Effect of Stage of Lactation and Parity on Mammary Gland Cell Renewal¹

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ABSTRACT

Milk production is a function of the number and activity of mammary epithelial cells, regardless of stage of lactation. Milk yield is generally higher in multiparous cows than in primiparous cows, but persistency is usually greater in the latter group. We compared several measures related to metabolic activity, apoptosis, and endocrine control of mammary cell growth in 8 primiparous and 9 multiparous cows throughout lactation. Mammary gland biopsies were taken in early [10 d in milk (DIM)], peak (50 DIM), and late (250 DIM) lactation to evaluate gene expression and determine DNA and fatty acid synthase (FAS) content. Milk samples taken the day before the biopsies were used to detect protease activities and to determine stanniocalcin-1 (STC) concentrations. Blood samples served to measure insulin-like growth factor-1, prolactin, and STC concentrations. Milk yield was higher in multiparous cows than in primiparous cows at the 10 DIM (32.8 ± 1.3 and 25.2 ± 0.8 kg/d) and 50 DIM (38.0 ± 1.2 and 29.8 ± 1.1 kg/d), but it was the same for both groups at 250 DIM (23.9 ± 1.5 and 23.8 ± 1.1 kg/d). Except for stearoyl-coenzyme A desaturase, expression of genes related to milk synthesis was not affected by stage of lactation. However, gene expression of acetyl-coenzyme A carboxylase, β -casein, and FAS was lower in early lactation in primiparous cows. Expression of both proapoptotic *bax* and antiapoptotic *bcl-2* genes was higher in primiparous cows, whereas the *bax*-to-*bcl-2* ratio was not changed. Mammary DNA concentration was higher in multiparous cows, as was the amount of FAS protein in early lactation. Two bands of protease activity were found in milk samples, and one of the bands had an apparent molecular weight similar to gelatinase A and was dependent on the stage of lactation. Serum insulin-like growth factor-1 increased with day of lactation and

was higher in primiparous cows. Serum prolactin decreased in late lactation, but peak values were observed in early lactation for primiparous cows and peak lactation for multiparous cows. Milk STC content increased with advancing lactation. The results are consistent with a lower degree of differentiation and a greater capacity for cell renewal in the mammary gland of primiparous cows.

Key words: lactation, stanniocalcin, dairy cow, persistency

INTRODUCTION

Current dairy practices have embraced the intensive production concept of maximizing peak lactation milk output and minimizing the calving interval. However, very high yields, especially in early lactation, cause general and widespread concerns about metabolic and reproductive diseases. Therefore, minimizing the proportion of lifetime spent in early lactation by extending lactation may be a strategy worth considering for improving the longevity of dairy cows. However, longer lactations are more profitable when they are combined with a strategy that reduces the rate of decline in yield after peak production (van Amburgh et al., 1997).

From a mammary gland perspective, milk production is simply a function of the number of secretory cells and the secretory activity of these cells. In goats, the number of secretory cells is maximal at the initiation of lactation, and the increase in milk production that occurs in early lactation is due to cell differentiation (Knight and Wilde, 1993). After peak lactation, the differentiation state of the tissue is maintained constant throughout declining lactation, and the loss of secretory cells accounts for the decrease in milk yield. It is well established in rodents and ruminants that mammary cell loss during involution occurs through programmed cell death (apoptosis; Wilde et al., 1997a). Thus, in cows, apoptosis accounts for the progressive loss of secretory cells in declining lactation (Wilde et al., 1997b; Capuco et al., 2001). As a result, there is considerable incentive to identify the factors that regulate mammary

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apoptosis, in particular during these early stages of gradual involution.

Although some approaches have slightly improved the persistency of dairy cows, none have really changed the shape of the lactation curve. In fact, the only factor that consistently affects persistency is parity. Indeed, primiparous cows are well known for being highly persistent. Therefore, the objective of this study was to analyze factors affecting the mammary gland of multiparous and primiparous cows during the different phases of lactation.

