Computer-assisted image analysis of neovascularization in thyroid neoplasms from dogs

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Objective—To develop a computer-assisted image analysis procedure for quantitation of neovascularization in formalin-fixed paraffin-embedded specimens of thyroid gland tissue from dogs with and without thyroid gland neoplasia.

Sample Population—47 thyroid gland carcinomas, 8 thyroid gland adenomas, and 8 specimens of thyroid tissue from dogs without thyroid gland abnormalities (normal).

Procedure—Serial tissue sections were prepared and stained with antibodies against human CD31 or factor VIII-related antigen (factor VIII-rag). The areas of highest vascularity were identified in CD31-stained sections, and corresponding areas were then identified in factor VIII-rag-stained sections. Image analysis was used to calculate the total vascular density in each section, and neovascularization, expressed as a percentage, was determined as the absolute value of the total vascular density derived from factor VIII-rag-stained sections minus the vascular density derived from CD31-stained sections.

Results—Mean vascular density of thyroid gland carcinomas derived from CD31-stained sections was significantly greater than density derived from factor VIII-rag-stained sections. This incremental difference was presumed to represent degree of neovascularization. However, significant differences were not detected between vascular densities derived from CD31 and factor VIII-rag-stained sections for either normal thyroid gland tissue or thyroid gland adenomas. No significant correlations were found between vascular density in thyroid gland carcinomas and survival time following surgery.

Conclusion and Clinical Relevance—A computer-assisted image analysis method was developed for quantifying neovascularization in thyroid gland tumors of dogs. This method may allow identification of dogs with tumors that are most likely to respond to treatment with novel antiangiogenesis agents. (Am J Vet Res 2002;63:363–369)

Neovascularization, or angiogenesis, is the formation of new blood vessels from existing vasculature. Neovascularization occurs during wound healing, embryogenesis, and nonneoplastic conditions such as diabetic retinopathy, arthritis, and macular degeneration. It is also required for tumor growth and metastasis. Neovascularization is implicated in the pathogenesis of diffuse toxic goiter (ie, Graves’ disease) and thyroiditis, as well as neoplasia of the thyroid gland.1,2

The process of angiogenesis is complex and depends on antagonistic activities of promoters and inhibitors. Tumor cell mitoses are generally found within 130 µm of vessels.3 Promoters of neovascularization include vascular endothelial growth factor, fibroblast growth factors, tumor necrosis factor α, transforming growth factor, thymidine phosphorylase, and platelet-derived growth factor. Inhibitors include angiostatin, endostatin, interferons α, β, and γ, interleukins 1 and 12, and platelet factor 4.4 Many of these factors are actually produced by the activated endothelial cells themselves.5 Other factors secreted by activated endothelial cells result in degradation of the extracellular matrix, allowing local ingrowth of the neovessels and tumor cells.

Tumor vasculature differs from that of nonneoplastic tissues in both morphologic and biochemical characteristics. Because a tumor is largely new tissue, a dominating feature of tumor growth is angiogenesis. Certain biochemical markers of angiogenesis that are shared between intratumoral blood vessels and regenerating vessels are not found in resting blood vessels. However, intratumoral blood vessels are typically tortuous rather than straight.6

Cell markers used to identify endothelial cells by use of immunohistochemistry include factor VIII-related antigen (factor VIII-rag), CD31, and CD34. Antibodies to these markers are available for use on formalin-fixed paraffin-embedded tissues. Intratumoral vessels can be quantitated in several ways after immunostaining. Direct counting of blood vessels by use of light microscopy is a common technique, but this method is limited by intra- and interobserver variability. Moreover, it is possible to repeatedly count a single tortuous vessel as it enters and exits the tissue section at multiple sites.7 Image analysis of immunostained tissue specimens is another method used to quantitate intratumoral vessels. Image analysis allows more objective quantitation of microvessel density, vessel perimeter, and endothelial area than direct counting.7

The role of angiogenesis in human breast cancer has been widely studied. Intratumoral vessel density is...
predictive of patient outcome or response to treatment, metastatic likelihood, and overall survival time.

In humans, intratumoral vessel density is also predictive of patient outcome in ovarian carcinoma, colorectal carcinoma, squamous cell carcinoma of the head and neck, non–small-cell lung cancer, and prostate cancer. We are aware of only 5 studies in veterinary medicine in which immunohistochemical methods were used to examine and quantify angiogenesis in tumors of companion animals. However, none of these studies distinguished neovessels from established vessels. Three studies focused on canine mammary tumors, focused on osteogenic sarcoma, and focused on transmissible venereal tumor. Results of these reports revealed significant correlations between angiogenesis and either patient outcome following treatment or metastatic probability.

