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Messenger RNA expression and immunolocalization of psoriasin in the goat mammary gland and its milk concentration after an intramammary infusion of lipopolysaccharide

G.W. Zhang [a,b], S.J. Lai [c], Y. Yoshimura [a], N. Isobe [a,*]

[a] Graduate School of Biosphere Science, Hiroshima University, Higashi-Hiroshima, 739-8528, Japan
[b] Department of Animal Science, Rongchang Campus, Southwest University, Rongchang, Chongqing, 402460, China
[c] Institute of Animal Genetics and Breeding, Sichuan Agricultural University, Ya'an, Sichuan, 625014, China

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ABSTRACT

Psoriasin (S100A7) is a member of the S100 protein family of calcium-binding proteins and plays a crucial role in local host defenses. The present study aimed to identify the expression of S100A7 in the goat mammary gland and in milk. The goat S100A7 coding DNA sequence was identified using direct sequencing. An S100A7 antibody was raised in rabbits by immunization with a synthetic S100A7 peptide consisting of 13 amino acids. Messenger RNA expression and protein localization in different regions of a healthy mammary gland were detected by reverse transcription-polymerase chain reaction and immunohistochemistry. Changes in the concentration of S100A7 in the milk after an intramammary infusion of *Escherichia coli* lipopolysaccharide (LPS) were examined by an enzyme immunoassay.

The goat S100A7 peptide had 98% and 86% sequence similarity to that of sheep and bovines, respectively. The *S100A7* mRNA expression was higher in the teat and udder skin than in the cistern and parenchyma of the mammary gland. Immunoreactive S100A7 was localized in the epithelial cells of the alveolus and gland cistern, and stratified squamous epithelium of the teat. Psoriasin as a secreted protein was detectable in healthy milk, and an intramammary LPS infusion increased the concentration of S100A7 in the milk. The results suggest that S100A7 is produced in the epithelial cells of the mammary gland and is secreted into the milk.

Introduction

Mastitis is an inflammatory process of the mammary gland, and commonly a consequence of infection caused by pathogens that colonize the lumen of the gland through the teat canal and then enter the cistern and alveolus (Zhao and Lacasse, 2008). The teat canal is normally tightly closed by sphincter muscles, which prevent the entry of pathogens. The stratified squamous epithelium of the teat contains antimicrobial agents, such as long-chain fatty acids and antimicrobial peptides (AMPs), which assist in combating infection (Capuco et al., 1992). The epithelial cells of the cistern and alveolus also express many types of AMPs (Isobe et al., 2009c; Tetens et al., 2010). Antimicrobial peptides display direct antimicrobial activity against a wide range of pathogens (Zasloff, 2002) and may play an important role in the defense mechanism of the mammary gland against mastitis-causing organisms.

Psoriasin (S100A7) is one of the AMPs that exhibit antimicrobial activity against *Escherichia coli* in humans (Glaser et al., 2005) and bovines (Regenhard et al., 2009). However, the mechanisms of S100A7's antimicrobial action remain unclear (Gallo and Hooper, 2012). Psoriasin was first identified in the epithelial cells of human psoriatic skin (Madsen et al., 1991; Al-Haddad et al., 1999) and is secreted by breast cancer cells (Enerback et al., 2002) and psoriatic keratinocytes (Madsen et al., 1991). In vitro and in vivo studies revealed that S100A7 was expressed in human mammary epithelial cells (Enerback et al., 2002) and, in bovines, *S100A7* mRNA was constitutively expressed in the streak canal (Tetens et al., 2010). As an intramammary infection with *E. coli* can induce S100A7 expression in the teat cistern, and S100A7 has been shown to be secreted into the milk in *E. coli*-infected dairy cows (Regenhard et al., 2010), S100A7 may play a crucial role in local host defenses against mastitis.

The goat is a useful model animal for cows to study immune function in the mammary gland. To the best of our knowledge, no study has demonstrated S100A7 expression in the goat mammary gland and its secretion into the milk. The present study was undertaken to identify the expression of S100A7 in the goat mammary gland.

* Corresponding author. Tel.: +81 82 424 7993.
E-mail address: niso@hiroshima-u.ac.jp (N. Isobe).

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Materials and methods

Animals

Nine crossbred goats (Siba × Saanen, parity 1–4, mid-lactation stage, milk yield 300–800 mL/day, bodyweight 20–30 kg) were used in the present study. The goats were fed 0.8 kg of hay cubes and 0.15 kg of barley per day and had free access to water and a trace mineralized salt block. Diets were offered twice daily at 0800 and 1500 h. Only goats with low somatic cell counts (SCC) in their milk were selected. Three goats were used for collection of mammary gland tissues and the other six for intramammary infusion of lipopolysaccharide (LPS).