MATERIALS AND METHODS

Animals and Experimental Procedures

Twenty Holstein cows, 10 primiparous and 10 multiparous, were housed at the Dairy and Swine Research and Development Center of Agriculture and Agri-Food Canada in Sherbrooke, Quebec. The animals were milked twice daily and exposed to a photoperiod of 15 h of light and 9 h of darkness. They received 6 meals per day with free access to water. The cows were fed TMR according to production level. Three different rations were used based on a mix containing 40% corn silage and 60% grass silage. Ration 1, with a fat content of 3.8% and a protein content of 3.4%, was formulated to meet the needs of a 600-kg cow producing 40 kg/d of milk. Ration 2, with a fat content of 3.9% and a protein content of 3.4%, was formulated to meet the needs of a cow producing 30 kg/d of milk. Ration 3, with a fat content of 3.95% and a protein content of 3.45%, was formulated to meet the needs of a cow producing 20 kg/d of milk. In addition to the silages, the cows received dry hay, barley, high-moisture corn, a commercial dairy supplement, and a mineral supplement. Soybean cake was provided for rations 1 and 2. Milk production was monitored for a complete lactation. The multiparous cows had 2 to 5 lactations. Biopsies were taken from the upper portion of the mammary gland in early (10 DIM), peak (50 DIM), and late (250 DIM) lactation, using the method of Farr et al. (1996) and alternating between the left and right hindquarters. Tissue obtained from the biopsies was rinsed in sterile saline solution to remove all traces of blood, cut into 3 parts, immediately frozen in liquid nitrogen, and stored at -80°C . Each of the 3 parts was intended for separate analyses of RNA, DNA, and protein. Milk samples (combined a.m. and p.m. milkings) were taken the day before the biopsy and once weekly for the following 2 wk. The SCC, protein, lactose, and fat contents were measured using the Program d'Analyse des Troupeaux Laitiers du Québec (Sainte-Anne-de-Bellevue, Quebec, Canada). Another portion of the milk samples was centrifuged ($5,000 \times g$, 10 min, 4°C) to remove the somatic

cells and fat before aliquots were made and frozen at -20°C for subsequent analyses. A blood sample was collected from the tail vein the day before the biopsies. It was necessary to withdraw 2 primiparous cows and 1 multiparous cow from the project for health reasons.

Real-Time Reverse Transcription-PCR

Extraction of RNA from mammary tissue samples and determination of its concentration were performed as described by Delbecchi et al. (2005). The integrity and concentration of the RNA were verified by analyzing 2 μg of each sample on a 1.2% agarose gel containing ethidium bromide (1 $\mu\text{g}/\text{mL}$) and taking a picture under UV light (305 nm) with Polaroid 57 film.

Treatment of RNA samples with RNase-free DNase I, reverse transcription, and real-time PCR analysis using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) were conducted as described by Delbecchi et al. (2005). Primers for real-time PCR were designed using Primer Express 1.0 software (Applied Biosystems; Table 1) and synthesized by PE Oligofactory (Applied Biosystems). For each gene analyzed, a standard curve made with serial dilutions of a pool of reverse transcripts was utilized for quantifying mRNA levels. These levels were normalized with values obtained for the housekeeping gene, GAPDH.

Quantification of DNA and Protein in Mammary Tissue

The method used for DNA quantification was developed by Labarca and Paigen (1980). Briefly, the mammary gland tissue samples were weighed, homogenized in 2 mL of PBS containing 2 mM EDTA with a Polytron homogenizer (Kinematica GmbH, Lucerne, Switzerland) at power 5 to 6 for approximately 10 s, and brought to a concentration of 5 mg/mL with PBS. One hundred microliters of homogenate (0.5 mg) was added to 1.5 mL of a 0.5 $\mu\text{g}/\text{mL}$ solution of Hoechst 33258 reagent (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada). After incubation for 1 h in the dark at room temperature, samples were analyzed in a spectrofluorimeter (PerkinElmer LS3B; PerkinElmer, Wellesley, MA) at an excitation wavelength of 356 nm and an emission wavelength of 458 nm. The concentration of DNA in samples was determined using a standard curve generated with different amounts of calf thymus DNA (Sigma-Aldrich). Measurements were performed in triplicate.

The portion of the mammary gland biopsy reserved for protein analysis was homogenized at 4°C in a buffer (15 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.2% SDS, 0.5% sodium deoxycholate, 150 mM NaCl) containing