The objectives of the study reported here were to develop a quantitative immunohistochemical procedure to identify neovascularization and to use that method to compare vascularity among thyroid carcinomas, thyroid adenomas, and normal thyroid tissue of dogs. By examining serial sections of specimens with an antibody that binds to a marker on established vessels (ie, factor VIII-rag) and another antibody that binds to markers on both established vessels and neovessels (ie, CD31), we could then use subtraction to estimate the degree of neovascularization.

**Materials and Methods**

Tissue specimens—Tissues were obtained from the pathology collection of the Veterinary Medical Teaching Hospital at the University of California, Davis. Only surgical biopsy specimens of the thyroid gland that were collected between 1983 and 1989 were evaluated, because needle biopsy specimens were deemed too small for study. In addition, we only used specimens from dogs that had received no other treatment prior to surgery. Normal thyroid tissue was obtained from specimens collected during necropsy of dogs that died or were euthanatized during the same time period for reasons unrelated to the thyroid gland. Eight thyroid gland adenomas, 47 thyroid gland carcinomas, and 8 normal thyroid gland tissue specimens were examined. Age, sex, and breed of dog, treatment outcome, and tumor volume were obtained from the medical records. When treatment outcome was not available from the primary record, the local veterinarian or owner was contacted to obtain that information.

Histologic evaluation and immunohistochemistry—Tissue specimens had been fixed in neutral-buffered 10% formalin and routinely processed to paraffin wax. New sections of tissue specimens had been fixed in neutral-buffered 10% formalin and examined microscopically to confirm the diagnosis. Thyroid gland carcinomas were further characterized histologically as follicular, solid, mixed, or papillary. For immunohistochemistry, 4 serial sections (+4-μm thick) were cut from paraffin blocks and mounted on positively charged slides. The first section was stained for the CD31 antigen, the second for factor VIII-rag, and the third and fourth slides were negative controls for CD31 and factor VIII-rag staining, respectively.

Factor VIII-rag immunohistochemistry was performed, using an autostainer as described. Briefly, to retrieve antigen, slides were treated with 0.05% pronase for 10 minutes at 37 C and 0.01% trypsin in 0.1% CaCl2 buffer for 20 minutes at 37 C. The primary antibody used was a polyclonal rabbit antibody against human factor VIII-rag diluted 1:500 in PBS solution (PBSS); slides were incubated with the primary antibody for 60 minutes at room temperature (approx 20 C). The secondary antibody was a biotinylated goat anti-rabbit IgG diluted 1:500 in PBSS, and the tertiary reagent was horseradish peroxidase-labeled streptavidin diluted 1:500 in PBSS. Slides were incubated with the secondary antibody and tertiary reagent for 30 minutes each at room temperature. The chromagen was amino-ethyl-carbazole, and the counterstain was Mayer hematoxylin.

The CD31 immunohistochemistry procedure was similar, but several antigen retrieval methods were first tested, including steaming, boiling, and digestion with enzymes. The chromagen was amino-ethyl-carbazole, and the counterstain was Mayer hematoxylin.

**Image analysis—** Image analysis was performed according to a described method. Briefly, tissue sections stained with anti-CD31 antibody were scanned at 200× magnification to select the areas of highest vascularization not within the tumor capsule. From these areas, 3 nonoverlapping fields were identified and captured, using a digital camera connected to a microscope, and saved on a computer in a tagged image file format without compression. These same areas were then identified in the adjacent sections stained with antibody against factor VIII-rag and imaged in the same manner.

A color filter was developed to allow specific detection of the chromagen on CD31- and factor VIII-rag-stained slides. Chromagen was detected on slides of normal thyroid gland tissue specimens, using a computer and image manipulation software. With this software, pixels stained with chromagen were selected, converted to black, and transferred to a white background to create a binary image. The total number of black and white pixels in each image was quantified, using an image analysis program. Vascular density was calculated by dividing the total number of black pixels by the total number of pixels within the image. Total vascular density was the mean of values obtained for 3 images captured from each tissue examined and was expressed as a percentage. Neovascularization, expressed as a percentage, was determined as the absolute value of the total vascular density derived from factor VIII-rag-stained sections minus the vascular density derived from CD31-stained sections.

**Statistical analyses—** Total vascular density was compared among tissue types by use of a 2-sample t-test assuming equal variances. Staining for CD31 in 1 serial section was compared with staining for factor VIII-rag in the adjacent section by use of a paired 2-sample t-test. Relationships between vascular densities derived from CD31- or factor VIII-rag-stained sections and tumor type, capsular invasion, vascular invasion, size of tumor, whether the tumor was freely movable at diagnosis, serum T4 concentration,
duration of abnormal clinical signs before treatment, presence of hemorrhage or necrosis within tissue specimens, vascular density, and survival time were assessed by use of the Pearson χ² statistic for categorical variables or a t-test or ANOVA and correlation analysis for continuous variables. Survival rates were computed by use of the product-limit method. Statistical analyses were performed, using software programs, and for all tests, significance was set at P ≤ 0.05.

