Expression of interleukin-1β in the digital laminae of horses in the prodromal stage of experimentally induced laminitis

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Objective—To study expression of interleukin-1β (IL-1β) in the digital laminae of horses in the prodromal stage of experimentally induced laminitis.

Animals—8 healthy adult horses with no signs of laminitis.

Procedure—Black walnut extract was administered via nasogastric tube to 4 horses, and water was administered to the remaining 4 (controls). Complete blood counts and physical examinations were performed every 30 minutes after administration of black walnut extract or water. General anesthesia was induced when total WBC count decreased by 30% in horses given the black walnut extract and 3 hours after water administration in control horses. The left forefoot was perfusion fixed with neutral-buffered 10% formalin, and paraffin-embedded sections of the digit were used for in situ hybridization with an equine-specific IL-1β probe.

Results—IL-1β mRNA expression was observed in perivascular cells of the small laminar venules and capillaries in all 4 horses given black walnut extract and in interstitial cells remote from the microvasculature in 1 of the 4. Other cellular components of the laminar tissue and cellular components of the digital arterioles and veins did not exhibit IL-1β mRNA expression. Expression of IL-1β mRNA was not detected in laminae from control horses.

Conclusions and Clinical Relevance—Results suggest that IL-1β mRNA is expressed by perivascular cells in the laminar tissues of horses in the prodromal stage of experimentally induced laminitis. This provides evidence of an inflammatory process during the prodromal stage of laminitis, indicating that local digital proinflammatory cytokine expression may be an initiating factor in laminitis. (Am J Vet Res 2001;62: 714–720)

Laminitis is a crippling disease of horses in which degeneration of the digital laminae commonly occurs secondary to numerous systemic disease processes. Although a great deal of research has focused on the importance of systemic factors in the initiation of laminitis, no study has successfully linked disease processes occurring elsewhere in the body to the severe degenerative changes that occur in the digit with laminitis. Endotoxin in particular has received much attention because of the close association of laminitis with systemic diseases in which endotoxemia appears to predominate (eg, colitis, carbohydrate overload, colonic torsion, and pleuritis). However, the relationship between the presence of endotoxin and the development of laminitis has been inconsistent. Recently, other bacterial toxins have been purported to be causative agents in laminitis, but again, a link between the disease process and the pathologic changes in the laminae has not been identified.

Because of this failure to detect the systemic factors responsible for the changes that occur in the digital laminae with laminitis and because the digit appears to be uniquely affected by several systemic disease processes, many investigators have concentrated on the physiologic and cellular changes occurring within the digit itself. Several studies identified laminitis primarily as a vascular disease involving a reduction in laminar perfusion. Proposed causes of these laminar vascular derangements have included arteriovenous shunting, constriction of the venous side of the laminar microvasculature, reduction in endothelium-derived vasodilation, and platelet aggregation and vascular thrombosis in the digital vasculature. Other authors have proposed that laminar separation results from degeneration of the basement membrane interposed between the dermal and epidermal laminae secondary to local release and activation of metalloproteinases.

Local expression and release of mediators likely to be involved in the vascular changes occurring with laminitis, including endothelin, nitric oxide, prostanoids, and adenosine, have been investigated in recent studies. However, although each of these mediators may cause individual processes that occur in laminitis, their involvement does not explain the variety of changes (vasodilatation, vasoconstriction, coagulation, basement membrane abnormalities) purported to be present in the digit at the onset of laminitis.

Central inflammatory cytokines such as interleukin-1 (IL-1β) and tumor necrosis factor-α (TNF-α) are synthesized by a wide variety of cells during numerous systemic diseases. In other species, IL-1β alters the expression of numerous vasoactive, procoagulant, and proinflammatory mediators. Therefore, vascular...
changes such as vasodilatation, vasoconstriction, or intracellular coagulation may be induced by IL-1β through the activation of other enzymes and mediators. In addition, IL-1β is a potent activator of the synthesis of metalloproteinases, and expression of IL-1β and TNFα is induced by systemic diseases (eg, endotoxemia) similar to those that cause laminitis. Thus, it is possible that these cytokines are the central mediators in the pathogenesis of laminitis. The purpose of the study reported here was to determine the expression of IL-1β in the digital laminae of horses in the prodromal stage of experimentally induced laminitis.

Materials and Methods

Induction of laminitis—A well-described method for induction of laminitis involving administration of black walnut extract was used. We elected to study IL-1β expression at the onset of the decrease in WBC count following administration of black walnut extract. Previous studies of horses with black walnut-induced laminitis have documented that early hemodynamic changes are occurring at this time even though clinical signs of laminitis may not be apparent for several more hours.

