PHYSIOLOGY AND MANAGEMENT

Milk Plasmin During Bovine Mammary Involution That Has Been Accelerated by Estrogen[1]

F. ATHIE,[2] K. C. BACHMAN,[3] H. H. HEAD, M. J. HAYEN, and C. J. WILCOX
Department of Dairy and Poultry Sciences, University of Florida, Gainesville 32611

ABSTRACT

The purpose of this study was to evaluate whether the plasminogen and plasmin system within bovine mammary secretions was influenced by an estrogen treatment that was used to accelerate mammary tissue involution. Holstein cows were injected with 4 ml of ethanol excipient (n = 21) or 15 mg of estradiol-17β (n = 23) on each of the 4 d that preceded final milk removal. Dates of final milk removal (d 0) were designated as 60 d prior to expected dates of calving. Each mammary quarter was sampled once to collect secretions that corresponded to d 0, 3, 11, and 25 or d 1, 7, 18, and 30 of the dry period. Concentrations of plasminogen, plasmin, and somatic cells in secretions increased earlier for treated cows than for control cows. The ratio of plasminogen to plasmin in secretions decreased earlier for treated cows than for control cows. These responses support the suggestion that the plasminogen and plasmin system is involved in the involution of bovine mammary tissue. Estrogen treatment increased the activation of plasminogen, which was evidenced by a precipitous decrease in the ratio of plasminogen to plasmin that occurred as concentrations of plasminogen and plasmin increased. The activation of plasminogen likely contributed to the increased rate of mammary tissue involution that was effected by exogenous estrogen. Endogenous estrogen secreted by the developing fetal and placental unit might mediate, in part, the gradual involution that occurs during lactation.

(**Key words**: estrogen, plasmin, mammary gland, involution)

INTRODUCTION

The milk plasmin system of bovine animals has been studied intensively because proteolysis can decrease the production and quality of dairy products (8, 13, 14, 26, 39, 52). Current interest in the plasminogen and plasmin system (2, 10, 19, 43) includes defining its role in mammary tissue remodeling, notably during involution (27, 31, 35, 42, 47, 50, 51). Remodeling between lactations is faster and more extensive in the mouse than in the dairy cow (20, 31, 53). This difference and the varied responses to hormones and growth factors of mammary epithelial cells from these species confirm that caution must be used when extrapolating information from one species to another (55, 56).

In dairy cows, gradual involution of mammary tissue occurs during an established lactation despite the frequent removal of milk, which should reduce the influence of the feedback inhibitor of lactation (7, 54). Consequently, a decline in daily milk volume ensues as the number of differentiated epithelial cells decreases. Plasmin is thought to be involved in this involution process because the activity of plasmin (36) and plasminogen activator in milk (13, 35) increases as days of lactation increase. A portion of the improved persistency of lactation that occurs when cows receive bovine somatotropin has been suggested to result from a decline in plasmin activity that was effected by the elevation of IGF-I (34, 46). In hepatocytes, IGF-I enhances the activity of plasminogen activator inhibitor-1, which, in turn, reduces the conversion of plasminogen to plasmin that is mediated by plasminogen activator (11). However, IGF-I stimulated the production of u-plasminogen activator by MAC-T bovine mammary epithelial cells (37), but these cells (18) and those of the mouse (27) only minimally produced plasminogen activator inhibitor-1. A similar response in vivo would favor involution and a decreased persistency of lactation. In a transgenic mouse model (29), involution of the lactating mammary gland was inhibited by the IGF system.

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[2]Present address: Rua Goias, 1782, Centro-Avare, SP 18.701-210, Brazil.
[3]Reprint requests.

Evidence supports the transfer of plasminogen and presumably some plasmin from blood to milk via a paracellular route (35, 44, 46). Thus, plasmin activity in milk could be increased by the disruption of tight junctions (25, 35, 44, 46) or by plasminogen activation, which is effected by plasminogen activator that is produced by mammary tissue (18, 27, 31, 37) or is associated with somatic cells in milk (13, 16, 17, 38, 57).

Cessation of milk removal, which occurs when dairy cows are dried off in preparation for their next lactation, initiates an active involution of mammary tissue that appears to be completed in 30 d (30). Based on analysis of mammary secretions (3), estrogen administered at dry-off increased the rate of this involution. This acceleration of involution would be beneficial economically if it allowed the length of the dry period to be shortened without a loss in subsequent milk production.

