Identification and correlation of cytologic criteria to histologic grade in canine cutaneous mast cell tumors

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Identification and correlation of cytologic criteria to histologic grade in canine cutaneous mast cell tumors

by

Jo Ann Morrison

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Veterinary Clinical Sciences

Program of Study Committee:
Albert E. Jergens, Major Professor
Claire Andreasen
Mary Ann Nieves

Iowa State University
Ames, Iowa
2007

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DEDICATION

There have been innumerable people who have been instrumental in my professional and personal life: family, friends, colleagues and mentors. This page is too short and their accomplishments too vast to be documented here. However, they have my utmost admiration, respect, gratitude, and thanks. You know who you are. And to Sam, my Rhodesian Ridgeback, who lost his own battle with mast cell disease. It was not for any lack of heart or courage and I still miss you every day.
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CHAPTER 1. INTRODUCTION

Mast cell tumors are the most common cutaneous tumors recognized in dogs, and are the second most common cutaneous tumors identified in cats. Tumors may be limited to the skin and subcutaneous tissue, may metastasize to other sites, and may cause systemic, fatal disease. Several factors have been identified as prognostic indicators in animals with cutaneous mast cell tumors: location of the tumor on the body, breed of the animal, stage of disease, presence or absence of systemic signs, and histologic grade of the tumor. However, numerous studies have conflicting results which makes mast cell disease characterization and determination of prognosis problematic. This remains a significant challenge in the clinical setting.

Cytology, literally the study of cells, has long been recognized as a valuable component of the diagnostic evaluation of both internal and external lesions in humans and animals. Specimen evaluation via cytology has several, well recognized, unique advantages in the clinical arena. Cytologic specimens are relatively easy to obtain and diagnostic samples may be obtained by several methods. Needle fenestration and fine needle aspiration are the most commonly employed methods to obtain specimens for cytologic analysis from cutaneous lesions. These techniques are minimally invasive and associated with little to no patient morbidity, in contrast to surgical excision. These techniques are also easy to perform and readily available to veterinary practitioners. Cytologic evaluation is relatively inexpensive, requires no specialized equipment, and results are rapidly available via interpretation by a cytopathologist. Lastly, the information that can be obtained through cytology can facilitate future treatment planning.
and may be crucial to patient outcome. While cytology has been traditionally utilized for making a tentative diagnosis of mast cell tumors, the potential correlation of cytologic characteristics to histologic grade has not been fully investigated.

Historically, histologic grade has been considered one of the most valuable factors, if not the gold standard, in determining prognosis for mast cell tumors. The most commonly utilized histologic grading scheme was introduced by Patnaik et al in 1984.¹ This histologic scheme assigned mast cell tumors to one of three biologic grades (I, II, or III), based on defined morphologic criteria. The Patnaik¹ study also showed that prognosis worsened with increasing histologic grade. Treatment protocols were limited to surgical excision. In this original study of 83 dogs, the percentage of animals with grade I tumors surviving 1500 days was 83%; with grade II disease, the 1500 day survival percentage was 44% and with grade III disease, the 1500 day survival percentage was only 6%. Thus, increasing histologic grade was inversely correlated with survival time.

Present therapeutic options for cutaneous mast cell tumors involve surgical excision, chemotherapy, and radiation therapy, or a combination of treatment modalities. The histologic grade of a mast cell tumor plays a significant role in treatment decisions, as a more aggressive histologic grade warrants a more aggressive therapeutic plan. This was clearly demonstrated in the Patnaik study¹ where survival times significantly worsened with increasing histologic grade. However, to obtain a histologic examination of a tumor, the tumor must be excised and submitted to the pathology laboratory for interpretation. This requires surgical planning, general anesthesia, and post-surgical hospitalization. The tissue must then be submitted for analysis, and histopathology
results are not as readily available compared to results obtained from cytology. Once the
tissue biopsy review is completed, it may be determined that additional, more invasive
therapies are required for more aggressive neoplasms, which may increase patient
morbidity. This becomes an even larger consideration in those animals with recurrent
disease or multiple mast cell tumors as these animals may undergo more morbidity than
the patient with a single mass.

Procurement of a cytologic specimen is a significantly less invasive and
expensive procedure when compared to surgical excision for mast cell disease. If
cytology could be shown to correlate to histologic grade, this could provide to an
attractive option to veterinarians and clients. Cytology could, theoretically, be utilized
for surgical planning and determining the need for clinical staging prior to surgery. For
example, if cytologic evaluation indicated a more benign tumor process, then a less
aggressive surgical procedure with no additional staging might be warranted. This could
mean decreased patient morbidity, decreased hospitalization time and complication rates,
and potentially decreased client costs. Conversely, if characteristics associated with more
aggressive disease were identified on initial cytology, then extensive surgical resection,
complete clinical staging, or more aggressive adjuvant therapy might be indicated to
induce clinical remission.

A search of the literature demonstrated numerous contradictory findings regarding
mast cell disease, especially related to prognosis. These conflicting results translate into
variability in clinical staging, treatment, and use of adjunctive therapy. The literature
review section is designed to highlight these contradictions and is organized based on the
primary prognostic factor evaluated. These sections are labeled as: histologic grade,
clinical stage, treatment, tumor location, systemic signs, and breed. Some studies have evaluated additional aspects of mast cell disease and these are listed in the literature review as: cellular proliferation indices, Grade II mast cell tumors, cellular proliferation indices and histologic grade, nuclear morphometry, and cytologic correlation with histopathology. Lastly, there is a brief mention of the feline aspects of mast cell disease.

The aim of the present study was to identify cytologic criteria of mast cell tumors that correlate to their histologic grade using the Patnaik\textsuperscript{1} scale. The potential significance of this correlation could be demonstrated clinically in reduced patient morbidity and improved clinical management. To aid in understanding for the reader, several terms used commonly during oncology discussions, and utilized in this thesis are briefly defined below.

Term Definitions:*

**Cytology**: the microscopic study of cells.

**Cytospin**: a method for evaluation of cytology samples whereby samples are placed in a centrifugation chamber and cells are concentrated on a glass slide.

**Disease free interval**: time from initial treatment of tumor to relapse or recurrence of tumor.

**Fine needle aspiration**: one of the methods to obtain a cytologic sample where a needle with attached syringe is advanced into the target tissue. While the tip of the needle is within the tissue, negative pressure is produced in the needle and syringe and cells are drawn into the needle. Cells are then expelled from the needle hub onto a glass slide for analysis.

**Histology**: the anatomic evaluation of microscopic tissue structure and composition.
**Histopathology:** the histologic evaluation of diseased tissue.

**Immunohistochemistry:** the process of identifying particular cellular antigens via specific antibody markers, often used in the diagnosis of cancer.

**Lesion:** any abnormality involving any tissue due to disease or injury.

**Mast cell:** a granulated, connective tissue cell that is responsible, in part, for mediation of inflammatory reactions. Granules contain and may release substances such as histamine, heparin, and proteolytic enzymes. A mast cell may be a normal component of tissue; an infiltration of mast cells in a cutaneous nodule is a mast cell tumor.

**Mitosis:** the process of cellular division.

**Needle fenestration:** a method to obtain a cytologic sample where a needle is directed into the target tissue and advanced in several different directions while still contained within the tissue. Cells that have collected in the needle are then expelled onto a glass slide for analysis.

