INFORMATION TO USERS

This was produced from a copy of a document sent to us for microfilming. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the material submitted.

The following explanation of techniques is provided to help you understand markings or notations which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting through an image and duplicating adjacent pages to assure you of complete continuity.

2. When an image on the film is obliterated with a round black mark it is an indication that the film inspector noticed either blurred copy because of movement during exposure, or duplicate copy. Unless we meant to delete copyrighted materials that should not have been filmed, you will find a good image of the page in the adjacent frame. If copyrighted materials were deleted you will find a target note listing the pages in the adjacent frame.

3. When a map, drawing or chart, etc., is part of the material being photographed the photographer has followed a definite method in "sectioning" the material. It is customary to begin filming at the upper left hand corner of a large sheet and to continue from left to right in equal sections with small overlaps. If necessary, sectioning is continued again—beginning below the first row and continuing on until complete.

4. For any illustrations that cannot be reproduced satisfactorily by xerography, photographic prints can be purchased at additional cost and tipped into your xerographic copy. Requests can be made to our Dissertations Customer Services Department.

5. Some pages in any document may have indistinct print. In all cases we have filmed the best available copy.
Meuten, Donald John

INVESTIGATIONS ON THE PATHOGENESIS OF HYPERCALCEMIA AND MALIGNANCY IN DOGS

The Ohio State University

Ph.D. 1981

University Microfilms International 300 N. Zeeb Road, Ann Arbor, MI 48106
PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark ✓.

1. Glossy photographs or pages ✓
2. Colored illustrations, paper or print ______
3. Photographs with dark background ✓
4. Illustrations are poor copy ______
5. Pages with black marks, not original copy ______
6. Print shows through as there is text on both sides of page ______
7. Indistinct, broken or small print on several pages ✓
8. Print exceeds margin requirements ______
9. Tightly bound copy with print lost in spine ______
10. Computer printout pages with indistinct print ______
11. Page(s) _______ lacking when material received, and not available from school or author.
12. Page(s) _________ seem to be missing in numbering only as text follows.
13. Two pages numbered _________. Text follows.
14. Curling and wrinkled pages ______
15. Other ____________________________________________________________

University
Microfilms
International
INVESTIGATIONS ON THE PATHOGENESIS OF HYPERCALCEMIA AND MALIGNANCY IN DOGS

Dissertation

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Donald John Meuten, B.S., D.V.M.

* * * * *

The Ohio State University

1981

Reading Committee:
Gary J. Kociba
Charles C. Capen
Steven E. Weisbrode

Approved By

Co-Adviser
Department of Veterinary Pathobiology

Co-Adviser
Department of Veterinary Pathobiology
DEDICATION

To all graduate students who ever struggled with writing.

"We are returning your manuscript along with two rejection slips. One for the present manuscript and one for the next one you submit to us".

Charles Schultz
ACKNOWLEDGEMENTS

"If he is indeed wise he does not bid you enter the house of his wisdom, but rather leads you to the threshold of your own mind".

Kahlil Gibran

I believe this philosophy is practiced by my advisers Drs. Charles C. Capen and Gary J. Kociba and adhered to by the Department of Veterinary Pathobiology at The Ohio State University. It has been a long time emerging but I now thank them and the department for exposing me to this philosophy and permitting me to grow within its boundaries.

Dr. Gino V. Segre not only permitted me to work in his lab in the Bullfinch building of the Massachusetts General Hospital, but he lifted my enthusiasm and rekindled my interests in research and academics at a very critical point. I am most grateful to have had some exposure to him and other members of the endocrine unit at MGH.

Dr. Steven Weisbrode deserves a great deal of credit for the training I and other graduate students received in morphologic pathology. I thank him for this, and for providing an example of someone who has maintained a good perspective on his work and life.

Much feeling goes out to my mother and other members of my family who never understood what I was doing but offered their support.

I acknowledge The Burroughs Wellcome Company which provided a fellowship that supported me through this study, and the State of Ohio Canine Research Funds that awarded out investigation yearly grants.
I thank Virginia Stump and Sally Murray for their excellent secretarial work.

Lastly, I acknowledge my wife, Pamela, who never wanted me to start this project and always wanted it finished. She left me alone at critical times and tolerated the fact that this study took away time that we could have shared together. She maintained my lifelines to the real world and reminded me through example that there is much more to life than work.
VITA

October 4, 1948
Born - Waterbury, Connecticut

1970
B.S., University of Connecticut
Storrs, Connecticut

1974
D.V.M., Cornell University,
Ithaca, New York

1978-1981
Burroughs Wellcome Fellow,
Department of Veterinary Pathobiology
The Ohio State University,
Columbus, Ohio

PUBLICATIONS


FIELDS OF STUDY

Major Field: Veterinary Pathology

Studies in calcium metabolism and endocrine pathology. Professor Charles C. Capen.

Studies in veterinary clinical pathology. Professor Gary J. Kociba.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>VITA</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>x</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
</tbody>
</table>

## CHAPTER

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. HYPERCALCEMIA ASSOCIATED WITH AN ADENOCARCINOMA DERIVED FROM THE APOCRINE GLANDS OF THE ANAL SAC.</td>
<td>4</td>
</tr>
<tr>
<td>Introduction</td>
<td>4</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>6</td>
</tr>
<tr>
<td>Results</td>
<td>8</td>
</tr>
<tr>
<td>Discussion</td>
<td>17</td>
</tr>
<tr>
<td>Summary</td>
<td>23</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>II. ULTRASTRUCTURAL EVALUATION OF ADENOCARCINOMAS DERIVED FROM APOCRINE GLANDS OF THE ANAL SAC ASSOCIATED WITH HYPERCALCEMIA IN DOGS.</td>
<td>45</td>
</tr>
<tr>
<td>Introduction</td>
<td>45</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>47</td>
</tr>
<tr>
<td>Results</td>
<td>49</td>
</tr>
<tr>
<td>Discussion</td>
<td>53</td>
</tr>
<tr>
<td>Summary</td>
<td>57</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS (Continued)

CHAPTER

III. HYPERCALCEMIA IN DOGS WITH ADENOCARCINOMAS DERIVED FROM APOCRINE GLANDS OF ANAL SAC: BIOCHEMICAL AND HISTOMORPHOMETRIC INVESTIGATIONS. 81

| Introduction | 81 |
| Materials and Methods | 83 |
| Results | 91 |
| Discussion | 96 |
| Summary | 102 |

IV. HYPERCALCEMIA IN DOGS WITH LYMPHOSARCOMA: BIOCHEMICAL, ULTRASTRUCTURAL AND HISTOMORPHOMETRIC INVESTIGATIONS. 115

| Introduction | 115 |
| Materials and Methods | 117 |
| Results | 125 |
| Discussion | 131 |
| Summary | 137 |

V. RELATIONSHIP OF TOTAL SERUM CALCIUM TO ALBUMIN AND TOTAL PROTEIN IN DOGS. 157

| Introduction | 157 |
| Materials and Methods | 158 |
| Results | 160 |
| Discussion | 164 |
| Summary | 167 |

BIBLIOGRAPHY 175
## LIST OF TABLES

### CHAPTER I

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 Biochemical data from dogs with adenocarcinoma derived from the apocrine glands of the anal sac.</td>
<td>24</td>
</tr>
<tr>
<td>1.2 Calcium and phosphorus values before and after tumor removal and with subsequent recurrence in five dogs with adenocarcinoma of the glands of the anal sac.</td>
<td>25</td>
</tr>
<tr>
<td>1.3 Summary of clinical features in 36 dogs with adenocarcinoma derived from the apocrine glands of the anal sac.</td>
<td>26</td>
</tr>
</tbody>
</table>

### CHAPTER III

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1 Serum and urine data from dogs with apocrine gland adenocarcinoma and control dogs.</td>
<td>103</td>
</tr>
<tr>
<td>3.2 Histomorphometric evaluation of lumbar vertebrae from dogs with hypercalcemia and adenocarcinomas derived from apocrine glands of anal sac compared to control dogs and dogs with primary hyperparathyroidism.</td>
<td>104</td>
</tr>
</tbody>
</table>

### CHAPTER IV

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1 Serum and urine data from dogs with lymphosarcoma and control dogs.</td>
<td>138</td>
</tr>
<tr>
<td>4.2 Histomorphometric evaluation of lumbar vertebrae from dogs with lymphosarcoma (hypercalcemic and normocalcemic) compared to control dogs and dogs with primary hyperparathyroidism.</td>
<td>139</td>
</tr>
<tr>
<td>4.3 Anatomical distribution of tumors and hematologic data in hypercalcemic and normocalcemic dogs with lymphosarcoma.</td>
<td>140</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

**CHAPTER I**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 Transverse section of perineum from female dog with hypercalcemia and adenocarcinoma of apocrine glands of anal sac. Anal sacs (A) on both sides of rectum (R). Tumor nodule (arrow) 1 cm in diameter in wall of left anal sac protrudes into its lumen. Bar represents 1 cm.</td>
<td>27</td>
</tr>
<tr>
<td>1.2 Adenocarcinoma (T) in apocrine glands (G) subjacent to stratified squamous epithelial lining of anal sac (A).</td>
<td>29</td>
</tr>
<tr>
<td>1.3 Adenocarcinoma derived from apocrine glands of anal sac from acini (A) and tubules (T) lined by columnar or cuboidal epithelial cells. Lumen contains eosinophilic material (arrow).</td>
<td>31</td>
</tr>
<tr>
<td>1.4 Solid pattern of adenocarcinoma derived from apocrine glands of anal sac with polyhedral and columnar cells forming pseudorosettes around small capillaries (C).</td>
<td>33</td>
</tr>
<tr>
<td>1.5 Prominent tubuloacini (A) lined by tall cylindrical cells with multiple apical cytoplasmic projections (arrow) into lumen in adenocarcinoma derived from apocrine glands of anal sac.</td>
<td>35</td>
</tr>
<tr>
<td>1.6 Bimorphic growth pattern in adenocarcinoma derived from apocrine glands of anal sac with adjacent solid (S) areas and acini (A) formed by neoplastic cells.</td>
<td>37</td>
</tr>
<tr>
<td>1.7 Tumor cell emboli (arrow) of apocrine adenocarcinoma of anal sac in lymphatic in bronchial lymph node.</td>
<td>39</td>
</tr>
</tbody>
</table>
LIST OF FIGURES (Continued)

CHAPTER I

Figure

1.8 Atrophic parathyroid gland from dog with hypercalcemia associated with adenocarcinoma of apocrine glands of anal sac. Narrow cords of inactive chief cells (arrow), with reduced cytoplasmic area and hyper-chromatic nuclei, are separated by prominent interstitial spaces (I) with increased fibrous connective tissue.

1.9 Osteoclastic osteolysis in ilium of dog with adenocarcinoma derived from apocrine glands of anal sac. Osteoclasts (arrows) aligned along trabecular bone surfaces with excavations.

CHAPTER II

2.1 Bimorphic pattern in an adenocarcinoma arising from apocrine glands of the anal sac in a dog with hypercalcemia illustrating characteristic acini with central lumens adjacent to solid (S) microlobules. (H&E X215).

2.2 Tall columnar cells with microvilli (V) and a prominent basement membrane (left) lining a tubule in an adenocarcinoma derived from apocrine glands of the anal sac. Adjacent tumor cells are joined by tight junctions and desmosomes (D). A Golgi apparatus (G) is present in most cells. Tumor cells contain scattered mitochondria and many small electron-dense granules (arrows). (Uranyl acetate and lead citrate, X3,900).

2.3 Characteristic apocrine-like cytoplasmic blebs protruding into the lumen (L) of an acinus. The cytoplasm is relatively electron-lucent, devoid of organelles, but contains many small electron-dense secretory granules (arrows). Apocrine gland adenocarcinoma in a dog with hypercalcemia. N = nucleus of tumor cell. (Uranyl acetate and lead citrate, X6,400).
LIST OF FIGURES (Continued)

CHAPTER II

Figure

2.4 Solid area from an apocrine gland adenocarcinoma in a dog with hypercalcemia composed of polygonal tumor cells that have long profiles of rough endoplasmic reticulum and numerous mitochondria. (Uranyl acetate and lead citrate, X9,700).

2.5 Adenocarcinoma derived from apocrine glands of the anal sac illustrating a well-developed Golgi apparatus (G), microtubules (arrows), short profiles of rough endoplasmic reticulum, free polyribosomes, and membrane-limited secretory granules. Desmosomes (D) join two adjacent tumor cells. (Uranyl acetate and lead citrate, X14,300).

2.6 Apical portion of a tumor cell containing cluster of electron-dense granules (S) that vary in size from 200-400 nm in diameter. Large, electron-dense bodies (500-1000 nm in diameter) are adjacent to the nucleus. Clusters of microfilaments are present in the cytoplasm (arrows). (Uranyl acetate and lead citrate, X8,900).

2.7 Small electron-dense secretory granules (arrows) (200-400 nm in diameter) in neoplastic cells with an electron-dense core, closely applied limiting membrane, and narrow submembraneous space. Dog with hypercalcemia associated with an adenocarcinoma derived from apocrine glands of the anal sac. (Uranyl acetate and lead citrate, X34,300).

2.8 Acinus in an apocrine gland of the anal sac from a control dog lined by tall columnar cells with basilar nuclei. Note the electron-lucent cytoplasmic blebs (arrows) with laminated bodies. Large osmiophilic bodies (B) of varying size are located at the basilar aspect of the cells. Subjacent to the epithelial cells is a cytoplasmic projection from a myoepithelial cell (M). (Uranyl acetate and lead citrate, X4,100).
LIST OF FIGURES (Continued)

CHAPTER II

Figure

2.9 Apocrine cells of the anal sac in an active stage of secretory activity with dilated cisternae (E) from a control dog. These large vesicles fused with the plasma membrane to secrete their product by a merocrine type of secretion. Apocrine gland of anal sac from a control dog. (Uranyl acetate and lead citrate, X5,500).

2.10 Inactive cuboidal apocrine cell with a large nucleus (N), prominent Golgi apparatus (G), small profiles of endoplasmic reticulum, electron-dense granules of different sizes and density from a control dog. Microvilli (arrowheads) and desmosomes (D) are present at the luminal surface and a basally located myoepithelial cell (M), attached to the epithelial cell by a desmosome (arrow), has a convoluted nucleus and dense clusters of filaments in the cytoplasm. (Uranyl acetate and lead citrate, X7,600).

2.11 Microvilli present on the surface of apocrine cells from glands of the anal sac in control dogs. Tight junctions (T) and desmosomes (D) join adjacent cells. The perinuclear Golgi (G) apparatus is associated with small granules of low electron density. Numerous microfilaments (arrowheads) are dispersed in the cytoplasm. Several types of cytoplasmic granules (arrows) of varying size and electron density are present in apocrine cells of control dogs. (Uranyl acetate and lead citrate, X15,000).
CHAPTER III

Figure

3.1 Urinary excretion of calcium, hydroxyproline, and cAMP in dogs with apocrine adenocarcinoma (CA) compared to normocalcemic controls with and without tumors, and dogs with primary hyperparathyroidism. Cyclic AMP and calcium excretion were significantly increased in dogs with CA compared to control dogs. Cyclic AMP excretion was significantly greater (P<0.05) in hyperparathyroid dogs than any other group. Hydroxyproline in dogs with CA (19.6 µg/100 dl GF ± 3.9) was greater than controls (9.3 µg/dl GF ± 1.4) but this difference was not significant. Horizontal lines indicate mean for the group. Significant differences (P<0.05) from control dogs is indicated by "a" and from tumor-controls by "b". 105

3.2 Dogs with hypercalcemia associated with adenocarcinomas derived from apocrine glands of the anal sac (CA) had a decreased concentration of plasma iPTH compared to control dogs. Dogs with hypoparathyroidism (HYPO-PTH) had undetectable concentrations of iPTH while dogs with primary (HYPER-PTH) and secondary (RENAL HYPER-PTH) hyperparathyroidism had increased concentrations of iPTH compared to control dogs. Four of 6 dogs with chronic renal disease had iPTH values greater than 8,300 pg/ml. The limit of detectability for the iPTH assay (112 pg/ml) is indicated by the shaded area. Horizontal lines indicate the mean for the respective groups. 107
CHAPTER III

Figure

3.3 Changes in serum phosphorus, calcium, 1,25-(OH)₂D, and plasma iPTH associated with tumor excision and tumor recurrence in dogs with adenocarcinomas derived from apocrine glands of the anal sac. Following tumor removal, phosphorus concentrations increased, calcium returned to the normal range, iPTH concentrations increased two- to 20-fold, and 1,25-(OH)₂D decreased two- to 8-fold. Open columns represent values at initial clinical evaluation and dark columns have post-surgical values. Repeating numbers represent tumor recurrence and pre-surgical values. Post-operative samples were obtained at 24-48 h following tumor excision except for dog 2 which was sampled at 12 and 24 hours post-surgery, and dog 5 which had calcium and phosphorus determined seven days after tumor removal. Shaded areas for P and Ca are normal ranges for dogs. Shaded areas for iPTH and 1,25-(OH)₂D are mean + one SD for control dogs. The limits of detectability for iPTH (112 pg/ml) and 1,25-(OH)₂D (4 pg/ml) are indicated by broken lines.

3.4 Inactive parathyroid gland from a dog with hypercalcemia (15.8 mg/dl) and adenocarcinoma derived from the apocrine glands of anal sac. Inactive chief cells have a reduced cytoplasmic area and clumped nuclear chromatin. Narrow cords of chief cells are separated by wide bands of collagen and prominent perivascular spaces (S). (H & E, X315).

3.5 Inactive chief cells in a parathyroid gland from a dog with hypercalcemia (20.8 mg/dl), hypophosphatemia (1.8 mg/dl), and apocrine adenocarcinoma of anal sac. Inactive chief cells have a reduced cytoplasmic area, straight plasma membranes with few uncomplicated interdigitations, lipid bodies, and an electron-lucent cytoplasm containing few secretory organelles. (Uranyl acetate, and lead citrate, X4,500).
CHAPTER IV

Figure

4.1 Urinary excretion of calcium, cAMP, and hydroxyproline in five groups of dogs. Urine calcium was significantly greater in dogs with lymphosarcoma and hypercalcemia (LSA H-Ca) than normocalcemic lymphosarcoma-dogs (LSA N-Ca), control dogs, and tumor-control dogs. Hydroxyproline was significantly increased in dogs with lymphosarcoma and hypercalcemia compared to control dogs and was greater in hypercalcemic dogs with lymphosarcoma than normocalcemic dogs with lymphosarcoma. Urine cAMP was significantly greater (P<0.05) in hyperparathyroid dogs than control dogs and dogs with lymphosarcoma and hypercalcemia. Horizontal lines indicate mean. Different letters indicate significant differences (P<0.05).

4.2 Dogs with lymphosarcoma and hypercalcemia had lower concentrations of plasma iPTH and serum 1,25-(OH)₂D than normocalcemic lymphosarcoma dogs (LSA N-Ca) but the differences were not significant. Dogs with hypoparathyroidism (HYPO-PTH) had undetectable concentrations of iPTH while dogs with primary (HYPER-PTH) and secondary (RENAL HYPER-PTH) hyperparathyroidism had increased concentrations of iPTH compared to control dogs. Four of 6 dogs with chronic renal disease had plasma iPTH concentrations greater than 8,300 pg/ml. The limit of detectability for the iPTH assay (112 pg/ml) and the 1,25-(OH)₂D assay (4 pg/ml) are indicated by the shaded areas. Horizontal lines indicate the mean for the respective groups. Different letters indicate significantly different means (P<0.05).
CHAPTER IV

Figure

4.3 Lumbar vertebra from a control dog has thick cortical bone (arrows) and numerous wide bone trabeculae. The trabecular bone volume was 22.7%. Longitudinal end of vertebra and intervertebral disc are at the top (Von Kossa-tetrachrome, X30).

4.4 Bone is absent (arrows) along one cortical margin of a lumbar vertebra from a dog with hypercalcemia (12.3 mg/dl) and lymphosarcoma. Bone trabeculae are reduced in number and thickness. The trabecular bone volume was 7.6% (compare to Fig. 4.3, same magnification). Longitudinal end of vertebra and intervertebral disc are at the top of the photo (Von Kossa-tetrachrome, X30).

4.5 Lumbar vertebra from a control dog with a large trabeculum of bone that has smooth surfaces, no osteoclasts and flattened osteoblasts (Von Kossa-tetrachrome, X700).

4.6 Lumbar vertebra from a dog with hypercalcemia and lymphosarcoma. Howship's lacunae with (O) and without (arrow) osteoclasts lining a thin bone trabeculae with scalloped margins. Opposite the resorptive surface are columnar osteoblasts and osteoid. The marrow contains neoplastic lymphoid cells. Compare to bone trabeculum in Fig. 4.5 taken from a control dog at the magnification. (Von Kossa-tetrachrome, X700).

4.7 Inactive chief cells in a parathyroid gland from a dog with hypercalcemia (15 mg/dl) and lymphosarcoma. Inactive chief cells have large nuclei in relation to the small cytoplasmic area and straight plasma membranes, increased lipid droplets, and an electronlucent cytoplasm containing few secretory organelles (Uranyl acetate and lead citrate, X4,000).
CHAPTER IV

Figure

4.8 Lymphoblasts from a dog with hypercalcemia (14.2 mg/dl) and lymphosarcoma. Tumor cells have large nuclei with prominent nucleoli. The cytoplasm contains scattered short profiles of rough endoplasmic reticulum, mitochondria and a small Golgi apparatus (Uranyl acetate and lead citrate, X5,000).

CHAPTER V

Figure

5.1 Significant relationship ($r=0.575; P<0.001$) between serum albumin and total calcium concentrations in 209 hospitalized dogs. The least square regression line (solid line), the 95% confidence limits for the regression line (2 broken lines), and the 95% confidence limits for the population (shaded area) are given. The numbers represent the number of values at superimposed points. As the concentration of albumin increases or decreases there is a concurrent increase or decrease in serum total calcium. One-third of the variability in calcium was attributable to the change in the concentration of albumin ($R^2=0.33$).

5.2 Significant relationship ($r=0.411; P<0.001$) between serum total protein and total calcium concentrations in 209 hospitalized dogs. The least square regression line (solid line), the 95% confidence limit for the regression line (2 broken lines), and the 95% confidence limits for the population (shaded area) are given. The numbers represent the number of values at superimposed points. Approximately 17% of the variability in calcium was attributable to the change in the concentration of serum total protein ($R^2=0.169$).
LIST OF FIGURES (Continued)

CHAPTER V

Figure

5.3 Ninety-one percent of dogs with disorders of calcium metabolism and 85% of young dogs were outside of the 95% confidence for serum albumin (Fig. A) and total protein (Fig. B) compared with serum total calcium in the 209 hospitalized dogs. ● = Hypercalcemia and malignancy; ■ = young dogs (6-24 weeks old); X = renal disease; ○ = hypoparathyroidism; ▲ = primary hyperparathyroidism.
INVESTIGATIONS ON THE PATHOGENESIS OF HYPERCALCEMIA AND MALIGNANCY IN DOGS

By

Donald J. Meuten, D.V.M.
The Ohio State University, 1981
Professor Charles C. Capen, Co-Adviser
Professor Gary J. Kociba, Co-Adviser

Hypercalcemia in patients with cancer may be the result of tumor metastases to bone, production of bone-resorbing factors by the neoplastic cells or concurrent parathyroid hyperplasia. Dogs with lymphosarcoma or perirectal adenocarcinomas derived from apocrine glands of the anal sac often have hypercalcemia. The objectives of these studies were to investigate the pathogenesis of the hypercalcemia associated with two naturally occurring neoplasms in dogs.