This study describes the plasminogen and plasmin system in bovine mammary secretions that were collected from d –4 to d 30 of dry periods that were initiated without and with estradiol-17β (3).

MATERIALS AND METHODS

Pregnant Holstein cows in late lactation were randomly assigned either to the control (n = 21) or treatment group (n = 23). Treatment consisted of four subcutaneous injections of 15 mg of estradiol-17β (Sigma Chemical Co., St. Louis, MO) dissolved in 4 ml of ethanol; injections were administered once daily (3, 15). Control cows received ethanol excipient. Injections were administered on the 4 consecutive d (d –4 to –1) that preceded final milk removal (d 0). Milk production for each of the three daily milkings was recorded before and during the injection period.

Composite samples, which were representative of the mammary secretions present in all four quarters, were collected on d –4 (before injection) and on d –1 (after last injection). In addition, four samples of mammary secretions (one per quarter) from each cow were collected throughout the nonlactating period. Cows were assigned randomly to a pattern of secretion collection that corresponded to sampling on d 0, 3, 11, and 25 or d 1, 7, 18, and 30 relative to final milk removal (d 0). Following manual milking, an antibiotic preparation for dry cows was infused into the sampled quarter.

Plasmin and plasminogen activities in serum from mammary secretions were determined using the method of Korycka-Dahl et al. (23) after it was modified to accommodate a microassay. After collection, samples of mammary secretion were centrifuged at 7900 $\times$ *g* for 10 min at 4°C to isolate the skimmed fraction, which was frozen until analysis. After thawing, skimmed samples (1 ml) were incubated with 50 m*M* ϵ-amino-n-caproic acid (Sigma Chemical Co.) for 2 h at 25°C. Concurrently, the samples were treated with 15 mg of sodium citrate to dissociate casein micelles. Incubated samples were centrifuged at 2600 $\times$ *g* for 15 min at 4°C to obtain the mammary secretion serum. Reaction mixtures that contained 0.05 ml of secretion serum, 0.65 ml of assay buffer, and 0.05 ml (0.6 m*M*) of the chromogenic tripeptide substrate H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide (Sigma Chemical Co.) were pipetted in triplicate into 96-well microplates. Plates were incubated at 37°C. Absorbance was measured at 405 nm using a microplate autoreader (model EL309; Bio-Tech Instruments, Burlington, VT) after 30 and 120 min of incubation. Change in absorbance per minute during this interval was used to estimate plasmin activity. One unit of plasmin or plasminogen activity was defined as the amount of the enzyme that produced a change in absorbance at 405 nm of 0.001 in 1 min when *p*-nitroanilide was measured in the reaction mixture. Total proteolytic activity (plasmin plus plasminogen) was measured similarly; however, 25 to 60 Plough units of human urine urokinase (Sigma Chemical Co.) were added to the reaction mixture.

The general linear models procedure of SAS (12) was used for least squares ANOVA. The model included treatment, number of days dry, their interactions, and cow nested within treatment; the design was a split plot. Quarter was removed from the model after its effect was found to be nonsignificant. After we determined the highest order of regression that was significant, heterogeneity of regression was used to determine whether response curves for days dry differed between treatments. Whenever this test was significant at $P < 0.05$, the model containing the interaction between number of days dry and treatment was used to fit curves for each treatment.

RESULTS AND DISCUSSION

Concentrations of plasmin increased in mammary secretions that were collected during the early dry periods from cows that did not receive injections of estrogen (Figure 1). This increase began with the cessation of milk removal (d 0) and reached an apex by d 10. Thus, elevation of plasmin within mammary secretions is a process that occurs normally during the early dry period when active involution is initiated. This observation supports the suggestion (35) that, in the dairy cow, the plasminogen and plasmin system is involved in the involution of mam-

