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Contribution of Macrophages to Proteolysis and Plasmin Activity in Ewe Bulk Milk

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ABSTRACT

A total of 225 bulk sheep milk samples were collected from 5 intensively managed flocks during early, mid, and late lactation to assess the contribution of macrophages to the regulation of the plasmin-plasminogen system. Samples were analyzed for composition, somatic cell counts, milk renneting characteristics, and for plasmin (PL), plasminogen (PG), and plasminogen activators (PA) activities. Isolation of macrophages from milk was performed using a magnetic positive separation and mouse antiovine macrophage antibody; separated cells were lysed by several freeze-thaw cycles, and activity of urokinase PA (u-PA) was determined. Plasmin activity decreased during lactation (42.06 ± 0.66, early; 31.29 ± 0.66, mid; 28.19 ± 0.66 U/mL, late). The reduction in PL activity recorded in the mid and late lactation milk matched the increase in PG:PL ratio. The activity of PA increased throughout lactation; the highest value being recorded in the late lactation milk (260.20 ± 8.66 U/mL). Counts of isolated and concentrated macrophages were higher in early and mid lactation milk (3.89 ± 0.08 and 3.98 ± 0.08 $\log_{10}$ cells/mL, respectively) than in late lactation milk (3.42 ± 0.08 $\log_{10}$ cells/mL). Stage of lactation did not influence the activity of u-PA detected in isolated macrophages. The activity of u-PA associated with isolated milk macrophages only minimally contributed to total PA activity detected in milk. Proteolytic enzymes, associated with isolated macrophages, act on α-casein hydrolysis, as shown by urea-PAGE electrophoresis analysis. Somatic cell counts did not exceed 600,000 cells/mL, and this threshold can be considered a good index of health status of the flock and of the ability of milk to being processed. Our results lend support to the hypothesis that macrophages in ewe bulk milk from healthy flocks only slightly contribute to the activation of the PL-PG system.

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INTRODUCTION

Plasmin is the main native proteolytic enzyme in milk and is part of a complex protease-protease inhibitor system in milk, consisting of plasmin (**PL**); its inactive form, plasminogen (**PG**); PG activators (**PA**), which convert PG to PL, PA inhibitor (**PAI**), which inhibits PA activity; and PL inhibitor, which inhibits PL activity (Nielsen, 2003).

The PL system derives from blood, where it is involved in the degradation of fibrin clots (Sidelmann et al., 2000); in milk PL, PG, and PA are associated with the CN micelles (Politis, 1996). Tissue-type PA (**t-PA**) appear associated with CN fractions, whereas urokinase-type activators (**u-PA**) appear associated with somatic cell fraction (Politis, 1996). The most important inhibitors of PL are α_2-anti-PL, a specific PL inhibitor, and α_2-macroglobulin, a nonspecific inhibitor that reacts with most proteases. Christensen et al. (1995) estimated that there are at least 6 inhibitors of PL. Two types of PAI have been identified in milk, PAI-1 and PAI-2 (Politis, 1996).

Evidence exists for a close association between changes in the PG-PL system and gradual involution of the mammary gland in late lactation (Politis, 1996; Silanikove et al., 2005). Milk somatic cells can convert PG to PL, and, in particular, milk macrophages, which are a type of somatic cell, produce u-PA in vitro (Politis et al., 1991). In sheep, blood macrophages and neutrophils express a specific u-PA (Politis et al., 2002); also, in individual ewe milk samples with high SCC, PL activity has been found to be dependent on PA connected to milk macrophages (Albenzio et al., 2004).

Bulk milk traits (i.e., SCC and composition) provide reliable indications for milk quality and for its suitability for consumption and processing. Ewe milk is used almost totally for cheese manufacture so that indigenous proteolytic enzymes, mainly PL, play a major role in milk CN breakdown, thus reducing the ability of milk to be processed into cheese (Albenzio et al., 2005).

Therefore, this study was undertaken to investigate the contribution of milk macrophages to CN hydrolysis and the conversion of PG into PL in ewe bulk milk throughout lactation.