Perirectal adenocarcinomas merged with normal apocrine glands encompassing the anal sac, and had light and electron microscopic features similar to the normal apocrine glands. It was concluded that these adenocarcinomas (CA) were derived from apocrine glands of the anal sac.

Ninety-six percent of dogs with CA had metastases to regional lymph nodes. Metastases to bone were present in only one dog. Ninety percent of the dogs were hypercalcemic and 71% were hypophosphatemic. The hypercalcemia could not be attributed to changes in the concentration of serum albumin or
total protein. Parathyroid chief cells were inactive to atrophic, suggesting a negative feedback and involution of the parathyroid glands from the persistent hypercalcemia.

Since excision of the CA resulted in a remission of hypercalcemia while recurrence or growth of metastases were associated with an increase in serum calcium it was concluded that the tumors produced a factor that induced hypercalcemia. Ultrastructurally CA cells had numerous profiles of rough endoplasmic reticulum, well developed Golgi apparatuses, and electron-dense granules with a limiting membrane and narrow submembranous space that resembled secretory granules in polypeptide hormone-secreting endocrine cells. Lumbar vertebrae from dogs with CA had histomorphometric evidence of decreased trabecular bone volume and increased osteoclastic osteolysis. These dogs also had increased urinary excretion of calcium, phosphorus and hydroxyproline.

Immunoreactive parathyroid hormone (iPTH) was undetectable or decreased in plasma compared with control dogs and was absent from tumor extracts. There was no increase in plasma concentration of the metabolite of prostaglandin E₂ (PGE₂M) in dogs with CA. These findings indicated that immunoreactive PTH and prostaglandin E₂ were not responsible for the development of hypercalcemia. Serum concentration of 1,25-dihydroxyvitamin D (1,25-(OH)₂D) in dogs with CA was not significantly different from that in control dogs and normocalcemic tumor controls. The inappropriately normal concentration of 1,25-(OH)₂D in spite of marked hypercalcemia suggested a possible relationship between the presence of CA and circulating levels of 1,25-(OH)₂D.
Dogs with lymphosarcoma and hypercalcemia had decreased trabecular bone volume, increased osteoclastic osteolysis and concentrations of urinary calcium, phosphorus and hydroxyproline. Only those dogs with neoplastic involvement of the bone marrow had increased osteoclastic bone resorption. Dogs with lymphosarcoma that were normocalcemic did not have increased bone resorption nor increased concentrations of urine calcium, phosphorus or hydroxyproline. The hypercalcemia in dogs with lymphosarcoma was not attributed to changes in the concentration of serum albumin or total protein. Dogs with lymphosarcoma and hypercalcemia had decreased concentrations of plasma iPTH and serum 1,25-(OH)\(_2\)D. Immunoreactive PTH was not detected in lymphosarcoma tissue. Parathyroid chief cells were inactive to atrophic in dogs with lymphosarcoma and hypercalcemia. Plasma concentration of PGE\(_2\)M was not different between either group of dogs with lymphosarcoma. We concluded that the hypercalcemia in dogs with lymphosarcoma was not due to the ectopic production of iPTH, PGE\(_2\)M and 1,25-(OH)\(_2\)D, and that the hypercalcemia may be related to osteoclastic osteolysis induced locally by a bone-resorbing factor produced by the neoplastic cells.

Apocrine adenocarcinoma and lymphosarcoma appear to be useful animal models to investigate the local and humorally mediated mechanisms of stimulating osteoclastic osteolysis in the pathogenesis of hypercalcemia associated with neoplasia.
CHAPTER I

HYPERCALCEMIA ASSOCIATED WITH AN ADENOCARCINOMA DERIVED FROM THE APOCRINE GLANDS OF THE ANAL SAC

INTRODUCTION

Neoplasms occur frequently in the perineum of the dog with adenomas derived from the circumanal gland the most common (1, 2, 3). Other glands in the perineum that may undergo neoplastic transformation include dermal sebaceous and apocrine glands, merocrine anal glands, and the apocrine glands of the anal sac (4, 5, 6, 7, 8, 10). We describe a tumor that originated from the apocrine glands of the anal sac. In the most complete description of this unique syndrome, only females were reported to have developed this neoplasm, the tumors were malignant, and all dogs had hypercalcemia (11).

A syndrome of hypercalcemia associated with malignancy has been seen in dogs (11, 12, 13), mice (14), rabbits (15), rats (16), and infrequently in the horse (17) and cat (18). Lymphosarcoma (13) is the tumor most frequently associated with hypercalcemia in dogs, although there are isolated reports implicating other neoplasms (12, 19, 20, 21). Malignant neoplasms with metastases to bone are the most common cause of hypercalcemia in hospitalized people (22, 23). Many hypercalcemic people, however, have neoplasms without evidence of osteolysis due to bone metastases (23, 24). Evidence suggests that these tumors secrete a bone-resorbing substance that produces hypercalcemia (25, 26, 27, 28).
When hypercalcemia is associated with neoplasia, inactive parathyroid glands, and the absence of bone metastases, the syndrome is referred to clinically as pseudohyperparathyroidism (13, 25). We describe pseudohyperparathyroidism associated with an adenocarcinoma arising in the apocrine glands of the anal sac in dogs.
MATERIALS AND METHODS

Thirty-six dogs with adenocarcinomas of the apocrine glands of the anal sac were studied, 15 by necropsy and 21 by biopsy. Of the 36 dogs examined, 18 were killed with an overdose of barbituate, 3 died, and the remaining 15 are still alive. For the purpose of this report, the duration of survival is defined as the number of months the dog lived after a histologic diagnosis of adenocarcinoma.

Biochemical profiles were done with an automated analyzer (Gilford 3500, Gilford Instrument Laboratories, Inc., Oberlin, Ohio) and sequential multiple analyzer (SMA 6/60, Technicon Instruments Corp., 511 Benedict Ave., Tarrytown, N.Y.) for dogs 1 to 8, a chemistry analyzer, (Coulter Electronics, Inc., 590 West Twentieth St., Hialeah, Fla) for dogs 9 to 12, and an SMA 6/60 and SMA 12/60 (Technicon Instruments Corp., 511 Benedict Ave., Tarrytown, N.Y.) for dogs 13 to 17. Calcium was determined by a cresolphthalein complexone method (29). Profiles included phosphorus, blood urea nitrogen, alkaline phosphatase, albumin, creatinine, sodium, potassium, chloride, total carbon dioxide, alanine aminotransferase, aspartate aminotransferase, glucose, and total bilirubin. Thirteen of the 17 dogs in table 1.1 had at least three separate calcium and phosphorus determinations. The individual values in table 1.1 and the calculated mean values were from the first profile obtained for each dog before excision of the neoplasm. Calcium and phosphorus values reported in table 1.2 represent changes seen after tumor ablation and subsequent tumor recurrence. Five dogs not included in table 1.1 had individual calcium and phosphorus measurements. Two of these dogs were normocalcemic and three had calcium values in the hypercalcemic range for the respective laboratories. Calcium x phosphorus product (Ca mg/dl x P mg/dl) was calculated from values
obtained on the last biochemical profile for each dog, which generally correlated with the day of necropsy. Serum and urine osmolality were determined with a freezing point depression osmometer. Clinical data on seven dogs have been reported elsewhere (4, 6).

At necropsy one humerus, femur, rib, mandible, thoracic vertebra, and metacarpus or metatarsus from each of eight dogs were collected and the muscle was removed. The bones were radiographed by conventional techniques and the rib, mandibles, vertebrae, and metacarpal or metatarsal of seven dogs were radiographed by a magnification (1.5 times) technique (30). One femur, humerus, tibia, and several sternebrae; several ribs; and the lumbar, sacral and cervical vertebrae from eight dogs were cut longitudinally and selected areas were processed for light microscopy. The wing of the ilium and a section of mandible (just rostral to the first molar tooth) were cut perpendicular to the longitudinal axis and processed for light microscopy.

All tissues were fixed in neutral phosphate-buffered 10% formalin, trimmed, embedded in paraffin, sectioned at 6 um, and stained with hematoxylin and eosin (HE). Sections of kidney (ten dogs), stomach (fundus) (eight dogs), lung and myocardium (nine dogs) were stained with von Kossa and alizarin red S stains. Selected sections of neoplastic tissue and adjacent anal sac were stained with periodic acid-Schiff (PAS), osmium tetroxide, alcian blue, Fontana-Masson stain for argentaffin granules, and Azzopardi's modification of the Bodian stain for argyrophil granules. Sections of proximal humerus, lumbar vertebrae, sternebrae, proximal and distal femur, rib, ilium and mandible were fixed in buffered 10% formalin and decalcified to effect in 8% formic acid and 8% hydrochloric acid.
RESULTS

Clinical biochemistry

Hypercalcemia was present before removal of the tumor in 20 of 22 dogs with adenocarcinoma arising from the apocrine glands of the anal sac. Seventeen dogs had complete biochemical profiles; their calcium values ranged from 11.4 to 24.0 mg/dl with a mean of 16.1 mg/dl (table 1.1). Five other dogs had serum calcium values of 14.4, 15.4, 13.4, 9.5 and 8.1 mg/dl. Dog 13 in table 1.1 was normocalcemic (9.8 mg/dl) at the time of tumor biopsy, but was hypercalcemic (13.8 and 14.2 mg/dl) six months later at necropsy. One intact and one castrated male (dogs 11 and 10) were hypercalcemic.

Tumor removal resulted in a prompt return to normocalcemia in five dogs (table 1.2). Four dogs were normocalcemic 24 hours after tumor removal, while in the fifth dog serum calcium was not measured until one week after surgery, when it was normal. Post-surgical hypocalcemia was not found. Two dogs were killed later because of disseminated tumor metastases and recurrence of hypercalcemia.

Hypophosphatemia (mean 3.2 mg/dl) was present in 12 of 17 dogs for which biochemical profiles were obtained (table 1.1). Increased serum alkaline phosphatase was present in nine dogs (table 1.1). Two dogs had hyperproteinemia (8.1, 8.4 mg/dl) together with hypercalcemia. Serum sodium, potassium, glucose, bilirubin, and total carbon dioxide values were within normal limits.

Eight dogs were hypostenuric, with urine specific gravity values of 1.005, 1.009, 1.004, 1.011, 1.008, 1.000, 1.009, and 1.003. The range of urine specific gravity was 1.000 to 1.020, with a mean of 1.012 for 15 hypercalcemic dogs. Urine osmolality ranged from 154 to 752 mOsm/kg, with a mean of 395 in 10
dogs. Serum osmolality ranged from 295 to 345 mOsm/kg, with a mean of 311 for 11 dogs. The mean urine/serum osmolality ratio was 1.27. Serum chloride was in the normal range and the mean value was 107 mEq/l for 14 dogs. The mean serum chloride:phosphorus ratio for all dogs in table 1.1 was 33. Eight of 11 non-uremic dogs had chloride:phosphorus ratios greater than 33 (table 1.1) (31).

Hematology

Hemograms on 14 hypercalcemic dogs showed neutrophilia, lymphopenia and monocytosis. Mean leukocyte values (and ranges), in cells/ul, were: total leukocytes 13,268 (7,050-28,000); segmented neutrophils 11,048 (5,964-24,640); band neutrophils 91 (0-540); lymphocytes 911 (0-2,380); monocytes 798 (0-2,520); and eosinophils 418 (0-1,212). The mean hematocrit of 14 dogs was 44% with a range of 32% to 56%.

Clinical signs

Of the 36 dogs with adenocarcinomas arising from apocrine glands of the anal sac, 33 were females and three were males (table 1.3). Many breeds and mongrel dogs were represented; their ages ranged from 7 to 13 years with a mean of 10 years (table 1.3). Twenty-two of 36 dogs had one or more clinical signs referable to the mass in the perineum. Fourteen had functional disturbances related to the hypercalcemia. Sixteen of 20 dogs with preoperative hypercalcemia had anorexia, polyuria, polydipsia, paresis, lethargy, or vomiting (table 1.3). Of the 16 dogs without preoperative calcium values, 15 were presented because of perirectal mass, tenesmus, constipation, fetid odor, or pruritis of the anal region. Clinical signs unique to the hypercalcemic dogs
included polyuria, polydipsia, anorexia, weight loss, paresis, lethargy and vomiting.

Fifteen tumors protruded caudally and were visible grossly, while the rest were occult and were found only after perirectal or rectal palpation. In six dogs, only after hypercalcemia had been identified was a rectal and perirectal examination made and the neoplastic mass located. Tumors were located in the perineal subcutaneous tissue, ventral or lateral to the anus. The overlying epidermis was freely movable and the skin was usually intact and haired. In two dogs the tumor protruded through the epidermis as a multilobular ulcerated mass in the perineal region. Sublumbar metastases were palpated in 16 dogs. There were no other significant physical findings.

Of the 36 dogs included in this study, 15 are still alive six months after surgical removal of the tumor, including 12 in which only the primary perirectal tumor was removed, two in which the primary and abdominal metastases were excised, and one in which removal of the primary and metastatic tumors was followed by chemotherapy. Of the 21 dogs no longer alive, nine were killed after diagnosis and one died two days after surgery. The other 11 dogs received various treatments. They survived from 2 to 21 months, with a mean of 8.8 months. Nine of these dogs were killed and two died.

Macrosco pic lesions

Adenocarcinomas of the apocrine glands of the anal sac were unilateral in 29 dogs, and bilateral in four dogs (fig. 1.1). In the remaining three there was insufficient information to determine the exact location of the mass, except that it was perirectal. The majority of tumors were located lateral or ventral to an anal sac. The epithelial lining of the anal sac was intact and often was
compressed by protruding tumor nodules. The tumors ranged in size from 7 mm to 6 x 8 cm. They either expanded cranially through the pelvic canal as a continuous large neoplastic mass, or skipped several centimeters to enter the abdomen as separate tumor nodules. Primary tumors and sublumbar metastases were thinly encapsulated and easily separated from adjacent tissues. Recurring carcinomas in the perirectal area and occasional sublumbar metastases had indistinct boundaries, were poorly encapsulated, and invaded adjacent tissues. Apocrine adenocarcinomas were multilobulated and tan, and had varied amounts of connective tissue stroma. Cross-sectional surfaces had irregular areas of necrosis of various sizes, and dilated cystic spaces from 2 to 10 mm in diameter. These cysts were either empty or filled with a serous blood-tinged fluid.

Sublumbar metastases were present in the sacral, iliac and lumbar lymph nodes in 14 of the 15 dogs necropsied, and in surgical biopsies from eight dogs. The only apocrine adenocarcinoma that had not metastasized was the smallest (7 mm in diameter); this dog also had a single enlarged parathyroid gland (dog 6, table 1.1). Sublumbar metastases were up to 12 cm in diameter and displaced abdominal viscera ventrally. Eight dogs had pulmonary metastases and metastatic foci were occasionally seen in the spleen, pancreas, mediastinum, adrenal, liver, and spinal cord. Metastatic nodules varied in size from approximately 1 to 6 cm in diameter, were tan, raised, and smooth, and had depressed umbilicated centers.

The parathyroid glands were small and difficult to locate or not visible macroscopically in 13 of 16 dogs. One dog had an enlarged external parathyroid gland approximately twice the size of the other three glands. Concomitant neoplasms were found in four dogs, including hemangiosarcoma of the spleen
and liver, multiple benign mixed mammary tumors, subcuticular lipoma, and a leiomyoma of the cecum.

Mineralization of soft tissues was not prominent on macroscopic examination and was found in only two of 15 dogs. Subendocardial mineralization appeared either as white, linear streaks or as inconspicuous pale foci. Both dogs had white, gritty areas subjacent to the corticomedullary junction of the kidney, and one had multiple, minute, white foci in the cortex that represented mineralization of groups of glomeruli. These two dogs had the highest blood urea nitrogen and phosphorus values. Their serum calcium x phosphorus products were 113 and 137, respectively.

Postmortem radiographs of the humerus, femur, rib, mandible, and metacarpus or metatarsus and the thoracic vertebrae in seven dogs had no evidence of increased bone resorption. No areas of increased bone resorption were found in seven dogs by magnification radiographs of mandible, vertebrae, metacarpus or metatarsus, and rib. Radiolucent lesions and gross metastases were found in several bones in one of eight dogs.

Light microscopy

Perirectal adenocarcinomas merged with the tubular apocrine glands of the anal sac (fig. 1.2). The mantle of normal glands encompassing an anal sac was disrupted by a contiguous neoplastic mass that expanded ventrolaterally from the anal sac. The stratified squamous epithelial lining of the anal sac was intact, but four dogs had ulceration and invasion into the anal sac. None of the 36 dogs evaluated histologically had concurrent neoplasms of other perianal structures.
The neoplastic tissue was arranged in either a glandular or a solid pattern (figs. 1.3, 1.4), both in the primary tumor and in metastases. Both patterns could be found in adjacent areas of the same tumor mass. The glandular arrangement (fig. 1.3) was characterized by well-differentiated acini and tubules lined by uniform tall columnar or cuboidal cells. The abundant cytoplasm was eosinophilic and there were occasional prominent apical cytoplasmic blebs (fig. 1.5) that protruded into the lumina of acini. Luminal cell apices had an inconspicuous double line of resolution which was interpreted as a rudimentary brush border. Nuclei were basilar and round to oval. Mitotic figures averaged nine for every ten fields with 400x magnification. The lumina of acini and tubules were either empty or filled with a dense, homogeneous, eosinophilic and PAS-positive material. Long ribbons or garland patterns of neoplastic cells were uncommon. In addition to well-differentiated acini and tubules, there were occasional cysts of various sizes lined by cuboidal epithelial cells. A glandular pattern of arrangement of neoplastic cells predominated in eight dogs.

The solid pattern of neoplastic cells was characterized by sheets, microlobules, and packets separated by a thin fibrovascular stroma. Pseudo-rosettes were common in solid areas and the cylindrical nature of lining cells was accentuated by a nucleus-free zone adjacent to central blood vessels (fig. 1.4). Nuclear chromatin was stippled and usually had one or two amphophilic nucleoli. Ten neoplasms were predominantly of the solid type, but thorough examination of either HE- or PAS-stained sections showed a few acini with lumina in all neoplasms. Eighteen neoplasms were a mixture of glandular and solid patterns (fig. 1.6).
Adenocarcinomas were partially surrounded by a thin fibrous capsule and were divided by fibrous connective tissue into lobules of various sizes. Desmosplasia was prominent in five tumors with acini and nests of tumor cells isolated by dense bands of fibrous connective tissue. Neoplastic cells within lobules were subdivided further into small groups by a thin fibrovascular septum. Invasion of adjacent tissues or emboli into endothelium-lined spaces was present in all adenocarcinomas. Tumor cell emboli were more common in lymphatic vessels than in blood vessels. Iliac lymph nodes often were effaced by proliferation of neoplastic cells (fig. 1.7). Pulmonary, pancreatic, splenic, hepatic, adrenal, spinal cord, and mediastinal metastases resembled the primary tumors. Large tumor masses had areas of necrosis with exfoliated cells mixed with cytoplasmic and nuclear debris.

The secretory product in the lumina of acini lined by neoplastic cells stained intensely with PAS. In solid areas, acini with small lumina, difficult to detect on HE-stained sections, could be identified easily on sections stained with PAS. A few neoplastic cells contained minute, intracellular PAS-positive granules. The adjacent apocrine glands of the anal sac had large PAS-positive globules in their lumina, between cells and within cells. Alcian blue stained the secretory product formed by neoplastic cells and normal apocrine glands a light blue-gray. The Fontana technique stained the globular isotropic pigment of normal apocrine glands of the anal sac brown-black. Osmium tetroxide-, Fontana-, and Bodian-stained sections of tumors were uniformly negative.

Parathyroid glands were atrophic in eleven of 16 dogs. Atrophic parathyroids were characterized by narrow cords of inactive chief cells with abundant fibrous connective tissue and markedly widened perivascular spaces (fig. 1.8). The inactive chief cells were packed closely together with a
markedly reduced cytoplasmic area and prominent hyperchromatic nuclei. The enlarged parathyroid gland in one dog was interpreted as a chief cell adenoma, based on the degree of enlargement of one gland and atrophy of the remaining glands. Parathyroid glands were considered to be normal histologically in the remaining four dogs (two with hypercalcemia, two with normocalcemia). Diffuse or nodular proliferation of the parafollicular cells (C cells) was present in six of the hypercalcemic dogs and was absent in five.

Microscopic mineralization was present in the kidneys of nine of 10 hypercalcemic dogs and was interpreted as marked in three, moderate in two and mild in four dogs. Mineralization was most pronounced in tubules subjacent to the corticomedullary junction but was present in cortical and deep medullary tubules, Bowman's capsule, and glomerular tufts. Mineralization was present in the mucosa of the fundus of the stomach (three of eight dogs) and endocardium (two of nine dogs), but not in the lung of nine hypercalcemic dogs. Dogs with a calcium x phosphorus product of 50 or more had moderate to marked renal mineralization, while dogs with a product below 50 had mild or no mineralization.

Osteoclastic activity was increased in one dog in the rib, vertebra and ilium (fig. 1.9). Numerous osteoclasts lined trabecular bone which contained multiple shallow superficial excavations. This dog had a high calcium x phosphorus product (113) and blood urea nitrogen (74 mg/dl). In six other dogs with hypercalcemia associated with an adenocarcinoma derived from apocrine glands of the anal sac, microscopic examination of selected vertebrae, ilium, rib, femur (proximal and distal), humerus, and mandible showed no increased osteoclastic bone resorption. Osteocytic osteolysis was not seen and cementing
lines were smooth and linear. One dog had multiple metastases (dog 17, table 1.1) with localized osteolysis.
DISCUSSION

The findings in this study document a unique clinicopathologic syndrome characterized by persistent hypercalcemia and hypophosphatemia associated with an adenocarcinoma derived from apocrine glands of the anal sac. As previously reported (11) these neoplasms occur predominantly in old female dogs and are invariably malignant.

Adenocarcinomas of the glands of the anal sac often are occult, lying in the subcutaneous tissue adjacent to the anus and covered by freely movable haired skin. They have a characteristic histologic appearance, consisting of multiple lobules of neoplastic cells arranged in a solid and glandular pattern, separated by a moderate to abundant collagenous stroma. They contain varied numbers of acini and tubules lined by cuboidal to columnar epithelial cells, some of which have apical cytoplasmic blebs. These glandular structures often contain a secretory product and a rudimentary microvillus border.

Other glandular structures located in the perineal region that could be considered progenitor tissues for this neoplasm are the circumanal glands, the merocrine anal glands, and sebaceous and sweat glands of the skin. Adenomas of the circumanal glands are common neoplasms that affect predominantly male dogs, produce no systemic signs, and usually are benign (1, 2, 3). They generally are visible as protruding, multilobulated masses adherent to or extending through the skin of the perineum. Neither adenomas nor carcinomas of the circumanal glands form distinct acinar or tubular structures like those described in adenocarcinomas derived from apocrine glands of the anal sac. The merocrine anal glands (7), in most dogs necropsied in this study, were arranged in clusters medial to the anal sac and adjacent to the rectal mucosa. They were
clearly separated from the primary tumor by the internal anal sphincter and various amounts of connective tissue. Neoplasms derived from these glands would be expected to lie medial to the anal sac, rather than ventral or lateral to the anal sac as found in the majority of dogs in this study. An origin from sebaceous glands can be eliminated on the basis of morphologic characteristics of the neoplastic cells. Neoplasms arising from sweat glands in the dermis could conceivably form a pattern similar to that described in dogs with adenocarcinomas of apocrine glands of the anal sac. A close association with the epidermis and the sweat glands, however, was not found in any of the tumors in this investigation. Based on these observations and the emergence of glands of the anal sac with the neoplasms, the tumors were interpreted as adenocarcinomas derived from the apocrine glands of the anal sac. Considering the distinctive histologic appearance and relatively common occurrence of these neoplasms, it is peculiar that they have not been recognized more frequently. In a retrospective search conducted during this study, we found a number of cases that had been diagnosed as undifferentiated carcinoma, carcinoma of circumanal gland, and perianal carcinoma.

