# Characterization of gelatinases in bronchoalveolar lavage fluid and gelatinases produced by alveolar macrophages isolated from healthy calves

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**Objective**—To characterize gelatinases in bronchoalveolar lavage fluid (BALF) and gelatinases produced by alveolar macrophages of healthy calves.

**Sample Population**—Samples of BALF and alveolar macrophages obtained from 20 healthy 2-month-old calves.

**Procedure**—BALF was examined by use of gelatin zymography and immunoblotting to detect gelatinases and tissue inhibitor of metalloproteinase (TIMP)-1 and -2. Cultured alveolar macrophages were stimulated with lipopolysaccharide (LPS), and conditioned medium was subjected to zymography. Alveolar macrophage RNA was used for reverse transcriptase-polymerase chain reaction assay of matrix metalloproteinases (MMPs), cyclooxygenase-2, and inducible nitric oxide synthase.

**Results**—Gelatinolytic activity in BALF was evident at 92 kd (14/20 calves; latent MMP-9) and 72 kd (18/20; latent MMP-2). Gelatinolytic activity was evident at 82 kd (10/20 calves; active MMP-9) and 62 kd (17/20; active MMP-2). Gelatinases were inhibited by metal chelators but not serine protease inhibitors. Immunoblotting of BALF protein and conditioned medium confirmed the MMP-2 and -9 proteins. Endogenous inhibitors (ie, TIMPs) were detected in BALF from all calves (TIMP-1) or BALF from only 4 calves (TIMP-2). Cultured alveolar macrophages expressed detectable amounts of MMP-9 mRNA but not MMP-2 mRNA.

**Conclusions and Clinical Relevance**—Healthy calves have detectable amounts of the gelatinases MMP-2 and -9 in BALF. Endogenous inhibitors of MMPs were detected in BALF (ie, TIMP-1, all calves; TIMP-2, 4 calves). Lipopolysaccharide-stimulated alveolar macrophages express MMP-9 but not MMP-2 mRNA. The role of proteases in the pathogenesis of lung injury associated with pneumonia has yet to be determined. (*Am J Vet Res* 2004;65:163–172)

**Bovine respiratory disease complex (BRDC)** involves multiple viral, bacterial, and toxic agents as well as husbandry factors and host responses to these stimuli.[1-7] The summation of these factors reduces the ability of animals to resist infectious diseases.[2-7] Bacterial agents involved in BRDC are commensal organisms and rapidly proliferate when cattle are stressed or exposed to viral or mycoplasmal agents. *Mannheimia haemolytica*, *Pasteurella multocida*, and *Haemophilus somnus* are the most common isolates from cattle with BRDC.[8] These bacteria produce multiple virulence factors that amplify injury to lung tissue. The lungs of cattle have several adaptations that retard the spread of infection while limiting resolution of pyogranulomatous lung lesions.[9,10] Of importance, complete lobar-lobular septation results in large numbers of activated cells in confined regions of tissue.[11,12]

Neutrophil accumulation in the lungs is associated with rapid onset of clinical signs of pneumonia.[13,14] Neutrophil degranulation releases proteases that degrade nearly all types of extracellular matrix proteins. Neutrophil collagenase (also called **matrix metalloproteinase [MMP]**-8), gelatinase B (ie, MMP-9), neutrophil elastase, and serine proteases are all involved in neutrophil-mediated tissue injury.[15] Leukocyte proteases facilitate migration from the vasculature and across tissue barriers.[16-19] Carefully regulated focal degradation of basement membrane type-IV and -V collagen by proteases allows passage of leukocytes into extravascular spaces.

Alveolar macrophages express a number of proteases in vitro and when activated. Human alveolar macrophages produce gelatinases (ie, MMP-2 and -9) in addition to MMP-1, -3, and -12.[20] Rat alveolar macrophages produce MMP-2, -9, and -12.[20] Lung cells such as fibroblasts and epithelial cells primarily produce MMP-2, whereas macrophages and neutrophils primarily produce MMP-9.[21] Inflammatory diseases of the lungs are characterized by increased expression or activity of metalloproteinases.[21-29] Characterization of the proteases produced by alveolar macrophages, neutrophils, and lung tissue cells in healthy cattle will provide comparative information for use in the study of these enzymes expressed in cattle with BRDC. Defining the roles of these proteases in healthy cattle may provide information leading to

Received May 16, 2003.
Accepted July 14, 2003.
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Supported by the University of Missouri Agriculture Experiment Station.
The authors thank Anthony Caldwell, Mary F. Dickerson, Liz Staley, Marion Butcher, and Lindsey Tempinson for technical assistance.
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treatments that reduce lung damage commonly seen in pneumonic cattle.

The purpose of the study reported here was to describe gelatinase A and B (ie, MMP-2 and -9) and their endogenous inhibitors (ie, **tissue inhibitors of metalloproteinases [TIMPs]**) in **bronchoalveolar lavage fluid (BALF)** collected from healthy calves and characterize the pattern of protease expression by bovine alveolar macrophages at the protein and mRNA levels in vitro in response to stimulation with **lipopolysaccharide (LPS)**. Because alveolar macrophages play a role as a direct effector in the inflammatory responses of the lungs, the ability of these cells to produce and secrete MMPs may be important to understanding the role of MMPs in cattle with BRDC.

## Materials and Methods

**Animals**—Twenty calves (18 Holsteins and 2 Guernseys) were acquired soon after birth for use in the study. All calves were provided colostrum within 4 hours after of birth. When calves were 24 to 36 hours old, serum was obtained from each calf and tested by use of the sodium sulfite turbidity and total serum protein determinations to measure absorption of maternally derived IgG. Calves that did not achieve a positive reaction for the sodium sulfite turbidity test or have a serum total protein concentration ≤ 5.2 g/dL were removed from the study. Calves were housed in separate stalls and fed milk replacer by bottle until 60 days of age. These studies were evaluated and approved by the University of Missouri Animal Care and Use Committee.

**Collection of BALF**—When calves were 2 months old, they were sedated with xylazine hydrochloride (0.1 mg/kg, IM) and positioned in lateral recumbency. A sterile open-ended bronchoalveolar lavage catheter was passed via the nares into the right caudal lung lobe until it lodged in a bronchus. Then, 180 mL of sterile PBS solution without calcium or magnesium (22°C) was instilled (3 aliquots of 60 mL) and aspirated by use of a syringe. The recovered lavage fluid was placed in 50-mL conical tubes, and the tubes were placed on ice for transport to the laboratory.

