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Disturbed Cell Arrangement, Increased Cell Membrane Permeability and Apoptotic Cell Death Occur in Adenomyotic Uterine Tissues in Mice

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ABSTRACT—The relationship of the development of uterine adenomyosis with the changes in the cell arrangement and cell membrane permeability, and incidence of DNA fragmentation was examined in mice. In uterine areas showing the invasion of endometrial tissues into musculature, rhodamine-phalloidin staining for actin fibers revealed that the stromal cells ran parallel to the direction of the infiltration, and the muscle cells lost their regular arrangement, unlike those in the normal uteri of control mice. Inner myometrium showed positive fluorescence with Evans blue, which is known to stain only cells with increased membrane permeability. Outer myometrium also became fluorescence-positive, when the disease was advanced to severe state bearing invasion of endometrial tissues into the outer myometrium. No fluorescence with Evans blue was observed in the normal uterine tissues. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL method)-positive nuclei were exclusively found in some smooth muscle cells near both the blood vessels and tip of the invading endometrial tissue. These findings indicate that in adenomyotic uteri, apoptotic cell death developed in certain cells in myometrium, though disrupted cell arrangement and increased cell membrane permeability occurred in almost all of the inner myometrial cells. The increased membrane permeability in the myometrial cells might participate in the local occurrence of cell death near the blood vessels. Therefore, endometrial tissue would invade the myometrium through the space produced by the cell death along the blood vessels.

INTRODUCTION

Uterine adenomyosis (i.e., endometriosis interna), is characterized by an abnormal growth of glands and stroma into and beyond the myometrial layers. With the invasion of endometrial tissues, smooth muscle bundles of myometrium become loose, and begin to thin. Associated with the advance of the histological disorder, the muscle cells of the inner and outer myometrium are markedly reduced in size and are irregular-shaped compared to those in the normal mice (Ohta *et al.*, 1985; Mori and Nagasawa, 1989a), which results in the widened intercellular space. Despite these previous observations, the cause of the occurrence of myometrial disintegration and an exact point where endometrium could penetrate into myometrium remained unclear. Here, we demonstrate that in the adenomyotic uterus, cell arrangement was disturbed in both the endometrial and myometrial tissues, cell membrane permeability increased, and apoptosis occurred in the myometrial smooth muscle cells, thus suggesting the trigger of the invasion of endometrium.

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MATERIAL AND METHODS

Animals

A SHN strain of mice maintained in our laboratory was kept under controlled lighting (12 hr/day) and temperature (25°C ± 0.5°C). They were provided with a commercial diet (CA-1:CE-7 = 1:2; CLEA Japan Inc., Tokyo, Japan) and tap water ad libitum. The following experimental procedures were approved by the Animal Care and Use Committee of the Graduate School of Science, University of Tokyo, and all experiments conformed to the regulations described in the NIH Guide to the Care and Use of Laboratory Animals. Female mice, 40 to 50 days of age, were divided into two groups. The experimental group of 55 mice was grafted with a single anterior pituitary gland into the lumen of the right uterine horn. The pituitary grafts were obtained from age-matched litter mates. Fifty-five mice receiving no pituitary grafts served as controls.

Preparation of tissues

To examine cell membrane permeability, a dye exclusion test was performed using Evans blue (Sigma, St. Louis, MO), which was dissolved in phosphate-buffered saline (PBS; 0.15 M NaCl, 10 mM phosphate buffer, pH 7.0), sterilized by passage through membrane filters with a pore size of 0.2 μm and kept at 4°C. Dye solution was injected intravenously through the tail vein (1 mg dye / 0.1 ml PBS /10 g body weight) 10, 20, 40 and 60 days or 4 and 6 months after pituitary grafting. Three hours after the injection, mice were killed by cervical dislocation. Control mice were also given the dye and killed at comparable ages. In addition, some mice of both experimental and