Table 1. Primers used during real-time PCR

Gene of interest ¹	GenBank number	Primer sequence (5'–3')	Orientation	Amplicon length, bp	Primer concentration, ² nM
ACC	AJ312201	TTTGTCCTCCTGGGTTTAAAGC	Sense	70	300
		GCTGTCGGCCTTGTAACACA	Antisense		300
bax	U92569	GACGGCCTCCTCTCCTACTT	Sense	67	300
		AGCACTCCAGCCACAAAGAT	Antisense		300
bcl-2	U92434	ACGCCTTTGTGGAGCTGTATG	Sense	69	300
		CCTTCAGAGACAGCCAGGAGA	Antisense		300
β -CN	X14711	TGAAGGTCCTCATCCTTGCCT	Sense	75	300
		CACCAGGTACATTGAGTTCTTCCAG	Antisense		300
FAS	AF479289	GTTCCTCCAGCTCAACGAA	Sense	67	300
		GGACATGCTGCTCAAAGGATG	Antisense		300
fos	AF069515	TCTGAGGAGGCCTTACCCT	Sense	70	300
		TCTTGACGGGCTCCACTGAG	Antisense		300
GAPDH	AJ000039	GCATCGTGGAGGGACTTATGA	Sense	67	300
		GGGCCATCCACAGTCTTCTG	Antisense		300
IGF-I	AF404761	CCTGCGCAATGGAATAAAGTC	Sense	82	300
		AGGCAAAAAGAAATCCAGGGA	Antisense		300
IGFBP-5	S52657	AAAGAGCTACCGCGAGCAAG	Sense	77	300
		CTGCCATCTCGGAGGTGGT	Antisense		300
LPL	M16966	CAGAAGCTCCAAGTCGCCTTT	Sense	73	300
		GACCCCTGGTGAATGTGTG	Antisense		300
PRLR		ACACACGGAGCAAGGCGT	Sense	*	300
Long ³		TGCCCCGGCCAGAGT	Antisense		300
PRLR		GAACCTCAGGCCCATCCCT	Sense	*	300
Short ³		TCCGGATTCTCCAGCTTCTC	Antisense		300
SCD	AF481915	CCTGTGGAGTCACCGAACCT	Sense	66	300
		GTGTTGCCAATGATCAGGAAGA	Antisense		300
STC	NM 176669	AGTGATTGCTGCCTCAACA	Sense	62	300
		TCTCCAGGCATGCAAAAGCT	Antisense		300
TGF β -1	M36271	AACAATTCTGGCGCTACCTC	Sense	71	300
		CTCAAAGGACAGCCACTCC	Antisense		300

¹ACC = Acetyl-coenzyme A carboxylase; FAS = fatty acid synthase; IGFBP-5 = IGF binding protein-5; LPL = lipoprotein lipase; PRLR = prolactin receptor; SCD = stearoyl-coenzyme A desaturase; STC = stannio-calcin; TGF β -1 = transforming growth factor β 1.

²In the real-time PCR reaction.

³Generously provided by G. Dahl, University of Illinois, Urbana-Champaign.

a Complete Protease Inhibitor Cocktail Tablet (Roche, Laval, Quebec, Canada) for 10 mL of solution. The volume of buffer used (in microliters) was equal to 15 times the weight of the tissue sample (in milligrams). The homogenate obtained was agitated at 4°C for 1 h, and then centrifuged at 14,000 $\times g$ for 10 min. The supernatant was collected and frozen in aliquots at –20°C. The quantity of protein contained in the supernatant was determined using the BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL), using BSA as standard and according to the manufacturer's recommendations.

Immunoblotting

Sodium dodecyl sulfate-PAGE was performed on 4 to 7% Tris-HCl gels under reducing conditions according to Laemmli (1970). Each gel (6 $\times$ 8 cm) contained 2 wells reserved for markers of molecular weight (MW), specifically the Kaleidoscope Precision Plus Protein Standards (Bio-Rad, Mississauga, Ontario, Canada)

and the ECL DualVue Marker (Amersham Biosciences, Baie d'Urfé, Quebec, Canada). Migration of samples (25 μ g of protein per well) was performed at 100 V for 90 min for the GAPDH protein analysis and for 150 min for the fatty acid synthase (FAS) protein analysis.

The proteins were then transferred to a nitrocellulose membrane (100 V, 1 h, 4°C, in 25 mM Tris, 192 mM glycine, and 20% methanol) using a Bio-Rad TransBlot Electrophoretic Transfer Cell. The membrane was stained with Ponceau Red to ensure that the quantity of protein transferred was equivalent from one well to the other. After rinsing with water, the membrane was blocked overnight at 4°C with agitation in a Tris-buffered saline (TBS) solution (20 mM Tris-HCl, 137 mM NaCl, pH 7.6) containing 0.1% Tween 20 and 10% powdered skim milk. After washing once for 10 min and twice for 5 min in TBS-0.1% Tween 20, the membrane was incubated for 1 h at room temperature with agitation with the primary antibody diluted in TBS-0.1% Tween 20 containing 5% powdered skim milk. The anti-GAPDH antibody (Abcam, Cambridge, MA) was used

at a 1:5,000 dilution, and the anti-FAS antibody (BD Transduction Laboratories, Mississauga, Ontario, Canada) was used at a 1:1,500 dilution. After washing as above, the membrane was incubated with the horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature with agitation. The secondary antibody used was the one provided with the ECL Chemiluminescence Detection System (Amersham Biosciences), and it was used at a 1:1,000 dilution in TBS-0.1% Tween 20. Membrane detection was performed with the ECL Chemiluminescence Detection System (Amersham Biosciences) according to the manufacturer's recommendations and using Kodak XAR-5 films (Sigma-Aldrich). Developed films were digitized with the FX Pro More Bio-Rad Molecular Imager. The Quantity One program, version 4.5.1 (Bio-Rad) was used to quantify the bands obtained.

