Novel Development of Mammary Glands in the Nursing Transgenic Mouse Ubiquitously Expressing WAP Gene

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Abstract: Although whey acidic protein (WAP) has been suggested to have some biological functions, its true function has not yet been clearly elucidated. We have generated transgenic mice ubiquitously and highly expressing the WAP gene. The pups born from one female among these transgenic mice showed low growth or died during nursing. This transgenic founder showed novel development of the mammary glands, and demonstrated normal parturition and nursing behavior. The mammary glands showed low-distended ductal structures, and poor development of lobulo-alveolar and acinous formations despite normal nursing, while mammary ducts were rather large in comparison with those of normal lactating females. Although this founder was found to be mosaic for transgenesis, it was shown to be a useful animal model for investigating WAP function.

Key words: mammary gland, transgenic mouse, WAP

Whey acidic protein (WAP) has been found in the milk of a limited number of species of rodents (mice [1, 2, 3] and rats [3]), rabbits [4], and camels [5]. Recently, WAP was also isolated from the milk of pig [6, 7] and wallaby [8]. The expression of the WAP gene in epithelial cells of the mammary gland is regulated by a variety of lactogenic hormones, including insulin, glucocorticoid, and prolactin [9, 10]. Therefore, WAP promoter has been utilized to produce pharmaceutical proteins in the milk of farm animals [11, 12]. WAP proteins have a four-disulfide-core (4-DSC) domain, which is comprised of eight cysteine residues in a conserved arrangement [13]. Various proteins containing 4-DSC domains have been identified as protease inhibitors [14–17]. It was therefore postulated that the 4-DSC may be related to the action of a particular class of protease inhibitors [18, 19].

Wen et al. [20] reported that the endogenous WAP gene is expressed in various tissues of the lactating mouse, although the expression levels were rather low among various tissues. It was also shown that the early production of WAP in mammary glands of WAP/WAP transgenic mice disrupts the timing of gene activation, leading to premature termination of the differentiation process [21]. These findings led us to wonder whether WAP might possess some biological functions in vivo. To date, few studies have investigated the functions of WAP. To obtain insight into the biological functions
of WAP in vivo, we generated transgenic mice ubiquitously expressing exogenous mouse WAP gene under the control of the ubiquitously active cytomegalovirus immediate early enhancer-chicken β-actin hybrid (CAG) promoter [22]. Here, we report the development of unique mammary glands in a nursing transgenic female.

Animals: Mice of the ICR and BDF1(C57BL/6J × DBA/2) strains were purchased from a local dealer (SLC Co., Shizuoka-ken, Japan) and were housed under regulated temperature (22–25°C), humidity (40–60%), and illumination cycles (14 h light, 10 h dark), and food and water were supplied ad libitum. The experiments were conducted according to the guidelines for the care and use of laboratory animals, College of Agriculture, the University of Tokyo.

Construction of CAG/β-WAP: Total RNA was extracted from mammary glands of C57BL/6J female mice in late pregnancy by TRIZOL Reagent (GIBCO BRL, NY). An aliquot corresponding to 2 μg of the total RNA was subjected to denaturation at 65°C for 10 min, then, the RNA was reverse-transcribed in a reaction mixture (total volume of 20 μl) containing 2.5 mM dNTP, 5 μM random hexamer, 0.1 M DTT, 20 U RNase inhibitor, and 200 U reverse transcriptase (Super Script II, GIBCO BRL, NY). A thermocycler program (1 min at 94°C, 1 min at 58°C, 1 min at 72°C) was run on a thermocycler. PCR was performed in a 50 μl reaction mixture containing 2 μl of the sample cDNA solution, 2.5 mM dNTP, PCR buffer, 1 μM of primers, and 0.5 U Taq polymerase (TAKARA Shuzo Co., Tsu, Japan). The primers used for this PCR reaction were 5'-AGT-GGT-TGC-CTC-ATC-AGC-C-3' (5' primers) and 5'-GAC-AGG-CAG-GGA-TGG-C-3' (3' primers). A 434 bp fragment of WAP cDNA (V00856) was subcloned to pGEM-T Easy vector (Promega Co., Madison, WI). The Eco RI/Eco RI DNA fragment (452 bp) was separated from the vector and subcloned to the pCX vector. The pCX-WAP plasmid contained CMV enhancer, chicken β-actin promoter with splicing sequences, WAP cDNA, and the rabbit beta-globin 3' flanking sequences (Fig. 1). The Ssp I Bam HI DNA fragment (2.9 kb) was excised from the vector and separated by electrophoresis using a 1% agarose gel, then purified by CsCl ultra-centrifugation according to the method of Hogan et al. [23]. The purified 2.9 kb DNA fragment (Fig. 1) was dissolved in a solution containing 10 mM Tris-HCl (pH 7.4) and 0.25 mM EDTA (pH 7.4), then used for pronuclear microinjection.