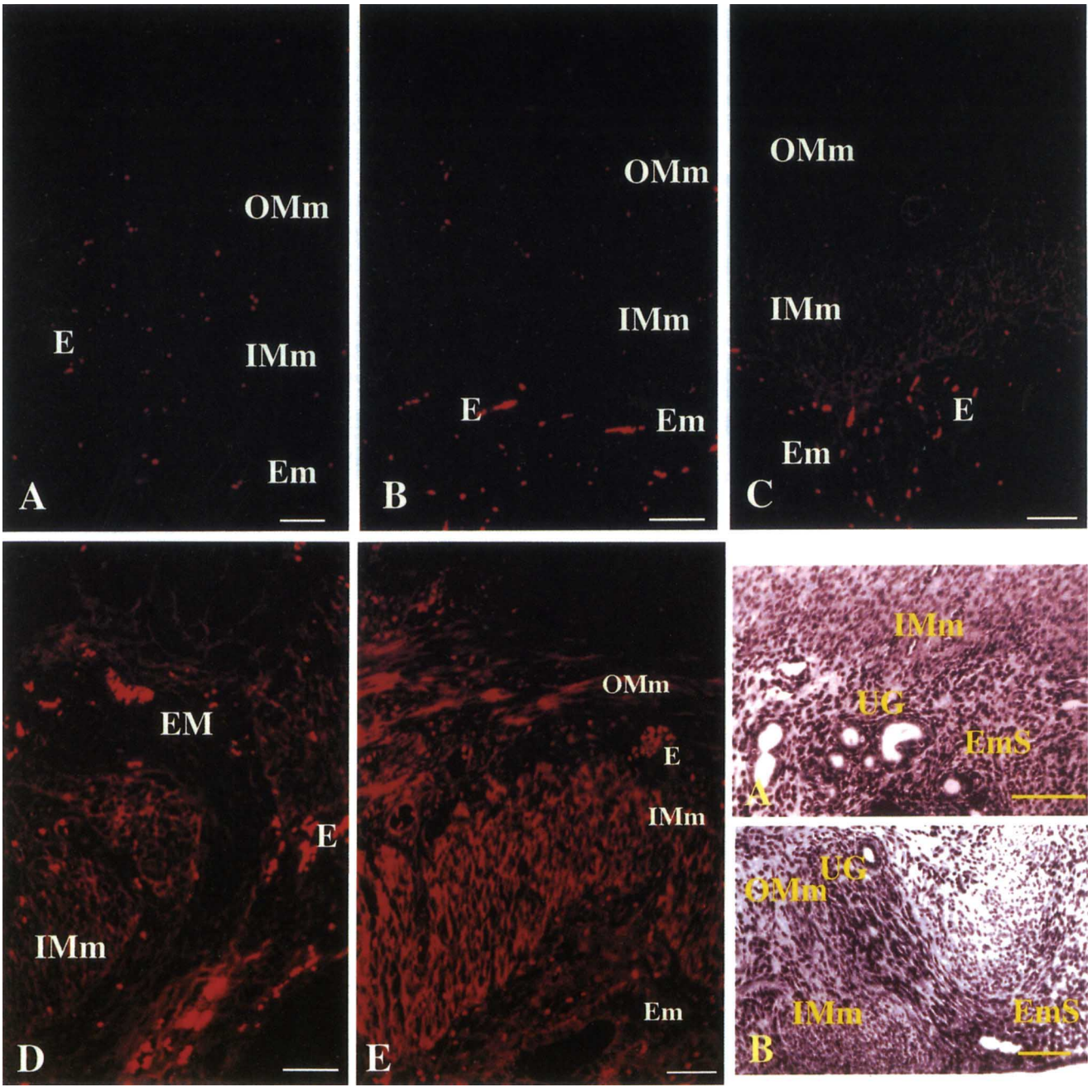

Fig. 1. Identification of Evans blue-stained cells. Positive staining with Evans blue preferentially observed in erythrocytes in all tissue samples. Normal uterus obtained from a 60-day-old control mouse showed no signal except erythrocytes (**A**). With a period of pituitary grafting, Evans blue-stained cells were seen in inner myometrium. Furthermore, outer myometrial cells also showed positive staining with advance of the adenomyotic change. The uteri were obtained from mice at 10 days (**B**), 20 days (**C**), 60 days (**D**) and 6 months (**E**) after pituitary grafting. Note that endometrial tissue was never stained, but myometrial tissue showed positive staining. Fluorescence in inner myometrial tissue was first observed at 20 days after the operation (**C**). Em, endometrium; IMm, Inner myometrium; OMm, outer myometrium; E, erythrocyte. Bar: 100 μm (**A**, **B**, **C**, **E**), 50 μm (**D**).

Fig. 2. Hematoxylin/eosin-stained sections of the normal uterus and pituitary-grafted uterus with adenomyotic change. Both tissues were obtained from a 100-day-old mouse (**A**). Normal uterus had no aberrant endometrial tissues. (**B**) In the uterus with adenomyosis, uterine gland (UG) invaded into inner myometrium (IMm) and reached the outer myometrium (OMm). UG, uterine gland; EmS, endometrial stroma; IMm, Inner myometrium; OMm, outer myometrium. Bar: 100 μm.

control groups receiving no injection of dye solution were autopsied at the same schedule as described and used to examine the changes in actin cytoskeleton arrangement by rhodamine-phalloidin staining. Each group of the differently treated preparations was obtained from more than three mice at different ages.

Immediately after sacrifice, the uteri were dissected out and fixed in 10% phosphate-buffered formalin for overnight at room temperature. Specimens were dehydrated through ethanol series, cleared in xylene and embedded in Paraplast Plus™ (Sherwood Medical, St. Louis, MO). Blocks were cut at 3 μm thickness and mounted on gelatin-coated glass slides. The preparations treated with Evans blue were