Expression of a transforming gene (E5) of bovine papillomavirus in sarcoids obtained from horses

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Objective—To determine expression of a transforming gene (E5) of bovine papillomavirus in sarcoids, other tumors, and normal skin samples collected from horses with and without sarcoids.

Sample Population—23 sarcoids and 6 samples of normal skin obtained from 16 horses with sarcoids, 2 samples of normal skin and 2 papillomas obtained from horses without sarcoids, and 1 papilloma obtained from a cow.

Procedure—Protein was extracted from tissue samples collected from horses and incubated with agarose beads covalently coupled to *Staphylococcus aureus* protein A and an anti-E5 polyclonal antibody. Following incubation, proteins were eluted from the beads and electrophoresed on a 14% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The E5 protein was detected by use of western blot analysis, using a chemiluminescence detection system.

Results—All 23 sarcoids had positive results for expression of E5 protein. Quantity of viral protein appeared to vary among sarcoids. All other tissues examined had negative results for E5 protein. Highest expression for E5 protein was observed in biologically aggressive fibroblastic variants of sarcoids, compared with expression in quiescent tumors.

Conclusions and Clinical Relevance—This study documented that activation and expression of the E5 gene is evident in sarcoids obtained from horses. These data support the conclusion that infection with bovine papillomavirus is important in the initiation or progression of sarcoids in horses. Treatment strategies designed to increase immune recognition of virally infected cells are warranted. (*Am J Vet Res* 2001;62:1212–1217)

Sarcoids are the most common cutaneous malignancy in horses and can result in substantial morbidity and economic loss.[1] The term sarcoid was first used in

1936 to describe the sarcoma-like characteristics of these tumors.[2] Sarcoids are categorized as occult, verrucous, fibroblastic, or malevolent on the basis of their clinical characteristics. The classic histopathologic description of sarcoids is that of a fibroblastic tumor with disorganized well-differentiated fibroblasts and pseudoepitheliomatous hyperplasia.[3,4] Histopathologic diagnosis of a sarcoid requires evidence that the tumor contains dermal and epidermal abnormalities.

The biologic behavior of sarcoids varies widely from benign quiescent variants to aggressive rapidly growing fibroblastic types. Nearly half of the horses with sarcoids develop multiple lesions over time. Studies[5,6] of horse populations suggested an infectious cause for sarcoids. Breed, family, and genetic predispositions also have been identified, with Quarter Horses and related breeds having the highest and Standardbreds having the lowest risk.[7,8] **Equine luekocytes antigen (ELA)** alleles are associated with breed and family predispositions for sarcoids.[9-12]

Papillomaviruses are a group of small double-stranded DNA viruses in the family Papovaviridae. They are the causative agents of benign papillomas in many vertebrate hosts.[13] Although most papillomavirus-induced lesions are benign, they can progress and become malignant. Malignant transformation is associated with Shope papillomavirus infection of rabbits,[14-17] certain human papillomaviruses,[18] and **bovine papillomaviruses (BPV)**.[19] Bovine papillomavirus 1 is the most extensively studied of these papillomaviruses. It is capable of inducing malignant transformation of several cell lines including bovine and equine fibroblasts and can induce fibrosarcomas in Syrian golden hamsters.[20-24]

The genome of BPV contains early and late coding regions. The early-coding genes are responsible for regulation of viral replication, maintenance of the episomal state, and activation of cell proliferation,[25] whereas the late-coding genes encode structural proteins that are only produced in the productive life cycle of well-differentiated keratinocytes in natural hosts. Bovine papillomavirus contains 2 genes (ie, E5 and E6), each of which is capable of inducing transformation in vitro.[24,26,27] The E5 gene product is a small protein (44 amino acids) that consists of a hydrophobic N-terminus (30 amino acids) and a hydrophilic C-terminus (14 amino acids).[28] It is primarily found as a membrane-associated dimer in the Golgi apparatus, plasma membrane, and other intracellular vacuoles.[29] The hydrophilic region appears to be the active region for transformation, because microinjection of a small pep-

tide from the C-terminus is sufficient to induce DNA synthesis and morphologic transformation.[30] Mutations that ablate the stimulation of DNA synthesis also result in loss of the transforming function, suggesting that these actions are the result of the same event.[31]

Because of its relatively small size and structure, it is unlikely that the E5 gene induces transformation by an enzymatic function. Studies[32,33] have documented that the E5 gene coprecipitates with the **platelet-derived growth factor-β (PDGF-β)** receptor and a 16-kd protein that is a subunit of the vacuolar H^+-ATPase pump. These proton pumps are critical for the function of cellular compartments that process growth factors, and binding of the E5 protein may result in alteration of this processing. The E5-induced transformation is associated with an increase in activation of mature and immature forms of the PDGF-β receptor.[34] Although the exact mechanism of action for E5-induced transformation and DNA synthesis is still unclear, its interaction with PDGF-β receptors is likely to be a critical step.