Despite the relative lack of anaplasia in these neoplasms, their biologic behavior was characteristically aggressive. Of the 23 dogs evaluated for metastasis, 22 had metastases to regional lymph nodes and occasionally to more distant sites. This observation is consistent with findings in the original report of this entity (11) in which metastases were present in seven of eight dogs. The rich supply of lymph vessels in the submucosa of the anal sac (7, 9) may account for this propensity for metastasis. Regardless of the well differentiated histologic appearance of this neoplasm, definitive evidence of malignancy always was found in the form of vascular or lymphatic or local tissue invasion.
Hypercalcemia is usually associated with this particular adenocarcinoma. A number of mechanisms can be postulated to explain persistent hypercalcemia in association with neoplasia. Hyperplasia or adenoma of the parathyroid glands is seen with increased frequency in people with malignant neoplasms (32, 33). Although one dog in this study had concomitant adenoma of the parathyroid gland and adenocarcinoma of the apocrine glands of the anal sac, this was the exception. A characteristic feature of this neoplasm was an associated trophic atrophy of all parathyroid glands, suggesting that these neoplasms do not produce a parathyroid-stimulating factor (34, 35) that would induce hypercalcemia secondary to stimulation of the parathyroid glands. The primary stimulus for parathyroid hormone secretion is decreased serum ionized calcium, although $\beta$-adrenergic stimulation also has been shown to mediate secretion both in vitro (36) and in vivo (37). The latter mechanism has been suggested as the explanation of hypercalcemia in one patient with a pheochromocytoma (34).

Hypercalcemia associated with a malignant neoplasm that does not metastasize to bone and with normal or inactive parathyroid glands often is referred to as pseudohyperparathyroidism or hypercalcemia of malignancy. Hypercalcemia is attributed to the production by the tumor of a humoral substance that induces resorption of calcium from bone (13, 25, 26). Rapid decline of serum calcium levels following tumor removal and the recurrence of hypercalcemia with tumor regrowth in man (24) and animals (11) supports this theory. Parathyroid hormone-like material has been identified in plasma, tumor venous effluent or neoplastic tissue in people with pseudohyperparathyroidism (26, 28, 38, 39, 40, 41). Increased levels of prostaglandin $E_2$ or its urinary metabolite also have been reported in association with hypercalcemia in people
with malignant neoplasms (42, 43, 44) in mice with fibrosarcoma (14), and in rabbits with VX₂ carcinoma (15). Osteoclast-activating factor has been identified in the medium from cultured myeloma and lymphosarcoma cells obtained from people with hypercalcemia (45). A recent report described an increased release of calcium from bone in vitro stimulated by the supernatant from cultured lymphosarcoma cells from dogs with hypercalcemia and normocalcemia (46).

The absence of primary renal lesions or markedly increased blood urea nitrogen and creatinine values, together with atrophic parathyroid glands, rules out secondary renal hyperparathyroidism or primary hyperparathyroidism as the mechanism responsible for the hypercalcemia in these dogs. Direct lysis of bone by metastatic tumor tissue was eliminated because metastases of the tumor to bone were found in only one dog in this study and none in previous reports (11, 47).

The presence of a humoral substance secreted by adenocarcinomas of the glands of the anal sac is strongly suggested by the rapid decline of serum calcium concentrations following excision of the tumor, and the subsequent return of hypercalcemia with tumor recurrence. A substance responsible for the development of hypercalcemia and hypophosphatemia in dogs with these tumors has not been reported; the most likely possibilities, however, include parathyroid hormone-like polypeptides, prostaglandins, and osteoclast-activating factor. One of the dogs with perirectal adenocarcinoma and hypercalcemia reported previously (11) had normal or elevated serum immunoreactive parathyroid hormone levels both before and after tumor removal. The production of active parathyroid hormone by adenocarcinomas derived from
tissues not normally associated with the secretion of hormone is not without precedent (25, 26, 38, 40).

Hypercalcemia was not always present in dogs with adenocarcinoma of the apocrine glands of the anal sac, as exemplified in this study by two tumor-bearing but normocalcemic dogs, and by other dogs that lacked clinical signs referable to hypercalcemia. Although several explanations are possible, two appear most likely. Firstly, hormone secretion may reflect a certain degree of differentiation and relatively poorly differentiated tumors may not be functional. Secondly, the level of hormone secreted may be low enough that a large mass of neoplastic tissue is necessary for detection of biologic activity. In support of the latter explanation is the observation that the parathyroid hormone content of malignant neoplasms in people with pseudohyperparathyroidism has ranged from only 0.75 to 8.9 μg per gram dry weight (26, 39, 40, 48) as compared to 200 to 600 μg per gram dry weight in normal and adenomatous parathyroid tissue (26, 48).

The absence of radiographically detectable bone loss in the dogs of this study is consistent with observations in people (49) and rabbits (50) with pseudohyperparathyroidism. In one study in rabbits bone loss was reported to be localized to bone immediately adjacent to the transplanted VX_2 carcinoma (50). Using morphometric techniques or magnification radiography, or both, two separate investigations have concluded that there was an increase in osteoclast numbers (51, 52) and generalized osteopenia (52) in rabbits with VX_2 carcinoma and hypercalcemia. Increased osteoclastic bone resorption was not a consistent finding in dogs with perirectal adenocarcinoma and hypercalcemia. Morphometric evaluation of bone in close proximity to the tumors and some distance
away may help quantitate the degree of bone loss in dogs with hypercalcemia associated with this malignant neoplasm in the perirectal region.

The lack of soft tissue mineralization may be attributable to low phosphorus values. Those dogs with the most pronounced soft tissue mineralization had the highest phosphorus, and calcium $\times$ phosphorus product. Soft tissue mineralization was moderate to marked when the calcium $\times$ phosphorus product was 50 or more, but was mild or absent when the product was below 50. Calcium $\times$ phosphorus values of 60 or more often are associated with soft tissue mineralization in man (53). Infusion of phosphate to normocalcemic and hypercalcemic people with either primary hyperparathyroidism or hypercalcemia and malignancy lowered serum calcium without increasing calcium excretion (54).

From a diagnostic viewpoint this syndrome is distinctive. It is important that this neoplasm be recognized as an entity distinct from adenoma of the circumanal gland because of the less favorable prognosis and the spectrum of associated functional disturbances. Recognition of hypercalcemia and associated clinical signs in an old female dog should suggest the possibility of adenocarcinoma of the apocrine glands of the anal sac. Serum calcium determinations are a useful index for the recurrence of this neoplasm. The occurrence of this unique syndrome in the dog provides the opportunity for future research on the pathogenetic mechanisms responsible for the development of hypercalcemia associated with neoplasia.
SUMMARY

Clinical, gross, and light microscopic findings are described for 36 dogs, 33 females and three males, with adenocarcinomas arising from the apocrine glands of the anal sac. All tumors had light microscopic features of malignancy and 22 of 23 metastasized to iliac and lumbar lymph nodes. Nine dogs had disseminated metastases, but bone metastases were found in only one dog. Differentiated neoplasms formed secretory acini and tubules lined by tall columnar or cuboidal epithelium. Most neoplasms were histologically bimorphic, with glandular areas and solid nests. Parathyroid glands were atrophic. Hypercalcemia (mean = 16.1 mg/dl) was present in 20 of 22 dogs and hypophosphatemia (mean = 3.2 mg/dl) in 12 of 17. Remission of hypercalcemia by tumor ablation and recurrence of hypercalcemia with tumor regrowth suggested that the tumor produced a substance that caused hypercalcemia. This unique clinicopathologic syndrome is characterized by hypercalcemia in old, predominantly female, dogs with an adenocarcinoma arising from the apocrine glands of the anal sac.
Table 1.1. Biochemical data from dogs with adenocarcinoma derived from the apocrine glands of the anal sac

<table>
<thead>
<tr>
<th>Dog</th>
<th>Ca (mg/dl)</th>
<th>P (mg/dl)</th>
<th>ALP&lt;sup&gt;1&lt;/sup&gt; (iu/l)</th>
<th>Albumin (gm/dl)</th>
<th>TPP&lt;sup&gt;2&lt;/sup&gt; (gm/dl)</th>
<th>BUN (mg/dl)</th>
<th>Creatinine (mg/dl)</th>
<th>AST&lt;sup&gt;3&lt;/sup&gt; (iu/l)</th>
<th>ALT&lt;sup&gt;4&lt;/sup&gt; (iu/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14.2</td>
<td>2.0</td>
<td>230</td>
<td>3.9</td>
<td>5.9</td>
<td>11</td>
<td>0.9</td>
<td>40</td>
<td>99</td>
</tr>
<tr>
<td>2</td>
<td>15.8</td>
<td>2.1</td>
<td>112</td>
<td>3.2</td>
<td>6.5</td>
<td>8</td>
<td>1.3</td>
<td>25</td>
<td>22</td>
</tr>
<tr>
<td>3</td>
<td>21.6</td>
<td>3.0</td>
<td>115</td>
<td>2.8</td>
<td>6.6</td>
<td>25</td>
<td>1.0</td>
<td>37</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>15.8</td>
<td>5.7</td>
<td>54</td>
<td>3.1</td>
<td>7.2</td>
<td>61</td>
<td>2.8</td>
<td>17</td>
<td>40</td>
</tr>
<tr>
<td>5</td>
<td>15.0</td>
<td>1.7</td>
<td>209</td>
<td>3.3</td>
<td>7.1</td>
<td>12</td>
<td>0.9</td>
<td>2</td>
<td>23</td>
</tr>
<tr>
<td>6</td>
<td>13.4</td>
<td>3.5</td>
<td>356</td>
<td>3.2</td>
<td>6.5</td>
<td>33</td>
<td>0.9</td>
<td>2</td>
<td>68</td>
</tr>
<tr>
<td>7</td>
<td>18.6</td>
<td>3.0</td>
<td>26</td>
<td>3.5&lt;sup&gt;5&lt;/sup&gt;</td>
<td>6.6</td>
<td>23</td>
<td>1.3</td>
<td>21</td>
<td>28</td>
</tr>
<tr>
<td>8</td>
<td>24.0</td>
<td>4.7</td>
<td>32</td>
<td>ND&lt;sup&gt;5&lt;/sup&gt;</td>
<td>6.8&lt;sup&gt;5&lt;/sup&gt;</td>
<td>74</td>
<td>1.4</td>
<td>44</td>
<td>18</td>
</tr>
<tr>
<td>9</td>
<td>12.2</td>
<td>5.5</td>
<td>36</td>
<td>3.8</td>
<td>ND&lt;sup&gt;5&lt;/sup&gt;</td>
<td>14</td>
<td>0.9</td>
<td>34</td>
<td>42</td>
</tr>
<tr>
<td>10</td>
<td>17.1</td>
<td>3.7</td>
<td>23</td>
<td>3.3</td>
<td>6.8</td>
<td>7</td>
<td>0.5</td>
<td>96</td>
<td>20</td>
</tr>
<tr>
<td>11</td>
<td>11.8</td>
<td>2.3</td>
<td>49</td>
<td>3.3</td>
<td>6.3</td>
<td>19</td>
<td>1.5</td>
<td>46</td>
<td>22</td>
</tr>
<tr>
<td>12</td>
<td>11.8</td>
<td>3.8</td>
<td>264</td>
<td>3.1</td>
<td>6.7</td>
<td>11</td>
<td>0.8</td>
<td>38</td>
<td>32</td>
</tr>
<tr>
<td>13</td>
<td>13.8</td>
<td>3.2</td>
<td>43</td>
<td>2.9</td>
<td>7.0</td>
<td>25</td>
<td>1.4</td>
<td>70</td>
<td>34</td>
</tr>
<tr>
<td>14</td>
<td>21.9</td>
<td>1.9</td>
<td>23</td>
<td>2.6&lt;sup&gt;5&lt;/sup&gt;</td>
<td>5.9</td>
<td>25</td>
<td>1.0</td>
<td>234&lt;sup&gt;5&lt;/sup&gt;</td>
<td>31</td>
</tr>
<tr>
<td>15</td>
<td>20.1</td>
<td>2.6</td>
<td>103</td>
<td>ND&lt;sup&gt;5&lt;/sup&gt;</td>
<td>8.4</td>
<td>62</td>
<td>2.5</td>
<td>ND&lt;sup&gt;5&lt;/sup&gt;</td>
<td>47</td>
</tr>
<tr>
<td>16</td>
<td>11.4</td>
<td>2.7</td>
<td>315</td>
<td>3.5</td>
<td>8.1</td>
<td>20</td>
<td>1.0</td>
<td>48</td>
<td>55</td>
</tr>
<tr>
<td>17</td>
<td>14.4</td>
<td>3.4</td>
<td>207</td>
<td>2.8</td>
<td>6.7</td>
<td>15</td>
<td>0.7</td>
<td>72</td>
<td>98</td>
</tr>
</tbody>
</table>

Mean 16.1 3.2 129 3.2 6.8 26 1.2 52 41
n 17 17 17 15 16 17 17 16 17
SE<sup>6</sup> 1.0 0.3 27 0.1 0.2 5 0.1 14 6
Range 11.8-24 1.7-5.7 23-356 2.6-3.9 5.6-8.4 7-62 5-2.8 <2-234 16-99
Normal<sup>7</sup> 9.6-11.2 3.6-6.0 0-67 2.3-3.2 6-7.5 9-29 0.4-1.0 8-29 9-38

<sup>1</sup> ALP = alkaline phosphatase  <sup>2</sup> TPP = total plasma protein  <sup>3</sup> AST = aspartate aminotransferase  
<sup>4</sup> ALT = alanine aminotransferase  <sup>5</sup> ND = not determined  
<sup>6</sup> SE = standard error  
<sup>7</sup> Normal laboratory range of the dog. Department of Veterinary Clinical Sciences, The Ohio State University.
Table 1.2. Calcium and phosphorus values before and after tumor removal and with subsequent recurrence in five dogs with adenocarcinoma of the glands of the anal sac

<table>
<thead>
<tr>
<th>Dog</th>
<th>Ca (mg/dl)</th>
<th>P (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tumor present</td>
<td>14.2</td>
</tr>
<tr>
<td></td>
<td>Removal</td>
<td>9.0</td>
</tr>
<tr>
<td>1</td>
<td>Recurrence (3)</td>
<td>16.8</td>
</tr>
<tr>
<td></td>
<td>Removal</td>
<td>10.4</td>
</tr>
<tr>
<td></td>
<td>Recurrence (15)</td>
<td>20.8</td>
</tr>
<tr>
<td>2</td>
<td>Tumor present</td>
<td>15.8</td>
</tr>
<tr>
<td></td>
<td>Removal</td>
<td>9.0</td>
</tr>
<tr>
<td>3</td>
<td>Tumor present</td>
<td>21.6</td>
</tr>
<tr>
<td></td>
<td>Removal</td>
<td>11.4</td>
</tr>
<tr>
<td></td>
<td>Recurrence (5)</td>
<td>16.0</td>
</tr>
<tr>
<td>4</td>
<td>Tumor present</td>
<td>15.8</td>
</tr>
<tr>
<td></td>
<td>Removal</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>Recurrence (12)</td>
<td>14.3</td>
</tr>
<tr>
<td></td>
<td>Removal</td>
<td>9.2</td>
</tr>
<tr>
<td>5</td>
<td>Tumor present</td>
<td>15.0</td>
</tr>
<tr>
<td></td>
<td>Removal</td>
<td>10.1</td>
</tr>
</tbody>
</table>

\(^1\) ND = not determined.

( ) = months following original excision of perirectal mass
Table 1.3. Summary of clinical features in 36 dogs with adenocarcinoma derived from the apocrine glands of the anal sac

<table>
<thead>
<tr>
<th>Sex</th>
<th>Female</th>
<th>33/36</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intact</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Castrated</td>
<td>22</td>
</tr>
<tr>
<td>Male</td>
<td>3/36</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Intact</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Castrated</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Age:</th>
<th>Range (years)</th>
<th>7-13</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (years)</td>
<td>10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Breed:</th>
<th>German Shepherd</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mongrel</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Poodle</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Schnauzer, Malamute, Irish Terrier, Keeshond, German Shorthair Pointer, Cocker Spaniel, Labrador Retriever, Dachshund, English Cocker, Golden Retriever, Springer Spaniel, Bassett, Welsh Corgi - 1 each</td>
<td></td>
</tr>
</tbody>
</table>

Clinical signs reported by owners:

- Anorexia: 14/36
- Weakness or lethargy: 12/36
- Polyuria, polydipsia: 12/36
- Perirectal mass: 15/36

<table>
<thead>
<tr>
<th>Tumor Location</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Unilateral</td>
<td>29</td>
</tr>
<tr>
<td>Bilateral</td>
<td>4</td>
</tr>
<tr>
<td>Not determined</td>
<td>3</td>
</tr>
</tbody>
</table>
Fig. 1.1: Transverse section of perineum from female dog with hypercalcemia and adenocarcinoma of apocrine glands of anal sac. Anal sacs (A) on both sides of rectum (R). Tumor nodule (arrow) 1 cm in diameter in wall of left anal sac protrudes into its lumen. Bar represents 1 cm.
Fig. 1.2: Adenocarcinoma (T) in apocrine glands (G) subjacent to stratified squamous epithelial lining of anal sac (A).
Fig. 1.3: Adenocarcinoma derived from apocrine glands of anal sac form acini (A) and tubules (T) lined by columnar or cuboidal epithelial cells. Lumen contains eosinophilic material (arrow).
Fig. 1.4: Solid pattern of adenocarcinoma derived from apocrine glands of anal sac with polyhedral and columnar cells forming pseudorosettes around small capillaries (C).
Fig. 1.5: Prominent tubuloacini (A) lined by tall cylindrical cells with multiple apical cytoplasmic projections (arrow) into lumen in adenocarcinoma derived from apocrine glands of anal sac.
Fig. 1.6: Bimorphic growth pattern in adenocarcinoma derived from apocrine glands of anal sac with adjacent solid (S) areas and acini (A) formed by neoplastic cells.
Fig. 1.7: Tumor cell emboli (arrow) of apocrine adenocarcinoma of anal sac in lymphatic in bronchial lymph node.
Fig. 1.8: Atrophic parathyroid gland from dog with hypercalcemia associated with adenocarcinoma of apocrine glands of anal sac. Narrow cords of inactive chief cells (arrow), with reduced cytoplasmic area and hyperchromatic nuclei, are separated by prominent interstitial spaces (I) with increased fibrous connective tissue.
Fig. 1.9: Osteoclastic osteolysis in ilium of dog with adenocarcinoma derived from apocrine glands of anal sac. Osteoclasts (arrows) aligned along trabecular bone surfaces with excavations.
CHAPTER II

ULTRASTRUCTURAL EVALUATION OF ADENOCARCINOMAS
DERIVED FROM APOCRINE GLANDS OF THE ANAL SAC
ASSOCIATED WITH HYPERCALCEMIA IN DOGS

Introduction

Carcinomas and sarcomas of nonparathyroid origin have been associated with the syndrome of hypercalcemia and malignancy in animals and human beings.\textsuperscript{1-7} When metastases to bone are not present, hypercalcemia has been attributed to the production and secretion of bone-resorbing substances by tumor cells. Hypercalcemic factors associated with this syndrome include parathyroid hormone\textsuperscript{1,8-10} parathyroid hormone-like peptides,\textsuperscript{11-13} prostaglandin E\textsubscript{2},\textsuperscript{14-17} osteolytic sterols,\textsuperscript{18} and osteoclast-activating factor.\textsuperscript{19,20} Another humoral factor recently has been described which shares certain biologic properties with parathyroid hormone but which is distinct from native parathyroid hormone (1-84 amino acid sequence).\textsuperscript{21} Presumably there are other factors which are not yet characterized. Detailed descriptions have been infrequently reported on the ultrastructural characteristics of these nonendocrine tumors associated with hypercalcemia.\textsuperscript{22-24}

An adenocarcinoma that occurs in the perirectal area of older female dogs results in persistent hypercalcemia in 90% of tumor-bearing animals but rarely metastasizes to bone.\textsuperscript{25,26} Tumor excision results in a return to
normocalcemia and tumor recurrence is associated with a return of hypercalcemia, suggesting that the adenocarcinomas produce a hypercalcemic factor. Assay of plasma and tumor tissue from these dogs revealed that the calcium-mobilizing factor produced by these tumors was neither immunoreactive parathyroid hormone nor prostaglandin E₂.²⁷ The adenocarcinomas associated with hypercalcemia appear to be derived from apocrine glands which encompass the anal sac. Apocrine glands of the anal sac are distinct from the merocrine anal glands which are found in this region in many species, including man.²⁸

The specific objectives of this investigation were to evaluate the ultrastructural characteristics of neoplasms derived from apocrine glands of the anal sac associated with hypercalcemia for evidence of synthetic and secretory activity, and to compare the ultrastructural features of neoplastic cells with apocrine glands of the anal sac from control dogs.
Materials and Methods

Ultrastructural evaluation was performed on 10 female dogs (mean 10 yr, range 7-13 yr) with hypercalcemia (mean 16.1 mg/dl, range 14.2 to 19.2 mg/dl) and adenocarcinomas derived from apocrine glands of the anal sac. Apocrine glands of the anal sac were studied in eight control dogs (mean 6 yr, range 3-12 yr; serum calcium 10.4 mg/dl, range 8.3 to 15 mg/dl). All dogs were killed with an overdose of barbiturate and small cubes of tumor tissue or wall of the anal sac from control dogs were immediately immersed in cold 3% glutaraldehyde. Tissues were trimmed into one mm³ blocks, fixed in 3% glutaraldehyde with 0.1 M sodium cacodylate buffer at pH 7.4 for 2 hours, washed twice in 0.1 M cacodylate buffer, and post-fixed in 1.33% osmium tetroxide with s-collidine buffer at pH 7.4 for 1 hour. They were dehydrated through ascending concentrations of ethyl alcohol, transferred to propylene oxide, and embedded in Epon 812 (Shell Chemical Co., New York, NY). One-micron thick sections were cut from each block and were stained with toluidine blue for light microscopic evaluation and selection of the most appropriate area of the block for sectioning. Thin sections were cut at 600 to 800 Å on a Reichert Om U2 ultramicrotome and mounted on 300 mesh copper grids. They were stained with uranyl acetate and lead citrate and examined with a Philips 200 or 300 transmission electron microscope.