Eight healthy adult horses ranging from 3 to 22 years old were used in the study. Prior to the study, all horses were examined and determined to be free of clinical signs of lameness or laminitis. Horses were housed in box stalls and fed a complete pelleted ration for 2 weeks. A complete physical examination was then performed, an IV catheter was placed in the left jugular vein, and an initial blood sample was obtained for a CBC. In 4 horses, 5 L of an aqueous solution of black walnut (Juglans nigra) extract was administered by nasogastric tube to induce laminitis as described. The other 4 horses (control horses) received 5 L of water by nasogastric tube. Physical examination, including assessment of rectal temperature, heart rate, and respiratory rate, abdominal auscultation, and evaluation of the gait, was performed every 30 minutes after administration of the black walnut extract or the water. Blood was collected for CBC and determination of total WBC count, compared with baseline count, was detected (2.5 to 3.5 hours after administration of the extract). In the control horses, anesthesia was induced 3 hours after administration of water. Horses were placed in lateral recumbency and ventilated with 100% oxygen; anesthesia was maintained with pentobarbital. Lactated Ringer’s solution was infused at a rate of 10 ml/kg of body weight, IV, throughout the study.

Tissue collection and fixation—A tourniquet was placed immediately proximal to the left metacarpophalangeal joint, and digital vessels of the foot were cannulated in the midpalmar region with polyethylene tubing. Digital arteries were perfused with heparinized sterile phosphate-buffered saline solution (PBSS) until the euvolemia from the veins became clear. The foot was then disarticulated at the level of the metacarpophalangeal joint, and the infusion was continued in the isolated digit with buffered 10% formalin for 30 minutes. Immediately following perfusion and fixation, 1-cm-thick sagittal sections of the digit were cut with a band saw. The laminar dermis and epidermis were carefully isolated from the dorsal surface of the distal phalanx and the deep aspect of the hoof wall. Each longitudinal band of laminar tissue was then sectioned into 0.5 cm segments that were immediately placed into buffered 10% formalin. Sections of digital vessels were harvested immediately distal to the cannulation site and placed in buffered 10% formalin. Tissue samples were preserved in buffered 10% formalin at 4 C for 18 hours after collection, placed in 70% ethanol for 24 hours, and then embedded in paraffin. Five-micrometer-thick tissue sections were mounted on charged glass slides.

Riboprobe synthesis—Oligomers were designed from the equine complementary DNA (cDNA) sequence for IL-1β, determined by Howard et al., to obtain a 320 base pair cDNA fragment. An equine-specific cDNA was produced by reverse transcriptase-polymerase chain reaction (RT-PCR), using lipopolysaccharide-exposed equine digital artery smooth muscle cell cDNA. The fragment was then extracted from the gel, ligated into a plasmid vector, and subcloned. A polyethylene glycol purification protocol was used for plasmid extraction, and the plasmid DNA was cut with EcoRI. A 100 base pair ladder was used to size the cloned DNA fragment on a 1.5% agarose gel stained with ethidium bromide. Deoxyribonucleic acid sequencing of both DNA strands was performed in an automatic sequencer. Homology of the IL-1β cDNA fragment’s sequence with the previously published equine IL-1β cDNA sequence was verified by computer alignment. The plasmid was cut by enzymes (BamHI and HindIII) at restriction sites flanking the DNA insert, and riboprobes in the sense (homologous to the mRNA sequence) and antisense (complementary to the mRNA sequence) direction were synthesized with a kit, using promoters (T3 and T7) located on either side of the multiple cloning site. The sense probe was synthesized to be used as a negative control, whereas the antisense probe was designed to hybridize to the mRNA for IL-1β. Riboprobes were labeled with 35S-uridine triphosphate. Probes were purified by means of filtration through microcentrifuge spin columns, and 1 µl each of the sense and antisense probes was electrophoresed on a 1.5% agarose gel. Autoradiography of the dehydrated gel was

Figure 1—Autoradiograph of sense and antisense equine interleukin-1β (IL-1β) riboprobes following gel electrophoresis of the probes. The RNA ladder indicated that the riboprobes were the expected size of approximately 350 base pairs.
used to ensure adequate incorporation of the radionuclide and to compare the size of the riboprobe with an RNA ladder (Fig 1).

