Recombinant Derp5 allergen with α_{S1} -casein signal peptide secreted in murine milk protects against dust mite allergen–induced airway inflammation

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ABSTRACT

Recent advances in recombinant technology make transgenic animals that produce pharmaceutical proteins in their milk more feasible. The group 5 allergen isolated from *Dermatophagoides pteronyssinus* (Derp5) is one of the most important dust mite allergens in humans. The aims of this study were to develop transgenic mice that could secrete recombinant Derp5-containing milk and to demonstrate that ingesting recombinant milk protects against allergic airway inflammation. Two transgenes were constructed separately. The α -LA-Derp5f transgene consisted of the bovine α -lactalbumin (α -LA) promoter and full-length Derp5 cDNA. The α -LA-CN-Derp5t transgene included the α -LA promoter, a leader sequence of α_{S1} -casein (CN), and signal peptide-truncated Derp5 cDNA. Both species of transgenic mice were confirmed to have successful transgene integration and stable germline transmission. Western blot analysis of the milk obtained from the offspring of transgenic mice demonstrated that recombinant Derp5 was secreted successfully in the milk of α -LA-CN-Derp5t transgenic mice but not in that of α -LA-Derp5f transgenic mice. This study provides new evidence that transgenic mice can secrete recombinant Derp5 efficiently in milk by adding a signal peptide of α_{S1} -casein. The antigenic activity of recombinant Derp5 milk was demonstrated to have a protective effect against allergic airway inflammation in a murine model in which the ingestion of recombinant Derp5-containing milk was used as pretreatment.

Key words: *Dermatophagoides pteronyssinus*, Derp5 allergen, transgenic mice, signal peptide, allergic airway inflammation

INTRODUCTION

The house dust mite, a common indoor allergen, has been considered an important risk factor associated with asthma attack in the domestic environment (Bateman et al., 2008). *Dermatophagoides pteronyssinus* (Derp) is the predominant species of house dust mite in areas of Taiwan, Australia, and Western Europe (WHO, 1988). Although elevated serum IgE against group 1 and 2 allergens isolated from *D. pteronyssinus* (Derp1 and Derp2) are common among individuals with allergies (van der Zee et al., 1988), numerous studies have indicated that group 5 allergens from *D. pteronyssinus* (Derp5) are another important group of dust mite allergens in humans (Lynch et al., 1997; Thomas et al., 2002; Weghofer et al., 2008). In a study by Lin et al. (1994), recombinant Derp5 peptide expressed in a pGEX vector system was demonstrated to have strong reactivity with serum IgE in over 50% of asthmatic patients. The Derp1 peptide has been shown to have cysteine protease activity (Simpson et al., 1989), but the actual functions of Derp2 and Derp5 peptides in dust mites are not clearly understood. In a study by Weghofer et al. (2008), Derp5 was demonstrated to be a product secreted from gut epithelia of *D. pteronyssinus*. This finding reveals the importance of Derp5 allergen studies because house dust mites are pervasive and could excrete this allergen into the domestic environment via their feces.

Allergen-specific immunotherapy (ASIT) is the process of repeatedly administering a relevant allergen to induce immune tolerance in patients with allergic diseases. In past decades, many modalities of ASIT were widely investigated and developed for patients with allergic rhinitis or asthma. Recent advances in the development of immunotherapy regimens and engineering technology have improved the specificity and efficacy of ASIT (Ring and Gutermuth, 2011; Calderón et al., 2012). Recombinant allergen has the advantages

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of convenience and a high productive yield compared with traditional allergen extracts. Advances in biotechnology have also made milk-producing animals excellent bioreactors for producing recombinant proteins in their mammary glands (Houdebine, 2000). Recombinant proteins from the mammary glands of transgenic animals have the advantage of proper posttranslational modifications, and these proteins can be conveniently collected and purified. The transgenic animal systems used to produce pharmaceutical proteins in milk have improved technically, and their clinical applications have become more prevalent (Houdebine, 2009). Some researchers have attempted to engineer dust mite allergen in bioreactor platforms such as bacteria and plants (Lin et al., 1994; Hsu et al., 2004), but production of recombinant dust mite allergen from milk of transgenic animals has not been demonstrated previously.

The aim of this study was to construct an optimal transgene and produce transgenic mice that can express and secrete recombinant Derp5 into their milk. We evaluated the pretreatment efficacy of ingestion of this recombinant Derp5-containing milk in a murine model of allergic airway inflammation.

MATERIALS AND METHODS

Construction of Derp5 Transgenes

A full-length Derp5 cDNA (396 bp) encoding a 19-AA signal peptide and a 113-AA functional peptide with a molecular weight of 14 kDa was a gift from C. H. Hsu at Chinese Medicine University, Taiwan (Hsu et al., 2004). To compare the efficiency of different signal peptides for Derp5 peptide secretion from mammary glands of transgenic mice, 2 DNA constructs were

engineered, each of which carried a 2.0-kb regulatory sequence of the bovine α -LA gene and either the full-length Derp5 (*Derp5f*) or the signal peptide-truncated Derp5 (*Derp5t*) cDNA sequences.

