Expression and Characterization of Bioactive Recombinant Human α -Lactalbumin in the Milk of Transgenic Cloned Cows

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ABSTRACT

Improvement of the nutritional value of cow milk with transgenic expression of recombinant human α -lactalbumin (α -LA) has been previously attempted. However, the detailed characterization of the recombinant protein and analysis of the transgenic milk components are not explored yet. Here, we first report production of healthy transgenic cows by somatic cell nuclear transfer, in which expression of up to 1.55 g/L of recombinant human α -LA was achieved. The recombinant human α -LA was purified from transgenic milk and displayed physicochemical properties similar to its natural counterpart with respect to molecular weight, structure, and regulatory activity for β -1,4-galactosyltransferase. Additionally, no *N*-glycosylation was found in the recombinant human α -LA, whereas the endogenous bovine α -LA was glycosylated at the unusual site ⁷¹Asn-Ile-⁷³Cys. Compared with milk from nontransgenic cows, expression of the transgene did not materially alter milk composition, such as fat and protein content. Our research thus provides scientific evidence supporting the feasibility of humanizing cow milk.

Key words: human α -lactalbumin, humanized milk, protein glycosylation, transgenic cow

INTRODUCTION

Cow milk provides important nutrients for the improvement of human health. However, due to the distinct composition of cow milk, it is essential to adjust the components of cow milk to resemble that of human milk to better satisfy the nutritional requirements of infants and adults. For instance, the ratio between CN and whey protein differs between species—approximately 80:20 (wt/wt) in cow milk and 40:60 (wt/wt) in human milk (Lien, 2003). The humanizing process of

cow milk should include selectively elevating the levels of α -LA, lactoferrin, and lysozyme, all of which are highly expressed in human milk, and introducing other proteins with nutraceutical or therapeutic benefit to humans, or removing β -LG, the primary allergen in cow milk.

α -Lactalbumin is a Ca^{2+} -binding protein present in the milk of all of mammals and functions as a modifier of β -1,4-galactosyltransferase in the mammary gland to produce lactose (Permyakov and Berliner, 2000). In human milk, α -LA is an abundant protein (2.44 ± 0.64 g/L; Jackson et al., 2004) that comprises approximately 25 to 35% of the total protein in contrast to its bovine counterpart, which only accounts for 2 to 5% of the total protein (Lönnerdal and Lien, 2003). α -Lactalbumin has physiological and nutritional significance in that it contains a relatively high proportion (63%) of the essential amino acids, particularly tryptophan, the precursor of serotonin, which is involved in nervous system activities such as mood regulation and cognitive function (Schmitt et al., 2005; Booij et al., 2006). α -Lactalbumin also contains a high percentage of branched-chain amino acids, such as isoleucine and leucine, which are related to protein synthesis in muscle (Layman, 2003). In addition, α -LA can stimulate mucus metabolism and may also be effective in preventing gastrointestinal infections among neonates (Bruck et al., 2006; Ushida et al., 2007). Interestingly, human α -LA made lethal to tumor cells, an α -LA folding variant that induces tumor cell apoptosis (Svensson et al., 2000), can effectively cure cutaneous warts caused by human papillomavirus (Gustafsson et al., 2004), suggesting that this folding variant might be a potential antitumor drug.

To enhance the nutritional value of cow milk and supply human α -LA for pharmaceutical research, expression of human α -LA in cow milk by transgenic technology appears to be promising. Transgenic cows containing recombinant human α -LA have been produced previously by means of pronuclear microinjection (Eyestone, 1999). Since the birth of Dolly, however, the greater efficiency and lower cost of somatic cell cloning

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by nuclear transfer makes this a preferable method for transgenic cow production (Polejaeva and Campbell, 2000).

Based on our previous generation of transgenic mice containing a genomic fragment of human α -LA (Yu et al., 2004), we implemented the expression of transgenic human α -LA in cow milk by somatic cell nuclear transfer (SCNT) with the aim of improving the nutritional value of the milk. The biochemical properties of the recombinant protein were systematically characterized by comparison with the natural human α -LA. Importantly, the composition of the transgenic milk did not differ substantially from that of nontransgenic milk.