**Pathology:** the study of disease.

**Survival time:** time from treatment of tumor to eventual death of the animal (from tumor or from any cause).

**Tumor:** a new growth of abnormal tissue which may be benign or malignant.

*Definitions were obtained / modified from Dorland’s Illustrated Medical Dictionary, 27th edition, 1988, W.B. Saunders Company, Philadelphia.*
LITERATURE REVIEW

Histologic grade

The seminal work that describes the histologic grading system used to this day for the diagnosis of mast cell tumors was done by Patnaik, Ehler, and MacEwen (1984).\textsuperscript{1} In this study, a new histologic grading system was developed that was notably different from the system, described by Bostock (1973)\textsuperscript{2} that had been used previously. The most notable difference was in the assignment of tumor grades, in that the Patnaik\textsuperscript{1} system completely reversed the grading scale for mast cell tumors. The current system for defining mast cell tumors describes three histologic grades of tumors. Grade I tumors are well differentiated, low grade tumors that contain a uniform population of mast cells. There is minimal extension of tumor cells beyond the skin and little evidence of edema or necrosis. Mitotic cells are not present in grade I tumors. Grade II tumors have intermediate differentiation. Mast cells are more variable in size and shape, extend somewhat deeper into the tissue, and may have associated edema and necrosis. Mitotic cells may be present, but are rare. Grade III tumors contain the most undifferentiated mast cells. These tumors extend deeply into surrounding tissues and commonly are associated with edema, necrosis, and hemorrhage. Mitotic cells are commonly observed in grade III mast cell tumors. In addition to defining histologic criteria of different tumor grades, the Patnaik\textsuperscript{1} study also demonstrated distinct differences in 1500 day survival times between the three histologic grades of mast cell tumors. This was one of the earliest studies that demonstrated the inverse correlation between histologic grade and long-term prognosis. The correlation of histologic grade to prognosis was also
investigated by Thamm, Mauldin, and Vail (1999) in a study of 41 cases of canine mast cell tumors. Similar to Patnaik, histologic grade was shown to be a significant prognostic factor in both univariate (p < 0.05) and multivariate (p < 0.001) analysis of the dogs studied. In another investigation, Turrel, Kitchell, and Miller et al (1988) evaluated prognostic factors for mast cell tumors in 85 dogs, and showed that histologic grade significantly influenced survival rate (p = 0.006). While histologic examination of biopsy specimens continues to be an extremely important determinant for mast cell tumor prognosis, there may be considerable variability in the interpretation of the histologic examination. For example, Northrup, Harmon, and Gieger et al (2005) showed significant interobserver variability between pathologists when they independently reviewed a set of 60 mast cell tumors. In this study, there was significant (p < 0.001) variation among 10 pathologists in assigning the same histologic grade to mast cell tumors. Indeed, all 10 pathologists at one institution agreed on the histologic grade for only 4 / 60 tumors, and 6 tumors were assigned all 3 grades by different pathologists. While the value of histology as a prognostic indicator has been shown, it would appear that agreement on histologic grade may be somewhat open to interpretation. Thus, it may be difficult to provide an accurate prognosis for all mast cell tumors on the basis of histologic grade alone.

Clinical stage

Clinical stage, which may be described as the extension of disease to involve regional lymph nodes or other metastatic sites, has also been documented to be an important prognostic indicator. A clinical staging system, defined by the World Health
Organization, is frequently utilized, in various modifications, in mast cell tumor
evaluation. This staging system is summarized in Table 1.

Table 1. World Health Organization Clinical Staging System.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Clinical Description</th>
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<tbody>
<tr>
<td>0</td>
<td>One dermal tumor, incompletely excised, without regional lymph node involvement</td>
</tr>
<tr>
<td>1</td>
<td>One dermal tumor, without regional lymph node involvement</td>
</tr>
<tr>
<td>2</td>
<td>One dermal tumor, with regional lymph node involvement</td>
</tr>
<tr>
<td>3</td>
<td>Multiple dermal tumors or a large, infiltrating tumor, with or without regional</td>
</tr>
<tr>
<td></td>
<td>lymph node involvement</td>
</tr>
<tr>
<td>4</td>
<td>Any tumor with distant metastasis, including blood or bone marrow involvement,</td>
</tr>
<tr>
<td></td>
<td>or tumor recurrence with metastasis</td>
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Turrel, Kitchell, and Miller et al (1988) performed one of the earliest studies that
investigated prognostic factors associated with clinical disease of mast cell tumors. In a
study of 85 dogs, clinical stage was found to be negatively correlated to median survival
time. Dogs with clinical stage 0 were shown to live significantly longer than dogs having
a more advanced clinical stage (p < 0.001). In a contrasting study, Chaffin and Thrall
(2002) found that dogs with stage 2 mast cell disease had similar median survival times
(1240 days) when compared to the median survival times of dogs with stage 0 disease
(1200 days) previously reported by Turrel. It is important to realize that differences in
results obtained by these separate studies could in part be explained by differing staging
and treatment protocols used by these authors. There is still controversy regarding the
optimal procedures that are appropriate for clinical staging (example, bone marrow
aspiration versus buffy coat evaluation of peripheral blood, or fine needle aspiration of
the spleen and liver) in animals with cutaneous mast cell disease. A study by Finora,
Leibman, and Fettman et al (2006)\(^8\) compared fine needle aspirates of liver and splenic tissues of normal dogs to dogs with cutaneous mast cell tumors. Healthy dogs were shown to have mast cells in both liver and splenic aspirates, similar to diseased dogs, thus, bringing the value of these protocols used for clinical staging into question. Additionally, McManus (1999)\(^9\) evaluated peripheral blood and buffy coat smears for mast cells in dogs with and without mast cell tumors in a study to further investigate the prevalence of systemic mast cell disease. Of the 120 cases that had mast cells in either a peripheral blood smear, buffy coat smear, or both, 94 dogs did not have mast cell tumors. This translated into 95.5% of peripheral blood smears that contained mast cells were obtained from dogs that did not have mast cell disease. Also, when considering buffy coat smears, dogs that did have mast cell tumors had an average of 71.4 mast cells / buffy coat while dogs that did not have mast cell tumors had an average of 276.2 mast cells / buffy coat. This study showed that mastocytemia is seen in conjunction with other disease states (e.g., inflammatory conditions, anemia). Clearly, there is a lack of uniform staging criteria that confounds the clinical evaluation of animals with mast cell disease. Thus comparisons between studies where clinical staging has been performed are problematic.

**Treatment**

Concurrent with the difficulty in uniform clinical evaluation is the non-standardized treatment protocols employed for animals with mast cell tumors. It is noteworthy that therapy recommendations may change over time, so animals with relatively similar disease burdens (clinical stage) may receive very different treatments.
and hence have disparate survival times. This makes evidence based determination of clinical management strategy problematic. Historically, surgery has been considered the mainstay of treatment and wide surgical excision has long been recommended for cutaneous mast cell tumors. Interestingly, the common, historical recommendation of a 3 centimeter surgical margin around the tumor does not appear to have any basis in the veterinary literature. A study by Simpson, Ludwig, and Newman et al (2004)\textsuperscript{10} prospectively evaluated 21 dogs histologically diagnosed with 23 cutaneous grade I and grade II mast cell tumors. Tissue was marked at 1, 2, and 3 centimeter margins from the tumor and evaluated for completeness of surgical excision. All grade I tumors were shown to be completely excised at all margins. Of the grade II tumors, 75\% were completely excised at the 1 centimeter margin and 100\% were completely excised at the 2 centimeter margin. Thus, pre-operative knowledge of tumor histologic grade has the potential to impact surgical planning and patient morbidity. Other investigations have shown that the adequacy of surgical excision may be open to interpretation. In another study, Weisse, Shofer, and Sorenmo (2002)\textsuperscript{11} defined a “clean” (disease-free) surgical margin as a 1 to 2 millimeter distance from tumor cells to normal tissue edge in 4 lateral margins. However, Gieger, Northrup, and Wall (2005)\textsuperscript{12} defined a clean margin as requiring at least 10 millimeters distance between tumor cells and normal tissue. The interpretation of the surgical margin and other factors such as histologic grade of the tumor, influence the decision for additional, or adjunctive therapy. Measures such as survival time and disease free interval may be significantly influenced by treatment modality. Treatment decisions in veterinary patients are also influenced by the availability of treatment modalities (radiation therapy units, chemotherapy
administration) and the associated costs of implementing such therapies. The variability in treatment protocols reported in the literature, as for clinical staging, makes comparisons of treatment efficacy and prognosis between studies difficult. This emphasizes the need to identify independent prognostic indicators that are minimally influenced by variables such as treatment.

