Apoptosis and oxidative stress of infiltrated neutrophils obtained from mammary glands of goats during various stages of lactation

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Objective—To examine apoptosis in infiltrated neutrophils during involution of mammary glands and compare them with those obtained during late and peak lactation, and to measure oxidative stress and activities of antioxidant enzymes and determine involvement of free radicals in apoptosis of infiltrated neutrophils.

Sample Population—Neutrophils from mammary gland secretions of 8 goats at 4 stages (late lactation, peak lactation, 1 and 2 weeks after end of lactation).

Procedure—DNA fragmentation was evaluated to characterize apoptosis. Concentration of thiobarbituric acid reactive substances (TBARS) was used to evaluate oxidative stress. Activities of superoxide dismutase and glutathione peroxidase were determined.

Results—Neutrophils from secretions obtained after end of lactation of all goats and from late-lactation milk of some goats underwent prominent apoptosis, whereas neutrophils from peak lactation secretions did not. Higher lipid peroxidation and lower antioxidant enzyme activities in neutrophils during involution were observed, compared with those during late and peak lactation. A significant negative correlation existed between TBARS concentrations and antioxidant enzyme activities during the nonlactating period.

Conclusions and Clinical Relevance—Apoptosis is a feature of infiltrated neutrophils during involution of mammary glands in goats. This feature may allow prompt resorption and clearance of infiltrated neutrophils without damaging surrounding tissues. Increased oxidative stress in infiltrated neutrophils from secretions obtained after end of lactation is probably related to a deficiency in antioxidant enzyme activities. Understanding the relationship between apoptosis and oxidative stress will lead to new strategies for manipulating involution and reducing tissue damage.

Polymorphonuclear leukocytes (PMN) are the predominant cell type in milk with high somatic cell count and in secretions obtained from nonlactating glands of dairy cattle and goats. The most important biological functions of PMN are to phagocytize particles and release various reactive oxygen intermediates (ROI). Microscopic examination of involuting bovine mammary glands has revealed fat, casein, and cellular debris in the cytoplasm of neutrophils and macrophages. Ingestion of milkfat globules by PMN at least partially contributes to their reduced phagocytic and bactericidal activities after infiltration. These results imply that phagocytes have a role in resorption of milk components and facilitate removal of degenerated epithelial cells from involuting mammary glands.

Neutrophils in blood samples obtained from humans were aged in vitro and were found to have impaired functions as a consequence of apoptosis. It also was documented in vitro that transmigration and phagocytosis ultimately accelerated apoptosis of human PMN. Apoptosis of PMN may repress inflammation that may otherwise be induced by liberation of proteolytic enzymes and other toxic products as a result of nonapoptotic death of PMN. Although the fate of neutrophils after diapedesis into mammary glands has not been extensively studied in dairy animals, it is reasonable to assume that apoptosis associated with aging also is evident in infiltrated neutrophils.

It has been suggested that oxidative stress is a modulator of apoptosis. This subsequently has been documented in several human cell lines. The correlation between phagocytosis-associated ROI production and the induction of apoptosis of circulating neutrophils was directly verified in humans, using an in vitro system. The objective of the study reported here was to examine...
whether apoptosis and oxidative stress were prominent events of infiltrated neutrophils in involuting mammary glands of goats. Internucleosomal degradation of DNA was used to assess apoptosis in neutrophils prepared from mammary gland secretions collected during a period of 2 weeks before and after cessation of milking as well as during a period of 4 to 6 weeks in the next lactation. During the same time, oxidative stress within neutrophils was assessed by measuring the extent of lipid peroxidation. The activities of 2 major antioxidant enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GPX), were measured to test their possible association with oxidative stress.

**Materials and Methods**

**Goats**—Eight goats (3 Toggenburg, 2 Alpine, 2 Nubian, and 1 Saanen) in their first or second lactation were used in the study. Goats were 2 to 3.5 years old and weighed 60 to 70 kg. Goats were enrolled in the study 2 weeks before the end of the current lactation and remained enrolled through 6 weeks of the next lactation. Throughout the study, goats were housed indoors in raised pens with slotted floors. A commercial concentrate that consisted mainly of maize grain (37%), soybean meal (21%), and wheat bran (10%) was fed twice daily (7 AM and 5:30 PM) at the rate of 0.6 kg/goat/d prior to parturition and 1.0 kg/goat/d after parturition. Pangola and alfalfa hay, water, and mineral salts were available ad libitum.

During the last 3 weeks of lactation, goats were milked once daily for 2 weeks and then once daily on alternate days for 1 week. Lactation ceased during the last month of gestation. After the final milking of the lactation, a dose of antibiotic was administered into each mammary gland via the teat canal. After parturition, goats were milked twice daily immediately preceding the feeding times. None of the goats had gross evidence of intramammary infection during the study.