The *Derp5* cDNA containing the original leader sequence from the pGEM7 plasmid was used to generate an intact *Derp5* coding sequence by PCR amplification using the primer set of pDerp5-1(+) and pDerp5-1(-) (Table 1). After PCR amplification, the products were digested with *XhoI* (cutting sites are underlined in the primer sequences shown in Table 1) and then ligated into the α -LA/pCR3 vector (Wu et al., 2007). A linear transgene was obtained through double digestion of the α -LA-Derp5f/pCR3 vector with *Bam*HI and *Bbs*I (New England Biolabs Inc., Ipswich, MA). The 3.1-kb transgene fragment consisted of the α -LA promoter (2.0 kb), the *Derp5* cDNA encoding a 19-AA signal peptide and a mature Derp5 peptide (*Derp5f*, 0.6 kb), and the bovine growth hormone (**bGH**) polyadenylation (**poly-A**) signal sequence (0.5 kb; Figure 1A).

To construct the α LA-CN-Derp5t transgene, the 19-AA signal peptide of *Derp5* cDNA was replaced with a 15-AA signal peptide from α S1-CN. The leader sequence of α S1-CN was amplified by PCR using the primer set of p α S1-CN(+) and p α S1-CN(-) (Table 1). The PCR products were inserted into an α -LA promoter-containing pGEM-T plasmid by digestion with *Sac*II and *Hpa*I. Then, the α -LA-CN sequence was ligated into the pCR3 vector via digestion with *Not*I. The constructed α -LA-CN/pCR3 vector was created for mammary gland-specific expression (Tung et al., 2011). The truncated *Derp5* cDNA in the pGEM-T plasmid (Promega, Madison, WI) was used to generate a truncated *Derp5* coding sequence that did not contain the original leader sequence via PCR amplification using the primer

Table 1. Oligonucleotide primer sets used to construct, detect, and analyze the α -LA-Derp5f and α -LA-Derp5t transgenes (containing the group 5 allergen isolated from *Dermatophagoides pteronyssinus*) in this study

Primer set	Oligonucleotide sequence (5' to 3') ¹	Melting temperature (°C)	PCR product size (bp)
α -LA-Derp5 transgene junction			
p α LA-124(+)	CTCTCTTGTTCATCCTCTTCC	47	480
pDerp5-213(-)	GAAATCTTGCGTCCACAGGC	49	
Bovine α -LA regulatory region			
p α LA-F(+)	CCTGACGCGTAGAATCGATTTCATGT	53	1,956
p α LA-R(-)	GGTTACGCGTCAAGATTCTGA	48	
p α S1-CN(+)	ACCGCGGAGTCTTGGGTTCAAG	53	91
p α S1-CN(-)	TGTTAACTGGCAAGAGCAACAG	50	
Derp5 cDNA 3'-coding region			
pDerp5-1(+)	TCTCGAGCATGAAATTCATCATTGC	49	250
pDerp5-1(-)	TTCTCGAGTGAAGGCAACAAG	46	
pDerp5-2(+)	GTGTTAACTGAAGATAAAAAACATGA	51	250
pDerp5-2(-)	TGGTGATATTTGGAACCTGA	43	
Mouse GAPDH internal control			
pGAPDH(+)	TACAGCAACAGGGTGGTGCA	62	250
pGAPDH(-)	TGGTACATGACGAGGCAGGT	62	

¹Restriction enzyme (*Xho*I) cutting sites are underlined.

Figure 1. Schematic map of the constructed α -LA-Derp5f and α -LA-CN-Derp5t transgenes (containing the group 5 allergen isolated from *Dermatophagoides pteronyssinus*), sequencing results of transgenes, and detection of their integration patterns in transgenic mice. (A) The structure of α -LA-Derp5f transgene consisted of the bovine (b) α -LA promoter, the full-length *Derp5* cDNA (*Derp5f*), and the bovine growth hormone (bGH) polyadenylation (poly-A) signal. (B) The structure of α -LA-CN-Derp5t transgene consisted of the α -LA promoter, the α _{S1}-casein leader sequence (CN), the truncated *Derp5* cDNA (*Derp5t*), and the bGH poly-A signal. (C) The sequence of the α -LA-Derp5f transgene encoded an original 19-AA signal peptide and a mature Derp5 peptide. (D) The sequence of the α -LA-CN-Derp5t transgene encoded a 15-AA α _{S1}-casein signal peptide and a mature Derp5 peptide. (E) Southern blots showing the integration patterns of the transgene in α -LA-Derp5f transgenic founder mice. (F) Southern blots showing the integration patterns of the transgene in α -LA-CN-Derp5t transgenic founder mice.

set of pDerp5-2(+) and pDerp5-1(-) (Table 1). The amplified products were double digested with *Hpa*I and *Xho*I and then ligated into the α -LA-CN/pCR3 vector. The 3.1-kb transgene fragment, which consisted of the α -LA promoter (2.0 kb), the α _{S1}-CN leader sequence, the truncated *Derp5* cDNA (*Derp5t*; 0.6 kb), and the bGH poly-A signal sequence (0.5 kb), was obtained from the α -LA-CN-Derp5t/pCR3 vector with double digestion with *Bam*HI and *Bbs*I (Figure 1B).