Tissues for light microscopy were fixed in 10% neutral phosphate-buffered formalin, routinely processed, and sectioned at 6 um. Selected sections of neoplastic tissue with adjacent anal sac from ten hypercalcemic dogs with adenocarcinomas and the anal sac region from the eight control dogs (5 females, 3 males; mean 6 yr., range 3-12 yr.) were stained with hematoxylin and eosin (HE), periodic acid-Schiff (PAS), alcian blue, Fontana-Masson for
argentaffin granules, and Azzopardi's modification of the Bodian stain for argyrophil granules. Sections of tumor and the normal apocrine glands of the anal sac were also stained with osmium tetroxide for fat.
Results

Adenocarcinomas derived from apocrine glands of the anal sac were in the subcutaneous tissue of the perineum and were closely situated to one or both anal sacs. The neoplasms did not directly contact the colon, rectum, merocrine anal glands or the overlying epidermis of the perineum. They expanded ventrally and laterally from their primary site and cranially into the pelvic canal. The adenocarcinomas merged with the apocrine glands which encompassed the anal sac and compressed the squamous epithelial lining of the anal sac.

Adenocarcinomas of the anal sac had a bimorphic histologic pattern characterized by distinct glandular areas and solid lobules (Figure 1). In glandular regions acini and tubules were lined by tall columnar cells which either had blebs of cytoplasm at cell apices or double lines of resolution, suggesting a brush border. Pseudorosettes were common in solid lobules and were characterized by a rim of neoplastic cells that had a nuclear-free zone adjacent to a central capillary. Distant metastases were histologically similar to the primary tumor and were present in iliac and sublumbar lymph nodes in all dogs. Detailed clinical, macroscopic and microscopic characteristics of dogs with this syndrome have been reported elsewhere.

Solid and glandular areas also were observed on ultrastructural evaluation of the adenocarcinomas. Acini were lined by tall cuboidal to columnar epithelial cells limited by a prominent basement membrane (Figure 2). Although acini were present in all adenocarcinomas derived from apocrine glands of the anal sac, their numbers varied considerably between neoplasms. Numerous microvilli partially covered the luminal surface of neoplastic cells.
forming acini. Prominent blebs of cytoplasm, characteristic of apocrine cells, often protruded from apices of tumor cells (Figure 3). These cytoplasmic protrusions were devoid of organelles and were relatively electron-lucent. Desmosomes were prominent along cell borders and tight junctions were present near cell apices (Figure 2). Plasma membranes of adjacent neoplastic cells often were interdigitated. Nuclei were round to oval and uniform in size and shape. They had a peripheral rim of dense nuclear chromatin and a pale central region containing one or two nucleoli.

The rough endoplasmic reticulum was well developed in neoplastic cells and consisted of short profiles, occasional lamellar arrays, and long individual profiles (Figure 4). Cisternae contained a homogeneous granular material. Free polyribosomes were dispersed through the cytoplasm. A prominent Golgi apparatus was observed in most neoplastic cells and consisted of two to four layers of agranular membranes (Figure 5). Small vesicles, approximately 80 to 200 nm in diameter, protruded from the membranes of the Golgi apparatus. These vesicles were either empty or partially filled with finely granular material. Microtubules were present in most neoplastic cells and microfilaments often were aggregated into clusters (Figure 6).

Two types of osmiophilic granules were observed in neoplastic cells (Figure 6). Small (150 to 400 nm in diameter) granules had a limiting membrane, narrow submembraneous space, and homogeneous dense core. They usually were associated with the Golgi apparatus or were situated near cell apices (Figure 7). Larger osmiophilic bodies (0.6 to 2.2 microns in diameter) had a poorly delineated limiting membrane and usually were situated near the nucleus or basilar aspect of the cell (Figure 6). These larger lysosome-like
granules were of variable electron density and often had prominent electron-lucent areas. Occasional intracytoplasmic granules were PAS-positive but cells from both neoplastic and normal apocrine glands of the anal sac did not contain granules which stained with either osmium tetroxide, Fontana-Masson, or Azzopardi's modification of Bodian silver reaction. Mitochondria were numerous in neoplastic cells and varied in size and shape.

Control dogs and dogs with adenocarcinomas had circumanl glands and merocrine anal glands which were distinct from apocrine glands of the anal sac. Circumanal glands were located in the connective tissue encompassing the anus and consisted of solid lobules containing large polyhedral eosinophilic cells. Circumanal glands also were present along the excretory duct of the anal sac and in one dog there was a small cluster of circumanl glands admixed with apocrine glands subjacent to the anal sac. Merocrine anal glands were subjacent to the anorectal mucosa and usually were medial to the internal anal sphincter, but occasionally penetrated through the sphincter. They had tortuous ducts which emptied at the mucocutaneous junction of the anus. Acini of merocrine glands were lined by low cuboidal cells that were devoid of cytoplasmic blebs. The circumanl glands and merocrine anal glands were located several centimeters from the tumor mass.

Apocrine glands in control dogs were subjacent to the excretory duct of the anal sac and formed a mantel in the substantia propria beneath the fundus of the anal sac. They were visible grossly as a two mm band of brown tissue encircling the anal sac. Ultrastructural evaluation revealed that apocrine glands of the anal sac varied in size and shape depending on their stage of secretory activity. Apocrine cells interpreted to be active were tall and
cylindrical, and had protrusions of cytoplasm which projected into acini (Figure 8). These apical cytoplasmic projections were electron-lucent and devoid of either secretory granules or organelles. Apocrine cells in the secretory phase were filled with dilated profiles of rough endoplasmic reticulum that contained an amorphous granular material. Membranes of endoplasmic reticulum appeared to fuse with the plasmalemma within the apical cytoplasmic projections and discharge their contents directly into the acinar lumen, characteristic of merocrine secretion (Figure 9). Inactive apocrine cells lined the majority of acini in control dogs. They were low cuboidal to squamous and had flat apical surfaces with short microvilli (Figure 10). Apocrine cells interpreted to be inactive had short profiles of rough endoplasmic reticulum, a small Golgi apparatus, numerous large osmiophilic bodies, and scattered microtubules and microfilaments (Figure 11). The osmiophilic bodies were up to 2.2 microns in diameter and were present in the basilar and perinuclear regions. Smaller electron-dense granules (150 to 400 nm in diameter) with a closely applied limiting membrane were uncommon and were located adjacent to the Golgi apparatus or near the luminal aspect of the cell.

Myoepithelial cells were present between epithelial cells and the basement membrane in apocrine glands of the anal sac from control dogs. They had an electron-dense cytoplasm that was packed with microfilaments (Figure 10). There was a small pale zone adjacent to the nucleus that was free of microfilaments and contained several short profiles of rough endoplasmic reticulum, mitochondria, and a small Golgi apparatus.
Discussion

Adenocarcinomas derived from apocrine glands of the anal sac occurred predominantly in female dogs and were consistently associated with hypercalcemia.\textsuperscript{25,26} The return to normocalcemia following tumor excision and the recurrence of hypercalcemia associated with tumor regrowth, suggested the tumors produced a hypercalcemic factor.\textsuperscript{25,26} Previous investigations have reported that the humoral factor in dogs with adenocarcinomas derived from apocrine glands of the anal sac was not immunoreactive parathyroid hormone or prostaglandin E\textsubscript{2}.\textsuperscript{27} Despite the finding that plasma levels of these calcium-mobilizing substances were not significantly elevated in dogs with hypercalcemia and adenocarcinomas as compared to control dogs, there was evidence of increased bone resorption in the lumbar vertebrae.\textsuperscript{27} Histomorphometric evaluation demonstrated that dogs with hypercalcemia and adenocarcinomas had reduced trabecular bone volume, increased resorptive surfaces and increased numbers of osteoclasts/mm of trabecular bone surface.

The present ultrastructural investigations demonstrated that adenocarcinoma cells from dogs with hypercalcemia had well developed synthetic and secretory organelles that are associated with the production of polypeptide hormones. The rough endoplasmic reticulum and free polyribosomes were abundant and a prominent Golgi apparatus was associated with numerous vesicles in neoplastic cells. Microtubules and microfilaments were numerous in tumor cells of dogs with hypercalcemia. Previous ultrastructural investigations of neoplastic cells in other animal models of hypercalcemia associated with malignancy have described the presence of rough endoplasmic reticulum and polyribosomes, but secretory granules were not reported.\textsuperscript{22-24} The tumor
cells in dogs with adenocarcinomas derived from apocrine glands of the anal sac had small (150-400 nm diameter) granules with a sharply delineated membrane and a narrow submembraneous space that were similar in size and structure to secretion granules in polypeptide hormone-secreting endocrine cells, such as parathyroid chief cells. These secretory granules were more common in neoplastic cells than normal apocrine cells, and were usually located near cell apices. Although they were compatible ultrastructurally with primary lysosomes or microperoxisomes, their resemblance to hormone-containing secretory granules suggests that immunocytochemical studies should be performed to determine if they contain parathyroid hormone or other bone-resorbing factors.

The well differentiated nature of apocrine adenocarcinomas derived from the anal sac was suggested by the numerous microvilli, desmosomal attachments, and production of a basement membrane. Desmosomes, tonofilaments, and microvilli have been reported in other neoplasms associated with hypercalcemia, probably due to the frequent association with epithelial tumors.

Previous studies suggested that the origin of perirectal adenocarcinomas associated with hypercalcemia in older female dogs was from apocrine glands of the anal sac. The electron microscopic observations in this report which support this conclusion were the apocrine-like cytoplasmic blebs, similar types of electron-dense granules, plus acini and tubules lined by columnar cells with a microvillar border in both neoplastic and normal cells of apocrine glands of the anal sac.

In normal glands of the anal sac, superficial blebs of cytoplasm protruded into the lumina of acini and eventually were detached from the subjacent
cytoplasm, which is characteristic of the apocrine secretion. These cytoplasmic protrusions and the detached blebs were electron-lucent and devoid of cytoplasmic organelles or secretory granules. Apocrine cells interpreted to be in the secretory phase contained large dilated profiles of rough endoplasmic reticulum filled with a granular material. They appeared to migrate to the cell apices where they fused with the plasma membrane and released their product directly into the lumina of acini. This process was more suggestive of merocrine secretion than an apocrine type, and was comparable to the secretion of the protein components of milk by mammary epithelial cells. The apocrine-like blebs in the glands of the anal sac were interpreted to be a form of ecdysis and represent cellular involution following the phase of secretory activity. Low cuboidal cells which had short profiles of rough endoplasmic reticulum and an electron-dense cytoplasm were interpreted to be in a resting phase of the secretory cycle.

Several characteristics suggested that the normal apocrine cells of the anal sac and the neoplasms derived from these cells are not part of the amine-precursor, uptake, decarboxylation (APUD) series. These features included a lack of staining for argentaffin and argyrophil granules, and the relatively infrequent occurrence and apical location of small electron-dense granules by electron microscopy. Furthermore, the large dilated profiles of rough endoplasmic reticulum, evidence for merocrine type of secretion, and the formation of glandular acini containing a secretory product are not characteristics of APUD cells.

Mediators of hypercalcemia associated with malignancy other than parathyroid hormone and prostaglandin E\textsubscript{2} include vitamin D sterols, osteoclast activating factor, and factors not yet characterized. There are a large
percentage of human patients with hypercalcemia and malignancy in which the hypercalcemic factor apparently is not parathyroid hormone or the other known bone resorbing substances. Similarly, dogs with adenocarcinomas derived from apocrine glands of the anal sac have hypercalcemia and increased bone resorption in the absence of increased circulating concentrations of either parathyroid hormone or metabolites of prostaglandin E₂. Ultrastructural studies reported here suggest that the neoplastic cells have well developed synthetic and secretory organelles necessary for the production of polypeptide hormones. Characterization of the contents of the secretory-like granules in tumor cells and additional biochemical studies designed to extract bone resorbing substances from the plasma or tumor tissue from dogs with this syndrome will further our understanding of the pathogenesis of hypercalcemia associated with malignancy in man and animals.
Summary

Adenocarcinomas derived from apocrine glands of the anal sac and associated with persistent hypercalcemia in dogs were composed of tumor cells with numerous profiles of rough endoplasmic reticulum, clusters of free ribosomes, and prominent Golgi apparatuses. Neoplastic cells contained microtubules, microfilaments, tonofibrils, and had two types of electron-dense granules. Large lysosome-like dense bodies ranged from 0.6 to 2.2 microns in diameter and had a poorly delineated limiting membrane. Small granules (150 to 400 nm in diameter) had a sharply delineated limiting membrane with a narrow submembranous space and a homogenous dense core. These smaller granules usually were located near apices of neoplastic cells whereas the larger granules were situated near the base of cells. Two types of granules were also present in cells from apocrine glands of control dogs but smaller granules were infrequent and the large lysosome-like bodies common. Apocrine cells in glands of the anal sac from control dogs that were in the secretory phase were columnar and had large dilated profiles of rough endoplasmic reticulum. Membranes of the endoplasmic reticulum fused with the plasmalemma and appeared to secrete their product directly into the lumina of acini, characteristic of merocrine secretion. Apical blebs of electron-lucent cytoplasm pinched off from non-neoplastic apocrine cells and were released into glandular lumens. Similar electron-lucent cytoplasmic blebs were present at apices of tumor cells. Myoepithelial cells were present between the epithelial cells and basement membrane in normal apocrine glands and were absent in neoplasms derived from these glands. Identification of the contents of the secretory-like granules in tumor cells and characterization of the hypercalcemic factor in the plasma or tumor tissue from dogs with this
syndrome may help explain the pathogenesis of hypercalcemia associated with this malignancy.
**Figure 2-1** - Bimorphic pattern in an adenocarcinoma arising from apocrine glands of the anal sac in a dog with hypercalcemia illustrating characteristic acini with central lumens adjacent to solid (S) microlobules. (H&E X215).
Fig. 2.1
Figure 2-2 - Tall columnar cells with microvilli (V) and a prominent basement membrane (right) lining a tubule in an adenocarcinoma derived from apocrine glands of the anal sac. Adjacent tumor cells are joined by tight junctions and desmosomes (D). A Golgi apparatus (G) is present in most cells. Tumor cells contain scattered mitochondria and many small electron-dense granules (arrows). (Uranyl acetate and lead citrate, X3,900).
Fig. 2.2
Figure 2-3 - Characteristic apocrine-like cytoplasmic blebs protruding into the lumen (L) of an acinus. The cytoplasm is electron-lucent and devoid of organelles. Apocrine gland adenocarcinoma in a dog with hypercalcemia. N = nucleus of tumor cell. (Uranyl acetate and lead citrate, X6,400).
Figure 2-4 - Solid area from an apocrine gland adenocarcinoma composed of polygonal tumor cells that have long profiles of rough endoplasmic reticulum and numerous mitochondria in a dog with hypercalcemia. (Uranyl acetate and lead citrate, X9,700.)
Figure 2-5 - Adenocarcinoma derived from apocrine glands of the anal sac illustrating a well-developed Golgi apparatus (G), microtubules (arrows), short profiles of rough endoplasmic reticulum, free polyribosomes, and membrane-limited secretory granules. Desmosomes (D) join two adjacent tumor cells. (Uranyl acetate and lead citrate, X14,300.)
Figure 2-6 - Apical portion of a tumor cell containing a cluster of electron-dense granules (S) that vary in size from 200-400 nm in diameter. Large, electron-dense bodies (500-1000 nm in diameter) are adjacent to the nucleus. Clusters of microfilaments are present in the cytoplasm (arrows). (Uranyl acetate and lead citrate, X8,900.)
Figure 2-7 - Small electron-dense secretory granules (arrows, 200-400 nm in diameter), in neoplastic cells with an electron-dense core, closely applied limiting membrane, and narrow submembraneous space. Dog with hypercalcemia associated with an adenocarcinoma derived from apocrine glands of the anal sac. (Uranyl acetate and lead citrate, X34,300.)
Figure 2-8 - Acinus in an apocrine gland of the anal sac from a control dog lined by tall columnar cells with basilar nuclei. Note the electron-lucent cytoplasmic blebs (arrows) with laminated bodies. Large osmiophilic bodies (B) of varying size are located at the basilar aspect of the cells. A cytoplasmic projection from a myoepithelial cell (M) is located subjacent to the epithelial cells. (Uranyl acetate and lead citrate, X4,100.)
Figure 2-9 - Apocrine cells of the anal sac in an active stage of secretory activity with dilated cisternae (E). These large vesicles fused with the plasma membrane and secreted their product by a merocrine type of secretion. Apocrine gland of anal sac from a control dog. (Uranyl acetate and lead citrate, X5,500.)
Figure 2-10-Inactive cuboidal apocrine cell with a large nucleus (N), prominent Golgi apparatus (G), small profiles of endoplasmic reticulum, electron-dense granules of different sizes and density from a control dog. Microvilli (arrowheads) and desmosomes (D) are present at the luminal surface. A basal myoepithelial cell (M) is attached to the epithelial cell by a desmosome (arrow) and has a convoluted nucleus with dense clusters of filaments in the cytoplasm. (Uranyl acetate and lead citrate, X7,600.)
Figure 2-11- Microvilli present on the surface of apocrine cells from glands of the anal sac. Tight junctions (T) and desmosomes (D) join adjacent cells. The perinuclear Golgi (G) apparatus is associated with small granules of low electron density. Numerous microfilaments (arrowheads) are present in the cytoplasm. Several types of cytoplasmic granules (arrows) of varying size and electron density are present in apocrine cells of control dogs. (Uranyl acetate and lead citrate, X15,000.)
CHAPTER III

HYPERCALCEMIA IN DOGS WITH ADENOCARCINOMA DERIVED FROM APOCRINE GLANDS OF ANAL SAC:
BIOCHEMICAL AND HISTOMORPHOMETRIC INVESTIGATIONS

INTRODUCTION

Hypercalcemia in patients with cancer may be the result of concurrent parathyroid hyperplasia, metastases to bone, or production of hypercalcemic factors by the tumor cells. Hypercalcemia frequently has been associated with malignant nonparathyroid neoplasms without metastases to bone that purportedly secrete bone-resorbing compounds (1,2). In vitro and in vivo studies have defined five bone-resorbing factors thought to be produced by tumors: parathyroid hormone (3,4), parathyroid hormone-like-peptides (5,6), prostaglandin E₂ (7,8), osteoclast-activating factor (OAF) (9), and vitamin D-like sterols (10). However, the substance produced by the tumor is not known in many human patients with solid neoplasms and hypercalcemia (11,12).

Hypercalcemia is present in 90% of dogs with adenocarcinomas derived from apocrine glands of the anal sac (CA) (13,14). Remission of hypercalcemia following tumor excision and recurrence of hypercalcemia with growth of tumor metastases suggested that these adenocarcinomas produce a hypercalcemic factor. The mechanism by which the CA produce hypercalcemia is not known, but the tumor cells contain numerous profiles of rough endoplasmic reticulum,
well-developed Golgi apparatuses, prominent microtubules and microfilaments, and electron-dense granules with a limiting membrane and narrow submembranous space that resemble secretory granules in polypeptide hormone-secreting endocrine cells (15). Therefore, dogs with apocrine adenocarcinomas may represent a valuable model for defining more completely the hypercalcemic factors produced by neoplasms of nonendocrine origin.

The specific objectives of this investigation were to: 1) determine plasma concentrations of immunoreactive parathyroid hormone (iPTH), prostaglandin E₂M (PGE₂M), and 1,25-dihydroxyvitamin D (1,25-(OH)₂D) in dogs with hypercalcemia and CA compared to age-matched controls with and without other neoplasms; 2) quantitate the concentration of iPTH in tumor tissue; 3) evaluate bone resorption by histomorphometric methods and correlate these findings with urinary hydroxyproline, cyclic adenosine monophosphate (cAMP), and electrolyte excretion; and 4) evaluate parathyroid chief cells ultrastructurally for secretory activity.
MATERIALS AND METHODS

Animals

Ten dogs with hypercalcemia and adenocarcinoma derived from apocrine glands of the anal sac (CA), 6 dogs ("tumor controls") with normocalcemia and various solid neoplasms (pulmonary carcinoma, circumanal gland carcinoma, apocrine gland adenocarcinoma of skin, hemangiopericytoma of skin, rectal adenoma, and Sertoli cell tumor of the testis), fifteen adult control dogs without clinical evidence of disease, 3 dogs with hypercalcemia (mean serum calcium 14 mg/dl) and primary hyperparathyroidism, 3 dogs with hypoparathyroidism and hypocalcemia (mean serum calcium 4.9 mg/dl), and 6 dogs with chronic renal disease (mean serum urea nitrogen 157 mg/dl; mean serum creatinine 8.2 mg/dl) were used in this study. Dogs were age- and weight-matched. Two dogs with hyperparathyroidism had primary nodular parathyroid hyperplasia and one had a chief cell adenoma. One dog with nodular parathyroid hyperplasia had concurrent neoplasia of the apocrine glands of the anal sac. However, the CA was only 7mm in diameter, and it was the only tumor of 23 which did not metastasize (14). Light microscopic and ultrastructural evaluation of the parathyroids from this dog revealed marked chief cell hyperplasia. Therefore, this dog was excluded from the group of 10 dogs with CA and included with dogs with primary parathyroid hyperplasia or adenoma. The clinical and pathologic characteristics of the dogs with hypoparathyroidism have been described previously (16). Dogs with primary hyper- and hypoparathyroidism and chronic renal disease were included in the study to determine if the immunoassay for parathyroid hormone could detect the
expected differences in circulating hormone in canine serum under conditions of increased or decreased parathyroid activity.

Dogs with hypercalcemia were evaluated for bone metastases by antemortem radiographs of vertebrae and long bones, and by postmortem radiographs of the thoracic and lumbar vertebrae, humerus, femur, rib, mandible, and metacarpal or metatarsal bones using conventional and magnification (1.5 times) techniques (17). One femur, humerus, tibia, sternebra, several ribs; and lumbar, sacral and cervical vertebrae from hypercalcemic dogs were cut longitudinally, examined for macroscopic metastases, and selected areas were processed for light microscopy.

**Histomorphometry**

Fourth or fifth lumbar vertebrae were sectioned longitudinally, fixed in neutral phosphate-buffered formalin, and transferred to 70% ethyl alcohol. Vertebrae were embedded in methacrylate without decalcification and two 4-5 μ sections were cut 200 μ apart on a Jung Model K Sledge microtome and stained with von Kossa-tetrachrome. Both sections of vertebrae were evaluated by point counting and line-intercept techniques using 10X and 20X objectives with a Zeiss Integrationsplatte I eyepiece. Cortical and trabecular bone volume were determined by point-counting in a 14.6 mm² area (7.3 mm²/section) based on the formula: volume = \( \frac{\text{no. hits} \times \text{grid area}}{\text{no. throws} \times 100} \) (18). All other parameters were measured by intersect-counting in a 7.8 mm² area (3.9 mm²/section). All intersections of trabecular bone with horizontal and vertical lines were counted and the trabecular surface perimeter was calculated from the formula: perimeter = \( \frac{\sqrt{2} \times \text{no. hits}}{2} \times \text{distance between grid lines} \) (18). The following parameters were measured in the center of vertebrae avoiding cortical margins and the longitudinal ends of bone:
1. **Cortical bone volume**, expressed as a percentage of the cortical space composed of mineralized and nonmineralized bone matrix.

2. **Trabecular bone volume**, expressed as a percentage of the trabecular space composed of mineralized and nonmineralized bone matrix.


5. **Osteoclastic resorptive surface**, percentage of trabecular surface perimeter covered by Howship's lacunae containing an osteoclast.


7. **Percent total resorptive surface**, osteoclastic and nonosteoclastic resorptive surfaces.

8. **Osteoclast**, number per millimeter of trabecular surface perimeter.