**Preparation of BALF cells**—Cells in BALF were prepared for use in the experiments. First, single aliquots of raw BALF were removed for use in differential cytologic examination. Microcentrifuge preparations were stained with modified Wright-Giemsa stain, and a minimum of 200 cells were counted. The remainder of the BALF was centrifuged at 400 × *g* for 10 minutes at 22°C. The cell pellet was resuspended in **Hank's buffered salt solution without calcium or magnesium (HBSS)**. The cells were again centrifuged, and the resulting pellet was resuspended in 10 mL of HBSS. Cells were enumerated by use of a standard hemacytometer. Viability was determined by exclusion of trypan blue dye. Cell numbers were converted to the number of viable cells per milliliter.

**Culture of alveolar macrophages**—After cells were counted and viability determined, the equivalent of $1 \times 10^6$ viable alveolar macrophages were placed into individual wells of a 12-well plate with **Dulbecco modified Eagle medium (DMEM)** containing 4% **fetal bovine serum (FBS)**, 100 U of penicillin G/mL, and 100 ng of dihydrostreptomycin/mL. Plates were incubated for 2 hours to allow cells to adhere. Then, media and nonadherent cells were removed; medium was replaced with fresh DMEM containing 4% FBS, penicillin, and dihydrostreptomycin; and cells were cultured undisturbed for 48 hours. Medium was removed and replaced with **serum-free medium (SFM)**, and cells were incubated for another 24 hours prior to the start of the experiment. At each change of medium, an inverted microscope ocular was used to examine the adherent alveolar macrophages in each well.

To compare gelatinase expression in bovine alveolar macrophages after in vitro stimulation with that of a cell type with known ability to express gelatinase A and B (ie, MMP-2 and -9), we cultured a bovine leukosis virus-positive lymphoblastoid cell line. This cell line (the **bovine pericardial effusion cell [BCC]** line) was derived from a cow with a tumor at the base of the heart that had positive results for bovine leukosis virus when tested by use of a **polymerase chain reaction (PCR)** assay and transmission electron microscopy. As determined by immunohistochemical analysis, BCCs express **cluster designation (CD)**79a and bovine leukocyte antigen 36 but not CD3, and they do not express CD3, CD4, CD8, CD11b, or CD40 as determined by use of flow cytometry. These positive-control cells were cultured in RPMI 1640 medium with 10% FBS, 100 U of penicillin G/mL, and 100 ng of dihydrostreptomycin/mL for 48 hours; medium was removed, and SFM was added to the cells. By use of these conditions, mRNA for gelatinase A (MMP-2) and the corresponding MMP-2 protein are expressed in culture. In our experience, BCCs respond to phorbol esters and LPS by upregulation of gene expression for inducible **cyclooxygenase (COX**; ie, COX-2) and MMP-2 and -9.

**Preparation of monomeric LPS**—The use of LPS in vitro requires soluble CD14 or fetal serum (which increases the solubility of LPS and provides soluble CD14) to provide necessary factors that enable leukocytes or macrophages to respond.[30,31] Thus, to solubilize the LPS (*Escherichia coli* O55:B5[a]), 10 mg of LPS was incubated in 1 mL of FBS in a sterile microfuge tube for 1 hour in a water bath set at 37°C.[30,31] After incubation, the microfuge tube was placed in a beaker of warm water and sonicated at 50 W for 10 minutes.[30,31] The LPS was returned to the 37°C water bath until an aliquot was removed for dilution. This procedure generates monomeric LPS and uniform stimulation of cells in vitro.[30,31]

**Stimulation of alveolar macrophages**—Quiescent alveolar macrophages were cultured in SFM (negative-control sample) or stimulated by the addition of a volume of LPS (10 mg/mL of FBS; final concentration, 0.5% [vol:vol]) to provide 100 ng of monomeric LPS/mL. The alveolar macrophages were incubated for 24 hours prior to harvesting of conditioned medium and alveolar macrophages for RNA isolation. Total cellular RNA was harvested by the addition of 1 mL of Trizol reagent,[b] followed by incubation for 5 minutes on ice to allow lysis of adherent cells. After lysis, the wells were scraped with sterile, RNase-free tissue culture scrapers. The cell lysate-reagent suspension was removed from each well, placed into separate Eppendorf-type microfuge tubes, and frozen at –70°C.

**SDS-PAGE and substrate-embedded enzymography**—We performed SDS-PAGE and substrate-embedded enzymography (ie, zymography) as described elsewhere,[20,32] with minor modifications. A commercial system[c] was used to separate proteins on a 10% SDS-PAGE gel containing 0.1% gelatin (10% polyacrylamide) incorporated at the time of gel polymerization. Samples were mixed with 2× sample application buffer without β-mercaptoethanol (nonreduced) and electrophoresed at a constant voltage of 150 V for 90 minutes. Gels were removed and incubated in 2 changes of 2.5% Triton X-100 in water on a rocker to remove the SDS and renature proteins. Gels were then placed into Tris buffer (pH,

7.5) containing 10mM $CaCl_2$, 0.5mM $ZnCl_2$, and 0.01% nonionic detergent[d] (30% [vol:vol]) and incubated for 24 hours at 37°C. Inhibitors (10mM EDTA, 10mM 1,10-phenanthroline, or 10mM **phenylmethylsulfonyl fluoride [PMSF]**) were added to gels prior to incubation. Gels were stained with Coomassie brilliant blue R-250,[e] destained, and imaged on a flat-bed scanner at a resolution of 300 dots/inch. Stored images were analyzed by use of an image-analysis program.[f] The area under the curve for each LPS-stimulated sample (gelatinase A and B) was compared with the area under the curve for the corresponding control sample, and induction was determined by dividing the LPS-induced area by the control area for each gelatinase. Relevant control samples included human affinity-purified gelatinases (MMP-2 and -9),[g] TIMP-1,[h] TIMP-2,[i] or 2mM aminophenyl mercuric acetate, which autocatalytically activated the latent form of the enzymes.