In situ hybridization protocol—In situ hybridization was performed as described. Briefly, slides were deparaffinized, rehydrated in gradually decreasing concentrations of

Figure 2—Micrographs of sections of the laminar tissues from horses in the prodromal stage of experimentally induced laminitis and control horses following in situ hybridization of sections with an equine-specific IL-1β probe. Interleukin-1β mRNA expression (white grains) was evident in specific cells in the subendothelial region of capillaries (C) of the primary dermal laminae (PDL) on dark-field microscopic examination of digital laminae from horses with laminitis (sections a and b; red arrowheads), but IL-1β mRNA expression was not observed in laminar sections from control horses in which only background signal similar to that obtained with a sense IL-1β riboprobe was detected (section c; white arrowheads indicate laminar capillaries). Light-field (section d) and dark-field (section e) microscopic examination of tissues from 1 horse with laminitis revealed IL-1β mRNA expression (black grains in section d and white grains in section e) in cells in the interstitium remote from vessels (orange arrows). A magnified view (section f) of the outlined region in section d clearly shows perivascular (red arrowhead) and interstitial (orange arrowheads) cells positive for IL-1β mRNA expression. PEL = Primary epidermal laminae. SL = Region of interdigitating secondary laminae. Bars = 10 μm in sections a and f and 50 μm in sections b, c, d, and e.
ethanol, and serially incubated in 0.2% Triton X-100 in PBSS, proteinase K, and acetic anhydride with 0.1 mol of tetraethylammonium/L; slides were washed with PBSS between incubations. Slides were then dehydrated in gradually increasing concentrations of ethanol, air-dried, and pre-hybridized for 2 hours. Hybridization with \(^{35}\)S-labeled ribo-probes was performed overnight, using duplicate sections for the sense and antisense probes. After hybridization, slides were washed in \(2\times\) saline-sodium citrate buffer (SSC), incubated in RNase A, and washed several times with \(2\times\) SSC at room temperature and at 55 C and with \(0.1\times\) SSC at 55 C. Slides were dehydrated in gradually increasing concentrations of ethanol, air-dried, and dipped in photographic emulsion. The slides were then placed in a light-tight box and exposed at 4 C for 3 weeks, after which they were developed and counterstained with H&E.

Endotoxin assay—Endotoxin activity was determined by use of a chromogenic limulus amoebocyte lysate microtiter plate assay.

Results
Total WBC count decreased by at least 30% in all 4 horses given the black walnut extract. Rectal temperature, heart rate, respiratory rate, and intestinal motility (assessed by means of abdominal auscultation) in horses given black walnut extract did not differ substantially from values for the control horses. None of the horses developed clinical signs of lameness prior to euthanasia.

In situ hybridization of laminar tissue sections from horses treated with the black walnut extract revealed striking IL-1\(\beta\) mRNA expression in perivascular cells of laminar capillaries in the primary dermal laminae (Fig 2) and at the base of the laminae (Fig 3). Expression was limited to a few cells around each vessel, and not all laminar capillaries within a section had evidence of IL-1\(\beta\) expression. Specific identification of the perivascular cell type that was involved was not possible, given the techniques used. Capillaries and venules located near perivascular cells strongly positive for IL-1\(\beta\) expression themselves had minimal expression of IL-1\(\beta\), indicating that these perivascular cells were more sensitive to induction of IL-1\(\beta\) expression than were the components of the vascular wall. Cells positive for IL-1\(\beta\) expression were most often seen in association with the microvasculature at the base of the laminae. These cells were most commonly seen in association with capillaries, and no components of the capillary, venular, or arteriolar wall (endothelial or smooth muscle cells) were...
Interleukin-1β belongs to the IL-1 gene family and is the principal secreted form of IL-1. Numerous studies in other species have demonstrated that central proinflammatory cytokines such as IL-1β or TNFα may be synthesized and released by a wide variety of cells, including inflammatory cells, and these cytokines are known to reproduce all the clinical signs of inflammation. Interleukin-1β induces intravascular coagulation by up-regulation of surface molecules such as intercellular adhesion molecule 1 and E-selectin and migration of leukocytes in the extracellular space in response to chemotactic stimuli such as IL-8 and platelet activating factor. These latter effects may explain the reported invasion of the laminar tissue by leukocytes in later stages of the disease. Vasoactive mediators and enzymes potentially regulated by IL-1β, such as nitric oxide synthase and endothelin, may be responsible for vascular changes such as vasorelaxation or vasoconstriction, respectively, which have been suggested to be important in the pathophysiology of laminitis. Furthermore, IL-1β has the potential to up-regulate the expression of matrix metalloproteinases, activation of which may induce laminar basement membrane degradation. Consequently, IL-1β (and other proinflammatory cytokines) may play a central role in the pathogenesis of laminitis.

Together with TNFα, IL-1β is recognized as being responsible for initiating many of the proinflammatory effects associated with endotoxemia. Lipopolysaccharide markedly up-regulates expression of IL-1β by vascular endothelial cells, and although the role of lipopolysaccharide in the pathogenesis of laminitis is controversial, there is substantial evidence that endotoxemia and laminitis are closely associated in horses. Endotoxin or other bacterial toxins that may be absorbed from the diseased gastrointestinal tract may trigger the systemic release of a vasoactive mediator that in turn interferes with digital blood flow, but such an agent has yet to be identified. It is more likely that absorption of bacterial toxins from the compromised gastrointestinal tract induces a cascade of events involving the systemic release of central inflammatory mediators such as IL-1β that ultimately induces the local digital production of vasoactive mediators.

