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Escherichia coli induces apoptosis and proliferation of mammary cells

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Abstract

Mammary cell apoptosis and proliferation were assessed after injection of *Escherichia coli* into the left mammary quarters of six cows. Bacteriological analysis of foremilk samples revealed coliform infection in the injected quarters of four cows. Milk somatic cell counts increased in these quarters and peaked at 24 h after bacterial injection. Body temperature also increased, peaking at 12 h postinjection. The number of apoptotic cells was significantly higher in the mastitic tissue than in the uninfected control. Expression of Bax and interleukin-1β converting enzyme increased in the mastitic tissue at 24 h and 72 h postinfection, whereas Bcl-2 expression decreased at 24 h but did not differ significantly from the control at 72 h postinfection. Induction of matrix metalloproteinase-9, stromelysin-1 and urokinase-type plasminogen activator was also observed in the mastitic tissue. Moreover, cell proliferation increased in the infected tissue. These results demonstrate that *Escherichia coli*-induced mastitis promotes apoptosis and cell proliferation. *Cell Death and Differentiation* (2001) **8,** 808–816.

Keywords: apoptosis; *Escherichia coli*; proliferation; mammary cell

Abbreviations: CFU, colony forming units; *E. coli*, *Escherichia coli*; ECM, extracellular matrix, ICE, interleukin-1β converting enzyme; IL-1, interleukin-1; LPS, lipopolysaccharide; MMP, matrix metalloproteinase; PA, plasminogen activator; PAI, plasminogen activator inhibitor; RT-PCR, reverse-transcriptase polymerase chain reaction; *S. agalactiae*, *Streptococcus agalactiae*; *S. aureus*, *Staphylococcus aureus*; SCC, somatic cell counts; SL, stromelysin; SV40 Tag, Simian virus 40 T antigen; TIMP, tissue inhibitor of metalloproteinase; TNF α, tumor necrosis factor α

Introduction

Mastitis is characterized as an inflammation of the mammary gland, primarily due to the bacterial infection through teat canals. In the past several decades, the incidence of bovine mastitis caused by contagious pathogens has been reduced dramatically. This has indirectly led to an increased percentage of mastitis caused by environmental pathogens such as Gram-negative *Escherichia coli* (*E. coli*).[1] In addition, *E. coli* may induce severe mastitis that results in extensive mammary tissue damage and even, in some cases, death of the animals.[2–5] Therefore, *E. coli*-induced bovine mastitis has become an area of intense investigation.

It is well known that two major types of cell death, namely apoptosis and necrosis, exist in almost all organisms. Apoptosis has been portrayed as a programmed cell death in response to various inducers including cytokines, bacterial toxins and loss of matrix atachment.[6] During *Streptococcus agalactiae* (*S. agalactiae*)-induced mastitis, the increased expression of an apoptosis marker, TRPM-2, has been reported.[7] Furthermore, *Staphylococcus aureus* (*S. aureus*), one of the major contagious pathogens leading to bovine mastitis, induced apoptosis of a bovine mammary epithelial cell line.[8] These studies suggest that mammary epithelial cells die via apoptosis during bovine mastitis induced by Gram-positive pathogens. However, whether *E. coli* causes mammary tissue damage and cell death through a similar mechanism has not been reported.

Apoptosis is regulated by a cascade of signaling events involving the coordination of many factors. The Bcl-2 family of proteins, including both pro-apoptotic (such as Bax, Bcl-x_S, Bak and Bad) and anti-apoptotic (such as Bcl-x_L and Bcl-2) factors, play important roles in mediating the balance between cell survival and loss in various physiological and pathological events.[9] Additionally, the death signals are conducted by a family of cysteine proteases termed caspases.[10] For example, interleukin-1β converting enzyme (ICE), otherwise called caspase-1, is pro-apoptotic to mouse mammary epithelial cells.[11]

Loss of matrix attachment can also initiate apoptosis.[6] Specifically, extracellular matrix (ECM) mediates cell survival and death in the mammary gland. For example, apoptosis occurred when mouse primary epithelial cells were cultured on plastic or type I collagen substrata, but the cell death was suppressed by basement membrane.[12] Additionally, Merlo *et al*[13] also demonstrated that purified laminin, tenascin C, or collagen IV from ECM reduced apoptosis of a human breast epithelial cell line MCF-10A and a mouse mammary epithelial cell line HC11. Maintenance and breakdown of ECM components are regulated by the balance between the ECM-degrading proteinases and their inhibitors. For instance, altered expression of matrix metalloproteinases (MMPs) such as

stromelysins and gelantinases and serine proteinases such as plasminogen activators (PAs) coincided with mammary cell apoptosis during involution. As well, expression of the inhibitors for these enzymes, such as tissue inhibitors of metalloproteinases (TIMPs) and plasminogen activator inhibitors (PAIs), was decreased during apoptosis of mammary cells.[14,15] Conversely, expression of TIMP-1 transgene rescued the unscheduled apoptosis during late pregnancy in stromelysin-1 (SL-1) transgenic mice.[16] Taken together, these results have confirmed the importance of ECM-degrading enzymes and their inhibitors in regulation of programmed mammary cell death. However, the association of ECM breakdown and cell apoptosis was mostly demonstrated during mammary involution. Whether ECM degradation is involved in tissue damage and cell death during pathological events such as *E. coli*-induced mastitis has not been extensively documented.

Cell number is dependent on not only cell death, but also proliferation. Therefore, it is also important to investigate the alteration of cell proliferation during mastitis. Although a previous *in vitro* study demonstrated inhibition of bovine mammary epithelial cell growth by *E. coli* endotoxin and *S. aureus* α-toxin,[17] impact of experimentally induced *E. coli* mastitis on cell proliferation in bovine mammary gland has not been elucidated.