**SDS-PAGE and immunoblot analysis of MMP proteins**—Proteins in BALF samples and conditioned medium from culture of alveolar macrophages (25 µg of protein/lane) were resolved by use of SDS-PAGE on 4%-to-12% gradient gels (proteins were reduced by use of 2-mercaptoethanol in SDS containing sample buffer) and then electrotransferred to nitrocellulose membranes by overnight incubation at 13 V. Membranes were blocked in Tris-buffered saline solution containing 0.1% Tween 20 and 5% nonfat dried milk.[j] After blocking at room temperature (22°C) for 1 hour, each membrane was incubated overnight at 4°C on a rocker with antibodies to MMP-2,[k] MMP-9,[l] TIMP-1,[m] and TIMP-2.[n] After incubation, excess unbound antibody was washed from the membranes and binding of primary antibody was determined by use of horseradish peroxidase-labeled anti-rabbit or anti-sheep antibodies. Unbound secondary antibody was removed by use of 3 sequential washes in Tris-buffered saline solution containing 0.1% Tween 20, followed by addition of chemiluminescence substrate[o] and exposure to film.[p]

**Reverse transcription-PCR assay for bovine MMP and inflammatory mediators**—Total cellular RNA isolated from cultured alveolar macrophages and BCCs were purified by use of phenol-chloroform extraction and ethanol precipitation, as described elsewhere.[33,34] Briefly, approximately 500 ng of total RNA isolated from macrophages incubated in SFM or SFM with LPS (100 ng/mL) was used to prepare first-strand cDNA by reverse transcription by adding 0.5 µg of oligo-$(dt)_{16}$[q] to the total cellular RNA samples. The PCR buffer (final concentration 1X), 2.5mM magnesium, 10mM dithiothreitol, 0.2mM of each **deoxynucleoside triphosphate (dNTP)**, and 200 units of **Muloney murine leukemia virus reverse transcriptase (MuLV-RT)** were added to achieve a final volume of 100 µL. A negative-control sample was assayed at the same time by substituting water for the MuLV-RT to control for contaminating genomic DNA. Cycling conditions for the cDNA synthesis were 10 minutes at 22°C, 15 minutes at 42°C, 5 minutes at 99°C, and 5 minutes at 5°C.

The PCR assay conditions for the MMPs, inflammatory mediators (ie, COX-2 and **inducible nitric oxide [iNOS]**), and housekeeping genes (β-actin or **glyceraldehyde 3-phosphate dehydrogenase [GAPDH]**) were as described elsewhere,[34-40] with slight modifications (**Appendix**). The PCR amplification consisted of 5 µL of reverse transcription template or water, 1.5mM $MgCl_2$, 1X PCR buffer, 0.2mM of each dNTP, 10µM of each primer, and 1.5 units of *Taq* polymerase in a final volume of 100 µL in various thermocycler conditions.

The PCR products were separated by use of electrophoresis in ethidium bromide-stained, 2% agarose[r] (ratio of 1 to 3) gels and imaged under ultraviolet light. Each treatment and stimulus was performed on 5 cell preparations, each of which was obtained from a different calf. Images were digitized by use of a gel-imaging system.[s] The PCR products from MMP-2, MMP-9, COX-2, and iNOS reactions were sequenced by use of a DNA sequencer,[t] and sequences were aligned and compared with established sequences by use of a software program.[u]

**Statistical analysis**—Mean ± SD differential cell counts for BALF samples were determined by counting a minimum of 200 cells/slide. Gelatin zymographic analysis of the activity of gelatinase A and B (MMP-2 and -9) was performed as described, and induction for each protease was determined by comparison with the control incubation for that calf by determining the area under the curve for that

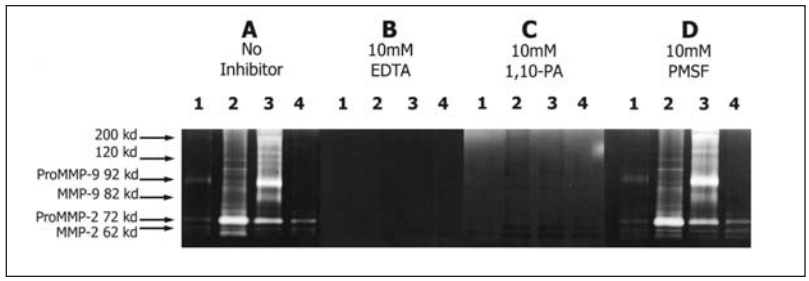


Figure 1—Gelatin zymogram of lyophilized bronchoalveolar fluid (BALF) protein obtained from 4 representative healthy calves. Lanes 1 to 4 represent each of the respective calves. Conditions were as follows: A, incubated with no inhibitor; B, incubated with 10mM EDTA; C, incubated with 10mM 1,10-phenanthroline (1,10-PA); and D, incubated with 10mM phenyl methylsulfonyl fluoride (PMSF). Notice the prominent bands for the latent form of gelatinase B (latent form of matrix metalloproteinase [MMP]-9 [proMMP-9]; approx 92 kd) in lanes 1 to 3, whereas lane 4 does not have a prominent band at this molecular weight. Active gelatinase B (MMP-9) is evident as a prominent 82-kd band in lane 1 but is more diffuse in lanes 2 and 3. Activity of the latent form of gelatinase A (latent form of MMP-2 [proMMP-2]) is apparent at approximately 72 kd in lanes 1 to 4, whereas active gelatinase A (MMP-2) is evident as a 62-kd band in lanes 2 to 4. Higher molecular weight forms of gelatinase (ie, aggregates) are evident in lanes 2 and 3. In the gels in which EDTA and 1,10-PA were included in the gel renaturation-development buffers, there is complete inhibition of gelatinase activity, which is indicative of metalloproteases. In gels in which PMSF (serine protease inhibitor) was included in the buffers, there is no effect on gelatinase activity.