MATERIALS AND METHODS

Production of Transgenic Cows

The expression vector pHLa4-EGFP-NEO (Figure 1A) was transfected into fetal oviduct epithelial cells (FOV) from Chinese Holstein cows by electroporation. Cell lines expressing transgenic human α -LA were selected using G418 and fluorescence screening. The procedure of nuclear transfer was performed according to Gong et al. (2004). Briefly, transgenic human α -LA epithelial cells were transferred into the enucleated oocytes from ovaries of adult yellow cattle obtained from a slaughterhouse. Electrofusion of reconstructed embryos was conducted by the BTX Electro Cell Manipulator 2001 (BTX, San Diego, CA) using a field power of 2.5 kV/cm and two 10 μ s of pulse with a 1-s interval. Artificial activation of the reconstructed embryos occurred 0.5 h after fusion. Then the embryos were cultured in vitro for 7 d to allow for blastocyst development. These transgenic blastocysts were then transferred to synchronized Chinese Luxi yellow cattle recipients 7 d after estrus. Fetal survival was monitored via rectal palpation on 60 and 90 d after embryo transfer. All animal procedures were approved by the Institutional Animal Care and Use Committee at the China Agricultural University.

Analysis of Nucleic Acids

Total genomic DNA was extracted from the ear biopsies of transgenic and nontransgenic cows. The presence of recombinant human α -LA and the marker genes *EGFP* and *NPT II* were assayed by PCR (Figure 1A). Two pairs of primers specific for human α -LA were designed: the forward primer 1: 5'-GAG TGA TGC TTC CAT TTC AG-3' and the reverse primer 1: 5'-CAG AGA TGT ACA GGA TCT GC-3', which amplified 790 bp of the target gene (P1), and the forward primer 2: 5'-TAG ATC TAG GGG TTA GGG GAA CT-3' and the reverse primer 2: 5'-TGC GGC CGC

ATT GAG TTG GTA CAG ACA GT-3', which amplified 1,161 bp of the target gene (P2). The primers for *EGFP* were as follows: the forward 5'-TGC AGT GCT TCA GCC GCT AC-3' and the reverse 5'-CTC AGG TAG TGG TTG TCG GG-3', which amplified 403 bp of the target gene (P3). The primers for *NPT II* were as follows: the forward 5'-AGG ATC TCC TGT CAT CTC ACC TTG CTC CTG-3' and the reverse 5'-AAG AAC TCG TCA AGA AGG CGA TAG AAG GCG-3', which amplify 493 bp of the target gene (P4).

The genomic DNA (10 μ g) was digested with *EcoRI* and analyzed by Southern blot using a 1.5-kb human α -LA hybridization probe labeled with [α - 32 P] deoxycytidine triphosphate (Figure 1A). Copy numbers of the transgene were evaluated by signal quantification of the Southern blot using a Storm 820 PhosphorImager system (Molecular Dynamics, Sunnyvale, CA).

To determine the chromosomal location of the transgenic α -LA insert, metaphase chromosomes were prepared from ear skin fibroblasts from each transgenic cow for fluorescence in situ hybridization. The plasmid pHLa4-EGFP-NEO was used as a probe and labeled with biotin-14-deoxyadenosine triphosphate (Invitrogen, Carlsbad, CA) following the protocol of the manufacturer. Hybridization was performed for 24 h at 37°C. The probe was detected with fluorescein isothiocyanate-conjugated avidin (Vector Laboratories, Burlingame, CA). Chromosomes were counterstained with propidium iodide. Images were acquired by using an epifluorescence microscopy equipped with a DP70 CCD camera (Olympus, Tokyo, Japan).

Milk Collection

Lactation of transgenic and nontransgenic cows (6 to 8 mo of age) was induced by lactating agents (National Caotan Pharmacy Company, Xi'an, China). The hormone-induced milk was collected 3 times at 6-h intervals each day for 1 mo and stored at -20°C.

