The Effect of Estradiol on Collagen Structure and Organization in the Immature Rat Uterus (42891)

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Abstract. **An examination of collagen ultrastructure in the lamina propria of the immature ovariectomized rat uterus revealed that a single injection of estradiol (40 μg/kg) produced a biphasic effect on collagen structure and organization. In saline-treated animals (controls) dense populations of collagen bundles were seen throughout the extracellular matrix (EX). Cross-sections of these bundles suggested that the bundles run parallel to the long axis of the uterus and thin filaments seemed to form cross-links between collagen fibers. In contrast, large clear spaces, collagen fragments, and loosely packed bundles of collagen were observed in the EX of animals injected with estradiol 24 hr earlier.**

In a time course study (0, 1, 2, 4, 24, and 48 hr), estradiol treatment altered collagen structure and organization 1 hr following administration. Collagen bundles did not appear to be as densely packed as in control tissues and large clear spaces were evident in the EX. Two hours following estrogen administration, collagen fibers appeared to be fragmented and seemed to be separating from the plasma membrane of stromal cells. Four hours following estrogen administration, large clear spaces occupied most of the EX in the lamina propria. Collagen fragments were diffusely distributed throughout the EX and small cross-sectional patches of collagen bundles were present. In 48-hr-treated animals, collagen bundles reappeared and were often closely associated with the plasma membrane of stromal cells. The collagen was not as abundant as in control animals.

An overview of the cellular organization of the lamina propria revealed that stromal cells in control tissues were more densely packed than in estradiol-treated tissues (4, 24, and 48 hr) and the stromal cell size appeared to increase in hormone-treated tissues. These responses provide a good model system to study the role of estradiol in the control of collagen structure and organization in the uterus. [P.S.E.B.M. 1989, Vol 191]

Estradiol-stimulated growth in the immature rat uterus can be characterized by a complex series of biochemical changes (1). Uterine growth develops as a biphasic response which includes a series of early events, that occur 4–6 hr after hormone administration, and a series of late events that occur 24–48 hr after hormone treatment (2). Early responses include increases in water uptake (3), an activation of RNA polymerase (4), elevated rates of glucose (2) and lipid (5) metabolism, and an infiltration of plasma eosinophils (6). The late responses (the hyperplasic stage) include increases in DNA, RNA, and protein syntheses (7). In a recent report (8), the immature rat uterus also has been utilized as a model system to study the role of growth factor receptors in the control of uterine growth. Since these changes are induced by the same molecular events that have been shown to occur in mature cycling animals (1), it appears that the immature rat uterus is a good model system to study the role of estrogens in the control of uterine growth and development.

The immature rat uterus is composed of multiple cell types which have been shown to respond to estradiol (9). Kirkland *et al.* (10) have reported that mitotic figures appear in endometrial and myometrial cells over a period of 24–36 hr after estradiol administration. Other reports (11, 12) also suggest that other hormones play "permissive" roles in the regulation of uterine growth.

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Electron micrographs of uterine tissue reveal a number of changes in cellular morphology that are associated with estradiol-induced growth. Endometrial cell growth is associated with alterations in nuclear morphology, development of complex nucleoli, formation of euchromatin, development of an extensive rough endoplasmic reticulum (13–15), and formation of gap junctions (16). Ultrastructural changes in the nuclear and cytoplasmic morphology of endometrial and myometrial tissues also have been characterized in the uterus of the immature rat model (17, 18).

Collagen metabolism occurs in the lamina propria of the endometrium and numerous reports have shown that collagen serves as an important structural unit in the uterus (19–21). Collagen biosynthesis and degradation have been closely linked to the timing of and processes associated with parturition (20). However, the precise role that estrogens play in regulating uterine collagen metabolism is not well-characterized due to conflicting reports, utilizing different experimental protocols, which suggest that estrogens either increase (21), have no effect on (22), or stimulate both the synthesis and breakdown of collagen (23).

Since estrogens are known to play a central role in the growth and development of uterine tissue, it is important to understand the role that they play in the regulation of collagen metabolism. Since the immature rat uterus has been effectively employed as a model system to study growth responses induced by estrogens (7), this study was initiated to examine the structure and organization of collagen in the immature rat uterus and to characterize changes in collagen structure that occur following estradiol administration.

