 49.1 years); 10 cases of adenomatous hyperplasia (age, 42–62 years; mean, 50.3 years); and 13 cases of atypical adenomatous hyperplasia (age, 42–70 years; mean, 60.7 years). 4) In 14 cases (age 55–72 years; mean, 63 years) endometrial adenocarcinoma was diagnosed. The carcinomas were classified according to WHO[26] into well, moderately, and poorly differentiated adenocarcinomas.

Immunoperoxidase Staining

Immunostaining was carried out as previously described.[17,21] Most of the tissues were fixed in neutral buffered formalin or in ethanol/acetic acid. Pretreatment of sections with pepsin (5 mg/ml, Sigma Chemical Co., St. Louis, Mo) or trypsin (0.5 mg/ml, Sigma) was used.[17,29] Polyclonal rabbit antiserum against rat laminin purified from the L2 yolk sac carcinoma[30] was produced and characterized as described.[31] A dilution of 1:500 of the antiserum was used. Affinity-purified anti-laminin antibodies[17] were used at a concentration of 1–5 μg/ml. Monoclonal antibody (4E10) against human laminin[32] was used in a dilution of 1:1000 of ascites fluid or 10 μg/ml of purified IgG1. Rabbit anti-human laminin receptor antiserum[33] was used at a dilution of 1:100. The antibodies were diluted in 50 mM Tris-HCl, pH 7.4, and incubated with the sections for 2 hours at room temperature. The secondary antisera, swine anti-rabbit IgG and peroxidase anti-peroxidase rabbit serum,[34] were purchased from Dakopatt, Copenhagen, Denmark, and used in a dilution 1:50. The peroxidase reaction was visualized by the use of 5 mg/10 ml 3,3′-diaminobenzidine in 50 mM Tris-HCl, pH 7.2, and 0.03% H_2O_2. The washing buffer was 0.25 M NaCl in 50 mM Tris-HCl, pH 7.2. In control sections the specific antisera were replaced by absorbed antisera[17] or nonimmunized rabbit serum.

Electron Microscopy

Endometrial biopsies from 6 cases of cystic hyperplasia were examined by electron microscopy. The specimens were processed as follows: Tissue blocks were promptly fixed at room temperature in Karnovsky's fixative (2.5% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2) for 1 hour. All specimens were treated with 1% tannic acid (Mallinckrodt, Inc., St. Louis, Mo) as described.[35] The samples were dehydrated in graded ethanols, postfixed in 2% osmium-tetroxide, and embedded in Epon. Ultrathin sections from selected areas were collected on copper grids, stained with uranyl acetate and lead citrate, and examined with a JEOL 500 electron microscope.

Results

Laminin in the Stromal Cells of the Normal Human Endometrium

In the proliferative phase, the stromal compartment of the endometrium consisted predominantly of small