Characterization of the Recombinant Protein

Purification and Identification of Recombinant Human α -LA. The whey was obtained by ultracentrifugation of transgenic whole milk at 100,000 $\times$ g, 1 h and 20°C, to precipitate CN and discard the fat. Purification of recombinant human α -LA was performed using an ÄKTApurifier 10 system (Amersham Biosciences, Piscataway, NJ). Ten milliliters of whey was injected onto an anion exchange HiLoad 16/10 Q Sepharose column (Amersham Biosciences) equilibrated with 50 mM Tris-HCl, pH 7.4. After removing the unbound proteins by washing (50 mM Tris-HCl, pH 7.4), the bound protein was eluted using a NaCl segment

Figure 1. Expression of recombinant human α -LA. (A) Schematic construct of phLa4-EGFP-NEO. The genomic human α -LA gene (9,459 bp) was inserted into the expression vector containing double selection marker gene *EGFP* and *NPT II*. The regions selected for PCR screening and Southern blot were indicated. P denoted product of PCR; P1 and P2 for human α -LA; and P3 and P4 for *EGFP* and *NPT II*, respectively. EGFP = enhanced green fluorescent protein; NPT II = neomycin phosphotransferase II; Amp = ampicillin-resistant gene; and ori = pBR322-derived origin. (B) PCR analysis of the transgenes. P1 and P2 represented the PCR products of recombinant human α -LA, respectively; P3 was for the PCR products of *EGFP*; and P4 for that of *NPT II*. (C) Southern blot analysis for transgenic human α -LA. The target DNA sequence was approximately 1.5 kb, and approximately 1 copy of transgene was integrated into the genome of transgenic cows as a reference with copy numbers of positive control. (D) Western blot analysis for expression of recombinant human α -LA. The whey protein from transgenic and non-transgenic cows was separated on native PAGE and then blotted and hybridized with antihuman α -LA antibody. Lane 1 = human α -LA; lane 2 = human whey; lane 3 to 5 = transgenic whey from Xingwa, Longwa, and Huiwa, respectively; lane 6 = nontransgenic whey control.

gradient in 50 mM Tris-HCl, pH 7.4. Recombinant α -LA was collected at 50 mM NaCl and further purified by gel filtration using a HiLoad 16/60 Superdex 75 prep grade column (Amersham Biosciences) in wash buffer (50 mM Tris-HCl, 0.1 M NaCl, pH 7.4). Fractions were collected and subjected to SDS-PAGE analysis, and the purity of recombinant human α -LA was estimated using SYPRO Ruby gel stain (Invitrogen).

Purified recombinant human α -LA and natural human and bovine α -LA (Sigma, St. Louis, MO) control were analyzed by native PAGE and transferred to nitrocellulose membrane for Western blot analysis. The N-terminal Edman sequencing was performed by Shanghai GeneCore BioTechnologies Co. Ltd. (Shang-

hai, China). The molecular mass of purified α -LA was measured by matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) at the linear pattern. Quantitative analysis of the amount of recombinant and endogenous α -LA in transgenic milk was achieved by RIA at the Institute of Atomic Energy Utilization (Beijing, China).

Calcium-Dependent Electrophoretic Shift of α -LA. The electrophoretic shift of α -LA was performed according to Thompson et al. (1989). Briefly, a final concentration of 10 mM CaCl_2 was respectively added to recombinant α -LA and bovine or human α -LA control. Also, calcium removal from the α -LA proteins was performed by using ethylene glycol tetraacetic acid at

a final concentration of 10 mM. The treated samples were separated by 15% native PAGE and visualized by Coomassie Brilliant Blue G250 staining.

Lactose Synthesis Induced by α -LA In Vitro.

Chemical synthesis of the lactose in vitro was conducted as described in Holpert and Cooper (1990) with modifications. Briefly, the reaction system was as follows: 6 mU of β -1,4-galactosyltransferase from bovine milk (EMD Biosciences, San Diego, CA); 50 mM HEPES, pH 6.63; 0.0245% Triton X-100; 0.0245% BSA; 9 mM MnCl_2 ; 25 mM glucose; 0.4 mM uridine diphosphate-galactose (EMD Biosciences); plus 20 μg of purified recombinant or native human α -LA in a final volume of 100 μL . The reaction was carried out at 37°C for 2 h. The synthetic lactose was detected using a Lactose/D-Galactose kit (R-Biopharm AG, Darmstadt, Germany) according to the instructions of the manufacturer.