**Electron Microscopy**

Parathyroid glands from 6 control dogs and 6 dogs with hypercalcemia and apocrine gland adenocarcinomas derived from the anal sac were examined by transmission electron microscopy. Dogs were killed with an overdose of barbiturate and one parathyroid gland was immediately immersed in cold glutaraldehyde. The tissues were trimmed into 1 mm³ blocks, fixed in 3% glutaraldehyde with 0.1M sodium cacodylate buffer at pH 7.4, post-fixed in 1.33% osmium with s-collidine buffer at pH 7.4, dehydrated through ascending concentrations of ethanol, transferred to propylene oxide, and embedded in Epon 812 (Shell Chemical Company, New York, NY). Thin sections were stained
with uranyl acetate and lead citrate, and examined with a Philips 200 or 300 electron microscope.

**Plasma Parathyroid Hormone**

Blood was collected from the jugular vein into chilled heparinized syringes, transferred to cold heparinized glass tubes, and centrifuged at 4°C. Plasma was collected and stored at -70°C. Immunoreactive parathyroid hormone was measured by a modification (8) of methods previously described (19), using antiserum GP-1 (final dilution 1:700,000). Partially purified human parathyroid hormone (20) was used as a standard. Final assay volume was 0.5 ml. Standard curves and plasma samples (300 µl) from each dog were assayed in triplicate. Duplicates of plasma samples were incubated without antiserum to correct for nonspecific effects due to incubation damage of radioiodinated hormone. The detection limit of the assay, defined as 85% of the bound-to-free ratio given by tracer-binding to the antiserum in the absence of added hormone, was 112 pg/ml.

**Tissue Extracts for iPTH**

The following tissues were collected for extraction procedures: 10 adenocarcinomas from apocrine glands of the anal sac; parathyroid glands collected from 50 adult dogs; lymph nodes from 6 control dogs; and 5 tumors (circumanal carcinoma, apocrine adenocarcinoma, hemangiopericytoma, rectal adenoma, and Sertoli cell tumor) from normocalcemic tumor-control dogs. All tissues were rapidly trimmed of fat and connective tissue, frozen in liquid nitrogen, and stored at -70°C. Tissues were lyophilized and approximately 0.5 to 1 g of lyophilized tumor tissue or 0.1 g of lyophilized parathyroid glands were extracted by methods previously described (21). Tissues were pulverized with
dry ice, homogenized with 8M urea - 0.2 N HCl, incubated on ice for 30 min, centrifuged at 30,000 X g for 15 min, and aliquots of the supernatant frozen for use in the iPTH assay. One ml aliquots of urea-HCl extracts were diluted with 6 ml of chilled water, precipitated with 2 ml of 80% trichloroacetic acid, and centrifuged at 3,000 X g for 10 min. Pellets were frozen, lyophilized, and stored at -70°C for use in the assay. Urea-HCl aliquots and trichloroacetic acid precipitates were neutralized with concentrated sodium hydroxide and brought to pH 8.6. Ten µl of these neutralized solutions were used in the iPTH assay.

Five and 2.5 µl-equivalents also were assayed from parathyroid gland extracts. Twenty µl-equivalents caused nonspecific displacement of the antibody, thus interfering with the assay. Immunoreactive PTH was measured as described earlier, with GP-1 antiserum (final dilution 1:700,000), at an assay volume of 0.5 ml. All samples were assayed in triplicate and damage controls were prepared in duplicate. Parathyroid hormone concentrations in tissue were expressed as µg/gm of dry weight.

**Plasma 13,14-dihydro 15-keto Prostaglandin E₂**

The major metabolite of PGE₂, 13,14-dihydro-15-keto-PGE₂ (PGE₂M), was measured in the plasma of 9 dogs with apocrine adenocarcinomas derived from anal sac, 10 control dogs, and 4 normocalcemic tumor-controls by radioimmunoassay (22,23). Plasma for determination of PGE₂M was collected simultaneously and in an identical manner as that collected for the iPTH assay. The corresponding PGF₂α metabolite, 13,14-dihydro-15-keto-PGF₂α, cross-reacts 5% with the anti-13,14-dihydro-15-keto-PGE₂. Several samples of dog plasma also were assayed with anti-13,14-dihydro-15-keto-PGF₂α. The 13,14-dihydro-15-keto-PGE₂ cross-reacts (3%) with this anti-PGF₂α metabolite.
These simultaneous radioimmunoassays demonstrated that it was the PGE$_2$ metabolite that was detected in the assay. The detection limit of the PGE$_2$M assay was 4 pg/ml.

**Serum 1,25-dihydroxyvitamin D (1,25-(OH)$_2$D).**

Five ml of serum containing 5000 cpm of (26,27-$^3$H) 1,25-(OH)$_2$D$_3$ (94 Ci/mM) were extracted with 30 ml dichloromethane by vortexing 5 min in 50 ml polypropylene tubes and collecting the organic phase after centrifugation at 8,000 X g (24). Two successive re-ex extractions (15 ml) were used for the aqueous phase. Pooled extracts were dried under nitrogen, re dissolved in 0.5 ml hexane: chloroform:methanol (9:1:1), placed on 0.7 x 9 cm columns of Sephadex LH-20 and eluted with that solvent system. The 1,25-(OH)$_2$D$_3$-containing region in the eluate was used for high pressure liquid chromatography (HPLC) on a 5 µm LiChrosorb Si 60 column (0.46 x 25 cm) (E. Merck, Darmstadt, Germany) using 9:1 hexane:isopropanol. Appropriate fractions of the HPLC eluate were dried and reconstituted with 95% ethanol for recovery determinations and competitive protein binding assays.

Intestinal cytosolic receptor protein was prepared from 4-week-old rachitic chicks using 50 mM phosphate buffer at pH 7.4 with 50 mM HCl and 1 mM dithiothreitol at 1.5 mg/ml. Assay tubes had 0.2 ml receptor, 0.02 ml standard or unknown, and 3000 total cpm ($^3$H) 1,25-(OH)$_2$D$_3$. The standard curve was from 5-200 pg 1,25-(OH)$_2$D$_3$. 1,25-(OH)$_2$D$_3$ for preparation of standard curves was courtesy of Dr. Anthony W. Norman, University of California, Riverside, with 5 ng 1,25-(OH)$_2$D$_3$ added to determine nonspecific binding. Tubes incubated 1 h at 25°C, were kept chilled thereafter, and 0.2 ml of 50% hydroxylapatite was added in 5 mM phosphate buffer. Tubes were
vortexed and allowed to equilibrate for 15 min. They were centrifuged at 10,500 X g for 2 min and the supernatant fluid discarded. The hydroxylapatite-receptor complex was washed 3 times with 0.1% triton X-100 in 5 mM phosphate buffer and the remaining ($^3$H) 1,25-(OH)$_2$D$_3$ was extracted from the complex and counted. The detection limit of the assay was 4 pg/ml of serum. Serum 1,25-(OH)$_2$D$_3$ concentrations were expressed in pg/ml. Serum 1,25-(OH)$_2$D was quantitated in 9 dogs with CA, 6 control dogs without tumors, and 6 normocalcemic dogs with tumors.

**Serum and Urine Electrolytes, Hydroxyproline, cyclic AMP**

Serum and urine biochemical profiles were performed utilizing sequential multiple analyzers (SMA 6/60 and SMA 12/60, Technicon Instruments Corp., 511 Benedict Ave., Tarrytown, NY 10591). Calcium was determined by a cresophthalein complexone method (25). Phosphorus was determined by a method based on the formation of phosphomolybdic acid (26). Serum, plasma and urine were collected simultaneously in the morning after a 24-hour fast. Urine values were obtained from spot urine samples and fractional values and excretion/dl glomerular filtrate (GF) calculated as previously described (27,28).

Urinary hydroxyproline was determined as described previously (29) and urinary cyclic adenosine monophosphate (cAMP) was determined by a competitive protein binding (30) technique (Diagnostic Products Corporation, 12306 Exposition Blvd., Los Angeles, CA 90064). Results for urinary hydroxyproline and cAMP were expressed as µg hydroxyproline (HOP)/dl GF and nM cAMP/dl GF.

**Statistical Analyses**

Statistical differences between the means for groups were evaluated by Duncan's multiple range with the level of significance at $P<0.05$. When the
concentration of iPTH, 1,25-(OH)_2D, or PGE_2M was undetectable it was reported as such in scattergram figures. In calculating the mean for a group and making statistical comparisons an undetectable concentration was assigned the numerical value of the limit of detectability for the assay.
RESULTS

Serum and urinary electrolytes, hydroxyproline, and cyclic AMP

Dogs with apocrine gland adenocarcinomas (CA) had serum calcium concentrations that ranged from 14.2 to 19.2 mg/dl (Table 3.1). Hypercalcemia in dogs with CA (15.7 ± 0.6 mg/dl) was significantly greater (P<0.05) than the hypercalcemia present in dogs with primary hyperparathyroidism (14.0 ± 0.5 mg/dl). Dogs with CA had significant hypercalciuria and increased urine phosphorus (per dl GF) as compared to control dogs and normocalcemic tumor control dogs (Table 3.1; Fig. 3.1). In addition, the results for fractional excretion of calcium (mean ± SE) indicated that the urinary excretion of calcium in dogs with apocrine carcinomas (0.021 ± 0.007) was significantly increased compared to control dogs (0.002 ± 0.0005) and increased compared to normocalcemic tumor controls (0.004 ± 0.0008).

Urinary cAMP/dl GF was significantly increased in dogs with apocrine carcinomas compared to control dogs but was less than dogs with primary hyperparathyroidism (Fig. 3.3). Urinary hydroxyproline and phosphorus were numerically higher in dogs with apocrine carcinomas than control dogs but the differences were not significant (Table 3.1; Fig. 3.1). There were no significant differences in the concentration of serum albumin, urea nitrogen, alkaline phosphatase, or phosphorus between the groups of dogs (Table 3.1).

Plasma PGE₂M

The mean concentration of PGE₂M in the plasma of dogs with apocrine adenocarcinoma and hypercalcemia was 15.9 pg/ml with a range of 4 (undetectable) to 30 pg/ml (Table 3.1). The concentration of PGE₂M in dogs
with CA was not significantly different from the concentration in control dogs (11.8 pg/ml) or normocalcemic tumor-controls (10.3 pg/ml).

**Plasma immunoreactive parathyroid hormone**

The mean concentration for iPTH in the plasma of dogs with hypercalcemia and apocrine adenocarcinomas was $168 \pm 40$ pg/ml with a range of undetectable (5 dogs) to 266 pg/ml (Fig. 3.2). The concentration of iPTH in dogs with CA was not significantly different from the concentration in control dogs ($322 \pm 33$ pg/ml) or normocalcemic tumor-controls ($264 \pm 46$ pg/ml), but was significantly decreased compared to dogs with primary hyperparathyroidism. The concentration of iPTH in the venous drainage from a large metastatic abdominal CA in one dog was 120 pg/ml while the concentration in the arterial supply was 136 pg/ml.

Concentration of iPTH in 6 dogs with chronic renal failure was markedly increased compared to control dogs (Fig. 3.2). Plasma iPTH levels were undetectable in dogs with primary hypoparathyroidism but increased in dogs with primary hyperparathyroidism (mean: 1540 pg/ml; Fig. 3.2).

**Effects of tumor excision**

Apocrine adenocarcinomas were excised in seven of 10 dogs with hypercalcemia. In six dogs the serum calcium returned to the normal range within 24 to 48 h of tumor removal (Fig. 3.3) and in another dog the serum calcium was normal when it was first measured one week after surgery. Postoperative hypocalcemia was not observed. Recurrence of the tumor in 3 dogs was associated with return of hypercalcemia and subsequent surgical removal of the tumor was followed by a return to normocalcemia. Serum phosphorus increased following tumor excision (Fig. 3.3). Serum calcium, phosphorus and plasma
iPTH concentrations were measured pre- and post-operatively in two dogs (Fig. 3.3). Twenty-four to 48 hours after tumor removal, the plasma iPTH concentration increased two- to 20-fold. Mean preoperative values for calcium and iPTH were 16.5 mg/dl and 183 pg/ml, respectively, and mean postoperative values were 10.0 mg/dl and 690 pg/ml.

**Immunoreactive parathyroid hormone in tumor extracts**

Urea-hydrochloric acid extracts from apocrine adenocarcinomas (10 dogs), tumors from normocalcemic control dogs, and lymph nodes from control dogs without tumors, were assayed for iPTH before and after precipitation with trichloroacetic acid. Immunoreactive PTH was not detected in tissue extracts from any tumor or lymph node. The iPTH concentrations in similar extracts of parathyroid glands from adult dogs were greater than 200 µg/gm.

**Serum 1,25-(OH)₂D**

The mean serum concentration of 1,25-(OH)₂D in 9 dogs with apocrine adenocarcinomas and hypercalcemia was 23 pg/ml with a range of 7-58 pg/ml (Table 3.1). Although these dogs had hypercalcemia and normophosphatemia, the mean serum 1,25-(OH)₂D was not significantly different from either group of normocalcemic control dogs (Table 3.1).

The concentration of 1,25-(OH)₂D decreased two- to eight-fold following excision of the apocrine adenocarcinomas in 3 dogs (Fig. 3.3). Mean preoperative serum calcium was 16.5 mg/dl and preoperative serum 1,25-(OH)₂D was 32 pg/ml; postoperative values were 10.0 mg/dl and 6 pg/ml, respectively.

**Light and electron microscopy of parathyroid glands**

Parathyroid glands from six dogs with hypercalcemia and apocrine adenocarcinomas were composed of inactive or atrophic chief cells. They were
arranged in narrow cords with prominent perivascular and interstitial spaces containing collagen fibers. Nuclei of inactive chief cells had clumped chromatin and the cytoplasm was lightly eosinophilic (Fig. 3.4). Ultrastructurally, the reduced cytoplasmic area of chief cells, straight cell borders with uncomplicated interdigitations between adjacent cells, and the poorly developed synthetic and secretory organelles, suggested chief cells in dogs with persistent hypercalcemia were in the inactive stage of the secretory cycle or atrophic (Fig. 3.5). Inactive chief cells had short profiles of rough endoplasmic reticulum, infrequent secretory granules, and a small Golgi apparatus. Intracytoplasmic lipid droplets were common in inactive chief cells. Parathyroid glands from control dogs had numerous chief cells in the active stage of the secretory cycle interspersed with inactive chief cells but no atrophic chief cells.

Bone histomorphometry

Histomorphometric analysis indicated that dogs with apocrine adenocarcinomas and hypercalcemia had significantly decreased trabecular bone volume as compared to age-matched control dogs (Table 3.2). Total resorptive surface (Howship's lacunae with and without osteoclasts) was significantly increased as were the number of osteoclasts per mm of trabecular bone. Dogs with primary hyperparathyroidism also had significantly increased total resorptive surface and numbers of osteoclasts (Table 3.2).

One of 10 dogs with apocrine adenocarcinoma had metastases in multiple bones. The dog was hypercalcemic (14.4 mg/dl) and had a low normal serum phosphorus concentration (3.4 mg/dl). There was marked osteoclastic osteolysis adjacent to and several mm from the tumor metastases. All counts for histomorphometric analysis were done in vertebrae free of metastases.
Differences in bone density were not detected between dogs with apocrine adenocarcinomas and control dogs on either antemortem or postmortem radiographs using conventional and magnification techniques.
DISCUSSION

Apocrine adenocarcinomas derived from glands of the anal sac appeared to produce a factor other than iPTH or PGE₂ that induced bone resorption and resulted in persistent hypercalcemia in dogs. The parathyroid glands had ultrastructural evidence of secretory inactivity in response to the hypercalcemia but the serum concentration of 1,25-(OH)₂D was not decreased. Lumbar vertebrae from hypercalcemic dogs were free of the metastases and had decreased trabecular bone volume and increased osteoclastic osteolysis. Evidence for increased humorally mediated osteoclastic bone resorption and decreased bone volume also has been reported in rabbits with the prostaglandin-producing VX₂ carcinoma (31) and in human patients with cancer-associated hypercalcemia (32). Increased bone resorption in dogs with apocrine adenocarcinomas appeared to be responsible for the increased urinary hydroxyproline, hypercalciuria, and hyperphosphaturia. Urinary calcium excretion in dogs with apocrine adenocarcinomas was approximately three times greater than that of dogs with primary hyperparathyroidism. The humoral factor produced by these tumors apparently does not stimulate tubular reabsorption of calcium, suggesting it may be different from parathyroid hormone.

The combination of undetectable to low normal concentrations of plasma iPTH and the absence of detectable iPTH in tumor extracts suggested that the hypercalcemic factor produced by apocrine adenocarcinomas was immunologically distinct from PTH. The antiserum used (GP-1) is multivalent (19,33) and recognizes antigenic sites throughout the PTH molecule. Therefore, humoral products similar to PTH synthesized by the tumor should have been detected by the assay system. The immunoassay used was able to discriminate on the basis
of plasma iPTH concentrations between hypoparathyroid dogs (undetectable iPTH), primary hyperparathyroid dogs (increased), and renal secondary hyperparathyroidism (markedly increased). Dogs with apocrine adenocarcinomas and hypercalcemia had consistently decreased concentrations of plasma iPTH compared to normocalcemic control dogs with and without other tumors.

The low normal concentrations of plasma iPTH in about half of the dogs with persistent hypercalcemia and apocrine adenocarcinomas was similar to that reported by Rijnberk et al. (13) and interpreted as being inappropriately high relative to the serum calcium concentration. Mechanisms other than ectopic production of iPTH also could explain these findings, such as: 1) continued secretion of a basal concentration of parathyroid hormone in spite of the hypercalcemia, 2) alteration of PTH metabolism resulting in the production of carboxy terminal fragments with longer half-lives, and 3) presence in the plasma of a substance which nonspecifically binds with the radioligand used in the assay. It has been previously reported that parathyroid glands in calves continue to secrete iPTH during a similar magnitude of hypercalcemia (34). The dogs with apocrine adenocarcinomas had increased serum creatinine, suggesting that a decreased clearance of iPTH by the kidney may contribute to the circulating concentrations of iPTH in some dogs with persistent hypercalcemia. High serum concentrations of calcium also have been reported to induce the secretion of fragments of iPTH by parathyroid glands (35). These fragments may contribute to the immunoheterogeneity of the circulating hormone and account for some of the iPTH measured in these dogs (36). Undetectable or low normal concentrations of iPTH are not uncommon findings in human beings with hypercalcemia and malignancy (37,38).
The interpretation of hypercalcemia and plasma iPTH values is further complicated by observations that approximately 10 to 40% of human patients with primary hyperparathyroidism also have neoplasms of nonparathyroid tissue (39,40,41). However, these patients usually have unequivocally high plasma iPTH values (42). The only dog in this study with persistent hypercalcemia, apocrine adenocarcinoma, and concurrent primary hyperparathyroidism had a markedly increased concentration of plasma iPTH (3860 pg/ml) and had light and electron microscopic evidence of primary hyperplasia involving all parathyroid glands. In contrast, the remaining dogs with apocrine adenocarcinomas had undetectable or low normal concentrations of plasma iPTH and parathyroid chief cells had ultrastructural evidence of secretory inactivity (43). Furthermore, removal of parathyroid glands from a dog with an apocrine adenocarcinoma and hypercalcemia has been reported not to reverse the hypercalcemia (13). These findings suggested the tumors are not secreting a humoral substance that stimulates chief cells leading to the development of parathyroid hyperplasia and persistent hypercalcemia in dogs with apocrine adenocarcinomas.

The increased concentrations of urinary cAMP in dogs with apocrine adenocarcinomas and primary hyperparathyroidism are comparable to observations made in human patients (12). Experimental infusion of PTH into dogs has been reported to increase urinary cAMP by glomerular filtration rather than by increased renal production of cAMP (44). Urinary cAMP was increased in dogs with apocrine adenocarcinomas compared to control dogs but not when compared to normocalcemic tumor-controls. Similar changes in urinary cAMP excretion have been reported in human beings with hypercalcemia and
malignancy (12). A bimodal distribution with high and low concentrations of urinary cAMP, as observed in some human beings with hypercalcemia and malignancy (11), was not seen in dogs with apocrine adenocarcinomas.

Dogs with apocrine adenocarcinomas had significant hypercalcemia, normal serum phosphorus concentration, and low iPTH concentrations, yet had normal concentrations of 1,25-(OH)_2D compared to control dogs. Possible mechanisms to explain the unexpected finding of normal 1,25-(OH)_2D levels in association with changes in calcium, phosphorus, and iPTH that would be expected to cause decreased values include: 1) stimulation of renal production of 1,25-(OH)_2D by the hypercalcemic factor secreted by the tumor, 2) production of 1,25-(OH)_2D by the tumor, 3) decreased uptake and degradation of 1,25-(OH)_2D, and 4) nonspecific competition for the 1,25-(OH)_2D receptor protein used in the assay by a substance in the serum of dogs with apocrine adenocarcinomas. The rapid decrease in serum 1,25-(OH)_2D concentration following tumor excision in 3 dogs suggested that the presence of the tumor or products produced by the tumor cells was responsible for maintaining the serum concentration of 1,25-(OH)_2D.

Concentration of plasma PGE_2M was not significantly different among dogs with apocrine adenocarcinoma and control dogs and normocalcemic tumor-controls. The absence of increased PGE_2M in the plasma of hypercalcemic dogs with CA suggests that PGE_2 was not the mediator of the hypercalcemia in these dogs. In animal models of hypercalcemia and malignancy where PGE_2 has been determined to be a mediator of hypercalcemia the plasma concentration of its metabolite, (PGE_2M), had a greater incremental increase above basal levels than the parent compound (PGE_2) (23). The greater increase in PGE_2
metabolites was attributed to the rapid conversion of PGE$_2$ to 13,14-dihydro-15-keto-PGE$_2$ and the longer half-life of this metabolite (23,45).

All six parathyroid glands from dogs with apocrine adenocarcinomas had ultrastructural evidence of secretory inactivity, presumably secondary to the persistent hypercalcemia. These findings were consistent with the undetectable or low normal levels of plasma iPTH found during hypercalcemia. The two-to 20-fold increase of plasma iPTH following tumor removal indicated that the suppression of synthetic and secretory activities of the parathyroid glands could be rapidly reversed when the blood calcium returned to the normal range. Similar observations have been made in human patients with hypercalcemia and malignancy (21), but the magnitude of increase in iPTH following surgical removal of the neoplasms was not as great as in dogs with apocrine adenocarcinomas. Treatment of dogs with lymphosarcoma and hypercalcemia by chemotherapy also resulted in a decrease of serum calcium and an increase in plasma iPTH (46).

