THE DEMONSTRATION AND IDENTIFICATION OF ACID MUCOPOLYSACCHARIDES IN CANINE MAMMARY TUMORS: A HISTOLOGICAL AND HISTOCHEMICAL STUDY

By

THOMAS EARL PALMER

Bachelor of Arts
Southern Illinois University
Edwardsville, Illinois
1969

Master of Science
Saint Louis University
Saint Louis, Missouri
1973

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Thesis Approved:

Andrew W. McMaster
Thesis Adviser

Jeffie F. Gossett

E. A. Sesser

Calvin G. Barnes, Jr.

George V. Odell

K. A. Carraway

Norman N. Durham
Dean of the Graduate College

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TABLE OF CONTENTS

TABLE

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Benign tumors and cancers arising from mammary tissues are among the most common group of neoplasms in the bitch and the most common cancer (7, 18, 26, 32, 41, 43). This is well documented by extensive literature reviews and a large number of studies (10, 31, 40, 42). Classification of these tumors by numerous authors have been difficult and complex. The complexity is well documented in dealing with the so-called "mixed mammary tumor" which implies multi-cell neoplastic origin. Recently Monlux et al. (41) have presented a simplified classification which eliminates some of this complexity. In that classification, the benign and malignant mixed mammary groups were eliminated. It was concluded that fibrous connective tissue, bone and cartilage associated with epithelial tumors were heterotopic and did not possess true neoplastic potential. The explanation for the appearance of these heterotopic tissues in this appreciable segment of the epithelial tumors of the canine mammary gland is investigated in the present paper.

Considerable controversy exists over the possible origins of the cartilage and bone in mammary tumors. Although a variety of theories have been proposed, none seem

to provide conclusive evidence. It is probable that there may be several and even overlapping mechanisms. Foremost among these are two conflicting views on the cell types actively contributing to the origin of cartilage; myoepithelial (ectodermal) versus stromal (mesodermal). Much of the bone is believed to be a result of ossification of the cartilage matrix.

Cartilage is composed of collagen fibers embedded in a ground substance rich in acid mucopolysaccharides. Similar acid mucopolysaccharides are found not only in the cartilage and pseudocartilage of certain mammary tumors but in their scattered intercellular spaces as well. Therefore, in this study the starting point was accepted that the histochemical classification of polysaccharides which identifies certain mucopolysaccharides as being derived only from mesodermal tissues and others only from ectodermal tissues is correct. By identifying the individual acid mucopolysaccharides and studying their occurrence in a variety of mammary tumors, more insight should be gained not only on the cell of origin but also on what may stimulate its production.

The classification of mucopolysaccharides or rather the polysaccharide-protein complexes studied is based on the summary text by Culling (11). He used the term acid mucopolysaccharide to identify all naturally occurring anionic heteroglycans as a group. These are essentially polymers of alternating units of hexosamine and uronic acid groups. His classification separates these from the neutral polysaccha-

rides (non-ionic homoglycans) and the glycoproteins. Even though some glycoproteins have many histochemical properties identical to those of the acid mucopolysaccharides, it is generally accepted that the former compounds are found in epithelial mucins and the latter in connective tissues (39, 55, 68, 77). This difference becomes the critical point of evaluation of this study.

In an attempt to standardize a nomenclature, many investigators favor the use of the more chemically precise terms glycosaminoglycan or glycosaminoglucuronoglycan. Because of its more favorable position in biological literature, acid mucopolysaccharide will be used in this study. This term also has continued preferential use by both the histochemist and the pathologist.

Throughout this study the terms precartilage and pseudocartilage may appear to be used interchangably. However, precartilage refers to the earliest mucopolysaccharide accumulation which is believed to lead step wise to the formation of pseudocartilage and then true hyaline cartilage. The pseudocartilage is primarily distinguished from the precartilage by its accumulation of collagen fibers and from true cartilage by an absence of lacunae.

CHAPTER II

REVIEW OF SELECTED LITERATURE

The fibrous, cartilage, bone and even myoid components of the "mixed" tumors of glandular tissues have traditionally puzzled investigators who have centered much attention on description and classification (10, 26, 22, 40, 43). Although most investigators advocate a relative minor role for these tissues in the final prognosis in relation to malignancy (7, 41), the origin of such mesenchymal elements has remained a subject of controversy and research concerning whether the cartilage and bone are mesodermal or ectodermal in origin and about the basis of their formation. Unfortunately there is great confusion today as to whether these components represent previous inflammatory or involutionary changes, hyperplastic changes or benign or malignant neoplastic changes. Most of the research referred to in this investigation has been in mixed tumors of the human salivary gland and to lesser extent in the human breast and the canine mammary gland. A historical review of the literature on the histogenesis of the mixed salivary tumor was published by Mylius in 1960 (49). Similar reviews on the mammary gland were presented by Allen (5) and Willis (76).