Enrichment and Purification of Glycosylated α -LA. The glycosylated α -LA was enriched using concanavalin A affinity chromatography from 100 mL of whey from transgenic whole milk. The column packed with concanavalin A Sepharose 4B (Amersham Biosciences) was equilibrated with 5 column volumes of the prebinding buffer (20 mM Tris-HCl, pH 7.4; 1 M NaCl; 1 mM CaCl_2 ; 1 mM MnCl_2 ; and 1 mM MgCl_2) followed by equilibration with 5 column volumes of the binding buffer (20 mM Tris-HCl, 0.5 M NaCl, pH 7.4). The whey solution (20 mL per run) was injected into the affinity column that was then washed extensively with the binding buffer. The glycosylated proteins were eluted with the binding buffer containing 0.5 M α -D-methylmannoside (Sigma) identified by glycoprotein-specific fluorescent staining Pro-Q Emerald 300 (Invitrogen) after analysis by SDS-PAGE. The glycosylated α -LA was separated from total enriched glycoproteins by gel filtration using a HiLoad 16/60 Superdex 75 prep grade column (Amersham Biosciences) in 50 mM Tris-HCl, 0.15 M NaCl, pH 7.4. The purified glycosylated α -LA was desalted and concentrated by ultrafiltration via Amicon Ultra-15 with 5 kDa molecular mass (M_r) cutoff (Millipore, Billerica, MA) and was stored at -20°C.

Glycosylation Analysis. Purified freeze-dried glycosylated α -LA (80 μg) was dissolved in 80 μL of digestion buffer (8 M urea and 50 mM NH_4HCO_3 , pH 7.8) and digested by trypsin (Sigma; 100:1 mass of protein to enzyme) for 16 h at 37°C. Subsequently, half of the trypsin digestion reaction was desalted via Ziptip (Millipore) and was further treated with Glu-C (Sigma; 50:1 mass of protein to enzyme) in a final volume of 40 μL containing 20 mM NH_4HCO_3 , pH 7.8, for 16 h at 37°C. The 2 α -LA hydrolysates were then deglycosylated using PNGase F (Sigma). The deglycosylated α -LA was analyzed with Finnigan LTQ linear quadrupole ion

trap MS (Thermo Fisher Scientific, Waltham, MA). A PicoFrit column packed with BioBasic C18 was used for the HPLC with a flow rate at 200 nL/min. The glycosylation pattern of peptides was initially screened by MS and then the determination of specific glycosylated residues was performed with tandem MS (MS/MS). Data were analyzed using BioWorks 3.1 (Thermo Fisher Scientific), and the standards for positive results were as follows: $\text{Xcor}/\Delta\text{Cn} > 2.2/0.08$ for a charge of +1; $\text{Xcor}/\Delta\text{Cn} > 2.5/0.08$ for +2; and $\text{Xcor}/\Delta\text{Cn} > 3.5/0.08$ for +3.

Analysis of Milk Components

The gross composition of whole milk samples from the lactation-induced transgenic and nontransgenic cows was determined using MilkoScan 4000 (Foss Electric, Hillerød, Denmark) for analysis of the percentage of total fat, total protein, total lactose, and total solids. Quantification of proteins was estimated by the method of Bradford (1976). Samples of whey proteins were obtained by ultracentrifugation of skim milk. For 1-dimensional gel electrophoresis, skim milk samples were separated by 15% SDS-PAGE and then visualized by Coomassie Brilliant Blue G250 staining. For 2-dimensional gel electrophoresis, equivalent amounts of skim milk protein (25 μg for pH 3 to 6 and 60 μg for pH 5 to 8) from transgenic and nontransgenic cows were subjected to isoelectric focusing using immobilized pH gradient gel strips (11 cm with a linear gradient of pH 3 to 6 or 5 to 8; Bio-Rad, Hercules, CA) and were subsequently separated by 15% SDS-PAGE. Equivalent amounts of whey protein (25 μg for pH 3 to 6 and 40 μg for pH 5 to 8) from transgenic and nontransgenic cows were similarly analyzed. Protein spots were visualized by staining with colloidal Coomassie Blue (Candiano et al., 2004). Scanned images of 2-dimensional gels were analyzed by PDQuest software (Bio-Rad). Protein spots were digested in-gel with trypsin (Sigma), and then the resulting peptides were extracted and analyzed by MALDI-TOF MS. Peptide mass fingerprint alignments were matched against the public database using the MASCOT program (Matrix Science, Boston, MA).