Zymograms

The procedure used was modified from Raser et al. (1995). Samples consisting of 5 μ L of skim milk were run through 4 to 10% Tris-glycine gels, with the 10% separating gel containing 2 mg/mL of gelatin (Sigma-Aldrich) as substrate. Samples were diluted 1:1 in the deposition buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 0.005% bromophenol blue, 20% glycerol) before loading. Two wells were reserved for controls, namely, 0.05 μ g of collagenase III (Invitrogen, Carlsbad, CA) and 2 μ g of human plasmin (Sigma-Aldrich). After electrophoresis (4 h at 100 V, 4°C), the gels were gently agitated in a 2.5% solution of Triton X-100 for 30 min at room temperature to permit renaturation of the proteases. The gels were allowed to equilibrate for 30 min in the development buffer (50 mM Tris-HCl, pH 7.6, 200 mM NaCl, 5 mM CaCl₂, 0.02% Brij 35). Fresh buffer was added for a subsequent incubation of 88 h at 37°C without agitation to permit digestion of the gelatin. The gels were then immersed for 30 min in the staining solution [0.5% R-250 Coomassie blue (Bio-Rad), 40% methanol, 10% acetic acid] and destained in a solution of 50% methanol and 10% acetic acid until the desired shade was obtained. The gels were digitized and the files converted into .tif and grayscale formats using the Canvas program (ACD System, Victoria, BC, Canada) before being analyzed with the Quantity One program, version 4.5.1.

Hormones

Serum concentrations of IGF-I and prolactin (PRL) were determined using a double-antibody RIA. Measurement of IGF-I was performed as described by Abribat et al. (1993), with the exception that IGF-I rabbit

antiserum was obtained from the Reproductive Laboratory, USDA, in Beltsville, Maryland. Measurement of PRL was performed using a procedure modified from Lapierre et al. (1990); an incubation for 24 h of serum samples with PRL rabbit antiserum (National Institutes of Health, Bethesda, MD) and iodinated PRL was followed by an incubation for 1 h with secondary antiserum and polyethylene glycol before centrifugation and counting. For both hormones, antirabbit goat antiserum (Linco Research Inc., St. Charles, MO) was used as secondary antiserum. Milk and serum concentrations of STC were determined by RIA in accordance with Niu et al. (2000). The inter- and intraassay coefficients of variation were 13.08 and 7.20% for PRL, 0.04 and 3.49% for IGF-I, and 15.0 and 10.0% for STC.

Statistical Analyses

Statistical analysis of the real-time PCR results was performed in accordance with the recommendations of User Bulletin #2 for the ABI PRISM 7700 Sequence Detection System, December 11, 1997 (Applied Biosystems). Briefly, an ANOVA on the base logarithm (10) of the values normalized individually in relation to GAPDH was performed using the MIXED procedure of SAS (SAS Institute, 2002) with repeated measurements. The results were expressed in the form of log ratios; hence, there is no standard deviation. It should be noted that the coefficients of variation of the Ct (cycle threshold) for each triplicate sample had to be less than 3%. Otherwise, the outlier value was eliminated.

The statistical analysis of the results for milk production, milk components, DNA, and immunoblot assays was performed using the MIXED procedure of SAS with repeated measurements. The PDIF option with Tukey adjustment was used to test differences among least square means. The statistical analyses of the zymogram results were performed with the number of somatic cells as a covariate.

RESULTS

Milk Production and Composition

Milk production was higher ($P < 0.001$; Figure 1) in multiparous cows in early lactation and at the peak of lactation. However, the level of production of multiparous cows decreased more quickly, becoming similar to that of the primiparous group toward the end of lactation. At 250 DIM, the milk yield of both groups was equivalent, 23.9 ± 1.5 kg/d for the multiparous group and 23.8 ± 1.1 kg/d for the primiparous group.

Milk fat and protein contents were not influenced by parity, but protein was at its lowest at the peak of lactation (Table 2). The lactose content varied with both

Figure 1. Milk production $\pm$ standard error of the means of primiparous and multiparous cows.

day of lactation and parity ($P < 0.05$). An interaction was observed between day of lactation and parity ($P < 0.01$) and was related to a lower lactose content for multiparous cows in late lactation ($P < 0.001$). The SCC was not affected by parity, but was at its lowest level at the peak of lactation ($P < 0.05$; Table 2).