**Tumor location**

Tumor location has also been reported to influence prognosis. Gieger, Theon, and Werner et al (2003)\(^\text{13}\) retrospectively evaluated 24 mast cell tumors on the muzzle of dogs. In this study, a larger percentage of tumors from the muzzle were histologically classified as grade III (29%) as compared to mast cell tumors localized to other anatomic locations. Dogs with grade III tumors had significantly shorter survival times (p = 0.0027) than did dogs with tumors classified as grade I or grade II, as would be expected with a more biologically aggressive form of the disease. Similarly, Turrel, Kitchell, and Miller et al (1988)\(^\text{4}\) showed that tumor location was an important prognostic variable, with tumors on the extremities having significantly longer survival times (p < 0.004) than tumors located on the trunk. However, a study by Sfiligoi, Rassnick, and Scarlett et al (2005)\(^\text{14}\) showed no significant difference in survival times or disease free intervals in 124 dogs with mast cell tumors in the inguinal / perineal regions compared to dogs with mast cell tumors in other sites.
**Systemic signs**

The presence or absence of systemic signs of mast cell disease is another area that has been associated with survival. The ability of mast cell tumors to cause systemic signs relates to the contents of the mast cell granules involving histamine, heparin and proteolytic enzymes. Release of these substances may result in gastroduodenal ulceration and perforation, which may be a significant cause of morbidity and mortality. Other systemic signs of mast cell disease include delayed wound healing, tissue ulceration and edema, hypotension, and coagulation abnormalities. A study by Mullins, Dernell, and Withrow et al (2006)\(^\text{15}\) of 54 cases of cutaneous mast cell tumors revealed that the presence of systemic signs at the time of diagnosis was the only variable associated with significantly shorter disease free intervals (p = 0.023). Other variables that were evaluated in this study, including incomplete tumor excision, local recurrence, and large tumor size were not found to be significantly associated with disease outcome. O’Keefe, Couto, and Burke-Schwartz et al (1987)\(^\text{16}\) also described systemic signs in 16 dogs with mast cell tumors. Eighty eight percent of the dogs in this study died or were euthanized as a result of their clinical disease. Common clinical signs in these dogs included anorexia, vomiting, diarrhea, edema, ulceration, and abscessation. The dogs in that study also had a higher percentage of grade III tumors (77%), compared to dogs in other studies. These accumulated data provide consistent evidence that grade III tumors have a more aggressive biologic behavior; thus it is not surprising that animals with grade III tumors might have more systemic signs.

One of the proposed mechanisms for the development of systemic signs of mast cell disease is the release of histamine from mast cell granules causing a generalized
increase in plasma histamine levels. Fox, Rosenthal, and Twedt et al (1990)\textsuperscript{17} measured histamine levels in dogs with mast cell tumors and in normal dogs. These authors found that tumor bearing dogs had significantly higher levels of histamine as compared to healthy controls. However, plasma histamine levels in dogs with mast cell tumors did not correlate to clinical disease stage, tumor volume, or histologic grade of the tumor in this study. Furthermore, no relationship could be detected between plasma histamine levels and disease progression. In contrast, a study by Ishiguro, Kadosawa, and Takagi et al (2003)\textsuperscript{18} showed a positive association between plasma histamine levels and disease progression in dogs with mast cell tumors. In this small study of 11 dogs, there was no difference noted in initial plasma histamine concentration between affected dogs and normal controls. However, significant increases in plasma histamine levels in animals with mast cell tumors were noted with clinical disease progression (p = 0.003). Lastly, dogs with terminal disease were noted to have the most significant elevations in plasma histamine, and these animals were refractory to anti-histamine therapy.

**Breed**

Certain breeds of dogs have been shown to have a higher incidence of mast cell tumors suggesting breed predispositions for the development of disease. In Patnaik’s study,\textsuperscript{1} Boxers showed an increased prevalence, representing 14\% of the total cases. The most common pure breeds affected in another study\textsuperscript{15} were the Labrador retriever (20\%) and the Boxer (11\%). It is generally accepted that brachycephalic breeds (Boxers, Boston terriers) have an increased incidence of mast cell tumors. Shar-Peis have also been shown to have an increased incidence of mast cell tumors as reported by Miller
In this study, Chinese Shar Peis were the most common breed, less than 2 years of age, diagnosed with mast cell tumors. Similarly, grade III tumors diagnosed in young dogs less than 2 years of age, were most prevalent in Shar-Peis. Despite these observations, it is still controversial as to what effect breed predisposition might have on prognosis.

**Cellular proliferation indices**

Most recently, other factors correlating to prognosis have been identified in an attempt to avoid the limitations and contradictory findings evidenced in previous studies. For example, silver nucleolar organizing regions (AgNOR), proliferating cell nuclear antigen (PCNA), and Ki-67 are all indicators of cellular proliferation and have prognostic significance. Another factor, c-kit, is a proto-oncogene that has also been evaluated as a prognostic variable. The increased expression of these factors corresponds to increased cellular activity, and presumably, more aggressive biologic behavior. The expression of these factors in tumor tissue, identified by immunohistochemical staining on histopathology samples, has been shown to correlate with shortened survival times and worsening prognosis in different studies. Bostock, Crocker, and Harris et al (1989) showed significant correlations between AgNOR scores, histologic grade, and disease outcome in a study of 50 mast cell tumors in dogs. Nucleolar organizer regions (NOR) act as sites for transcription of rRNA (ribosomal RNA, which is involved in the process of protein synthesis). As cellular proliferation increases, the cell cycle shortens and RNA synthesis increases. In this study, as histologic grade increased, AgNOR expression also increased and increased AgNOR expression correlated to shortened survival time.
Simoes, Schoning, and Butine (1994) also evaluated AgNOR counts and PCNA labeling, via immunohistochemistry, along with histologic grade and mitotic index in 122 canine mast cell tumors. Proliferating cell nuclear antigen (PCNA) is a protein required for DNA synthesis and is associated with cellular growth fraction. Their results demonstrated that AgNOR and PCNA staining was significantly increased in recurrent versus non-recurrent mast cell disease and also in metastatic versus non-metastatic mast cell disease. In a separate investigation, Preziosi, Morini, and Sarli (2004) investigated the expression of c-kit in 31 cutaneous mast cell tumors in dogs. The c-kit is a proto-oncogene that encodes for a type III tyrosine kinase protein (KIT). This protein functions as a receptor for stem cell factor, also known as mast cell growth factor, that stimulates mast cell growth and differentiation. Their study demonstrated a highly significant correlation (p < 0.00000) between c-kit expression and histologic grade. Abadie, Amardeilh, and Delverdier (1999) investigated the expression of Ki-67 in 120 mast cell tumors. The role of Ki-67 is not completely elucidated; however, it is thought to be associated with nucleolar RNA. In this study, Ki-67 expression was shown to be significantly higher in dogs that died of mast cell disease (p < 0.001). The expression of Ki-67 was also significantly different between tumors when tumors were grouped by histologic grade. Ki-67 expression increased as tumor histologic grade increased. Another indicator of potential biologic activity is intratumoral microvessel density (IMVD) which was investigated by Preziosi, Sarli, and Paltrinieri (2004) in an evaluation of 32 cutaneous mast cell tumors. In this study, tumor microvasculature was identified by immunohistochemistry and its density was shown to negatively correlate with survival time (p = 0.046). Another factor that has been evaluated for biologic...
behavior as it relates to prognosis is DNA ploidy status. Ayl, Couto, and Hammer et al (1992) investigated DNA ploidy in 44 canine mast cell tumors. The DNA content of neoplastic cells was measured via flow cytometry and tumors were determined to be either diploid or aneuploid. The rationale for this study was diploid status would indicate relatively consistent DNA content among the nuclei of tumor cells while aneuploid status would indicate more variable DNA content within the nuclei of tumor cells, suggesting biologic aggressiveness. Results from this investigation showed that aneuploid status was more common in animals with advanced mast cell disease.