RESULTS

Transgene Expression

The transgene vector pHLa4-EGFP-NEO was constructed to contain a 9,459-bp genomic DNA fragment of human α -LA, including 6.9 kbp of 5' flanking region, 2.4 kbp of coding sequence, and 159 bp of 3' flanking region. The vector also carried 2 selection marker genes, *EGFP* and *NPT II* (Figure 1A). Through SCNT

Table 1. Production of transgenic cloned cows

Transgenic cell line	Cloned blastocysts ¹	Pregnant cattle at 90 d ²	Viable heifers ³	
			At full term	At 8 mo
FOV-LA (fetal oviduct epithelial cells)	362/824 (44%)	16/72 (22%)	6/121 (5%)	3/121 (2.5%)

¹Expressed as proportions of reconstructed embryos in vitro developing into the blastocysts.
²Proportions of pregnant recipients in total amounts transferred.
³Proportions of embryos transferred developing into calves.

and embryo transfer, the transgenic cloned embryos were transplanted into recipients of Chinese Luxi yellow cattle. Six transgenic cows were born at full term. Three transgenic cows, Xingwa, Longwa, and Huiwa (about 30 mo of age at the time of this study), were apparently healthy (Table 1) and were milked for characterization of the recombinant protein and analysis of milk composition.

Polymerase chain reaction was performed to detect integration of human α -LA, *EGFP*, and *NPT II* in the bovine genome. We designed 2 pairs of primers specific for human α -LA encompassing the full length of coding sequence (Figure 1A). The PCR results showed that these 3 genes were all inserted into the genome of the above-mentioned cows (Figure 1B). The copy number of the human α -LA transgene was determined by Southern blot analysis. Both transgenic cows, Xingwa and Longwa, had 1 copy of this transgene integrated into their genomes (Figure 1C; 1 copy of this transgene was also identified in Huiwa, data not shown). To investigate the chromosome localization of the human α -LA transgene, we conducted fluorescence in situ hybridization, which indicated that the transgene was integrated on only 1 chromosome in transgenic cows (data provided in supplementary Figure S1A, B, and C, which can be found online at <http://jds.fass.org/content/vol91/issue12/>). Recombinant human α -LA was highly expressed in milk from Xingwa and Longwa (up to 1.55 g/L), but lower expression was determined in Huiwa as measured by RIA (Table 2) and confirmed by Western blot analysis (Figure 1D). Meanwhile, levels of endogenous bovine α -LA in these transgenic cows were stable and consistent with expression in nontransgenic controls (Table 2).

Physicochemical Properties of Recombinant Human α -LA Purification of the Expressed Human α -LA.

We used 2 steps of chromatography to purify recombinant human α -LA from the transgenic cow milk. Recombinant human α -LA was first separated from bovine α -LA by anion exchange chromatography. Further purification was performed using gel filtration to separate recombinant human α -LA from the rest of proteins in transgenic milk (Figure 2A and B). The purified recombinant human α -LA was identified by Western blot (Figure 2B, left insert) and analyzed by gel electrophoresis with SYPRO Ruby staining (Invitrogen; Figure 2B, right insert); it was free of bovine α -LA. Sequencing by the N-Edman degradation confirmed that the N-terminal residues of the purified recombinant protein were intact (KQFTKCELSQ, GenBank accession no. CAA28800, data not shown). To further verify the integrity of the primary structure, the molecular weight of the recombinant human α -LA was assessed by MALDI-TOF MS and was shown to be very close to that of natural human α -LA (calculated 1,4078.2 Da; Table S1, which can be found online at <http://jds.fass.org/content/vol91/issue12/>), suggesting that neither deletion of amino acid residues nor post-translational modifications occurred for the purified recombinant protein.

Recombinant Human α -LA Showed the Same Bioactivity and Conformation as Its Natural Counterpart.

In the mammary gland, α -LA promotes the synthesis of lactose. By simulating the biochemical reaction in vitro, recombinant human α -LA was shown the similar capacity with a natural counterpart to interact with β -1,4-galactosyltransferase to synthesize lactose (Figure 3A). To evaluate whether the recombi-

Table 2. Expression of recombinant human (rHuman) and bovine α -LA in transgenic milk

α -LA	Transgenic cows (mean $\pm$ SD, g/L)			Nontransgenic cows (Mean $\pm$ SD, g/L)
	Xingwa	Longwa	Huiwa	
rHuman α -LA	1.13 $\pm$ 0.06	1.55 $\pm$ 0.26	0.17 $\pm$ 0.02	—
Bovine α -LA	1.01 $\pm$ 0.01	0.78 $\pm$ 0.01	1.2 $\pm$ 0.04	1.03 $\pm$ 0.09