Materials and Methods

Female Sprague-Dawley rats (Ancare Corp., Manhasset, NY) were ovariectomized at 22 days of age. Six days later animals were injected intraperitoneally with 17β-estradiol (40 μg/kg; Sigma Chemical Co., St. Louis, MO) at 1, 2, 4, 24, and 48 hr prior to sacrifice. Estradiol was injected in a vehicle containing 0.9% sodium chloride and 5% ethanol and control animals were vehicle injected. Animals were sacrificed by decapitation and uterine horns were carefully removed and cleaned of adhering fat. Twelve rats were used as control animals, eight rats were used in the 24-hr treatment group, and four rats were used in the remaining treatment groups.

Uteri removed from experimental animals were fixed in a 2.5% gluteraldehyde solution (in 0.1 *M* phosphate buffer, pH 7.3; Polysciences Inc., Warrington, PA) for 3 hr. Tissues were then post fixed in 1% osmium tetroxide (in 0.1 *M* phosphate buffer; Polysciences Inc.) for 1 hr and rinsed three times in buffer. Fixed tissues were dehydrated in a graded series of ethanol solutions and then immersed in two changes of 100% propylene oxide (Polysciences Inc.) over a period of 20 min. Dehydrated tissues were infiltrated with 50% Epon-type resin (1/1 dilution of PolyBed 812 resin with 100% propylene oxide; Polysciences Inc.) and then embedded in 100% PolyBed. Each uterine horn was placed in a flat mold containing 100% PolyBed and the horn was oriented in a standard position so that tissue sections could be made perpendicular to the long axis of the uterus. Each step performed in the tissue preparation was carried out at room temperature and mixing was done with a Penetron rotator (Sunkay Labs, Tokyo, Japan). Embedded tissues were sectioned using a diamond knife (Diatome; US, Fort Washington, PA) and an ultramicrotome (LKB Ultrotome V; Gaithersburg, MD). Thin sections (900–1000 Å) mounted on uncoated 300-mesh copper grids were stained with uranyl acetate and Reynold's lead citrate. Stained sections were viewed with a Hitachi H600 transmission electron microscope (Hitachi, Mt. View, CA) at an accelerating voltage of 75 kV.

Light micrographs were prepared from tissues that were embedded in 100% PolyBed and 1-μm-thick sections were made with a glass knife on an ultramicrotome (LKB Ultrotome V). Sectioned tissues were applied to clean microscope slides, and after staining coverslips were affixed with Permount (Fisher Scientific Co.). Tissues were stained with an epoxy tissue stain containing toluidine blue and basic fuchin (Electron Microscopy Sciences) and photographs were taken with a 35-mm Nikon camera attached to a Nikon Labophot microscope with AFX Unit.

Electron micrographs and light micrographs presented in this study are typical observations made from sections prepared from at least four different animals.

Results

The generalized morphologic characteristics of uterine endometrial stromal cells can be seen in Figures 1 and 2. In Figure 1, tissues were prepared from saline-treated (control) animals whereas tissues seen in Figure 2 were prepared from animals treated with estradiol 24 hr before fixation. In control animals, the stromal cells were generally spindle shaped and possessed long, cytoplasmic processes. Nuclei, which occupied most of the area within cells, were pleomorphic and contained dense patches of heterochromatin along the inner borders. Because of the electron density of the cytoplasm, organelles were difficult to characterize. Dense collections of collagen bundles were seen in virtually every extracellular space. Many longitudinal as well as cross-sectional bundles of collagen were observed in each tissue but the majority of bundles appeared to be oriented in a direction that was parallel to the long axis of the uterus. Longitudinal sections of collagen showed the typical banding pattern.

Stromal cells from 24-hr estradiol-treated animals (Fig. 2) were much larger in size than cells observed from control animals. Long complex cytoplasmic processes were common and were present at all times fol-