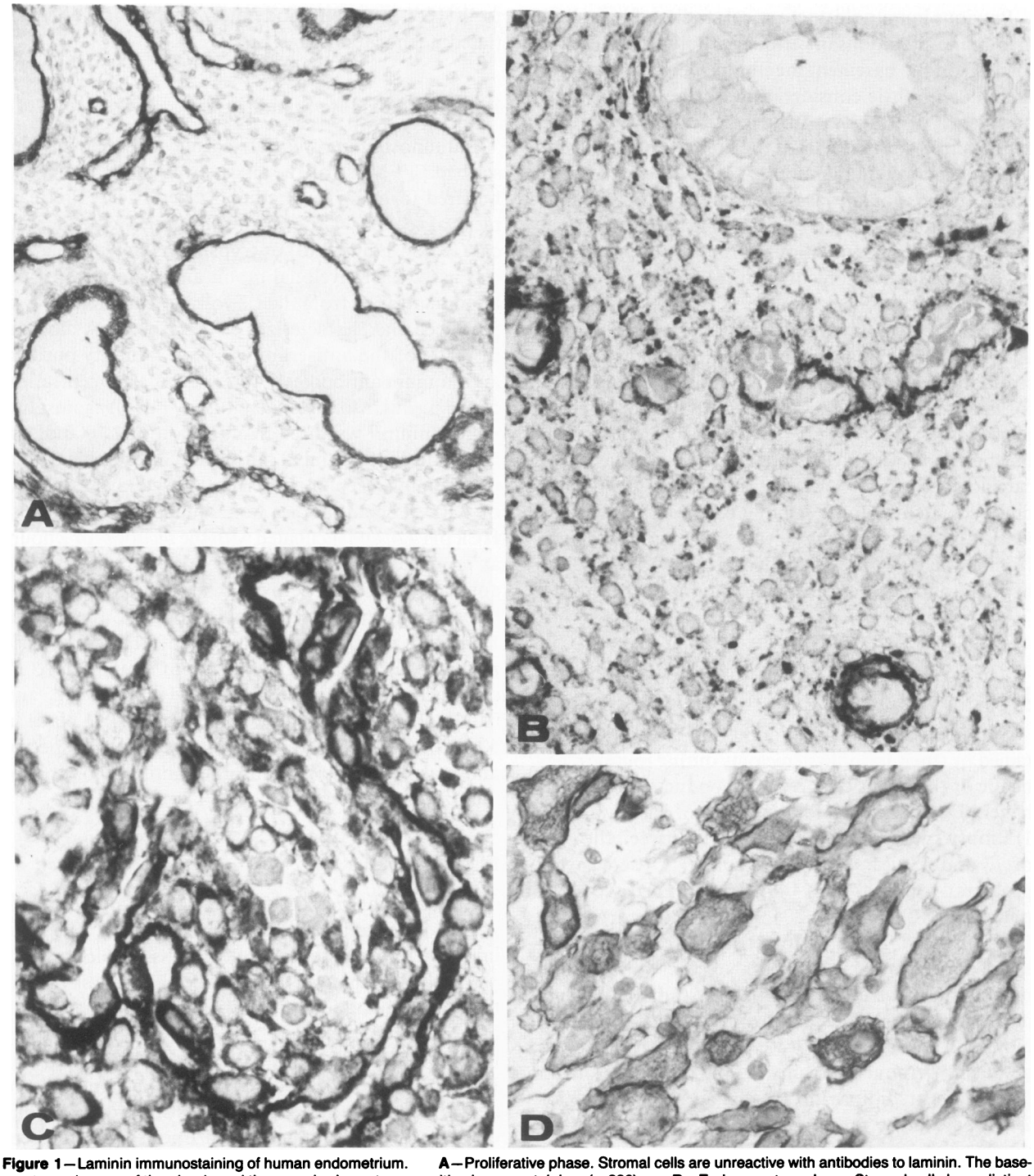

Figure 1—Laminin immunostaining of human endometrium. **A**—Proliferative phase. Stromal cells are unreactive with antibodies to laminin. The basement membranes of the glands and the vessels show strong positive immunostaining. (×230). **B**—Early secretory phase. Stromal cells have distinct laminin-positive material mostly confined to the cytoplasm, but small deposits are also recognized along the outer side of the cell membrane. (×440) **C**—Late secretory phase. Numerous stromal cells express very intense positive cytoplasmic immunostaining. (×400) **D**—Endometrium that has been influenced for prolonged periods of time with gestagen. Note that the large fusiform stromal cells are encircled by a rim of distant laminin-positive material. The laminin distribution in this case is indistinguishable from that seen of decidual cells of early pregnancy. (×600)

spindle-shaped cells.[6,7,36] Immunostaining with anti-laminin antibodies failed to identify detectable amounts of laminin associated with these cells, either within the cytoplasm or in the pericellular matrix (Figure 1A). In this respect, the endometrial stromal cells of the proliferative phase did not differ from fibroblastlike stromal cells in other tissues.

With the onset of the secretory phase, the stromal cells of the endometrium gradually undergo major morphologic changes, which is apparent at Day 24 and is

Table 1—Immunostaining for Laminin of Stromal Cells in Normal, Hyperplastic, and Carcinomatous Endometrium

Histologic diagnosis	No. cases	Laminin in stromal cells (no. cases positive)
Proliferative phase	15	0 (0%)
Early secretory phase	9	7 (78%)
Late secretory phase	20	20 (100%)
Cystic hyperplasia	29	20 (69%)
Adenomatous hyperplasia	10	3 (30%)
Atypical, adenomatous hyperplasia	13	0 (0%)
Endometrial carcinomas	14	0 (0%)

termed predecidualization.[6,7,36] Predecidualization was accompanied by the appearance of intracellular and pericellular laminin. At Days 14–17, before histologically recognizable predecidualization could be identified, scattered immunoreactive laminin was seen in the cytoplasm of stromal cells close to the small arteries and underneath the endometrial surface (Figure 1B).