One advantage that utilizing various cellular proliferation indices provides is that the evaluation of these factors is considered to be more objective than other currently utilized prognostic criteria. While studies are currently ongoing, these factors may prove to be important for overall disease prognosis and may provide an advantage over traditional histopathologic grading. However, their routine use by histopathology laboratories on a widespread basis is yet to be performed.

**Grade II mast cell tumors**

The majority of mast cell tumors are identified as grade II on histologic evaluation. Cahalane, Payne, and Barber et al (2004) reviewed 68 cases of mast cell tumors and found 64 (94%) of tumors were histologically classified as grade II tumors. Sfiligoi, Rassnick, and Scarlett et al (2005) evaluated 124 cases of mast cell tumors. In that study, 82 (66%) of tumors were classified as grade II. Scase, Edwards, and Miller et al (2006) retrospectively analyzed 121 dogs with mast cell disease. Eighty six of the 121 dogs (71%) were classified as having grade II tumors. Even in smaller studies, the
majority of mast cell tumors are identified as grade II tumors. Gerritsen, Teske, and Kraus et al (1998) evaluated 17 dogs with mast cell tumors, and 12 / 17 (71%) were diagnosed with grade II disease. In a study of 22 dogs by Rassnick, Moore, and Williams et al (1999), 10 dogs (53%) were determined to have grade II tumors. Michels, Knapp, and DeNicola et al (2002) retrospectively reviewed 31 cases of mast cell tumors. In that study, 20 / 31 (65%) of tumors received a histologic classification of grade II.

The significance that the majority of mast cell tumors receive a histologic classification of grade II is clear when considering their diverse biologic behavior. Biologic behavior of these tumors has been shown to vary widely with regards to local recurrence rates, development of additional cutaneous disease, and metastatic rates. Weisse, Shofer, and Sorenmo (2002) evaluated 31 dogs with grade II mast cell tumors that had complete surgical excision as determined by histopathology. In that study, the local recurrence rate was 11% and the rate of distant tumor recurrence (regional lymph node, new cutaneous or other sites) was 22%. In a separate study, thirty two dogs with grade II mast cell tumors that had incomplete surgical excision and follow up radiation therapy at the surgical site were evaluated prospectively. Four percent of dogs had local recurrence of mast cell disease and 4% had fatal metastatic mast cell disease.

Additionally, Seguin, Leibman, and Bregazzi et al (2001) retrospectively evaluated fifty five dogs that had complete surgical excision of grade II tumors. These data showed that 5% of tumors recurred locally, 11% developed mast cell tumors at a distant cutaneous location, and another 5% developed metastatic disease. Furthermore, Poirier, Adams, and Forrest et al (2006) retrospectively reviewed 45 cases of grade II tumors having incomplete surgical excision followed by local radiation therapy at the surgical site.
Local tumor recurrence was documented in less than 7 months for 6.7% of cases; metastatic disease developed in 4.4%, and distant cutaneous disease developed in 31%. Taken together, these accumulated studies would seem to demonstrate the variability in biologic behavior and disease progression observed with grade II mast cell tumors. It is therefore reasonable to investigate other factors, beyond histologic grade alone, that may play a significant role in predicting disease progression in animals with grade II mast cell tumors.

Cellular proliferation indices and histologic grade

Applying PCNA, AgNOR, and Ki-67 tissue staining may allow grade II mast cell tumors to be more specifically evaluated. An initial study by Bergman, Monette, and Northrup et al (2005)\textsuperscript{35} designated grade II mast cell tumors into low, medium, or high categories, based on their staining intensity for PCNA, AgNOR, and Ki-67. These authors showed that increased expression of these proliferation markers was positively associated with the development of metastatic disease. In addition, the up-regulated expression of these markers predicted development of metastatic disease better than histologic grade and was negatively associated with survival. Even indirect measures of cellular proliferation may prove useful for delineating grade II mast cell tumors. Romansik, Reilly, and Kass et al (2007)\textsuperscript{36} retrospectively reviewed histopathology samples from 148 mast cell tumors for their mitotic activity. The mitotic index was determined by number of mitoses / 10 high powered fields and the region of the tumor with the highest overall mitotic activity was used for histologic evaluation. The results showed significant relationships between mitotic index and histologic grade, and between
increasing mitotic index and the risk for metastatic disease. Also, mitotic index was shown to be predictive of survival time for animals with grade II mast cell tumors (p < 0.001). Lastly, a study by Seguin, Besancon, and McCallan et al (2006) investigated AgNOR, PCNA, and Ki-67 staining in a review of 30 cutaneous grade II mast cell tumors in dogs. The combination of PCNA and Ki-67 scores were shown to be significant for prediction of local tumor recurrence. The presence of local tumor recurrence was negatively associated with disease survival.

**Nuclear morphometry**

As previously described, obtaining specimens for cytologic evaluation has several advantages over obtaining specimens for histopathology. One salient advantage is the ability to obtain important information about a tumor via this minimally invasive procedure. While cytologic diagnosis of mast cell tumors has been shown to have 100% correlation with the histopathologic diagnosis (Griffiths, Lumsden, and Valli (1984)), there have been few attempts to correlate specific cytologic (nuclear or cytoplasmic) criteria to histopathologic grade. De Vico, Sfacteria, and Maiolino et al (2002) used an automated nuclear morphometry system to compare morphologic characteristics of cytology samples to histopathology samples from different spontaneous tumors in dogs. The automated system used a computerized image analysis system to load cellular images into a digital memory system. The system then allowed cytoplasmic perimeters and nuclear outlines to be traced, and these parameters could be numerically calculated. This study showed that nuclear form factor (a value calculated by measuring nuclear perimeter and nuclear area) was not statistically different (p < 0.01) between the cytologic and
histopathologic samples. In a subsequent study, Strefezzi, Xavier, and Catao-Dias (2003), evaluated 24 cutaneous mast cell tumors with automated nuclear morphometry. Tumors had been previously assigned a histopathologic grade. Parameters such as nuclear area, mean diameter, and perimeter were measured via morphometry and these data showed that those values did increase with increasing severity of histopathologic grade. However, statistical significance was only reached between grade II and grade III tumors, and between grade I and grade III tumors, using specific staining techniques. A similar study was performed by Maiolino, Cataldi, and Paciello et al (2005) on 35 mast cell tumors analyzed via nuclear morphometry. Results were similar to the Strefezzi (2003) study in that some parameters (mean nuclear area and mean nuclear perimeter) were different between mast cell tumors of varying histologic scores. However, these differences were only significant between grade I and grade III tumors and between grade II and grade III tumors.

