Expression of cyclooxygenase-1 and -2 in naturally occurring squamous cell carcinomas in horses

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Objective—To assess expression of cyclooxygenase (COX)-1 and -2 in naturally occurring squamous cell carcinomas (SCCs) and the analogous normal tissues in horses.

Sample Population—Tissue samples collected from 3 conjunctival, 2 vulvar, 4 preputial, and 5 penile SCCs during surgical excision in 14 horses and from corresponding body regions (conjunctiva [n = 5 horses], vulva [2], prepuce [3], and penis [3]) in 5 horses euthanized for reasons unrelated to neoplasia.

Procedures—Tissue samples were snap frozen in liquid nitrogen and stored at –80°C until analysis. Protein was extracted from the frozen tissues, and western blot analyses were performed. Nonneoplastic and abnormal tissues from each body region were run on the same blot, and blots were run in triplicate. Molecular-weight markers and COX-1 and -2 ovine standards (positive control samples) were run concurrently on the gels; negative control samples were not used.

Results—All tissues, including the nonneoplastic and SCC tissues, expressed both COX-1 and -2 proteins.

Conclusions and Clinical Relevance—Results indicated that the expression of COX proteins in both nonneoplastic and SCC-affected tissues in horses is markedly different from that in other species. The reason for the potential benefit of COX-2 inhibitors in horses and other species is unknown. Further research needs to be performed to evaluate the efficacy of COX-2 inhibitors as cancer treatments in horses. Investigation of the mechanisms of tumor development in horses should be performed to increase understanding of this disease and ascertain how the mechanisms differ from those in other animals. (Am J Vet Res 2007;68:76–80)

Squamous cell carcinoma is the most common tumor of the eye and adnexal structures and the urogenital system in horses.[1-3] As the population of geriatric horses increases as a result of improved management and health care, the incidence of neoplasia will also increase.[4] There are many treatments for equine SCC; treatment modalities include excision, laser ablation, radiation therapy, chemotherapy (involving 5-fluorouracil, cisplatin, or carboplatin), cryotherapy, and hyperthermia treatment.[5-10] The success rates of these treatments range from 30% to 90% (with success being defined as nonrecurrence of the neoplasm for a period of > 1 to 5 years). Excision alone is one of the least successful approaches, whereas combining surgery with radiation therapy or chemotherapy offers the best chance of success.[5,6,11,12] The rate of success also depends on the extent and location of the tumor at diagnosis.[6] However, despite the treatment success achieved via combination of surgery with radiation therapy or chemotherapy, certain SCCs (eg, gastric, pharyngeal, or bladder tumors) develop in locations that are inaccessible to surgery and radiation therapy. As well, many institutions do not offer radiation therapy for horses. Recent reports, both published and anecdotal, describe successful resolution of SCCs in horses by oral administration of piroxicam, a nonselective COX inhibitor.[13,a] This is an attractive treatment option because piroxicam is readily available, inexpensive, and easy to administer. The use of COX inhibitors for treatment of tumors in horses is extrapolated from their use in other species.

Received January 19, 2006.
Accepted July 17, 2006.
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Presented in part at the 25th Annual Veterinary Cancer Society Conference, Huntington Beach, Calif, October 2005 and at the 2005 American College of Veterinary Surgeons Veterinary Symposium, San Diego, Calif, October 2005.
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Abbreviations

SCC	Squamous cell carcinoma
COX	Cyclooxygenase
TBS	Tris-buffered saline (0.9% NaCl)
TBS-T solution	TBS solution containing 0.0005% Tween

In rats, dogs, and humans, tumors (including SCCs) express COX-2, whereas the corresponding normal nonneoplastic tissues of those species principally express COX-1.[14,15] The expression of COX-2 has been linked to the grade and rate of metastasis in humans.[16] In dogs and humans with a variety of neoplasms, treatment with COX-2 inhibitors in combination with chemotherapy has been used successfully.[17-21] The mechanism of action of the COX inhibitors is not fully understood. The COX-2 inhibitors do not have a direct cytotoxic effect on tumor cells.[22] The COX-2 enzyme is thought to aid tumor growth and invasion through increasing angiogenesis, increasing metastasis and invasiveness, inducing resistance to apoptosis and cisplatin, and causing suppression of the natural immune response.[15,23,24] Many of these tu-