In the present study, mammary cell proliferation and death during *E. coli*-induced bovine mastitis were investigated. Expression of Bcl-2-related proteins and ICE was evaluated. In addition, alteration of ECM-degrading enzymes and their inhibitors was examined.

Results

E. coli challenge induced intramammary infection

Five hundred colony-forming units (CFU) of *E. coli* were injected into the left quarters of six healthy lactating cows. As a result, the left quarters of four cows were infected by *E. coli*, as proven by bacteriological analysis of foremilk samples obtained 12 h and 24 h after bacterial injection, as well as the increases in milk somatic cells counts (SCC) and rectal temperature. Compared to samples collected prior to injection ($1.79 \pm 0.63 \times 10^5$ cells/ml), mean milk SCC increased at 12 h postinjection ($3.51 \pm 0.46 \times 10^6$ cells/ml, $P<0.01$) and peaked at 24 h postinjection ($1.10 \pm 0.25 \times 10^7$ cells/ml, $P<0.01$), decreasing gradually thereafter. By 144 h after bacterial injection, milk SCC ($1.83 \pm 1.10 \times 10^5$ cells/ml) did not differ from uninfected controls (Figure 1). Rectal temperature also increased after bacterial injection (Figure 2). The temperature peaked at 12 h postinjection, which was significantly higher than that of preinfected cows (40.1 ± 0.5°C vs 38.5 ± 0.3°C, $P<0.05$).

Apoptosis occurred in *E. coli*-infected mammary glands

In order to investigate whether mammary cells died by apoptosis, the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay was performed. For each sample, cells were classified into three types (epithelial, stromal, and lumenal cells) and apoptotic cells were counted in 10 microscopic fields (400× magnification). *E. coli* mastitis increased the percentage of apoptotic epithelial and stromal cells compared with that in uninfected glands ($P<0.05$, Table 1 and Figure 3). On the other hand, percentage of apoptotic cells in the lumina did not differ between infected and uninfected quarters ($P>0.05$).

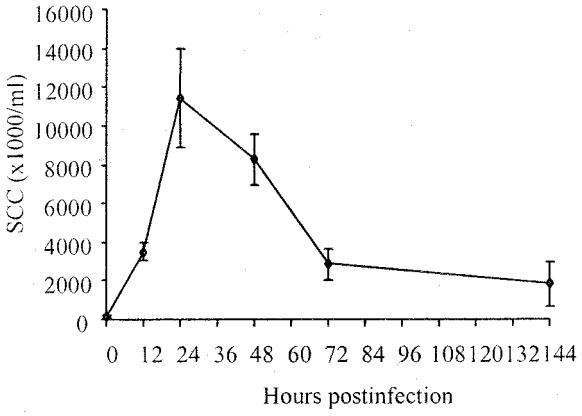

Figure 1 Milk SCC upon bacterial challenge. Left quarters were injected with 500 CFU of *E. coli*, and SCC was measured in foremilk samples collected from the infected glands. Data are presented as the means ± S.E.M.

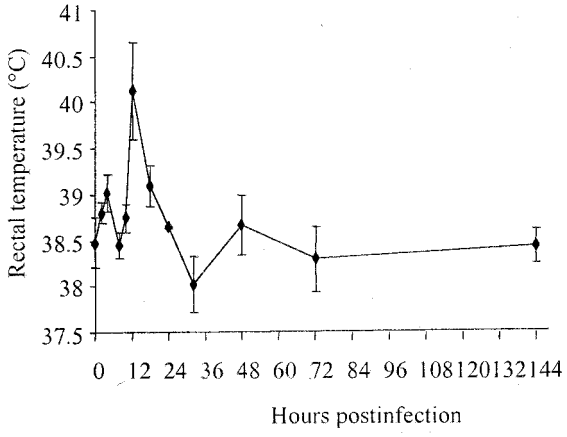

Figure 2 Rectal temperature of cows upon bacterial challenge. Left quarters were injected with 500 CFU of *E. coli*, and rectal temperature was measured. Data are presented as the means ± S.E.M.

Table 1 Mammary cell apoptosis induced by *E. coli* infection

	Apoptotic cells/10 microscopic fields[a]	
Cell type	**Control**	**Mastitic**
Epithelial	1.80 ± 0.5^b	8.80 ± 2.85^c
Stromal	7.33 ± 5.7^b	18.00 ± 6.79^c
Lumenal	5.17 ± 3.6^b	5.00 ± 1.83^b

[a]Values are means ± S.E.M. for numbers of apoptotic cells in 10 microscopic fields. [b,c]Values in the same row with different superscripts statistically differ ($P<0.05$)

Expression of Bcl-2 family of proteins and ICE was altered during *E. coli*-induced mastitis

Next, participation of Bcl-2 family of apoptotic regulatory genes in the induction of apoptosis during *E. coli*-induced mastitis was investigated by examining the expression of Bax, Bcl-2 and Bcl-x at both protein and RNA levels. Compared to uninfected controls, Western blot analysis revealed a 130 and 100% increase in Bax expression at 24 and 72 h after bacterial injection, respectively (Figure 4A). Conversely, Bcl-2 protein level decreased by 75% at 24 h postinfection, but did not differ from uninfected tissues at 72 h (Figure 4B). There was no significant difference in Bcl-x_L expression between the infected and uninfected mammary tissue samples (Figure 4C).

The RNA levels of the above-mentioned genes were also detected using the reverse-transcriptase polymerase chain reaction (RT–PCR). Bax expression increased by 140 and 100% at 24 and 72 h postinfection, in comparison with the controls (Figure 5A). At 24 h after *E. coli* challenge, the RNA level of Bcl-2 decreased 50% compared to controls, but did not differ 72 h postinjection (Figure 5B). Consistent with protein expression, RNA level of Bcl-x_L did not differ between control and infected tissues. Bcl-x_S was not detectable even after 40 cycles of PCR (Figure 5C). In all cases, equal RNA loading was verified by amplifying a housekeeping gene, GAPDH (Figure 5E).