rehydrated through ethanol series, washed in water for 1.5 hr to remove excess dye, dehydrated and mounted in Entellan (Merck, Darmstadt, Germany) . The other tissue preparations obtained from mice without Evans blue injection were rehydrated and preincubated in PBS for 5 min at 37°C. The specimens were immersed in 20 U rhodamine-phalloidin (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) in 40 μl PBS on the slide glasses, covered with the parafilm and incubated for 30 min at 37°C in a moisture chamber. Slides were washed with PBS for 5 min twice at room temperature, dehydrated and mounted in Entellan. Some sections were stained with hematoxyline/eosin regardless of Evans blue treatment.

To detect apoptotic cell death by the TUNEL method, every 3 experimental mice were killed 10, 20, 40 and 60 days or 4 months after pituitary grafting. Consisting of 3 mice each, 5 groups were also autopsied at comparable ages. The uterus was dissected out and fixed in metharcarn solution (methanol:chloroform:acetic acid=6:3:1). Deparaffinized sections were prepared as previously described. TUNEL was carried out using an In Situ Cell Death Detection Kit, AP (Boehringer Mannheim, Germany) according to the manufacture's protocol. In brief, the sections were washed with PBS and then incubated in a mixture of TdT and fluorescein-labeled dUTP for 60 min at 37°C. After washing with PBS, sections were treated with anti-fluorescein antibody conjugated with alkaline phosphatase, and incubated for 20 min with NBT, BCIP (Promega, Madison, USA).

Microscopic observation

The sections treated with Evans blue were observed under a fluorescent microscope (BH-RFL-LB; Olympus, Tokyo, Japan) equipped with a green activation filter (546 nm) and barrier filter (610 nm) since the dye was activated with green light. The sections treated with rhodamine-phalloidin were observed with the same equipment using a different barrier filter (570 nm). Sections stained with hematoxyline/eosin were observed through bright-field optics. Specimens for TUNEL method were observed under a differential interference microscope (BH2-RFCA; Olympus).

