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Original Article

Enhanced myometrial autophagy in postpartum uterine involution

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ABSTRACT

Objective: To understand the mechanisms of postpartum uterine involution, we investigated the uterine myometrial changes during pregnancy and the postpartum period.
Materials and methods: Nine groups of uterine myometrial samples from mice ($n = 4$) were collected on gestational Day 0 (nonpregnant), Day 1, Day 2, Day 7, Day 14, and Day 21 and on postpartum Day 1, Day 2, and Day 7. Human samples of uterine myometrium on term ($n = 1$) and postpartum Day 1 ($n = 2$) were also collected. Ki-67 immunostaining was used to determine myometrial proliferation. For cell hypertrophy analysis, organelle proteins, β-actin, prohibin, calnexin, and golgin-97 were analyzed by Western blotting. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) and evaluation of activated caspase-3 expression by Western blot analysis assay were used to detect apoptosis. Autophagy was assayed via the evaluation of LC3 expression by Western blotting, immunohistochemistry, and autophagosomes by electron microscopy.
Results: Uterine myocytes proliferated during the early stage of gestation with a peak at Day 2, whereas myocyte hypertrophy with increased cellular organelle production occurred gradually in later stages of pregnancy. Postpartum autophagy developed abruptly in uterine myocytes without obvious apoptosis.
Conclusion: Autophagy of myocytes may play an important role in uterine involution. These results have implications for our understanding of myometrial functional adaptations during pregnancy and the physiological role of autophagy in the uterine remodeling events in the postpartum period.

Introduction

The human uterus undergoes profound physiological tissue remodeling during pregnancy and involution during the postpartum period. Dramatic increases in uterine size to accommodate the growing fetus occur and the uterus very rapidly returns to the initial (before pregnancy) level after delivery. It has been estimated that the nonpregnant parous uterus weighs approximately 70 g, and at term, the uterus weighs approximately 1100 g, representing an almost 20-fold increase in mass [1,2].

The myometrium, the distinct muscular layer of the uterine wall that is involved in contraction during labor, is the main component in the enlargement of the uterus during pregnancy. It consists predominantly of smooth muscle cells but also contains fibroblasts, blood and lymphatic vessels, immune cells, and connective tissue. The connective tissue, or stroma, provides a supportive matrix for the bundles of smooth muscle, and a framework that expands as the uterus distends during gestation. The size and number of myometrial smooth muscle cells are determined by the stage of pregnancy [3].

The cellular and molecular responses of myometrial cells to both biochemical and biophysical changes during this period are not fully known. At the cellular level, an individual myometrial cell might enlarge almost 100-fold to approximately 500 μm in length at term. This is a reversible process, and the cell is maintained in a differentiated status and undergoes hypertrophy instead of hyperplasia in response to various stimuli during pregnancy. As pregnancy progresses, the cell body steadily enlarges but involutes

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and returns to approximately its original size by 6 weeks after delivery in humans.

Following parturition, the uterus recovers its prepregnancy size with some weight gain. Although the postpartum involution of the uterus has been studied previously [4–7], the mechanisms responsible for the reduction in size and number of muscle cells are still not fully understood. Myocyte apoptosis and/or autophagy in the postpartum period has been suggested in uterine involution. Takamoto et al [4] and Shkurupiy et al [5] suggested that uterine smooth muscle undergoes apoptosis in uterine involution, whereas Gray et al [6] suggested that it did not. Using electron microscopy examination, Henell et al [7] suggested that autophagy is a likely mechanism responsible for the size reduction observed in uterine involution.

Autophagy is a lysosomal degradation pathway for cytoplasmic material, which is activated during stress conditions, such as amino acid starvation [8,9]. In general, mammalian cells use autophagy during short periods of starvation to degrade nonessential cellular components to liberate nutrients for vital biosynthetic reactions. The process is characterized by the formation of double membrane vacuoles containing cytoplasmic constituents, the autophagosomes. These autophagosomes are fused with lysosomes to form the autolysosomes, which subsequently degrade the sequestered material. It is a survival mechanism for cells under nutrient starvation. Excessive autophagic activity may lead to cellular death [10–12]. However, the role of autophagy in uterine involution in the postpartum period has not been fully explored.