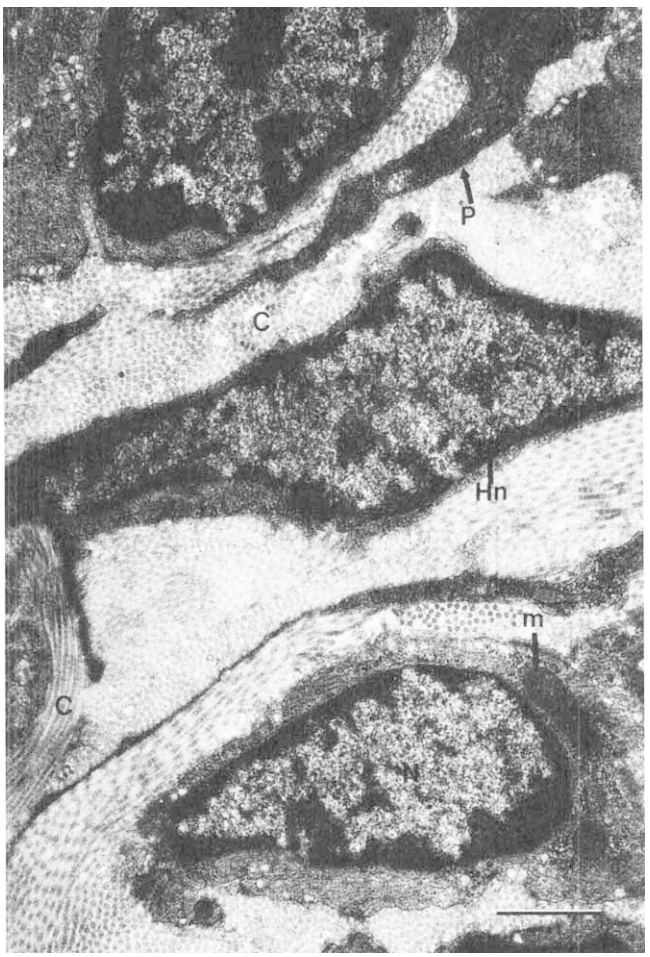

Figure 1. Electron micrograph of stromal endometrium isolated from an immature saline-treated rat. Labeled structures include nucleus (N), heterochromatin (Hn), mitochondria (M), collagen (C), cytoplasmic process (P) (magnification ×13,700). Bar= 1 μm.

lowing estrogen treatment. Nuclei from treated animals were oval and contained large amounts of euchromatin which was evenly dispersed throughout the nucleus. Well-developed nucleoli were present and were composed of fibrillar regions. The cytoplasm contained large numbers of free ribosomes and an extensive rough endoplasmic reticulum. Vacuoles were present and the mitochondria were swollen in appearance. The collagen network in the extracellular spaces was not nearly as well-developed as that seen in control tissues. For example, longitudinal collagen bundles appeared to be fragmented and less densely packed. These bundles were oriented parallel to the long axis of the uterus but were not as numerous as in control animals.

Figure 3 illustrates structural characteristics of collagen fibers at high magnification in control and estradiol-treated (24 hr) tissues. In control (Fig. 3A) and estradiol-treated tissues (not shown), cross-sectional views of collagen bundles showed that individual fibers appeared to be cross-linked by a network of filaments. In estrogen-treated tissues (Fig. 3B), longitudinal sections of collagen fibers appeared to be fragmented and composed of large collections of fibrils. A network of filaments was also present between collagen fragments. In contrast, control tissues (Fig. 4A) revealed no collagen fragmentation.

Having characterized the structure and organization of collagen in control and 24-hr estradiol-treated rats, the time course of changes in collagen morphology following estradiol administration is shown in Figures 4–6. One hour after hormone administration (Fig. 5A), collagen bundles appeared to be less densely packed than in control tissues and large clear spaces appeared between the collagen bundles and stromal cells. In addition, patches of a filamentous network became apparent in the extracellular spaces. Two hours after estradiol administration (Fig. 5B), longitudinal and cross-sectional bundles of collagen began to disappear and individual collagen fibers within each bundle became less densely packed and organized. A network of filaments was also seen in extracellular spaces. The stromal cells contained a large number of free ribosomes and smooth endoplasmic reticulum was seen.