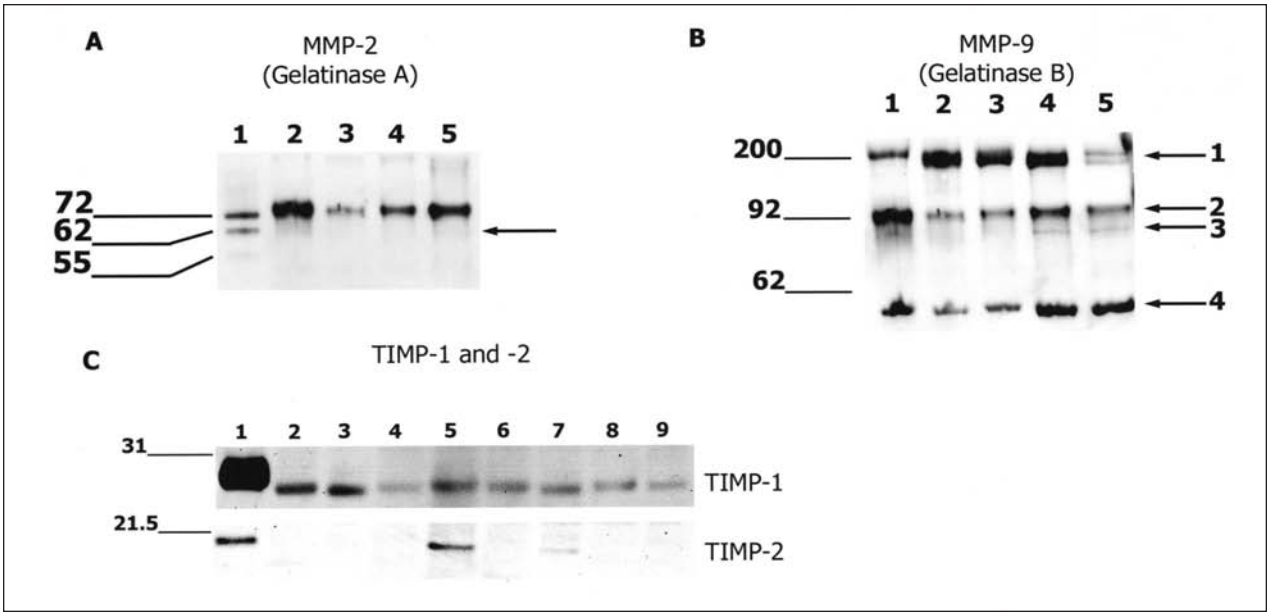


Figure 2—Immunoblot analysis of BALF protein obtained from healthy calves and analyzed under reducing conditions by use of polyclonal anti-MMP-2 antibodies (A), polyclonal anti-MMP-9 antibodies (B), and polyclonal antibodies to tissue inhibitor metalloproteinase (TIMP)-1 and -2 (C). A—Lane 1 is human MMP-2 (positive-control sample) for confirmation of protein and size comparison, whereas lanes 2 to 5 reveal activity of primarily the latent form of MMP-2 (72-kd bands), although active MMP-2 is evident as less-intense, 62-kd bands in only a few BALF samples (arrow). B—Lane 1 is human MMP-9 (positive-control sample) for confirmation of protein and protein molecular weights, whereas aggregate MMP-9 (200-kd band; arrow 1), the latent form of MMP-9 (92-kd band; arrow 2), active MMP-9 (band at approx 82 kd; arrow 3), and a smaller molecular weight degradation product of MMP-9 (56-kd band; arrow 4) are evident in lanes 1 to 5. C—Lane 1 contains human proteins with approximate molecular weights of 29 and 21 kd (positive-control sample). Lanes 2 to 9 reveal TIMP-1 (29-kd band) in BALF from all calves, whereas TIMP-2 (21-kd band) was evident in BALF from only 2 calves (only 4/20 calves in the study). Numbers on left side represent size in kilodaltons.

gelatinase in control and LPS-induced conditions. The ratio of LPS-induced to control results was used as the induction value. Mean ± SD of induction values was determined.

## Results

**Differential cytologic evaluation**—Cytologic examination of raw BALF samples obtained from the calves in the study revealed a mean ± SD of 9.1 ± 11.6% (range, 0.9% to 48%) neutrophils and 90.7 ± 11.6% (range, 52.0% to 99.0%) alveolar macrophages and lymphocytes. The BALF sample from 1 Guernsey calf that did not have clinical evidence of pneumonia contained 48% neutrophils, whereas the remaining calves had < 12% neutrophils. The amount of infused lavage fluid recovered varied from 56% to 88% (100.8 to 158.4 mL of the instilled volume of 180 mL).

**Zymography of BALF**—Gelatin zymography revealed that BALF samples from most of the 20 calves included in this study contained gelatinases. Gelatinase A (MMP-2) was evident as 72-kd (18/20 calves) and 62-kd (17/20) bands, and gelatinase B (MMP-9) was evident as 92-kd (14/20) and 82-kd (10/20) bands (**Fig 1**).

Samples of BALF from most (18/20) calves also had prominent gelatinase activity at approximately 120 kd and > 200 kd (Fig 1). Several distinct gelatinase bands at < 60 kd were also apparent in BALF from most calves. In all 20 calves, gelatinase activities were not apparent when 10mM EDTA or 10mM 1,10-phenanthroline was added to gel renaturation-development buffers. In contrast, addition of 10mM PMSF did not interfere with gelatinase activities. Furthermore, intensity of the bands at 92 and 72 kd decreased when 2mM aminophenyl mercuric acetate was added to the samples prior to electrophoresis (data not shown).

**Immunoblot analysis of MMP proteins in BALF**—To further characterize the specific gelatinases within BALF samples, immunoblot analysis was performed by use of anti-MMP-2 and anti-MMP-9 polyclonal antibodies. Results revealed that both gelatinases were found in BALF obtained from healthy calves (**Fig 2**). The pattern of expression for immunologically reactive MMP-2 in BALF obtained from the calves was compatible with the predominant 72-kd latent form of MMP-2 (15/20 samples), with slight immunoreactivity at approximately 62 kd (18/20). Immunoblots of BALF proteins with anti-MMP-9 antibodies had the same pattern in samples obtained from most calves. A prominent band at 92 kd (14/20 calves) and less intense immunoreactivity at approximately 82 kd (10/20) indicated both the latent and active forms of MMP-9. Prominent bands observed at 56 kd in the immunoblots were not observed by use of zymography. In addition, in most samples, at least 1 band was evident at approximately 200 kd, representing aggregates of MMP-9 molecules released from cells.

Immunoblot analysis of BALF proteins from the calves in this study also contained TIMP-1 and -2 proteins (Fig 2). Detection of TIMP-1 in BALF was confirmed by immunoblot analysis, which revealed bands at approximately 113, 55, and 30 kd. These bands represented TIMP-1 bound to MMP-9, TIMP-1