The number of stromal cells exhibiting laminin-positive immunostaining as well as the intensity of the staining reaction gradually increased throughout the secretory phase. In late secretory phase most (>90%) predecidualized stromal cells were positive. The positive immunostaining was found in the cytoplasm and also discontinuously in the pericellular matrix (Figure 1C). Stromal cells located in the basal part of the endometrial biopsy specimens consistently appeared unreactive. All controls using preimmune or absorbed sera were also negative. In a given section with abundant stromal cells the extent of extracellular laminin-positive material varied from scattered punctate deposits on the cell surface to a "semicircular" rim of immunoreactive material surrounding portions of individual cells.

In 4 cases investigated, women had been treated for prolonged periods of time with gestagen. This treatment is known to induce the formation of predecidualized stromal cells indistinguishable from decidual cells of pregnancy.[11] In several areas of these four endometrial biopsy specimens, stromal cells were surrounded by a continuous pericellular sheath of laminin (Figure 1D).

Laminin in Stromal Cells of Endometrial Hyperplasia and Endometrial Carcinomas

Immunohistochemical localization of laminin was studied in 52 cases of endometrial hyperplasia, includ-

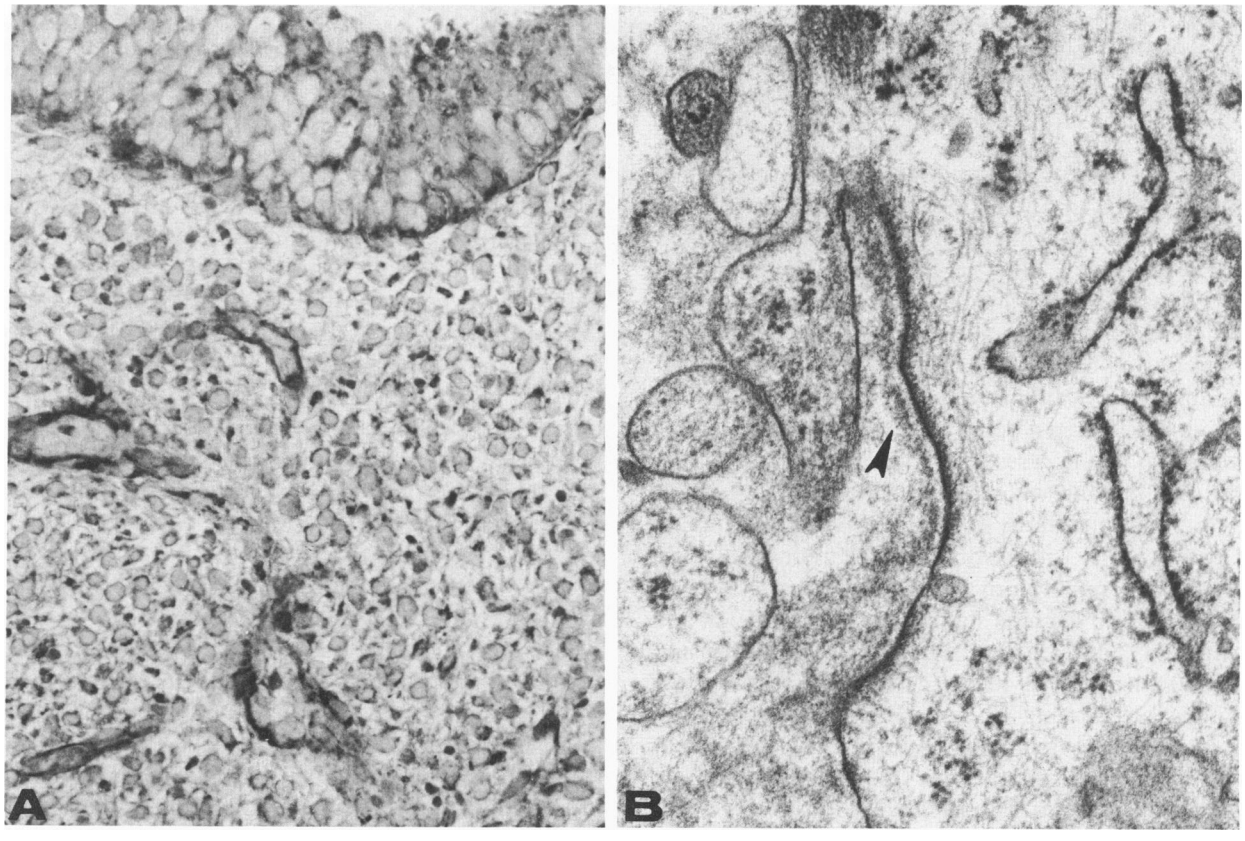

Figure 2—Human endometrial biopsy specimens with hyperplasia. **A**—Adenomatous hyperplasia. Immunostaining with antibodies to laminin. The stromal cells show granular laminin-positive material in the cytoplasm and also along the cell surface. (×420) **B**—Cystic hyperplasia. Electron-microscopic demonstration of the presence of a fragmented pericellular basement membrane-like material (*arrow*) close to the plasma membrane of a stromal cell. (×40,000)