We hypothesize that autophagy is an important mechanism in uterine involution. Therefore, in this study, we investigated the uterine myometrial changes during pregnancy including myometrium hyperplasia, as demonstrated by nuclear Ki-67 expression and hypertrophy, as measured by uterine weight gain and increased cellular organelle production. Because of a potential role in uterine involution, we also examined myometrial apoptosis via a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay and caspase-3 activation. Finally, we studied the autophagy activity of myometrium derived from mice as well as from human samples. We found that myometrial hyperplasia occurs mainly in early gestation, whereas cellular hypertrophy with increase cellular organelle production occurs in later stages of pregnancy. After parturition, autophagy occurs abruptly in uterine involution without an increase of myocyte apoptosis. The results have implications for our understanding of myometrial functional adaptations during pregnancy, and the physiological role of autophagy in the uterine remodeling events in the postpartum period.

Materials and Methods

Cell culture and reagents

The cervical cancer HeLa cell line was purchased from American Type Culture Collection (ATCC; Rockville, MD, USA). The cells were maintained in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 mg/mL streptomycin (Life Technologies, Inc., Carlsbad, CA, USA) in a humidified atmosphere of 5% CO_2. Cisplatin was obtained from Sigma Chemical Company (St Louis, MO, USA).

Antibodies against calnexin (1:2000), golgin-97, and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anticleaved caspase-3 (Asp175) and antiprohibin antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA) and Lab Vision Products Thermo Fisher (Fremont, CA, USA). Antimicrotubule-associated protein 1 light chain 3 antibody (LC3, 1:1000, Code No. PM036) was obtained from MBL International Corporation (Nagoya, Japan). Rat monoclonal anti-mouse Ki-67 antibody (clone TEC-3) was obtained from Dako (Carpinteria, CA, USA). Mouse monoclonal antisera raised against smooth muscle calponin (C2687, clone hCP) and a β-actin antibody were obtained from Sigma-Aldrich (Oakville, ON, Canada). Calponin was used as a standard control because calponin expression levels are consistent throughout pregnancy [13,14].

Animals

Female Imprinting Control Region mice (ICR mice) (8 weeks old) were mated with male ICR mice. The different stages of the estrous cycle in the mice were determined by cytologic examination of vaginal smears. Day 1 of gestation was designated as the day a vaginal plug was observed. The average time to delivery under these conditions was approximately 3 weeks. Term was on Day 21. The mice were maintained in a specific pathogen-free animal care facility. The experimental protocol adhered to the rules of the Animal Protection Act of Taiwan and was approved by the Laboratory Animal Care and Use Committee of National Cheng Kung University, Tainan, Taiwan.

Tissue collection and preparation

The mice were sacrificed and the uterine myometrial samples were collected on gestational Day 0 (nonpregnant), Day 1, Day 2, Day 7, Day 14, and Day 21 as well as on postpartum Day 1, Day 2, and Day 7. On each day of gestation, tissue was collected from four different mice and processed separately ($n = 4$). Because we examined mice segments of the uterine horn and one mouse uterus has 8–10 segments, we sacrificed only four mice in each group. To determine the myometrial weight of each segment of the uterine horn (1 uterine segment contains 1 mouse fetus, as shown between the red arrows in Fig. 1A), the segments of uterine horns from different gestational days and postpartum days were cut. The endometrium, mouse fetus, and placenta in the fertilized uterus were carefully removed by scraping the luminal surface of the uterus using a scalpel blade under a dissecting microscope (Nikon SMZ-2T, Nikon Corp, Tokyo, Japan). For protein extraction, the uterine horns were placed into ice-cold phosphate buffered saline (PBS) and bisected longitudinally. After removing the endometrium, fetus, and placenta, the remaining mouse myometrial tissues were immediately snap frozen in liquid nitrogen and stored at −80°C for subsequent protein analyses. For immunohistochemical studies, the intact uterine horns were cross-sectioned, cut into segments using a scalpel blade, and placed in formalin for fixation and embedded in paraffin. For the human uterine samples, three archival formalin-fixed, paraffin-embedded uterine specimens were retrieved from the Department of Pathology, Chi Mei Medical Center, Liouying, Tainan, Taiwan. The myometrial tissue was obtained either at the term, during a scheduled cesarean section or from samples obtained during postpartum hysterectomies. The sampled patients received a hysterectomy 1 day after delivery because of uncontrolled postpartum uterine atony. A histopathologic examination of the samples indicated a normal myometrium.

Immunohistochemistry

Immunohistochemistry was performed as previously reported with some modifications [15]. Briefly, 4-μm-thick sections were cut sequentially from the archival specimens, dewaxed with xylene, and rehydrated through the use of graded alcohol. After blocking endogenous peroxidase activity, the sections were subjected to heat-induced antigen retrieval using an autoclave and then incubated with Ki-67 antibodies at 4°C overnight. The LC3 immunohistochemical detection procedure was based on a protocol that