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Iron Metabolism-Related Genes and Mitochondrial Genes Are Induced during Involution of Mouse Mammary Gland

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To understand molecular mechanisms of mammary gland involution, several clones were isolated after the primary differential screening of a total 40,000 pfu of involution-specific cDNA library, and further characterized. The partial sequences and Northern analysis revealed that iron metalbolism-related genes and mitochondrial genes were induced during mammary gland involution. The expression of the lactoferrin gene was induced at involution days 1, 2, and 3. The expression of ferritin heavy chain gene was induced at involution days 1, 2, 3 and 4. Cytochrome oxidase subunit 1 and cytochrome oxidase subunit 2 genes were induced at involution days 4 and 7. The expression of cytochrome b gene was induced at involution day 7. These results imply that iron metabolism and mitochondrial function may be altered during mammary gland involution.

Apoptosis or programmed cell death is of fundamental importance to biological processes ranging from embryogenesis to the development of several systems in organisms. The mammary glands is a unique tissue with a developmental potential after birth, since following the onset of pregnancy, epithelial cells in the mammary gland proliferate and differentiate into milk-secreting cells during lactation. Involution of the lactating mammary gland following weaning is accompanied by a reduction in the number of epithelial cells. Mammary glands can repeat this cycle periodically between the pregnant and the nonpregnant stage. Therefore, mammary glands are an excellent model organ for researching natural cell involution events.

After completion of lactation, the mammary gland undergoes involution, regressing to a state resembling that of a virgin animal. This phase of mammary gland development is characterized by dramatic epithelial cell death and tissue remodelling. The previous studies suggest that the involution of mammary gland requires active gene expression. In other studies including us, the expression of stromelysin, sulfated glycoprotein-2, Fas antigen, and interleukin-1 β converting enzyme was induced during involution of the mammary gland (1, 2, 3). But, many questions for molecular mechanisms by which regulate mammary epithelial cell death remain unanswered. In the present studies, involution-induced cDNAs from mouse mammary gland were cloned and characterized. Our results demonstrate that genes involved in iron metabolism and mitochondrial function are induced during mammary gland involution.

MATERIALS AND METHODS

Animals. Tissues obtained from ICR mice were used in all experiments. For the induction of involution, the young were removed 10 days after parturition, and the mammary tissues were obtained at the indicated time after weaning.

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Abbreviations: PCR, polymerase chain reaction. CO1, cytochrome oxidase subunit 1. CO2, cytochrome oxidase subunit 2. Cb, cytochrome b.

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cDNA library construction. Total RNA was extracted by the acid/guanidinium thiocyanate/phenol chloroform method (4). For construction of involution-specific cDNA library, poly A RNA was extracted from mammary gland at 6, 12, 24, 36, 48, 60, 72, and 96 hours after weaning. The first strand cDNA was synthesized using the pooled poly A RNA, Xho1-$(dT)_{15}$ primer and AMV reverse transcriptase. The second strand cDNA was synthesized using RNaseH, DNA polymerase and DNA ligase. The EcoRI adapted cDNAs were ligated to the λZAP II vector, and the recombinant vectors were subjected to *in vitro* packaging, and transfected into the XLI-Blue cells. The unamplified titers were 1.3×10^6 plaque-forming units per milliliter (pfu/ml). The clear plaques were estimated as 90%. The insert size distribution of the library were determined by polymerase chain reaction (PCR) using ZAP insert screening amplifiers (Clontech, USA). The insert size range of 14 plaques was 0.5∼2.8 kb.