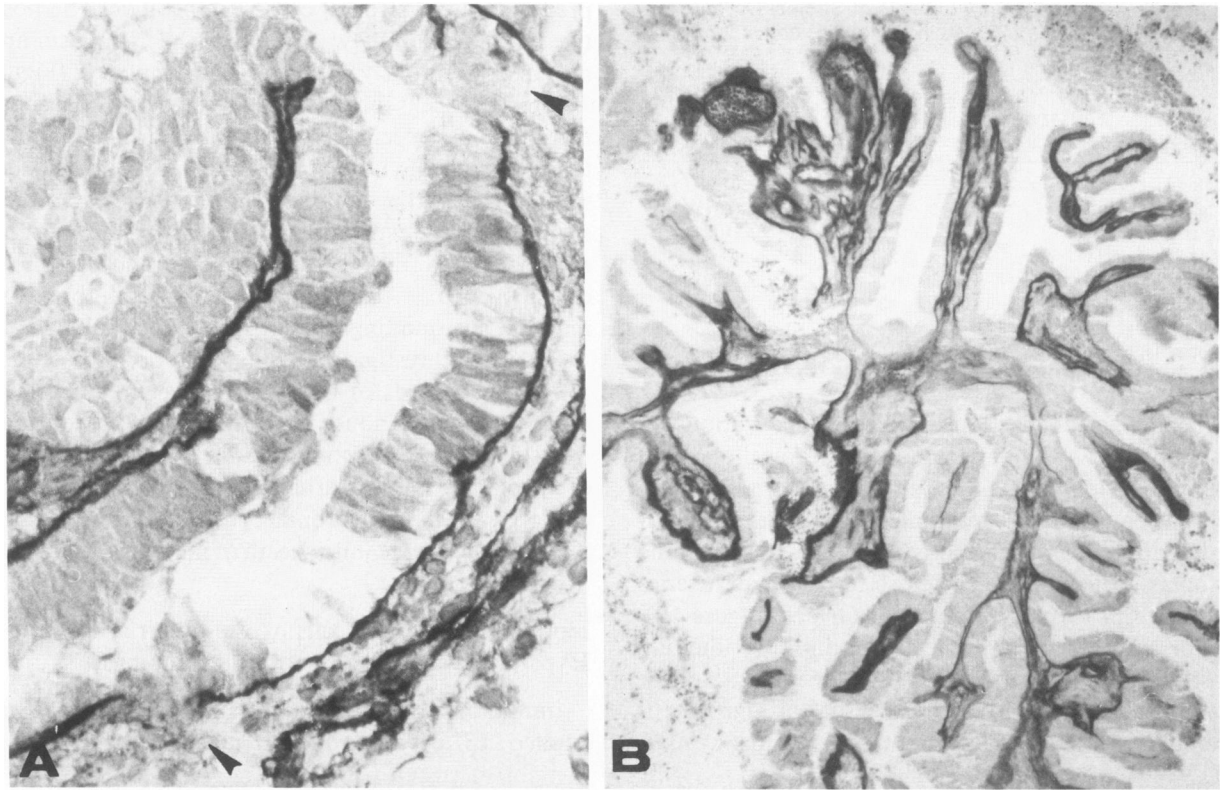

Figure 3—Human endometrial biopsy specimens immunostained with antibodies to laminin. **A**—A case of atypical adenomatous hyperplasia with areas of defective basement membrane at the points of early invasion (*arrows*). Scant stroma contains laminin-negative stromal cells. (×400) **B**—A case of highly differentiated adenocarcinoma. Note that the basement membranes separating the carcinoma cells from the stroma are strongly positive. Stromal cells are unreactive. (×110)

ing 29 cases of cystic hyperplasia, 10 cases of adenomatous hyperplasia, and 13 cases of atypical adenomatous hyperplasia. Furthermore, 14 adenocarcinomas were investigated. All types of hyperplasia contained densely packed, spindle-shaped stromal cells indistinguishable morphologically from stromal cells of the proliferative phase.[37-40] However, immunohistochemical staining for laminin revealed a distinct difference among these three types of hyperplasia groups (Table 1). In 69% of the cases of cystic hyperplasia laminin-positive material was seen both in the cytoplasm of stromal cells and in the form of small extracellular aggregates. Thus, with antibody to laminin one could identify a population of stromal cells, not otherwise appreciated on sections stained with hemotoxylin and eosin. Only 30% of the adenomatous (Figure 2A) and none (0%) of the atypical adenomatous hyperplasia demonstrated laminin-immunoreactive material. All 14 cases of adenocarcinomas examined were devoid of laminin-positive stromal cells (Figures 3A and B).

In an effort to further correlate the localiztion of the laminin-positive material with a subcellular structure at the ultrastructural level, we performed electron microscopy on six endometrial biopsy specimens characterized by cystic hyperplasia. At the ultrastructural level, stromal cells in hyperplastic endometrium resembled fibroblasts. In the nuclei, euchromatin predominated and several nucleoli were recognized. In the