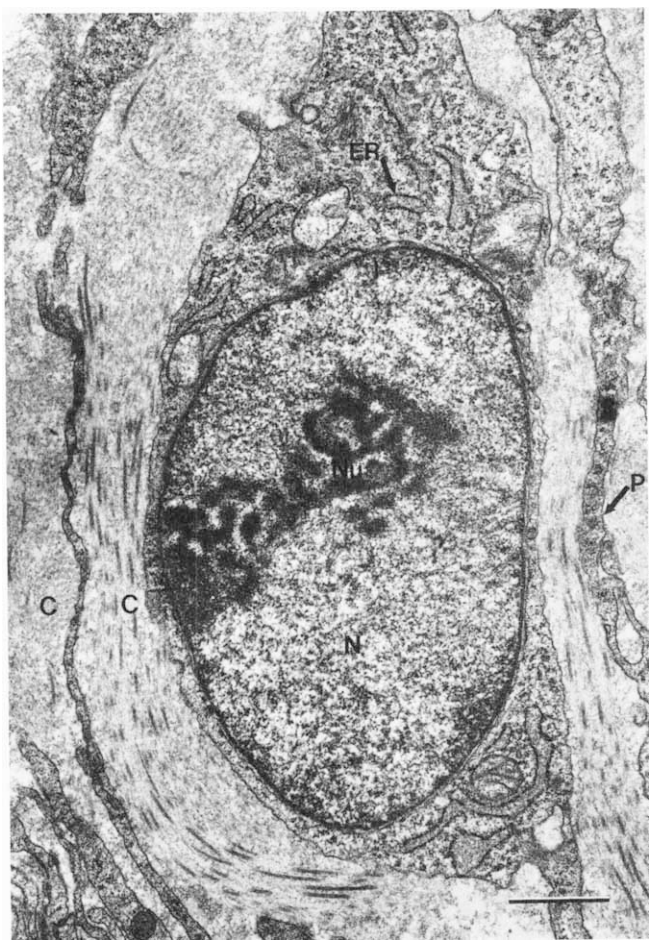

Figure 2. Electron micrograph of stromal endometrium isolated from an immature estradiol-treated (24-hr) rat. Labeled structures include nucleus (N), nucleolus (Nu), endoplasmic reticulum (ER), collagen (C), cytoplasmic process (P) (magnification ×13,500). Bar= 1 μm.

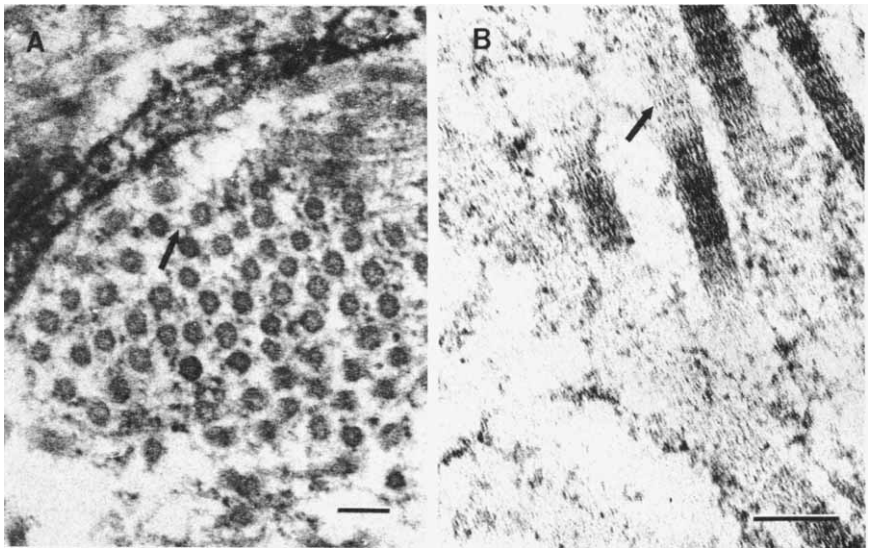

Figure 3. Electron micrograph of collagen fibers located in the extracellular matrix of immature saline-treated (A) and 24-hr estradiol-treated (B) rats. Arrow in A shows possible cross-links between collagen fibers and arrow in B shows fibril components of a collagen fragment (magnifications of A and B are ×67,470 and ×110,288, respectively). Bars = 0.1 μm.