Phylogenetic and Bioinformatics Analyses of Signal Peptides

The Basic Local Alignment Search Tool (BLAST; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to search signal peptide-containing proteins in *Arthropoda* species using the signal peptide of Derp5 as a query.

A total of 9 signal peptides similar to that of Derp5 in *Arthropoda* species (Derp5 SP group) were used for phylogenetic analysis with the 16 signal peptides of mammalian milk proteins, including 9 signal peptides of casein (Casein SP group) and 7 signal peptides of lactoferrin, α -LA, and early lactation protein. The sequences of the signal peptides were aligned by ClustalW (Larkin et al., 2007) and adjusted manually. The phylogenetic tree was constructed by using the unweighted pair-group method with arithmetic mean (UPGMA) and Dayhoff model using MEGA6 software (www.megasoftware.net/).

The conservation of the signal peptides in Casein SP group and Derp5 SP group phylogenies was created by the WebLogo program (<http://weblogo.berkeley.edu>). The overall height of the stack indicates the sequence conservation at each position, whereas the height of

symbols within the stack indicates the relative frequency of each AA at that position, and the AA were colored according to their chemical properties: green for polar AA (S, T, Y, C), purple for polar uncharged AA (Q, N), blue for basic AA (K, R, H), red for acidic AA (D, E), and black for hydrophobic AA (A, V, L, I, P, W, F, M). The hydrophobicity of the AA in the signal peptides of Casein SP and Derp5 SP groups were calculated by Kyte-Doolittle method (Kyte and Doolittle, 1982) using ProtScale program (<http://web.expasy.org/protscale/>).

Purification of Transgenes and Production of Transgenic Mice

The in-frame sequences of the transgenes from the leader sequence through the Derp5 junction were determined using the Dye Terminator sequencing system (Applied Biosystems Inc., Foster City, CA). The transgene DNA was purified by CsCl₂ gradient ultracentrifugation, and transgenic mice (CD-1 strain) were generated by pronuclear microinjection as described previously (Chen et al., 2008a,b). The animal trial in this study was approved by the Institutional Animal Care and Use Committee of National Chung Hsing University, Taiwan (IACUC No.98-52).

Detection of the Transgenes in Transgenic Mice

Tail DNA from the founder mice was rapidly screened for the Derp5 transgenes by PCR amplification. The PCR was performed using one set of primers, p α LA-124(+) and pDerp5-213(-) (Table 1), which defined a 480-bp (*Derp5f*) or a 472-bp (*Derp5t*) region spanning the α -LA promoter and *Derp5* cDNA junctional sequence. The PCR was performed as follows: 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s in a thermal cycler (AG-9600; AcuGen Systems, Lowell, MA). The PCR products were analyzed on a 1.5% agarose gel and detected by UV transillumination. Positive PCR screening results were further confirmed by Southern blot hybridization as described previously (Shen et al., 2012). Genomic DNA (10 μ g) was digested with *Xho*I at 37°C overnight, electrophoresed on a 0.8% agarose gel, and transferred to a Durose membrane (Stratagene, La Jolla, CA). A radiolabeled fragment of Derp5 cDNA was used as a probe to hybridize to the membrane, and the blots were subjected to autoradiography.

Reverse Transcription-PCR Analysis of Derp5 mRNA from Mammary Gland Tissues of Transgenic Mice

Total RNA from mammary gland tissues of female transgenic mice during lactation periods was extracted

using the RNazol B reagent (Tel-Test, Friendswood, TX). One microgram of total RNA was treated with DNase I and then extracted with phenol-chloroform. The RNA pellets were resuspended in diethylpyrocarbonate-treated water and used to synthesize first-strand cDNA with random primers and SuperScript reverse transcriptase (Gibco BRL, Gaithersburg, MD). The reverse transcription (RT) products were used for further PCR amplification. The PCR was performed for 35 cycles as previously described (Wen et al., 2013). The primers used included 2 pairs of *Derp5*-specific primers, pDerp5-1(+)/(-) and pDerp5-2(+)/(-), and a pair of *GAPDH*-specific primers, pGAPDH(+)/(-) (Table 1). The *GAPDH* transcript was used as an internal control for RT-PCR.