MATERIALS AND METHODS

Experimental Design and Milk Sampling

The experiment was conducted from March to July 2005 in 5 intensively managed commercial flocks of Comisana ewes in southern Italy and consisting of about 200 ewes per flock. Ewes involved in the trial had lambed in the winter of 2005 and were in parity 2 to 4. Ewes were housed on straw litter; they grazed in the morning and were supplemented with hay and concentrate. Ewes were healthy at the beginning of the experiment and were monitored for health by veterinarians throughout the study. In each flock, 5 sampling cycles were performed during early, mid, and late lactation (<70 d, from 110 to 130 d, and >160 d in lactation, respectively). Ewes exhibiting any sign of mastitis were excluded from milking. The ewes were milked at 0800 and 1500 h using a highline milking machine (44 kPa; 120 pulsations/min). Both milkings were included in each sampling cycle. Milk samples were collected in 200-mL sterile plastic containers, carried to the laboratory by transport tankers at 4°C, and immediately analyzed. For each sampling cycle, samples were collected in triplicate. A total of 225 bulk milk samples were collected and analyzed for fat, total protein, and lactose (MilkoScan 133B; Foss Electric, Hillerød, Denmark). Total N, noncasein N (**NCN**), and NPN were determined by standard procedures using the Kjeldahl method (IDF, 1993). Casein N was calculated as the difference between total N and NCN; whey N was calculated as the difference between NCN and NPN. All N data were expressed as protein equivalent using a conversion factor of 6.38. Somatic cell count was determined using a Fossomatic 90 (Foss Electric) according to the International Dairy Federation standard (IDF, 1995). Renneting characteristics (clotting time, rate of clot formation, and clot firmness after 30 min) were measured by a Foss Electric formagraph. Plasmin and PG activities in milk were determined by the method of Baldi et al. (1996); the dissociation of PL and PG from CN micelles was obtained by incubation of skim milk with 50 m*M* of ε-aminocaproic acid for 2 h at room temperature (Korycka-Dahl et al., 1983). The solution consisted of 250 μL of 0.1 *M* Tris-HCl buffer, pH 7.4; 0.6 m*M* Val-Leu-Lys-*p*-nitroanilide (V7127, Sigma Chemical Co., Milan, Italy); 30 plough units (2.5 μL) of urokinase (U0633, Sigma Chemical Co.); and 30 μL of milk serum. Plasmin activity was measured in the same solution without adding urokinase. Plasminogen activity was the difference. A similar mixture without sample was used as negative control. Activity of total u-PA was performed in a solution containing 100 m*M* Tris buffer, pH 8.0; 50 μg/mL of PG (P5661, Sigma Chemical Co.); 0.6 m*M* Val-Leu-Lys-*p*-nitroanilide (Sigma Chemical Co.); and 10 μL of sample. The reaction solution was incubated at 37°C for up to 3 h, and absorbance at 405 nm was measured at 30-min intervals using a microtiter plate reader. A sample without PG served as a negative control. One unit of PL, PG, and total u-PA activity was defined as the amount of the enzyme that produces a change of 0.1 in absorbance at 405 nm in 60 min.

Macrophage Isolation and Total Macrophage-Associated u-PA Activity

Milk samples (~400 mL) were centrifuged at 2,000 × *g* for 30 min at 4°C; then the fatty fraction and supernatant were removed. Cells from the bottom layer were suspended in 500 μL of PBS (pH 7.4) containing 0.02% NaN_3 and centrifuged twice (400 × *g* for 15 min at 4°C) to concentrate cells. A cell concentration of at least 1×10^7 cells/mL was obtained, which was measured using Fossomatic 90 (Foss Electric).

Isolation of macrophages from milk cells was performed by a magnetic positive separation (EasySep, StemCell Technologies, Vancouver, Canada) using mAB mouse IgG (MCA919; Serotec, Oxford, UK) directed against macrophage surface antigens. The assay was performed according to Caroprese et al. (2006). After separation of macrophages, cells were lysed by at least 3 freeze-thaw cycles. Activity of u-PA was determined in lysed cells as previously reported. The mixture was incubated for up to 3 h, and absorbance at 405 nm was measured at 30-min intervals using a microtiter plate reader. A sample without PG served as a control.