RESULTS

Vital staining with Evans blue

To clarify the changes in cell membrane permeability by incorporation of dye, uterine sections of mice treated with Evans blue were observed under a fluorescent microscope. Interstitial spaces in myometrium and endometrium were Evans blue-positive in the preparations without washing, but the fluorescence disappeared after washing with tap water for 1.5 hr. However, the fluorescence in myometrium remained without fading after washing, suggesting that Evans blue became insoluble in the cytoplasm of myometrial tissues. Positive fluorescence with Evans blue was always observed in erythrocytes but not endometrial stromal cells.

Control uteri with normal histology showed negative staining in the myometrium at any ages examined (Fig. 1A). Ten days after pituitary grafting, the uteri showed negative staining as well as controls (Fig. 1B). Twenty days after the operation, fluorescence-positive cells were found in the inner myometrium, and the intensity of staining became stronger 40 days after the operation (Fig. 1C). Fluorescence-positive cells were not restricted to the area where endometrium were invading, but existed throughout the inner myometrial layer (Fig. 1D). When the disorder was advanced to severe state bearing the invasion of endometrium into outer myometrium, positive staining was observed finally in the outer layer (Fig. 1E).

Cellular actin filament

The relationship between the development of adenomyotic changes and the change in cell arrangement was studied with rhodamine-phalloidin staining. Actin fibers visualized by rhodamine-phalloidin indicated the direction of cell arrangement. In longitudinal sections of control uterus showing normal histological characteristics (Fig. 2A), the arrangement of cells showed no polarity in endometrial stroma, concentric structure in inner myometrium and a definite direction parallel to the long axis of the uterine horn in outer myometrium (Fig. 3A). In adenomyotic uteri, the cell arrangement markedly changed; the endometrial stromal cells were disposed parallel to the direction of the invading endometrium (Figs. 2B, 3B). Simultaneously, the inner myometrial cells lost their concentric structure and became arranged irregularly. When the invasion of endometrium reached the outer myometrium as an advanced state of adenomyosis, the outer myometrial cells also lost their regular alignment (Fig. 3C).

DNA fragmentation

TUNEL method stained the cell nuclei undergoing DNA fragmentation. No positive signals of DNA fragmentation were found in the control uterus at any ages (Fig. 4A). Along with a period of pituitary grafting, positively stained nuclei appeared as follows: 10 days after pituitary grafting, no signal was obtained; 20 and 40 days after the operation, nuclei of some smooth muscle cells near the blood vessels in the inner myometrium were positively stained (Fig. 4B); and some myometrial cells touching the tip of the endometrial invasion had positively stained nuclei 60 days and 4 months after pituitary grafting when adenomyosis advanced to severe state (Fig. 4C, D, E).

DISCUSSION

The dye exclusion test has been widely used to distinguish dead cells in cell preparations. In this test, dead cells with permeable cytoplasmic membranes are stained by several types of dye. Evans blue is used frequently for dye exclusion tests (Gaff and Okong'o-Ogola, 1971). For example, it specifically stained degenerated skeletal muscle fibers in dystrophine deficient muscle (Matsuda *et al.*, 1995). In the present study, myometrial cells in the adenomyotic uteri of mice with pituitary graft were stained with Evans blue. Thus, hyperprolactinemia induced by pituitary grafting (Mori and Nagasawa, 1989b; Mori *et al.*, 1989, 1991) may be respon-