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mor characteristics are reduced via COX inhibition. The effect of COX-2 is thought to be mainly mediated through increased concentrations of prostaglandin E_2. The beneficial effect of treatment associated with both selective and nonselective COX inhibitors may be attributable to a decrease in the overall production of prostaglandins, regardless of their source.[15,17,25]

The purpose of the study reported here was to assess expression of COX-1 and -2 in naturally occurring SCCs and the analogous nonneoplastic tissues in horses. The hypothesis was that in horses, nonneoplastic skin would mainly express COX-1 and SCC tissue would mainly express COX-2. The intention was that the results of these COX expression analyses could then be examined for similarity to those of other species in which COX inhibitors have been used successfully to treat neoplastic diseases.

Materials and Methods

During excision, tissues were harvested from 3 conjunctival, 2 vulvar, 4 preputial, and 5 penile SCCs removed from 14 client-owned horses. Samples were snap-frozen in liquid nitrogen and stored at –80°C until analysis. A veterinary pathologist confirmed that each tumor was an SCC via examination of H&E-stained sections of collected tissue. Tissue was also harvested from corresponding body regions (conjunctiva [n = 5 horses], vulva [2], prepuce [3], and penis [3]) in 5 horses that were euthanized for reasons unrelated to neoplasia. These tissues were also snap-frozen in liquid nitrogen and stored at –80°C until analysis. All procedures were approved by the North Carolina State University Institutional Animal Care and Use Committee.

Protein was extracted from the frozen tissue, and western blot analysis was performed. Briefly, tissues were homogenized on ice in radioimmunoprecipitation buffer, aprotinin, and sodium orthovanadate and then incubated for 30 minutes with phenylmethylsulfonylfluoride. The samples were then centrifuged at 4°C for 10 minutes. The supernatant was collected and centrifuged at 4°C for 10 minutes. The supernatant was again collected, and the protein content was determined by use of a spectrophotometer and protein assay.[b] Equal amounts of protein were then loaded into a precast gel,[c] resolved, and electrophoretically transferred to a nitrocellulose membrane. Kaleidoscope staining markers[d] (molecular weight indicators) and COX-1 and -2 ovine standards[e] (positive control samples) were run concurrently on the gels. Negative control samples were not used. The membranes were blocked at room temperature (approx 20°C) for 2 to 3 hours in TBS-T solution and 5% nonfat milk. The membranes then were incubated in a refrigerator overnight (approx 15 hours) with goat anti-human COX-1 or -2 polyclonal antibodies[f] diluted (1:500) in TBS solution with 5% nonfat milk. The membrane was washed 3 times in TBS-T solution. The membrane was then incubated with donkey anti-goat IgG polyclonal antibodies linked to horseradish peroxidase[g] diluted (1:2,000) in TBS-T solution with 5% nonfat milk for 1 hour at room temperature. The membrane was washed again in TBS-T solution and then in TBS solution alone. A chemiluminescence system[h] was used, and the membranes were exposed to radiographic film. Blots were run in triplicate.

The radiographic film was scanned, and the images were analyzed via densitometry.[i] Statistical analysis was performed on the densitometric results. Because both nonneoplastic and abnormal tissues from each body region were run on the same blot, the densitometric data were statistically analyzed within each blot by use of a 2-tailed *t* test. A value of $P < 0.05$ was considered significant. The power and normality of distribution were determined for each data set during calculation of the *t* test.[j] The power of each test was calculated, and the test result was considered meaningful if the power of the test was > 0.8.