Proteolytic Activity of Macrophages on Na Caseinate

A 4% ovine Na caseinate in a Tris buffer solution, pH 8.0, was used to investigate the proteolytic activity of lysed macrophages during early, mid, and late lactation. Whole CN and Na caseinate were prepared from a sample of ovine skim milk according to Mulvihill and Fox (1977). Sodium caseinate was dissolved in 0.1 *M* Tris buffer (Sigma Chemical Co.) and 0.05% NaN_3. Lysed macrophages were incubated in Na caseinate buffer from 0 to 24 h at 37°C. The effects of macrophage proteolytic activity on Na caseinate were evaluated by urea-PAGE using a Protean II xi vertical slab gel unit (Bio-Rad, Watford, UK). The stacking and resolving gel system was prepared as described by Andrews (1983).

Table 1. Least squares means ± SEM of chemical composition and renneting characteristics of ewe bulk milk

Item	Stage of lactation			SEM	Effect
	Early	Mid	Late		
pH	6.69	6.62	6.7	0.05	NS
Fat, %	5.6	6.16	6.26	0.2	NS
Protein, %	5.03	5.17	4.77	0.16	NS
Lactose, %	4.51[a]	4.37[a]	4.04[b]	0.05	***
CN, %	3.64	4.01	3.53	0.14	NS
Whey protein, %	1.08[a]	0.88[b]	0.88[b]	0.03	**
SCC log cells/mL	5.25[b]	5.72[a]	5.29[b]	0.09	**
Clotting time, min	22.05[b]	16.94[c]	28.86[a]	0.6	***
Rate of clot formation, min	1.76[b]	1.47[b]	2.57[a]	0.16	**
Clot firmness at 30 min, mm	39.53[b]	52.09[a]	30.05[b]	2.8	**

[a–c]Means within a row with different superscripts differ ($P < 0.05$).
**$P < 0.01$; ***$P < 0.001$.

The gels were stained using a modification of the method of Blakesley and Boezi (1977) with Coomassie Brilliant Blue G250. The distained gels were acquired by the Gel Doc EQ system (Bio-Rad) using a white light conversion screen and analyzed with the Quantity One software (Bio-Rad) to determine the signal intensity (optical density) of the defined bands. Identification of bands was done by comparison with the Na caseinate standard. The relative quantity of each defined band in a single lane was determined as the percentage of the optical density, considering 100% the sum of the optical density measured in all bands of that lane.

Statistical Analyses

Data were processed by ANOVA, using the GLM procedure of SAS Institute (1999). The model utilized was:

$$y_{ij} = \mu + \alpha_i + \beta_{ij} + \varepsilon_{ij},$$

where μ = the overall mean; α = the effect of stage of lactation (i = 1 to 3); β = flock variation within stage of lactation; and ε = the error.

The variation due to stage of lactation was tested. Bulk milk SCC and isolated macrophage counts were transformed into logarithms to normalize their frequency distributions before performing statistical analysis. Percentage composition of the CN and CN degradation products was tested for stage of lactation, time of incubation, and their interaction. When significant effects were found (at $P < 0.05$), the Student t-test was used to locate significant differences between means.

RESULTS AND DISCUSSION

Composition of ovine bulk milk was not subjected to severe changes during lactation, probably because of both the good health of ewe udders through lactation and the bulk milk ability to reduce fluctuations of individual milk samples. Indeed, no differences were observed for pH values and for fat, protein, and CN content in milk during lactation (Table 1). Stage of lactation influenced the lactose content, which was lower in late lactation than in early and mid lactation ($P < 0.001$), and the whey protein content, which decreased from early to mid and late lactation ($P < 0.01$). Somatic cell count was affected by stage of lactation ($P < 0.01$), the highest SCC being detected in the mid lactation milk. Moreover, SCC did not exceed 600,000 cells/mL, suggesting this is an indicator of good udder health and good milk characteristics for cheese making (Sevi et al., 1999). Considering the good milk quality, as suggested by the SCC, and whey protein concentrations recorded in the late lactation milk, the decrease in the lactose content during late lactation could be due to the reduction in milk yield with advancing lactation and the increase of microbial fermentation in summer milk (Sevi et al., 2004; Albenzio et al., 2005). Stage of lactation affected milk-coagulating behavior, with the shortest clotting time ($P < 0.001$) in mid lactation milk. Rate of clot formation was highest ($P < 0.01$) in late lactation, whereas curd firmness was highest in mid lactation ($P < 0.01$). The reduction of milk-renneting traits with advancing lactation agrees with Sevi et al. (2003).