Figure 2. Purification of recombinant human α -LA. (A) Anion exchange chromatography. The arrow indicates the fraction containing recombinant human α -LA free of bovine α -LA. (B) Gel filtration. The purified recombinant human α -LA was achieved by gel filtration chromatography. Purified recombinant protein was separated with native PAGE (upper insert in left panel) and then blotted for identification (lower insert in left panel). Human and bovine natural α -LA (hLA and bLA) were used as a positive and negative controls, respectively. The inset of gel electrophoresis showed the purity of recombinant protein separated by decreasing SDS-PAGE and stained by SYPRO Ruby gel stain (Invitrogen, Carlsbad, CA).

nant human α -LA was correctly folded, we compared the electrophoretic shift of α -LA treated with CaCl_2 or ethylene glycol tetraacetic acid using native PAGE. The Ca^{2+} -binding loop is important for correct folding of α -LA (Rao and Brew, 1989) and is located at the junction of the protein α - and β -subdomains. It is formed by 4 aspartic acid residues, 1 lysine residue, and 1 or 2 water molecules and is closed by a disulfide bridge (Permyakov and Berliner, 2000). Mutation of these residues or breakage of the disulfide bond destroys the Ca^{2+} -binding loop and prevents proper folding (Redfield et al., 1999; Permyakov et al., 2001). Upon loss of Ca^{2+} , the negative charges on the aspartic acid residues are exposed and are responsible for a

Figure 3. Lactose synthesis and Ca^{2+} binding shift. (A) Lactose synthesis stimulated by recombinant human α -LA in vitro. Human α -LA (hLA) was used as positive control. (B) Ca^{2+} binding shift on native PAGE. Human α -LA, bovine α -LA, and recombinant human α -LA were treated with CaCl_2 and ethylene glycol tetraacetic acid (EGTA), respectively. Conc = concentration.

change of electrophoretic mobility on native PAGE. As demonstrated in Figure 3B, the Ca^{2+} -induced change in mobility was identical between recombinant and natural human α -LA, indicating that the Ca^{2+} -binding loop in the recombinant protein was perfectly constructed and the recombinant human α -LA was similar to its natural counterpart with respect to Ca^{2+} binding ability.

Glycosylation Modification. In humans, a relatively small proportion of α -LA (about 1%) is glycosylated at ^{71}Asn in the unusual triplet sequence $^{71}\text{Asn-Ile-}^{73}\text{Cys}$ rather than in the general consensus sequence Asn-X-Ser/Thr (Giuffrida et al., 1997). The glycosylated bovine α -LA (3 to 5% of total protein) also has been reported (Barman, 1970), but the specific glycosylation site is unknown. To verify whether the recombinant human α -LA present in the transgenic cow milk was glycosylated, all of the *N*-linked glycoproteins in the transgenic whey were enriched by means of concanavalin A affinity chromatography, and then the enriched fractions containing glycosylated α -LA were separated via gel filtration (Figure S2A and B, which can be found online at <http://jds.fass.org/content/vol91/issue12/>). The purified glycosylated α -LA was digested by trypsin. To obtain appropriate length of

Figure 4. Identification of glycosylation site of α -LA. (A) Electrospray ionization mass spectrometry for deglycosylated peptides. The circle indicated characteristic peptide for glycosylation, which was selected as a precursor ion for tandem mass spectrometry (MS/MS). (B) MS/MS for glycosylated peptide. Charge of fragment ions was +3, and Xcor was equal to 5.82. Glycosylation sequence and site (71 Asn) are indicated on the top of this figure.

peptides adapted to subsequent identification by MS, these trypsin-digested peptides were further treated by endoproteinase Glu-C. The resulting peptides were deglycosylated by PNGase F (Sigma), causing release of the oligosaccharide moiety from asparagine with a concomitant asparagine-to-aspartic acid transition. Peptide fragments were then analyzed by electrospray ionization MS/MS combined with microscale reverse-phase high performance liquid chromatography. The peptides affected by PNGase F(Sigma) treatment were screened by electrospray ionization MS (Figure 4A) and further deduced by MS/MS (Figure 4B). The glycosylated peptide was identified and determined to contain the sequence IWCKDDQNPHSSNICNISC DK. This

was matched with amino acid sequence of the bovine α -LA and the carbohydrates attached at 71 Asn in the sequence 71 Asn-Ile- 73 Cys. Taken together, these results suggested that the recombinant human α -LA might not be glycosylated in transgenic cows.