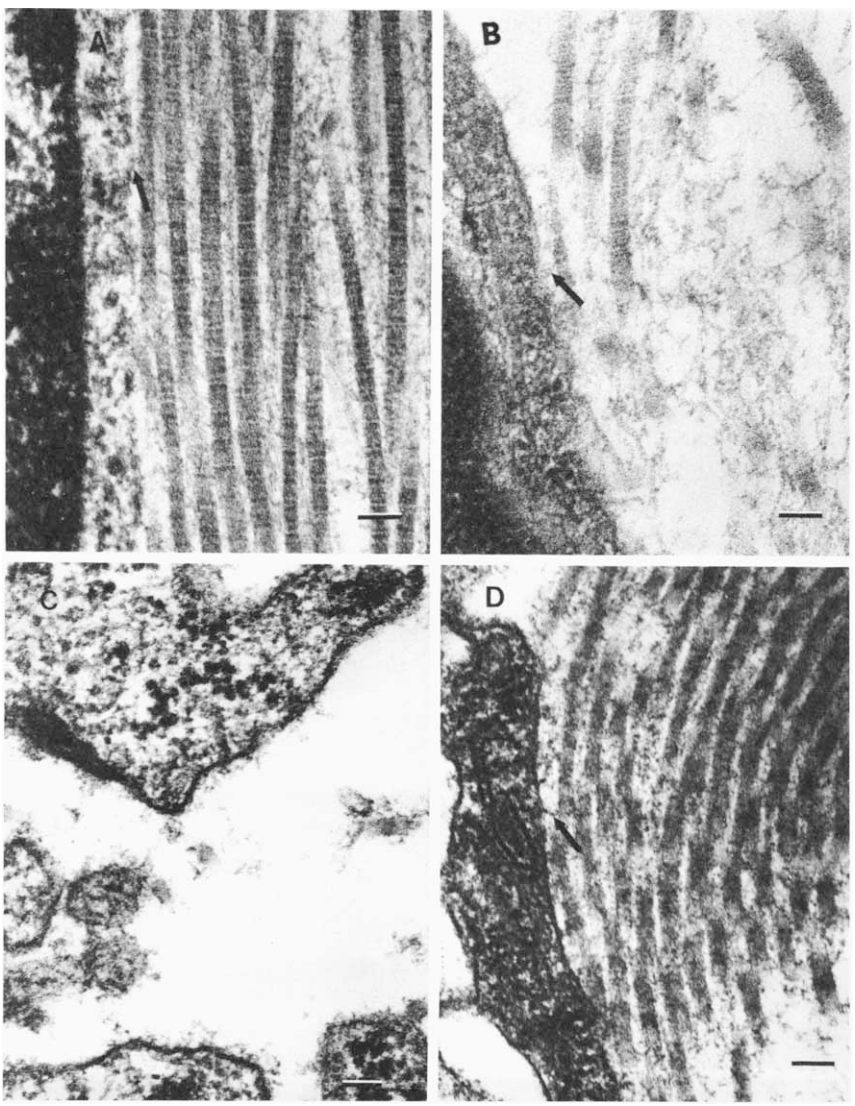

Figure 4. Electron micrograph of stromal endometrium isolated from immature saline-treated (A) and estradiol-treated rats. B–D illustrate stromal tissues prepared from animals treated with estradiol 1, 4, and 48 hr, respectively, before tissue fixation. Arrows show possible links between collagen fibers and the plasma membranes of stromal cells (×52,800). Bars = 0.1 μm.

In uteri prepared from animals treated with estradiol 4 hr prior to tissue fixation (Fig. 6A), collagen fragments were diffusely distributed throughout the extracellular spaces. The extracellular matrix contained clear spaces and regions of loosely organized filaments. The cytoplasm of stromal cells contained aggregates of free ribosomes and vesicles. Forty-eight hours after estradiol administration (Fig. 6B), collagen bundles were present in the extracellular matrix of the lamina propria. Longitudinal sections of collagen bundles showed densely packed fibers revealing the typical banding pattern seen in control animals. Clear spaces between collagen bundles were present and as a result the collagen matrix appeared to be less dense than those in control animals. Areas containing a network of filaments were seen closely associated with collagen bundles. The cytoplasm of stromal cells had become electron dense and appeared to be similar to control cells.

Figure 4 illustrates the progressive dissociation of collagen from the plasma membranes of stromal cells following estradiol administration. In control uteri (Fig. 4A), collagen fibers were closely associated with the plasma membrane of stromal cells. Collagen fibers showed the typical banding patterns and a network of filaments can be seen between individual collagen fibers within a bundle. A filament also appeared to link a collagen fiber with the plasma membrane (see arrow). One hour following estradiol administration (Fig. 4B), collagen fibers seemed to be dissociating from the surface of the plasma membrane. Filaments projecting outward from the surface of the plasma membrane were attached to collagen fibers while other filaments remained unattached. Individual collagen fragments appeared to be disintegrating into a granular electron-dense material. Filaments were also seen between collagen fragments. Figure 4C illustrates the loss of collagen fibers associated with the plasma membrane of stromal cells isolated from animals treated with estradiol 4 hr before tissue fixation. A network of filaments attached to the plasma membrane of stromal cells was seen to project outward into extracellular spaces but they did not appear to be attached to collagen. In tissues from the 48-hr treatment group (Fig. 4D), the close association of collagen fibers with the plasma membrane was again evident. Also observed was a network of filaments that seemed to link collagen fibers to each other and to the plasma membrane of stromal cells. As in control tissues, collagen possessed the typical banding pattern.