Count of isolated and concentrated macrophages was higher ($P < 0.01$; Table 2) in early and mid than in late lactation milk. When lysed macrophages were incubated on Na caseinate, changes in percentage composition of intact CN and CN degradation products (γ-CN and α-CN products) after 24 h of incubation were observed (Table 3), according to the electrophoretic profile (Figure 1). An effect ($P < 0.05$) of stage of lactation was found for α-CN products, whereas no significant changes were found for α-, γ- and β-CN. After 24 h of incubation, β-CN did not undergo appreciable degradation, whereas α-CN underwent hydrolysis (about 20%), suggesting that the former is more resistant to the enzymes associated with macrophages isolated from ewe bulk milk. A more intense hydrolysis of the α-CN was observed in early than in late lactation, consistent with the levels of isolated macrophages, and resulted in a higher amount of α-CN degradation products, suggesting that the enzymes associated with macrophages could contribute to CN breakdown in ewe bulk milk with <600,000 cells/mL. Macrophages are a somatic cell population whose main physiological function is to defend the udder from infection and contain lysosomes with active proteolytic enzymes as elastase, collagenase, and cathepsins (Kelly and McSweeney, 2003).

Stage of lactation influenced ($P < 0.001$) the total PA activity detected in milk, whereas no differences were found for u-PA activity detected in isolated macro-

Table 2. Least squares means ± SEM of plasmin, plasminogen, plasminogen:plasmin, total plasminogen activator (PA), total macrophage-associated urokinase-PA (u-PA), and macrophage counts in ewe bulk milk during lactation

Item	Stage of lactation			SEM	Effect
	Early	Mid	Late		
Plasmin, U/mL	42.05[a]	31.29[b]	28.19[c]	0.66	***
Plasminogen, U/mL	4.77[c]	38.91[a]	31.51[b]	1.07	***
Plasminogen:plasmin	0.12[b]	1.31[a]	1.16[a]	0.05	***
Total PA, U/mL	37.43[c]	76.53[b]	260.20[a]	8.66	***
Total macrophage-associataed u-PA, U/mL	7.07	6.75	6.09	1.41	NS
Macrophage count, $\log_{10}$ cells/mL	3.89[a]	3.98[a]	3.42[b]	0.08	**

[a–c]Means within a row with different superscripts differ ($P < 0.05$).

**$P < 0.01$; ***$P < 0.001$.

phages (Table 2). In a previous study, Albenzio et al. (2004) found a positive relation between PG conversion and the concentration of macrophages in individual ewe milk with high SCC and ascribed such a relation to the activity of u-PA associated with the macrophage membranes. The majority of the cell associated u-PA in ovine macrophages and neutrophils is bound to a specific u-PA receptor of the cell membrane. Resting macrophages and neutrophils isolated in sheep blood have numerous free and unoccupied u-PA binding sites on their cell membrane, so they produce very low amounts of u-PA (Politis et al., 2002). In this study, macrophage-associated u-PA activity (Table 2) was lower than total PA activity in milk throughout lactation. In addition, the activity of u-PA detected in milk macrophages remained relatively constant, whereas total PA activity increased markedly with advancing lactation, suggesting u-PA associated with macrophages only slightly contributed to total PA activity. Further investigations are needed to verify the role played by u-PA associated with milk neutrophils and t-PA to total PA activity in sheep milk.

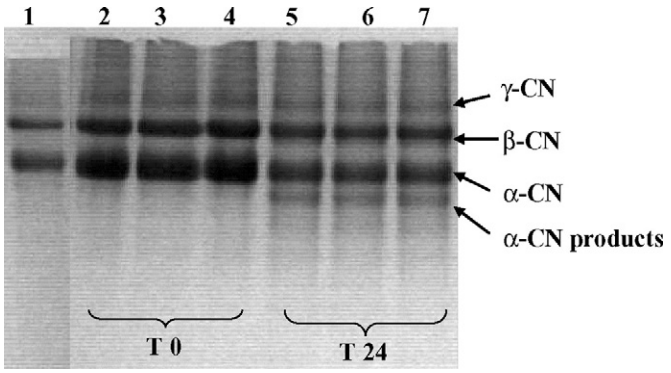

Figure 1. Urea PAGE of Na caseinate incubated with lysed macrophages at 0 and 24 h at 37°C. Lane 1: ovine Na caseinate purified. Lanes 2 and 5: protein profiles at 0 and 24 h after incubation, in early lactation. Lanes 3 and 6: protein profiles at 0 and 24 h after incubation, in mid lactation. Lanes 4 and 7: protein profiles at 0 and 24 h after incubation, in late lactation.

