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The Expression Changes of Casein mRNAs in Mammary Epithelial Cells Recovered from Bovine Milk during the Lactation Period

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ABSTRACT : The aim of this study was to examine the correlation between bovine casein (CN) mRNA expression levels in mammary epithelial cells and lactation period, the yields of milk proteins and other parameters. The cells were collected from each cow's milk, which contained somatic cell counts (SCC) of less than 100,000 cells/ml. The levels of αs1-, αs2-, β- and κ-CN mRNA expression were significantly correlated with each other in mammary epithelial cells ($p<0.01$). All cows produced either less than 30 kg/day/cow or a over 30 kg/day/cow level of milk yield (MY). It was shown that the CN mRNA expression levels decreased gradually from the calving period to late lactation, when MY was over 30 kg/day/cow. The SCC tended to increase gradually during the course of lactation, but it was negatively correlated with milk protein and CN yields ($p<0.01$) when MY was less than 30 kg/day/cow. Moreover, there was a tendency for a negative correlation between SCC and αs1-CN and β-CN mRNA expression level, when MY was less than 30 kg/day/cow ($p<0.05$). (**Key Words :** Casein mRNA Expression, Mammary Epithelial Cells, Lactation Period, Milk Yield, Casein Yield, SCC)

INTRODUCTION

Caseins are the major protein components in bovine milk. Bovine casein micelle are made up of αs1-, αs2-, β- and κ-casein (CN), while genetic polymorphisms have also been found (Grosclaude et al., 1979). The casein genes exist on chromosome 6 in the order αs1-CN, β-CN, αs2-CN, and κ-CN within the 200-kb casein locus cluster (Mercier and Vilotte, 1993). The mouse casein micelles consist of α-, β-, γ- and κ-CN, and these genes have a high sequence similarity with those of bovine αs1-, β-, αs2- and κ-CN respectively (Mercier and Vilotte, 1993; Kuraishi et al., 2000). In mouse β- and γ-CN mRNA, the length of the poly-(A) tail was decreased by weaning during 24 h after parturition, whereas the poly-(A) tail length for both CN mRNAs was increased after nursing during 12 h (Kuraishi et al., 2000). Travers et al. (1996) reported that the level of CN mRNA declined after weaning in rats. Not enough studies have been done on CN mRNA expression in bovine mammary epithelial cells, which are collected from raw milk, but also from rats and mice. As dairy cows are usually separated from their calf immediately after calving, they do not receive suckling from their calf, but this differs in the case of nursing or weaning of rats. It is of great interest to establish whether the CN mRNA expression levels in bovine mammary epithelial cells, which were collected from raw milk, change during the course of lactation and correlate with the milk yield, milk protein and CN yield, and the somatic cell count (SCC) level.

The aim of this study was to investigate whether the bovine CN mRNA expression levels in mammary epithelial cells collected from raw milk, in which the SCC level was below 100,000 cells/ml, changed during the course of lactation and also whether this increase or decrease was correlated with milk yield and other parameters.

MATERIALS AND METHODS

Animals and sample collection

Seventeen dairy cows with an SCC of less than 100,000 cells/ml in each of four milk teats, which are free of

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Table 1. Sequence of RT-PCR primer pairs for bovine CN mRNAs

Primer		Sequence (5'→3')
αs1-CN	Sense	GTCAAGTGAATTCTGAGG
	Antisene	TGGCACTTACAGGAGAAG
αs2-CN	Sense	AAGAAGACCGTTGACATGGAATC
	Antisene	GCTGGTTGTATGAAGTAAAGTGG
β-CN	Sense	TGTGGTGGTGCCGCCTTTCCTTTCA
	Antisene	CTCTGGGGATAGGGCACTGCTTTCT
κ-CN	Sense	GCAAAACCAAGAACAACCAATACGC
	Antisene	TAAATGATAAATGTGGGTGTGGGTG
GAPDH	Sense	GGGCTGCTTTTAATTCTGG
	Antisene	TGACAATCTTGAGGGTGTTA

Table 2. PCR profile times, temperature and the cycles (for αs1-, αs2-, β-, κ-CN: 25 cycles, GAPDH: 40 cycles)

Intial step	Melting	Annealing	Extension	Final step
3 minutes	30 seconds	30 seconds	60 seconds	7 minutes
94°C (one cycle)	94°C	55°C	72°C	72°C

subclinical mastitis, were selected and a total of 58 samples of foremilk were collected during lactation period. The SCC in each teat's milk was measured by Fossomatic 5500 (Foss Electric, Hillerod, Denmark) on the day prior to sampling. About 45 ml of the milk sample was collected into a 50 ml sterilized vessel from the four teats of each cow, and then was immediately cooled in ice water, followed by examination within 5 h using RT-PCR analysis.