Results

The median age of dogs from which normal thyroid gland tissue was obtained was 7 years (mean, 6.9 years; range, 2 to 11 years). The median age of dogs with thyroid gland adenomas was 11 years (mean, 10.9 years; range, 9.75 to 14 years), whereas that of dogs with thyroid gland carcinomas was 10 years (mean, 10.5 years; range, 4 to 17 years). Of dogs with carcinoma, 9 were sexually intact males, 11 were castrated males, 3 were sexually intact females, and 24 were spayed females. Dogs in the carcinoma group comprised 10 Labrador Retrievers, 10 Golden Retrievers, 15 other purebred dogs of various breeds, and 12 mixed-breed dogs.

Vascular invasion was identified histologically in 31 carcinomas, whereas capsular invasion was detected in 40 carcinomas. Tumor volume was determined for 45 of the 47 dogs with thyroid carcinoma. Median volume was 33.75 cm³ (mean, 62 cm³; range, 1.65 to 360 cm³). Ten of 47 dogs were hyperthyroid on the basis of T₄ concentration; results of imaging studies confirmed this diagnosis. Concentration of T decreased to within reference range after excision of the tumor. Eight of 10 hyperthyroid dogs had clinical signs of hyperthy-
roidism, including polyuria, polydipsia, weight loss, and diarrhea, at the time the diagnosis was made.

Complete follow-up information was available for 23 of 47 dogs with thyroid gland carcinoma. Seven of 23 had local tumor recurrence after surgery, and 9 had distant metastases (range, 0 to 1,739 days). Median survival time in this group after surgery was 670 days (range, 92 to 1,739 days). Eight of the 23 dogs received adjuvant treatment after surgery, including radiation or chemotherapy (carboplatin or doxorubicin). Four dogs were still alive when this study was performed and were censored for the purposes of statistical analyses.

Figure 4—Photomicrographs of serial sections of a canine thyroid gland adenoma stained with an anti-human factor VIII-rag antibody (a) or an anti-human CD31 antibody (b). Notice that vascular density is similar between sections. See Figure 3 for key.

Figure 5—Photomicrographs (a and b) and binary digital images (c and d) of serial sections of a canine thyroid gland carcinoma stained with an anti-human factor VIII-rag antibody (a and c) or an anti-human CD31 antibody (b and d). Binary digital images were created by use of computer-assisted image analysis of the photomicrographs. Notice that vascular density (black areas) is greater in sections stained with anti-CD31, indicating a high degree of neovascularization in this tumor. See Figure 3 for key.
Mean total vascular density derived from CD31-stained sections of thyroid gland carcinomas (4.22%) was significantly greater than that of normal thyroid gland tissue (0.98%; P < 0.001) or thyroid gland adenomas (1.53%; P = 0.002). Total vascular density of thyroid adenomas, however, was not significantly (P = 0.053) different from that of normal tissue (Fig 1). Mean total vascular densities derived from factor VIII-rag-stained sections were 2.17, 1.26, and 0.86% for carcinomas, normal tissue, and adenomas, respectively (Fig 2). Total vascular density derived from CD31-stained sections was not significantly different from that derived from factor VIII-rag-stained sections for normal thyroid gland tissue (P = 0.19) or thyroid gland adenomas (P = 0.09; Fig 3 and 4). However, vascular density derived from CD31-stained sections of thyroid gland carcinomas was significantly (P < 0.001) greater, compared with factor VIII-rag-stained sections (Fig 5). Finally, neovascularization in normal thyroid gland tissue was 0.28%, whereas that in thyroid gland adenomas was 0.7% and in carcinomas was 2.05%.

Capsular invasion was inversely correlated with vascular density in CD31-stained sections of thyroid carcinomas (P = 0.018). Mean vascular density of tumors with capsular invasion was 3.86%, whereas that of tumors without capsular invasion was 0.86%. There were no significant associations between capsular invasion and vascular density in factor VIII-rag-stained sections. No other significant associations were found between vascular density derived from either CD31 or factor VIII-rag-stained sections and the other histologic features examined (ie, histologic diagnosis [tumor type], tumor volume, tumor necrosis, or vascular invasion).

Survival times did not correlate with tumor type, capsular invasion, vascular invasion, size of tumor, whether the tumor was freely movable at diagnosis, T4 concentration, duration of abnormal clinical signs before treatment, or the presence of hemorrhage or necrosis in the tissue specimen. There were also no significant correlations between vascular densities derived from CD31- or factor VIII-rag-stained sections and overall survival time.