**Preparation of infiltrated neutrophils from mammary gland secretions**—Samples of mammary gland secretions were collected once weekly during the period of 2 weeks before and 2 weeks after end of lactation and between 4 and 6 weeks after parturition. An alternative collection scheme was used after end of lactation to avoid disturbing the normal process of involution. We collected samples from the mammary glands only on the left side on day 7 after end of lactation and only on the right side on day 14 after end of lactation. During lactation, 100 ml of milk was collected prior to the morning milking, using aseptic procedure. During the nonlactating period, 20 ml of mammary gland secretions were obtained, using the same aseptic procedure. Teat ends were cleaned, using gauze moistened with a 70% solution of ethanol. Samples were collected into sterile plastic bottles, placed on ice, and transferred immediately to the laboratory.

Neutrophils were prepared as described elsewhere. Aliquots (30 ml of milk or 20 ml of nonlactating secretion) were centrifuged (500 X g for 20 minutes at 4°C). After careful decanting, the fat and cream layers were wiped off the wall of each tube. The cell pellet then was washed twice, using 50 ml of RPMI, and resuspended in 1 ml of RPMI. Cytocentrifuge slides were prepared from 5 µl of the cell suspension, using centrifugation at 60 X g for 3 minutes. The remainder of the suspension was stored at –20°C until used for other neutrophil assays, which were performed within 1 week after sample collection.

Cytocentrifuge slides were stained, using a modified Wright stain. Differential cell counts revealed that, on average, 90% of the cell population was neutrophils. Morphologic characteristics of infiltrated neutrophils were examined, using oil-immersion light microscopy at 1,000X magnification. Because of the consistency of secretions obtained during the nonlactating period and cell clustering, enumeration of cells with a hemacytometer was not practical. Therefore, neutrophil counts in mammary gland secretions were represented by DNA content per milliliter of volume, based on results of the diphenylamine reaction.

**Analysis of DNA internucleosomal fragmentation**—Apoptosis of infiltrated neutrophils was monitored by detection of DNA. Agarose gel electrophoresis of the extracted total genomic DNA was conducted to reveal internucleosomal fragmentation (ie, DNA ladder), using a procedure described elsewhere, with slight modifications. Briefly, 1 ml of the previously prepared neutrophil suspension was incubated in 1.5 ml of lysis buffer (10 mM Tris, 20 mM EDTA, 0.5% Triton X-100, pH 8.0) for 30 minutes at 4°C. A portion of the cell lysate was digested, using proteinase K (300 µg/ml) and 1% sodium dodecyl sulfate at 37°C for 3 hours. The DNA in the solution was extracted, using a mixture of phenol and chloroform (1:1, vol:vol) followed by a mixture of chloroform and isoamyl alcohol (24:1, vol:vol). The aqueous phase was mixed with an equal volume of isopropanol and stored overnight at –20°C. The next day, DNA was recovered by centrifugation and washed with a 70% solution of ethanol. Electrophoresis of neutrophil DNA was performed in a 2% agarose gel in the Tris-EDTA-boric acid buffer (1 mM EDTA, 45 mM Tris and 40 mM boric acid, pH 8.0). A DNA ladder that ranged from 0.2 to 3.0 kilobase pairs was used as the standard marker. Each gel was stained with ethidium bromide (0.5 µg/ml), developed, and photographed under UV light.

**Assay of DNA cleavage**—A DNA cleavage assay served as a supplement to the DNA ladder assay for use in detecting DNA fragmentation. The DNA cleavage assay is capable of detecting large molecular weight DNA fragments that initially are formed during the early stage of apoptosis and oligonucleosomal fragments recognizable as a DNA ladder on agarose gel electrophoresis. A portion of the previously prepared cell lysate was centrifuged (14,000 X g for 60 minutes at 4°C). The DNA content in the supernatant, the pellet, and the original uncentrifuged lysate was measured, using the diphenylamine reaction. The percentage of DNA fragmentation was calculated as the amount of DNA in the supernatant divided by the amount of DNA in the supernatant and the pellet.

**Lipid peroxidation in neutrophils**—The extent of lipid peroxidation in neutrophils was expressed as the concentration of thiobarbituric acid reactive substances (TBARS) in accordance with the method of Shindo and Hashimoto. Cell lysate was boiled in butylhydroxytoluene-supplemented acidic thiobarbituric acid reagent. The TBARS were extracted, using a mixture of isomyl alcohol and pyridine (2:1, vol:vol), and measured spectrophotometrically at 535 nm, using 1,1,3,3-tetramethoxypropane as a standard.