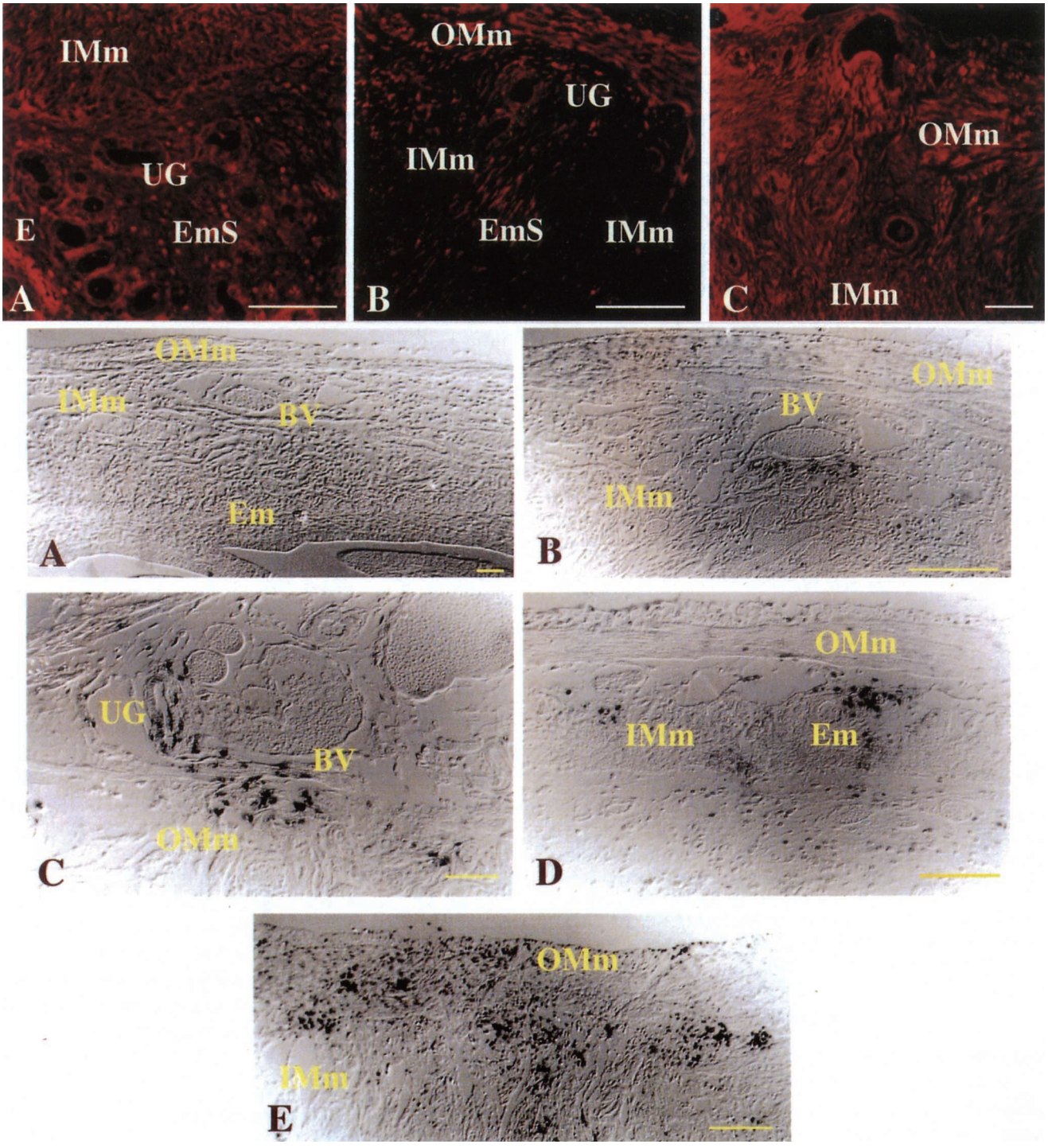

Fig. 3. Actin fibers visualized by rhodamine-phalloidin. Actin cytoskeleton in the normal uterus of a 100-day-old mouse is seen in **A**. In adenomyotic uterus of a pituitary-grafted mouse at 100 days of age (**B**), endometrium penetrated into inner myometrial tissue, and the endometrial stromal cells were rearranged to turn toward the invading direction. When the disease advanced to severe state at 4 months after the operation, endometrium invaded the outer myometrium, and the myometrial cells in the outer layer were disrupted and lost their regular alignment (**C**). UG, uterine gland; EmS, endometrial stroma; IMm, Inner myometrium; OMm, outer myometrium; E, erythrocyte. Bar: 100 μm (**A**, **B**, **C**).

