Comparison of distributions of survivin among tissues from urinary bladders of dogs with cystitis, transitional cell carcinoma, or histologically normal urinary bladders

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Objective—To compare distributions of survivin among tissues from urinary bladders of dogs with cystitis, transitional cell carcinoma (TCC), or histologically normal urinary bladders.

Sample Population—24 archived and 7 fresh-frozen specimens of urinary bladders from dogs with cystitis.

Procedures—Immunohistochemical analysis of archived tissue specimens was performed to identify survivin protein in the nucleus and cytoplasm of cells by use of polyclonal rabbit anti-survivin antibody. Tissues that contained $\geq 5\%$ immunoreactive cells were considered positive for survivin protein. Reverse-transcription PCR analysis was performed on fresh-frozen tissues to identify survivin mRNA. Data on tissues from dogs with TCC or histologically normal urinary bladders that were obtained during another study were used for statistical comparisons.

Results—Twelve of 24 (50\%) cystitic tissues were positive for nuclear survivin, compared with 28 of 41 (68\%) TCC tissues and 0 of 46 (0\%) normal tissues. Two of 24 (8\%) cystitic tissues were positive for cytoplasmic survivin, compared with 7 of 41 (17\%) TCC tissues and 17 of 46 (37\%) normal tissues. Proportions of specimens that contained nuclear or cytoplasmic survivin were significantly different between cystitic and normal tissues but not between cystitic and TCC tissues. Four of 7 cystitic tissues were positive for survivin mRNA, which was comparable with results for TCC and normal tissues.

Conclusions and Clinical Relevance—Nuclear survivin was detected in TCC and cystitic tissues but not in normal urinary bladder tissues. Additional studies are needed to determine whether nuclear survivin contributes to the development or progression of TCC. (Am J Vet Res 2008;69:1073–1078).
survivin-ΔEx3 usually localizes to the nucleus. Although survivin splice variants have not been reported in canine tissues, these characteristics of splice variants suggest that various isoforms and their patterns of localization play a role in regulation of cell proliferation and apoptosis in healthy as well as neoplastic tissue.

Because defective apoptosis can lead to persistence of mutated neoplastic cells, it is possible that survivin may have a role in preneoplastic lesions or early carcinogenesis. In human tissues, survivin has been detected in hyperplastic and dysplastic lesions of the prostate, skin, colon, lung, cervix, oral cavity, and endometrium. Some studies have revealed that expression of survivin is increased in highly dysplastic or neoplastic tissues, compared with expression in hyperplastic or low-grade dysplastic tissues of the same type.

Not only are hyperplastic tissues associated with neoplastic lesions, but there also is an increasing amount of research linking inflammation and cancer. In human tissues, inflammation can lead to various cancers, including cancer of the breast, liver, endometrium, and colon. Cytokines and other cellular signaling proteins expressed during inflammation can promote carcinogenesis, and expression of survivin is upregulated via inflammatory cytokines. Additionally, treatments intended to suppress expression of inflammatory cytokines also suppress expression of survivin. These findings suggest a link between survivin, inflammation, and carcinogenesis.

Although cystitis has not been established as a risk factor for TCC of the urinary bladder in dogs, chronic irritation and infection are risk factors for urinary bladder tumors in humans. Because canine TCC shares many similar features with human TCC, we wanted to determine whether expression of survivin in tissues of urinary bladders of dogs with cystitis would be associated with urinary bladder tumorigenesis.

The objective of the study reported here was to compare distributions of survivin in tissues from urinary bladders of dogs with cystitis to those of tissues from histologically normal urinary bladders and urinary bladders with TCC that were evaluated in another study. We hypothesized that nuclear survivin would be detected in cystitic tissues and therefore that nuclear survivin plays a role in cell proliferation and tumor growth in the urothelium of urinary bladders in dogs.

**Materials and Methods**

**Sample population**—For immunohistochemical analysis, archived, formalin-fixed (24 to 48 hours), paraffin-embedded specimens of urinary bladders from 24 dogs with cystitis (cystitic tissue) were obtained from the Veterinary Medical Diagnostic Laboratory of the University of Missouri. One pathologist (JRT) confirmed the diagnosis of cystitis via histologic examination of tissues for inflammatory leukocytes.