Most of the theories of origin include the process of metaplasia. Some investigators proposed that the cartilage was derived from epithelial cell metaplasia (5, 32, 66). Others disagreed saying the cartilage and bone developed from metaplasia of the connective tissue stromal elements (18, 76). In support of this latter theory some proposed that the neoplastic epithelium in some way stimulated the connective tissue metaplasia (38, 43, 70). Still others reported that the epithelial mucins and/or degenerative epithelial cells and products contributed to the formation of cartilage and bone (10, 16, 17, 76).

In recent years the myoepithelial origin of the cartilage has received considerable attention. Peyron in 1924 (56) was one of the first to call attention to the role myoepithelial cells played in tumors of the canine mammary gland and pointed out the similarities between these tumors and those of the salivary glands in man. Hamperl (27) described the myoepithelia as multipotential cells and did much to advance their role in the cartilage formation in the mixed tumors. The significance of their presence and absence in a variety of human breast tumors has been reported by Ahmed (1, 2, 3, 4) and by Murad and vonHaam (46, 47, 48). Similar studies in the salivary gland have been reported by Mylius (49) and by Hubner et al. (28). All of these support the definite role that the myoepithelium plays in the formation of the myxoid portions

of the tumors. They suggest the same cells may have a role in cartilage formation. Welsh and Meyer (75) supported the role of the myoepithelium in the formation of the mixed salivary tumors but found no evidence to suggest a direct transformation of epithelium or myoepithelium to cartilage forming cells. Pulley (57) in his study of the myoepithelium in the formation of the canine mixed mammary tumor suggested there is a direct transition of the myoepithelium first to cartilage producing cells and then to cells which produce bone. Cotchin (10) agreed with the myoepithelial theories for the formation of cartilage but suggested the bone found in the mixed tumors results from either endochondrial calcification of pre-existing cartilage or intramembranous calcification of the connective tissue stroma. There is apparent wide acceptance of this theory on bone formation as there has been very little reported in the literature on this aspect in either the salivary or mammary tumors. In a more recent publication Monlux et al. (41) described the cartilage and bone in most canine epithelial mammary tumors as heterotopic and suggested that their formation may be a result of stimulation of the connective tissues by escaped epithelial secretions or direct contact of the epithelium with the stroma.

Under normal conditions, both the collagen and the mucopolysaccharides characteristic of the connective tissue ground substance are synthesized by the connective tissue

(mesodermal) cells (21, 22, 29, 33). The histochemical identification of these polysaccharides is often difficult and complex. Spicer et al. (68) suggested that the main confusion was caused by the fact that biochemically identified mucopolysaccharides can rarely be identified unequivocally in specific tissue sites by histochemical methods. In the same respect, many carbohydrate rich substances detected histochemically are not related to the mucopolysaccharides known to the biochemist. The development of new histochemical techniques and the adaption of biochemical techniques into histochemical methods, along with the suggested terminologies by Spicer et al. (68) has revolutionized the identification of mucopolysaccharides. Further modifications of the classification system as well as detailed methods are found in accepted histochemical texts (11, 55, 71, 76).

The specific derivation of certain mucopolysaccharides is supported by the work of Sorvani (67) in his study of endometrial and cervical adenocarcinoma. The epithelial mucins in this study were not hyaluronidase labile and were characterized as sialomucins, sulfomucins and neutral mucins. Leppi and Spicer (35) reported similar findings in the salivary glands of certain primates including man.

Histochemical studies of the mucopolysaccharides in "mixed" tumors are very limited. Utilizing somewhat primitive histochemical methods, Grishman (25) and Erichsen (16, 17) identified chondroitin sulfate in human

and canine mixed mammary tumors respectively. Ozzello and Speer (54) followed with a broad study of the acid and neutral mucopolysaccharides distributed in the human breast but made no attempt to identify specific mucopolysaccharides. In 1962 Spicer et al. (69) followed with a more specific study of the mucins in the luminal secretions found in various lesions of the human breast. The only mucin of any significance was identified as an epithelial derived sialomucin. Lovell et al. (36) reported that chemical analysis of the acid mucopolysaccharide content of mixed salivary tumors indicated a high content of hyaluronic acid and chondroitin sulfates B and C.

One of the most significant histochemical studies utilizing modern techniques was reported by Quintarelli and Robinson (59) in which they characterized the glycosaminoglycans (mucopolysaccharides) of the salivary gland mixed tumors. The techniques used indicated two different mucopolysaccharides were being produced in the tumors; one was typical of epithelial (neutral) glycoproteins and the other characteristic of the connective tissue protein-polysaccharides. The latter was found to be a mixture of hyaluronic acid, chondroitin-4 and chondroitin-6 sulfate. Their findings indicated the acid mucopolysaccharide to be of mesenchymal derivation and there was no suggestion of any transformation from an epithelial to a connective tissue mucin.