RNA preparation

RNA extraction : Seven hundred fifty μl of TOLIZOL LS reagent (Invitrogen, USA) was added into 250 μl of each milk sample, followed by stirring heavily for 15 sec, and then the solution was placed for 5 min at room temperature. Trichloromethane ($CHCl_3$) was added into this mixture and the solution was then centrifuged for 15 min at 4°C, at 14,000 rpm. Five hundred μl of 2-propanol was added into the upper layer which contained RNA, and the solution was centrifuged for 30 min at 4°C, at 14,000 rpm. Subsequently 500 μl of 80% ethanol was added into the precipitate, and then the solution was centrifuged for 10 min at 4°C, at 14,000 rpm. Then 50 μl of sterilized-distilled water was added into the precipitate, followed by sufficient mixing. To quantify the amount of total RNA extracted, the optical density (OD) of the RNA stock solution was determined at 260 nm. In addition, the $OD_{260\ nm}/OD_{280\ nm}$ (nucleic acid/protein) absorption ratio was measured.

DNase digestion : Eight μl DNase reaction mixture (1 U DNase, 5 μl DNase buffer and 20 U RNase inhibitor) (Promega, Madison) were added into the 6 μg of RNA stock solution. This mixed solution was filled up to 50 μl by adding sterilized-distilled water, and then the solution was incubated for 60 min at 37°C. Subsequently 100 μl of phenol/$CHCl_3$/2-propanol (25:24:1, v/v/v) solution was added, followed by stirring heavily for 15 sec, and then was centrifuged for 1 min at 4°C, at 14,000 rpm. Ten μl of 3 M NaOAc and 300 μl of 99.5% ethanol were added into the upper layer, and then the solution was well stirred, followed by centrifugation for 30 min at 4°C, at 14,000 rpm. Then 150 μl of 80% ethanol was added into the precipitate, and the solution was centrifuged for 5 min at 4°C, at 14,000 rpm. Then the precipitate was mixed into 42 μl of sterilized-distilled water.

RP-PCR

RT-PCR reaction : Seven μl of the solution which contained 1 μg RNA and 0.5 μg oligo (dT) primer (GIBCO BRL, USA) was incubated for 10 min at 70°C, and then was immediately cooled for 1 min with ice water. The primer sequences used for target genes are shown in Table 1. Then 20 μl of RT reaction mixture (4 μl 5×1st standard buffer (GIBCO BRL, USA), 4 μl 2.5 mM dNTPs (TaKaRa, Japan), 2 μl 0.1 M dithiothreitol (GIBCO BRL, USA), 0.5 U RNase inhibitor (Promega, USA), and 200 U MMLV Reverse Transcriptase (GIBCO BRL, USA) were added, followed by incubation for 50 min at 42°C. Subsequently the solution was incubated for 15 min at 70°C, and was then immediately cooled for 1 min with ice water. Thereafter 1 μl RNase H (GIBCO BRL, USA) was added into the mixture, which was then incubated for 20 min at 37°C. One μl cDNA was added to 49 μl of PCR mixture (5 μl 10×PCR buffer (TaKaRa, Japan), 4 μl 2.5 mM dNTPs (TakaRa, Japan), and 0.25 μl of each specific primer listed in Table 1), and then 0.25 μl ExTaq DNA polymerase (TakaRa, Japan) and 37.25 μl distilled water were added to this solution. This prepared reaction solution was amplified in an RT-PCR Thermal Cycler (Gene Amp. PCR System 24000, Perkin Elmer). All PCR reactions are listed in Table 2.

Agarose gel electrophoresis : The electrophoresis of

Table 3. Contents of milk and mRNA expression of 4 major casein constitutions (mean±s.d.) in the 3 tested groups (all cows, <30 kg/day and >30 kg/day)

	Milk yield (kg/day)		
	All cows (n = 58)	<30 kg/day (n = 43)	>30 kg/day (n = 15)
Milk yield (kg/day)	29.2±7.6	25.2±4.0	38.5±5.3
Lactation period[1]	172.1±94.6	211.0±83.8	81.3±40.3
αs1-casein mRNA[2]	0.99±0.32	1.03±0.31	0.88±0.34
αs2-casein mRNA[2]	0.95±0.51	1.05±0.52	0.72±0.41
β-Casein mRNA[2]	1.21±0.55	1.31±0.54	0.96±0.53
κ-CaseinN mRNA[2]	1.02±0.59	1.13±0.59	0.76±0.50
Protein (%)	3.33±0.23	3.34±0.25	3. 30±0.19
Casein (%)	2.46±0.25	2.49±0.25	2.39±0.24
Protein yield (kg)	0.97±0.25	0.84±0.14	1.27±0.19
Casein yield (kg)	0.72±0.18	0.63±0.12	0.92±0.14
SCC	4.41±0.31	4.46±0.32	4.30±0.25