Gene Expression

Results concerning the expression of genes related to the synthesis of milk components, namely β -CN, lipoprotein lipase (LPL), FAS, acetyl-coenzyme A carboxylase (ACC), and stearoyl-coenzyme A desaturase (SCD) are shown in Table 3. An interaction between stage of lactation and parity was observed for the expression of the ACC, β -CN, and FAS genes ($P < 0.05$). These interactions were essentially the result of a lower expression of these genes in the mammary gland of primiparous cows in early lactation ($P < 0.05$). No signifi-

cant effect of parity was found on the expression of these genes in peak and late lactation. There was an effect of stage of lactation ($P < 0.001$) on SCD expression.

With respect to the genes related to apoptosis, the expression of the *bax* and *bcl-2* genes was higher in the primiparous group ($P < 0.05$; Table 3), and the expression of the *fos* gene tended to be higher ($P = 0.06$). In addition, the expression of the *bax* gene decreased over time ($P < 0.05$). The apoptotic index, which is calculated as the ratio of the expression of the proapoptotic gene *bax* over the expression of the antiapoptotic gene *bcl-2*, did not vary significantly with stage of lactation or parity, and there was no stage of lactation $\times$ parity interaction (data not shown).

Mammary expression of IGF binding protein-5 (IGFBP-5) did not change during lactation, although it tended to decrease in late lactation ($P = 0.08$; Table 3). Expression of IGF-I, stanniocalcin-1 (STC), transforming growth factor β -1, and long and short forms of

Table 2. Composition (%) of milk from primiparous and multiparous cows at different stages of lactation

Variable	Primiparous			Multiparous			SEM	<i>P</i>		
	Early	Peak	Late	Early	Peak	Late		Time	Parity	Time $\times$ parity
Fat	3.97	3.73	4.27	4.15	3.81	4.14	0.20	0.06	NS	NS
Protein	3.08	2.78	3.64	3.12	2.83	3.57	0.08	0.001	NS	NS
Lactose	4.62	4.69	4.67	4.60	4.69	4.35	0.08	0.01	0.04	0.01
SCC ¹	1.96	1.44	1.81	2.17	1.56	2.07	0.17	.01	NS	NS

¹Log₁₀ transformed values.

Table 3. Gene expression¹ in the mammary gland of primiparous and multiparous cows at different stages of lactation

Gene ²	Primiparous			Multiparous			<i>P</i>		
	Early	Peak	Late	Early	Peak	Late	Time	Parity	Time × parity
ACC	0.11 ^a	0.99	1.06	0.98	0.85	0.25	NS	NS	0.03
β-CN	0.08 ^a	0.35	0.88	0.97	1.20	0.26	NS	0.20	0.05
FAS	0.10 ^a	0.32	1.59	0.72	1.25	0.41	NS	0.20	0.05
SCD	0.03	0.28	2.36	0.33	1.18	0.69	0.001	NS	0.09
LPL	0.11	0.45	1.48	0.72	1.39	0.31	0.15	NS	0.07
bax	2.28	1.87 ^a	1.31	1.59	0.90	0.97	0.02	0.01	NS
bcl-2	1.51	1.35 ^a	1.15	1.00	0.51	0.85	NS	0.02	NS
fos	0.75	0.81	0.87	0.54	0.33	0.34	NS	0.06	NS
IGF-I	1.71	0.23	0.64	0.23	0.80	0.10	NS	0.20	0.09
IGFBP-5	1.89	2.53 ^a	1.14	2.09	0.90	0.85	0.08	0.12	0.10

^aDifferent from multiparous cows ($P < 0.05$).

¹Values obtained were normalized with respect to the levels of GAPDH cDNA. Ratios were transformed to log₁₀ values for statistical analysis. Data are presented as antilog values of the calculated means.

²ACC = Acetyl-coenzyme A carboxylase; FAS = fatty acid synthase; SCD = stearoyl-coenzyme A desaturase; LPL = lipoprotein lipase; IGFBP-5 = IGF binding protein-5.

the PRL receptor (**PRLR**) genes was not affected by parity or stage of lactation (data not shown).

DNA Concentration in Mammary Tissue

A portion of the biopsies was reserved for quantifying DNA to obtain an index of the number of cells with respect to parity and lactation period. A higher concentration of DNA was measured in the biopsies collected from the multiparous cows than the primiparous cows ($P < 0.05$; Table 4). There was no significant effect of stage of lactation on DNA concentration.

Evaluation of the Quantity of FAS

Proteins extracted from mammary biopsies were analyzed by immunoblotting. The quantity of FAS evaluated on immunoblots was normalized to the quantity of GAPDH protein detected on the same immunoblots. Stage of lactation did not significantly influence the value of the FAS-to-GAPDH ratio. However, analysis of this ratio demonstrated an interaction between parity and stage of lactation ($P < 0.03$; Table 4). In early lactation, the FAS-to-GAPDH ratio was lower in primiparous cows than in multiparous cows ($P < 0.05$).