In conclusion, dogs with adenocarcinomas derived from the apocrine glands of the anal sac have hypercalcemia secondary to the production of a humoral factor that appears to be distinct from iPTH or PGE$_2$. It increases bone resorption distant from the tumor and increases urinary calcium, cAMP and hydroxyproline excretion. The inappropriately high serum concentration of 1,25-(OH)$_2$D in tumor-bearing hypercalcemic dogs and the rapid decrease following excision of the tumor suggested a relationship between the tumor and circulating 1,25-(OH)$_2$D levels. The persistent hypercalcemia caused secretory inactivity of the parathyroid glands and decreased production of iPTH. Surgical removal of the tumor was followed by an increased secretion of iPTH that
prevented postoperative hypocalcemia. The unknown factor(s) secreted by this unique adenocarcinoma in dogs induced osteoclastic osteolysis and hypercalciuria apparently by a mechanism not involving the secretion of either iPTH or PGE$_2$. Further investigations into the pathogenesis of hypercalcemia in dogs with apocrine adenocarcinomas may further our understanding of the mechanisms responsible for cancer-associated hypercalcemia in human beings and animals.
SUMMARY

Hypercalcemia, hypercalciuria and hyperphosphaturia were present in 10 female dogs with adenocarcinomas derived from apocrine glands of the anal sac (CA). Excision of the neoplasm resulted in remission of hypercalcemia while recurrence or growth of metastases was associated with a return of hypercalcemia. Before tumor removal, the plasma concentration of immunoreactive parathyroid hormone (iPTH) was near the lower limits of control dogs or undetectable. Immunoreactive PTH was not detected in extracts from tumors. Parathyroid glands from dogs with CA had ultrastructural characteristics of secretory inactivity. Plasma concentrations of 13,14-dihydro-15-keto-prostaglandin E\(_2\) (PGE\(_2\)\(\text{M}\)) and serum 1,25-dihydroxyvitamin D were not significantly different from control dogs. Urinary cyclic adenosine monophosphate and hydroxyproline were increased in dogs with CA. Lumbar vertebrae from hypercalcemic dogs had decreased trabecular bone volume and increased osteoclastic osteolysis compared with age-matched control dogs. Following tumor excision, serum total calcium returned to the normal range while iPTH increased two- to twenty-fold and 1,25-dihydroxyvitamin D decreased two- to eight-fold. Post-operative hypocalcemia was not observed. These results indicated that CA produced a hypercalcemic factor other than immunoreactive parathyroid hormone or prostaglandin E\(_2\) that increased osteoclastic osteolysis and urinary cAMP, and resulted in hypercalcemia, hypercalciuria, and hyperphosphaturia.
### Table 3.1. Serum and urine data from dogs with apocrine gland adenocarcinoma and control dogs.

<table>
<thead>
<tr>
<th></th>
<th>Control Dogs (n=15)</th>
<th>Normocalcemic Tumor-Control Dogs (n=6)</th>
<th>Apocrine CA Hypercalcemia (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Serum Calcium (mg/dl)</strong></td>
<td>9.7 ± 0.1</td>
<td>9.4 ± 0.1</td>
<td>15.7 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Serum Phosphorus (mg/dl)</strong></td>
<td>4.1 ± 0.1</td>
<td>4.2 ± 0.3</td>
<td>2.9 ± 0.6</td>
</tr>
<tr>
<td><strong>Serum Albumin (g/dl)</strong></td>
<td>3.1 ± 0.1</td>
<td>2.8 ± 0.1</td>
<td>3.1 ± 0.1</td>
</tr>
<tr>
<td><strong>Serum Creatinine (mg/dl)</strong></td>
<td>0.9 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td><strong>Serum ALP (IU/L)</strong></td>
<td>40 ± 4</td>
<td>54 ± 9</td>
<td>116 ± 34</td>
</tr>
<tr>
<td><strong>Urine P (mg/dl GF)</strong></td>
<td>0.76 ± 0.07</td>
<td>0.54 ± 0.12</td>
<td>2.00 ± 0.72</td>
</tr>
<tr>
<td><strong>Plasma PGE&lt;sub&gt;2M&lt;/sub&gt; (pg/ml)</strong></td>
<td>11.8 ± 2.8&lt;sup&gt;*(10)&lt;/sup&gt;</td>
<td>10.3 ± 3.6&lt;sup&gt;* (4)&lt;/sup&gt;</td>
<td>15.9 ± 2.7&lt;sup&gt;*(9)&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Serum 1,25-(OH)&lt;sub&gt;2&lt;/sub&gt;D (pg/ml)</strong></td>
<td>26 ± 5&lt;sup&gt;* (6)&lt;/sup&gt;</td>
<td>16 ± 4</td>
<td>23 ± 5</td>
</tr>
</tbody>
</table>

<sup>n</sup> = number of dogs; *( ) indicates a different number of dogs.

Values are expressed as mean ± standard error.

Abbreviations: apocrine CA = apocrine gland adenocarcinoma; ALP = alkaline phosphatase; PGE<sub>2M</sub> = 13,14-dihydro-15-keto-prostaglandin E<sub>2</sub>; GF = glomerular filtrate; 1,25-(OH)<sub>2</sub>D = 1,25-dihydroxyvitamin D.

<sup>a</sup> Significant difference (P<0.05) as compared to control dogs and normocalcemic control dogs.
Table 3.2. Histomorphometric evaluation of lumbar vertebrae from dogs with hypercalcemia and adenocarcinomas derived from apocrine glands of anal sac compared to control dogs and dogs with primary hyperparathyroidism.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Cortical Bone (%)</th>
<th>Trabecular Bone (%)</th>
<th>Trabecular Surface (mm)</th>
<th>Resorptive Surfaces without Osteoid/Osteoclasts (%)</th>
<th>Resorptive Surfaces with Osteoclasts (%)</th>
<th>Total Resorptive Surface (%)</th>
<th>Osteoclasts (/mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control dogs</td>
<td>6</td>
<td>50.34* (3.12)</td>
<td>21.03 (1.81)</td>
<td>25.55 (1.82)</td>
<td>12.38 (3.26)</td>
<td>1.63 (0.49)</td>
<td>0.42 (0.18)</td>
<td>2.06 (0.50)</td>
</tr>
<tr>
<td>Apocrine</td>
<td>6</td>
<td>42.75 (3.67)</td>
<td>12.68* (0.80)</td>
<td>23.41 (3.12)</td>
<td>11.12 (4.33)</td>
<td>6.31+ (0.59)</td>
<td>1.68 (0.46)</td>
<td>7.99+ (0.83)</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>3</td>
<td>60.28 (0.84)</td>
<td>18.50 (2.81)</td>
<td>24.43 (0.82)</td>
<td>4.63 (1.40)</td>
<td>9.54 (4.63)</td>
<td>2.23 (0.35)</td>
<td>7.95+ (0.65)</td>
</tr>
<tr>
<td>Hyperparathyroidism</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mean * (+ SE)

* Significantly different (P<0.05) from control dogs.

n = number of dogs.
Fig. 3.1  Urinary excretion of calcium, hydroxyproline, and cAMP in dogs with apocrine adenocarcinoma (CA) compared to normocalcemic controls with and without tumors, and dogs with primary hyperparathyroidism. Cyclic AMP and calcium excretion were significantly increased in dogs with CA compared to control dogs. Cyclic AMP excretion was significantly greater (P<0.05) in hyperparathyroid dogs than any other group. Hydroxyproline in dogs with CA (19.6 µg/100 dl GF ± 3.9) was greater than controls (9.3 µg/dl GF ± 1.4) but this difference was not significant. Horizontal lines indicate mean for the group. Significant differences (P<0.05) from control dogs is indicated by "a" and from tumor-controls by "b".
FIG. 3.1

FIG. 3.1
Dogs with hypercalcemia associated with adenocarcinomas derived from apocrine glands of the anal sac (CA) had a decreased concentration of plasma iPTH compared to control dogs. Dogs with hypoparathyroidism (HYPO-PTH) had undetectable concentrations of iPTH while dogs with primary (HYPER-PTH) and secondary (RENAL HYPER-PTH) hyperparathyroidism had increased concentrations of iPTH compared to control dogs. Four of 6 dogs with chronic renal disease had iPTH values greater than 8,300 pg/ml. The limit of detectability for the iPTH assay (112 pg/ml) is indicated by the shaded area. Horizontal lines indicate the mean for the respective groups.
FIG. 3.2
Changes in serum phosphorus (P), calcium (CA), 1,25-(OH)$_2$D, and plasma iPTH associated with tumor excision and recurrence in dogs with adenocarcinomas derived from apocrine glands of the anal sac. Following tumor removal, phosphorus concentrations increased, calcium returned to the normal range, iPTH concentrations increased two- to 20-fold, and 1,25-(OH)$_2$D decreased two- to 8-fold. Open columns represent values at initial clinical evaluation and dark columns have post-surgical values. Repeating numbers represent tumor recurrence and pre-surgical values. Post-operative samples were obtained at 24-48 h following tumor excision except for dog 2 which was sampled at 12 and 24 h post-surgery, and dog 5 which had calcium and phosphorus determined seven days after tumor removal. Shaded areas for P and Ca represent laboratory reference values (mean ± 2SD for 100 normal adult dogs). Shaded areas for iPTH and 1,25-(OH)$_2$D are mean ± one SD for control dogs. The limits of detectability for iPTH (112 pg/ml) and 1,25-(OH)$_2$D (4 pg/ml) are indicated by broken lines.
FIG. 3.3
Fig. 3.4 Inactive parathyroid gland from a dog with hypercalcemia (15.8 mg/dl) and adenocarcinoma derived from the apocrine glands of anal sac. Inactive chief cells have a reduced cytoplasmic area and clumped nuclear chromatin. Narrow cords of chief cells are separated by wide bands of collagen and prominent perivascular spaces (S). (H & E, X315).
Fig. 3.5 Inactive chief cells in a parathyroid gland from a dog with hypercalcemia (20.8 mg/dl), hypophosphatemia (1.8 mg/dl), and apocrine adenocarcinoma of anal sac. Inactive chief cells have a reduced cytoplasmic area, straight plasma membranes with few uncomplicated interdigitations, lipid bodies, and an electron-lucent cytoplasm containing few secretory organelles. (Uranyl acetate, and lead citrate, X4500).
HYPERCALCEMIA IN DOGS WITH LYMPHOSARCOMA: BIOCHEMICAL, ULTRASTRUCTURAL AND HISTOMORPHOMETRIC INVESTIGATIONS

INTRODUCTION

Hypercalcemia has been associated with the production of bone-resorbing compounds by hematologic and solid nonparathyroid neoplasms (1,2). In vitro and in vivo studies have demonstrated that hypercalcemic factors produced by tumors are generally one of five compounds: parathyroid hormone (3,4), parathyroid hormone-like peptides (5,6), prostaglandin E₂ (7,8,9), osteoclast activating factor (OAF (10)), and osteolytic sterols (11). Production of osteoclast-activating factor by tumor cells has been implicated in the pathogenesis of hypercalcemia associated with multiple myeloma and lymphosarcoma in human beings (10,12).

Supernatants from cultured lymphosarcoma cells from dogs with hypercalcemia or normocalcemia have been shown to stimulate increased release of calcium from bone in vitro (13). Neither the characteristics of this apparent bone-resorbing substance nor its similarities with OAF are known. Radiographic, gross and histologic evidence of bone destruction were not detected in these dogs although histomorphometric analysis of bone was not done. It was concluded that most lymphosarcomas from dogs, whether hypercalcemic or
normocalcemic, produce a bone-resorbing substance in vitro and that ectopic secretion of iPTH was not involved in the pathogenesis of hypercalcemia in dogs with lymphosarcoma (13).

The objectives of this investigation were to characterize the pathogenesis of the hypercalcemia associated with lymphosarcoma in dogs and specifically to: 1) determine plasma concentrations of immunoreactive parathyroid hormone (iPTH), 13,14-dihydro-15-keto-prostaglandin E₂ (PGE₂M), and 1,25-dihydroxyvitamin D (1,25-(OH)₂D) in dogs with lymphosarcoma and hypercalcemia compared to dogs with lymphosarcoma and normocalcemia and age-matched controls with and without solid neoplasms; 2) quantitate the tissue concentration of iPTH in lymphosarcoma; 3) evaluate bone resorption by histomorphometric methods between dogs with lymphosarcoma and hypercalcemia, normocalcemia, control dogs, and dogs with primary hyperparathyroidism; 4) determine if there are differences in the concentration of urinary electrolytes, cAMP and hydroxyproline between hypercalcemic and normocalcemic dogs with lymphosarcoma; and 5) evaluate parathyroid chief cells and lymphosarcoma cells ultrastructurally for evidence of secretory activity.
MATERIALS AND METHODS

Animals

Eighteen dogs with hypercalcemia and lymphosarcoma, 9 dogs with lymphosarcoma and normocalcemia, 6 dogs ("tumor-controls") with normocalcemia and various solid neoplasms (pulmonary carcinoma, circumanal gland carcinoma, apocrine gland adenocarcinoma of skin, hemangiopericytoma of skin, rectal adenoma, and Sertoli cell tumor of the testis), 15 adult control dogs without clinical evidence of disease (normal controls), 3 dogs with hypercalcemia (mean serum calcium 14 mg/dl) and primary hyperparathyroidism, 3 dogs with hypoparathyroidism and hypocalcemia (mean serum calcium 4.9 mg/dl) and 6 dogs with chronic renal disease (mean serum urea nitrogen 157 mg/dl; serum creatinine 8.2 mg/dl) were used in this study. There were no significant differences (P>0.05) in age or body weight between groups. Two dogs with hyperparathyroidism had primary nodular parathyroid hyperplasia and one had a chief cell adenoma. The clinical and pathologic characteristics of the dogs with hypoparathyroidism have been described previously (14). Dogs with primary hyper- and hypoparathyroidism and chronic renal disease were included in the study to determine if the immunoassay for parathyroid hormone could detect differences in circulating hormone levels in canine serum under conditions of increased or decreased parathyroid activity.

Dogs with hypercalcemia were evaluated for bone metastases by ante-mortem radiographs of vertebrae and long bones, and by postmortem radiographs of the thoracic and lumbar vertebrae, humerus, femur, rib, mandible, and metacarpal bones or metatarsal bones using conventional and magnification (1.5 times) techniques (15). One femur, humerus, tibia, and sternebra, several ribs;
and lumbar, sacral and cervical vertebrae from hypercalcemic dogs were cut longitudinally, examined for macroscopic metastases, and selected areas were processed for light microscopy.

**Histomorphometry**

Fourth or fifth lumbar vertebrae were sectioned longitudinally, fixed in neutral phosphate-buffered formalin, and transferred to 70% ethanol. Vertebrae were embedded in methyl methacrylate without decalcification and two 4-5 μ sections were cut 200 μ apart on a Jung Model K Sledge microtome and stained with von Kossa-tetrachrome. Both sections of vertebrae were evaluated by point counting and line-intercept techniques using 10X and 20X objectives with a Zeiss Integrationsplatte I eyepiece. Cortical and trabecular bone volume were determined by point-counting in a 14.6 mm² area (7.3 mm²/section) based on the formula: volume = \( \frac{\text{no. hits} \times \text{grid area}}{\text{no. throws} \times 100} \) (16). All other parameters were measured by intersect-counting in a 7.8 mm² area (3.9 mm²/section). All intersections of trabecular bone with horizontal and vertical lines were counted, and the trabecular surface perimeter was calculated from the formula: perimeter = \( \frac{\pi}{2} \times \frac{\text{no. hits}}{2} \times \text{distance between grid lines} \). The following parameters were measured in the center of vertebrae avoiding cortical margins and longitudinal ends of bone:

1. **Cortical bone volume**, expressed as a percentage of the cortical space composed of mineralized and nonmineralized bone matrix.

2. **Trabecular bone volume**, expressed as a percentage of the trabecular space composed of mineralized and nonmineralized bone matrix.


5. **Osteoclastic resorptive surface**, percentage of trabecular surface perimeter covered by Howship's lacunae containing an osteoclast.


7. **Percent total resorptive surface**, osteoclastic and nonosteoclastic resorptive surfaces.

8. **Osteoclast**, number per millimeter of trabecular surface perimeter.

**Electron microscopy**

Lymphoid tissue from ten hypercalcemic dogs with lymphosarcoma, and 8 normocalcemic lymphosarcoma dogs were examined by transmission electron microscopy. Parathyroid glands from 6 control dogs, 10 dogs with hypercalcemia and lymphosarcoma, and 8 dogs with lymphosarcoma and normocalcemia were examined by transmission electron microscopy. Dogs were killed with an overdose of barbiturate and one parathyroid gland and three sections of lymphosarcoma were immediately immersed in cold glutaraldehyde. The tissues were trimmed into 1 mm³ blocks, fixed in 3% glutaraldehyde with 0.1M sodium cacodylate buffer at pH 7.4, post-fixed in 1.33% osmium with s-collidine buffer at pH 7.4, dehydrated through ascending concentrations of ethanol, transferred to propylene oxide, and embedded in Epon 812 (Shell Chemical Company, New York, NY). Thin sections were stained with uranyl acetate and lead citrate and examined with a Philips 200 or 300 electron microscope.

**Plasma parathyroid hormone**

Blood was collected from the jugular vein into chilled heparinized syringes, transferred to cold heparinized glass tubes and centrifuged at 4°C. Plasma was collected and stored at -70°C. Immunoreactive parathyroid
hormone (iPTH) was measured by a modification (8) of methods previously described (17), using antiserum GP-1 (final dilution 1:700,000). Partially purified human parathyroid hormone (18) was used as standard. Final assay volume was 0.5 ml. Standard curves and plasma samples (300 μl) from each dog were assayed in triplicate. Duplicates of plasma samples were incubated without antiserum to correct for nonspecific effects due to incubation damage to radioiodinated hormone. The detection limit of the assay, defined as 85% of the bound-to-free ratio given by tracer binding to the antiserum in the absence of added hormone, was 112 pg/ml.

**Tissue extracts for iPTH**

The following tissues were collected for extraction procedures: 7 lymph nodes and 8 mediastinal masses from hypercalcemic dogs with lymphosarcoma; 8 lymph nodes and one mediastinal mass from normocalcemic dogs with lymphosarcoma; 6 lymph nodes from 6 control dogs; 5 tumors (circumanal carcinoma, apocrine adenocarcinoma, hemangiopericytoma, rectal adenoma and a Sertoli cell tumor) from normocalcemic tumor-control dogs, and parathyroid glands collected from 50 adult dogs. All tissues were rapidly trimmed of fat and connective tissue, frozen in liquid nitrogen, and stored at -70°C. Tissues were lyophilized and approximately 0.5 to 1 g of lyophilized tumor tissue or 0.1 g of lyophilized parathyroid glands were extracted by methods previously described (19). Tissues were pulverized with dry ice, homogenized with 8M urea - 0.2 N HCl, incubated on ice for 30 min, centrifuged at 30,000 X g for 15 min, and aliquots of the supernatant were frozen for use in the iPTH assay. One ml aliquots of urea-HCl extracts were diluted with 6 ml of chilled distilled water, precipitated with 2 ml of 80% trichloroacetic acid, and centrifuged at 3,000 X g
for 10 min. Pellets were frozen, lyophilized, and stored at -70°C for use in the assay. Urea-HCl aliquots and trichloroacetic acid precipitates were neutralized with concentrated sodium hydroxide and brought to pH 8.6. Ten microliter-equivalents of the neutralized solutions were used in the iPTH assay.

Five and 2.5 microliter-equivalents were assayed from parathyroid gland extracts. Twenty microliter-equivalents caused nonspecific displacement of the antibody, thus interfering with the assay. Immunoreactive PTH was measured as described earlier with GP-1 antiserum (final dilution 1:700,000) at an assay volume of 0.5 ml. All samples were assayed in triplicate and damage controls were prepared in duplicate. Parathyroid hormone concentrations in tissue were expressed in μg/gm of dry weight.

Plasma 13,14-dihydro-15-keto prostaglandin E<sub>2</sub> (PGE<sub>2</sub>M)

The major metabolite of PGE<sub>2</sub>, 13,14-dihydro-15-keto-PGE<sub>2</sub> (PGE<sub>2</sub>M), was measured in the plasma of 10 hypercalcemic dogs with lymphosarcoma, 9 normocalcemic dogs with lymphosarcoma, 10 control dogs, and 4 normocalcemic tumor-controls by radioimmunoassay (20,21). Plasma for determination of PGE<sub>2</sub>M was collected simultaneously and in an identical manner as that collected for the iPTH assay. The corresponding PGF<sub>2</sub>α metabolite, 13,14-dihydro-15-keto-PGF<sub>2</sub>α, cross-reacts slightly (5%) with the anti-13,14-dihydro-15-keto-PGE<sub>2</sub>. Several samples of dog plasma also were assayed with anti-13,14-dihydro-15-keto-PGF<sub>2</sub>α. The 13,14-dihydro-15-keto-PGE<sub>2</sub> cross-reacts slightly (3%) with this anti-PGF<sub>2</sub>α metabolite. These simultaneous radioimmunoassays demonstrated that it was the PGE<sub>2</sub> metabolite that was detected in the assay. The detection limit of the PGE<sub>2</sub>M assay was 4 pg/ml.
Serum 1,25-dihydroxyvitamin D (1,25-(OH)₂D)

Five ml of serum containing 5000 cpm of (26,27²⁻H) 1,25-(OH)₂D₃ (94 Ci/mM) were extracted with 30 ml dichloromethane by vortexing 5 min in 50 ml polypropylene tubes and collecting the organic phase after centrifugation at 8,000 X g (22). Two successive re-extractions (15 ml) were used for the aqueous phase. Pooled extracts were dried under nitrogen, redissolved in 0.5 ml hexane: chloroform:methanol (9:1:1), placed on 0.7 x 9 cm columns of Sephadex LH-20 and eluted with that solvent system. The 1,25-(OH)₂-D-containing region in the eluate was used for high pressure liquid chromatography (HPLC) on a 5 μ LiChrosorb Si 60 column (0.46 x 25 cm) (E. Merck, Darmstadt, Germany) using 9:1 hexane:isopropanol. Appropriate fractions of the HPLC eluate were dried and reconstituted with 95% ethanol for recovery determinations and competitive protein binding assays.

Intestinal cytosolic receptor protein was prepared from 4-week-old rachitic chicks using 50 mM phosphate buffer at pH 7.4 with 50 mM HCl and 1 mM dithiothreitol at 1.5 mg/ml. Assay tubes had 0.2 ml receptor, 0.02 ml standard or unknown, and 3000 total cpm (³H) 1,25-(OH)₂D₃. The standard curve was from 5-200 pg 1,25-(OH)₂D₃ (l,25-(OH)₂D₃ for preparation of standard curves was courtesy of Dr. Anthony W. Norman, University of California, Riverside), with 5 ng 1,25-(OH)₂D₃ added to determine nonspecific binding. Tubes incubated 1 h at 25°C were kept chilled, thereafter, and 0.2 ml of 50% hydroxylapatite was added in 5 mM phosphate buffer. Tubes were vortexed and allowed to equilibrate for 15 min. They were centrifuged at 10,500 X g for 2 min and supernatant fluid discarded. The hydroxylapatite-receptor complex was washed 3 times with 0.1% triton X-100 in 5 mM
phosphate buffer and the remaining \(^{3}\text{H}\) \(1,25-(\text{OH})_2\text{D}_3\) was extracted from the complex and counted. The detection limit of the assay was 4 pg/ml of serum. Serum \(1,25-(\text{OH})_2\text{D}\) was quantitated in 12 dogs with hypercalcemia and lymphosarcoma, 6 dogs with lymphosarcoma and normocalcemia, 6 control dogs without tumors, and 6 normocalcemic dogs with tumors.

**Serum and urine electrolytes, hydroxyproline, and cyclic AMP**

Serum and urine biochemical profiles were performed utilizing sequential multiple analyzers (SMA 6/60 and SMA 12/60, Technicon Instruments Corp., 511 Benedict Ave., Tarrytown, NY 10591). Calcium was determined by a cresolphthalein complexone method (23). Phosphorus was determined by a method based on the formation of phosphomolybdic acid (24). Serum, plasma and urine were collected simultaneously in the morning after a 24-hour fast. Urine values were obtained from spot urine samples or from 24-hour collections and fractional values, mg Ca/dl glomerular filtrate (GF) and mg Ca per 24 hours/kg body weight were calculated as previously described (25,26).