[1] Days post partum.
[2] Each casein mRNA expression level is indicated by the rate of the strength of the stained band compared with that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.
[3] The yields of milk protein and casein were calculated by the equation (the milk yield×(%) of each component).

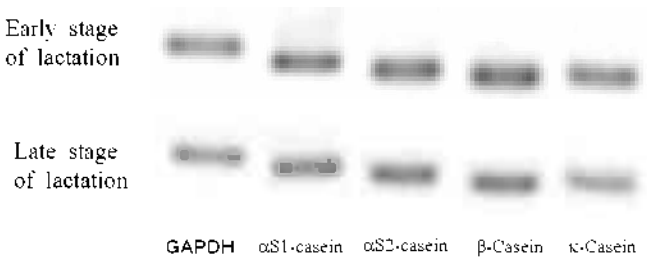

Figure 1. The mRNA expression patterns of GAPDH and casein components in bovine mammary epithelial cells in milk of >30kg/day during the lactation period.

PCR products were performed in 2% agarose gels, and bands were stained with ethidium bromide (0.1 μg/ml), and photographed. Thereafter the bands of these CN mRNAs were determined by an ATTO Densitograph software (ATTO, Tokyo, Japan).

The ratio of each CN mRNAs' expression level was calculated in comparison with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level as the standard of mRNA expression.

Milk analyses

Milk samples were analyzed for the concentration of milk protein and SCC with a Milkoscan 6000 and a Fossomatic 5500 (Foss Electric, Hillerod, Denmark), respectively. Casein was prepared at pH4.6, by titrating with 0.01 N HCl. The nitrogen (%) of milk protein and CN were measured using semimicro-Kjeldahl techniques (AOAC). Casein components were separated by Urea-PAGE electrophoresis, and the bands were stained with mix-staining solution (acetic acid:methanol:distilled water = 1:3:6, v/v/v, with 0.25% brilliant blue G coloring added), and then photographed. The composition of CN was calculated from the peak area of each stained band which was measured by ATTO Densitograph software (ATTO, Tokyo, Japan).

Statistical analysis

The data are demonstrated as the mean±standard deviation (Table 3). Statistical analysis was carried out using a student's t-test (SAS program). Differences were considered to be significant at $p<0.01$ or $p<0.05$.

RESULTS

Milk yield (MY), lactation period, CN mRNA expression level, CN yield and SCC

Cows with an SCC of less than 100,000 cells/ml in all teats' milk were used in this study. The SCC in teat milk was measured on the day prior to sampling. The data in Table 3 were analyzed in 3 groups (all cows, MY <30 kg/day/cow, and MY >30 kg/day/cow). A typical profile of each CN mRNA expression and GAPDH is shown in Figure 1. In this study, cows in which MY was >30 kg/day/cow were between early and mid lactation, whereas those in which MY was <30 kg/day/cow tended to be in the mid to late stage of lactation. The CN mRNA expression levels were higher in MY at <30 kg/day/cow than at MY >30 kg/day/cow.

Correlation between CN mRNA expression levels and other parameters

Casein mRNA expression levels were significantly correlated with each other ($p<0.01$) in all cows, at MY <30 kg/day/cow and at MY >30 kg/day/cow. The correlations between αs1-CN, αs2-CN, β-CN, and κ-CN mRNA expression levels and the course of lactation at MY >30 kg/day/cow are shown in Figure 2-5. It was recognized that