Components of Transgenic Milk

Both transgenic cow milk and control cow milk were collected at 3 different time points during 1 mo of hormone-induced lactation. We compared the lactose, total protein, total fat, and total solids in milk from transgenic and nontransgenic cows (Figure 5A) and found that the composition of the 2 milk types did not

Figure 5. Analysis of milk composition. (A) Total fat, total protein, lactose, and total solids in transgenic and nontransgenic milk. (B) Total protein profile in skim milk from transgenic recombinant human α -LA (rhLA for lanes 4 to 6) and nontransgenic cows (bMilk for lanes 8 to 10) separated by decreasing SDS-PAGE. Lane 1 = molecular mass markers; lane 2 = the purified natural human α -LA (hLA); lane 3 = human skim milk (hMilk); lane 7 = transgenic cow skim milk containing recombinant human lactoferrin (rhLF). Ig_H = heavy chain of immunoglobulin; LF = lactoferrin; Ig_L = light chain of immunoglobulin; SA = serum albumin.

differ substantially and these parameters were within the normal range of milk components (Walstra and Jenness, 1984).

We then compared the individual protein components in transgenic milk with nontransgenic milk. Protein components in skim milk were initially separated by SDS-PAGE. The major proteins in skim milk are shown in Figure 5B. Four kinds of CN, α _{S1}-CN, α _{S2}-CN, β -CN, and κ -CN, and the major whey proteins α -LA, β -LG, serum albumin, and immunoglobulins had similar distribution patterns in milk from transgenic and nontransgenic cows. To make more specific comparisons, we performed 2-dimensional gel electrophoresis to separate the proteins in either skim milk or whey using

immobilized pH gradient gel strips with a narrow pH range of 3 to 6 or 5 to 8 (Figure 6). The major proteins from skim milk were not qualitatively different between transgenic and nontransgenic samples except that both A and B variants of β -LG were present in transgenic milk as identified by MALDI-TOF MS (Figure 6A, B, E, and F). The whey protein profiles of transgenic and nontransgenic samples presented in Figure 6C, D, G, and H were also similar with respect to protein distribution after removal of the majority of CN by precipitation.

DISCUSSION

Although production of transgenic cattle harboring the human α -LA gene was reported by Eyestone (1999) using pronuclear microinjection, the detailed characterizations of the expression and properties of recombinant human α -LA have not been published. In the current study, we first produced healthy transgenic cows containing human α -LA using the efficient method of SCNT. We compared the physicochemical properties of the resulting recombinant human α -LA and found that they were very similar to those of natural human α -LA. Furthermore, the overall composition of the transgenic milk did not differ from that of nontransgenic milk.

The transgene construct containing 6.5 kbp of 5' flanking sequence and only 159 bp of 3' flanking sequence allowed efficient expression of human α -LA in transgenic cow milk. In this study, the expression level in transgenic cows was comparable with that in transgenic rats and transgenic mice (Fujiwara et al., 2003; Yu et al., 2004) but varied among the 3 transgenic cows, likely due to random integration of the transgene in the genome of transgenic cows. Expression of the transgene did not interfere with expression of bovine α -LA, implicating that the recombinant human α -LA and bovine α -LA can be independently coexpressed in mammary glands under the same gene-regulatory niches. We are currently selecting transgenic cows as founders for propagation of a transgenic population using multiple ovulation and embryo transfer, and many transgenic male and female calves containing the human α -LA gene have been obtained.