Data from formalin-fixed, paraffin-embedded tissue specimens from histologically normal urinary bladders (normal tissue; n = 46) and urinary bladders with TCC (TCC tissue; 41) that had been evaluated for survivin immunoreactivity in another study were used for comparison. These tissues had been examined by 1 pathologist (JRT) to confirm the diagnosis of TCC in neoplastic tissues and ensure that no neoplastic or inflammatory changes were evident in the normal tissues.

For PCR assays, when available, fresh specimens of normal urinary bladders were obtained from dogs with cystitis that underwent cystoscopy or cystotomy during sterile surgical procedures. Specimens were snap-frozen in liquid nitrogen immediately after collection and stored at −80°C. Fresh specimens from dogs with TCC of the urinary bladder were collected and stored in the same manner. Fresh specimens of normal urinary bladder tissues were obtained from dogs that underwent terminal surgeries with approval from the Animal Care and Use Committee of the University of Missouri.

**Immunohistochemical analysis**—Rabbit polyclonal anti-survivin antibody was used to detect survivin protein in tissue; all tissues were processed and stained as described elsewhere. One pathologist (SET) examined all tissues to evaluate immunoreactivity in another study that had been evaluated for survivin (normal tissue; n = 46) and urinary bladders with TCC that were evaluated in another study. One pathologist (JRT) confirmed the diagnosis of cystitis via histologic examination of tissues for inflammatory leukocytes.

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**Immunohistochemical analysis**—Rabbit polyclonal anti-survivin antibody was used to detect survivin protein in tissue; all tissues were processed and stained as described elsewhere. One pathologist (SET) examined all tissues to evaluate immunoreactivity to survivin in tissue specimens from urinary bladders of dogs with cystitis. For all specimens, tissues were considered positive for survivin protein when ≥5% of cells were judged to be immunoreactive. Tissues were considered to be negative for survivin protein when <5% of cells were immunoreactive. Specimens were classified as positive for cytoplasmic survivin when cytoplasmic immunoreactivity was detected in ≥5% cells and positive for nuclear survivin when nuclear immunoreactivity was detected in ≥5% of cells. For all assays, bovine fetal kidney was used as a positive control. Cystitic tissue treated with rabbit IgG instead of anti-survivin antibody was used as a negative control.

**PCR assay**—Isolation of RNA, synthesis of complementary DNA, and traditional qualitative reverse-transcription PCR were performed as described elsewhere. Primers used were developed from the canine survivin sequence (GenBank AB180206). Five specimens of fresh-frozen canine testicular tissue were used as positive controls. Deionized water without complementary DNA and tissue specimens from normal urinary bladders known to be negative for survivin mRNA were used as negative controls. β-Actin was used as a housekeeping gene for each tissue specimen, and data from specimens with positive PCR bands for β-actin were included in the final statistical analysis.

**Statistical analysis**—For immunohistochemical testing, tissue specimens were classified as positive or negative for survivin immunoreactivity and grouped into those with nuclear, cytoplasmic, or no expression of survivin. Proportions of positive test results were compared among the 3 types of tissues (normal, cystitic, or TCC) with a χ² test. When cells of the 2 × 2 contingency tables used for the χ² test contained <5 observations, a Fisher exact test was used instead. For the PCR assay, tissue specimens were classified as positive or negative for the survivin gene. Differences among cystitic, TCC, and normal tissues were evaluated by use of a Fisher exact test. For all comparisons, differences were considered significant at P < 0.05.
Results

Dog characteristics—Twenty-four specimens of urinary bladders from dogs with cystitis that were examined at the University of Missouri Veterinary Medical Diagnostic Laboratory from September 2004 through October 2006 were included in the immunohistochemical analyses. Ten of the 24 (42%) dogs were spayed females, 9 (38%) dogs were castrated males, and 4 (17%) dogs were sexually intact males, and the sex of 1 dog was unknown. Five of 24 (21%) dogs were mixed-breed dogs, 2 (8%) were Labrador Retrievers, 2 (8%) were Golden Retrievers, 2 (8%) were Boxers, and 2 (8%) were Pugs. Other breeds represented by 1 (4%) dog included Bichon Frise, Dalmatian, Doberman Pinscher, German Shorthaired Pointer, Lhasa Apso, Maltese, Miniature Pinscher, Miniature Schnauzer, Schnauzer, Yorkshire Terrier, and Standard Poodle. Median age of the dogs with cystitis was 6.4 years (range, 1.7 to 12.8 years).