**Cytologic correlation with histopathology**

Correlation between cytologic markers and histopathology in other tumor types has been evaluated. In human oncology, Khan, Haleem, and Hassani et al (2003) identified 6 distinct cytologic criteria that showed excellent correlation to histopathologic grade in human ductal carcinoma in situ: pleomorphism, nuclear size, nuclear margin, nucleoli, naked tumor nuclei, and mitotic count (p < 0.001 for each criteria). Limited work in the veterinary field has examined the potential application of advanced staining techniques for markers of cellular proliferation in cytologic samples obtained from tumors and lymph nodes. Vajdovich, Psader, and Toth et al (2004) investigated the use
of AgNOR staining in cytology and histopathology of canine lymph nodes in 16 dogs diagnosed with lymphoma versus healthy dogs. Results showed that AgNOR scores in both cytology and histology samples were significantly different (p < 0.001) between control dogs and dogs with lymphoma. Kravis, Vail, and Kisseberth (1996) found a similar linear relationship (p < 0.001) between AgNOR staining of fine needle aspirates and histopathology sections from 32 canine mast cell tumors. They also demonstrated that AgNOR staining of cytology samples correlated to tumor histologic grade.

**Feline disease**

Histologic grade as determined by the Patnaik scale has no influence on survival time in cats with cutaneous mast cell disease. This study by Molander-McCrary, Henry, and Potter et al (1998) examined 32 cases of feline mast cell tumors that were treated surgically. Completeness of surgical excision and histologic grade had no correlation to survival time or recurrence of disease. Johnson, Schulman, and Lipscomb et al (2002) evaluated pleomorphic mast cell tumors in 15 cats. Pleomorphic tumors contained mast cells with widely variable cellular and nuclear diameters. The large majority (79%) of cats had no local recurrence, no additional cutaneous tumors, and no metastasis in the follow up period (range 5-25 months). Feline cutaneous mast cell tumors are considered to have a largely benign course.

**Summary**

In summary, mast cell tumors have a complicated, variable disease progression. While these tumors are commonly diagnosed in veterinary medicine, the prediction of
biologic behavior and the ability to reliably predict their disease progression remain
problematic. The numerous limitations of the previous studies to date make comparisons
between these studies difficult. Histopathology has long been considered the gold
standard for prognosis of canine mast cell tumors but cytologic criteria may be equally
important. The goal of the study described here was to evaluate specific cytologic
morphologic features of mast cell tumors which might correlate to histopathologic grade.
STUDY DESIGN AND MATERIALS AND METHODS

The study design was retrospective in nature, with cases that had a cytologic diagnosis of mast cell tumor identified from computerized searches of Iowa State University medical records and pathology reports in patients evaluated at the College of Veterinary Medicine. All cytology samples included in the study were evaluated at Iowa State University College of Veterinary Medicine from 1995 – 2000. Clinical cases were presented to the teaching hospital and cytopathology samples were mailed in to the clinical pathology laboratory for evaluation. All cases were required to have at least one cytology slide of the tumor available for review. All slides were required to have at least 10 high power fields available for diagnostic evaluation. One microscope was used for cytologic evaluation the entire study (Nikon, Alphaphot 2, YS2 with 4x, 10x, 40x, and 100x (oil immersion) objectives).

Location of the sample was recorded, either as an aspirate from a discrete, cutaneous mass, or from aspiration of a lymph node. The sample processing was also recorded as either a direct smear (via fine needle aspiration or needle fenestration) or as a cytospin sample. All samples were stained by clinical pathology laboratory personnel with Wright-Giemsa stain using a standard protocol.

Fourteen separate cytologic criteria were evaluated on each slide. Cytologic criteria that are well known to be universal indicators of malignancy (e.g. nuclear to cytoplasmic ratio)\textsuperscript{47} were initially included. Criteria unique to mast cells (e.g. granule staining properties) were additionally included after discussion with board certified pathologists. Table 2 lists the individual cytologic criteria and their associated
parameters evaluated during this study. The individual criteria have been grouped into larger sections for ease of understanding.

Table 2. Cytologic criteria evaluated in 52 cases of mast cell tumors.

<table>
<thead>
<tr>
<th>Mast Cell Granule Characteristics</th>
<th>Associated Blood Cell Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granule staining properties</td>
<td>Red blood cells</td>
</tr>
<tr>
<td>Well</td>
<td>Lowest number</td>
</tr>
<tr>
<td>Intermediate</td>
<td>Highest number</td>
</tr>
<tr>
<td>Poor</td>
<td>Neutrophils</td>
</tr>
<tr>
<td>Granule percentage within the cell</td>
<td>Lowest number</td>
</tr>
<tr>
<td>Lowest percentage</td>
<td>Highest number</td>
</tr>
<tr>
<td>Highest percentage</td>
<td>Eosinophils</td>
</tr>
<tr>
<td>Cellular Proliferation Criteria</td>
<td>Lowest number</td>
</tr>
<tr>
<td>Multinucleated cells</td>
<td>Highest number</td>
</tr>
<tr>
<td>Lowest number</td>
<td>Mast Cell Cytoplasmic Criteria</td>
</tr>
<tr>
<td>Highest number</td>
<td>Clear and distinct</td>
</tr>
<tr>
<td>Binucleated cells</td>
<td>Intermediate</td>
</tr>
<tr>
<td>Lowest number</td>
<td>Variable and indistinct</td>
</tr>
<tr>
<td>Highest number</td>
<td>Overall cell size (estimated in microns)</td>
</tr>
<tr>
<td>Mitotic figures</td>
<td>Smallest</td>
</tr>
<tr>
<td>Lowest number</td>
<td>Largest</td>
</tr>
<tr>
<td>Highest number</td>
<td></td>
</tr>
<tr>
<td>Mast Cell Nuclear Criteria</td>
<td></td>
</tr>
<tr>
<td>Nuclear size (estimated in microns)</td>
<td></td>
</tr>
<tr>
<td>Smallest</td>
<td></td>
</tr>
<tr>
<td>Largest</td>
<td></td>
</tr>
<tr>
<td>Predominant nuclear shape</td>
<td></td>
</tr>
<tr>
<td>Round</td>
<td></td>
</tr>
<tr>
<td>Intermediate</td>
<td></td>
</tr>
<tr>
<td>Irregular</td>
<td></td>
</tr>
<tr>
<td>Presence and prominence of nucleoli</td>
<td></td>
</tr>
<tr>
<td>Few and indistinct</td>
<td></td>
</tr>
<tr>
<td>Intermediate</td>
<td></td>
</tr>
<tr>
<td>Multiple and prominent</td>
<td></td>
</tr>
<tr>
<td>Nuclear to cytoplasmic ratio</td>
<td></td>
</tr>
<tr>
<td>Uniform and fixed</td>
<td></td>
</tr>
<tr>
<td>Variable</td>
<td></td>
</tr>
<tr>
<td>Highly variable</td>
<td></td>
</tr>
</tbody>
</table>

The reviewer was blinded to the histologic grade of each sample. Some cytologic criteria (n = 5) were calculated as an overall average while other criteria (n = 9) were recorded as
the highest and lowest values documented per slide (each slide having at least 10 high powered fields available for diagnostic evaluation). Table 3 lists the cytologic criteria grouped by method of evaluation.