Discussion

In this study, we applied computer-assisted image analysis to quantitate blood vessel density within formalin-fixed paraffin-embedded thyroid tissues of dogs. Endothelial cells were stained by use of immunohistochemical techniques, and we were able to discriminate neovascularization from established vasculature within thyroid gland carcinomas.

Vascular density of carcinomas derived from sections stained with antibody against CD31, a marker on endothelial cells of neovessels and established vessels, was significantly greater than that of normal thyroid tissue or thyroid gland adenomas. However, there was no difference in vascular density between normal thyroid tissue and thyroid adenomas. These results are consistent with vascular densities reported for many neoplasms and support the concept that neovascularization accompanies the growth of malignant tumors and acquisition of an angiogenic phenotype may be an important step in carcinogenesis.24,25

Direct microscopic examination of CD31 and factor VIII-rag-stained tissue sections revealed that large blood vessels were labeled with both antibodies, and an additional population of small pleomorphic vascular channels was labeled with anti-CD31 antibody. We presumed that these additional stained vessels represented neovessels. This assumption was further supported by the observation that neovessels were not observed in benign tumors (ie, adenomas) or normal thyroid tissues.

Endothelial cells express different antigens at different stages of development and differentiation. For example, intensity of staining for factor VIII-rag increases within blood vessels of the endometrium of humans as the endometrium progresses from early to late proliferative stages during menstruation.26 Further, CD31 is expressed early in vascular development; monoclonal antibodies directed against CD31 inhibit endothelial tube formation in vitro.27 The expression of CD31 on endothelial cells of immature vascular structures explains the labeling of neovessels that we observed in the present study.

In another study,38 staining of formalin-fixed paraffin-embedded normal and neoplastic human tissue specimens with both anti-factor VIII-rag and CD31 antibodies revealed uniform staining within healthy tissues and benign tumors but heterogeneity within malignant tumors stained with anti-factor VIII-rag antibody. Factor VIII-rag-stained sections of malignant tumors (ie, carcinomas) also revealed greater heterogeneity in both staining intensity and distribution, compared with adjacent CD31-stained sections. The heterogeneous staining identified in factor VIII-rag-stained sections was presumed to be related to neovessels within malignant tumors that were not expressing factor VIII-rag.

Although there are numerous studies describing the relationship between vascular density of tumors and malignancy in humans, there have been few similar studies in companion animals. The results of this study indicate that angiogenesis correlates with a malignant phenotype in the thyroid gland of dogs, and that image analysis of adjacent sections stained with anti-CD31 and factor VIII-rag antibodies allowed discrimination of established blood vessels from neovessels. These findings suggest that neovessels within thyroid gland carcinomas of dogs may serve as targets for anticancer treatment strategies. Although we anticipated that high vascular density would also correlate directly with other clinical or histologic characteristics of malignancy (eg, tumor volume, tumor capsular invasion, tumor vascular invasion) or prognosis following treatment, those associations were not evident from the data examined here. In 1 study of human papillary thyroid gland carcinomas, vascular density did not serve as a predictor of patient response after treatment.29 In another study, vascular density was only predictive of outcome for medullary carcinomas (C-cell origin) but not for tumors of other histologic classifications.30

Perhaps examination of a larger number of thyroid gland carcinomas would have allowed us to detect significant associations between tumor vascular density and other features of malignancy. In humans with thyroid gland carcinomas, poor prognostic indicators
include increased age, tumor volumes > 4 cm, extravascular extension of tumor, distant metastases, nodal involvement, and certain histologic tumor variants.32-34 We are aware of only 1 study7 in veterinary medicine that linked histologic features (ie, capsular and vascular invasion by the tumor) to survival times in dogs. Certainly there is room for more detailed investigations of prognostic factors for thyroid gland tumors in dogs.

In the present study, median survival time (670 days) of dogs with thyroid gland carcinomas that were treated was greater than survival times previously described for dogs with thyroid gland carcinomas.34-37 Ten of 31 dogs tested were hyperthyroid, although no significant differences were found in survival times between hyper- and euthyroid dogs. Two dogs with C-cell tumors of the thyroid gland had survival times of only 132 and 144 days, which was brief, compared with the favorable outcome reported elsewhere for dogs with C-cell thyroid gland tumors that were treated surgically.35

In this study, we described the use of static images to define neovascularization in thyroid gland tumors of dogs, using specimens from pathology archives. Interest in microscopic methods to assess angiogenesis is underscored by the observation that one commercial vendor35 provides histologic laboratory reagents for 70 markers under the category angiogenesis. For prospective studies, rapid changes in technology now allow direct examination of microvasculature in real time by use of magnetic resonance imaging, positron emitting tomography, video microscopy, nuclear scintigraphy, and Doppler ultrasonography.36-38

References


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