Fig. 4. TUNEL-positive nuclei are localized near the blood vessels and the invading endometrial tissue. Sections were observed under a differential interference microscope, and black spots represent TUNEL-positive nuclei. Normal uterus of a 60-day-old control mouse had no obvious signals (**A**). Along with the duration of pituitary grafting, positively stained nuclei appeared. Twenty days after the operation, cells near the blood vessels often had signals (**B**). At 60 days when endometrium penetrated into myometrial tissue, the muscle cells near the blood vessels and around the invasion of endometrial tissues were stained positively (**C**, **D**). Four months after pituitary grafting, the disease advanced to severe state, and many signals were observed in the musculature (**E**). Em, endometrium; IMm, Inner myometrium; OMm, outer myometrium; BV, blood vessel. Bar: 100 μm (**A**, **B**, **D**, **E**), 50 μm (**C**).

sible for the increased cell membrane permeability, which causes the cell death in the myometrium, since prolactin plays a role in an increase of cell membrane permeability (Russel, 1989; Kelly *et al.*, 1991).

Apoptosis takes place during the development of some organs and various pathological processes (Evan *et al.*, 1995; Thompson, 1995; Hale *et al.*, 1996). In this study, DNA fragmentation occurred selectively in the smooth muscle cells. TUNEL preferentially labels apoptosis in comparison to necrosis (Gorczyca *et al.*, 1993; Gold *et al.*, 1994), but the possibility that some of the TUNEL-positive signals resulted from the necrotic cell death was not excluded. TUNEL method revealed that apoptotic cell death occurred strictly around the blood vessels at the early stage of adenomyosis. With advance of the disease, myometrial cells touching the tip of the invading endometrium underwent DNA fragmentation. The increased intracellular space derived from the cell death near the blood vessels in the myometrium allowed the endometrium to penetrate into the musculature. Though many cells in the myometrium were Evans blue-positive, certain stained-cells were TUNEL-positive. Increased cell membrane permeability was often linked to the markers of cell death. Therefore, some other factors may lead to cell death near the blood vessels.

In our previous study, expression of prolactin receptor mRNA increased along with the advance of adenomyosis (Yamashita *et al.*, 1997). Our immunohistochemical study demonstrated that the uterine myometrial cells were prolactin positive (Mori and Nagasawa, 1989a). Prolactin triggers Ca^{2+} mobilization from intracellular stores and also stimulates Ca^{2+} entry probably through voltage-insensitive nonspecific channels in CHO cells (Prevarskaya *et al.*, 1994). Increase of the cytoplasmic calcium level can cause cellular toxicity, resulting in cell death by activating a number of enzymes that cause degradation of intracellular proteins, cellular membranes and nuclear DNA (Rasmussen, 1986; Nicotera *et al.*, 1992; McConkey and Orrenius, 1996). Increments of Ca^{2+} causes the disruption of actin cytoskeleton which plays a role in stabilizing cell morphology (LeBaron *et al.*, 1988), and disorganization of actin filaments involves the subsequent influences on gene expression (Bissell and Barcelos-Hoff, 1987). Actin filament disruption might reflect a structural change of smooth muscle cell. In the muscle cells near the blood vessel, intrinsic matrix metalloprotease, macrophage or other factors may be related to the local occurrence of apoptosis.

In this study, increased cell membrane permeability indicated by Evans blue could serve as a possible marker for the risk of the invasion of endometrial tissues into the musculature. Staining of actin filament by rhodamine-phalloidin revealed where the myometrium was invaded and suggested a movement of the endometrial stromal cells. Our findings also suggested that the endometrium invade along the blood vessels into the myometrium. Clarification of the mechanism for the occurrence of apoptosis in the muscle cells near the blood vessels could raise a possible method for the prevention of the development of uterine adenomyosis.

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