In addition to the Bcl-2 family of proteins, expression of ICE was also examined by RT–PCR. Compared to the control samples, ICE expression increased by approximately 170% at both 24 and 72 h postinfection (Figure 5D).

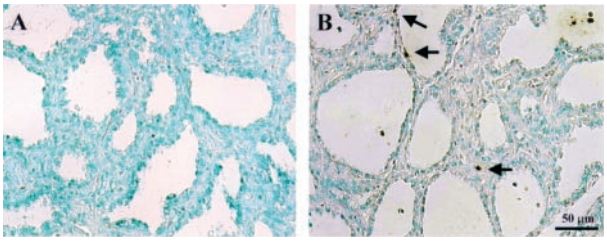

Figure 3 Detection of apoptotic cells by the TUNEL method. The apoptotic cell nuclei with brown staining (arrows) were visualized under microscope. (**A**) Uninfected control mammary tissue; (**B**) *E.coli* infected mammary tissue

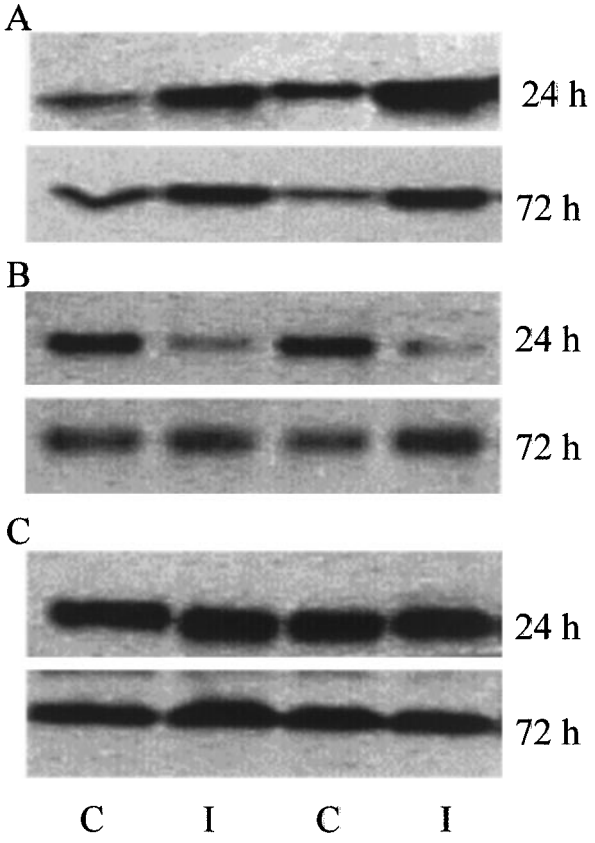

Figure 4 Protein expression of apoptosis-related genes in mammary tissue. *E. coli* infected mammary glands were biopsied at either 24 h or 72 h after bacterial challenge. The contralateral quarters were biopsied prior to challenge and served as controls. Proteins were extracted and Western blot analysis performed. C: control; I: infected. (**A**) Bax; (**B**) Bcl-2; (**C**) Bcl-x

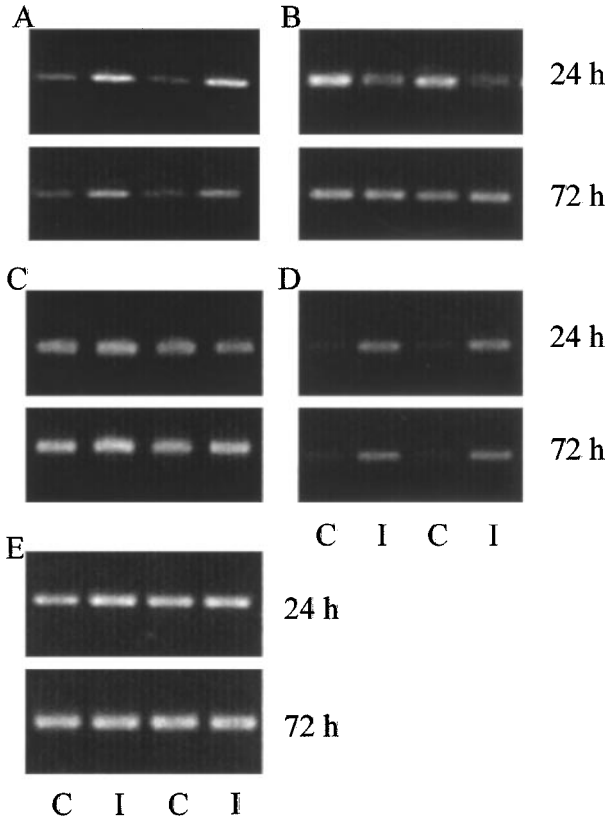

Figure 5 RNA expression of apoptosis-related genes in mammary tissue. *E. coli* infected mammary glands were biopsied at either 24 h or 72 h after bacterial challenge. The contralateral quarters were biopsied prior to challenge and served as controls. RNA was extracted and subjected to RT–PCR. Equal amount of RNA loading was verified by the expression of GAPDH gene. C: control; I; infected. (**A**) Bax; (**B**) Bcl-2; (**C**) Bcl-x; (**D**) ICE; (**E**) GAPDH

Association of ECM-degrading enzyme expression with *E. coli*-induced mastitis

Expression of ECM-degrading enzymes and their inhibitors during *E. coli*-induced mastitis was also investigated in the current study. Gelatin zymography revealed an increase in the activity of a 92-kDa gelatinase by 10- and seven-fold at 24 and 72 h after bacterial challenge, as compared to the uninfected control samples (Figure 6). According to its apparent molecular weight, this enzyme was postulated as gelatinase B (MMP-9). To partially confirm this, expression of MMP-9 at the RNA level was investigated by RT–PCR. MMP-9 mRNA increased by approximately 280 and 250% at 24 and 72 h postinfection, as compared to control samples (Figure 7A). Furthermore, expression of SL-1, another MMP that was induced during mouse mammary involution, was investigated. SL-1 RNA increased to a lesser extent (about 50%) than that of MMP-9 at 24 and 72 h after bacterial challenge (Figure 7B). Besides, uPA expression also increased by 150 and 200% at 24 and 72 h after *E. coli* injection (Figure 7C). Equal amount of RNA loading was verified by amplifying a housekeeping gene, GAPDH (Figure 7D).