In order to provide an overview of endometrial organization and stromal cell morphology, Figure 7 shows low magnification (×800) photomicrographs of the lamina propria from control and estradiol-treated animals. All photographs show a portion of the epithelial lining of the endometrium and a section of the lumen of the uterus. In control tissues (Fig. 7A), the stromal cells were spindle shaped and smaller in size than cells seen in hormone-treated tissues. Four hours

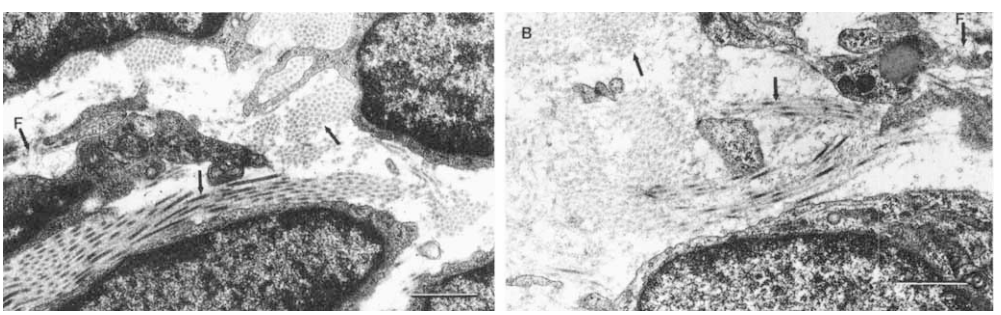

Figure 5. Electron micrograph of stromal endometrium isolated from immature estradiol-treated rats. Figure parts illustrate stromal tissues prepared from animals pretreated with estradiol 1 hr (A) and 2 hr (B) prior to tissue fixation. Arrows show longitudinal and cross-sectional bundles of collagen fibers and filaments labeled (F) (original magnification ×8,800). Bars = 1 μm.

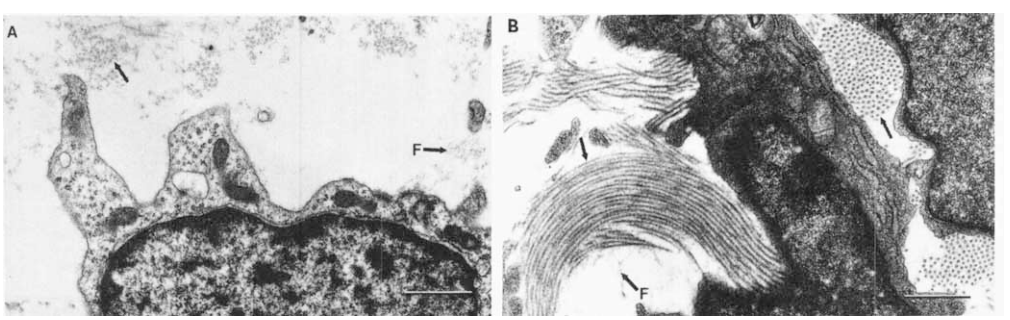

Figure 6. Electron micrograph of stromal endometrium isolated from immature estradiol-treated rats. Figure parts illustrate stromal tissues prepared from animals treated with estradiol 4 hr (A) and 48 hr (B) prior to tissue fixation. Arrows show longitudinal and cross-sectional bundles of collagen fibers and filaments labeled (F) (magnification ×8,820). Bars = 1 μm.