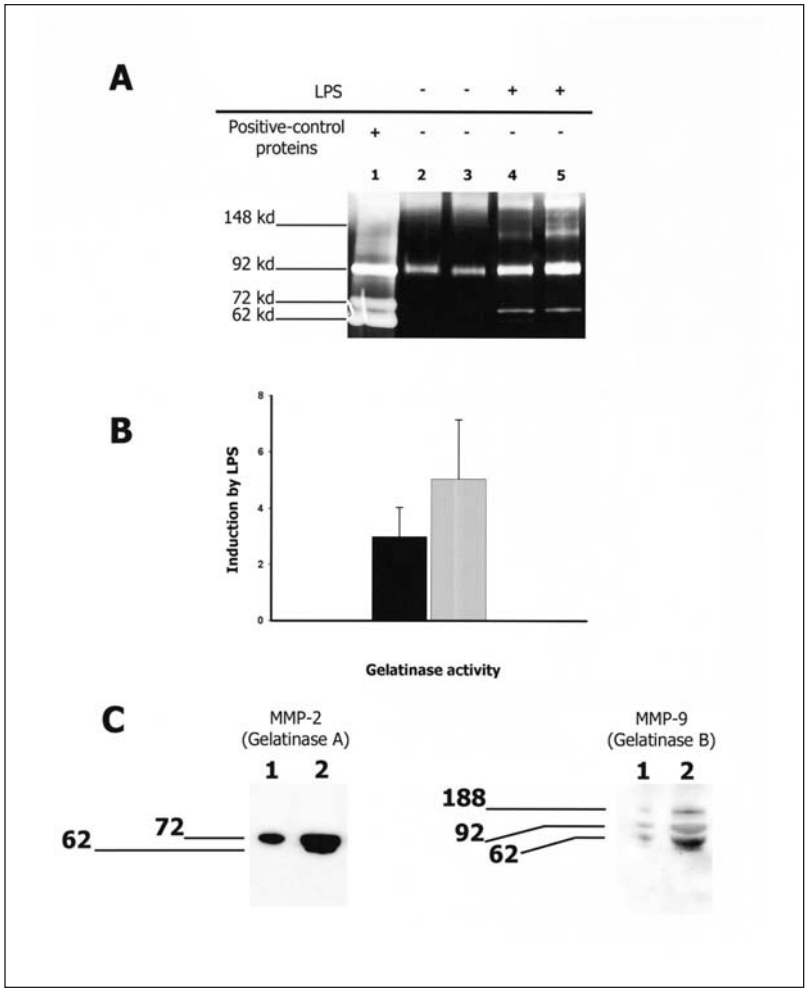


Figure 3—Results for gelatin zymography of conditioned medium obtained from alveolar macrophages cultured with (+) and without (–) lipopolysaccharide (LPS; 100 ng/mL) and immunoblot analysis of conditioned medium protein by use of MMP-2 and -9 antibodies. A—Gelatin zymogram of conditioned medium obtained from alveolar cells cultured without (lanes 2 and 3) and with (lanes 4 and 5) LPS. Lane 1 is a positive-control sample for proMMP-2, active MMP-2, and active MMP-9. Notice that MMP-2 activity is evident only in conditioned medium from the LPS-stimulated culture. B—Induction of gelatinase A (MMP-2; black bar) and gelatinase B (MMP-9; gray bar) by LPS stimulation of bovine alveolar macrophages. The area under the curve for each LPS-stimulated sample for the specific MMP was compared with that for the corresponding sample incubated without LPS (control sample). Results represent the mean ± SD fold-increase for LPS-stimulated samples in comparison with amounts for samples incubated without LPS (control sample). C—Immunoblot analysis of conditioned medium obtained from cultured alveolar macrophages under nonreducing conditions and polyclonal antibodies to MMP-2 and -9. Lane 1 in both immunoblots is conditioned medium from alveolar macrophages incubated in medium alone, whereas in both immunoblots lane 2 represents conditioned medium from LPS-stimulated alveolar macrophages. Notice the latent (92-kd band) and active (82-kd band) forms and the degradation products (56-kd band) of MMP-9. Numbers to the left of each immunoblot represent size in kilodaltons.

dimers, and free TIMP-1, respectively. Free TIMP-1 was detected in all BALF samples. The pattern for TIMP-2 proteins revealed free TIMP-2 (band at approx 21 kd). Free TIMP-2 was detected in only 4 of 20 samples.

**Analysis of cultured alveolar macrophages**—Conditioned medium from alveolar macrophages cultured for 24 hours in media alone (control sample) or media that contained LPS (100 ng/mL) was examined. Higher concentrations of LPS were toxic to some cultures, and LPS at 100 ng/mL caused measurable responses with minimal addition of serum. Gelatinase B (MMP-9) activity was evident (92-kd band) in most samples, including control samples (**Fig 3**). Incubation with LPS resulted in an increase (range, 1- to 12-fold; mean, 5.0- ± 2.1-fold) of gelatinase B (MMP-9) over amounts of MMP-9 for control samples incubated without LPS. Gelatinase A (MMP-2) activity (62-kd band) was not evident in most control samples, whereas LPS-induced gelatinase A (MMP-2) activity was increased (range, 0.88- to 7-fold; mean, 3- ± 1-fold).