Apart from the above-mentioned genes, the expression of tPA, TIMP-1, TIMP-2 and PAI-1 was also measured by RT–PCR. No significant difference between the infected and uninfected samples was observed (data not shown).

Cell proliferation was promoted in *E. coli*-infected mammary glands

In order to investigate the alteration of cell number during *E. coli*-induced mastitis, cell proliferation was also investigated by Ki-67 immunohistochemistry. Percentage of proliferating cells labeled with Ki-67 over total cells was arcsine-transformed to normalize the data distribution. Proliferation of all three cell types (epithelial, stromal, lumenal) was much higher in the infected glands than in controls ($P<0.05$, Table 2 and Figure 8).

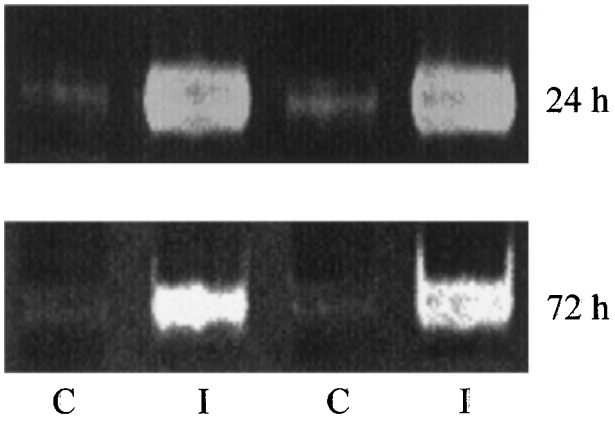

Figure 6 Expression and activity of a 92-kDa gelatinase (MMP-9). *E. coli* infected mammary glands were biopsied at either 24 h or 72 h after bacterial challenge. The contralateral quarters were biopsied prior to challenge and served as controls. Proteins were extracted and subjected to SDS–PAGE containing 0.1% gelatin. C: control; I: infected

Discussion

The present study demonstrated cell apoptosis in *E. coli*-infected bovine mammary glands, which was accompanied by increased expression of Bax and ICE, and decreased expression of Bcl-2. Expression of several ECM-degrading enzymes was also increased in the infected glands. Additionally, proliferation of mammary cells was increased during *E. coli*-induced mastitis. To our knowledge, this is

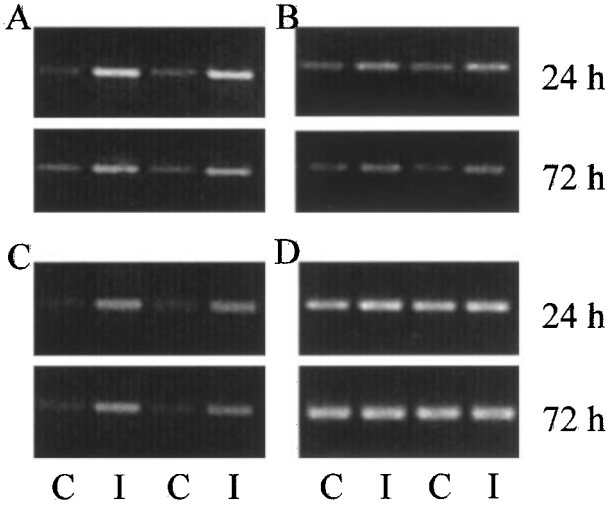

Figure 7 RNA expression of ECM-degrading enzymes in mammary tissue. *E. coli* infected mammary glands were biopsied at either 24 h or 72 h after bacterial challenge. The contralateral quarters were biopsied prior to challenge and served as controls. RNA was extracted and subjected to RT–PCR. Equal amount of RNA loading was verified by the expression of GAPDH gene. C: control; I: infected. (**A**) MMP-9; (**B**) SL-1; (**C**) uPA; (**D**) GAPDH

Table 2 Mammary cell proliferation induced by *E. coli* infection

	Arcsine transformed % Ki-67 labeled cells[a]	
Cell type	**Control**	**Mastitic**
Epithelial	0.034 ± 0.003^{b}	0.107 ± 0.01^{c}
Stromal	0.046 ± 0.01^{b}	0.114 ± 0.02^{c}
Lumenal	0.007 ± 0.01^{b}	0.046 ± 0.0216^{c}

[a]Values are arcsine transformed percentage of Ki-67 labeled cells over total cells. [b,c]Values in the same row with different superscripts statistically differ ($P<0.05$)

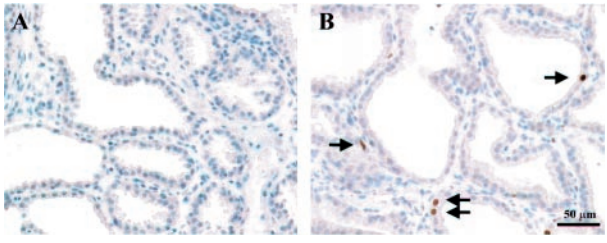

Figure 8 Detection of cell proliferation by Ki-67 staining. The Ki-67 labeled cells with brown staining (arrows) were visualized under microscope. (**A**) Uninfected control mammary tissue; (**B**) *E. coli* infected mammary tissue

the first study to provide evidence that *E. coli* infection induces cell death via apoptosis in lactating mammary glands.