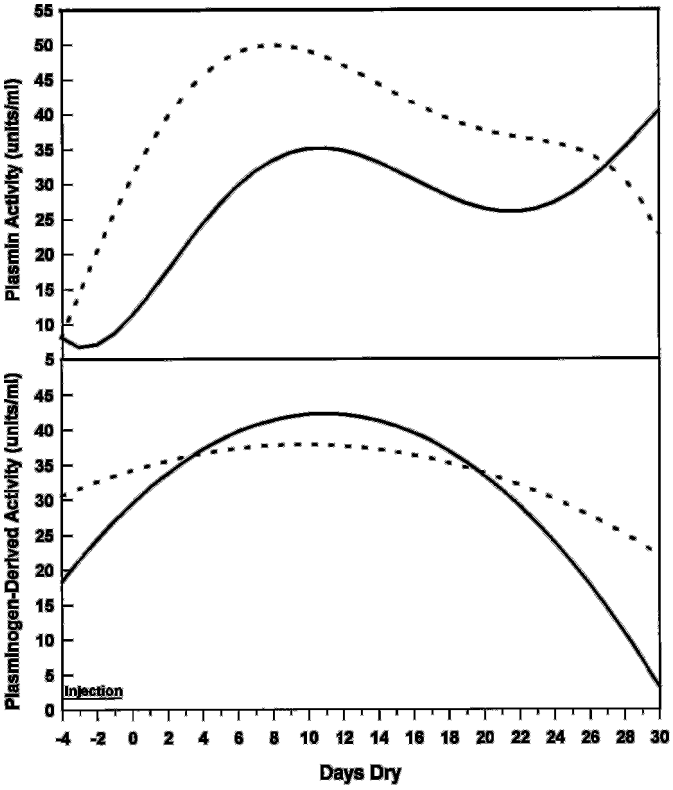

Figure 1. Relationship between the concentrations of plasmin and plasminogen in mammary secretions and the number of days dry for control cows (—) and cows treated with estrogen (---) prior to final milk removal. Regression equations for curves are as follows: control cows, $y_1 = 11.21 + 2.822x + 27.72 \times 10^{-2}x^2 - 5.049 \times 10^{-2}x^3 + 2.108 \times 10^{-3}x^4 - 2.67 \times 10^{-5}x^5$ and $y_2 = 29.50 + 2.348x - 1.076 \times 10^{-1}x^2$; cows treated with estrogen, $y_1 = 30.95 + 4.871x - 27.68 \times 10^{-2}x^2 - 1.255 \times 10^{-2}x^3 + 1.188 \times 10^{-3}x^4 - 2.17 \times 10^{-5}x^5$ and $y_2 = 34.25 + 0.742x - 0.382 \times 10^{-1}x^2$, where x = days dry, y_1 = plasmin activity, and y_2 = plasminogen-derived activity. Curves differed for plasmin ($P < 0.017$) but not for plasminogen ($P < 0.227$). A unit of enzyme activity is defined as a change in absorbance at 405 nm of 0.001 per minute.

mary tissue, as has been observed for the mouse (27, 31). Additional supporting evidence for this proposed involvement was provided by the earlier and greater ($P < 0.02$) increase in plasmin from secretions that were collected from cows that received estrogen (Figure 1). Based on comparisons of the rates and magnitudes of changes in the composition of mammary secretions (3), these same cows underwent accelerated involution of mammary gland tissue.

Changes in concentrations of plasmin and its inactive proenzyme, plasminogen (Figure 1; Table 1), were influenced by the decrease in secretion volume that occurred with the onset of active involution. Furthermore, initial decreases in secretion volume were larger for cows that received estrogen (3), which would contribute to the higher concentrations of plasmin and plasminogen that occurred earlier in these cows. Adjustment according to the amount of milk produced was possible for treatment means that were calculated for before (d −4) and after (d −1) injection (Table 1). After this adjustment, treatment means differed for plasmin only ($P < 0.047$). Therefore, a reduction in the secretion volume was not the sole reason for the increased concentration of plasmin that occurred as an immediate response to estrogen treatment. The ratios of plasminogen to plasmin in mammary secretions that were collected during the dry period were compared to assess the effect of estrogen treatment on the plasminogen and plasmin system (Figure 2). Changes in these ratios were independent