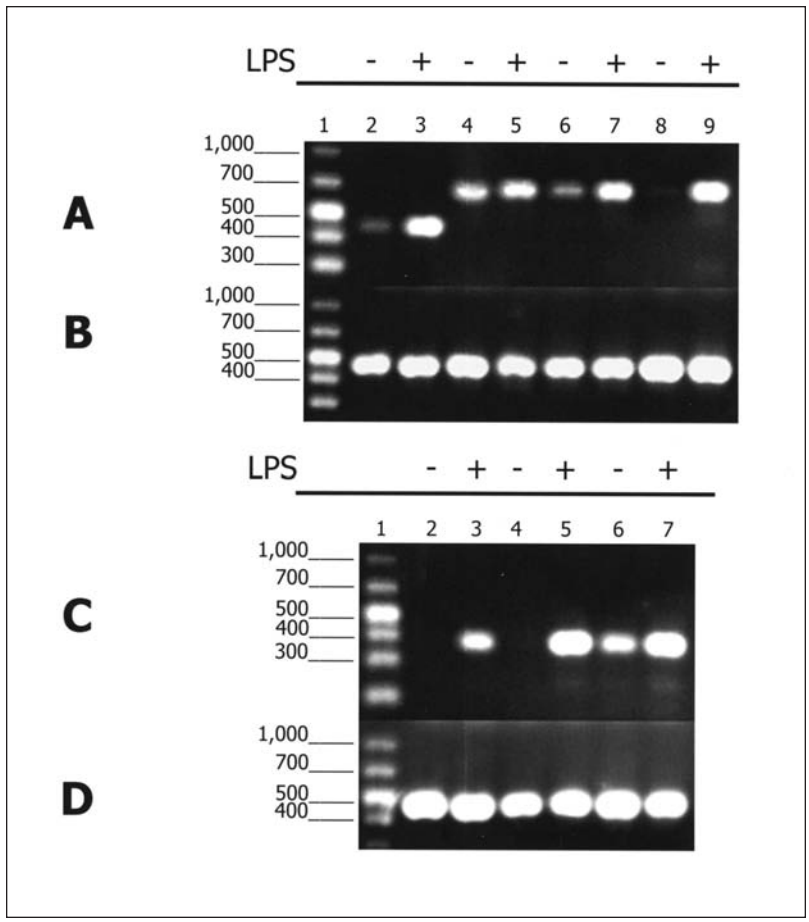


Figure 4—Products of a polymerase chain reaction (PCR) assay for inducible cyclooxygenase (COX; ie, COX-2) and MMP-9 (A), the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH; [B and D]), and inducible nitric oxide synthase (iNOS; [C]) from mRNA isolated from calf alveolar macrophages incubated with (+) or without (–) LPS (100 ng/mL). A—Notice the expression of COX-2 mRNA by alveolar macrophages incubated without LPS (lane 2), whereas results differ for alveolar macrophages incubated with LPS (lane 3). Lanes 4 and 5, 6 and 7, and 8 and 9 are the expression of MMP-9 mRNA by alveolar macrophages from 3 calves (including the calf in lanes 2 and 3). Although alveolar macrophages incubated without LPS express some message for genes associated with inflammation, LPS dramatically increases basal expression. The predicted size of COX-2 and MMP-9 products is 449 and 658 bp, respectively. B—Notice the PCR products from mRNA isolated from control and LPS-induced alveolar macrophages for GAPDH (housekeeping gene). B and D—Expression of the housekeeping gene (GAPDH) was determined in each of these samples as a control for loading of gels (predicted size is 468 bp). C—Notice that calf alveolar macrophages respond similarly to LPS for expression of bovine iNOS. The predicted size of the iNOS product is 372 bp. Numbers to the left of each gel represent number of base pairs.

Migration of bovine MMP-9 was slightly slower than that for commercially available human MMP-9, and MMP-9 was constitutively expressed by bovine alveolar macrophages in culture.

Conditioned medium from alveolar macrophages cultured with or without LPS expressed immunoreactivity compatible with MMP-2 and -9 proteins, whereas only medium from cells cultured with LPS had immunoreactivity at 62 kd (ie, active form of MMP-2; Fig 3). Matrix metalloproteinase-9 was detected at approximately 92, 82, and 56 kd by the anti-MMP-9 antibodies.

Expression of MMP-9 mRNA was observed in cultured (quiescent) alveolar macrophages obtained from some calves (**Fig 4**). Culture after addition of LPS increased mRNA for bovine MMP-9. The expression of MMP-9 mRNA was observed in association with other genes responsive to LPS stimulation (COX-2 and iNOS), documenting concurrent expression of multiple genes in association with the MMP-9 expression.

In contrast to the expression of MMP-9 mRNA, MMP-2 mRNA was not expressed in bovine alveolar macrophages cultured with or without LPS in this study (**Fig 5**). The BCCs that expressed MMP-2 mRNA and were induced by LPS in vitro were used as a positive-control sample. This cell line expresses MMP-2 and COX-2 when stimulated with LPS, concanavalin A, and phorbol esters, as evaluated by use of bovine-specific primers.

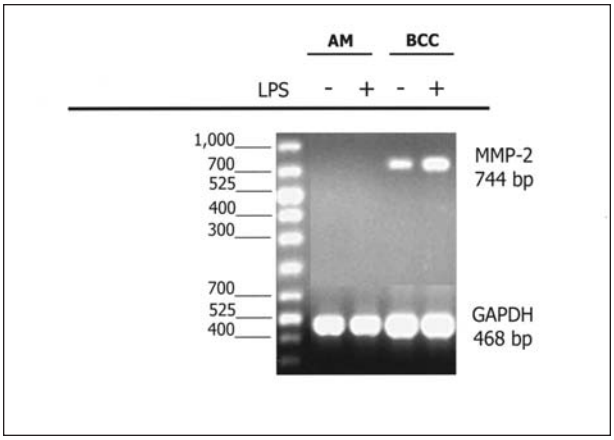


Figure 5—Ethidium bromide-stained agarose gel of the product of a reverse transcription-PCR assay revealing lack of expression of MMP-2 mRNA (approx 744 bp) by bovine alveolar macrophages (AM; lanes 2 and 3) obtained from 1 calf in comparison with expression in positive-control cells (bovine pericardial effusion cell [BCC] line; lanes 4 and 5) when cells were incubated in medium with (+; lanes 3 and 5) or without (–; lanes 2 and 4) LPS (100 ng/mL). Lane 1 is a base-pair ladder for use in determining the approximate size of the amplified product. Notice the results for the housekeeping gene, GAPDH (approx 468 bp).

## Discussion

Our evaluation of MMP in BALF indicated substantial expression of gelatinases in lungs of healthy calves (Fig 1). These gelatinases have characteristics of MMPs as determined on the basis of the migration of these proteins in comparison with that of human MMP-2 and -9, inhibition of gelatinolytic activity by EDTA and 1,10-phenanthroline but not PMSF, and cross-reactivity with antibodies to human MMP-2 and -9 proteins (Fig 2). In most of the calves, the latent form of the enzymes predominated. The latent form of gelatinase B (MMP-9) was found in BALF obtained from 14 of 20 calves, whereas the active form of MMP-9 was found in BALF obtained from 10 of 20 calves in this study. Some calves did not have detectable MMP-9 gelatinolytic activity in BALF.

Expression of MMP-2 and -9 is upregulated by cytokines released in response to LPS as a result of oxidants produced by leukocytes and expression of cellular integrins. These proteinases readily cleave basement membrane (type-IV and -V) collagen and elastin.[41,42] Basement membranes are critical for maintaining selective permeability and regeneration of epithelial integrity after injury.[43] Loss of barrier functions and epithelial integrity leads to accumulation of exudate and, eventually, fibrosis. In normal tissues and during tissue repair, expression and activity of these proteases are carefully regulated such that there is a local balance between degradation and inhibition. During inflammation, uncontrolled recruitment of cells and damage to structural proteins result in excessive activity of tissue proteases and tissue damage. In inflamed lungs, MMP-9 is a key contributor to degradation of lung tissue and endogenous inhibitors (eg, $\alpha_1$-proteinase inhibitor, antitrypsin, and C1-inhibitor) and it potentiates activation of neutrophil chemotactic chemokines.[44-47] Matrix metalloproteinase-9 is overexpressed in many inflammatory disorders of the lungs in humans, including idiopathic pulmonary fibrosis,[22] emphysema,[23] asthma,[24] cystic fibrosis,[25] and adult respiratory distress syndrome.[41] Investigators have detected MMP-9 in healthy animals and animals with disease conditions of the lungs, mammary glands, synovial fluids, CSF, and reproductive tract.[26-28,42,48-51] Sources of MMP-9 include leukocytes, although activation of epithelial cells, endothelial cells, and fibroblasts may also contribute to protease activity in BALF.[52] To our knowledge, evaluation of the expression and function of MMPs in the lungs of ruminants has not been reported.