Increased apoptosis of mammary cells in mastitic glands was probably induced by the up-regulation of pro-apoptotic factors, Bax and ICE. Involvement of Bax in regulation of mammary cell apoptosis has been documented extensively. For instance, Bax expression was induced at the onset of mouse mammary involution, which was accompanied by programmed cell death.[18–20] Premature apoptosis during pregnancy in mice carrying a Simian virus 40 T antigen (SV40 Tag) transgene was associated with a steady level of Bax expression.[21,22] Loss of functional Bax reduced apoptosis by 20% in the first stage of mouse mammary involution.[23] Participation of ICE in mammary cell apoptosis has also been suggested in previous studies. At day 2 of mouse mammary involution, when programmed cell death started to be observed, ICE expression was up-regulated.[14] Moreover, inhibition of ICE reduced apoptosis induced by loss of ECM or anti-Fas in mouse or human mammary epithelial cells.[11,24]

In addition to the alteration of Bax and ICE expression, downregulation of Bcl-2 may also contribute to apoptosis during *E. coli* mastitis, since its anti-apoptotic effect has been very well documented. Ectopic expression of Bcl-2 in mammary epithelial cells completely prevented apoptosis induced by serum and grow-factor withdrawal.[13] In MCF-7 cells, induction of apoptosis by bFGF overexpression was accompanied by downregulation of Bcl-2 expression.[25] Moreover, apoptosis was suppressed by 50 and 70% in Bcl-2 transgenic mice during the first and second stages of involution, respectively.[23]

Bcl-2 expression in mouse lactating mammary gland was detected by Merlo *et al*,[13] but not by other researchers.[19,20] In our experiments, Bcl-2 expression was detected in bovine lactating mammary glands at both protein and RNA levels. The discrepancy from different studies may be due to differences in genetic regulation between different species, or different breeds within the same species. Weak Bcl-2 expression was also detected in sheep mammary tissue at the first day of cessation of lactation, when alveolar structure remained almost as intact as that of the lactating stage.[26] It has been suggested that Bcl-2 is expressed by ductal but not secretory alveolar epithelial cells in mice,[12] thus, ruminants and some mouse strains may have higher percentages of ductal cells during lactation than other mouse strains, which results in variation of Bcl-2 expression. Nevertheless, the difference in Bcl-2 expression reported by different research groups may also be ascribed to distinct experimental conditions. In the Western blot analyses conducted by us and Merlo *et al*,[13] at least 80 μg of proteins were used in SDS-gel electrophoresis, whereas Metcalfe *et al*[19] only applied 20 μg of total proteins in their study.

The unaltered expression of Bcl-x_L suggested that this gene did not regulate the apoptosis induced by *E. coli* infection of the mammary tissue. Consistent with another investigation, Bcl-x_S was undetectable in lactating mammary glands by RT–PCR.[19] However, Heermeier *et al*[18] reported low level expression of Bcl-x_S in mouse lactating mammary gland. This discrepancy may be due to higher assay sensitivity in the latter study by combining DNA hybridization with RT–PCR.

Expression of MMP-9, SL-1 and uPA was increased in association with apoptosis during *E. coli* mastitis. MMP-9 can be produced by bovine neutrophils as well as human mammary and airway epithelial cells.[27–30] Furthermore, an established bovine mammary epithelial cell line, MAC-T also produced MMP-9, as detected by gelatin zymography in the current study (data not shown). As shown in the current study, MMP-9 was expressed at a very low level in healthy bovine lactating glands. Upon *E. coli* infection, it is possible that mammary epithelial cells increase the expression of MMP-9 upon stimulation by pro-inflammatory mediators such as interleukin-1 (IL-1) and tumor necrosis factor α (TNF-α), which are induced during *E. coli* mastitis.[31] As reported by Roh *et al*,[32] MMP-9 production by human smooth muscle cells was increased by IL-1β and TNF-α. Alternatively, elevated MMP-9 expression may be due to an increase in the neutrophil population of the mammary tissue since neutrophils are recruited and rapidly migrate to the site of local infection,[4] as evidenced by increased milk SCC after exposure to *E. coli*. Cytokines induced by *E. coli* challenge could also stimulate neutrophils to express more MMP-9. It is also worth noting that gelatin zymography revealed a much higher increase (10- and sevenfold at 24 and 72 h postinfection, respectively) of MMP-9 than did RT–PCR (3- and 2.5-fold increase at 24 and 72 h postinfection, respectively). This may be a reflection of increased enzyme activity in addition to the up-regulation of gene expression. However, it can also be due to differences in translational efficiency or mRNA half-life of this enzyme between the uninfected and infected glands. SL-1, whose expression correlated with apoptosis during mammary involution,[14] contributed to the breakdown of most ECM components including laminin and collagen type IV.[33] These components have been viewed as survival factors in mammary tissue.[13] SL-1 expression could also be induced by IL-1, as shown in cardiac fibroblasts and human endometrial cells.[34,35] Expression of uPA was shown to be stimulated in bovine mammary epithelial cells by *S. aureus*, a major contagious pathogen resulting in mastitis.[36] Likewise, the stimulatory effect of *E. coli* mastitis on uPA expression has been demonstrated in the current study. In PC-3 human prostatic cancer cells, lipopolysaccharide (LPS), a bacterial endotoxin which can be released by *E. coli*, increased uPA expression.[37] It remains to be demonstrated whether LPS or the pro-inflammatory cytokines induced by *E. coli* are responsible for the production of ECM-degrading enzymes in the bovine mammary gland.