**Antioxidant enzyme activity in neutrophils**—Functions of antioxidant enzymes in neutrophils were monitored by assaying the activities of SOD and GPX (EC 1.11.1.9), as described by Marklund and Marklund and Lawrence and Burk, respectively. Briefly, 1 unit of SOD activity represented the ability to inhibit the auto-oxidation rate of 8 mM pyrogallol by 50% in a system of 100 mM Tris-cacodylic buffer (pH 8.2) at 25°C. Optical density was monitored at 412 nm for 3 minutes. Cytosolic and mitochondrial SOD activities were measured by use of this assay. Activity of GPX in the cell lysate was expressed as the amount of 1 mM reduced glutathione that was oxidized by 1.5 mM H2O2 to form oxidized glutathione, using a coupled system of 2 mM reduced form of nicotinamide adenine dinu-
Adenosine diphosphate (ADP) and 1 U of glutathione reductase/ml at pH 7.0 and 25 C.

Statistical analysis—Least-squares ANOVA was performed to examine the effect of physiologic stage of mammary glands on the properties of infiltrated neutrophils such as DNA cleavage, lipid peroxidation, and antioxidant enzyme activity. The 4 physiologic stages of the mammary gland compared in the study were late lactation (1 to 2 weeks before end of lactation), early during nonlactation (1 week after end of lactation), late during nonlactation (2 weeks after end of lactation), and peak lactation (4 to 6 weeks after parturition). Data for weeks 1 and 2 before end of lactation did not differ significantly and were pooled for the late-lactation stage. Similarly, data for weeks 4, 5, and 6 after parturition did not differ significantly and were pooled for peak-lactation stage. Percentages of DNA cleavage and TBARS concentrations were used to evaluate the relationship between DNA degradation and oxidative stress. On the other hand, TBARS concentrations and SOD or GPX activity were paired to explore the correlation between oxidative stress and antioxidant enzyme activities of infiltrated neutrophils. For all analyses, values of \( P \leq 0.05 \) were considered significant.

**Results**

In the study reported here, cellular DNA content in mammary gland secretions was measured as an indicator of neutrophil number. During the first 2 weeks after end of lactation, neutrophil DNA concentration in mammary gland secretions was significantly higher than those of late (mean ± SEM, 1.43 ± 0.12 mg/ml) and peak stages (1.22 ± 0.11 mg/ml) of lactation (Fig 1). Furthermore, the DNA concentration in secretions obtained 2 weeks after end of lactation (8.89 ± 2.07 mg/ml) was significantly higher than that in secretions obtained 1 week after end of lactation (4.22 ± 0.46 mg/ml). Volume of the secretions obtained during the nonlactating period was < 1% of the volume obtained during late and peak lactation.

Agarose gel electrophoresis of genomic DNA extracted from neutrophils of secretions obtained 1 and 2 weeks after end of lactation revealed a laddering pattern (Fig 2). Size of these DNA fragments was consistent with oligonucleosomes of 180 to 200 base pairs generated by endonuclease cleavage during apoptosis. The laddering pattern of genomic DNA also was seen in some neutrophil preparations from milk obtained during late lactation (data not shown).

The proportion of neutrophil chromatin DNA that was cleaved into lower molecular weight fragments that did not sediment after centrifugation at 14,000 \( \times \) g for 60 minutes was determined in this study as an indication of the extent of DNA degradation. Mean proportion of cleaved neutrophil chromatin 2 weeks after end of lactation (75.6 ± 1.3%) was significantly higher than that 1 week after end of lactation (69.4 ± 1.3%) and during late and peak lactation (Fig 3). Cleavage of neutrophil DNA in milk obtained during late lactation (57.9 ± 1.9%) was significantly higher than that during peak lactation (17.0 ± 0.8%).

The extent of oxidative stress in neutrophils was represented by the concentration of TBARS as products of lipid peroxidation. In the first 2 weeks after end of lactation, TBARS concentration in neutrophils increased significantly (11.07 ± 0.83 and 11.40 ± 1.25 nmol/mg of DNA 1 and 2 weeks after end of lactation, respectively).
respectively), compared with concentrations from late (7.92 ± 1.05 nmol/mg of DNA) or peak (7.72 ± 0.76 nmol/mg of DNA) lactation (Fig 4). When the thiobarbituric acid test was applied to the noncellular fraction of milk recovered after centrifugation and pelleting of cells, a similar increase in TBARS concentration was observed for secretions obtained during the nonlactating period (results not shown). However, peroxidation products found in the noncellular fraction of milk only represented less than 10% of the total peroxidation products in mammary gland secretions from all stages.