Immunohistochemistry Assay of Recombinant Derp5 Expression in Mammary Gland Tissues of Lactating Transgenic Mice

Mammary gland tissues taken from both groups of transgenic mice (α -LA-Derp5f and α -LA-CN-Derp5t) during mid lactation (d 15) were fixed in paraformaldehyde as described previously (Yen et al., 2009). Tissue sections (5 μ m) of mammary glands were stained with hematoxylin and eosin (H&E) and photographed under a light microscope (Carl Zeiss, Jena, Germany). An immunohistochemical assay of recombinant Derp5 expression in these tissue sections was conducted as previously described (Liu et al., 2013). Briefly, rabbit anti-Derp5 polyclonal antibody was diluted 1:20 (1% BSA in PBS), and 50- μ L aliquots of anti-Derp5 antibody were incubated with the tissue slides for 30 min at 37°C. After washing with 10 volumes of BSA-PBS buffer, the mammary gland slides were incubated with goat anti-rabbit IgG antibody conjugated with fluorescein isothiocyanate (FITC; Boehringer-Mannheim, Mannheim, Germany) at a dilution of 1:100 (1% BSA in PBS) for 30 min at 37°C. After washing, these slides were observed under a Nikon Optiphot microscope (Nikon, Tokyo, Japan) equipped with epifluorescence optics.

Detection of Recombinant Derp5 Secretion in the Milk of Transgenic Mice

To identify the successful secretion of recombinant Derp5 in the milk, lactating female progeny from both groups of transgenic mice were injected with oxytocin (China Chemical & Pharmaceutical Co., Taipei, Taiwan), and the milk from 2 different transgenic mice was collected under anesthesia. Ten-fold dilutions of the milk samples collected during mid lactation (d 15) were subjected to 12% SDS-PAGE and stained with

Coomassie blue. All milk proteins from the transgenic mice were electrotransferred from the gel to a polyvinylidene difluoride membrane (MEN Life Science Products, Boston, MA). The expression of Derp5 was detected with a rabbit anti-Derp5 antibody (1:2,000 dilution) and an anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase (HRP; 1:10,000 dilution). The chemiluminescent ECL detection system (ECL, Amersham, UK) was used in the detection of these blots as previously described (Tung et al., 2014). The concentrations of recombinant Derp5 in the milk were analyzed using ELISA.

Pretreatment Effect of Recombinant Derp5-Containing Milk Ingestion in a Murine Model of Allergic Airway Inflammation

The protocol and protective use of Derp5-containing milk ingestion in a murine model of allergic airway inflammation were the same as those used in our previous study (Liu et al., 2013). Three-week-old CD-1 mice were divided into 3 experimental groups, as follows.

- (1) Group A: unsensitized mice fed wild-type (**WT**) milk (3.0 mL/kg of BW per day), which served as the normal control group; after being fed WT milk for 3 wk, these mice did not receive sensitization with Derp5.
- (2) Group B: sensitized mice fed recombinant Derp5 milk (adjusted to 1.0 mg/mL recombinant Derp5 concentration in milk; 3.0 mL/kg of BW per day), which served as the pretreatment group; after being fed recombinant Derp5-containing milk for 3 wk, these mice received sensitization with Derp5.
- (3) Group C: sensitized mice fed WT milk (3.0 mL/kg of BW per day); after being fed WT milk for 3 wk, these mice also received sensitization with Derp5.

The sensitizations were performed by intraperitoneal injection of 10 µg of purified Derp5 emulsified in 4 mg of Al(OH)₃ on both d 21 and 35. The Derp5 was produced and purified from the recombinant *Escherichia coli* system (Hsu et al., 1996), and Al(OH)₃ was used as an adjuvant to induce a T-helper 2 (Th2) response. On d 42, all mice received an allergen challenge by exposure to an aerosol of 0.1% purified Derp5 for 30 min to induce allergic airway inflammation. The animal trials were repeated for 2 independent experiments.

Analysis of Pulmonary Function

After aerosol challenge to induce allergic airway inflammation, the mice were placed into a baromet-

ric plethysmograph (Buxco Electronics, Troy, NY) to measure their pulmonary function as previously described (Liu et al., 2013). Maximal forced expiratory flow (**MFEF**) at 50% total lung capacity (**50% TLC**) was measured using different doses of acetylcholine, as described previously (Lin, 2001). The measurement of enhanced pause (Penh) values when mice were exposed to increasing doses of nebulized methylcholine was identified as airway hyper-responsiveness in these mice, as previously described (Hamelmann et al., 1997). The Penh values were calculated as means ± standard errors of the mean.

Analysis of Airway Inflammation by Histopathological Examination of Lung Tissues

After the pulmonary function test, these mice were killed for histopathological analysis of airway inflammation. Sections from the left lung of these mice were stained with hematoxylin and eosin and photographed under a light microscope. The degrees of alveolar hemorrhage, inflammatory cell infiltration, and destruction of tracheal epithelia in these 3 groups of mice were assessed and compared as previously described (Vilotte et al., 1989; Liu et al., 2013).