Bovine papillomavirus was initially proposed to be the causative agent of sarcoids in the early 1950s when experimental inoculation of horses with wart extracts obtained from cattle induced lesions that were clinico-pathologically similar to sarcoids in horses.[35,36] Although most studies support the conclusion that BPV plays a role in the induction of sarcoids, proof of a cause-and-effect relationship is lacking. If infection with BPV is critical for the initiation or progression of malignancy in sarcoids of horses, then expression of 1 or more of the early-coding genes, specifically the transforming genes, should be detectable in sarcoid tissue. The objective of the study reported here was to evaluate the expression of the E5 gene in tissues obtained from horses with and without sarcoids.

Materials and Methods

Sample collection—Tissue samples were collected by incisional or excisional biopsy from horses admitted to the Veterinary Medical Teaching Hospital at the University of California, Davis. At the time of evaluation, information was collected regarding the time of appearance, type of sarcoid, and its growth characteristics. Tissues were cut into 0.5-cm fragments, snap frozen in methylbutane, chilled in liquid nitrogen, and stored at –70 C until analyzed. In addition to 23 sarcoids, 6 normal skin samples (3 obtained from a site 2 to 4 cm from the margin of the nearest sarcoid and 3 from a site ≥ 20 cm from the margins of the nearest sarcoid) from sarcoid-affected horses, 2 samples of normal skin from unaffected (healthy) horses, 2 papillomas from horses without sarcoids, and 1 papilloma from a cow were collected for study. Samples of normal skin from unaffected horses were used as negative-control samples, and the papilloma obtained from the cow was used as a positive-control sample.

Polymerase chain reaction—All samples were tested for BPV type-1 or -2 DNA, using a **polymerase chain reaction (PCR)**. Amplification of an approximately 280-base pair fragment of the E5 genome was performed as described elsewhere.[37] Restriction fragment-length polymorphism analysis was used to determine the specific subtype of papillomavirus. Positive-control tissues were papillomas from cattle. Negative-control samples were papillomas from horses and samples of normal skin from unaffected horses.

Protein extraction—An aliquot (0.1 to 0.4 g) of tissue from each sample was pulverized while frozen and immediately suspended in **sodium dodecyl sulfate (SDS)** lysis buffer (50 mM HEPES-NaOH, 100 mM NaCl, 2 mM EDTA, 0.4% SDS). Additional destruction of tissue was accomplished with a glass homogenizer until a finely ground sample was obtained. After homogenization, samples were sonicated for 10 seconds and incubated for 15 minutes at 95 C. After incubation, Triton X-100 was added to all tubes to achieve a final concentration of 20%. Samples were mixed and then centrifuged for 5 minutes at 2,000 $\times$ g for 30 seconds. Supernatant was removed, and the quantity of protein was determined by use of a protein assay kit.[a] Absorbance was measured at 562 nm. After protein quantification, samples were immediately used for immunoprecipitation experiments.

Immunoprecipitation—An aliquot (20 µl) of agarose beads with covalently bound *Staphylococcus aureus* protein A[b] was rinsed 2 times in 1 ml of PBS buffer. Following each rinse, beads were pelleted by centrifugation (1,100 $\times$ g for 30 seconds), and the supernatant was removed. One milligram of protein from each sample was suspended in 1 ml of sample buffer (SDS lysis buffer with 20% Triton X-100). Protein samples were added to 20 µl of agarose beads and 5 µl of polyclonal anti-E5 antiserum (provided by 1 of the investigators [RS]), and the mixture was incubated in a rolling mixer overnight at 4 C. After incubation, beads were washed 3 times, using the following procedure: samples were immediately centrifuged (1,100 $\times$ g for 30 seconds), the supernatant was removed, and 1 ml of modified RIPA buffer (20 mM 3-morpholinopropanesulfonic acid, 1 mM EDTA [pH 7.0 to 7.2] followed by addition of 150 mM NaCl, 3.5 mM SDS, 25 mM deoxycholic acid, and 1% NP40[c]) was added, followed by incubation for 5 minutes on a rolling mixer at 4 C. After incubation, samples were rinsed with 3 washes of sterile PBS buffer. After completion, beads were resuspended in SDS elution buffer (750 µl of 1$\times$ dye mix [150 mM Tris-HCl, 15% sucrose, 2 mM EDTA, 0.01% bromphenol blue], 150 µl of 20% SDS, and 20 µl of dithiothreitol) and incubated for 15 minutes at 37 C with intermittent mixing. Following incubation, beads were heated to 95 C to denature the proteins, and 3 µl of 1M iodoacetamide was added prior to a final incubation in the dark at 37 C for 30 minutes to elute proteins. Beads subsequently were pelleted, and the supernatant was collected for SDS-polyacrylamide gel electrophoresis.