cytoplasm, distended rough endoplasmic reticulum contained granular material. Collagen fibers were seen extracellularly close to the cell surface. Some stromal cells retained granular basement membrane-like material confined to the cell surfaces. In some areas we were able to identify fragments of what appeared to be a typical basement membrane with a structure resembling lamina densa and rara (Figure 2B).

Distribution of Laminin in Epithelial Basement Membranes

Epithelial basement membranes of the endometrial glands (Figure 1A), vessels, and smooth muscles were positive for laminin, as expected.[18] In cystic and adenomatous hyperplasia the epithelial basement membranes could be visualized by laminin staining as a continuous band around the glands (not shown). In atypical adenomatous hyperplasia, the staining of basement membranes was often discontinuous (Figure 3A). Lo-

Table 2—Immunostaining for Laminin in Glandular Epithelial Basement Membranes of Hyperplastic and Carcinomatous Endometrium

Histologic diagnosis	No. cases	Laminin staining in epithelial basement membrane (no. cases positive)	Laminin staining in epithelial cytoplasm (no cases positive)
Cystic hyperplasia	29	29 (continuous)	–
Adenomatous hyperplasia	10	10 (continuous)	–
Atypical adenomatous hyperplasia	13	13 (some areas defective)	2
Well-differentiated carcinomas	2	2 (defective)	–
Moderately differentiated carcinomas	8	8 (defective)	2
Poorly differentiated carcinomas	4	4 (defective, and present only in a few areas)	4

calization of laminin in the carcinomas of the endometrium revealed that several tumor elements, especially in the well or moderately differentiated type of carcinomas, possessed basement membrane-like structures, but the staining was in a discontinuous pattern (Figure 3B). In the poorly differentiated carcinomas, only focal remnants of basement membranes were seen. Cytoplasmic staining for laminin was found in several adenocarcinomas but most intensely in poorly differentiated tumors (Table 2).