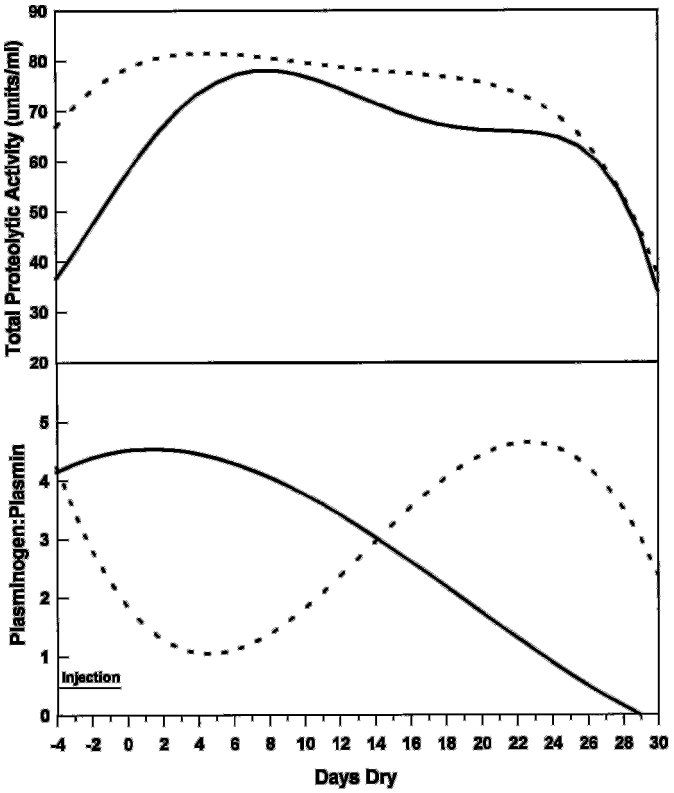

Figure 2. Relationship between the concentration of total proteolytic activity from plasmin and plasminogen and of the ratio of plasminogen to plasmin in mammary secretions and days dry for control cows (—) and cows treated with estrogen (---) prior to final milk removal. Regression equations for curves are as follows: control cows, $y_1 = 36.78 + 5.213x + 51.11 \times 10^{-2}x^2 - 1.042 \times 10^{-1}x^3 + 4.99 \times 10^{-3}x^4 - 7.6 \times 10^{-5}x^5$ and $y_2 = 4.51 + 3.359 \times 10^{-2}x - 1.325 \times 10^{-2}x^2 + 2.316 \times 10^{-4}x^3$; cows treated with estrogen, $y_1 = 67.02 + 4.971x - 56.15 \times 10^{-2}x^2 + 2.233 \times 10^{-2}x^3 - 0.14 \times 10^{-3}x^4 - 0.66 \times 10^{-5}x^5$ and $y_2 = 1.841 - 37.821 \times 10^{-2}x + 4.976 \times 10^{-2}x^2 - 12.186 \times 10^{-4}x^3$, where x = days dry, y_1 = total proteolytic activity, and y_2 = ratio of plasminogen to plasmin. Curves differed for total proteolytic activity ($P < 0.006$) and ratio ($P < 0.008$). A unit of enzyme activity is defined as a change in absorbance at 405 nm of 0.001 per minute.

TABLE 1. Least squares mean concentrations of mammary secretion parameters before and after injection of excipient and estradiol.

Secretion parameter	Experimental group[1,2]						Treatment comparison[3]	
	Control			Estradiol				
	Before	After	SEM	Before	After	SEM	T	T × D
							$P <$	
Plasmin, units[4]/ml	8.35	9.80	1.23	9.84	18.07	1.17	0.005	0.007
Plasminogen, units/ml	24.72	20.60	4.38	29.10	39.11	4.19	0.023	0.107
Total activity, units/ml	33.07	30.40	4.59	38.94	57.18	4.38	0.025	0.003
Plasminogen:plasmin	4.37	3.23	0.69	4.19	2.26	0.66	0.466	0.565
SCC/ml, $\times 10^6$	1.02	1.52	0.28	0.94	2.29	0.27	0.568	0.129

[a]Number of observations was 21 and 23 for each mean presented for control and estradiol groups, respectively.

[2]Values for before and after injection were not adjusted for milk.

[3]T = Treatment (excipient or estradiol); D = date of observation (before or after injection).

[4]A unit of enzyme activity is defined as a change in absorbance at 405 nm of 0.001 per minute.

of changes in the secretion volume that occurred during the study period.

Within each treatment group, relative concentrations of plasminogen and plasmin in mammary secretions varied during the study (Figure 2). Furthermore, because the regression curves were not parallel and differed in magnitude ($P < 0.009$), estrogen treatment influenced the ratio of plasminogen to plasmin. In contrast to the relatively unchanged ratio from d –4 to d 5 for control cows, a precipitous decline occurred in the ratio for cows treated with estrogen. This decline occurred as concentrations of plasmin and plasminogen increased (Figure 1). Obviously, in cows that received estrogen, the rate of increase for plasmin exceeded that for plasminogen, which suggests that activation of plasminogen was effected by the estrogen treatment.