Statistical Analysis

To assess the different levels of airway hyper-responsiveness, repeated-measures ANOVA was performed. A value of $P < 0.05$ was used to indicate statistical significance.

RESULTS

Generation of α -LA-Derp5f Transgenic Mice

The 3.1-kb linear transgene fragment (Figure 1A), which consisted of the α -LA promoter (2.0 kb), a *Derp5* cDNA encoding a 19-AA signal peptide and a mature Derp5 peptide (Derp5f; 0.6 kb), and the bGH poly-A signal sequence (0.5 kb), was used for pronuclear embryo microinjection. Sequencing confirmed that the transgene included the leader sequence through the Derp5 junction (Figure 1C). Six newborn mice were identified as transgenic founder mice by positive PCR screening results. The integration patterns of the transgene in these mice were further analyzed by Southern blotting using *Xho*I restriction enzyme digestion. The results showed that all 6 transgenic founders had inverted repeats of transgene (6.0-kb product) and that 5 of 6 had direct repeats (3.1-kb product; Figure 1E). Some offspring of these transgenic founder mice were demonstrated to have stable germline transmission.

Generation of the α -LA-CN-Derp5t Transgenic Mice

To produce α -LA-CN-Derp5t transgenic mice, the following construct was used for pronuclear microinjection: a 3.1-kb transgene fragment (Figure 1B) consisting of the α -LA promoter (2.0 kb), the α_{S1} -CN leader sequence, the truncated *Derp5* cDNA (Derp5t; 0.6 kb), and the bGH poly-A signal sequence (0.5 kb). The sequence of transgene DNA from the leader sequence through the Derp5 junction was also demonstrated to be correct (Figure 1D). Seven newborn mice were identified as transgenic founder mice according to the positive PCR screening results. The integration patterns of the transgene in these mice were further analyzed by *Xho*I digestion and Southern blotting. The results demonstrated that all 7 transgenic founders had inverted repeats of transgene (6.0-kb product), and only 2 (#51 and #79) also had direct repeats (3.1-kb product) of transgenic integration (Figure 1F). Some offspring of these transgenic mice were also demonstrated to have stable germline transmission.

Expression and Secretion of Recombinant Derp5 in the Mammary Glands of Transgenic Mice

To identify the expression of *Derp5* mRNA transcripts, total tissue RNA was extracted from mammary gland tissues of α -LA-Derp5f and α -LA-CN-Derp5t transgenic mice during mid lactation (d 15). As shown in Figures 2A and 2B, the *Derp5* transcripts of the 250-bp RT-PCR products were found in mammary gland tissues from both α -LA-Derp5f and α -LA-CN-Derp5t female transgenic offspring. To further determine the cellular location of recombinant Derp5 synthesis in these transgenic mice, tissue sections of mammary glands were immunostained with anti-Derp5 primary antibodies. Immunohistochemical assay of the mammary gland sections from both groups of transgenic mice was performed by hematoxylin-eosin and immunohistochemistry staining. The results showed a significant accumulation of recombinant Derp5 (FITC signals) in mammary acini but not in the lumen of lactiferous tubules in α -LA-Derp5f transgenic mice (Figure 2C). However, we observed significant accumulation of recombinant Derp5 in both mammary acini and the lumen of lactiferous tubules in α -LA-CN-Derp5t transgenic mice (Figure 2D).

For the further detection of recombinant Derp5 secretion, milk collected from lactating female offspring of these 2 groups of transgenic mice was subjected to SDS-PAGE (Figures 2E and 2F, upper panel) and Western blot (Figures 2E and 2F, lower panel) analyses. The results showed that none of the 6 lines of offspring of α -LA-Derp5f transgenic mice could secrete recombinant

Derp5 into their milk. Furthermore, recombinant Derp5 was successfully expressed and secreted into the milk of the 7 lines of offspring of α -LA-CN-Derp5t transgenic mice after the Derp5 signal peptide was replaced by the α_{S1} -casein signal peptide.