Plasmin and PA were affected by stage of lactation ($P < 0.001$, Table 2); higher values of PL activity were recorded in early (42.1 U/mL) than in mid (31.3 U/mL) and in mid than in late (28.2 U/mL) lactation milk. In contrast, the highest PG activity values were detected in mid lactation compared with early and late lactation. Both PA (t-PA and u-PA) can convert the proenzyme PG to active PL (Politis, 1996). The PL-PG system in ewe milk is mainly influenced by stage of lactation and number, type, and activity of milk somatic cells (Albenzio et al., 2004). The reduction in PL activity registered in the mid and late lactation milk resulted in an increase in the PG:PL ratio that indicates PG activation efficiency (Weng et al., 2006). In cows, evidence exists of a relationship between the activation of the PL-PG system and the gradual involution of the mammary gland, which occurs during the decline phase of lactation and is controlled by physiological factors connected to the tissue remodeling processes (Flint et al., 2005). Bianchi et al. (2004) observed an increase in PL activity and a decrease in PG activity as lactation progressed in individual hand-milked samples from Sardinian ewes. Albenzio et al. (2004) found a decrease in PL activity during lactation with individual machine-milked Comisana ewes. The results on PL activity of the present

Table 3. Changes (%) in CN (α- and β-CN) and CN degradation products (γ-CN and α-CN products) after 24 h of incubation of lysed macrophages on Na caseinate

Item	Stage of lactation			SEM	Effect
	Early	Mid	Late		
γ-CN	1.0	0.7	1.6	0.47	NS
β-CN	11.4	10.9	9.8	0.45	NS
α-CN	–20.2	–19.9	–19.2	0.5	NS
α-CN products	8.5[a]	8.2[ab]	7.8[b]	0.17	*

[a,b]Means within a row with different superscripts differ ($P < 0.05$).

*$P < 0.05$.

trial carried out on ewe bulk milk agree with findings on individual ewe milk. Results from Flint et al. (2005) and Bianchi et al. (2004) may not be comparable with the present results, because the former were obtained in bovine milk and the latter in a different sheep breed. The PL system behaves individually in different species or breeds, and it was reported that mammary gland involution has considerable interspecies differences. Indeed, although in cows an overlap between lactation and gestation occurs, sheep are nongestating at drying off (Lamote et al., 2004). Plasmin activity, however, is the result of the balance between the actions of activators and inhibitors. The reduction of PG conversion into PL is not easy to explain; a possible explanation could be the increase in milk of PAI, such as retinoic acid, glucocorticoids, β-LG, α-LA, α1-antitrypsin, and α2-macroglobulin, as suggested by Le Roux et al. (2003). As an alternative, the decrease in PG activity during the last stage of lactation could be attributed to the reduced transfer of PG from blood to milk. The functional state of the mammary gland and the hormone actions are the main regulators of molecular transfers in milk. McManaman and Neville (2003) reported that exogenous substances can be transferred from blood to milk directly through paracellular pathways or indirectly through the transcytosis pathway, which includes the transport of serum proteins, albumin, hormones, and cytokines. Finally, according to Weng et al. (2006), local factors involved in the regulation of PG activation cannot be excluded.

CONCLUSIONS

In ewe bulk milk from healthy flocks, the enzymes associated with macrophages hydrolyze CN according to macrophages levels during lactation, whereas the contribution of macrophages to the regulation of the PL-PG system is independent of macrophage concentrations and stage of lactation. In addition, the activity of u-PA associated with macrophage cells only minimally contributed to total PA activity in milk. It would be interesting to further investigate the different role played by the other components of the PA system (i.e., u-PA associated with milk neutrophils and t-PA) on PL activity in sheep milk during lactation.

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