The antioxidant enzyme functions of infiltrated neutrophils were monitored by measuring activities of SOD and GPX. Amount of total SOD activity, including CuZn-SOD in the cytosol and Mn-SOD in the mitochondria, were significantly lower 1 (148.3 ± 20.3 U/mg of DNA) and 2 (135.6 ± 29.1 U/mg of DNA) weeks after end of lactation, compared with those of late (227.8 ± 30.0 U/mg of DNA) or peak (226.1 ± 14.7 U/mg of DNA) lactation (Fig 4). Similarly, GPX activity at 1 and 2 weeks after end of lactation (99.2 ± 16.6 and 90.3 ± 22.1 U/mg of DNA, respectively) was significantly lower than activity of late (156.6 ± 21.7 U/mg of DNA) or peak (159.3 ± 12.0 U/mg of DNA) lactation. Activities of SOD and GPX were positively correlated throughout the study ($r^2 = 0.97; P < 0.001$). Less than 0.1% of total activity of SOD or GPX in mammary gland secretions was distributed in the noncellular fraction of milk (results not shown), and activities of both enzymes in the noncellular fraction of milk did not differ significantly among the various stages of lactation (data not shown).

Correlation analysis also revealed a significant ($P < 0.001$) negative relationship between TBARS concentration and activity of SOD ($r^2 = -0.75$) or GPX ($r^2 = -0.74$) during the 2 weeks after end of lactation. However, such a correlation did not exist during peak and late lactation. To analyze the possible relationship between DNA degradation and oxidative stress of infiltrated neutrophils, proportion of neutrophil DNA cleavage was used to calculate the correlation with corresponding TBARS concentrations. An obvious correlation was not detected ($r^2 = 0.11$) between these 2 variables during the involution of mammary glands in goats.

**Figure 3**—Mean ± SEM percentage of DNA cleavage in infiltrated neutrophils prepared from mammary gland secretions obtained from 8 goats during late and peak lactation and 1 and 2 weeks after end of lactation. Values with different letters differ significantly ($P < 0.05$). See Figure 1 for remainder of key.

**Figure 4**—Oxidative stress as represented by the concentration of thiobarbituric acid reactive substances (TBARS; top) and activities of superoxide dismutase (SOD; middle) and glutathione peroxidase (GPX; bottom) in infiltrated neutrophils prepared from mammary gland secretions obtained from 8 goats during late and peak lactation and 1 and 2 weeks after end of lactation. Data are reported as mean ± SEM. See Figure 1 for key.
Discussion

In the study reported here, we observed apoptosis in infiltrated neutrophils of mammary glands of goats during the first 2 weeks after end of lactation and, in some goats, during late lactation. In addition, the possible role of antioxidant enzymes in induction of this final event of neutrophils in the mammary glands was examined. Although the importance of infiltrated neutrophils in resorption of milk components and removal of degenerated epithelial cells during involution is obvious, there is scarce information about the clearance of infiltrated neutrophils in the literature. On the contrary, abundant evidence exists about the fate of mammary epithelial cells, indicating that elimination of secretory cells during mammary tissue involution is by apoptosis. Ultrastructural change indicative of apoptosis and DNA internucleosomal fragmentation has been documented in involuting mammary tissues of mice and rats. Also, DNA laddering has been detected in goat mammary tissue at the end of lactation. Adaptation of a sensitive nick-end DNA labeling technique in tissue sections suggested that apoptosis is a normal physiologic event during peak and declining lactation in rodents and ruminants.

Phagocytes engulf alveolar epithelial cells shed into the lumen and residual milk components during involution of mammary glands. Monitoring the fate of infiltrated neutrophils in mammary gland secretions will help us to understand the involution process. Whereas the neutrophil DNA concentration in secretions obtained during the nonlactating period increased by approximately 4- to 8-fold of the concentrations in milk samples obtained during late or peak lactation, the volume of the secretions during the nonlactating period was less than 1% that of peak yield. Thus, the total number of neutrophils in milk during involution was reduced. Neutrophils are recruited from the bloodstream and migrate through the vascular endothelium in response to specific cytokines. Neutrophils produce large amounts of ROI and proteolytic enzymes necessary for host defense. It was initially documented by Savill et al that PMN in blood samples obtained from humans undergo apoptosis in vitro. It has subsequently been postulated that down-regulation of a number of neutrophil functions (chemotaxis, phagocytosis, and granule release) associated with apoptosis promotes resolution of inflammation and limits tissue injury. In the study reported here, the laddering pattern of neutrophil genomic DNA was evident in samples obtained from all goats and 2 weeks after end of lactation. This was further confirmed by the increase of DNA cleavage in neutrophils during the early nonlactating period. To our knowledge, these results are the first evidence that infiltrated neutrophils in mammary glands of goats undergo apoptosis during involution in vivo.