Specimens of cystitic tissue were obtained via cystotomy in 17 of 24 (71%) dogs and via necropsy in 5 (21%) dogs, and methods were unknown for 2 (8%) dogs. Of the dogs that underwent cystotomy, 5 of 17 had cystic calculi, 2 had a thickened bladder wall or bladder wall mass, and 8 had cystotomies for unknown reasons. The remaining 2 patients had cystotomies for a ruptured bladder (one secondary to trauma and the other secondary to cystic calculus obstruction). Of the 5 dogs with cystitis from which bladder samples were obtained at necropsy, 2 were euthanatized because of spinal tumors that caused urinary incontinence, and others were euthanatized for conditions unrelated to the urogenital system (1 was hit by a car, 1 had a mediastinal mass, and 1 had suspected immune-mediated hemolytic anemia).

Characteristics of dogs from which normal TCC tissues were obtained are summarized elsewhere. Briefly, the 41 dogs with TCC were evaluated at the University of California (n = 21), University of Missouri (13), and Purdue University (7). Median age of dogs for which signalment information was available (n = 39) was 11.3 years (range, 5 to 16 years). The 46 dogs from which normal tissues were obtained consisted of 42 dogs that underwent terminal surgery (ages unknown) and 4 dogs that were euthanatized for diseases unrelated to the urinary tract at the University of Missouri.

Immunohistochemical analysis—Nuclear survivin immunoreactivity in cystitic tissues was compared with results obtained for normal and TCC tissues. Twelve of the 24 (50.0%) cystitic tissues were positive for nuclear survivin, whereas the remainder were negative (Figure 1). Proportions of immunoreactive specimens among the 3 different groups were significantly (P < 0.001) different (Figure 2). A Fischer exact test revealed that the proportion of TCC tissues with positive test results for nuclear survivin was significantly different from the proportion of normal tissues with positive test results. A significant (P < 0.001) difference was also detected between normal and cystitic tissues with respect to proportions for which positive test results for nuclear survivin were obtained. The difference between TCC and cystitic tissues with respect to nuclear immunoreactivity was not significant (P = 0.23); however, the power of that comparison was low (β = 0.17).

Cytoplasmic survivin immunoreactivity was variable among the 3 types of tissues (Figure 3). Two of the 24 (8.3%) cystitic tissues had positive results for cytoplasmic survivin, whereas 22 (91.7%) cystitic tissues were negative. Overall χ² analysis revealed that proportions of immunoreactive tissue specimens among the 3 groups were significantly (P = 0.008) different. The differences between normal versus TCC tissues and cystitic versus TCC tissues with respect to proportions of specimens that contained cytoplasmic survivin were
not significant ($P = 0.07$ and $P = 0.7$, respectively); however, the power of these comparisons was low ($\beta = 0.43$ and $\beta = 0.06$, respectively). A significant ($P = 0.02$) difference was detected between normal and cystitic tissues with respect to proportions that contained cytoplasmic survivin.

**PCR assay**—Specimens of urinary bladder tissue from 7 dogs with cystitis were available for PCR analysis. Of these 7 specimens, 3 also had sufficient quantities of formalin-fixed tissue to include in immunohistochemical analysis. Four of the 7 tissues contained survivin mRNA (Table 1). Of the 3 specimens that were tested via immunohistochemical analysis and PCR assay, 2 were positive for nuclear survivin protein and survivin mRNA. The remaining specimen was negative for survivin protein and survivin mRNA.

Data from 22 histologically normal bladder tissues and 6 urinary bladders with TCC from another study were used for comparisons. Differences were not significant between survivin mRNA expression in cystitic versus TCC tissues ($P = 0.19$) or cystitic versus normal tissues ($P = 1.0$); however, again the power was low ($\beta = 0.007$ and $0.032$, respectively).