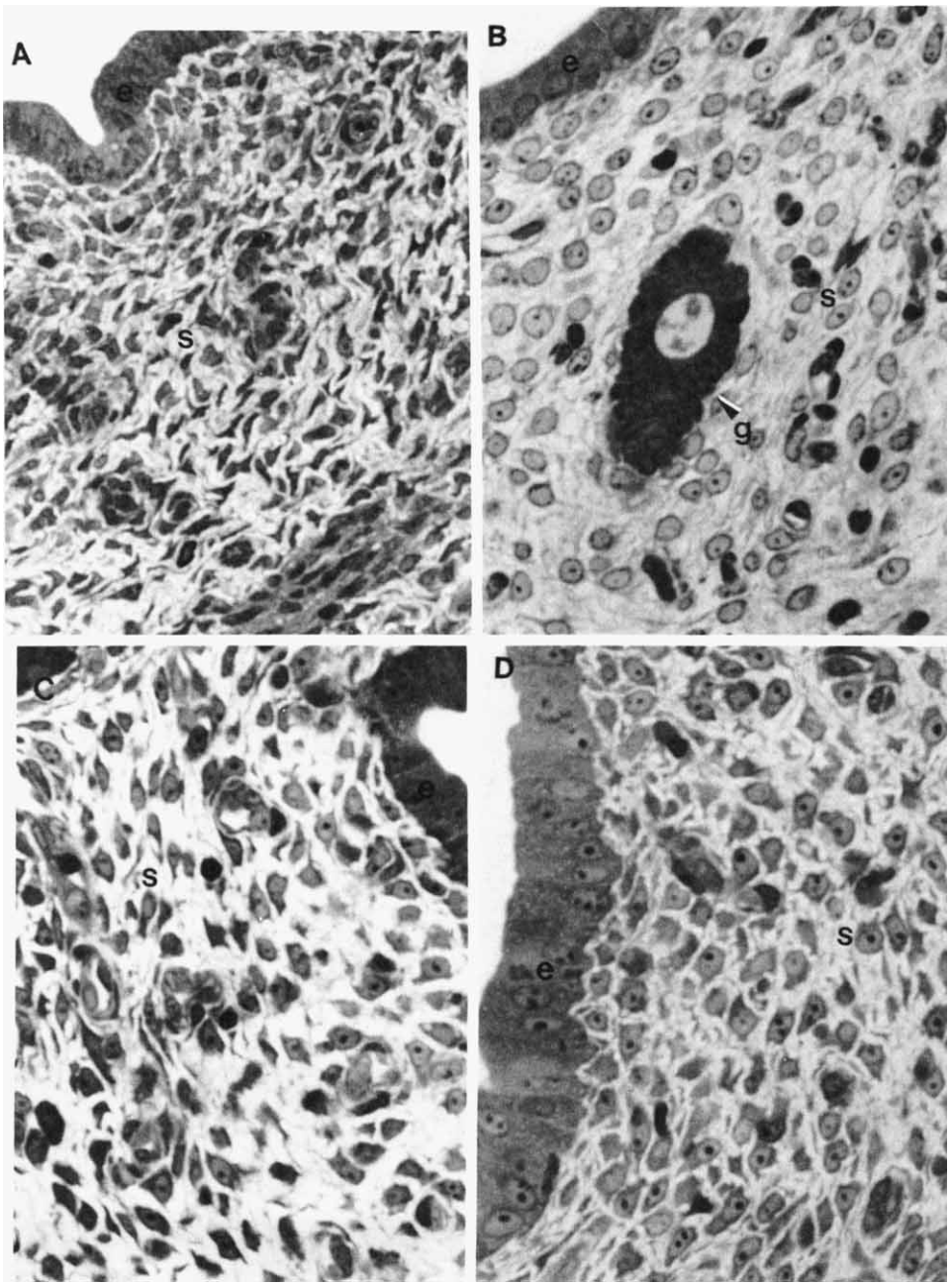

Figure 7. Light photomicrographs of the endometrium isolated from immature saline-treated (A) and estradiol-treated rats. B–D illustrate the endometrium of tissues removed from animals treated with estradiol 4, 24, and 48 hr, respectively, before tissue fixation. Labeled cells include the epithelial cells (e), stromal cells (s), and epithelial glands (g) (original magnification ×800).

after estradiol administration (Fig. 7B), stromal cells appeared to be swollen in appearance and the nuclei were oval. The cytoplasm and the plasma membranes were difficult to see and the cells did not appear to be as densely packed as seen in control tissues. Twenty-four and 48 hr after estradiol treatment (Fig. 7, C and D), stromal cells appeared to regain some of their spindle shape and the cytoplasm and the plasma membrane became more distinct. The stromal cells contained numerous cytoplasmic projections.

Discussion

The turnover of collagen, which is a major extracellular constituent of tissues, has been shown to be associated with development (24, 25), regeneration (26), and cell division (27). In uterine tissue, collagen degradation and biosynthesis undergo dramatic changes during different physiologic states (28–30). The role that estrogens play in these processes is not well-defined because of conflicting results (21–23). In this study, we examined the effect of a single dose of estradiol on

collagen structure and organization in the immature rat uterus because this model system has been successfully employed to characterize the role of estrogens in the regulation of uterine cell growth and function (1).

The uterine growth response induced by estradiol in the immature rat occurs as a biphasic process which includes a series of early responses that occur 4–6 hr after hormone administration and a series of late responses that occur 24–36 hr after hormone exposure (2). In this study, the estradiol-induced stromal cell growth response was clearly seen as changes in cell size and morphology. The initial growth response seen 4 hr after estrogen administration probably reflected tissue and cell edema since this is the same time point following estrogen administration when water uptake by the uterus has been reported (1). As the process of growth continued, alterations in stromal cell size seen 24 and 48 hr after estrogen administration probably reflected the stage in the growth response that has been associated with cell division (10) and increases in protein and DNA synthesis (7) in the immature rat uterus.