In addition to DNA laddering, DNA cleavage is frequently used to assess DNA integrity. Wyllie reported that results from this quantitative assay were associated with morphologic changes of chromatin condensation. In a study of apoptosis of aged neutrophils obtained from humans, a time-related increase in percentage of cleaved DNA (up to 30%) was observed. In our study, we observed DNA cleavage of up to 75%. A similar high percentage of DNA cleavage has been reported for activation-induced death of T-cell hybridomas. Differences in the percentages for DNA cleavage may be attributable to differences in treatments, cell types, or preparation methods of the DNA fragments. Despite a lack of neutrophil DNA laddering in some milk samples obtained during late lactation in our study, mean DNA cleavage ratio of infiltrated neutrophils was prominently high at this stage of lactation, compared with that of peak lactation. A gradual decrease in frequency of milk removal was practiced during late lactation prior to cessation of lactation; it was noticed that some goats ceased total lactation more quickly than other goats. The differing responses to the procedure at end of lactation among goats may be ascribed as part of the cause of inconsistency between DNA laddering and the DNA cleavage assay for neutrophils from milk obtained during late lactation. It is plausible that large DNA fragments derived from loops or rosettes of chromatin during the initial degradation of DNA contributed to the DNA cleavage assay but were not evident in the DNA laddering assay.

Possible involvement of ROI in the course of apoptosis in infiltrated neutrophils was studied by measuring the extent of oxidative stress as indicated by TBARS concentrations and antioxidant enzyme activities reflected by total SOD and GPX activities. Depletion of antioxidant defense or an increase in ROI production can tip the ROI-antioxidant balance and cause oxidative stress. Oxidative stress has been proposed as a mediator of apoptosis in many cell types, including T cells, human breast cells, and neutrophils. In those studies, antioxidants or free radicals were added in vitro to assess inhibition or induction of apoptosis. The oxidative or respiratory burst in neutrophils also can be triggered by phagocytosis. During this process, abundant molecular oxygen is consumed, resulting in formation of superoxide anion by plasma membrane-bound NADPH oxidase. The major role of the superoxide anion is to serve as a precursor of H2O2 and to generate other oxygen metabolites such as hydroxyl radicals and singlet oxygen. Although these ROI are important for killing bacteria, they also initiate a chain reaction to induce peroxidation of unsaturated lipids in the cell membrane. The enzymes SOD and GPX work together to reduce those ROI. The former reduces superoxide anions to H2O2 at an accelerated rate and, thus, prevents their toxic effects. On the other hand, GPX regulates the amount of intracellular H2O2 by reducing it to water.

During involution of mammary glands in goats, infiltrated neutrophils have more oxidative stress than their counterparts from late or peak lactation. This increased oxidative stress is likely caused by a simultaneous depression in antioxidant enzyme function during involution, because a negative correlation between the activity of SOD or GPX and the corresponding TBARS concentration was documented. On the other hand, a similar extent of peroxidation stress was found in infiltrated neutrophils from late and peak lactation. These cells also have similar activities of SOD and GPX. It is likely that factors other than oxidative stress also contribute to apoptosis of infiltrated neutrophils during late lactation in some goats.
Direct evidence was not provided in this report to support the induction of apoptosis by oxidative stress in infiltrated neutrophils during involu-

mmary glands. Concurrent detection of DNA ladder and increased oxidative stress or depressed antioxidant enzyme function only implied the possible involve-
mnt of ROI in induction of apoptosis at this stage. However, lack of a direct correlation between oxidative stress and DNA cleavage also suggested the involve-
mment of induction factors other than oxidative stress. In a study that used cultured cerebellar granule cells, it was found that apoptosis triggered by nitric oxide was accompanied by induction of lipid peroxidation, as measured by the thiobarbituric acid assay. That same report further revealed an attenuation of lipid peroxidation and prevention of apoptosis following prior treatment with an antioxidant. Similarly, Shindo and Hashimoto declared the importance of antioxidant cellular defenses, especially SOD, for protecting cuta-
neous cell lines from ultraviolet B-induced apoptosis. Evidence that oxidized lipid has a direct role in induc-
tion of apoptosis is relatively rare, except that an oxygen
generate derivative of arachidonic acid, hydroperoxy eicosatetraenoic acid, has been implicated in tumor necrosis factor-induced apoptosis.27

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