This study was carried out in accordance with the Guideline for Animal Experimentation, Hiroshima University, Japan (C09–23 approved 23 March 2012).

Collection of mammary gland tissues

Udder tissue samples (teat, udder skin, gland cistern, and parenchyma) from three goats were collected within 20 min after euthanasia by exsanguination under sedation with Xylazine (Bayer HealthCare) and anesthesia with pentobarbital (Somnopentyl; Kyoritsu Seiyaku). The tissues were divided into two parts: the first part was frozen immediately on dry ice and then stored at –80 °C to examine *S100A7* mRNA expression by reverse transcription-polymerase chain reaction (RT-PCR); the second part was immediately fixed with neutralized 10% (vol/vol) formalin. Fixed tissues were washed with running water and dehydrated for paraffin embedding then used for protein localization by immunohistochemistry.

Isolation of total RNA and RT-PCR

Total RNA was isolated with the Sepasol-RNA I Super (Nacalai Tesque), and treated with DNase I (Promega). Purified RNA samples were reverse-transcribed using ReverTra Ace (Toyobo). The process for total RNA isolation and cDNA synthesis followed the manufacturer's instructions.

According to the bovine *S100A7* gene sequence (GenBank number NM_174596.2), the primer was designed to identify the coding DNA sequence (CDS) of the goat *S100A7* gene as 5′-GAAGCCAAGATGAGCAGCTCTC-3′ for forward and 5′-GGAGGCCTCTGGGCTCACT-3′ for reverse. The PCR amplification was carried out using a programmable thermal controller PTC-100 (MJ Research) in a 25-μL reaction mixture containing 0.5 μM specific primers, 0.5 μL aliquot of cDNA, 1 × PCR buffer (1.5 mM $MgCl_2$), 0.2 mM deoxyribonucleotide triphosphate mixture, and 1.25 U Takara Ex Taq (Takara Bio). The PCR reaction was performed with the following conditions: 1 denaturation cycle at 94 °C for 3 min; 35 cycles at 94 °C for 30 s, 65 °C for 30 s and 72 °C for 30 s, and then an extension cycle at 72 °C for 7 min. *β-actin* (GenBank number NM_173979.3) expression was analyzed as an internal control for reaction efficiency and the primers were 5′-CATCACCATCGGCAATGAG-3′ for forward and 5′-CCGTGTTGGCGTAGAGGTC-3′ for reverse.

The PCR reaction volume and PCR reaction of *β-actin* were also identical to the step listed above, with an annealing temperature of 55 °C. The PCR products were electrophoretically separated on a 2% (wt/vol) agarose gel containing 0.5 mg/mL ethidium bromide and examined under UV illumination. The PCR products used the direct sequencing method with the 3130xl Genetic Analyzer (Applied Biosystems). The same primer pairs described above were used for sequencing.

Antibody preparation

As previously described (Isobe et al., 2009a), the antibody was raised in rabbits against a synthetic S100A7 peptide consisting of 13 amino acids (FEKQDKNKDRKID; **Fig. 1**) from its C-terminal classical EF-hand. After whole blood collection from the immunized rabbit, the S100A7 antibody was purified using a HiTrap Protein G High Performance affinity column (GH HealthCare) according to the manufacturer's directions. The affinity-purified S100A7 antibody was then used for enzyme immunoassay (EIA) and immunohistochemistry as described below.

Enzyme immunoassay

The specificity of the S100A7 IgG antibody was determined by EIA as described previously (Morimoto et al., 2012) with minor modifications. A 96-well microtiter plate was coated with 4 μg/mL of anti-rabbit S100A7 antibody in carbonate buffer (pH 9.7) at room temperature for 2 h; this was followed by blocking with 150 μL of assay buffer supplemented with 0.2% bovine serum albumen (BSA) at pH 7.8 for 30 min. Fifty microliters of the synthetic S100A7 standard (0, 0.1, 0.3, 1, 3, 10, 30, and 100 ng/mL) were then added to the wells and incubated for 3 h at room temperature. A total of 50 μL of horseradish peroxidase (HRP)-labeled S100A7 (total dilution ×60,000) was then added and the plate was incubated for 30 min at room temperature.

The S100A7 was conjugated with HRP using the peroxidase-labeling kit-SH (Dojindo Laboratories). After washing three times with phosphate buffered saline (PBS), the wells were incubated with 150 μL of tetramethylbenzidine solution for 30 min. Optical density was measured at a wavelength of 655 nm using a microplate reader (Model 550; Bio-Rad Laboratories). Assays were performed five times The sensitivity of the assay, calculated as the concentration of S100A7 that can detect two standard deviations below the mean optical density at 0 ng/mL S100A7, was estimated as 0.35 ng/mL.