Quantification of Milk Proteolytic Activity

Gelatin zymograms revealed 2 bands of proteolytic activity with apparent MW of 106 and 68 kDa (Figure 2A). The densitometric analysis of the lower MW band (Figure 2B) showed that the band varied during lactation ($P < 0.01$), with the highest activity observed in late lactation ($P < 0.05$). In addition, an interaction between stage of lactation and parity was present ($P < 0.05$) because of higher activity in the milk of multiparous cows in early lactation ($P < 0.05$). The intensity of this band was not affected by the number of somatic cells in the milk.

There was no effect of stage of lactation or parity on the intensity of the upper proteolytic band (data not shown). However, there was a very strong relationship ($P < 0.001$) between this band and the SCC in the milk.

Hormones

Serum concentration of IGF-I increased as lactation advanced ($P < 0.001$; Table 5). In addition, higher concentrations were observed in primiparous cows than in multiparous cows ($P < 0.01$). Prolactin concentration declined in late lactation ($P < 0.01$; Table 5), but peak

Table 4. DNA and fatty acid synthase (FAS) content in the mammary gland of primiparous and multiparous cows at different stages of lactation

Variable	Primiparous			Multiparous			SEM	<i>P</i>		
	Early	Peak	Late	Early	Peak	Late		Time	Parity	Time × parity
DNA, mg/g	0.60	1.25	0.80	2.11	1.99	1.39	0.20	NS	0.005	NS
FAS ¹	0.18 ^a	0.29	0.67	0.62	0.61	0.52	0.12	NS	0.10	0.03

^aDifferent from multiparous cows in early and peak lactation ($P < 0.05$).

¹Log₁₀ [mm² of FAS band/mm² of GAPDH band].

Figure 2. Gelatin zymography of milk from a primiparous cow and a multiparous cow at different stages of lactation. A) Representative zymogram. 1: Plasmin; 2: collagenase; 3, 4, 5: milk from a primiparous cow in early, peak, and late lactation, respectively; 6, 7, 8: milk from a multiparous cow in early, peak, and late lactation, respectively; 9: kaleidoscope prestained standard from Bio-Rad (Mississauga, Ontario, Canada). B) Least squares means of densitometric analysis of the 68-kDa band. Standard error of the least squares means = 9.7. Int = intensity.

values were observed in early lactation for primiparous cows and in peak lactation for multiparous cows (time $\times$ parity, $P < 0.05$). There was an increase ($P < 0.05$) in milk STC with advancement of lactation.

DISCUSSION

As expected, the lactation curve of primiparous cows was much flatter than that of multiparous cows. Despite the higher peak of milk production in multiparous cows,

their lower persistency resulted in similar production for both groups in late lactation.

Knight and Wilde (1993) and Capuco et al. (2001) attributed the increase in milk production to cell differentiation, because indicators of metabolic activity increase during this period. In this experiment, several indicators suggest that the mammary gland was more metabolically active in multiparous cows than in primiparous cows, especially at the onset and peak of lactation. These indicators include the expression of genes related to metabolic activity, and the level of FAS protein in mammary tissue. Indeed, the values determined for these parameters in early lactation were lower in primiparous cows than in multiparous cows. On the other hand, the DNA content of the mammary gland was lower in primiparous cows. Mammary DNA content has been used to evaluate the number of epithelial cells, because they are smaller than stromal cells. This suggests that the lower milk production observed in primiparous cows in early lactation could be related, at least in part, to a lower density of secretory cells.

Changes in the expression of pro- and antiapoptotic factors were not clear enough to provide a definitive interpretation of their involvement in gradual involution. However, late lactation was associated with an increase in gelatinase activity in milk. Politis et al. (1989) indicated that plasmin activity is correlated with gradual involution, but the bands produced by milk on the gelatin zymograms do not correspond to plasmin. The apparent MW of the lower band, 68 kDa, is close to the MW of gelatinase A (72 kDa; Uria and Werb, 1998). Gelatinase A or matrix metalloproteinase-2 has a spectrum of target proteins that gives it the capacity to degrade the basal membrane. In rodents, the activity of this enzyme increases during involution (Talhouk et al., 1992). The extracellular matrix is an important survival factor for mammary epithelial cells (Farrelly et al., 1999), and premature epithelial cell apoptosis was observed in transgenic mice overexpressing a matrix metalloproteinase (stromelysin-1) in the mammary gland (Alexander et al., 1996). Matrix metalloproteinase involvement in cattle involution has not been