![Figure 3](image)

**Figure 3**—Histogram of the proportions of specimens of urinary bladders from dogs with TCC ($n = 41$), cystitis (24), or histologically normal urinary bladders (46) that tested positive (black bars) or negative (white bars) for cytoplasmic survivin. Specimens were classified as positive for cytoplasmic survivin when cytoplasmic immunoreactivity was detected in $\geq 5\%$ cells. Proportions of specimens that contained cytoplasmic survivin differed between cystitic and normal tissues ($P = 0.02$) but not between TCC and cystitic tissues ($P = 0.7$) or TCC and normal tissues ($P = 0.09$).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>No. of tissues positive for survivin mRNA</th>
<th>Total No. of tissues evaluated</th>
<th>Proportion of tissues positive for survivin mRNA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urinary bladder with TCC</td>
<td>6</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>Urinary bladder with cystitis</td>
<td>4</td>
<td>7</td>
<td>57</td>
</tr>
<tr>
<td>Normal urinary bladder</td>
<td>11</td>
<td>22</td>
<td>50</td>
</tr>
</tbody>
</table>

Differences between pairs of proportions were not significant ($P > 0.05$).

**Discussion**

The objective of the study reported here was to characterize survivin expression in tissues from the urinary bladders of dogs with cystitis and compare the proportion of tissues expressing survivin with proportions in tissues from histologically normal urinary bladders or urinary bladders of dogs with TCC. The results support our hypothesis that nuclear survivin exists in cystitic tissues in dogs. Survivin is not likely to be a specific diagnostic marker for TCC of the urinary bladder in dogs, but nuclear survivin can be detected in tissues from urinary bladders of dogs with TCC or cystitis. Additional investigation is warranted to better define a role for survivin in proliferation of cells and regulation of apoptosis.

Nuclear survivin may have a role in proliferation of hyperplastic and neoplastic tissues. None of the tissues from histologically normal urinary bladders contained nuclear survivin, whereas 50% of cystitic tissues and 68% of TCC tissues were immunoreactive. This finding is supported by results of a study of human TCC tissues, in which tissues with higher proportions of cells with nuclear survivin had significantly higher cell proliferation fractions (assessed via Ki-67 scores) than did tissues with lower nuclear survivin scores. In addition, the proportion of cells that are immunoreactive to survivin is higher in human hyperplastic prostatic tissue, hyperproliferative skin lesions, hyperplastic colonic mucosa, and hyperplastic endometrium, compared with the proportion of cells that are immunoreactive to survivin in normal tissue counterparts. The expression of survivin is also higher in malignant tissues, compared with expression in hyperplastic or inflamed tissues. These findings suggest survivin may play a role in cell proliferation and tumor progression via regulation of cell proliferation or by indirect inhibition of apoptosis.

The differential distribution of survivin within the cell in cystitic and TCC tissues versus normal tissues from urinary bladders of dogs may reflect different functions of survivin on the basis of its location. Studies in human tissues have revealed that nuclear survivin generally promotes cell proliferation, whereas cytoplasmic survivin is involved in cell survival and maintenance. In addition, because survivin localizes to the mitotic spindle in the G2/M phase of cell division, nuclear survivin is believed to regulate the cell cycle and cell proliferation, whereas cytoplasmic survivin is hypothesized to regulate apoptosis. Therefore, cytoplasmic survivin in canine bladder tissues may function to maintain cell survival in normal bladder urothelium, whereas nuclear survivin in hyperplastic uri-
nary bladder and urinary bladders with TCC may facilitate cell proliferation.

Another explanation for subcellular localization of survivin in the study reported here is the possible existence of survivin splice variants in dogs. A few survivin splice variants that encode for slightly different proteins with different cellular localization sites and functions have been described in humans. Survivin-ΔEx3 localizes to the nucleus and has antiapoptotic properties, whereas survivin-2B localizes to the cytoplasm and has reduced antiapoptotic properties, compared with properties of survivin-ΔEx3. Additionally, survivin-ΔEx-3 is associated with a higher rate of lung tumor recurrence in humans, compared with the rate associated with survivin-2B. The existence of survivin splice variants may account for differential expression patterns in various types of canine bladder tissues; however, additional evaluation is necessary to determine whether distinct survivin isoforms are associated with various subcellular localization patterns in dogs.