Results

Western blot analysis revealed that all the tissues analyzed (nonneoplastic and SCC tissue) expressed both COX-1 and -2 proteins **(Figures 1 and 2)**. The expression of the COX enzymes detected via western blotting was confirmed by use of the molecular-weight indicators and positive control samples. Data were normally distributed, but because of low sample numbers, the statistical analysis was not powerful enough to truly detect a difference. There were only 2 and 3 samples of vulvar and conjunctival tissues; therefore, the findings for these tissues were not analyzed. For the penile and preputial samples, nonneoplastic and abnormal tissues were compared by use of the *t* test; data were normally distributed, but the sample numbers were not sufficiently great to provide enough power to detect a statistical difference **(Table 1)**. The COX-2 expression in preputial tissue affected by SCC was significantly ($P = 0.04$) greater than the expression level in nonneoplastic preputial tissue.

Discussion

An important finding in the present study was that

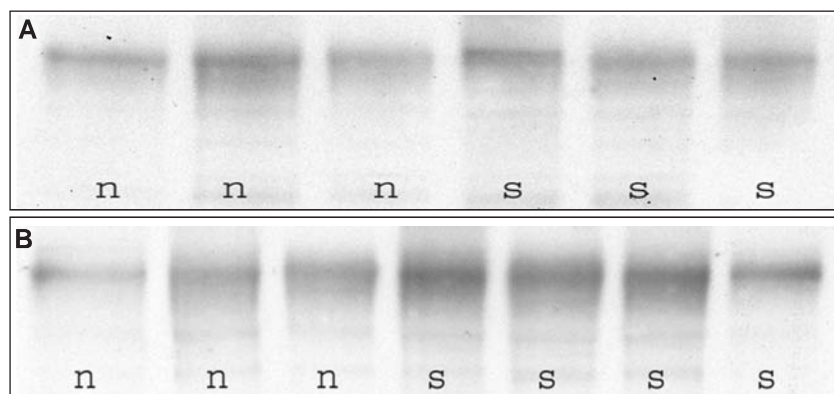

Figure 1—Results of western blot analyses involving antibodies against COX-1 (A) or -2 (B) in nonneoplastic (n) penile tissue and samples of penile SCCs (s) obtained from horses. Each lane represents 1 tissue sample obtained from 1 horse.

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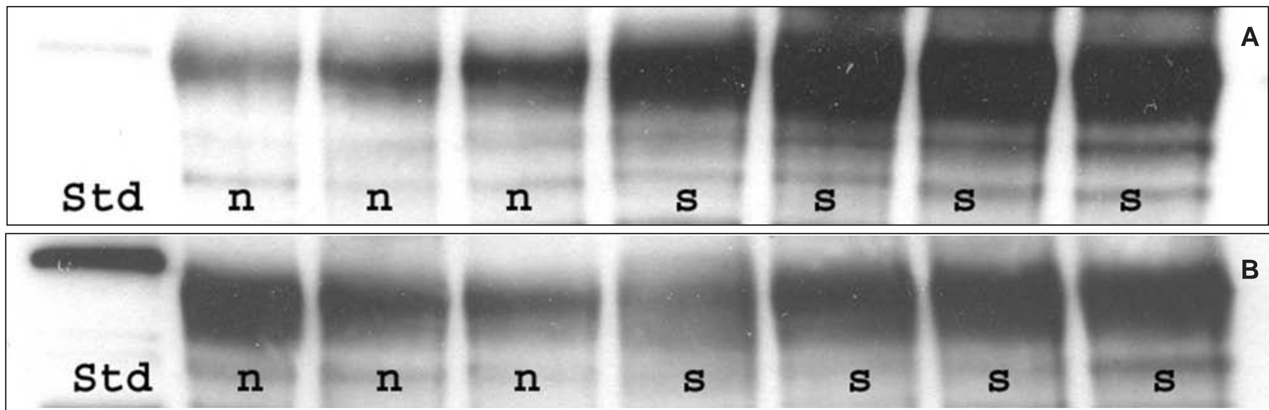

Figure 2—Results of western blot analyses involving antibodies against COX-1 (A) or -2 (B) in nonneoplastic (n) preputial tissue and samples of preputial SCCs (s) obtained from horses. Ovine COX-1 (std) is present as positive control sample. Each lane represents 1 tissue sample obtained from 1 horse.