Laminin Receptor in the Human Endometrium

In normal endometrial glands, immunoreactive material was confined to the basal region of the glands and near the zone of the basement membrane (Figure 4A). In the proliferative phase the endometrial stromal cells were negative, whereas in secretory phase they were slightly positive for the laminin receptor. As the stromal cells gradually changed to predecidual cells, an increasing laminin receptor staining was found (Figure 4B). In the hyperplasia, several glands showed more diffuse cytoplasmic staining (not shown). This type of staining reaction was markedly increased in the carcinomas, especially when poorly differentiated tumor elements were present (Figure 5).

Discussion

The present study indicates that accumulation of laminin in normal human endometrial stroma may be

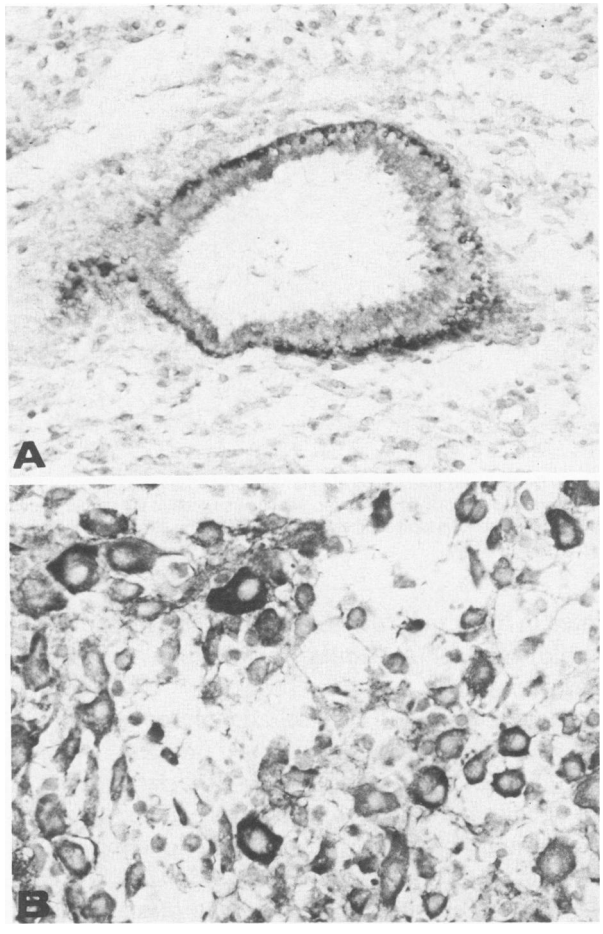

Figure 4—Immunohistochemical examination of the distribution of laminin receptor in normal human endometrium. **A**—Proliferative phase of the normal menstrual cycle. Note that the polyclonal antibody reacts in a polarized manner with material corresponding to the basal part of the cells toward the basement membrane. Stromal cells appear unreactive at this stage. (×275) **B**—Secretory phase of the normal menstrual cycle. Note that stromal cells have now attained a diffusely distributed positive cytoplasmic staining with antibodies against human laminin receptor. (×460)

intimately linked to the cyclic changes in the endometrial glands. The stromal cells of the proliferative phase do not exhibit laminin-positive material, as assessed by immunohistochemistry. In the second half of the normal menstrual cycle, the secretory phase, stromal cells begin to accumulate laminin in an intracellular and extracellular location. Morphologically, predecidualization of stromal cells is seen from Day 24,[7] at which time the laminin immunoreactivity of the stromal cells is already very intense. The laminin immunostaining further identified stromal cells in the early secretory phase which morphologically could not yet be classified as predecidual cells. Thus, the expression of laminin discriminates stromal cells of proliferative phase from those in secretory phase. The exact timing of the onset of laminin expression in the stromal cells may be eluci-