The detection of nuclear survivin in tissues from urinary bladder of dogs with cystitis or TCC supports the supposition that hyperplasia or inflammation of the urinary bladder can lead to malignant transformation of bladder tissues in dogs. Investigators in other studies have proposed that inflammation plays a role in the development of bladder tumors in humans. To our knowledge, the existence of survivin in human cystic tissues has not been investigated, but survivin has been detected in several types of preneoplastic lesions in humans such as breast adenomas, oral epithelial dysplasias, actinic keratoses, colorectal adenomas, and cervical intraepithelial lesions. Expression of survivin is upregulated in human keratinocytes and mouse skin exposed to UV-B radiation, which is a risk factor for skin cancer. In a study in humans, survivin was detected in only 33% of precancerous oral lesions that did not have malignant transformation but was detected in 94% of oral lesions that progressed to squamous cell carcinoma. When normal, inflammatory, and malignant pleural lesions in human tissues are compared, survivin expression appears to increase with the severity of disease, and inflammatory cytokines such as granulocyte-macrophage colony-stimulating factor increase survivin expression in human tissues. These findings indicate that survivin may be a mechanism for development of tumors in hyperplastic or inflamed tissues. Additional research is necessary to determine whether cystitis may be a precancerous lesion in dogs and whether survivin may be a target for antitumor therapy.

The differences between TCC and cystic tissues with respect to nuclear and cytoplasmic survivin immunoreactivity were not significant in the study reported here. This lack of significance may have been attributable to a small number of specimens evaluated (particularly in the cystitis group), as reflected by low powers of the statistical tests. In addition, TCC may have induced secondary cystitis, and therefore immunoreactivity to survivin may have been attributable to concurrent inflammation in TCC tissue specimens. In our study, it was not possible to histologically evaluate all specimens for degree of inflammation because many tumor specimens were too small. In addition, because this was a retrospective analysis, we could not evaluate changes in the degree of survivin expression as cystitis progressed to TCC. Additional studies are needed to determine the degree of survivin expression in urinary bladder tissues to determine whether cystic tissues have lower degrees of survivin expression than do TCC tissues.

Detection of survivin mRNA in 4 of 7 cystitic tissue specimens, 6 of 46 TCC tissue specimens, and 11 of 22 normal tissue specimens confirmed survivin expression in these tissues. Although no differences were detected among the samples, the powers of the tests were low because of small sample sizes.

Detection of survivin mRNA corresponded with detection of nuclear survivin in 3 cystic tissue specimens and 2 TCC tissue specimens. One cystic tissue specimen was negative for survivin mRNA, nuclear survivin, and cytoplasmic survivin. However, normal tissue specimens that were positive for survivin mRNA were negative for cytoplasmic and nuclear survivin. The concentration of survivin mRNA in cystic and TCC tissue specimens may have been high enough to be translated into detectable protein; however, that concentration may not have been high enough in normal tissue specimens, or existing concentrations of survivin protein may have been too low for detection. Because the objective of this study was to identify survivin expression in cystic tissues, we did not perform quantitative analysis of mRNA.

The results of our study indicated that survivin existed in tissues from the urinary bladders of dogs with cystitis. Specifically, nuclear survivin was detected in cystic and TCC tissues, but not in normal tissues from urinary bladders. In light of rapidly growing research that links inflammation and carcinogenesis, it is possible that survivin may be another mechanism—in addition to or independent of cytokines—for the development of bladder cancer. Other studies are needed to determine whether cell proliferation is higher in tissues with higher ratios of nuclear to cytoplasmic survivin. If so, then nuclear survivin may be an early marker for bladder tumors or a potential target for treating dogs with chronic cystitis or urinary bladder tumors.

References


a. NOVUS Biologicals Inc, Littleton, Colo.