Within control cows, initiation of the activation of plasminogen occurred less rapidly, but this activation caused a gradual decline in the ratios of plasminogen to plasmin that continued throughout the period studied (Figure 2). The slight change in the ratios from d –4 to d 5 indicated that, in control cows, the initial rates of increase for plasminogen and plasmin (Figure 1) were similar. This initial maintenance of the ratios suggests that plasminogen and plasmin present in secretions of control cows at dry-off (d 0) were concentrated uniformly as resorption of mammary secretion ensued with little associated activation of plasminogen. Also, if additional plasminogen and plasmin were transferred from blood to milk during this early period, the transfer process, which likely was nonselective, did not result in an alteration of the relative concentrations of plasminogen and plasmin within the mammary secretions. Consequently, if plasminogen transfer exceeded plasmin transfer, which is likely because of the higher concentration of plasminogen in blood (24), sufficient activation of plasminogen would have had to occur post-transfer to maintain the existing ratio of plasminogen to plasmin (Figure 2). Transfer of plasminogen and possibly of plasmin occurred to contribute to the elevations in the control cows that were observed. This conclusion is supported by the evidence showing that both groups of cows reached similar peak values for total proteolytic potential from plasmin and plasminogen (Figure 2).

Maximum proteolytic potential from plasmin and plasminogen was reached earlier in secretions from cows that received estrogen (Figure 2). Because estrogen treatment disrupts tight junctions in mammary tissue (3), the transfer of plasminogen and plasmin from blood into secretion is facilitated. In the absence of any evidence that plasminogen or plasmin is synthesized by mammary tissue, the elevations in concentrations of these entities in treated cows (Figure 1) were likely in response to both the decrease in secretion volume and this facilitated transfer. Therefore, in order for secretions from control cows to approach a similar maximum concentration at d 10 (Figure 2), transfer of plasminogen and plasmin would also have had to occur in the control cows. In control cows, this transfer was facilitated by the disruption of tight junctions that occurred when

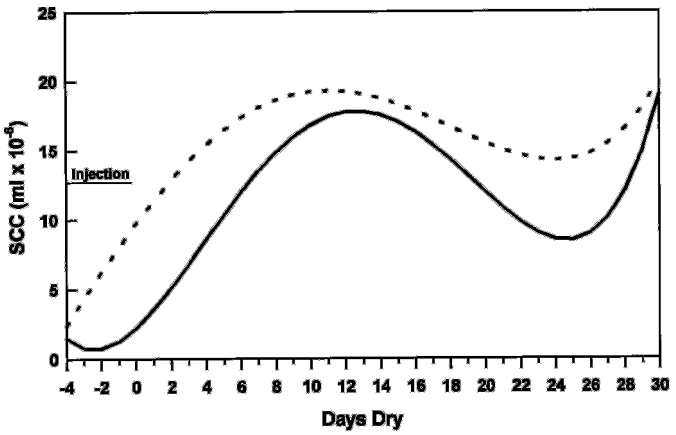

Figure 3. Relationship between the concentration of somatic cells in mammary secretions and days dry for control cows (—) and cows treated with estrogen (---) prior to final milk removal. Regression equations for curves are as follows: control cows, $y_1 = 2.25 + 1.170x + 16.82 \times 10^{-2}x^2 - 17.76 \times 10^{-3}x^3 + 3.83 \times 10^{-4}x^4$; cows treated with estrogen, $y_1 = 9.76 + 1.673x - 5.42 \times 10^{-2}x^2 - 2.90 \times 10^{-3}x^3 + 1.07 \times 10^{-4}x^4$, where x = days dry and y_1 = SCC ($\times 10^{-6}$). Curves differed ($P < 0.027$).

milking frequency was decreased (46). With complete cessation of milk removal at dry-off (d 0), this disruption occurred for both groups of cows. However, in treated cows, the abrupt disruption that was mediated by estrogen preempted the effects of the more gradual disruption that followed cessation of milk removal. Consequently, more immediate increases in concentrations of plasmin and plasminogen (Figure 1) were observed for cows that received estrogen. As indicated previously, the increase in plasmin also involved activation of plasminogen, which was more extensive in cows that received estrogen (Figure 2).