Phylogenetic Analysis of Derp5 and Casein Signal Peptides

To identify the differences between Derp5 and casein signal peptides, Derp5 signal peptide and its 8 BLAST results in *Arthropoda* were aligned with the signal peptides of lactated proteins in mammals, and then the phylogenetic tree was constructed. Results showed that Derp5 signal peptide and similar peptides were clustered with the signal peptides of α -, β -, and κ -CN (casein SP group) and separated into 2 major groups: Derp5 SP group and casein SP group; the other milk protein signal peptides (including the phylogenies of lactoferrin, α -LA, and early lactation protein) were performed as out-groups of the phylogenetic tree (Figure 3A). To evaluate the differences between the Derp5 and casein signal peptides, the sequences of the signal peptides were analyzed by conservation and hydrophobicity of the AA at each position. Both the casein and Derp5 signal peptide groups showed 3 conservative regions: the N-terminal region (showed predominantly M-K or M-R AA), and middle and C-terminal regions (primarily composed of nonpolar AA and few polar AA; Figure 3B and 3D). Notably, 2 hydrophilic AA (T-C) were present only in the middle region of casein signal peptides (positions 10 and 11 in Figure 3B), which thus showed the lower hydrophobicity (~ 2) in the middle region compared with the higher hydrophobicity (> 2.5) of Derp5 signal peptides (Figure 3C and 3E).

Pretreatment with Recombinant Derp5-Containing Milk

Recombinant Derp5-containing milk samples were collected from different female offspring of α -LA-CN-Derp5t transgenic mice. The concentration of Derp5 in pooled recombinant milk was quantified by ELISA, and the milks were diluted and adjusted to a concentration of 1.0 mg/mL Derp5 before feeding. The feeding amount of milk was 3.0 mL/kg of BW per day. Thus, the pretreatment dose of recombinant Derp5 was estimated to be 60 μ g daily in sensitized mice fed recombinant Derp5 milk (group B). In our murine model of dust mite allergen-induced airway inflammation (Figure 4), histopathological analysis of airway inflammation was used to identify the effect of pretreatment involving the ingestion of recombinant Derp5-containing milk. The results showed that sensitized mice fed recombi-

Figure 2. Expression and secretion of recombinant Derp5 (the group 5 allergen isolated from *Dermatophagoides pteronyssinus*), in mammary glands and milk. (A) Total RNA was isolated from mammary gland tissues of α -LA-Derp5f transgenic mice offspring (#19, 24, 25, 12, 36, and 27) and wild-type mice (W-) during mid lactation (d 15); Mr = molecular weight marker. Derp5 transcripts were found in 6 lines of offspring by detecting the reverse transcription (RT)-PCR products. Mammary gland RNA from a nonlactating mouse was used as a negative control (NC), and a *GADPH* primer set was used as an internal control. (B) Total RNA was isolated from mammary gland tissues of α -LA-CN-Derp5t transgenic mice offspring (#113, 81, 44, 51, 79, 80, and 108) and wild-type mice; Mr = molecular weight marker. Derp5 transcripts were found in all 7 lines of offspring using RT-PCR. (C) The mammary gland sections from α -LA-Derp5f transgenic mice offspring were fixed and stained with hematoxylin and eosin (H&E; left panel) and anti-Derp5 antibodies for immunohistochemistry (IHC; right panel) analysis (200 $\times$ magnification). The structures of mammary acini are labeled as MA, and lactiferous tubules are labeled as LT. (D) The mammary gland sections from α -LA-CN-Derp5t transgenic mice offspring were fixed and stained with H&E and immunohistochemistry staining (200 $\times$ magnification). (E) Milk from α -LA-Derp5f transgenic female mice was subjected to SDS-PAGE (upper panel). The secretion of recombinant Derp5 into milk was detected by Western blot analysis using anti-Derp5 antibodies (lower panel). (F) Milk from α -LA-CN-Derp5t transgenic female mice was subjected to SDS-PAGE (upper panel). The secretion of recombinant Derp5 into milk was detected by Western blot analysis using anti-Derp5 antibodies (lower panel).

nant Derp5 milk (group B) had attenuated destruction of tracheal epithelia compared with that of sensitized mice fed WT milk (group C; Figure 5A). In addition, the degrees of alveolar hemorrhage and inflammatory cells infiltration were decreased in the lung parenchyma of sensitized mice fed recombinant Derp5-containing milk (group B) compared with sensitized mice fed WT milk (group C) (Figure 5B).

Pulmonary function tests were conducted using mice from 3 experimental groups to evaluate the influence of recombinant Derp5 milk ingestion on airway hyper-

responsiveness. The decrease in the percentage change of MFEF at 50% TLC from baseline in group C was higher than that in group A when acetylcholine doses were given at 25 and 75 μ g/kg ($P < 0.05$) or 150 and 500 μ g/kg ($P < 0.01$). However, the MFEF 50% TLC values in sensitized mice fed recombinant Derp5 milk (group B) were significantly higher compared with the values in sensitized mice fed WT milk (group C; $P < 0.05$, Figure 6A).

We observed dose-dependent increases in Penh values in response to aerosolized methylcholine in all 3 groups.