While it is clear that *E. coli* infection induced programmed cell death in the mastitic mammary tissue, the extracellular mediators of this effect are unknown. One candidate is LPS released by *E. coli*. Although the total pathogenesis of *E. coli* mastitis was probably not reproducible by LPS administration, infusion of LPS did lead to many clinical aspects of *E. coli* infection.[4] The pro-apoptotic effect of LPS has been shown on Caco-2 human intestinal epithelial cells[38] and bovine glomerular endothe-

lial cells.[39] Accordingly, LPS may exert a similar effect on cell death in the mammary tissue. However, *in vitro*, LPS did not cause tissue damage in explants of lactating bovine mammary tissue[40] but stimulated the expression of IL-1 by MAC-T cells.[41] Furthermore, *E. coli* infusion into bovine mammary glands also induced production of pro-inflammatory cytokines, including IL-1 and TNF-α.[31,42] TNF-α and IL-1 induced programmed cell death in a variety of cell types, such as bovine endothelial cells[39] and human mammary, intestinal, bronchial and endometrial epithelial cells.[38,43–45] Therefore, the increased apoptosis in *E. coli*-infected mammary gland may be attributable to *E. coli*- or LPS-induced proinflammatory cytokines.

Apart from increased apoptosis, cell proliferation was also promoted during *E. coli* mastitis. This may as well be accounted by the signaling mediators triggered by LPS or the cytokines. It is not uncommon that one gene may have a dual effect on proliferation and apoptosis. For instance, expression of SV40 Tag in mouse mammary glands induced unscheduled apoptosis of a large amount of cells during the pregnancy period. Nevertheless, the surviving cells underwent deregulated growth and resulted in tumor formation after the first pregnancy.[21,22] As well, Bcl-2 was shown to be anti-proliferative in addition to its anti-apoptotic function.[46] In our study, the decrease of Bcl-2 expression at 24 h postinfection may in part account for the increased rate of cell proliferation. Furthermore, apart from its role in ECM degradation and apoptosis induction, SL-1 was suggested to play an important role in mammary tumorigenesis,[33] indicating its potential function in promoting cell proliferation.

Increased cell proliferation during coliform mastitis may serve as a mechanism to ameliorate tissue damage. Although *E. coli* may induce severe clinical symptoms, most infected cows will eliminate the bacteria and undergo self-cure.[2–5] While bacterial toxins, or the proinflammatory mediators, may cause cell death by apoptosis, increased cell proliferation may compensate for the cell loss during inflammation. Interestingly, a previous study also showed an increase in DNA synthesis with the induction of bovine mammary involution by milk stasis, when mammary cell apoptosis was typical.[47]

The signaling events involved in mammary cell apoptosis and proliferation induced by *E. coli* infection are still not fully understood. The ligands, receptors, and genes participating in transmitting the death and proliferating signals have not been clarified. Recently, it was reported that *S. aureus* induced mammary cell apoptosis through a mechanism involving caspases 3 and 8.[48] Elucidation of mechanims leading to apoptosis and cell proliferation during *E. coli* mastitis requires further study.

In summary, *E. coli* infection caused programmed cell death in mastitic tissue. Bax, ICE and Bcl-2 may play important roles in regulating the cell death and survival. Alteration of MMP-9, SL-1 and uPA expression was associated with cell apoptosis, suggesting that ECM degradation may partially account for the cell loss during mastitis. Proliferation of cells was also stimulated, which may be part of the self-cure mechanism after *E. coli* infection.

Materials and Methods

Animals

Six healthy mid-lactating Holstein cows from the Beltsville Agricultural Research Center dairy herd (Beltsville, MD, USA) were selected for this study. Selection criteria included noninfected mammary quarters and milk SCC less than 250×10^3 cells/ml at the time of bacterial challenge exposure. Use of animals for this investigation was approved by the Beltsville Agricultural Research Center's Animal Care and Use Committee.

Sample collection

Quarter foremilk samples for the determination of SCC and for bacteriological examination were collected aseptically twice a week for several weeks before bacterial challenge exposure. Milk SCC in all mammary quarters were consistently less than 250×10^3 cells/ml and mammary quarters were free from intramammary infection.

E. coli challenge exposure was performed immediately after a specified morning milking. Foremilk samples for bacteriological examination and SCC determination were collected immediately before *E. coli* challenge exposure, to establish a base line, and at the next five milkings (12, 24, 48, 72 and 144 h). Clinical observations, including rectal temperature, milk appearance, udder palpation and the general condition of the cows, were made just before bacterial injection and at the same time points, after injection, as the milk sample collections. Rectal temperatures were additionally taken at 2, 4, 8 and 10 h postinjection.

The challenged rear quarters of three cows were biopsied 24 h after bacterial injection from the upper half of the gland, while biopsies from the other three cows were obtained 72 h after bacterial injection, using the biopsy method described by Farr *et al*.[49] The biopsies contained cores of the secretory tissue. A portion of each tissue biopsy was immediately snap frozen in liquid nitrogen and stored at -80°C until protein or RNA extraction. The remaining tissue was placed in 10% neutral-buffered formalin for immunohistochemical analyses. The tissue was fixed at 4°C overnight and then processed for paraffin embedding and sectioning. Biopsies from the right rear quarters of all six cows were obtained prior to *E. coli* injection as control samples.

Intramammary challenge exposure

The organism used was a serum-resistant *E. coli*, strain P-4, serotype O32:H37, which originally had been recovered from a cow with clinical mastitis[50] and had been used in studies of *E. coli* mastitis.[51] Before the challenge exposure, a tube of brain–heart infusion broth (Baltimore Biological Laboratories, Division of Becton, Dickinson & Co., Cockeysville, MD, USA) was inoculated with lyophilized *E. coli* and incubated for 18 h at 37°C. The resulting broth culture was streaked onto a Trypticase soy blood agar plate to determine its purity. After incubation, several colonies were transferred to 10 ml of Todd–Hewett broth, incubated for 6 h at 37°C and refrigerated. On the basis of consistency of bacterial population in cultures, the challenge–exposure inoculum was prepared, using serial dilutions in sterile distilled water and a final dilution in low-endotoxin Earle's balanced salt solution. The desired challenge–exposure inoculum was 250 CFU/ml. Bacterial challenge exposure in each cow was performed immediately after the specified milking, by gentle injection of a 2-ml volume (500 CFU) of the prepared inoculum into the gland sinus of left front and left rear mammary quarters.