Differential screening. For the primary screening, the library was plated with *E. coli* XL1-Blue cells in a low density (about 2,500 pfu/150 mm plate). Following an 8h incubation at 37℃, the plates were cooled at 4℃ for 2h. Phage DNA from each plate was transferred onto the nylon membranes in duplicate with the first filter for 1 min and the second filter for 10 min. DNA on the filters was denatured in 0.5 N NaOH, 1.5 M NaCl for 3 min, neutralized in 0.5 M Tris-HCl (pH 8.0), 1.5 M NaCl for 3 min and fixed onto the filters by baking at 80℃ for 2h. The membranes were prehybridized at 68℃ for 2h in a solution containing 6 × SSC, 10 mM EDTA, 0.5% SDS, 100 ug/ml of salmon sperm DNA, 10% dextran sulfate, and poly A. The differential hybridization was carried out at 68℃ for overnight with [^{32}P] labeled cDNA probe prepared from mRNAs of lactating tissues and of involuted tissues, respectively. The [^{32}P] labeled first-strand cDNA probes were synthesized using 3 ug of poly A RNA, 0.5 ug of oligo (dT) primer, 45 U AMV reverse transcriptase, and α-[^{32}P] dCTP. The membranes were washed twice in 2× SSC/0.1% SDS at room temperature for 15 min, twice in 2× SSC/0.1% SDS at 42℃ for 30 min, and twice in 0.1 × SSC/0.1% SDS at 65℃ for 30 min. After comparing signals from the two films, the involution-induced positive plaques were identified and picked with sterile pipet tips and stored at 4℃ in 200 ul of phage dilution buffer with 2% chloroform. Second differential screening was also performed as described above.

PCR/Southern differential screening. Twenty microliters of positive phage plaques obtained from the primary screening were subjected to two cycles of freezing-thawing treatment, and the released lambda DNA was used as templates for PCR. The primers used for PCR were T3 and T7 primers corresponding to the T3 and T7 promoters in lambda ZAP II vector. After denaturation for 5 min at 95℃, PCR was performed for 1 min at 95℃, 1.5 min at 60℃, and 2 min at 72℃ for 30 cycles. Twenty microliters of each of the PCR products were run on 1% agarose gels in duplicate. The gels were denatured in 0.5 N NaOH, 1.5 M NaCl for 30 min, neutralized in 0.5 M Tris-HCl (pH 8.0), 1.5 M NaCl for 30 min, and DNAs in the gels were transferred onto two membranes by capillary reaction. Differential hybridization was carried out as described above in the primary screening. After comparing signals from the two membranes, induced clones were identified, selected, and proceeded for further analyses.

Northern analysis. Twenty micrograms of total RNA were electrophoresed on an 1% agarose gel containing formaldehyde, and blotted onto a membrane. The lambda DNA containing the cDNA insert was converted into the phagemid, pBluescript, by Lambda ZAP II Automatic Excision Process (Stratagene, USA). The plasmid was digested with EcoR I and Xho I, and the insert was obtained after low melting agarose gel electrophoresis. The insert of cDNA clone was labeled using a Prime-It Random Primer Labeling Kit (Stratagene). The membrane was hybridized with the labeled insert of the indicated cDNA clone. The equal amount of RNA loading was confirmed by the intensities of 28S and 18S band (data not shown), and the efficiency of transfer were monitored by ethidium bromide staining.

Sequencing. Partial sequencing of clones was performed by the dideoxy nucleotide chain-termination method using [^{35}S] dATP, T3 primer, and the Sequenase DNA Sequencing Kit (USB, USA). The sequences of each clone were compared to the sequence data of GenBank in NIH, USA.

RESULTS AND DISCUSSION

To isolate involution-induced genes, the directional cDNA library was constructed in the λZAP II vector using pooled poly A RNA extracted from mammary gland at 6, 12, 24, 36, 48, 60, 72, and 96 hours after weaning. Differential screeing method was used to select involution-induced clones. For the primary screening, the library was plated in a low density (about 2,500 pfu/150 mm plate). After the primary screening of a total 40,000 pfu of cDNA library, 200 positive phage candidates were isolated. Secondary differential screening was performed for some plaques. Twenty clones were rescreened using a PCR/Southern differential hybridization, and ten clones were further characterized by Northern analysis and partial sequencing. The sequences of each clone were compared with the GenBank database. Seven known genes were induced in the process of mammary gland involution: the apoptosis-associated gene sulfated glycoprotein-2 (2), the WDNM1 gene, a member of a family of protease inhibitor (5), and three mitochondrial genes and two iron metabolism-related genes reported in this paper. Three unknown genes are under characterization.