In this study, expression of IL-1β mRNA was evident around laminar capillaries. This expression represents a local inflammatory phenomenon in the digit at the onset of laminitis that may occur in response to increased circulating concentrations of other agents such as lipopolysaccharide. The marked decrease in circulating WBC count prior to the onset of laminitis suggests that black walnut extract causes systemic toxemia and inflammation. Additionally, in recent preliminary experiments, we have detected IL-1β expression in the subcapsular macrophages of mesenteric lymph nodes collected from 1 of the black walnut-exposed horses at the same time the laminar tissue was perfused and fixed. If we find this result to be consistent, it would suggest that absorption of toxins from the compromised gastrointestinal tract may induce production of proinflammatory cytokines by the reticu-
loendothelial system. When released into the systemic circulation, these cytokines may stimulate further expression of proinflammatory cytokines in peripheral tissues such as the cellular components of the laminae. Although we did not detect circulating endotoxin in the horses given black walnut extract in this study, it is possible that the toxin released is not endotoxin, is absorbed to a greater extent by the portal system (eg, mesenteric lymph nodes and liver) before it is released into the systemic circulation, or is present in the circulation only for short periods.

Expression of IL-1β mRNA in this study was limited to a few perivascular cells per vessel, and not all vessels exhibited expression of the cytokine. A possible explanation for this specific limited expression of IL-1β mRNA is that tissue sections were collected during an early stage of laminitis, and it is possible that more cellular components of the laminae would be found to express IL-1β at later times, owing to paracrine stimulation via the perivascular cells. Further studies assessing IL-1β expression at later times in the development of the laminitis are necessary to address this issue. The marked expression of IL-1β by perivascular cells at the base of the laminae is interesting, because most of the arteriovenous anastomoses in the equine digit are in this region. Thus, expression of IL-1β in this area may lead to local production of vasoactive mediators (eg, nitric oxide, prostacyclin), resulting in aberrant dilation of arteriovenous anastomoses.

The perivascular cells in which expression of IL-1β mRNA was identified may represent fibroblasts or pericytes or possibly be of leukocyte origin. A somewhat similar phenomenon of preferential expression by a perivascular cell type is observed in the glomerulus, where mesangial cells undergo greater expression of IL-1β than do other vascular cell components of the glomerulus when exposed to lipopolysaccharide and other biologically active components of gram-negative bacterial cell walls. Detection of cells positive for IL-1β expression in other areas of the dermal laminar interstitium remote from the microvasculature in 1 horse in this study indicates that the cell type may be present in multiple areas throughout the dermal lamina, that there is more than 1 cell type induced to express IL-1β with laminitis, or that the cell type is capable of migration (eg, a marginated leukocyte). We are currently investigating the identity of these cells by means of transmission electron microscopy.

In a recent review of equine laminitis, it was stated that an important issue for any event found to occur in horses with laminitis is determining whether the event is a critical step in the development of the disease, a noncontributing consequence of some other step, or merely a coincidence. It is possible that IL-1β mRNA expression is a consequence of another event occurring in the laminitis process. Interleukin-1β expression has been found to be up-regulated in numerous studies of ischemia and expression may, therefore, be induced by digital hemodynamic changes previously reported to be occurring at the same time when we collected our tissues. However, the striking IL-1β expression at the base of the laminae, an area that is not normally considered to be ischemic with laminitis, does not support this possibility. Additionally, IL-1β expression in 2 anatomically distant tissues, the digital interstitium and the mesenteric lymph node, does not support the suggestion that expression of IL-1β is a consequence of a local hemodynamic event in the digit. Finally, it is difficult to believe that expression of IL-1β is simply a coincidence because of the documented potency of the central proinflammatory cytokines in inducing a plethora of other mediators in numerous other disease processes.

In situ hybridization allowed us to study expression of IL-1β at the mRNA level. However, to assess the correlation between mRNA expression and protein synthesis, a technique such as immunohistochemistry, using an equine-specific antibody, would be necessary. At this time, such an antibody is not available, and nonspecies-specific antibodies used in other laboratories have failed to provide satisfactory results. Therefore, future investigations may benefit from the synthesis of an equine-specific anti–IL-1β antibody.

In conclusion, we have documented local up-regulation of IL-1β mRNA expression in the digital laminae of horses in the prodromal stage of experimentally induced laminitis. This fact, in combination with the knowledge that IL-1β can induce expression of most of the mediators previously detected in the laminic digit, suggests that IL-1β expression may be a central factor in the cascade of events that leads to clinical laminitis.

References