Evidence that disruption of tight junctions occurred in the mammary tissue of all cows during the early dry period was provided by the elevation of somatic cells in mammary secretions (Figure 3; Table 1). The entrance of somatic cells into the secretions via this paracellular route required the disruption of tight junctions (25, 46). Changes in the sodium and potassium contents of mammary secretions also suggested that this disruption occurred (3). Elevation of somatic cells in mammary secretions appears to be a normal characteristic of the involution process, regardless of infection status at dry-off (22, 28). Although intramammary infection status prior to injection of estrogen was not determined, no cows with clinical signs of mastitis entered this study. Notably, estrogen has caused symptoms related to mastitis (e.g., clotted milk and high SCC) to develop in cows that were classified as free of mastitis based on Feulgen-DNA values (4). The mechanism by which estrogen elicits this chemotactic response in the absence of intramammary infection warrants further investigation. Of particular interest is the possible involvement of cytokines (6) and lactoferrin (45, 48).

The magnitude of the response curves for SCC differed (Figure 3; $P < 0.03$); however, tests for heterogeneity of regression provided insufficient evidence for us to state that the curves were not parallel. Thus, as observed for the plasmin and plasminogen responses (Figure 1), estrogen effected an earlier influx of somatic cells (Figure 3). The migration of somatic cells into mammary secretions facilitates an associated movement of plasminogen and plasmin through disrupted tight junctions and damaged alveolar cells (1, 25, 46). In the absence of estrogen treatment, the transfer of somatic cells, plasminogen, and, presumably, at least some plasmin was slower. Furthermore, based on the ratios of plasminogen to plasmin (Figure 2), much less of the initially transferred plasminogen was activated to plasmin. Consequently, plasminogen reached a higher concentration in mammary secretions that were collected from control cows (Figure 1). After reaching this higher concentration, plasminogen decreased to very low concentrations because of its activation to plasmin, which was evidenced by the resultant decrease in the ratio of plasminogen to plasmin to a very low value (Figure 2). Thus, extensive activation of plasminogen apparently is a characteristic of the involution process because activation occurred when exogenous estrogen was not present. When exogenous estrogen was provided, activation of plasminogen appeared to be enhanced, which caused an early nadir (d 3) in the ratio (Figure 2). The subsequent elevation and decline in the ratio response curve agreed with the relative changes that occurred in the concentrations of plasmin and plasminogen (Figure 1).

The origin, site, and regulation of the activity of plasminogen activator that effected the decrease in the ratio of plasminogen to plasmin in both groups of cows (Figure 2) are not known. Clearly, estrogen treatment increased the activity of plasminogen activator to a greater extent than that normally present during the initial phase of active involution of bovine mammary tissue. Because blood and mammary tissue are the two possible origins of the plasminogen activators found in the samples of milk (8, 26) and mammary secretions that were collected during the dry period (21), estrogen treatment would have had to augment the transfer of plasminogen activator from one or both of these locations into mammary secretions to allow the observed activation of the

blood-derived plasminogen to occur. In bovine milk, native plasminogen activators are associated with casein micelles (39, 52), where plasmin and plasminogen are primarily located (33). Activity of plasminogen activator also is associated with milk somatic cells (35, 52, 57); however, plasminogen and plasmin were absent from somatic cell extracts (33). The respective locations of these entities in bovine mammary tissue and its associated secretions during involution are unknown. Furthermore, involvement of plasminogen activator inhibitor (2, 51) would have to be considered in any attempt to explain the increase in the activity of plasminogen activator associated with mammary involution as it occurs in the absence or presence of exogenous estrogen.

In vitro studies with bovine mammary epithelial cells (18, 37), milk macrophages, and blood monocytes (38) have shown that each can produce u-plasminogen activator. Bovine mammary epithelial cells also produce plasminogen activator inhibitors (18). The contribution of these sources to our observed increase in activity of plasminogen activator is unknown. Based on immunoreactivity and in situ hybridization results (27), the fibroblast-like stromal cells in mouse mammary tissue synthesized and secreted u-plasminogen activator, which apparently became bound to epithelial cells; the coproduced proteinases, gelatinase A, and stromelysin-1 became enriched in myoepithelial cells. Also, both myoepithelial cells and macrophages were found not to be major producers of u-plasminogen activator.