When examining gelatin zymograms, several molecular weight bands were identified, including bands at approximately 200, 120, 92, and 82 kd. The latent form of MMP-9 is stored and released from neutrophil granules, often in complexes of multiple MMP-9 molecules (> 200 kd) or complexed with neutrophil granule lipocalin (approx 120 kd), but it is not complexed with TIMP-1 because neutrophils do not express TIMPs.[52,53] In several calves, gelatinolytic bands observed at higher molecular weights were also identified by use of immunoblot analysis, confirming detection of forms in BALF of calves. Several calves expressed single bands at approximately 200 kd, and in 1 calf, 2 distinct bands at 200 kd suggested that the antibodies recognized dimers and free enzyme.

In half of the BALF samples, immunoblot analysis revealed faint bands with a slightly faster migration rate (82 kd) that were the active form of gelatinase B (MMP-9). In nearly all BALF samples, the immunoblot analysis included a band at approximately 56 kd that did not appear on the gelatin zymograms. The antibodies we used recognized a 56-kd protein that does not possess enzyme activity but remains immunologically reactive.

Because there were few neutrophils in the BALF in 19 of 20 calves, we suspect that gelatinase B (MMP-9) was produced primarily by alveolar macrophages or lung epithelial cells. There were also few RBCs in BALF samples, so we believe that blood contamination of the BALF was minimal and that serum MMPs or TIMPs did not confound our results. However, we did not determine the protein content of the raw BALF, nor did we evaluate urea concentration in the serum or BALF as a marker of dilution of BALF; therefore, blood leukocytes or plasma proteins may have contributed slightly to our results. These issues are also relevant to the amount of MMP-2 in BALF.

Activity and immunoreactivity of MMP-2 was also detected in BALF samples obtained from healthy calves. Healthy calves express the latent form of MMP-2 (72 kd; 18/20 calves) as well as the active form of MMP-2 (62 kd; 17/20). Matrix metalloproteinase-2 can be upregulated in experimental models of LPS-induced pulmonary fibrosis.[22] In another study,[29] it was suggested that a lack of MMP-2 expression in MMP-2 knock-out mice is associated with lethality of allergic respiratory disease as a result of reduced inflammatory cell egress from the lungs. In that study, investigators documented that the enzymatic action of MMP-2 is critical for removal of inflammatory cells from the lung parenchyma, and persistence of the cells in lung tissue is lethal in mice lacking expression of MMP-2. Expression of this protein in BALF obtained from healthy calves, in combination with a lack of macrophage production of MMP-2,

suggests that this protein is contributed to the lining fluid by many cell types found in the distal portions of the lungs. Airway epithelial cells as well as fibroblasts are sources of MMP-2 in cattle.[54,55] As stated previously, further study of MMP-2 in lung parenchyma is required to confirm this speculation in calves.

Endogenous inhibitors of MMPs (ie, TIMPs) were also detected in BALF obtained from calves. Tissue inhibitors of matrix metalloproteinases are produced by nearly all cell types, except for neutrophils. Monocytes, macrophages, and tumor cells produce MMP-9 and TIMP-1 after specific activation processes.[53] Epithelial cells, fibroblasts, and chondrocytes produce TIMP-2, but neutrophils or peripheral blood mononuclear cells do not.[53,56] Tissue inhibitors of metalloproteinases bind specifically to MMPs, with TIMP-2 binding to MMP-2 in a 1:1 stoichiometry and TIMP-1 binding to MMP-9 in 1:1 or 1:2 stoichiometry.[49,50] We detected TIMP-1 in BALF obtained from all calves examined in the study reported here, whereas TIMP-2 was detected in BALF from only 4 of 20 calves. Analysis of these data suggests that TIMP-1 is produced in large part by alveolar macrophages, whereas TIMP-2 may be produced by other cells in the distal portion of the lungs.

Alveolar macrophages are resident phagocytes in the alveolar region of the lungs, and they secrete a number of proteases including the MMPs. Analysis of our results documents that bovine alveolar macrophages express MMP-9 but not MMP-2. Although other proteases, such as macrophage metalloelastase (ie, MMP-12) or collagenase-3 (ie, MMP-13), have been implicated in chronic obstructive pulmonary disease of horses and are expressed by alveolar macrophages obtained from rats, our evaluation of these proteins by use of zymography did not reveal activity compatible with 35- or 22-kd gelatinases (MMP-12) in BALF or cultured alveolar macrophages obtained from healthy calves. However, our protocols called for incubation of gelatin zymograms for 24 hours, whereas other investigators have used much longer incubation periods to detect less abundant proteases.[20,28] In addition, the use of casein or κ-elastin zymography to further analyze BALF and conditioned medium may be required to confirm our findings.[20,26-28] Our results also documented that LPS readily induces MMP-9 expression by calf alveolar macrophages, which is similar to results for rat and human alveolar macrophages. Expression of MMP-9 is often induced concurrent with the expression of TIMP-1.[53] Analysis of our results for healthy calves suggests that alveolar macrophages are cells that produce MMP-9 and are a source of TIMP-1.

Expression of MMP-9 mRNA in alveolar macrophages cultured with and without LPS was easily determined, and LPS induced more than a 2-fold increase for most calves. Although MMP-9 must be induced in alveolar macrophages, the process of cellular isolation or in vitro culture of these cells was a sufficient stimulus to increase the expression of this message in our system. We attempted to verify that LPS-induction of MMP-9 protein was related to gene expression by comparison with expression of genes we knew would be upregulated by LPS. In our results, simultaneous amplification of MMP-9, COX-2, and iNOS mRNA was observed in cultures in addition to housekeeping genes (GAPDH), indicating that MMP-9 is expressed by alveolar macrophages after LPS-induction. This was especially important when considering the lack of corresponding MMP-2 mRNA and the potential effects of in vitro culture on gene expression.