Table 5. Concentration (ng/mL) of IGF-I and prolactin in the serum and of stanniocalcin (STC) in the serum and milk from primiparous and multiparous cows at different stages of lactation

Variable	Primiparous			Multiparous			SEM	<i>P</i>		
	Early	Peak	Late	Early	Peak	Late		Time	Parity	Time $\times$ parity
IGF-I	91.0	132.9	169.4	74.2	103.6	130.1	9.0	0.001	0.01	NS
Prolactin	10.8	8.2	6.4	9.5	16.1	3.5	2.1	0.01	NS	0.03
STC serum	3.8	3.3	3.5	4.0	3.5	3.9	0.45	0.06	NS	NS
STC milk	0.15	0.35	0.33	0.20	0.22	0.59	0.11	0.03	NS	0.15

assessed previously, but it is likely that matrix metalloproteinases are important for the control of involution.

The finding that milk STC increased in late lactation confirmed our previous results for this calciotropic hormone and lent support to the hypothesis that it could play a role in the regulation of milk secretion (Delbecchi et al., 2005). Calcium is crucial for the mammary epithelium, as demonstrated by in vitro experiments in which depletion of intracellular calcium inhibits milk protein synthesis (Duncan and Burgoyne, 1996) and the response to prolactin (Bolander, 2002) in mouse mammary epithelial cells. In mammals, the highest levels of STC expression are in the ovaries, and this expression was shown to be greatly enhanced in mice during gestation and lactation. Moreover, STC expression during lactation is entirely dependent on the presence of a suckling litter (Deol et al., 2000). Recently, we found that STC concentration increased in the milk of cows during milk stasis (Tremblay et al., 2006). Although there is still no direct evidence for this, it is tempting to speculate that STC may play a role in mammary involution.

Serum concentration of IGF-I was higher in primiparous cows than in multiparous cows. This hormone is a strong mitogen for the growing mammary gland (Weber et al., 2000) and is an important survival factor for mammary cells (Flint and Knight, 1997). To our knowledge, the effect of chronic administration of IGF-I has not been tested directly on the lactating bovine mammary gland. However, bST administration to lactating cows, which increases the IGF-I circulating level, was shown to increase cellular proliferation without affecting the rate of cell loss by apoptosis (Capuco et al., 2001). Changes in IGF-I concentration are positively correlated with persistency (Sorensen and Knight, 2002). Serum and mammary concentrations of IGF-I are well correlated (Weber et al., 2000) and, in our study, it is likely that mammary cells from primiparous cows were exposed to higher levels of IGF-I.

Because the number of cells present in the mammary gland of cows at calving is already at its peak, little proliferation was assumed to occur during lactation. However, Capuco et al. (2001) showed in multiparous cows that the mammary cell population is much more dynamic than originally believed, with most cells that are present in the gland at the end of lactation having been formed after calving. Therefore, we propose that the lower but flatter lactation curve of primiparous cows is due to the presence of a smaller population of secretory cells in the gland in early lactation. The action of mitogenic or survival factors would induce a higher rate of proliferation in the mammary gland of primiparous cows. These cells would differentiate into lactating cells as lactation progresses under the stimulation of the

milking-induced release of prolactin. Accordingly, an approach resulting in higher circulating levels of mitogenic or survival factors could result in better lactation persistency and allow longer lactation.

In conclusion, lower secretory activity and DNA concentrations were found in the mammary gland of primiparous cows in early lactation. Serum IGF-I increased with the day of lactation and was higher in primiparous cows. These results are consistent with a lower degree of differentiation and a greater capacity for cell renewal in the mammary gland of primiparous cows.