One feature of the involution process that was common to both treatment groups was the influx of somatic cells (Figure 3). Understandably, the relative distribution of cell types might have differed between treatments and from the distribution associated with mastitis (57). Regardless, this influx process likely contributed to the elevation in the activity of plasminogen activator (57) that apparently occurred in both groups; this elevated activity caused the decreases in ratios (Figure 2) that characterized the mammary gland involution (35). Interestingly, in the study of Politis et al. (35), no differences were found between the ratios of plasminogen to plasmin in milk samples that were isolated from healthy and mastitic quarters despite the higher plasmin activity in milk with a high SCC. This observation, which acknowledged an increase in the activity of plasminogen activator during mastitis (13, 57), prompted the suggestion that the key phenomenon during mastitis was alveolar damage that facilitated increased transport of plasminogen from blood to milk (35). Therefore, in mastitic quarters, the quantity of plasminogen that was transported would have had to have been sufficient to maintain the ratio of plasminogen to plasmin after the activation of a portion of plasminogen had caused an increase in plasmin concentration.

This same scenario could be used to explain the very early events that occurred when involution was initiated by cessation of milk removal in the absence of exogenous estrogen. From d 0 through 5, SCC increased as did the concentrations of plasmin and plasminogen; ratios of plasminogen to plasmin were maintained (Figures 1, 2, and 3). The subsequent decrease in the ratios began prior to the peaks at d 10 for somatic cells, plasmin, and plasminogen. Apparently, the activity of plasminogen activator increased to activate a greater percentage of the transferred plasminogen. The source of this activity of plasminogen activator is unknown, but evidence exists that the activity of plasminogen activator might not have been associated entirely with the influx of somatic cells. Reduction in milking frequency increased plasminogen activator, although SCC did not exceed 250,000 (46). Furthermore, estrogen treatment, although effecting more rapid and extensive increases in somatic cells, plasmin, and plasminogen (Figures 1 and 3), caused an even faster and more precipitous decrease in the ratio of plasminogen to plasmin (Figure 2). Thus, estrogen likely increased the activity of plasminogen activator that was unassociated with somatic cells (52). Importantly, estrogen stimulated synthesis of plasminogen activators in human breast tissue (41, 43) and decreased milk production via oral contraceptives (32). Whether estrogen increases production of plasminogen activator in bovine mammary tissue is unknown, but evidence suggests this possibility. Bovine mammary epithelial cells can produce u-plasminogen activator (18, 37), and the high concentrations of milk plasminogen activator that have been observed during late lactation in dairy cows (13, 35) were not entirely attributable to somatic cell contents (57). Endogenous estrogen produced by the developing bovine fetal and placental unit (9, 40, 49) might have stimulated synthesis of plasminogen activator by mammary tissue in quantities sufficient to explain partially this increase in milk plasminogen activator and the gradual mammary involution that contributed to the decline in milk production as days of lactation increased (35). After d 100 of gestation, when estrogen release began (9, 40), days pregnant had a negative effect on milk production, which likely involved a gradual involution of mammary secretory tissue (5).

Teleologically, it would be appropriate if the developing bovine fetus, through secretion of estrogen, could initiate the involution of the mammary gland

upon which its postpartum survival depends. Involution initiated in such a manner would be essential to the survival of the newborn calf and its species, because the developing but unborn calf could affect the gradual weaning of a nursing sibling, which, in turn, would ensure that the mammary gland at parturition would contain colostrum and the antibodies needed to nourish and immunologically protect the newborn calf. In modern dairy cows that have been selected to produce quantities of milk that greatly exceed the amounts required to nourish their offspring, judicious augmentation of this survival mechanism might allow the length of the dry period to be decreased profitably (3).

CONCLUSIONS

Concentrations of plasmin and its proenzyme plasminogen increased in mammary secretions that were collected after final milk removal. The rates of these elevations in concentrations were increased when estradiol was administered prior to final milk removal. Estradiol treatment also accelerated the activation of plasminogen as evidenced by a precipitous decrease in the ratio of plasminogen to plasmin. These observations suggest that the acceleration of involution that was effected in the bovine mammary gland by estradiol (3) involved an increased activation of plasminogen.

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