Table 1—Expression levels (based on densitometric analyses of western blots) of COX-1 and -2 in SCCs excised from 14 horses and nonneoplastic tissues collected from the corresponding body regions in 5 horses euthanized for reasons unrelated to neoplastic disease.

Western blot	Antibody probe	Tissue type (No. of samples)	Densitometric reading (mean ± SD)	Fold difference*
Penile tissue	COX-1	Nonneoplastic (3)	444 ± 76	0.94
		SCC (5)	417 ± 28	
	COX-2	Nonneoplastic (3)	339 ± 97	1.63
		SCC (5)	551 ± 130	
Preputial tissue	COX-1	Nonneoplastic (3)	237 ± 39	1.5
		SCC (4)	355 ± 107	
	COX-2	Nonneoplastic (3)	79 ± 48	3.8
		SCC (4)	300 ± 127	
Conjunctival tissue	COX-1	Nonneoplastic (5)	2,149 ± 59	1.03
		SCC (3)	2,207 ± 212	
	COX-2	Nonneoplastic (5)	1,668 ± 360	1.07
		SCC (3)	1,781 ± 302	
Vulvar tissue	COX-1	Nonneoplastic (2)	484 ± 175	0.89
		SCC (2)	432 ± 218	
	COX-2	Nonneoplastic (2)	212 ± 134	0.81
		SCC (2)	172 ± 119	

*The fold difference in COX expression between the nonneoplastic and SCC tissue is given, but it is not known what fold change would be clinically relevant.

COX-1 and -2 were expressed in equine SCCs. In 1 published account[13] of the use of oral piroxicam for nonresectable tumors in horses, results have been favorable. Piroxicam is a nonselective COX inhibitor in other species,[26] but to our knowledge, studies to assess the selectivity and toxic effects of this drug in horses have not been performed. Drugs that target COX-2, which has been implicated in progression of neoplasia in other species, may ultimately be of value in treating SCC in horses, particularly if high levels of COX-2 expression occur within those tumors. Cyclooxygenase-2 inhibitors are thought to have antineoplastic activity through inhibition of the synthesis of prostaglandin E_2, a product of COX-2. This prostaglandin increases angiogenesis, thereby promoting metastasis and invasiveness of many tumors; it also suppresses the natural immune response and can cause production of enzymes that combat chemotherapeutic drugs.[15,23,24] By inhibiting prostaglandin E_2 synthesis through inhibition of COX-2, many characteristics of tumors that allow them to grow and invade tissues are inhibited. The advantage of the use of selective COX-2 antagonists (to avoid undesirable adverse effects) is clear in other species; however, in horses, the expression of COXs appear quite different and any advantage of selective versus nonselective COX inhibition in cancer treatment is unknown. Additionally, the reasons that COX inhibitors, particularly piroxicam, empirically appear to be of value in the treatment of neoplasia warrant further investigation. It may be that any inhibition of either COX enzyme decreases prostaglandin production in horses and thus combats tumor growth and metastasis.

With regard to the findings of the present study, no conclusive statement can be made about changes in COX expression between nonneoplastic and SCC tissue in any of the body regions examined because of the low power of the statistical analyses. The fold difference in expression of COX-1 or -2 between nonneoplastic and SCC tissue was calculated because there appeared to be greater COX expression in the abnormal tissue; however, the clinical importance of this is unknown. The main conclusion drawn from our study is that the level

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of COX-2 protein expression in horses appears to differ markedly from expression levels in other species that have been studied. In particular, COX-2 was expressed in both nonneoplastic and SCC tissues, whereas in other species, COX-2 is typically expressed primarily in neoplastic tissues and not in normal tissue. Also in horses, COX-1 expression was detected at similar levels in nonneoplastic and abnormal tissues, as would be expected for this constitutive enzyme. In other species, COX-1 expression is decreased in abnormal tissues.[14] In horses, COX-2 expression has also been detected in other nonneoplastic and abnormal tissues; for example, samples of equine large and small intestines express both COX-1 and -2 under normal conditions.[27,k] Therefore, it appears that COX-2 should also be regarded as a constitutively expressed enzyme in urogenital and intestinal tissues in horses. Studies relating to COX expression in other species should, therefore, be extrapolated to horses only with great caution. The reason for the difference in COX-2 expression in horses, compared with that in other species, is unknown. There may be a difference in the equine promoter region of the gene so that it is expressed constitutively and inflammatory mediators are not required to activate expression. However, gene sequencing and investigation need to be carried out before any conclusions can be drawn.