Bacterial culture

Each foremilk sample was plated in a 0.05-ml volume onto Trypticase soy blood agar for identification of *E. coli* and purity. Infection was defined as recovery of *E. coli* from at least two consecutive samples with a concurrent increase in SCC.

Determination of SCC

Foremilk samples were heated for 15 min at 60°C and maintained at 40°C until being counted (Fossomatic 90; Foss Electric, Hilleroed, Denmark). The cell counter was calibrated monthly with bovine milk somatic cell standards (Dairy Quality Control Institute Services, Mountain View, MN, USA). Duplicate counts were made on each milk sample.

In situ detection of apoptotic cells and Ki-67 immunolocalization

After fixation at 4°C overnight, tissues were placed in 70% ethanol, embedded in paraffin and sectioned at 6 μm according to standard procedures.

Apoptotic cells in histological sections were detected by the TUNEL assay using the Apoptag kit (Intergen, Purchase, NY, USA), as previously described with slight modifications.[52] In brief, tissue sections were deparaffinized and hydrated, next they were treated with 20 μg/ml of proteinase K in phosphate-buffered saline (PBS) for 15 min and quenched in 2% H_2O_2 in PBS for 10 min at room temperature. Tissue sections were incubated with terminal deoxynucleotidyl transferase for 60 min at 37°C, washed, and then incubated with anti-digoxigenin-peroxidase for 30 min at room temperature. Following the reaction and color development with diaminobenzidine, sections were counter-stained with methyl green (0.1% in acetate buffer, pH 4.0) and mounted with Permaslip (Alban Scientific Inc., St. Louis, MO, USA). Apoptotic cells were visualized and counted microscopically.

Immunolocalization of Ki-67 antigen was performed following the instruction provided by the manufacturer of the primary antibody (Zymed Laboratories, San Francisco, CA, USA). Briefly, slides were deparaffinized, hydrated, quenched with 3% H_2O_2 in phosphate buffered saline (PBS) for 10 min, washed in PBS, and heat-treated in a microwave oven for antigen retrieval in 10 mM citrate buffer (pH 6.0). Slides were washed in PBS and blocked with 5% non-immune goat serum in PBS. The MIB-1 monoclonal antibody (Zymed) and Histostain SP kit (Zymed) was used for immunolocalization of Ki-67 antigen. Slides were incubated with the primary antibody for 60 min at room temperature with the biotinylated secondary antibody, washed in PBS, and then incubated with the steptavidin-peroxidase-conjugate for 10 min at room temperature. After washing in PBS, sections were incubated with diaminobenzidine, counter stained with hematoxylin and mounted with Permaslip.

Protein extraction

Ten mg of frozen tissues were homogenized in 500 μl of RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet-P 40, 0.5% sodium deoxycholate and 0.1% SDS, pH 8.0) containing 2 mM PMSF, 20 μg/ml of leupeptin, 10 μg/ml of pepstatin, 10 μg/ml of aprotinin and 1 mM NaF, using a high shear homogenizer (Tissuemizer Mark II T25, Tekmar Company, Cincinnati, OH, USA). Samples were kept on ice for 15 min, vortexed vigorously for 1 min, and then centrifuged at 13 000 $\times$ *g* for 15 min at 4°C. Supernatants were collected and protein concentrations were quantitated by BCA protein assay (Pierce, Rockford, IL, USA).

Western blot analysis

Expression of Bcl-2, Bax and Bcl-x proteins in the mammary tissues was investigated by Western blot analysis. A mouse anti-Bax monoclonal antibody (clone 2D2, Zymed), a mouse anti-Bcl-2 monoclonal antibody (NCL-bcl-2, Novacastra, Newcastle, UK) and a rabbit anti-Bcl-x polyclonal antibody (Transduction Laboratories, Lexington, KY, USA) were used. Except for Bcl-2 detection, where 100 μg of proteins were applied, 50 μg of proteins from each sample were electrophoretically separated on a 14% SDS-polyacrylamide gel (NOVEX, San Diego, CA, USA). Afterwards, proteins were transferred onto a P-immobilon PVDF membrane (Millipore, Bedford, MA, USA) using an electro-transblot apparatus (NOVEX). The membrane was blocked in Tris-buffered saline containing 0.05% Tween-20 (TBS-T, pH 7.6) with 5% non-fat milk powder for 1 h at room temperature, and followed by incubation with the primary antibodies at different dilutions (1 : 500 for anti-Bax, 1 : 50 for anti-Bcl-2 and 1 : 1000 for anti-Bcl-x) in TBS-T with 1% bovine serum albumin for 1 h. After three washes with TBS-T for 5 min each, the membrane was incubated with secondary antibodies (1 : 1000 diluted in TBS-T, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 45 min. The membrane was then washed three times in TBS-T for 10 min each. Proteins were detected by the enhanced chemiluminescence (ECL) method following the manufacturer's instructions (Amersham, Arlington Heights, IL, USA). Intensity of the protein bands was densitometrically quantitated using the Kodak 1D Image Analysis Software (Eastman Kodak Company, Rochester, NY, USA).