In a recent paper Takeuchi and co-workers (70) presented

a limited biochemical and histochemical study of the glycosaminoglycans of certain human breast tumors. They were able to correlate the variations of the individual acid mucopolysaccharide content with a histological pattern. Their findings indicated that the glycosaminoglycan synthesis was due to mesenchymal cell activity which may have been promoted by the presence of neoplastic epithelium.

CHAPTER III

MATERIALS, METHODS AND EXPERIMENTAL DESIGN

Materials

The case material used in this study was obtained from the Tulsa Registry of Canine and Feline Neoplasms located in the Department of Veterinary Pathology and supported by the National Institute of Health. The only exceptions were three cases obtained from the Oklahoma State University Small Animal Clinic. All tumors included in this study occurred naturally and their classification is according to that advanced by the Tumor Registry. Unless otherwise specified, control tissues used in the histochemical procedures were obtained from clinically normal female dogs in which no neoplasms were recognized.

A total of 34 tumors were used in this study and included 17 ductal carcinomas, 6 adenomas, 5 intraductal carcinomas, 4 ductal papillomas and 2 lobular carcinomas. The classification of these tumors is based on their most malignant features as a whole and therefore does not reflect the presence of cell groupings suggesting secondary more benign classifications. For an example, many of the ductal carcinomas had co-existing areas suggesting adenoma

and papilloma components as well. With very few exceptions all the tumors had some areas of fibrous, cartilage and/or bone metaplasia within them.

Methods

For the light microscopic and histochemical studies, all tissues were fixed in 10% phosphate buffered neutral formalin, processed overnight on an automatic tissue processor and embedded in Paraplast.[1] Sections were cut at 4 or 6 micron and the following histological and histochemical procedures were performed on all cases considered; hematoxylin and eosin (H&E), Alcian blue (pH2.5)-Periodic acid Schiff (AB-PAS) and Elbadawi's hexachrome (14). On selected cases the following additional procedures were used; Periodic acid Schiff with and without Diastase[2] (PAS-D and PAS), Alcian blue (pH 2.5)-nuclear fast red with and without hyaluronidase[3] (AB-NFR-H and AB-NFR), Alcian blue (pH 2.5)-nuclear fast red with and without KOH-neuraminidase[4] (AB-NFR-N and AB-NFR), Periodic acid-phenylhyrazine Schiff (PAPS), Alcian blue (pH 1.0) - nuclear fast red (AB - 1.0 - NFR) and Alcian blue (pH 2.5)-Aldehyde Fuchsin (AB-AF). With the exception of the hexachrome all procedures are according to methods described in Culling's Handbook of Histopathological and Histochemical Techniques (11).

Tissues for electron microscopy were fixed in 2% glutlaradehyde in 0.08% cacodylate buffer (pH 7.2) for

2 hours on ice. Following a brief washing in the cacodylate buffer with 0.18 m sucrose added, the tissues were post fixed in 2% osmium tetroxide, dehydrated through graded ethanols, infiltrated with propylene oxide, embedded in DER 732[5] and hardened at 60°C under vacuum for approximately 48 hours. Sectioning was done on a Sorvall MT-2 ultramicrotome with glass knives. Thick sections (1-1.5 micron) were collected on 3x1 glass slides and stained with Richardson blue stain (62) for light microscopic examination and orientation. Thin, silver sections were collected on copper grids, stained with 5% aqueous uranyl acetate and lead citrate (74), viewed and photographed with a Philips 200 electron microscope.

Duplicate paraffin sections were used in the histochemical digestion procedures. One section was treated with the appropriate digestion solution and the duplicate with buffer only. Both slides were incubated under the same conditions. The digestion solutions used included:

- Bovine testicular hyaluronidase, 50 mg per ml of acetic-acetate buffer, pH 6.0 at 37°C for six hours.
- Vibrio cholera neuraminidase, 100 units per ml of 0.05 M acetate buffer with 0.1% CaCl, pH 5.5 at 37°C for twenty-four hours. Pretreatment with 1% potassium hydroxide in 70% ethanol was used to enhance the digestive action of the neuraminidase.

- Diastase in the form of 0.1% amylase in phosphate buffered saline (37), pH 6.0 at 37°C for one hour.
- Acid hydrolysis with 0.02 sodium acetate-Hcl buffer, pH 2.5 at 75°C for two hours.

Control sections for the hyaluronidase procedure included canine aorta, skin and tracheal cartilage. Neuraminidase controls included sections of canine large and small intestine and bovine sub-maxillary gland. Sections from a hyperglycemic liver were used as diastase controls. The hematoxylin-eosin and hexachrome procedures were used only for morphological evaluation of the tumors studied.