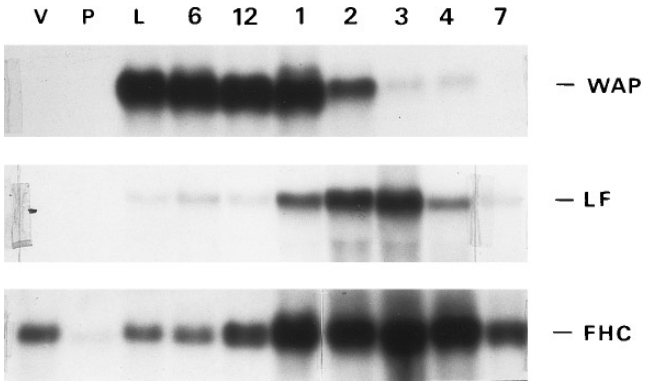

FIG. 1. Northern analysis of the mammary involution-induced genes. The 20 ug of total RNA isolated at virgin (V), pregnant day 10 (P), lactating day 10 (L), and involution 6h, 12h, 1, 2, 3, 4, 7 days of mouse mammary gland were separated on a 1% formaldehyde/agarose gel and transferred onto the membrane. The blot was hybridized with the indicated [^{32}P] labeled clone. WAP, whey acidic protein; LF, lactoferrin; FHC, ferritin heavy chain.

Genes involved in iron metabolism were induced during mammary gland involution. The expression levels of lactoferrin were low in virgin, pregnant, and lactating mammary tissues (Fig. 1). The expression of lactoferrin was induced at involution day 1, reached the peak at involution day 3, then reduced after involution day 4. The expression of the ferritin heavy chain gene was induced at involution days 1, 2, 3, and 4 (Fig. 1). The expression of whey acidic protein RNA, milk protein gene, was induced at lactation period, maintained until involution day 2, but dramatically reduced after involution day 3. Although the precise functional role of the genes in mammary gland involution presently remains unclear, it is possible that iron metabolism is altered during mammary involution. Lactoferrin function as a factor of resistance to infection of the mammary gland (6). The increase in lactoferrin levels may contribute to prevent microbial infection during mammary involution at which stage the residual milk in the mammary gland provides good nutritional sources for microbial growth. Lactoferrin can also exert anti-inflammatory properties by inhibition of formation of toxic oxygen radicals (7). Ferritin heavy chain mRNA levels were increased by oxidative damage in arsenate-exposed HeLa cells (8). It has been suggested that ferritin protects cells from oxygen stress (9). Recently, several observations suggest that oxidative mechanisms are involved in the induction of apoptosis (10, 11). In the present study, it is not clear whether oxygen radicals are generated as a consequence of cell death during involution and that these induce expression of the iron metabolism-related genes. Alternatively, it is also possible that oxidative stress is a result of the induction of the genes by effective trapping of ferrous ion. Additional studies will be required to delineate the cause for the induction of the genes and to understand functional role of the gene products in the involution processes of mammary gland.

The induction of mitochondrial genes involved in electron transport and respiratory chain was observed. As shown in Fig. 2, the basal levels of cytochrome oxidase subunit 1 (CO1) and cytochrome oxidase subunit 2 (CO2) mRNAs were detected in virgin- and lactating-mammary glands. This low levels of CO1 and CO2 mRNAs were maintained from involution 6h through involution day 3. The expression of CO1 and CO2 genes was induced at involution days 4 and 7. The cytochrome b (Cb) gene was expressed at the basal levels in virgin-, lactating-, and involuted- mammary glands from involution 6h through involution day 4. The expression of Cb gene was induced at involution day 7. Recent studies suggest that mitochondrial function and respiratory chain are related to apoptosis (12, 13, 14). It is possible that mitochondrial genes are upregulated to reduce the reactive oxygen species generated by mam-