Gel electrophoresis—Samples were loaded onto a 14% polyacrylamide gel and electrophoresed at 150 V until separation was complete. Molecular weight markers as well as positive- and negative-control samples were included on each gel. Following electrophoresis, gels were placed in 200 ml of transfer buffer (192 mM glycine, 20 mM Tris, 20% methanol) for 15 minutes to equilibrate. Proteins were transferred to a polyvinylidene difluoride membrane,[d] using an overnight transfer protocol at 30 V for 15 hours. Following transfer, each membrane was rinsed in transfer buffer.

Western blot analysis—Membranes were incubated in blocking solution (2% bovine serum albumin added to wash buffer [140 mM NaCl, 10 mM NaPO4 {pH 7.4}, 0.5% Triton X-100]) and incubated for 30 minutes. Following blocking, each membrane was rinsed in wash buffer (2 rinses, 15 min/rinse) and incubated at 20 C for 90 minutes in primary antibody (rabbit anti-E5; provided by 1 of the investigators [RS]). Following incubation with the primary antibody, the membrane was washed in wash buffer as described previously and incubated in secondary anti-rabbit antibody[e] conjugated to horseradish peroxidase for 90 minutes at 20 C.

Primary and secondary antibodies were diluted 1:10,000 and filtered through a 0.2-μm filter prior to use. After a final series of washes, an enhanced chemiluminescence system[f] was used to detect antigen-antibody complexes. After incubation for 5 minutes, excess chemiluminescence reagent was removed, and membranes were exposed to autoradiography film[g] to reveal E5 protein bands. Film was developed, using an automated developing system.

Results

Twenty-three sarcoids from 16 horses were examined. Results of PCR revealed that 22 of 23 sarcoids (15 of 16 horses) contained BPV type-1 or -2 DNA. Of the 15 horses with sarcoids that had positive results for BPV DNA, 7 had BPV type-1 DNA, and 8 had BPV type-2 DNA. The BPV subtype was the same for all sarcoids obtained from a particular horse. Of the 6 samples of normal skin obtained from sarcoid-affected horses, 2 obtained from the same horse (1 sample close to the sarcoid and 1 sample from a distant site) had positive results for BPV DNA.

Fourteen sarcoids were classified as fibroblastic, 7 were verrucous, 1 was a mixed verrucous and fibro blastic, and 1 was derived from a tumor that metastasized from a regional lymph node. Tumor growth rates were described as rapid for 8 sarcoids and quiescent for 2 sarcoids; growth rates for the remaining 7 tumors were unknown.

Four horses had multiple tumors, 3 of which appeared to have regional metastasis. Tumor locations included the pinna (n = 5), neck and poll (5), margin of the eyelid (3), limb (3), inguinal area (2), facial crest (2), flank (2), and submandibular lymph node (1).

All sarcoids had positive results when tested for expression of E5 protein, although the quantity of viral protein appeared to vary among sarcoids **(Fig 1)**. Tissues with negative results for E5 protein included 2 papillomas obtained from horses, 2 samples of normal skin from unaffected horses, and 6 samples of normal skin from 4 sarcoid-affected horses. Of the latter 6 skin samples, 2 had positive results for a portion of the E5
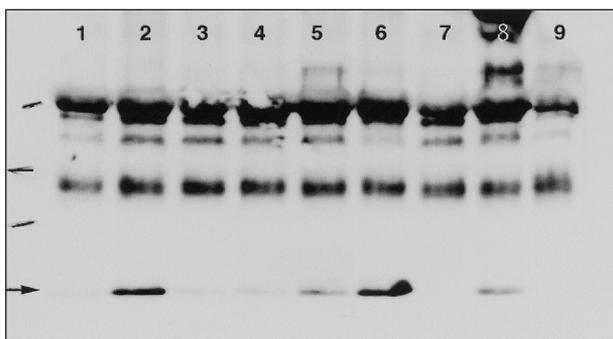