Table 3. Grouping of mast cell cytologic criteria based on method of evaluation

<table>
<thead>
<tr>
<th><strong>Criteria determined as overall average</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Granule staining properties</td>
<td></td>
</tr>
<tr>
<td>Predominant nuclear shape</td>
<td></td>
</tr>
<tr>
<td>Presence and prominence of nucleoli</td>
<td></td>
</tr>
<tr>
<td>Nuclear to cytoplasmic ratio</td>
<td></td>
</tr>
<tr>
<td>Cytoplasm border</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Criteria determined as low to high range</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Granule percentage within cell</td>
<td></td>
</tr>
<tr>
<td>Multinucleated cells</td>
<td></td>
</tr>
<tr>
<td>Binucleated cells</td>
<td></td>
</tr>
<tr>
<td>Mitotic figures</td>
<td></td>
</tr>
<tr>
<td>Nuclear size</td>
<td></td>
</tr>
<tr>
<td>Red blood cells</td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td></td>
</tr>
<tr>
<td>Eosinophils</td>
<td></td>
</tr>
<tr>
<td>Overall cell size</td>
<td></td>
</tr>
</tbody>
</table>

The methods of evaluation were developed with statistician consultation.
Granule staining was semiquantitated by evaluating at least 10 high powered fields on a slide. If the majority of granules were well stained, then the slide received a score of 1. Intermediate staining granules received a score of 2, and poorly stained granules received a score of 3. Figure 1 shows 3 well stained mast cells.

Figure 1. Mast cell granule staining. Three well stained mast cell tumors showing abundant dark purple granules. (Wright Giemsa stain, magnified, 100x)

Clearly, granules within the cytoplasm might obscure nuclear and cytoplasmic characteristics of a particular cell. In that event, that cell was not included in the determination of cytologic criteria for that particular high powered field.

Nuclear shape was determined as an overall score for each slide after evaluating at least 10 high powered fields. If the majority of the nuclei were determined to be round, regular, and well defined, then the slide was given a score of 1 for that particular criteria. If the predominant nuclear shape was judged to be irregular, with the nuclei having indentations, or an irregular outer margin, then the slide was given a score of 3. Lastly, if the nuclei in the majority of mast cells were judged to be variable between the 2 extremes of round (scored as 1) and irregular (scored as 3), then the slide was given a score of 2. Figure 2 shows 2 examples of mast cell nuclear shapes.
Nucleoli characteristics were investigated on cytology samples and judged to be either indistinct and few, intermediate, or multiple and prominent. These slides were scored in a similar fashion with one overall score assigned to each slide. Indistinct and few nucleoli were scored as 1, intermediate as 2, and multiple and prominent nucleoli scored as 3. Figure 3 shows a mast cell with a single, clearly defined nucleolus.
Nuclear to cytoplasmic ratio was determined as a mean score for each slide (average of at least 10 high powered fields). If the nuclear to cytoplasmic ratio was judged to be fixed and uniform, the slide received a score of 1. If the ratio was variable, the slide received a score of 2. If the nuclear to cytoplasmic ratio was judged to be highly variable across the slide, the slide was given a score of 3.

Cytoplasm border was the last parameter where overall impression of the slide was recorded as a mean score. If the cytoplasm was judged to be clear and distinct, then a score of 1 was given. If the cytoplasm was determined to be somewhat intermediate, then a score of 2 was recorded. A score of 3 was given when the cytoplasm was determined to be overall variable and indistinct.

Seven criteria were recorded as both a low and high number: granule percentage within the cell, multinucleated and binucleated cells, mitotic figures, red blood cells, eosinophils, and neutrophils. Two criteria were recorded as smallest and largest size: nuclear size and overall cell size.

Granule percentage within the cell was evaluated with a lowest and highest number. The lowest number was defined as the lowest or smallest proportion of cytoplasm that contained granules. The lowest estimation was $1/8^{th}$ (or 0.125) percentage of the cell contained granules. The highest proportion was seen in those cells that appeared to be completely filled with granules, and in those cases, the value of 99% (0.99) was assigned. Figure 4 shows an example of a mast cell with the highest granule percentage recorded.
Multinucleated cells and binucleated cells were counted in a similar fashion, with
the lowest and highest number being recorded on each slide. In a similar manner, mitotic
figures were counted as a low and high number. Figure 5 shows an example of a
binucleated mast cell.

Figure 5. Binucleated mast cell (black arrow). Nuclei appear as pale blue, symmetric
structures within pale pink / purple cytoplasm. (Wright Giemsa stain, magnified, 100x)
Counts were obtained for the lowest number of cells visible in each high powered field and the highest number of cells visible in each high powered field for red blood cells, neutrophils, and eosinophils. The lowest value assigned for any of the cell lines was 0. The highest value was given when the field had cells that were too numerous to count, in which the value of 999 was assigned. This value was used to aid in statistical analysis of the data. Figure 6 shows a mast cell and associated blood constituents.

![Figure 6. Associated blood cell components. Mast cell (white arrow), red blood cells (black arrow), and an eosinophil (blue arrow). (Wright Giemsa stain, magnified, 100x)](image)

When evaluating cell size, the smallest and largest cell sizes seen in an average of 10 high powered fields were recorded. The cell sizes were measured by comparing the diameter of the mast cells to adjacent red blood cells, which are estimated to have a diameter of 7 microns (in the dog). Figure 7 shows the measurement of a mast cell with an adjacent red blood cell.
Figure 7. Mast cell size. Mast cell (white arrow) measuring approximately 21 microns compared to a nearby red blood cell (black arrow) measuring 7 microns. (Wright Giemsa stain, magnified, 100x)

Nuclear size was measured in like manner with the smallest nucleus and the largest nucleus measured and recorded. These cytologic criteria were developed prospectively and were only minimally modified after the start of the study.

The cytologic evaluation technique was as follows. Each slide was initially scanned at low-power (10x objective) to determine slide quality, cellularity, and staining quality. Once the slide was determined to be of overall acceptable diagnostic quality, the slide was evaluated under high-powered (100x-oil) objective. Each high-powered field was initially scanned for diagnostic quality (cellularity, staining quality, absence of cell clumping) and at least 10 separate, distinct high-powered fields were identified per slide. If there were not at least 10 diagnostic fields present, the slide was removed from the study. Once the slide was reviewed and determined to be acceptable, the cytologic criteria were individually measured. The order in which the cytologic criteria were
evaluated and recorded was consistent throughout the study. The order of data collection and recording is presented in Table 4.

Table 4. Collection and recording order of mast cell cytology data.