The purified recombinant human α -LA shows no difference with the natural counterparts regarding molecular weight, conformation, and bioactivity. Such analyses suggested that recombinant human α -LA from transgenic cow milk is substantially equivalent to its natural counterpart. Furthermore, we did not detect glycosylated recombinant human α -LA in the transgenic milk even though all *N*-linked glycoproteins were enriched with concanavalin A affinity chromatography; however, a portion of natural human α -LA has been

Figure 6. Analysis of protein composition by 2-dimensional electrophoresis. Transgenic and nontransgenic skim milk and whey samples were characterized at linear pH gradient 3 to 6 and 5 to 8. For comparison of skim milk, major proteins in the nontransgenic (A and B) and the transgenic (E and F) were separated. For comparison of proteins in whey, whey proteins in the nontransgenic (C and D) and the transgenic (G and H) were compared. LA = α -LA; LG A and B = A and B variants of β -LG; and Ig_H and Ig_L = heavy or light chains of immunoglobulin; hLA = human α -LA.

reported to be glycosylated at 71 Asn in atypical tripeptides 71 Asn-Ile- 73 Cys (Giuffrida et al., 1997). Interestingly, we identified glycosylation of bovine α -LA at 71 Asn rather than at the predicted 45 Asn, at which site other species, such as rats, glycosylated α -LA (Prasad et al., 1979). There might exist 2 ways that incur the absence of glycosylation of human α -LA in transgenic cows. Due to formation of the disulfide bond between 73 Cys and 91 Cys in nonglycosylated α -LA, which would block the glycosylation reaction, the conformation of glycosylated α -LA is unstable in the endoplasmic reticulum. Thus, the glycosylated recombinant human α -LA might be degraded more severely than the bovine α -LA by a stringent quality control system in mammary gland epithelial cells, as indicated in yeast *Pichia pastoris* that greatly secreted glycosylated recombinant goat α -LA (Saito et al., 2002). Alternatively, it seems likely that the bovine oligosaccharyltransferase would prefer the endogenous α -LA to the recombinant form during the substrate competition.

Although recent reports suggest that milk and meat derived from cloned cattle is as safe to eat as conventional products (Tian et al., 2005; Yamaguchi et al., 2007; Yang et al., 2007), and the final risk assessment of animal cloning is available on the US Food and Drug Administration Web site (<http://www.fda.gov/cvm/cloning.htm>), the safety concerns of transgenic foods such as transgenic milk must be considered in different ways due to genomic alteration of livestock. Several transgenic researches, such as β / κ -CN-transgenic cows

(Laible et al., 2007) and human insulin-like growth factor-I transgenic pigs (Monaco et al., 2005), demonstrated that the components of milk from nontransgenic and transgenic animals were not significantly different. These studies indicate the safety of transgenic cow milk; however, the safety of each specific transgene needs to be evaluated on a case-by-case basis. In the present study, the total protein, fat, lactose, and solids in the transgenic milk also show no material difference with those of nontransgenic milk. In addition, we found only 2 variants of β -LG present in transgenic milk with no qualitative change of the major proteins, such as the CN and endogenous α -LA. The variants A and B of β -LG identified in our transgenic milk have been previously reported to increase milk yield and possibly protein content (Tsiaras et al., 2005; Molina et al., 2006), which might be involved in greater amounts of proteins in transgenic milk and further investigation need to be explored. Taken together, these results established that random insertion of transgenic human α -LA did not lead to altered expression of the major components in milk of transgenic cows.

The concentration of the lactose in transgenic cow milk was not remarkably increased, whereas the physiological function of the recombinant human α -LA to react with β -1,4-galactosyltransferase to produce lactose was normal. In transgenic pigs containing bovine α -LA, greater levels of lactose were only detected early in lactation (d 0), and subsequent samples were not significantly different from controls (Bleck et al., 1998). This

suggests that a threshold of lactose content may exist to maintain proper osmotic pressure, thus controlling the total milk volume. But whether the overexpression of α -LA could accelerate the synthetic velocity of lactose in the Golgi apparatus of the transgenic epithelial cells and thus increase the milk yield remains to be investigated.

In summary, recombinant human α -LA expressed in milk from transgenic cows had the same physicochemical characteristics as the natural counterpart and did not show any side effect on transgenic milk. Because the recombinant human α -LA is equivalent to natural human α -LA, utilization of this recombinant protein as an additive to infant formula and health-promoting foods is likely to be safe. Increased expression of α -LA has great implications, such as increasing milk production and ultimately increasing growth of offspring in transgenic pigs and mice (Boston et al., 2001; Noble et al., 2002). In this respect, expression of human α -LA in transgenic cow milk would benefit the dairy industry and more importantly would increase the nutritional value and suitability of milk for human consumption.

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