The initial disappearance of collagen observed in the lamina propria in this study followed the same time course of change as the early uterine growth responses. Coupled with this observation, estradiol has recently (31) been demonstrated to stimulate the turnover of proteoglycans in the mouse uterus. Since the metabolism of collagen and its associated proteoglycans have been associated with water uptake in developing tissues (32), the processes associated with the disappearance of collagen seen in this study may have also resulted in the development of an extracellular osmotic environment that contributed to water uptake by the uterus. Further studies are, of course, needed to examine this possibility.

The attachment of collagen fibers to the plasma membranes of cells appears to be mediated by the proteoglycans (27) and collagen receptors (33). These contacts appear to play important roles in cell and tissue morphology and in cell division (34). In this study we found a close association between collagen fibers and stromal endometrial cells in saline-treated and 48-hr estradiol-pretreated animals. This close association was lost shortly after estradiol administration (1-, 2-, and 4-hr posttreatment) and these changes could have resulted from a loss in plasma membrane receptors for collagen which has been demonstrated to occur during different physiologic states (34), or it could have resulted from the metabolism of proteoglycans which has been linked to tissue growth and development (35). Collagen resorption and then reformation has been reported to occur in numerous tissues that are undergoing the processes of growth and regeneration (36–39) and thus the changes observed in collagen structure and organization seen in this study may reflect a similar sequence of events in which tissue remodeling is associated with uterine growth.

Collagen catabolism has been reported to occur by both extracellular and intracellular mechanisms. Extracellular catabolism is associated with the release of collagenase into extracellular spaces (40) whereas intracellular catabolism involves phagocytosis of collagen fibers followed by lysosomal digestion (41). In this study we found no evidence of intracellular collagen catabolism by stromal cells since vacuoles containing collagen fibers or fragments were not observed at any of the time points associated with collagen resorption. Macrophages have been associated with uterine collagen catabolism (42) and eosinophil infiltration into uterine tissue has been linked to early responses induced by estradiol (6). These cell types may have contributed to collagen catabolism in the immature uterus through the release of collagenase or through the process of phagocytosis. No ultrastructural evidence was found to support their widespread distribution in the uterus or their presence in regions of the lamina propria associated with generalized collagen catabolism.

Perez-Tamayo (43) noted that during collagen resorption in carrageenin granulomas, collagen fibers undergo fragmentation, fibril separation, and fibril disintegration. The same changes in collagen morphology appeared to occur in uterine tissue following estrogen treatment. Until additional studies are performed, however, this interpretation must be questioned since tangential-sectioned portions of intact collagen fibers may have produced collagen fragments that appeared to be similar to collagen during the process of resorption.

The disappearance and reappearance of collagen fibers seen in this study can be explained either by the catabolism and anabolism of collagen or by a reorganization process that involves the disassembly and reassembly of collagen and proteoglycan fragments. Estradiol has been demonstrated to increase the amount of collagen in the immature rat uterus (44) and the production of a collagenase inhibitor also has been shown to be regulated by estradiol (45). These studies provide evidence that the biosynthesis of collagen in the uterus can be controlled directly or indirectly by estradiol. In contrast, Harkness (20) has reported that early changes in the organization of cervical collagen that occur following parturition are not associated with large changes in the content of tissue collagen. The disappearance and the eventual reappearance of collagen seen in this current study can be explained either by changes in collagen turnover or by alterations in collagen structure and organization that are independent of changes in tissue collagen content. Further studies are needed to characterize the possibilities.

In addition to the roles collagen may play in the process of tissue remodeling in the uterus, collagen has also been shown to play an important role in tissue structure and organization. The directional orientation of collagen fibers has been demonstrated to affect tissue tensile strength and rigidity in uterine tissue (46, 47).

Results from our study suggest that many bundles of collagen are not randomly organized but rather are oriented parallel to the lumen of the uterus. This organization may play a role in regulating the longitudinal tensile strength of the uterus in the immature rat. Numerous investigators have also reported that proteoglycans are closely associated with collagen in a variety of tissues (48, 49). Proteoglycans provide cross-links between collagen fibers that are thought to increase the structural integrity of the extracellular matrix (50). In the immature rat uterus, we also observed a fine network of fibers that appeared to cross-link collagen fibers. The fine network of fibers that appeared in the lamina propria of uterine tissue was similar to proteoglycan networks that have been reported by others (51).

Results from this study demonstrate that a single dose of estradiol induces both the disappearance and reappearance of collagen bundles in the lamina propria of the immature rat uterus. These changes correspond, in time, with well-characterized early and late uterine growth responses. As a consequence, these changes in collagen structure and organization may serve as a good model system to study the role of steroid hormones in the regulation of collagen structure and organization in the uterus.

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