Table 4. Correlation coefficient between each parameter

	kg/day	Lactation period[1]	αs1-CN mRNA	αs2-CN mRNA	β-CN mRNA	κ-CN mRNA	Protein yield (kg)[3]	Casein yield (kg)[3]	SCC[4]
Milk yield (kg)	<30	-0.72**	-0.18	-0.26	-0.28	-0.26	0.96**	0.92**	-0.46**
	>30	0.15	-0.38	-0.29	-0.42	-0.24	0.91**	0.74**	-0.05
Lactation period[1]	<30		-0.003	0.14	0.07	0.12	-0.63**	-0.65**	0.53**
	>30		-0.64**	-0.60**	-0.65**	-0.53*	0.17	0.13	-0.07
αs1-CN mRNA[2]	<30			0.65**	0.72**	0.67**	0.14	0.07	-0.37*
	>30			0.70**	0.79**	0.70**	-0.40	-0.42	-0.11
αs2-CN mRNA[2]	<30				0.88**	0.91**	0.05	-0.14	-0.25
	>30				0.90**	0.95**	-0.27	-0.21	-0.33
β-CN mRNA[2]	<30					0.92**	0.05	-0.05	-0.36*
	>30					0.92**	-0.48	-0.36	0.18
κ-CN mRNA[2]	<30						-0.02	-0.12	-0.26
	>30						-0.29	-0.17	0.27
Protein yield (kg)[3]	<30							0.87**	-0.49**
	>30							0.67**	0.12
Casein yield (kg)[3]	<30								-0.53**
	>30								-0.20

[1] Days post partum.
[2] Each casein mRNA expression level is indicated by the rate of the strength of stained band compared with that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.
[3] Calculation by the rate (%) of each component×milk yield.
[4] SCC in the rage of <100,000/ml. ** p<0.01, * p<0.05.

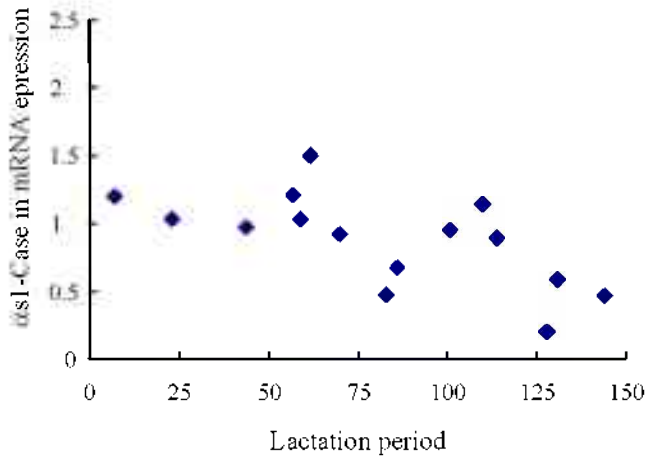

Figure 2. The distribution of the rates of αs1-CN mRNA expression compared with GAPDH mRNA expression in bovine mammary epithelial cells in milk of >30 kg/day/cow during the lactation period.

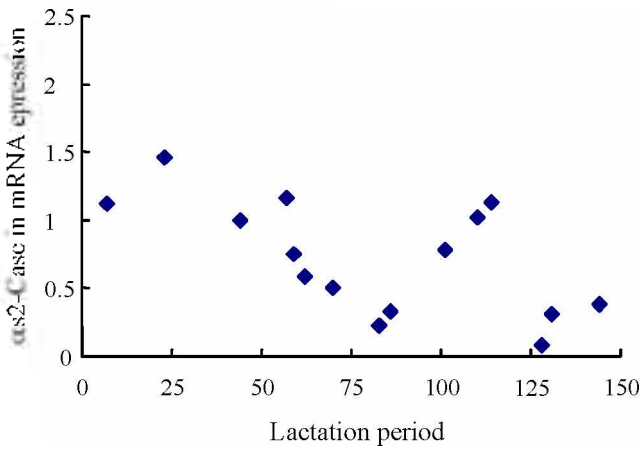

Figure 3. The distribution of the rates of αs2-CN mRNA expression compared with GAPDH mRNA expression in bovine mammary epithelial cells in milk of >30 kg/day/cow during the lactation period.

CN mRNA expression levels at MY >30 kg/day/cow were negatively correlated with the course of lactation ($p<0.01$). When MY was <30 kg/day/cow, it seemed to be negatively correlated between αs1-CN and β-CN mRNA expression levels and SCC ($p<0.05$).

Milk yield was negatively correlated with the course of lactation and SCC in <100,000 cells/ml under MY at 30 kg/day/cow ($p<0.01$). Milk yield was positively correlated with milk protein and CN yields ($p<0.01$). When MY was <30 kg/day/cow, SCC showed a positive correlation with the course of lactation, but was negatively correlated with protein and CN yields ($p<0.01$).