Figure 1—Results of immunoprecipitation on western blot hybridization of samples obtained from sarcoids, other tumors, and normal skin of horses and a papilloma obtained from a cow. Each lane contained 1 mg of protein. Lanes are as follows: 1, 3, and 4 are clinically benign slow-growing verrucous sarcoids; 2, 5, and 6 are clinically aggressive fibroblastic sarcoids; 7 is a papilloma obtained from a horse; 8 is a papilloma obtained from a cow; and 9 is a sample of normal skin obtained from an unaffected horse. Lines on the left of the figure correspond to protein molecular weight markers of 46, 30, and 14 kd.[i] Notice that the E5 protein bands in the samples correspond to a 7-kd molecular weight marker (arrow).

genome when tested by use of PCR. Although expression of E5 protein was not quantified, chemiluminescence intensity relates directly to the quantity of E5 protein on the western blots, and relative expression of protein for each sample was consistent in repeated experiments. Highest expression of E5 protein was observed in biologically aggressive fibroblastic variants of sarcoids, compared with expression in the 2 quiescent tumors, both of which were categorized histopathologically as verrucous.

Discussion

All 23 sarcoids expressed the E5 gene of BPV. Detection of BPV mRNA in sarcoids has been reported[38]; however, to our knowledge, the study reported here represents the first report of expression of a BPV protein in sarcoids obtained from horses and supports the hypothesis that viral infection and protein expression are associated with development of sarcoids.

Other studies[37,39-45] have documented BPV type-1 or -2 DNA in sarcoids, using PCR, DNA in situ hybridization, or Southern hybridization techniques,but data supporting expression of viral transforming genes has been lacking. The fact that viral DNA was found only in sarcoid tissues in most studies infers a relationship between viral infection and neoplastic transformation. In another study,[37] we detected viral DNA in normal samples of skin obtained from sarcoid-affected horses, suggesting a more complex relationship between viral infection and cell transformation. Although experimental inoculation of BPV in skin of horses resulted in tumors morphologically similar to naturally developing sarcoids in horses, several important differences were evident. Experimentally induced sarcoids had a spontaneous regression rate of 100%. Furthermore, they differed histologically from naturally developing sarcoids; there was a lack of classic epithelial changes and increased inflammatory cell infiltrates in some sarcoids.[36] In other studies,[46,47] injection of bovine wart extracts induced warts in cattle and sarcoid-like tumors in horses, but injection of sarcoid extracts did not. Experimentally inoculated horses developed a humoral immune response to BPV, whereas horses with naturally developing sarcoids did not. In addition, papillomavirus particles were not found in sarcoids obtained from horses. It is conceivable that the inflammatory response and high rate of regression in the experimentally induced sarcoids reflected differences between naturally developing and experimentally induced infections. Most of the investigators used extracts of warts obtained from cattle, which were crude cell extracts containing many bovine cellular proteins as well as papillomaviral particles. Presentation of multiple bovine antigens to the immune system of horses could result in a strong immune response against the virus and bovine cellular proteins. In addition, those extracts contained large numbers of infectious virions, whereas natural infection may evolve from a single virus particle entering a cell and inducing a latent or nonproductive viral infection. Strong cellular and humoral immune responses observed in the experimentally infected

horses were most likely a result of the large antigen exposure in the inoculation.

Most papillomaviruses are extremely species- and tissue-specific viruses. Papillomaviral infection of a heterospecies has been reported but is uncommon. In cases in which a nonhost species has been infected, the virus does not enter the productive life cycle and exists episomally as a stable piece of DNA, transcribing early-coding genes to maintain viral copy number and regulate cell growth.[25] Papillomaviruses appear to require a specific cellular environment to enter the productive life cycle and generate infectious virions. This environment appears to exist only within well-differentiated keratinocytes of its natural host. Because of these requirements, it is difficult to produce viral particles in tissue cultures and, thus, to our knowledge, has not been performed in a nonhost species. Although experimental infection of hamsters with BPV-1 results in formation of malignant growths with viral DNA persisting in transformed cells, viral structural proteins are not transcribed, and viral particles are not produced.[48] Even experimental infection of domestic rabbits with the cottontail rabbit papillomavirus results in poor viral production, compared with infection of its natural host, the closely related cottontail rabbit.[49] Therefore, lack of BPV structural proteins and virus-like particles in sarcoids of horses is likely the result of the strict cellular requirements needed for the virus to enter the productive life cycle. Because sarcoids do not contain infectious virions, experimental inoculation of sarcoid extracts would be unlikely to result in induction of warts in cattle.