Gelatinase A (MMP-2) is produced by lung epithelial and mesenchymal cells and may be found in BALF as a result of normal epithelial turnover in young animals.[56,57] Expression of MMP-2 has been documented in alveolar macrophages and T lymphocytes of humans and is believed to mediate migration of these cells into tissues.[19,20] Gelatinase A (MMP-2) is synthesized and released at the cell membrane where it interacts with $\alpha_v\beta_3$ integrins (membrane-type MMP [ie, MT1-MMP]) and TIMP-2 to regulate the activity of TIMP-2 at the surface of secreting cells, which allows proteolysis in carefully regulated locations. With this type of production, it would be expected that release into media in vitro would be limited, compared with that observed in our cultures to which fetal serum was added.[57,58]

Characterization of expression of MMPs in lung tissues is needed to determine the contribution of other cell types to the protease component of BALF. Examination of diseased lung cells and tissues will also be necessary to further characterize the contribution of proteases to inflammation of the lungs in cattle. Models of experimentally induced inflammation may be superior to natural infection because bacterial proteases play important roles in the pathogenesis of pulmonary inflammation and may complicate the interpretation of zymography results. The production of MMPs by healthy lungs of cattle, compared with that produced during pulmonary injury, should provide useful information on the pathogenesis of lung lesions.

[a]LPS, *Escherichia coli* O55:B5, Sigma Chemical Co, St Louis, Mo.
[b]Trizol reagent, Gibco-Life Technologies, Carlsbad, Calif.
[c]NuPage Tris-glycine SDS zymogram gels, Invitrogen, Carlsbad, Calif.
[d]Brij-35, 30% solution, Pierce Biotechnology, Rockford, Ill.
[e]Coomassie brilliant blue R-250, Bio-Rad Laboratories, Hercules, Calif.
[f]Image for Windows, version 1.0.0.1, Scion Corp, Frederick, Md.
[g]Control 1 protein, catalogue No. M2928, Sigma Chemical Co, St Louis, Mo.
[h]TIMP-1 protein, Biomol Research Laboratories Inc, Plymouth Meeting, Pa.
[i]TIMP-2 protein, Biomol Research Laboratories Inc, Plymouth Meeting, Pa.
[j]Carnation instant nonfat dried milk, Nestle-Carnation, North York, ON, Canada.
[k]Sheep anti-MMP 2 polyclonal antibody, catalogue No. 5980-0211, Biogenesis Inc, Kingston, NH.
[l]Sheep anti-MMP 9 polyclonal antibody, catalogue No. 5980-0911, Biogenesis Inc, Kingston, NH.
[m]Rabbit anti-TIMP 1, catalogue No. SA-373, BioMol Research Laboratories Inc, Plymouth Meeting, Pa.
[n]Rabbit anti-TIMP 2, catalogue No. SA-374, BioMol Research Laboratories Inc, Plymouth Meeting, Pa.
[o]LumiGLO reagent and peroxidase, Cell Signaling Technology, Beverly, Mass.
[p]Hyperfilm ECL, Amersham Pharmacia Biotech, Buckingshire, UK.
[q]Oligo-dt-16, Promega Corp, Madison, Wis.
[r]Nu-Sieve 1:3 agarose, FMC Bioproducts, Rockland, Me.
[s]Kodak DC290 zoom gel-imaging system, Kodak Inc, New Haven, Conn.
[t]ABI model 377 DNA sequencer, Applied Biosystems, Foster City, Calif.
[u]Vector NTI suite 6.0 for Windows NT, Informax Inc, Bethesda, Md.

## Appendix

Primers and cycling conditions used in a polymerase chain reaction assay to detect gelatinases, inflammatory mediators, and housekeeping genes in RNA isolated from alveolar macrophages obtained from healthy calves

| Gene | GenBank accession No. | Product length (bp) | Direction | Primer sequence | Cycling conditions* | Reference |
|---|---|---|---|---|---|---|
| GADPH | U85042 | 468 | Sense | GAT GCT GGT GCT GAG TAT GTA GTG | 1 cycle at 94°C for 10 minutes; 32 cycles at 94°C for 1 minute and 54°C for 1 minute; 1 cycle at 72°C for 2 minutes; 72°C for 10 minutes | 35 |
| | | | Antisense | ATC CAC AAC AGA CAC GTT GGG AG | | |
| β-actin | K00622/ K00623 | 890 | Sense | ACC AAC TGG GAC GAC ATG GAG | 1 cycle at 94°C for 10 minutes; 35 cycles at 94°C for 1 minute and 60°C for 1 minute; 1 cycle at 72°C for 2 minutes; 72°C for 10 minutes | 36 |
| | | | Antisense | GCA TTT GCG GTG GAC AAT GGA | | |
| COX-2 | AF004944 | 449 | Sense | TCC AGA TCA CAT TTG ATT GAC A | 1 cycle at 94°C for 10 minutes; 35 cycles at 94°C for 1 minute and 55°C for 1 minute; 1 cycle at 72°C for 2 minutes; 72°C for 10 minutes | 37 |
| | | | Antisense | TCT TTG ACT GTG GGA GGA TAC A | | |
| iNOS | U14640 | 372 | Sense | TAG AGG AAC ATC TGG CCA GG | 1 cycle at 94°C for 3 minutes; 35 cycles at 94°C for 45 seconds and 54°C for 30 seconds; 1 cycle at 72°C for 90 seconds; 72°C for 7 minutes | 38 |
| | | | Antisense | TGG CAG GGT CCC CTC TGA TG | | |
| MMP-2 | AF290428 | 744 | Sense | GAC CGA ATA GAA TAG CCC | 1 cycle at 94°C for 3 minutes; 35 cycles at 94°C for 45 seconds and 54°C for 30 seconds; 1 cycle at 72°C for 90 seconds; 72°C for 7 minutes | 39 |
| | | | Antisense | TCT CAA TGG TGC TCT GG | | |
| MMP-9 | X78324 | 658 | Sense | CGA CGA TGA AGA GTT GTG GT | 1 cycle at 94°C for 3 minutes; 30 cycles at 94°C for 45 seconds and 55°C for 30 seconds; 1 cycle at 72°C for 90 seconds; 72°C for 7 minutes | 40 |
| | | | Antisense | GTA CAT GGG GTA CAT GAG CG | | |

*Cycling conditions represent denaturation; annealing; extension; and terminal elongation.

GAPDH = Glyceraldehyde-3-phosphate dehydrogenase. COX-2 = Cyclooxygenase-2 (ie, inducible cyclooxygenase). iNOS = Inducible nitric oxide synthase. MMP-2 = Matrix metalloproteinase-2 (ie, gelatinase A). MMP-9 = Matrix metalloproteinase-9 (ie, gelatinase B).

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