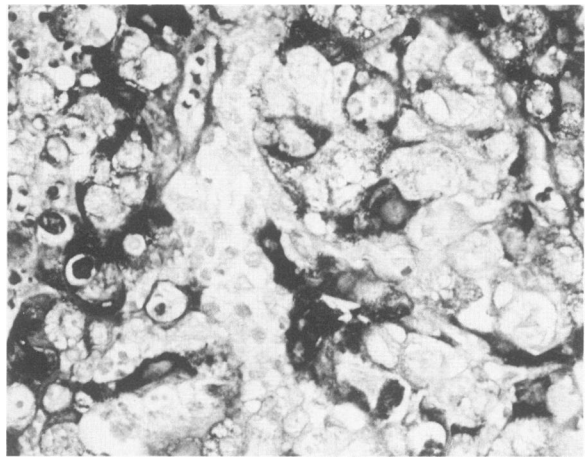

Figure 5—Immunohistochemical examination of the distribution of laminin receptor in a poorly differentiated adenocarcinoma of the uterus. Tumor cells show intense cytoplasmic staining, whereas the stromal cells appear unreactive. (×355)

dated in the future with the use of *in situ* hybridization employing cDNA clones for laminin or laminin receptor.

Adenomatous and atypical adenomatous hyperplasia, without adequate therapy may progress to adenocarcinoma of the uterus.[27,28,38,40] In the past, studies on the biology and grading of hyperplasia did not generally employ immunohistochemistry. With this approach, we demonstrated that stromal cells in 69% of the cases classified as endometrial cystic hyperplasia exhibited laminin-immunoreactive material. On the other hand, adenomatous hyperplasia, considered to be a more ominous form of hyperplasia, had significantly less laminin in the stroma (only 30%). In the cases of atypical adenomatous hyperplasia and in adenocarcinomas, no laminin-positive stromal cells were identified. These results suggest that the progression from cystic to atypical adenomatous hyperplasia and overt carcinoma is associated with decreasing amounts of stromal laminin. These findings may be of diagnostic significance.

We further investigated the stromal cells in cystic hyperplasia at the ultrastructural level. The cytologic features observed with distended rough endoplasmic reticulum was as previously described by More et al.[41] We also showed that the granular material seen at the cell surfaces, previously thought to be fibrinoid,[9] in fact represent basement membrane material with features reminiscent of lamina rara and densa. It thus appears that the stromal cells of cystic hyperplasia are comparable to the stromal cells in the early secretory phase of the normal menstrual cycle. The stromal cells of atypical adenomatous hyperplasia, on the other hand, appear to be devoid of pericellular material[39] and, as shown in the present study, also devoid of laminin-immunoreactive material. These morphologic data indicate that one is dealing with two different diseases, which is further supported by clinical evidence, since cystic hyperplasia does not evolve into either atypical adenomatous hyperplasia or carcinoma.

Previous studies of the tissue distribution of laminin and Type IV collagen have shown that these components are present in all normal epithelial basement membranes.[18] In carcinomas a variable degree of basement membrane disorganization has been described.[17,21-25] However, such studies have not included endometrial tumors. We therefore studied several endometrial carcinomas and found that well to moderately well differentiated endometrial adenomcarcinomas consistently contained laminin-positive discontinuous/defective basement membrane, whereas little or no basement membrane staining was seen in most poorly differentiated carcinomas. This is in accordance with previous ultrastructural studies.[39] These poorly differentiated carcinomas, however, exhibited tumor cell cytoplasmic staining for laminin, which suggests that tumor cells retain the ability to synthesize basement membrane components even though the extracellular assembly of this structure may be defective. These results are in concordance with previous studies of nonendometrial carcinomas.[17,21-25]

Antibody to laminin receptor was used in the study of the presence of this protein in the endometrium under normal and pathologic conditions. In the normal endometrial glands, this antigen appeared to be polarized toward the basal region of the cells near the basement membrane. Adenocarcinoma cells, on the other hand, expressed a significant amount of laminin receptor, both diffusely distributed in the cytoplasm and also along the cell surfaces. This result suggests that the laminin receptor might be less polarized and may be more abundant in the cytoplasm of malignant cells, as compared with their normal counterparts. Stromal cells of the proliferative phase of the normal menstrual cycle were unreactive with the anti-laminin receptor antiserum. In the secretory phase and in the case of hyperplasia, however, stromal cells appeared to express laminin receptor. Thus, we conclude that laminin and laminin receptor appear to be codistributed in the human endometrium.

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