<table>
<thead>
<tr>
<th>Table 4. Collection and recording order of mast cell cytology data.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lymph node vs. discrete mass</strong></td>
</tr>
<tr>
<td><strong>Granule staining</strong></td>
</tr>
<tr>
<td><strong>Percentage of cell filled with granules</strong></td>
</tr>
<tr>
<td><strong>Cell size</strong></td>
</tr>
<tr>
<td><strong>Nuclear size</strong></td>
</tr>
<tr>
<td><strong>Nuclear shape</strong></td>
</tr>
<tr>
<td><strong>Cytoplasm border</strong></td>
</tr>
<tr>
<td><strong>Binucleate cells</strong></td>
</tr>
<tr>
<td><strong>Multinucleate cells</strong></td>
</tr>
<tr>
<td><strong>Mitotic figures</strong></td>
</tr>
<tr>
<td><strong>Eosinophils</strong></td>
</tr>
<tr>
<td><strong>Neutrophils</strong></td>
</tr>
<tr>
<td><strong>Red blood cells</strong></td>
</tr>
<tr>
<td><strong>Nucleoli</strong></td>
</tr>
<tr>
<td><strong>Nuclear : cytoplasmic ratio</strong></td>
</tr>
<tr>
<td><strong>Smear vs. cytospin</strong></td>
</tr>
</tbody>
</table>

Data sheets were developed to record cytologic findings and the end results were recorded to a spreadsheet for statistical analysis. Each criteria was assigned a numeric value for statistical analysis at the end of data collection. As an example, if the cytology sample was obtained from a cutaneous mass, the data was recorded as 1 for mass and 0 for lymph node on the spreadsheet. Kendal-Tau non-parametric analysis was performed on the initial data set and the value for statistical significance was set at \( p < 0.1 \). This \( p \) value was chosen in an attempt to increase the number of statistically significant findings. Logistic regression was then performed on those cytologic criteria identified as significant from the initial statistical analysis. All statistical analysis was performed with a commercially available statistical software package (JMP, Cary, NC, version 4).
RESULTS AND DISCUSSION

Fifty two total cases fulfilled the study inclusion criteria. Thirty nine of the 52 cases had multiple slides available, and so 132 total slides were reviewed. Eighty slides were direct smears, and 52 slides were cytospin samples. All cytologic criteria were evaluated on each of the 132 slides. Twenty six of the cases had concurrent histopathologic diagnosis and histologic grade recorded, based on the Patnaik\textsuperscript{1} scale. Twenty of the those 26 cases had multiple cytology slides, so there was a total of 64 histologic grades.

The histologic grades of the tumors that were evaluated were as follows: grade I tumors (7/26 = 26.9\%), grade II tumors (14/26 = 53.9\%), and grade III tumors (5/26 = 19.2\%). All statistically significant findings between cytologic criteria and histologic grade were identified from samples that were processed as cytospin samples. Four cytologic factors were determined to be significantly related to histologic grade in the initial, non-parametric analysis. Those factors were: presence of multinucleated cells [Kendal-Tau value = 0.42, p-value = 0.04], mitotic index [Kendal-Tau value = 0.39, p-value = 0.06], large cell size [Kendal-Tau value = 0.31, p-value = 0.09], and nuclear shape [Kendal-Tau value = 0.45, p-value = 0.03]. Once these values had been identified, logistic regression was performed on those four cytologic criteria and three cytologic factors were determined to be statistically significant. Those factors were: presence of multinucleated cells (p-value = 0.02), large cell size (p-value = 0.07), and nuclear shape (p-value = 0.06). The resultant value for mitotic index was determined to be p = 0.1, which did not reach statistical significance.
The recorded values on cytologic analysis for multinucleated cells ranged from a low of 0 cells to a high of 3 cells. The lowest number of multinucleated cells was not determined to be significant, but the highest number of multinucleated cells did reach statistical significance. The distribution of multinucleated cell numbers is illustrated in figure 8.

Figure 8. Graphical representation of highest numbers of multinucleated cells.

The presence of an increased number of multinucleated cells was positively correlated to increasing histologic grade (p-value = 0.02).

The largest mast cells measured ranged from 21-70 microns in diameter. The distribution of mast cell sizes are illustrated in figure 9. Results of correlation analysis indicated that larger cell size correlated positively to increasing histologic grade (p-value = 0.07).
The final cytologic factor that was determined to have a statistically significant relationship to histologic grade was nuclear shape. The distribution of nuclear shape scores are illustrated in figure 10. The presence of irregular nuclear shape positively correlated to increasing histologic grade (p-value = 0.06).
In the current study, 3 cellular characteristics were found to be significantly correlated to histologic grade: presence of multinucleated cells, large cell size, and irregular nuclear shape. These cytologic and histologic criteria would seem to correlate well based on previous descriptions of the mast cells in the histologic scoring system. A summary of the descriptions for histologic grades I, II, and III for mast cell tumors (as defined by Patnaik) is provided in Table 5.

Regarding histologic evaluation, multinucleated cells are more commonly observed in grade III mast cell tumors. In the current study, the presence of multinucleated cells was also associated with increasing histologic grade. The same can be said for the presence of large cell size (giant cells) in histologically confirmed grade III tumors. Furthermore, cytologically the present study demonstrated that the largest mast cells were seen in grade III tumors.
Table 5. Summary of Patnaik histologic grading system for mast cell tumors.

<table>
<thead>
<tr>
<th>Grade</th>
<th>Histologic Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Cells are round and similar in size and shape and have distinct cytoplasmic borders with fine, intracytoplasmic granules; nuclei are round and chromatin is condensed; no mitotic cells are noted.</td>
</tr>
<tr>
<td>II</td>
<td>Cells are moderately variable in size and shape; cells are round to ovoid and there are scattered giant cells and spindle cells; most cells have distinct cytoplasm with fine, intracytoplasmic granules; in some, the cytoplasm is indistinct and granules are large and hyperchromatic; nuclei are round to indented with scattered chromatin and some nuclei have multiple nucleoli; mitotic figures are present, but rare.</td>
</tr>
<tr>
<td>III</td>
<td>Cells have wide variety in shape and size, including round, ovoid, or spindle shaped; cytoplasm is indistinct with fine, intracytoplasmic granules or granules are not obvious; binucleated cells are common; giant cells and multinucleated cells are seen; mitotic cells are also common.</td>
</tr>
</tbody>
</table>

Both multinucleated cells and large cell size can be considered relatively objective criteria. Simplistically, the presence of multinucleated cells may be ascertained as a simple ‘present or absent’ determination. Cell size may be measured directly, by use of an eyepiece micrometer, or can be estimated by comparing the diameter of mast cells to the diameter of other cells (such as red blood cells = 7 microns) in the same microscope field.

On the other hand, nuclear shape is a less objective parameter, as compared to multinucleated cells and cell size. This parameter was determined as an opinion of the predominant nuclear shape on the slide. There was no defined measurement of nuclear shape or objective comparison to other parameters on the slide.

It is noteworthy that no granular characteristics evaluated in this study (e.g., granule staining properties or granule percentage within the cell) were significantly correlated to prognosis. Based on the histologic description, especially of the staining characteristics of grade III tumors, one might have expected to see some positive
relationship between granule characteristics and histologic grade. However, no such relationships were discovered.

Another cytologic criteria that was examined was the presence of other blood cell constituents, such as red blood cells, eosinophils, and neutrophils. Since mast cell tumors may have associated hemorrhage from tissue invasion, as described in the Patnaik\(^1\) grading scheme, cytologic evidence of red blood cells may or may not correlate with histologic grade. Eosinophils and neutrophils were evaluated for somewhat similar reasons, namely due to associated hemorrhage (especially with neutrophils), or concurrent inflammation. Eosinophils are commonly identified in cytologic and histologic samples from mast cells tumors.\(^{48,49}\) While the presence of eosinophils has not been evaluated in correlation with histologic grade, cytologic evaluation of eosinophil numbers has also not been determined. Regardless, in this current study, there was no significant relation between mast cell histologic grade and cytologic red blood cell, neutrophil, or eosinophil numbers.