Urine hydroxyproline was determined as described previously (27) and urine cyclic adenosine monophosphate (cAMP) was determined using a competitive protein binding (28) technique (Diagnostic Products Corporation, 12306 Exposition Blvd., Los Angeles, CA 90064). Results for urinary hydroxyproline and cAMP were expressed as \(\mu g\) hydroxyproline (HOP)/dl GF and nanomoles (nM) cAMP/dl GF. Urine specific gravity was determined with a refractometer.

**Statistical analyses**

Statistical differences between the means for groups were evaluated with Duncan's multiple range with the level of significance at \(P<0.05\). When the concentration of iPTH, \(1,25-(\text{OH})_2\text{D}\), or PGE\(_2\)M was undetectable it was
reported as such in scattergram figures. In calculating the mean for a group and making statistical comparisons an undetectable concentration was assigned the numerical value of the limit of detectability for the assay.

Statistical differences for hematologic parameters (Table 4.3) were evaluated with the Student t-test (P 0.01).
RESULTS

Serum and urine electrolytes, hydroxyproline, and cyclic AMP

Dogs with lymphosarcoma and hypercalcemia had serum calcium concentrations that ranged from 12.1 to 15.8 mg/dl. The mean serum calcium in this group (13.9 ± 0.3 mg/dl) was significantly greater than in control dogs and dogs with lymphosarcoma and normocalcemia (Table 4.1). Dogs with hypercalcemia and lymphosarcoma had a significant hypercalciuria and hyperphosphaturia compared to normocalcemic lymphosarcoma dogs, tumor-controls, and control dogs (Table 4.1; Fig. 4.1). These differences were detected when urine calcium was calculated as mgCa/dl GF, as fractional clearance, or as mgCa/kg/day. Fractional urinary excretion of calcium (mean ± SE) for the dogs were: controls (n=15), 0.002 ± 0.0005; tumor controls (n=6) 0.004 ± 0.0008; hypercalcemic lymphosarcoma (n=15) 0.030 ± 0.005; normocalcemic lymphosarcoma (n=8) 0.009 ± 0.006. Urine calcium excretion in mg/kg/day (mean ± SE) was: controls (n=9) 0.96 ± 0.35; hypercalcemic lymphosarcoma (n=4) 4.5 ± 1.7; normocalcemic lymphosarcoma (n=4) 0.35 ± 0.05. One normocalcemic dog with lymphosarcoma had a slightly increased urine calcium (0.24 mg/dl GF), but normal urine phosphorus (1.29 mg/dl GF) and normal urine hydroxyproline (11 µg/dl GF).

Hypercalcemic lymphosarcoma dogs excreted significantly increased concentrations of urinary hydroxyproline as compared to control dogs and increased concentrations compared to normocalcemic dogs with lymphosarcoma (Fig. 4.1). The concentration of urine cAMP was significantly greater in dogs with lymphosarcoma and normocalcemia than those with lymphosarcoma and hypercalcemia (Fig. 4.1). Urinary cAMP was significantly greater in dogs with primary hyperparathyroidism than control dogs and dogs with lymphosarcoma
and hypercalcemia (Fig. 4.1). Normocalcemic lymphosarcoma dogs had urinary concentrations of cAMP that were not significantly different from the concentration of cAMP in hyperparathyroid dogs.

Mean urine specific gravity in dogs with hypercalcemia and lymphosarcoma was 1.013 and in normocalcemic lymphosarcoma dogs was 1.026.

Dogs with hypercalcemia and lymphosarcoma had significantly greater concentrations of serum urea nitrogen (41 + 6 mg/dl) than did normocalcemic lymphosarcoma dogs (15 ± 3 mg/dl), tumor-control dogs (19 ± 5 mg/dl), and control dogs (17 ± 3 mg/dl). Dogs with lymphosarcoma and normocalcemia had decreased serum albumin and increased serum alkaline phosphatase (Table 4.1).

**Concentration of iPTH, 1,25-(OH)₂D₃, and PGE₂M**

Although the mean concentration of plasma iPTH was lower in dogs with hypercalcemia and lymphosarcoma than in normocalcemic lymphosarcoma dogs, control dogs and tumor-control dogs the differences were not significant (Fig. 4.2). Dogs with primary hyperparathyroidism had significantly increased concentrations of iPTH (mean: 1560 pg/ml) while dogs with hypoparathyroidism had undetectable concentrations of iPTH, and dogs with chronic renal disease had markedly increased concentrations of iPTH (Fig. 4.2). Four of the dogs with renal disease had iPTH values greater than 8,300 pg/ml.

The concentration of 1,25-(OH)₂D was undetectable in 8 of 12 dogs with hypercalcemia and lymphosarcoma and the mean concentration was 6 pg/ml. 1,25-(OH)₂D was detected in all 6 dogs with normocalcemia and lymphosarcoma and the mean concentration was 11 pg/ml (Fig. 4.2).

Although the plasma concentration of PGE₂M was high in dogs with lymphosarcoma and hypercalcemia compared to control dogs, normocalcemic
tumor-controls, and normocalcemic lymphosarcoma dogs (Table 4.1) there were no significant differences in the plasma concentration of PGE₂ among the groups.

**Immunoreactive parathyroid hormone in tumor extracts**

Urea-hydrochloric acid extracts from lymphosarcoma (both normocalcemic and hypercalcemic dogs), tumors from normocalcemic control dogs, and lymph nodes from 6 controls dogs without tumors were assayed for iPTH before and after precipitation with trichloroacetic acid. Immunoreactive PTH was not detected in tissue extracts from any tumor or lymph node. The iPTH concentrations in similar extracts of parathyroid glands from adult dogs were greater than 200 μg/gm.

**Bone histomorphometry**

Dogs with hypercalcemia and lymphosarcoma as a group had significantly (P<0.05) decreased trabecular bone volume (Figs. 4.3, 4.4) and increased numbers of Howship's lacunae, percent total resorption, osteoclasts per mm trabecular bone, and percent osteoid as compared to control dogs and dogs with normocalcemia and lymphosarcoma (Table 4.2). None of the normocalcemic lymphosarcoma dogs had increased resorptive indices. Hypercalcemic lymphosarcoma dogs often had osteoclasts on trabecular bone surfaces opposite a surface lined by osteoid and large columnar osteoblasts (Figs. 4.5, 4.6). Dogs with primary hyperparathyroidism also had significantly increased osteoclastic activity (Table 4.2).

Of the 9 dogs with hypercalcemia and lymphosarcoma, 6 had decreased trabecular bone volume and increased numbers of osteoclasts. The bone marrow from these 6 dogs contained lymphosarcoma. The three hypercalcemic dogs
without increased bone resorption did not have lymphosarcoma in the bone marrow of the vertebra used for histomorphometric evaluation nor in the marrow of several other decalcified bones (femur, humerus, rib, lumbar vertebrae, ilium). All three of these dogs were hypercalciciruric, two were hyperphosphaturic, and one had increased urinary hydroxyproline.

Two of 7 normocalcemic dogs with lymphosarcoma had neoplastic bone marrow. Bone resorbing indices were not increased in any of the dogs in this group. Urine calcium was slightly increased in one normocalcemic dog with lymphosarcoma but urine phosphorus and hydroxyproline were normal.

Peritrabecular fibrosis was noted in bones of dogs with increased resorption. Dogs with hypercalcemia and lymphosarcoma had increased osteoclastic osteolysis in cortical bone but these changes were not quantitated.

One dog with hypercalcemia and lymphosarcoma had 0.5-1 cm masses of lymphosarcoma in lumbar and thoracic vertebrae. These tumors were visible grossly and in postmortem radiographs. With the exception of this dog, evidence of bone loss from antemortem and postmortem (conventional and 1.5X magnification) radiographs were not detected. Examination of decalcified bones stained with hematoxylin and eosin did not reveal differences in bone volume or osteoclast activity between the groups of dogs studied.

**Light and electron microscopy of parathyroid glands**

By light microscopic examination, 7 of 10 hypercalcemic dogs with lymphosarcoma and one of 8 normocalcemic dogs with lymphosarcoma had inactive parathyroid glands. Inactive parathyroids were composed of chief cells that were crowded closely together and had prominent perivascular and interstitial spaces containing collagen fibers. Inactive chief cells had a reduced cytoplasmic area and clumped nuclear chromatin.
Ultrastructurally, 9 of 10 hypercalcemic and 0 of 8 normocalcemic dogs with lymphosarcoma had parathyroid glands composed predominately of inactive parathyroid chief cells. Inactive chief cells had a reduced cytoplasmic volume, straight plasma membranes, and increased intracytoplasmic lipid (Fig. 4.7). The Golgi apparatus was small and associated with a few small granules, the rough endoplasmic reticulum consisted of short profiles, and secretory granules were scarce. Although similar cells were present in parathyroid glands from normocalcemic lymphosarcoma dogs they were admixed with other chief cells that were in various stages of secretory activity. Chief cells that were interpreted to be in an active stage of the secretory cycle had a larger Golgi apparatus, lamellar arrays of rough endoplasmic reticulum, more numerous secretory granules, an expanded cytoplasmic area and more complex interdigitations of plasma membranes between cells.

Parathyroid glands from control dogs had numerous chief cells in the active stage of the secretory cycle interspersed with few inactive chief cells.

Ultrastructure evaluation and distribution of lymphosarcoma

Lymphosarcoma was present in the anterior mediastinum from 14 of 16 dogs with lymphosarcoma and hypercalcemia and in 1 of 9 dogs with lymphosarcoma and normocalcemia (Table 4.3). Anterior mediastinal masses were large (usually greater than 6 cm in length) and occupied the area normally containing the thymus. These tumor masses were distinct in their location from tracheobronchial and sternal lymph nodes, which were also replaced by neoplastic tissue in the majority of dogs.

The bone marrow contained neoplastic lymphoid cells in 11 of 16 dogs that were hypercalcemic and in 4 of 9 dogs that were normocalcemic. Increased
lymphocyte numbers and circulating immature lymphocytes were detected in the peripheral blood of one dog in each group. Lymphosarcoma was identified on both sides of the diaphragm in all hypercalcemic and normocalcemic dogs (Table 4.3). By a combination of cytologic, histologic and ultrastructural evaluation it was determined that 23 of 25 tumors consisted of lymphoblasts or prolymphocytes, one tumor was lymphocytic and another was histiocytic.

Ten lymphosarcomas from hypercalcemic dogs and 8 from normocalcemic dogs were examined ultrastructurally. No obvious differences between these two groups were found. The majority of tumor cells had large nuclei and a small amount of cytoplasm with poorly developed organelles that were assessed to be lymphoblasts or prolymphocytes (Fig. 4.8). Nuclei were usually round to oval but in 4 tumors nuclei had folded, convoluted borders that formed bizarre shapes. Nuclear pockets were common. The cytoplasm contained few organelles and in many cells the only discernible structures were mitochondria, a small Golgi apparatus, and free polyribosomes. The Golgi apparatus was perinuclear in location and consisted of short folds of smooth membranes. Small vesicles were associated with its surface and occasionally granules (200-400 nm in diameter) were seen with a limiting membrane and electron-dense core. A few profiles of rough endoplasmic reticulum were found in some cells. Cells comprising the lymphosarcomas did not appear to have well developed organelles concerned with the active synthesis or storage of polypeptide hormones.
DISCUSSION

Dogs with lymphosarcoma and hypercalcemia appeared to produce a factor other than iPTH, PGE₂ or 1,25-(OH)₂D that induced bone resorption and resulted in persistent hypercalcemia. They had evidence of increased bone resorption and increased urinary excretion of calcium, phosphorus and hydroxyproline compared to normocalcemic lymphosarcoma dogs and control dogs. Only dogs with lymphosarcoma in the bone marrow had evidence of increased bone resorption. Three dogs had hypercalcemia and hypercalciuria without increased bone resorption or lymphosarcoma in the lumbar vertebrae used for histomorphometric evaluation. Since only one lumbar vertebra was examined by histomorphometry and although the bone marrow was examined by light microscopy from several bones, it is probable that these three dogs had increased bone resorption and neoplastic bone marrow in bones not examined. Neither osteoclastic bone resorption nor decreased trabecular bone volume were found in any of the normocalcemic dogs with lymphosarcoma. These data suggest that: 1) hypercalcemic dogs with lymphosarcoma produce a bone-resorbing factor that acts locally; 2) increased bone resorption occurs only in those bones which have lymphosarcoma in the bone marrow, and 3) normocalcemic lymphosarcoma dogs do not produce a bone-resorbing factor in vivo or if a factor is produced, it is in such low concentrations or so distant from bone that it does not mobilize significant amounts of calcium.

Previous investigations indicated that lymphosarcoma cells from 3 of 5 hypercalcemic and 2 of 2 normocalcemic dogs produced a bone resorbing substance in vitro (13). The authors concluded that most canine lymphosarcomas produced a bone resorbing factor, but only some dogs became
hypercalcemic and cited the comparable results that are found in human beings with multiple myeloma. Although in vitro bone resorption was not evaluated in the dogs we studied, our histomorphometric results indicated that in vivo bone resorption was not present in the bones examined from normocalcemic lymphosarcoma dogs. The possibility still exists that most canine lymphosarcomas do produce a bone resorbing factor in vitro and in vivo. However, if this substance is produced in vivo in normocalcemic dogs with lymphosarcoma it appears that it does not result in morphometric changes in bone, nor in a significant mobilization and excretion of calcium, phosphorus or hydroxyproline.

The marked hypercalcemia coupled with the decreased concentration of plasma iPTH in hypercalcemic dogs with lymphosarcoma and the absence of detectable iPTH in tumor extracts suggests that the hypercalcemic factor produced by tumor cells was immunologically distinct from PTH. The antiserum used (GP-1) is multivalent and recognizes antigenic sites throughout the PTH molecule (17,29). Therefore, any humoral product synthesized by the tumor similar to PTH should have been detected by the assay. The immunoassay used was able to discriminate on the basis of plasma iPTH concentrations, between normal dogs and dogs with primary hyperparathyroidism (increased iPTH), hypoparathyroidism (undetectable iPTH), and renal disease (markedly increased iPTH). Our interpretation that the mechanism of hypercalcemia in dogs with lymphosarcoma is not due to the excessive production of iPTH is consistent with the data in human patients with lymphosarcoma (10,13).

Previous reports have indicated that iPTH was detectable in the plasma of some dogs with lymphosarcoma and hypercalcemia (13,30). Similar observations also have been reported for dogs with adenocarcinomas derived from apocrine
glands of the anal sac (31,32), and for human patients with hypercalcemia and malignancy (33,34). Although the low normal concentrations of plasma iPTH has been interpreted as inappropriately high relative to the serum calcium (30,31,33), the concentration of plasma iPTH in dogs with hypercalcemia and lymphosarcoma may be explained by mechanisms other than ectopic production of iPTH. First, the continued secretion of a basal concentration of parathyroid hormone has been reported in calves with experimental hypercalcemia (35). Second, the plasma from the dogs may contain a substance which nonspecifically binds with the radioligand used in the assay. Third, alteration of parathyroid hormone metabolism associated with the lymphosarcoma may result in the production of carboxy terminal fragments with a longer half-life. The dogs with lymphosarcoma and hypercalcemia had increased concentrations of serum creatinine suggesting that a decreased clearance of iPTH by the kidney may contribute to the plasma iPTH in some dogs with persistent hypercalcemia. High serum concentrations of calcium also have been reported to induce the secretion of fragments of iPTH by parathyroid glands (36). These fragments may contribute to the immunoheterogenity of the circulating iPTH and account for some of the iPTH measured in these dogs (37). Undetectable or low normal concentrations of iPTH are not uncommon findings in human beings with cancer-associated hypercalcemia (33,34).

Additional evidence that ectopic PTH production is not involved in the pathogenesis of hypercalcemia in dogs with lymphosarcoma is that hypercalcemic dogs had lower concentrations of urinary cAMP than did normocalcemic dogs with lymphosarcoma and that serum 1,25-(OH)_2D levels were decreased in hypercalcemic dogs with lymphosarcoma. 1,25-(OH)_2D was detected in all
all normocalcemic lymphosarcoma dogs, while 8 of 12 hypercalcemic dogs with
lymphosarcoma had undetectable concentrations. These observations are
consistent with an inhibition of the renal 1α-hydroxylase by the increased serum
calcium.

Although the concentration of plasma PGE₂M was increased in dogs with
lymphosarcoma that were hypercalcemic, this change was not significant
compared to normocalcemic dogs with lymphosarcoma. In animal models of
hypercalcemia and malignancy where PGE₂ has been determined to be a
mediator of hypercalcemia, the plasma concentration of its metabolite (PGE₂M)
had a greater incremental increase above basal levels than the parent compound
(PGE₂) (20). The greater increase in PGE₂ metabolites was attributed to the
rapid conversion of PGE₂ to PGE₂M and the longer half-life of this metabolite
(20,38). Previous results have indicated that the plasma concentration of PGE₂
was not different between normocalcemic and hypercalcemic dogs with
lymphosarcoma (13). It appears that circulating concentrations of PEG₂ or
PGE₂M do not differentiate between hypercalcemic and normocalcemic dogs
with lymphosarcoma. Whether there are differences in the local production of
prostaglandin E₂ in dogs with lymphosarcoma was not determined. It has been
demonstrated that PGE₂ stimulates the production of osteoclast-activating
factor from lymphocytes cultured in vitro (39).

Parathyroid glands from dogs with lymphosarcoma and hypercalcemia
were composed of chief cells interpreted to be in the inactive stage of the
secretory cycle or atrophic. The straight plasma membranes, reduced
cytoplasmic area, increased intracellular lipid, and poorly developed Golgi
apparatus and rough endoplasmic reticulum were consistent with secretory
inactivity (40). Presumably these changes in the chief cells from dogs with lymphosarcoma and hypercalcemia were secondary to the effects of persistent hypercalcemia on chief cells.

Lymphosarcoma cells from hypercalcemic and normocalcemic dogs were lymphoblasts or prolymphocytes and there was extensive neoplastic involvement of lymphoid organs on both sides of the diaphragm, similar to previous reports on lymphosarcoma in dogs (41). The extent of lymphosarcoma and the total tumor burden did not appear to be different between the two groups; however, hypercalcemic dogs more consistently had neoplastic involvement of the anterior mediastinum and the bone marrow (88% and 69%, respectively) than did the normocalcemic dogs (11% and 44%). No differences were noted in the ultrastructural morphology between tumor cells from hypercalcemic and normocalcemic dogs. Tumor cells from both groups had large nuclei and a small amount of cytoplasm that contained few secretory organelles.

In conclusion, the hypercalcemia in dogs with lymphosarcoma was attributed to the production of a factor distinct from iPTH, PGE$_2$ or 1,25-(OH)$_2$D that increased bone resorption and the urine excretion of calcium phosphorus, and hydroxyproline. Only dogs with neoplastic infiltration of the bone marrow had increased osteoclastic bone resorption and hypercalcemia. Normocalcemic lymphosarcoma dogs did not have increased bone resorption nor increased urinary excretion of calcium, phosphorus or hydroxyproline. An explanation compatible with these results is that lymphosarcomas from normocalcemic dogs do not produce a bone resorbing factor which mobilized sufficient bone calcium to increase urine or serum calcium concentrations. Decreased urine cAMP, serum 1,25-(OH)$_2$D, and plasma iPTH, combined with
the absence of detectable concentrations of iPTH in tumor tissue, suggested that lymphosarcomas in dogs did not produce iPTH. Future investigations that correlate in vitro and in vivo bone resorption in dogs with lymphosarcoma as well as the identification of the local bone resorbing factor produced by lymphosarcomas will help to further clarify the pathogenesis of the hypercalcemia associated with lymphosarcoma.
SUMMARY

Dogs with lymphosarcoma and hypercalcemia had decreased trabecular bone volume combined with increased osteoclastic osteolysis and increased concentrations of urinary calcium, phosphorus and hydroxyproline. Only dogs with neoplastic bone marrow had increased osteoclastic bone resorption. Dogs with lymphosarcoma that were normocalcemic did not have increased bone resorption nor increased concentrations of urinary calcium, phosphorus or hydroxyproline compared to hypercalcemic dogs or age-matched control dogs. Hypercalcemic dogs with lymphosarcoma had decreased concentrations of plasma iPTH and serum 1,25-(OH)_{2}D compared to normocalcemic dogs and control dogs. Immunoreactive PTH was not detected in lymphosarcoma tissue. Light and electron microscopic examination of parathyroid glands revealed inactive or atrophic chief cells and evidence of secretory inactivity in dogs with lymphosarcoma and hypercalcemia. Plasma concentration of PGE_{2}M was not significantly different between groups of dogs with lymphosarcoma. Ultrastructurally, lymphosarcomas were composed of tumor cells with large nuclei and a paucity of cytoplasmic cell organelles. Lymphosarcoma in dogs with hypercalcemia appeared to produce a bone-resorbing substance that was immunologically distinct from iPTH, PGE_{2}M, and 1,25-(OH)_{2}D. This factor induced the resorption of bone and the mobilization of calcium when the bone marrow was infiltrated with tumor cells.
TABLE 4.1 Serum and urine data from dogs with lymphosarcoma and control dogs.

<table>
<thead>
<tr>
<th></th>
<th>Control dogs (n=15)</th>
<th>Normocalcemic tumor-control dogs (n=6)</th>
<th>Hypercalcemic lymphosarcoma dogs (n=18)</th>
<th>Normocalcemic lymphosarcoma dogs (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Calcium (mg/dl)</td>
<td>9.7 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.4 ± 0.1&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>13.9 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.7 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum Phosphorus (mg/dl)</td>
<td>4.1 ± 0.1</td>
<td>4.2 ± 0.3</td>
<td>4.6 ± 0.3</td>
<td>5.1 ± 0.3</td>
</tr>
<tr>
<td>Serum Albumin (g/dl)</td>
<td>3.1 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.8 ± 1&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>3.0 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.6 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum ALP (IU/L)</td>
<td>40 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>54 ± 9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>150 ± 38&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>260 ± 86&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum Creatinine (mg/dl)</td>
<td>0.9 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.0 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.7 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.9 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Urine Phosphorus (mg/dl GF)</td>
<td>0.76 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.53 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.10 ± 0.53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.12 ± 0.19&lt;sup&gt;a&lt;/sup&gt;           *(14)</td>
</tr>
<tr>
<td>Plasma PGE&lt;sub&gt;2&lt;/sub&gt;M (pg/ml)</td>
<td>11.8 ± 2.8&lt;sup&gt;(10)&lt;/sup&gt;</td>
<td>10.3 ± 3.6&lt;sup&gt;(4)&lt;/sup&gt;</td>
<td>32.3 ± 8.8&lt;sup&gt;(10)&lt;/sup&gt;</td>
<td>14.7 ± 4.4</td>
</tr>
</tbody>
</table>

n = number of dogs. *<sup>( )</sup> indicates a different number of dogs.

Values are expressed as mean ± standard error.

Abbreviations: ALP = alkaline phosphatase; GF = glomerular filtrate; PGE<sub>2</sub>M = 13,14-dihydro-15-keto-prostaglandin E<sub>2</sub>.

Means with different lettered superscripts are different (P<0.05).
TABLE 4.2 Histomorphometric evaluation of lumbar vertebrae from dogs with lymphosarcoma (hypercalcemic and normocalcemic) compared to control dogs and dogs with primary hyperparathyroidism.