Production and screening of CAG/β-WAP transgenic mice: Transgenic mice were produced by the DNA microinjection procedure according to the standard protocol [23]. In brief, fertilized one-cell embryos were collected from the oviducts of superovulated BDF1 females that had been mated to males. The purified DNA fragment (approximately 2,000 copies) was microinjected into the pronuclei of zygotes in a microdrop of M2 medium (Sigma, St. Louis, MO). The injected embryos were cultured in a microdrop of M16 medium (Sigma, St. Louis, MO) in a humidified atmosphere of 5% CO2 and 95% air at 37°C until embryo transfer. The surviving embryos were transferred into the oviducts of pseudopregnant ICR females prepared by mating to vasectomy adult males of the same strain. Genomic DNA of the pups born was prepared from the tips of tails according to the standard protocol [23]. The pups were screened for transgenesis by Southern blotting analysis as follows. The extracted DNA (10 μg) was digested with Eco RI, subjected to agarose gel electrophoresis, blotted to nylon membrane (Pall Gelman Sciences, Ann Arbor, MI, USA), and hybridized with the 32P-labeled 452 bp fragments of mouse WAP cDNA.

Preparation of whale mount species of mammary
glands: The third thoracic mammary glands were removed from the transgenic and the control mice. Each mammary gland was dissected from the inner surface of the skin, retaining some of the peripheral connective tissues, spread on a cork board, and fixed in freshly prepared Bouin’s solution. The glands were then defatted overnight in acetone. The whole mounts were stained with Mayer’s hematoxylin, embedded in Canadian Balsam, and photographed.

Growth of offspring of transgenic founder: Six (4 females and 2 males) of forty-two pups born were found to be transgenic mice by Southern blotting analysis of tail DNAs (Fig. 2). The transgenesis in pups was also confirmed by PCR using the primers spanning WAP cDNA (data not shown). In the process of producing the offspring of these founder mice, we noticed poor growth of the pups born from one transgenic female (the $\#\varphi 2$ founder) at 10 days after birth. We measured the body weight of the pups being nursed by her, and observed that the growth rate of the pups was significantly lower than that of the pups from normal females (data not shown). In her second parturition, all of the pups born and nursed by her died within 3 days after birth. In her third parturition, we exchanged her pups for those born from a normal female at 3 days after birth. All of the 7 pups born from the normal female showed significantly retarded growth after being nursed by the transgenic founder, and died 3 days after the exchange (Fig. 3).

Mammary glands of the $\#\varphi 2$ founder: We sacrificed the founder, collected tissue samples, and prepared whole-mount specimen of her mammary glands. This nursing founder female showed a high level expression of the exogenous WAP transgene in various tissues, including the mammary gland, the brain, the skeletal muscle, and the kidney, but not in the liver, among those analyzed (Fig. 4). In later experiments, one other transgenic mouse, a male, was found to express the transgene only in the brain, and the 4 other transgenic mice did not express the transgene in any of the tissues analyzed. The mammary glands of this female founder showed very unique development. The typical figures of the mammary glands with poor development are
shown in Fig. 5 C and D; they had low-distended ductal structures, and poor development of the lobulo-alveolar and acinous formation for the middle lactating period. It is interesting that the mammary ducts of this founder were much larger in size than those of normal lactating females. Histological features of the mammary glands of the #2 founder suggested that her synthesis of milk proteins was inadequate, causing low growth or death of her pups.

It is noteworthy that this founder showed no abnormality in physiological functions other than novel development of the mammary glands. Despite high expression of the exogenous WAP gene in various tissues, she grew normally, and showed normal parturition and nursing behavior. The present result may help to explain our previous observation of no histological or physiological changes in transgenic mice in which WAP gene was overexpressed in the liver under the control of the metallothionein-I gene promoter [24]. Although WAP has been isolated in the milk of a limited number of mammalian species [1-8], the isolation of WAP from the milk of the wallaby [8] indicates that the synthesis and secretion of WAP is widespread in many mammalian species. A variety of mucosal proteins that comprise one or multiple copies of a 4-DSC domain have shown protease inhibitor functions [14, 25, 26]. Robinson et al. [21] reported that the early production of WAP in WAP/WAP mammary glands disrupts the timing of gene activation, leading to premature termination of the differentiation process. The present results indicate that early and forced expression of WAP in mammary glands caused novel development of the mammary glands. Simpson et al. [6] suggested that WAP is possibly glycosylated, based on the fact that the molecular size of porcine WAP in SDS-PAGE was greater than that determined from amino acid sequences. These findings, taken with our present results, clearly reveal that WAP plays an important role in development of the mammary glands of female mice in a tissue-specific fashion throughout lactation. Based on
the limited sequence identity of WAP with known protease inhibitors, it has been thought that WAP may be a protease inhibitor [19].

Although the present founder female showed interesting phenotypes by which to conjecture the biological function of WAP, this mouse was unfortunately found to be mosaic for transgenesis, based on the finding that no transgenic mice were detected among her 19 offspring. This may explain the absence of expression of the transgene in the liver of the founder female, as shown in Fig. 4. Therefore, we were unable to obtain further detailed data from this transgenic mouse line. We are now generating more transgenic mice with high ubiquitous expression of exogenous WAP gene in order to investigate their phenotypes in detail.

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References