From data collected for other species, it is evident each tumor type in each species needs to be individually investigated. For instance, some human neoplasms (ovarian adenocarcinomas and cervical carcinomas) overexpress COX-1, compared with COX-2, but the latter is commonly overexpressed in other human neoplasms.[28] Also, human COX variants have been discovered, which may cause an under- or overestimation of COX-1 or -2 expression when analyses are performed.[28] In horses, such variants have not been examined but may have also affected the results of the present study. Furthermore, because the homologies of the COX enzymes between humans and horses have not been assessed, it is not known whether the use of goat anti-human COX antibodies affected our study results.

Densitometric analyses of western blots have to be interpreted with caution because densitometry readings assume absolute equal protein loading. The amount of protein in each sample was determined by use of a spectrophotometer, which only provides an estimate of the protein quantity. Real-time PCR assays would be able to accurately quantify COX-1 and -2 RNA transcripts, but such assessments may not accurately reflect the amount of protein that is ultimately expressed by the tissue because of posttranscriptional modification.

One reason for the lack of uniform results in COX expression between nonneoplastic and abnormal tissues may be the fact that the abnormal tissue samples were not controlled for size or duration of the tumor or location of the sample within the tumor itself. The effect of size and chronicity of equine tumors on expression of COX is unknown. In tumors in humans, the expression of COX-2 has been correlated to the grade and metastases.[16] A more controlled sample population and consistent location (peripheral or central) of samples within the tumors may more accurately reflect enzyme expression. Histologic examination of the tumor samples did reveal variable amounts of tissue necrosis, which will affect the amount of protein production in a tissue sample.

The expression levels of COX-1 and -2 were not compared to each other because separate blots were probed with each antibody, and interblot comparisons are not recommended because of differences in handling and developing, even if the total amounts of protein loaded were identical. Therefore, we were unable to make any statements regarding the relative levels of COX-1 to -2 in the equine tissues examined.

The findings of the present study have indicated that horses have a different pattern of COX expression in nonneoplastic skin and SCC-affected tissues than other species that have been evaluated. Further work is required to investigate the reasons for this difference, the possible mechanisms of action of COX inhibitors (both selective and nonselective) with regard to equine tumors, and the presence or absence of any changes in COX expression with varying tumor location or size.

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b. Biorad protein assay, Bio-Rad Laboratories, Hercules, Calif.
c. Criterion XT Precast Gel, Bio-Rad Laboratories, Hercules, Calif.
d. Kaleidoscope staining markers, Bio-Rad Laboratories, Hercules, Calif.
e. COX-1 and COX-2 ovine standards, Cayman Chemical, Ann Arbor, Mich.
f. Goat anti-human COX-1 or COX-2 polyclonal antibodies, Santa Cruz Biotechnology Inc, Santa Cruz, Calif.
g. Horseradish peroxidase, Santa Cruz Biotechnology Inc, Santa Cruz, Calif.
h. ECL western blotting detection reagents and analysis system, Amersham Biosciences, Piscataway, NJ.
i. Scanolytics Zero D, version 1.1, Scanalytics, Rockville, Md.
j. Sigmastat, version 3.0, Systat Software Inc, Richmond, Calif.
k. Morton AJ, Rotting AK, Freeman DE, et al. Characterization of cyclooxygenase 1 and cyclooxygenase 2 expression in normal and ischemic-injured equine left dorsal colon (abstr), in *Proceedings*. 8th Annu Int Equine Colic Res Symp 2005;147–148.

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