N-terminal EF-hand

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      goat    MSSSQLEQAISALIDLFHKHSGPDDTIEKEALLQLLKENFPNFLSACEKR 50
98% sheep     MSGSQLEQAISALIDLFHKHSGPDDTIEKEALLQLLKDNFPNFLSACEKR 50
84% bovine    MSSSQLEQAITDLINLFHKYSGSDDTIEKEDLLRLMKDNFPNFLGACEKR 50
63% human     MSNTQAERSIIGMIDMFHKYTRRDDKIEKPSLLTMMKENFPNFLSACDKK 50
              **.:* *::*  :*::***::  **.***  ** ::*:******.**:*:
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C-terminal EF-hand

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      goat    GRDYLSNIFEKKDKNKDQKIDFSEFLSLLADIASDYHNHSHGEELCSGGNK 101
98% sheep     GRDYLSNIFEKKDKNKDQKIDFSEFLSLLADIASDYHNHSHGEELCSGGNK 101
84% bovine    GRDYLSNIFEKQDKNKDRKIDFSEFLSLLADIATDYHNHSHGAQLCSGGNQ 101
63% human     GTNYLADVFEKKDKNEDKKIDFSEFLSLLGDIATDYHKQSHGAAPCSGGSQ 101
              * :**:::***:***:*:***********.***:***::***    ****.:
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Fig. 1. Alignment of the psoriasin (S100A7) protein from different species (GenBank number: goat, KC345019; sheep, XM_004002524.1; bovine, NM_174596.2; human, NM_002963.3). The underlined sequences were synthesized and the S100A7 antibody was raised in rabbits against this peptide. The EF-hand is a helix–loop–helix structural domain or motif found in a large family of calcium-binding proteins. The asterisks represent that the amino acid was absolutely conserved among different species.

Immunohistochemistry

Fixed and dehydrated mammary gland tissues were embedded in paraffin in the usual manner. Sections (2-μm thick) were air dried on slides. After deparaffinization, sections were boiled in citric acid (0.01 M) solution and were blocked with goat normal serum for 30 min. Sections were incubated with the anti-S100A7 antibody (1 μg/mL) in PBS for overnight at 4 °C. Sections were then washed with PBS for 10 min. Immunoreaction products were identified using Histofine (Nichirei) for 1 h at 37 °C and then washed with PBS for 10 min. Immunoreaction products were then visualized by incubating the sections with a diaminobenzidine reaction mixture. The sections were counterstained with hematoxylin and dehydrated and covered. The anti-S100A7 antibody was replaced with normal rabbit IgG at 1 μg/mL for the negative control. Stained sections were examined under a Nikon Eclipse E400 microscope (Nikon).

Intramammary infusion of LPS and milk concentration of S100A7

The LPS stock solution (1.0 mg/mL) was prepared by dissolving LPS from *E. coli* O111:B4 (Wako Pure Chemical) in saline (0.9% NaCl). The left mammary gland of six goats was infused with LPS at a dose of 5 μg/5 mL and the right mammary gland was infused with 5 mL of saline, as described previously (Isobe et al., 2009b; Huang et al., 2012; Olumee-Shabon et al., 2013). Milk samples were collected by hand before infusion (0 h, healthy milk) and 24 h after the intramammary infusion of LPS or saline.

Milk samples were divided into two parts: the first part was spread on a glass slide for SCC using the Breed method (Schalm et al., 1971); the second sample of milk was used to collect skim milk by centrifugation at 1700 *g* for 30 min at 4 °C. Skim milk was stored at –20 °C until the concentration of S100A7 was measured.

The concentration of S100A7 in the milk was determined by EIA as described above. Skim milk samples were diluted 5000 times with assay buffer. Assays were performed in duplicate.

Statistical analysis

The milk concentrations of S100A7 were expressed as mean fold changes ($n = 6$) compared with 0 h for each individual udder. The significance of the S100A7 concentration and SCC between 0 h and 24 h after the infusion of LPS or saline was analyzed using a two-way repeated-measures ANOVA, followed by the Tukey multiplex analysis; $P < 0.05$ was considered significant.

Results

Gene sequence of S100A7

The complete CDS of the goat *S100A7* gene was determined by direct sequencing using reverse-transcripted cDNA. The nucleotide sequence of the goat *S100A7* gene (GenBank number KC345019)