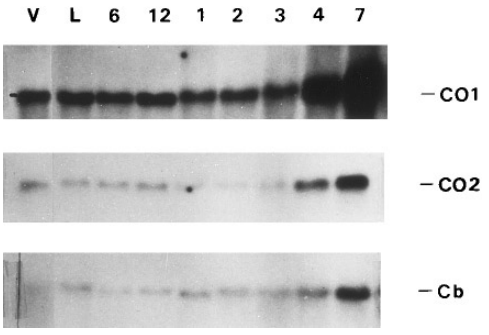

FIG. 2. Northern analysis of mitochondrial genes. The 20 ug of total RNA isolated at virgin (V), lactating day 10 (L) and involution 6h, 12h, 1, 2, 3, 4, 7 days of mouse mammary gland were separated on a 1% formaldehyde/agarose gel and transferred onto the membrane. The blot was hybridized with the indicated [^{32}P] labeled clone. CO1; cytochrome oxidase subunit 1, CO2; cytochrome oxidase subunit 2, Cb; cytochrome b.

mary epithelial cell death. Another possibility is that the induction of the genes may represent changes in mitochondrial energy metabolism.

The lactoferrin was expressed only in the mammary gland (Fig. 3). It was not expressed in thymus, spleen, uterus, ovary, liver, brain, heart, lung, kidney, testis, and mammary gland of male. The ferritin heavy chain was highly expressed in the involuted mammary tissues, whereas it was expressed at a moderate levels in kidney, heart, uterus, and lung (Fig. 3). In mammary gland and uterus, mouse lactoferrin is hormonally regulated (15), but its regulation of expression in mammary gland is distinct from the uterus. In the uterus and vagina, the ovarian sex steroid, 17β-estradiol, is the inducer (15). Lactoferrin gene is regulated by estrogen through an estrogen-responsive DNA element in the uterus (16). In this study, lactoferrin gene was induced at the involution stages of mammary gland in which prolactin levels are decreased. But, lactoferrin gene was not induced in the mammary gland during pregnancy (Fig. 3) at which stage high levels of estrogen and progesterone are produced. The expression of ferritin heavy chain in the mammary gland is also quite distinct from the uterus. The expression of the ferritin heavy chain mRNA in the uterus rose dramatically at the onset of pregnancy with the surge of progesterone (17). They suggest that ferritin heavy chain is a progesterone-inducible marker in the uterus during pregnancy. But, ferritin heavy chain gene was not induced in the mammary gland during pregnancy (Fig. 1). Different regulation mechanisms of lactoferrin and ferritin gene expression between mammary gland and uterus should be studied in the future.

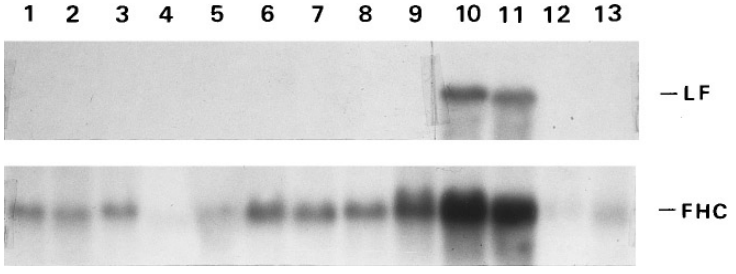

FIG. 3. Tissue-specific expression of lactoferrin (LF) and ferritin heavy chain (FHC) gene. The 20 ug of total RNA isolated from thymus (lane 1), spleen (lane 2), uterus (lane 3), ovary (lane 4), liver (lane 5), brain (lane 6), heart (lane 7), lung (lane 8), kidney (lane 9), mammary gland at involution day 2 (lanes 10 and 11) of female mouse and from testis (lane 12) and mammary gland (lane 13) of male mouse were separated on a 1% formaldehyde/agarose gel and transferred onto the membrane. The blot was hybridized with the indicated [^{32}P] labeled clone.

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