Figure 3. Analysis of the differences between signal peptides (SP) of mite Derp5 (the group 5 allergen isolated from *Dermatophagoides pteronyssinus*) and mammary gland casein. (A) Phylogenetic analysis between the Derp5 SP group (Derp5 signal peptide and its 8 BLAST results in *Arthropoda*) and the signal peptides of lactated proteins in mammals. (B) and (D) The predominant AA of each position in signal peptides of casein SP and Derp5 SP groups, respectively. The sequence logos of AA were created by the WebLogo program (<http://weblogo.berkeley.edu>) and colored according to their chemical properties as described in the Materials and Methods. (C) and (E) The hydrophobicity of the signal peptides (AA 8–13, highlighted in panels B and D) in casein SP and Derp5 SP groups, respectively. The hydrophobicity was calculated by Kyte-Doolittle method of ProtScale program (<http://web.expasy.org/protscale/>).

Figure 4. Experimental design of animal trials. Transgenic Derp5 (containing the group 5 allergen isolated from *Dermatophagoides pteronyssinus*)-containing milk and wild-type (WT) milk were collected and fed to 3-wk-old pups for 21 d. Mice were divided into 3 experimental groups. Two of the 3 groups of mice were sensitized by intraperitoneal injection of 10 μ g of purified Derp5 on d 21 and 35 (groups B and C). All mice were then challenged with 0.1% Derp5 aerosols on d 42. Mice underwent pulmonary function testing (PFT) 18 h after aerosol challenge. Bronchoalveolar lavage fluid and lung sections were collected on d 43 for an analysis of the airway inflammation level.

The results showed that sensitized mice fed WT milk (group C) had higher Penh values than did unsensitized mice (group A) after exposure to >10 mg/mL of nebulized methylcholine ($P < 0.01$). These findings suggest that group C mice had higher airway hyper-responsiveness. The Penh values in sensitized mice fed recombinant Derp5 milk (group B) were significantly lower compared with those in sensitized mice fed WT milk (group C; $P < 0.05$, Figure 6B).

DISCUSSION

In this study, we engineered 2 constructs of Derp5-containing transgenes to produce 2 strains of transgenic mice. Compared with the original leader sequence of *Derp5* cDNA in the α -LA-Derp5f construct, the α -LA-CN-Derp5t construct was designed to have a different leader sequence—that of α_{S1} -casein. Both strains of transgenic mice were produced successfully by micro-injection technology, and stable germline transmission was shown in the offspring (Figure 1). Female α -LA-CN-Derp5t transgenic mice both expressed and secreted recombinant Derp5 in mammary glands, whereas female α -LA-Derp5f transgenic mice expressed but did not secrete Derp5 peptide in mammary glands. This finding demonstrates that a signal peptide of α_{S1} -casein plays an important role in the external secretion of recombinant Derp5 in the mammary glands of mice (Figure 2). Moreover, the antigenic activity of recombinant Derp5 milk was demonstrated by its protective ef-

fect against allergic airway inflammation in this murine model through ingesting recombinant Derp5 milk.

α -Lactalbumin is a whey protein in mammalian milk. Casein, a family of related phosphoproteins (α_{S1} , α_{S2} , β , and κ), accounts for up to 80% of the proteins in cow milk. The expression of foreign protein in the milk of transgenic animals can be achieved using promoters from milk protein genes. Many previous studies have demonstrated that the expression of transgenes by a bovine α -LA promoter has the advantage of mammary-specific expression and good protein production in the milk of mice (Vilotte et al., 1989; Soulier et al., 1992; Bleck and Bremel, 1993). In the phylogenetic and conservative analyses, we found 2 hydrophilic AA (T-C) that were present only in the middle region of casein signal peptides (positions 10 and 11 in Figure 3B) but not in the Derp5 SP group, which thus showed the lower hydrophobicity (~ 2) in the middle region compared with the higher hydrophobicity (>2.5) of Derp5 signal peptides (Figure 3C and 3E). The concentrations of target proteins in mouse milk ranged from 0.0025 to 1.5 mg/mL in those studies mentioned above. Because the mammary cellular machinery has a limited capacity for glycosylation, proteins produced at higher concentrations may not be mature or adequately glycosylated (Houdebine, 2009), and the bioactivity of recombinant protein could depend on adequate glycosylation. In our previous study, the secretion of recombinant coagulation factor FVIII (rFVIII) in the milk of transgenic mice was produced by regulatory sequences of a bovine

(A) Trachea **(B) Alveoli** **Group A****Group B****Group C**

Figure 5. Histopathological analysis of allergic airway inflammation. (A) Trachea sections from 3 different groups (group A = unsensitized mice fed wild-type (WT) milk as the normal control group; group B = sensitized mice fed recombinant Derp5 (containing the group 5 allergen isolated from *Dermatophagoides pteronyssinus*) milk as the pretreatment group; group C = sensitized mice fed WT milk as the allergic airway inflammation group) with 200 $\times$ magnification. Intact ciliated epithelia can be observed in group A and B (arrows), which are different from the damaged epithelia and inflammatory cells infiltration (arrowheads) observed in group C. (B) Alveoli sections from 3 different groups with 200 $\times$ magnification. Group B mice show attenuated red blood and inflammatory cell infiltration within alveoli (asterisks) compared with alveolar hemorrhage and inflammation observed in group C. Scale bar = 200 μ m. Representative photomicrographs of tracheal and lung sections are shown; n = 5 mice per group.