DISCUSSION

The αs1-CN, αs2-CN, β-CN and κ-CN mRNA expression levels were significantly correlated with each other in mammary epithelial cells which were collected from teat milk samples ($p<0.01$). It was confirmed that the SCC was less than 100,000 cells/ml in all quarters tested, and that the tested teat milk were free of subclinical mastitis. It was found that αs1-CN, αs2-CN, β-CN and κ-CN mRNA expression levels in mammary epithelial cells in bovine milk decreased gradually from the calving period to late lactation, in this study. The strength of CN mRNA

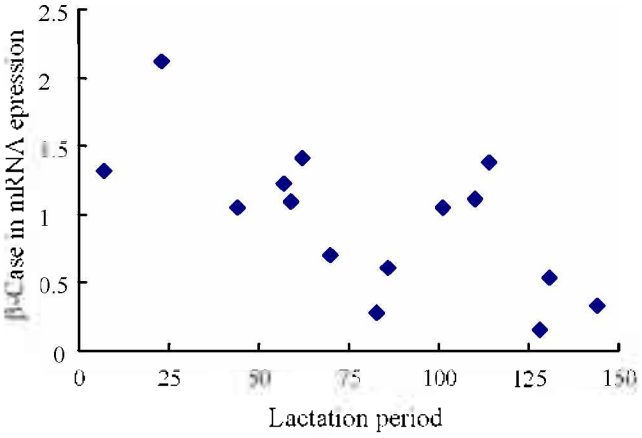

Figure 4. The distributions of the rates of β-CN mRNA expression compared with GAPDH mRNA expression in bovine mammary epithelial cells in milk of >30 kg/day/cow during the lactation period.

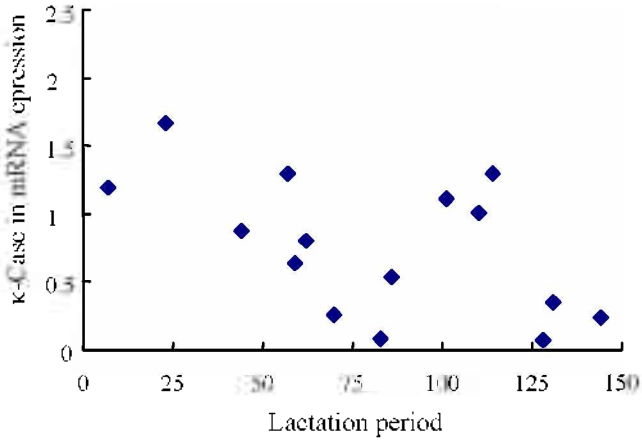

Figure 5. Distribution of the rates of κ-CN mRNA expression compared with GAPDH mRNA expression in bovine mammary epithelial cells in milk of >30 kg/day/cow during the lactation period.

expression was observed at an MY of >30 kg/day/cow. But there was no relation between CN mRNA expression level and MY, because CN mRNA expression levels decreased linearly from the calving period to late lactation, whereas MY increased gradually from the calving period to about 60 days post partum, and then decreased gradually toward the dry stage. It was of importance that there was a significant negative correlation between CN mRNA expression levels and the course of lactation at MY >30 kg/day/cow. The effect of the stage of lactation on MY was probably due to physiological changes in the number and activity of secretory cells within the mammary gland (Auldist et al., 1998). Travers et al. (1996) stated that the effects of hormone deprivation on milk protein synthesis could be explained in terms of the loss of secretory cells, and that this loss of cells involved programmed cell death. Schmitz et al. (2004) reported that κ-CN concentration in milk was different between SCC less than 150,000 cells/ml and an SCC of 150,000-300,000 cells/ml in quarters, whereas the other parameters referring to mRNA expression and concentration in milk were not different between quarters. Schmitz et al. (2004) also reported that milk cells did not show significant differences in mRNA expression, comparing SCC less than 150,000 cells/ml with those having elevated SCC during subclinical mastitis. In this study, cows with SCC of less than 100,000 cells/ml were used (these cows were free of subclinical mastitis), and it was recognized that αs1-CN and β-CN mRNA expression levels in bovine mammary cells in teat milk tended to correlate negatively with SCC, at MY <30 kg/day/cow ($p<0.05$). But these results of CN mRNA expression levels in this study were different from the observations by Schmitz et al. (2004) and Didier and Bruckmaier (2004), who examined the features of subclinical mastitis milk with an SCC between 150,000 and 300,000 cells/ml, and with an SCC <150,000 and an SCC >150,000 cells/ml, respectively. Ng-Kwai-Hang et al. (1987) reported that the genotypic variants of CN resulted in a great variation in the concentration of CN. In this study, the genotypes of each CN component in bovine mammary epithelial cells were not analyzed.

In the present study, it was indicated that CN mRNA expression levels tended to decrease gradually from the calving period to late lactation. But the mechanisms and factors resulting in these trends were not elucidated. Thus it is necessary to further investigate this phenomenon.

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