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REFERENCES

- Abribat, T., P. Brazeau, I. Davignon, and D. R. Garrel. 1993. Insulin-like growth factor-I blood levels in severely burned patients: Effects of time post injury, age of patient and severity of burn. *Clin. Endocrinol. (Oxf.)* 39:583–589.
- Alexander, C. M., E. W. Howard, M. J. Bissell, and Z. Werb. 1996. Rescue of mammary epithelial cell apoptosis and entactin degradation by a tissue inhibitor of metalloproteinases-1 transgene. *J. Cell Biol.* 135:1669–1677.
- Bolander, F. F., Jr. 2002. Prolactin activation of mammary nitric oxide synthase: Molecular mechanisms. *J. Mol. Endocrinol.* 28:45–51.
- Capuco, A. V., D. L. Wood, R. Baldwin, K. McLeod, and M. J. Paape. 2001. Mammary cell number, proliferation, and apoptosis during a bovine lactation: Relation to milk production and effect of bST. *J. Dairy Sci.* 84:2177–2187.
- Delbecchi, L., N. Miller, C. Prud'homme, D. Petitclerc, G. F. Wagner, and P. Lacasse. 2005. 17 β -estradiol reduces milk synthesis and increases stanniocalcin gene expression in the mammary gland of lactating cows. *Livest. Prod. Sci.* 98:57–66.
- Deol, H. K., R. Varghese, G. F. Wagner, and G. E. Dimattia. 2000. Dynamic regulation of mouse ovarian stanniocalcin expression during gestation and lactation. *Endocrinology* 141:3412–3421.
- Duncan, J. S., and R. D. Burgoyne. 1996. Characterization of the effects of Ca²⁺ depletion on the synthesis, phosphorylation and secretion of caseins in lactating mammary epithelial cells. *Biochem. J.* 317:487–493.
- Farr, V. C., K. Stelwagen, L. R. Cate, A. J. Molenaar, T. B. McFadden, and S. R. Davis. 1996. An improved method for the routine biopsy of bovine mammary tissue. *J. Dairy Sci.* 79:543–549.
- Farrelly, N., Y. J. Lee, J. Oliver, C. Dive, and C. H. Streuli. 1999. Extracellular matrix regulates apoptosis in mammary epithelium through a control on insulin signaling. *J. Cell Biol.* 144:1337–1348.
- Flint, D. J., and C. H. Knight. 1997. Interactions of prolactin and growth hormone (GH) in the regulation of mammary gland function and epithelial cell survival. *J. Mammary Gland Biol. Neoplasia* 2:41–48.

- Knight, C. H., and C. J. Wilde. 1993. Mammary cell changes during pregnancy and lactation. *Livest. Prod. Sci.* 35:3–19.
- Labarca, C., and K. Paigen. 1980. A simple, rapid, and sensitive DNA assay procedure. *Anal. Biochem.* 102:344–352.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685.
- Lapierre, H., D. Petitclerc, G. Pelletier, L. Delorme, P. Dubreuil, J. Morisset, P. Gaudreau, Y. Couture, and P. Brazeau. 1990. Effect of human growth hormone-releasing factor and (or) thyrotropin-releasing factor on hormone concentrations in dairy calves. *J. Anim. Sci.* 68:2436–2449.
- Niu, P., D. P. Radman, E. M. Jaworski, H. Deol, R. Gentz, J. Su, H. S. Olsen, and G. F. Wagner. 2000. Development of a human stanniocalcin radioimmunoassay: Serum and tissue hormone levels and pharmacokinetics in the rat. *Mol. Cell. Endocrinol.* 162:131–144.
- Politis, I., E. Lachance, E. Block, and J. D. Turner. 1989. Plasmin and plasminogen in bovine milk: A relationship with involution? *J. Dairy Sci.* 72:900–906.
- Raser, K. J., A. Posner, and K. K. Wang. 1995. Casein zymography: A method to study mu-calpain, m-calpain, and their inhibitory agents. *Arch. Biochem. Biophys.* 319:211–216.
- SAS Institute. 2002. Statistical Analysis System: Release 9.1. SAS Institute Inc., Cary, NC.
- Sorensen, A., and C. H. Knight. 2002. Endocrine profiles of cows undergoing extended lactation in relation to the control of lactation persistency. *Domest. Anim. Endocrinol.* 23:111–123.
- Talhok, R. S., M. J. Bissell, and Z. Werb. 1992. Coordinated expression of extracellular matrix-degrading proteinases and their inhibitors regulates mammary epithelial function during involution. *J. Cell Biol.* 118:1271–1282.
- Tremblay, G., L. Delbecchi, G. F. Wagner, B. G. Talbot, and P. Lacasse. 2006. Increase in stanniocalcin in milk of cows at involution. *J. Dairy Sci.* 89(Suppl. 1):333. (Abstr.)
- Uria, J. A., and Z. Werb. 1998. Matrix metalloproteinases and their expression in mammary gland. *Cell Res.* 8:187–194.
- van Amburgh, M. E., D. M. Galton, D. E. Bauman, and R. W. Everett. 1997. Management and economics of extended calving intervals with use of bovine somatotropin. *Livest. Prod. Sci.* 50:15–28.
- Weber, M. S., S. Purup, M. Vestergaard, R. M. Akers, and K. Sejrsen. 2000. Regulation of local synthesis of insulin-like growth factor-I and binding proteins in mammary tissue. *J. Dairy Sci.* 83:30–37.
- Wilde, C. J., C. V. Addey, P. Li, and D. G. Fernig. 1997a. Programmed cell death in bovine mammary tissue during lactation and involution. *Exp. Physiol.* 82:943–953.
- Wilde, C. J., L. H. Quarrie, E. Tonner, D. J. Flint, and M. Peaker. 1997b. Mammary apoptosis. *Livest. Prod. Sci.* 50:29–37.