α -LA promoter and an α -LA 19-AA signal peptide (Chen et al., 2002), and the concentrations of rFVIII in recombinant milk ranged from 7.9 to 50 μ g/mL. However, only 5 to 10% of rFVIII was demonstrated to have bioactivity by clotting assay. In contrast to the cassette of bovine α -LA promoter and α -LA signal peptide, the recombinant Derp5 peptide was secreted more efficiently into milk at a concentration of up to 2.0 mg/mL in female α -LA-CN-Derp5t transgenic mice by replacing the α -LA signal peptide with the signal peptide of α S1-casein. This comparison revealed that the α -LA-CN/pCR3 vector could be a mammary gland-specific expression vector with high yields of the target protein.

In the murine experiments of allergic airway inflammation, recombinant Derp5 in milk was demonstrated to have antigenic activity based on its protective effect against the development of Derp5-induced allergic airway inflammation and airway hyper-responsiveness (Figures 5 and 6). Today, the immunological mechanisms of immunotherapy and oral tolerance induction

are well understood. Ingestion of low doses of antigen favors tolerance driven by regulatory T (Treg) cells, whereas ingestion of high doses favors anergy-driven tolerance (Burks et al., 2008). The dosage of antigen ingestion in mice fed recombinant milk was estimated to be 60 μ g of recombinant Derp5 per day. The protective effect of recombinant Derp5-containing milk ingestion was more likely to be associated with Treg cells for the low doses of allergen intake in this study. Further experiments are needed to determine the precise immunological mechanism.

No recombinant Derp5 was detected in the milk of female α -LA-Derp5f transgenic mice by Western blot analysis. Immunohistochemistry analysis of mammary gland tissues of female α -LA-Derp5f transgenic mice showed that recombinant Derp5 was expressed but that it accumulated only within the mammary acini. This finding revealed that the signal peptide of Derp5 cDNA cannot cause the secretion of the recombinant protein into the lactiferous tubules. In a study using re-

Figure 6. Pretreatment effect of ingesting recombinant Derp5 (the group 5 allergen isolated from *Dermatophagoides pteronyssinus*)-containing milk on airway hyper-responsiveness. (A) The dose-response curve of the maximal forced expiratory flow (MFEF) at 50% total lung capacity (50% TLC) in 3 experimental groups that were given different acetylcholine (Ach) concentrations intravenously (i.v.). (B) Barometric whole-body plethysmography was used to measure pulmonary function in these experimental mice, and enhanced pause (Penh) values were measured in response to aerosol challenges with different concentrations of nebulized methylcholine (Mch). These data are presented as mean $\pm$ SEM; $n = 5$ mice per group. * $P < 0.05$ for group B compared with group C; ** $P < 0.01$ for group C compared with group A; # $P < 0.05$ for group B compared with group A.

combinant Derp5-specific antibodies and immunogold electron microscopy, Derp5 was found to be a secretory product of gut epithelial cells and a component of fecal material in dust mites (Weghofer et al., 2008). Although the signal peptide of Derp5 cDNA could cause secretion of Derp5 from gut epithelia into the gut lumen of dust mite, it does not have the capacity to secrete recombinant Derp5 into the lactiferous tubules and milk of transgenic mice.

Compared with bacteria and yeast systems, the use of a transgenic animal system to produce recombinant proteins has the advantages of proper posttranslational modifications and glycosylation of proteins. Large

dairy animals have been demonstrated to produce high amounts of recombinant protein at low cost through recombinant milk production (Houdebine, 2009). However, the successful generation of transgenic animals depends on high levels of biotechnology. The time required to obtain recombinant protein in transgenic animals is greater compared with that of other systems. Transgenic animal systems to produce pharmaceutical proteins in milk have improved technically over the past 2 decades, and a broad variety of recombinant proteins have been produced experimentally (Houdebine, 2000). Furthermore, the studies of biological properties and clinical applications of the recombinant proteins are needed before the products can be put on the market. A recombinant antithrombin (ATryn, GTC Biotherapeutics, Framingham, MA), the first pharmaceutical protein produced from the milk of transgenic goats, has opened a new era in the production of recombinant proteins from ruminant animals (Adiguzel et al., 2009).

In conclusion, this study demonstrated that recombinant Derp5 can be secreted efficiently into the milk of transgenic mice by adding a signal peptide of α_{S1} -casein. Pretreatment involving the ingestion of recombinant Derp5-containing milk prevented the development of allergic airway inflammation in the murine model. This study may pave the way to design an economical strategy of immunotherapy from large dairy animals to decrease the development of allergic asthma in humans.

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