<table>
<thead>
<tr>
<th>n</th>
<th>Control dogs</th>
<th>Lymphosarcoma Hypercalcemia</th>
<th>Lymphosarcoma Normocalcemia</th>
<th>Primary Hyperparathyroidism</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Cortical Bone (%)</td>
<td>Trabecular Bone (%)</td>
<td>Trabecular Surface (mm)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(%)</td>
<td>(%)</td>
<td>(mm)</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>50.34*</td>
<td>21.03</td>
<td>25.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(3.12)</td>
<td>(1.81)</td>
<td>(1.82)</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>53.44</td>
<td>15.48a,b</td>
<td>21.83</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(6.37)</td>
<td>(1.61)</td>
<td>(1.01)</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>56.19</td>
<td>20.36b</td>
<td>26.89</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.94)</td>
<td>(1.25)</td>
<td>(2.44)</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>60.28</td>
<td>18.50</td>
<td>24.43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.84)</td>
<td>(2.81)</td>
<td>(0.82)</td>
</tr>
</tbody>
</table>

Mean * (+ SE)

a Significantly different (P<0.05) from controls.

b Significant difference (P<0.05) between hypercalcemic and normocalcemic dogs with lymphosarcoma.

n = number of dogs.
### TABLE 4.3 Anatomical distribution of tumors and hematologic data in hypercalcemic and normocalcemic dogs with lymphosarcoma.

<table>
<thead>
<tr>
<th>LYMPHOSARCOMA</th>
<th>Hypercalcemia</th>
<th>Normocalcemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone Marrow</td>
<td>11/16</td>
<td>4/9+</td>
</tr>
<tr>
<td>Anterior Mediastinum</td>
<td>14/16</td>
<td>1/9</td>
</tr>
<tr>
<td>Liver</td>
<td>13/15</td>
<td>6/9</td>
</tr>
<tr>
<td>Spleen</td>
<td>12/15</td>
<td>7/9</td>
</tr>
<tr>
<td>Tonsil</td>
<td>12/14</td>
<td>7/7</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>18/18</td>
<td>9/9</td>
</tr>
<tr>
<td>Packed cell volume (%)</td>
<td>42.4 ± 2.4^a</td>
<td>32.7 ± 2.9</td>
</tr>
<tr>
<td>Total plasma protein (g/dl)</td>
<td>6.7 ± 0.1^a</td>
<td>5.4 ± 0.3</td>
</tr>
<tr>
<td>Total leukocytes X 10^9 (/µl)</td>
<td>10.5 ± 1.4</td>
<td>20.4 ± 5.3</td>
</tr>
<tr>
<td>Lymphocytes X 10^3 (/µl)</td>
<td>1.7 ± 0.4</td>
<td>4.6 ± 2.3</td>
</tr>
<tr>
<td>*Leukemia</td>
<td>1/18</td>
<td>1/9</td>
</tr>
<tr>
<td>**Lymphopenia</td>
<td>8/18</td>
<td>1/9</td>
</tr>
</tbody>
</table>

* Numerator represents the number of dogs that had tumors in the location listed and denominator represents the number of dogs evaluated.

^a Significant (P<0.01) difference from normocalcemic group.

* Leukemia = lymphocyte count greater than 5,000 cells/µl and immature cells present in blood film.

** Lymphopenia = lymphocyte count less than 1,200 cells/µl.
Fig. 4.1  Urinary excretion of calcium, cAMP, and hydroxyproline in five groups of dogs.

Urine calcium was significantly greater in dogs with lymphosarcoma and hypercalcemia (LSA H-Ca) than normocalcemic lymphosarcoma-dogs (LSA N-Ca), control dogs, and tumor-control dogs. Hydroxyproline was significantly increased in dogs with lymphosarcoma and hypercalcemia compared to control dogs and was greater in hypercalcemic dogs with lymphosarcoma than normocalcemic dogs with lymphosarcoma. Urine cAMP was significantly greater (P<0.05) in hyperparathyroid dogs than control dogs and dogs with lymphosarcoma and hypercalcemia. Horizontal lines indicate mean. Different letters indicate significant differences (P<0.05).
FIG. 4.1
Fig. 4.2  Dogs with lymphosarcoma and hypercalcemia (LSA H-Ca) had lower concentrations of plasma iPTH and serum 1,25-(OH)$_2$D than normocalcemic lymphosarcoma-dogs (LSA N-Ca) but the differences were not significant. Dogs with hypoparathyroidism (HYPO-PTH) had undetectable concentrations of iPTH while dogs with primary (HYPER-PTH) and secondary (RENAL HYPER-PTH) hyperparathyroidism had increased concentrations of iPTH compared to control dogs. Four of 6 dogs with chronic renal disease had plasma iPTH concentrations greater than 8,300 pg/ml. The limit of detectability for the iPTH assay (112 pg/ml) and the 1,25-(OH)$_2$D assay (4 pg/ml) are indicated by the shaded areas. Horizontal lines indicate the mean for the respective groups. Different letters indicate significantly different means (P<0.05).
FIG. 4.2
Fig. 4.3 Lumbar vertebra from a control dog has thick cortical bone (arrows) and numerous wide bone trabeculae. The trabecular bone volume was 22.7%. Longitudinal end of vertebra and intervertebral disc are at the top (Von Kossa-tetrachrome, X30).
FIG. 4.3
Fig. 4.4 Bone is absent (arrows) along one cortical margin of a lumbar vertebra from a dog with hypercalcemia (12.3 mg/dl) and lymphosarcoma. Bone trabeculae are reduced in number and thickness. The trabecular bone volume was 7.6% (compare to Fig. 4.3, same magnification). Longitudinal end of vertebra and intervertebral disc are at the top of the photo (Von Kossa-tetrachrome, X30).
Fig. 4.5  Lumbar vertebra from a control dog with a large trabeculum of bone that has smooth surfaces, no osteoclasts and flattened osteoblasts (von Kossa-tetrachrome, X700).
Fig. 4.6 Lumbar vertebra from a dog with hypercalcemia and lymphosarcoma. Howship's lacunae with (O) and without (arrow) osteoclasts lining a thin bone trabeculae with scalloped margins. Opposite the resorptive surface are columnar osteoblasts and osteoid. The marrow contains neoplastic lymphoid cells. Compare to bone trabeculum in Fig. 4.5 taken from a control dog at the same magnification. (Von Kossa-tetrachrome, X700).
Fig. 4.7  Inactive chief cells in a parathyroid gland from a dog with hypercalcemia (15 mg/dl) and lymphosarcoma. Inactive chief cells have large nuclei in relation to the small cytoplasmic area and straight plasma membranes, increased lipid droplets, and an electron-lucent cytoplasm containing few secretory organelles (Uranyl acetate and lead citrate, X 4,000).
Lymphoblasts from a dog with hypercalcemia (14.2 mg/dl) and lymphosarcoma. Tumor cells have large nuclei with prominent nucleoli. The cytoplasm contains scattered short profiles of rough endoplasmic reticulum, mitochondria and a small Golgi apparatus (Uranyl acetate and lead citrate, X 5,000).
CHAPTER V

RELATIONSHIP OF SERUM TOTAL CALCIUM TO ALBUMIN AND TOTAL PROTEIN IN DOGS

INTRODUCTION

Total serum calcium consists of ionized, complexed, and protein-bound fractions. Ionized calcium is the physiologically important fraction, but it is seldom measured, presumably because of strict collection requirements and the unreliability of ion-specific analyzers. In most laboratories, only serum total calcium is measured. Because approximately half of the calcium is protein-bound, the interpretation of total calcium depends on the concurrent values for serum albumin and total protein. In human beings, there are significant linear relationships between serum total calcium and albumin and between serum total calcium and total protein. Adjustment formulas have been derived for serum total calcium based on the concentration of albumin and total protein. The purpose of the study reported here was to determine whether a similar relationship existed in dogs.
MATERIALS AND METHODS

The serum total calcium and protein concentrations used for the calculation of linear regression, correlation coefficient, coefficient of determination, and confidence intervals were obtained from 209 patient records which had biochemical profiles performed by the clinical laboratory of The Ohio State University Veterinary Teaching Hospital. The criteria for inclusion in the study were an age of ≥1 year and serum urea nitrogen value of ≤30 mg/dl. The serum samples were from hospitalized dogs with varied clinical conditions, including 42 dogs presented for elective surgical procedures. Only the first biochemical profile was used from each dog. In addition to the 209 biochemical profiles from dogs which fulfilled the above criteria, another 66 biochemical profiles were obtained from dogs with disorders of calcium metabolism, including hypercalcemia associated with lymphosarcoma (15 dogs), hypercalcemia and adenocarcinomas derived from the apocrine glands of the anal sac (13 dogs), hypercalcemia associated with an anaplastic pulmonary neoplasm, giant cell type (1 dog), hypoparathyroidism (5 dogs), renal disease (15 dogs), and primary hyperparathyroidism (3 dogs), as well as from 14 dogs between 2.5 and 6 months old. The clinical, laboratory, and pathologic findings useful in the diagnosis of these disorders of calcium metabolism have been discussed in detail elsewhere. The data from dogs with calcium disorders were compared with the linear regression lines and the confidence intervals from the hospitalized dogs.

Hyperproteinemia (serum total protein ≥ 8 g/dl) was detected in 18 of the 209 hospitalized dogs. An additional 11 dogs with hyperproteinemia were
selected from the laboratory files. Hyperalbuminemia (≤3.7 g/dl) was detected in 6 of the 209 hospitalized dogs and in an additional 22 dogs after reviewing our files.

All biochemical profiles were determined by use of sequential multiple analyzers (SMA 6/60 and SMA 12/60, Technicon Industrial Systems, Inc., Corporate Headquarters, 511 Benedict Ave., Tarrytown, NY 10591). Total calcium was determined by a cresophthalein complexone method. The reference values for serum calcium for our laboratory were a mean of 10.1 mg/dl and a range (+ 2 standard deviations) of 8.8 to 11.3 mg/dl. The coefficient of variation for calcium controls for a 5-month period was 1.6%. Albumin was determined by a brom cresol green method. The mean normal serum albumin was 2.9 g/dl, with a range (+ 2 standard deviations) of 2.1 to 3.6 g/dl. The coefficient of variation for albumin for a 5-month period was 2.1%. Total protein was determined by the biuret method. The mean normal total protein was 6.8 g/dl, with a range (+ 2 standard deviations) of 5.7 to 7.8 g/dl.

Least squares regression lines were computed along with the 95% confidence intervals for the regression line and the 95% confidence intervals for the population of 209 dogs. The correlation coefficient (r) and the coefficient of determination ($R^2$) were calculated from the regression analysis. The analyses of variance were performed to determine whether the lines significantly fitted the data. The results indicated that the estimated slope was significantly different from zero ($P<0.001$) (i.e., that serum total calcium and albumin and serum total calcium and total protein were related).
RESULTS

The adjustment formulas for calcium were derived by adding the difference between the y intercept value from the regression line and the normal mean for serum calcium (10.1 mg/dl) to each intercept value. The y intercept for albumin was 6.57 and the difference was 3.53. For serum total protein, the y intercept was 6.81 and the difference was 3.29. The slope of the regression line was multiplied by the absolute value for albumin or serum total protein. The slope for serum total protein was 0.41. The slope for albumin was approximately one (1.04), thereby eliminating this step. Therefore, the final formulas were: adjusted calcium (mg/dl) = measured serum total calcium (mg/dl) - serum albumin (g/dl) + 3.5; and adjusted calcium (mg/dl) = measured serum total calcium (mg/dl) - 0.4 (serum total protein g/dl) + 3.3.

A positive linear relationship was found between serum total calcium and albumin (Fig. 5.1) for the 209 hospitalized dogs (r=0.575, P ≤ 0.001). One-third of the variability in calcium was attributable to the change in the concentration of albumin (R²=0.33). Fourteen of the 209 dogs had a low concentration of serum albumin (≤ 2.0 mg/dl). Of these 14 dogs, 4 (29%) were normocalcemic and 10 (71%) were hypocalcemic (6.9-8.7 mg/dl). After adjustment of total calcium for albumin concentration, 9 of the 10 hypocalcemic dogs had values of calcium within the normal range. None of the dogs with hypocalcemia in association with hypoalbuminemia had evidence of diseases of calcium metabolism.

In 28 dogs that had hyperalbuminemia (serum albumin concentrations ≥ 3.7 g/dl), the measured and adjusted concentrations of serum total calcium were normal. The mean (and range) for serum albumin concentration in these 28 dogs
was 4.0 (3.7 to 5.1) g/dl. One dog with an albumin concentration of 5 g/dl had a measured calcium of 11.3 mg/dl and an adjusted calcium of 9.8 mg/dl.

A positive linear relationship was found between serum total calcium and serum total protein (Fig. 5.2) for the 209 dogs (r=0.411, P ≤ 0.001). Approximately 17% of the variability in calcium was attributable to the change in the concentration of serum total protein (R^2 = 0.169). Twenty-two dogs had a low serum total protein concentration (≤ 5.6 g/dl) and 7 of these dogs also had a low concentration of serum albumin. Sixteen of the 22 dogs (73%) had a low serum calcium concentration and 6 dogs (27%) were normocalcemic. After adjustment of total calcium for the concentration of serum total protein, 14 (88%) of the 16 hypocalcemic dogs had values of calcium within the normal range. None of the dogs had diseases of altered calcium metabolism. Twenty-nine dogs with high concentrations of serum total protein (≥ 8 g/dl) had normal measured and adjusted calcium values. The mean serum total protein in these dogs was 8.6 and the range was 8.0-10.8 g/dl). The highest serum albumin value was 5.1 g/dl and the highest total protein concentration was 10.8 g/dl. No calcium concentration ≥ 12 mg/dl was ascribed to hyperalbuminemia or hyperproteinemia, nor was there a total calcium concentration ≤ 6.5 mg/dl attributable to hypoalbuminemia or hypoproteinemia.

After the use of the correction factors for albumin and total protein, 8 of the 209 hospitalized dogs had serum total calcium concentrations above normal. Two of the dogs had measured hypercalcemia (15.0 mg/dl and 12.5 mg/dl) with normal serum albumin and total protein concentrations associated with malignant neoplasms (adenocarcinoma of apocrine glands of the anal sac and lymphosarcoma). The adjusted calcium values were only slightly increased in
the other 6 dogs and ranged from 11.4-11.8 mg/dl. The clinical diagnoses for these dogs were chronic renal disease, hyperadrenocorticism, hip dysplasia, gastritis, vestibular disease and urethral disease. Their albumin values ranged from 3.0-3.6 g/dl and serum total protein ranged from 5.3-7.7 g/dl. A relationship was not determined between these diseases and the adjusted slight hypercalcemia.

Thirty-two of the 209 dogs had low serum calcium concentrations (6.9-8.7 mg/dl). After applying the correction formula for serum albumin, 27 (84%) of the 32 hypocalcemic dogs had normal values for adjusted serum total calcium. When the correction formula for serum total protein was applied to these 32 dogs, 28 (88%) of them had normal values for adjusted serum calcium. After use of adjustment formulas for both serum albumin and total protein concentration, 29 (91%) of the 32 dogs with measured hypocalcemia had adjusted calcium concentrations within the normal range. The relative hypocalcemia was attributed to low protein concentrations. None of the 32 dogs had diseases of calcium metabolism.

Ninety-one percent of dogs with disorders of calcium metabolism and 86% of young dogs (6 to 24 weeks of age) had calcium values outside of the 95% confidence limits calculated for albumin and total protein for the 209 hospitalized dogs (Fig. 5.3). Data from 95% of dogs with hypercalcemia and malignancy (lymphosarcoma, 15 dogs; adenocarcinoma of the apocrine glands of the anal sac, 13 dogs; and giant cell carcinoma of the lung, 1 dog) were outside of the 95% confidence limits for albumin and total protein. Similarly, data from the 14 young dogs were clustered in a relatively uniform group outside of the 95% confidence limits. The mean values for the 14 young dogs were total
calcium, 11 mg/dl; albumin, 2.7 g/dl; serum total protein, 5.2 g/dl; and phosphorus, 8.4 mg/dl. Adjusting calcium for albumin resulted in a mean value of 11.9 mg/dl and adjusting for serum total protein gave a mean calcium of 12.3 mg/dl. Data from 5 dogs with hypoparathyroidism and from 3 dogs with primary hyperparathyroidism were always outside of the 95% confidence limits. Seventy-nine percent of the dogs with renal disease had calcium values outside of the 95% confidence limits for albumin and total protein, but the group was widely scattered and did not follow a consistent pattern. Dogs with serum total calcium values outside of the 95% confidence limits were interpreted to have metabolic disorders of calcium metabolism.
DISCUSSION

Our findings established that there was a positive linear relationship between serum total calcium and albumin, and calcium and total protein in dogs. These findings were similar to studies with human samples correlating serum calcium with albumin, total protein and plasma specific gravity. In studies involving human beings the correlation coefficient of serum albumin to serum calcium varied from 0.40 to 0.94. The highest correlation coefficients were found in patients with hepatic disease. This group of patients often had protein disturbances, but rarely primary calcium disorders. The hypoalbuminemia characteristic of hepatic disease resulted in a relative hypocalcemia that could be corrected by an adjustment formula for calcium based on the concentration of serum albumin. Apparently the increased gammaglobulins in patients with hepatic disease do not bind sufficient calcium to compensate for the hypoalbuminemia. Approximately 80 to 90% of the protein-bound calcium in man is bound to albumin, and the remainder to globulins. In vitro studies have demonstrated that albumin, beta globulins, and alpha globulins have similar calcium binding characteristics, and all bind a greater amount of calcium than do gammaglobulins.

Presumably, the relationship between calcium and albumin and calcium and total protein would have been stronger if our study had only included dogs with hepatic disease, and dogs with hypoalbuminemia and hypoproteinemia. However, we believe that by not selecting for specific abnormalities that the sample population was less biased, permitting extrapolation of the information to a larger population of dogs. The derivation of separate correlation
coefficients and adjustment formulas for dogs with hepatic, renal, or gastrointestinal diseases would necessitate diagnosis of the primary disease before the appropriate adjustment formula could be applied. Serum samples were included from dogs with various clinical problems. This allows application of the adjustment formulas to a serum total calcium value before the clinical diagnosis is established for the patient. Therefore, the formulas may be helpful in making an early decision regarding a hypocalcemic or hypercalcemic disorder and thus aid in formulating differential diagnoses.

The principal use of calcium-adjustment formulas is to determine whether a dog has hypocalcemia or hypercalcemia that might otherwise be masked by the serum protein concentration. In most of the hospitalized dogs, the formulas corrected for the relative hypocalcemia caused by decreased concentrations of serum albumin and total protein. Dogs with total calcium concentration below 6.5 mg/dl or above 12 mg/dl had calcium metabolic disorders which could not be attributed to changes in the concentration of albumin or total protein.

Presumably, there are dogs with hypercalcemia that, because of low albumin or total protein concentrations, have a normal concentration of measured serum calcium. A dog with an albumin concentration of 1 g/dl and a calcium concentration of 10.5 mg/dl would have an adjusted calcium concentration of 13 mg/dl and, therefore, would be hypercalcemic.

Comparison of values for serum total calcium with albumin and serum total calcium with total protein were helpful in the identification of dogs with disorders of calcium metabolism. Dogs with hypercalcemia of malignancy, hypoparathyroidism, primary hyperparathyroidism, and renal disease had serum total calcium values that did not correlate with the concentration of serum.
albumin or total protein. The calcium values from these dogs were easily identified on graphs on which serum calcium was plotted against albumin or serum total protein. The pattern of high serum total calcium values in the young dogs in this study was interpreted as a physiologic increase in blood calcium and was similar to reported findings in calves, pups and human infants.14-16. The mechanism for this physiologic hypercalcemia and hyper-phosphatemia is not clearly established but is probably related to the positive calcium balance and rapid skeletal remodeling that occurs during growth. We conclude that adjustment of measured total serum calcium for albumin and total serum protein is essential for detection of abnormal calcium values, and that it is helpful in the identification of dogs with disorders of calcium metabolism.
SUMMARY

A positive linear relationship was found between total calcium and albumin and between total calcium and total protein in the serum of 209 dogs. Total calcium concentration correlated with the concentrations of albumin ($r=0.575; P<0.001$) and with the concentration of total protein ($r=0.411; P<0.001$). A correction formula for calcium was derived on the basis of the concentration of albumin: adjusted calcium (mg/dl) = calcium (mg/dl) - albumin (g/dl) + 3.5. The correction formula for calcium, based on the concentration of serum total protein was: adjusted calcium (mg/dl) = calcium (mg/dl) - 0.4 total serum protein (g/dl) + 3.3.

Hypocalcemia (≤8.7 mg/dl) was detected in 32 of the dogs. After adjustment of the measured total calcium for albumin and serum total protein, 29 (91%) of the dogs had calcium concentrations within the normal range. Hypercalcemia was not associated with hyperalbuminemia or hyperproteinemia. Ninety-one percent of dogs with disorders of calcium metabolism, including dogs with hypercalcemia associated with malignancy, hypoparathyroidism, hyperparathyroidism, and renal disease, and 85% of dogs less than 6 months old had calcium concentrations outside of the 95% confidence intervals for albumin and total protein calculated from the 209 dogs. It was concluded that adjustment of serum total calcium for protein concentration is essential for correction interpretation of calcium values and detection of abnormalities in calcium metabolism.
Figure 5.1. Significant relationship ($r=0.575; P < 0.001$) between serum albumin and total calcium concentrations in 209 hospitalized dogs. The least square regression line (solid line), the 95% confidence limits for the regression line (2 broken lines), and the 95% confidence limits for the population (shaded area) are given. The numbers represent the number of values at superimposed points. As the concentration of albumin increases or decreases there is a concurrent increase or decrease in serum total calcium. One-third of the variability in calcium was attributable to the change in the concentration of albumin ($R^2=0.33$).
Figure 5.2. Significant relationship \( r=0.411; P<0.001 \) between serum total protein and total calcium concentrations in 209 hospitalized dogs. The least square regression line (solid line), the 95% confidence limit for the regression line (2 broken lines), and the 95% confidence limits for the population (shaded area) are given. The numbers represent the number of values at superimposed points. Approximately 17% of the variability in calcium was attributable to the change in the concentration of serum total protein \( R^2=0.169 \).
Figure 5.3. Ninety-one percent of dogs with disorders of calcium metabolism and 85% of young dogs were outside of the 95% confidence for serum albumin (Fig. A) and total protein (Fig. B) compared with serum total calcium in the 209 hospitalized dogs. ○ = Hypercalcemia and malignancy; ■ = young dogs (6-24 weeks old); X = renal disease; O = hypoparathyroidism; △ = primary hyperparathyroidism.
Fig. 5.3A
Fig. 5.3B

CALCIUM (mg/dl)

TOTAL SERUM PROTEIN (g/dl)
CHAPTER I


BIBLIOGRAPHY

CHAPTER II

1 Sherwood LM, O'Riordan JLH, Aurbach GD, Potts JT Jr: Production of parathyroid hormone by nonparathyroid tumors. J Clin Endocrinol Metab 27:140-146, 1967


BIBLIOGRAPHY

CHAPTER III


30. Tovey KC, Oldham KG, Whelan JAM: A single direct assay for cyclic AMP in plasma and other biological samples using an improved competitive protein binding technique. Clinica Chimica Acta 56:221-224, 1974


44. Fox J, Heath H III: Parathyroid hormone does not increase nephrogenous cyclic AMP excretion by the dog. Endocrinology 107:2124-2126, 1980


BIBLIOGRAPHY

CHAPTER IV


28. Tovey KC, Oldham KG, Whelan JAM: A single direct assay for cyclic AMP in plasma and other biological samples using an improved competitive protein binding technique. Clinica Chimica Acta 56:221-224, 1974