Total RNA isolation

Ten mg of frozen tissues were lysed in 1 ml of RNA STAT-60 (Tel-test Inc, Friendswood, TX, USA) using a Takmar homogenizer. Two hundred μl of chloroform were added to each ml of cell lysate, and mixed thoroughly by vigorous shaking for 15 s. After 2–3 min, the sample mixture was centrifuged at 12 000 $\times$ *g* for 15 min at 4°C. The aqueous phase was transferred into a clean tube and incubated with 500 μl of isopropyl alcohol for 5 min. The RNA was precipitated by centrifugation at 12 000 $\times$ *g* for 10 min at 4°C. Afterwards, the RNA was washed with 1 ml of 75% ethanol by gentle vortexing, and then centrifuged at 7500 $\times$ *g* for 5 min at 4°C. RNA pellets were air-dried for 5–10 min, dissolved in 0.1% diethylpyrocarbonate-treated water, and stored at -80°C.

RT–PCR

The mRNA levels of pro-apoptotic and anti-apoptotic factors (ICE, Bax, Bcl-2 and Bcl-x), as well as ECM-degrading enzymes and their inhibitors were examined by RT–PCR. Reverse transcription of total RNA was performed following the instructions provided by the manufacturer (Gibco BRL, Burlington, Ont, Canada) of the Superscript II^{TM} RNase H$^-$ reverse transcriptase (RT). Briefly, 2 μg of total RNA were incubated with 0.5 μg of Oligo (dT)$^{12-18}$ (Gibco BRL) in 20 μl of reaction mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 500 μM dNTPs (Pharmacia, Baie d'Urfé, Que, Canada) and 200 U of RT at 42°C for 50 min. The RT reaction product was then amplified by polymerase chain reaction (PCR) with gene-specific primers and annealing temperatures (Table 3). The number of PCR cycles for each gene amplification was optimized to lie within the linear range and hence was also gene-dependent (Table 3). The 50 μl reaction mixture consisted of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.5 μM primers, 2 μl of RT reaction product and 2.5 U of *Taq* DNA polymerase (Gibco BRL). The PCR amplification was preceded by sample denaturation for 5 min at 94°C. Each cycle contained a denaturation

Table 3 Primers and annealing temperatures used in RT–PCR

Gene	Genbank Access No[a]	Primer sequence (5′–3′)	No. of cycles	Annealing Temperature
Bax	U92569	S[b]:TGAGCAGATCATGAAGACAGGG A[b]:TGAGCACTCCAGCCACAAAGA	28	60°C
Bcl-2	U92434	S:GATGACTTCTCTCGGCGCTAC A:AGTGCCTTCAGAGACAGCCAG	34	60°C
Bcl-x[c]		S:GGCAGCCGATGAGTTTGACCTG A:CATGCCCGTCAGGAACCAGC	32	64°C
ICE[c]		S:TTCATGTCTCATGGAATTCGG A:TCCTCCAAGTCACAAGACCAG	34	58°C
SL-1	AF069642	S:TCTTCCTTCAGGCATAGATGC A:AACAGCATCCACCTCTGATTC	34	56°C
UPA	X80851	S:GGAATGGTCACTCTTACCGAG A:AACAATCTTAAAGCGAGGCCT	32	58°C
TPA	X85800	S:GAAGACAGAGTTCCTGTGCGTG A:CACAGTACCTCCCAGGTCAGCTG	32	60°C
TIMP-1	S70841	S:TGAAACTGCCTTATACCAGCG A:AGTGAGTGTCGCTCTGCAGTT	32	58°C
TIMP-2	AF144764	S:ACTCTGGCAACGACATCTACG A:TCTTGATGCAGGCGAAGAACT	32	58°C
PAI-1	X16383	S:ATCCAAGAGGCAATGCAATTC A:GCTGAGAGTGTTCCCGTGGTA	32	60°C
GAPDH	U85042	S:CTGGCAAAGTGGACATTGTCGCC A:CTTGGCAGCGCCGGTAGAAGC	24	60°C

[a]Except for Bcl-x and ICE, all primers were designed based on the sequences obtained from GenBank (NCBI/EMBL). [b]S: sense primer; A: antisense primer. [c]Bcl-x and ICE primers were designed based on the sequences reported by Rueda *et al*.[53]

period for 45 s at 94°C, an annealing step for 30 s (temperature was gene-dependent) and an extension period for 90 s at 72°C. Following the requisite number of cycles, samples were incubated for a final 7 min at 72°C. Ten μl of PCR products were electrophoresed on a 1.8% agarose gel and ethidium bromide-stained DNA was photographed under transmitted ultraviolet light using a Kodak DC120 zoom digital camera. Intensity of the bands was densitometrically quantitated using the Kodak 1D Image Analysis Software.

Gelatin zymography

Expression and activity of gelatinases were evaluated by gelatin zymography. Fifty μg of protein lysates were subjected to non-reducing SDS-polyacrylamide gel electrophoresis (PAGE) on a pre-cast 10% Tris-Glycine gel with 0.1% gelatin (NOVEX). After electrophoresis, the gel was incubated in 1 × NOVEX zymogram renaturing buffer (2.5% Triton X-100 in water) with gentle agitation for 30 min at room temperature, followed by equilibration with zymogram developing buffer (10 mM Tris Base, 40 mM Tris HCl, 200 mM NaCl, 5 mM $CaCl_2$ and 0.2% Brij 35 in 1 l water) for 30 min at room temperature. Fresh zymogram developing buffer was then replaced and incubated with the gel at 37°C overnight. The next day, the gel was stained with 0.1% Coomassie Blue R-250 in acetic acid/methanol/water (1/4/5) for 30 min, and destained in acetic acid/methanol/water (1/3/6) until the bands were clearly visualized. Intensities of the bands were quantitated using the Kodak 1D Image Analysis Software.

Statistical analysis

Statistical analysis was performed using NCSS 97 software (JL Hintze, Kaysville, Utah, USA). For milk SCC and rectal temperature of the cows, comparison of two treatment groups (infected and uninfected samples) was carried out by a paired *t*-test. For other assays, difference between the infected and uninfected samples was analyzed by the student's *t*-test. Data were expressed as means ± standard errors of the means (S.E.M.).

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