While there were cytologic nuclear criteria that did correlate with histologic grade, namely nuclear shape and multinucleated cells, there were other nuclear criteria that did not have a statistically significant relationship identified. The other criteria included nuclear size, nuclear to cytoplasmic ratio, binucleate cells, presence and prominence of nucleoli, and the presence of mitotic figures (or mitotic index). The smallest nucleus measured 7 microns, and the largest was estimated to be 35 microns. Anisokaryosis (variation in nuclear shape) is considered to be a criteria of malignancy. We hypothesized that evidence of anisokaryosis seen on cytology samples might correlate to worsening histologic grade. However, this association was not found to be
significant. Even though increased nuclear to cytoplasmic ratio is a known cytologic criteria of malignancy, this factor in this cytologic study did not correlate to histopathologic grade.

Binucleated cells are typically included as part of the histologic description of grade III mast cell tumors. The lowest number of binucleated cells seen per high powered field in this study was 0, and the highest number was 4. Neither of these correlated to histopathologic grade.

The presence of irregular, or multiple nucleoli is considered another cytologic criteria of malignancy. There was no significant correlation found between nucleoli criteria and histologic grade of mast cell tumors.

The lowest value recorded for mitotic figures in our samples was 0, and the highest was 3. In the initial (univariate) analysis, mitotic index did attain statistical significance. However, this significance did not survive multivariate analysis so ultimately, neither binucleated cells, presence and prominence of nucleoli, nor mitotic index were shown to be significantly correlated to histologic grade.

Increased nuclear activity would appear to correlate to increased biologic behavior and thus, potentially, increased histologic grade. While various specialized staining techniques (e.g., AgNOR staining) have been shown to indicate increased nuclear activity, it may be difficult to demonstrate increased nuclear activity on the basis of routine cytologic evaluation of nuclear morphologic criteria. In this study, nuclear shape and the presence of multinucleated cells were statistically significant nuclear morphologic criteria that correlated to histologic grade.
The other, primarily cytoplasmic characteristic that was evaluated was the cytoplasm shape and border description. When reviewing the descriptions of histologic grade in the Patnaik\textsuperscript{1} scale, cytoplasmic descriptions are present with each grade (see Table 5). Thus, it was decided to include cytoplasm in the cytologic evaluation, to determine a possible relationship to histologic grade. This was another parameter where overall impression of the slide was recorded as a mean score. The data of the present study showed that this cytoplasmic parameter did not attain statistical significance.

In summary, 3 cytologic characteristics significantly correlated to histologic grade: presence of multinucleated cells, large cell size, and irregular nuclear shape. These cellular criteria are all included in the current histologic grading scale of mast cell tumors, so these correlations were not surprising. However, significant correlations were only found for 3 of the 14 studied cytologic criteria.

Several questions come to mind in explaining why certain cytologic criteria were not positively correlated to histopathologic grade. One limitation of our study is that sample numbers were small, as compared to earlier published reports. The cytology samples of the present report were small, and the tissue samples that were available for histopathologic evaluation were even less numerous. Thus, there were fewer comparisons available for certain tumor grades and there were several reasons for the small number of tissue samples. First, some cytology samples were obtained as mail in samples without the benefit of histopathologic examination performed at ISU. Secondly, there were no histopathology samples for some animals that had recurrent mast cell tumors, that had metastatic disease, or that were otherwise not surgical candidates.
Certain cytologic criteria were also more difficult to ascertain because of the subjective nature of the evaluation. Factors that were subjectively measured versus being directly measured included the nuclear to cytoplasmic ratio, the granule staining properties and granule percentage within the cells, the presence and prominence of nucleoli, the nuclear shape, and the cytoplasmic border description. It is possible that more precise differentiations, via objective criteria, for cytologic parameters might have led to greater histologic correlations. As an example, nucleoli were placed into 1 of 3 main classifications: few / indistinct, intermediate, or multiple / prominent. Perhaps more distinct differentiations could have allowed for a more precise description of this variable, such as: no nucleoli noted, 1-2 nucleoli visible, more than 2 nucleoli present within the nucleus.

Another study limitation is that only one observer recorded all cytologic evaluations. As evidenced by the previous description of variations in histopathologic grading between pathologists, there may be distinct differences in the manner in which slides are read and tissues are evaluated. The study could be strengthened by having multiple observers review the slide sets independently and determine what, if any, significant criteria remained after this “consensus” independent review.

Admittedly, this was a retrospective study with defined limitations. These limitations included a lack of standardization of sample procurement; a lack of follow up with histopathologic evaluation; incomplete medical records and / or incomplete medical record coding that limited the number of cases identified through medical record searches. All the cytologic criteria that were deemed significant in this study were observed on samples undergoing cytospin preparation. The cytospin technique increases
the number of cells available for evaluation in microscopic fields, as compared to direct smear samples. While cytospin slide preps concentrate cell populations and maintain cell integrity, this cytologic preparation method is not routinely available to most veterinary practitioners. One of the stated advantages of cytologic evaluation is the ease of sample procurement and the ability of general practitioners to obtain diagnostic samples. An obvious follow up to the current study is to prospectively enroll cases of cutaneous mast cell tumors and evaluate increased numbers of direct smear and cytospin samples. This would address the question as to whether cytologic criteria are only significantly related to histopathologic grades in cytospin samples and would be of relevance to veterinary practitioners in the field.
GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

In conclusion, there are a few known, accepted, prognostic factors for clinical progression of mast cell tumors. These factors include histologic grade, presence of clinical or systemic signs of mast cell disease, and increased expression of cellular markers of proliferation. To date, while there has been excellent correlation shown between cytologic and histopathologic diagnosis of mast cell tumors, there has not been a proven correlation between defined cytologic criteria and histopathologic grade of mast cell tumors. This study identified 3 defined cytologic criteria based on cytospin samples with statistically significant relations to histologic grade including the presence of multinucleated cells, the presence of large cell size, and the presence of an irregular nuclear shape.

Ideally, prospective evaluation of cytologic criteria of mast cell tumors could be initiated which included evaluation of the following parameters: sampling technique (e.g., needle fenestration versus needle aspiration techniques) and follow up histopathologic examination on all tissues (when deemed appropriate for the patient). This would assist in answering (1) whether there is value in direct smear preparations (as compared to cytospin samples); (2) whether the initial findings presented in this study can be confirmed by a separate, independent observer; (3) whether there are other cytologic criteria that correlate to histology; and (4) whether those criteria that have been identified might show greater significance through increased sample numbers.

Future investigations, primarily centered on those factors indicating cellular proliferation, should be evaluated. AgNOR, PCNA, and Ki-67 staining could be
performed on cytology samples. There would be great clinical impact if there would be a correlation to cytologic criteria, histologic grade, and known indicators of cellular proliferation. This could be even more significant if evidence was found to further elucidate grade II mast cell tumors. In the context of a prospective study, disease free intervals, survival times, and time to recurrent disease could be more readily documented, thus increasing the potential prognostic value of cytology. This could also allow for complete documentation of other variables such as dog breed, tumor location, tumor size, and the presence of associated clinical signs. If such valuable information could be obtained on the basis of a needle aspiration of cytology samples, then patient morbidity would be significantly decreased since surgical planning and adjunctive therapy could be more appropriately tailored to the patient. This information could then translate directly into defined therapeutic interventions.

While this study is limited in scope and there are some weaknesses in study design, it is notable that these data identified distinct cytologic criteria that significantly correlated to histologic grade of cutaneous mast cell tumors.
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