The amino acid sequence of the E5 protein is identical in BPV-1 and -2 DNA,[28,h] which allows use of a single polyclonal antisera to evaluate tumors containing both virus subtypes. The E5 protein was detected in tumors containing BPV-1 and -2 DNA without apparent differences between the 2 viral subtypes.

The E5 protein was not quantified in this study, because tissue homogenates contained a mixture of tumor, stromal, and inflammatory elements; however, there appeared to be a subjective correlation between the amount of E5 protein expressed per gram of tumor tissue and the relative growth rate and aggressiveness of the tumor. The E5 protein can increase the activation of the PDGF-β receptor of fibroblasts.[34,50] Although a small region of homology between the E5 protein and PDGF-β led researchers to hypothesize that activation was in a ligand-dependent manner, it subsequently was documented that binding requires only the hydrophobic region, suggesting that activation is a ligand-independent process.[51] Activation of PDGF-β receptors in fibroblasts results in stimulation of mitogenesis in nearby epithelial cells. The E5-mediated activation of the PDGF-β receptors in neoplastic fibroblasts of horses may explain the characteristic pseudoepitheliomatous hyperplasia observed in sarcoids.

In other studies[40,41] in which investigators used PCR, viral DNA was detected in 91 to 100% of sarcoids from horses. Although all 23 sarcoids evaluated in the study reported here had positive results for E5 protein, only 22 had positive results for E5 DNA, using the PCR technique. Given the large amount of connective tissue in certain types of sarcoids, it is possible that a particular section may contain little or no viral DNA and yield a false-negative result. Finding protein-positive, DNA-negative sarcoids suggests that the sensitivity of PCR for these tissues is less than optimal. On the basis of these results, it is possible that viral DNA is in a greater percentage of sarcoids than previously reported. Bovine papillomaviral DNA also has been detected in a small number of normal samples of skin from sarcoid-affected horses.[37] In the study reported here, viral DNA coding for a region of the E5 gene was detected repeatedly from 2 samples of normal skin obtained from 1 sarcoid-affected horse, but expression of E5 protein was not detected. Expression of BPV E5 protein was limited to histopathologically confirmed sarcoids. Perhaps papillomaviruses are capable of infecting the skin of horses and remaining latent until some factor triggers viral transcription and translation. A latent viral phase could explain the reason that sarcoid-affected horses often develop multiple tumors at distant sites without visible signs of metastasis. It also would explain the apparent predisposition for tumors to develop at sites of injury, because the growth stimulation associated with wound healing may trigger viral activation.

Viral DNA also was detected in normal samples of skin collected from several other sarcoid-affected horses, but because of the small size of the biopsy specimens procured, we were unable to evaluate expression of viral proteins in those samples. Additional evaluation of BPV-positive samples of normal skin from sarcoid-affected horses to determine expression of BPV E5 appears warranted.

Distribution of the location of sarcoids in this study is unlikely to be a true reflection of the distribution of sarcoids on horses in California, because our population consisted primarily of horses donated to our facility or that were euthanatized because of this disease; consequently, it would not reflect a random sample. Additional analysis of expression of viral proteins in various types of sarcoids would be important to determine whether expression correlates with clinicopathologic characteristics of these tumors.

Analysis of results of the study reported here revealed infection with BPV and translation of the E5 protein in sarcoids of horses. Because E5 is a transforming protein in vitro, it seems likely that it plays a role in fibroblastic transformation in horses. If expression of the E5 protein is required for initiation, promotion, or progression of naturally developing sarcoids in horses, then 1 treatment strategy would be to target the E5 protein and accomplish immune degradation of virally infected tumor cells. Because E5 is a membrane-associated protein, it would provide a reasonable target for immune-mediated antitumor activity. Additional exploration of strategies designed to increase immune recognition of viral proteins is warranted.

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