Experimental Design

The cases selected in this study included representative samples of the epithelial mammary tumor types classified according to Monlux et al. (41) with the largest proportions having cartilaginous and precartilaginous changes. Tumors with inadequate fixation, inadequate histories and those with complications (e.g. inflammation, other tumor types, etc.) were excluded.

The primary objective of this study was to obtain more information on the origin of cartilage (and bone) by identifying the characteristics of the acid mucopolysaccharides which contribute to its formation. According to the histochemical classification as outlined in Culling (11) and in Pearse (55), acid mucopolysaccharides are derived from either epithelial (ectodermal) cells or mesenchymal (meso-

dermal) cells. The differential staining and localization of specific acid mucopolysaccharides in the expanding matrix of mammary tumors is indicative of subsequent origin of the precartilage and cartilage within these tumors.

The basis for the identification of the major acid mucopolysaccharides is summarized in a simplified chart presented in Table I. This chart also shows the basic differentiation of epithelial and connective tissue mucins. The results of these four basic procedures were subsequently verified by other procedures mentioned in the methods. The Appendix lists all the histochemical procedures used, the chemical reactions and the histochemical results. Electron microscopy was included in this study in an attempt to verify and support the histological and histochemical localization of the acid mucopolysaccharides identified and the identification of the cells involved.

TABLE I

SUMMERIZED EXPERIMENTAL DESIGN
FOR THE IDENTIFICATION OF
MUCOPOLYSACCHARIDES

Type of Mucin and Reactive group(s)	Substance identified	PAS-AB pH 2.5	AB-ph 1.0	Neuraminidase AB-pH 2.5	Hyaluronidase AB-pH 2.5
Neutral Mucins (1:2 glycol)	glycogen	Red	Negative	--	--
Carboxylated Mucins (COOH)	Hyaluronic Acid	Blue-Purple	Negative	Blue	Negative or Reduced
Sulfated Mucins (COOH and OSO_3H)	Chondroitin-4 and Chondroitin-6 Sulfates	Blue-Purple	Blue	Blue	Negative or Reduced
Carboxylated Mucins (1:2 glycol and COOH)	Sialomucin	Blue-Purple	Blue or Negative	Negative or Reduced	Blue
Connective Tissue Mucins		Blue-Purple	Blue	Blue	Negative or Reduced
Epithelial Mucins		Blue-Purple	Blue or Negative	Negative or Reduced	Blue

*Taken in part and modified from Culling (11), p. 264-265.

FOOTNOTES

[1]Scientific Products, McGaw Park, Illinois.

[2]Sigma Chemical Co., Saint Louis, Missouri.

[3]Sigma Chemical Co., Saint Louis, Missouri.

[4]Grand Island Biological Co., Grand Island, New York.

[5]Polysciences, Inc., Warrington, Pennsylvania.

CHAPTER IV

RESULTS

Tumor Morphology

Light Microscopic

The epithelial neoplastic characteristics observed within the variety of tumors studied were those commonly used to identify each particular type. While it is not the intent of this study to include a detailed morphological description of each tumor it is necessary to characterize those general changes associated in some manner with the occurrence of acid mucopolysaccharides. It should be noted however that no relationship could be established between the occurrence of acid mucopolysaccharides and a specific tumor type.

Ductal carcinomas made up the largest group of tumors studied and provided the greatest variety of morphological changes within a single group. Many of these changes appeared to directly or indirectly involve acid mucopolysaccharide accumulation. Without exception all the ductal carcinomas of this study had areas of precartilage, pseudocartilage and/or mature cartilage associated with them. There also were accumulations of tissues resulting from

squamous, fibrous and osteoid transformation and bone heterotopia. It was not possible to determine whether these mesenchymal-like changes were associated with the later developmental appearance of carcinoma alone or had appeared when the tumor was in a adenoma and papilloma stage, or with both. Some of the more malignant infiltrating carcinomas had considerable intercellular mucinous material closely associated with the neoplastic epithelium (Figure 1). In metastases, the tissue elements often were present to support late origin even if there were earlier changes. These changes were seen in late infiltrations in the mammary tissue and within metastases identified in lymph nodes, lung, brain, etc (Figure 2). Many of the tumors had moderately to greatly distended ducts containing variable amounts of secretory material sometimes mixed with necrotic debris within their lumens (Figure 3). Still other cases with limited cartilaginous changes, had dense fibrous to osteoid material as well as true bone which appeared to completely encase the neoplastic epithelium (Figure 4). Frequently metastastic sites in lymph nodes and lung possessed the same carcinoma and mesenchymal patterns found at the primary site (Figure 5).

With the obvious exception of the epithelium, the adenomas (Figure 6) and papillomas of this study had many of the same changes observed in the ductal carcinomas. Heterotopia of cartilage and bone and fibrous proliferation

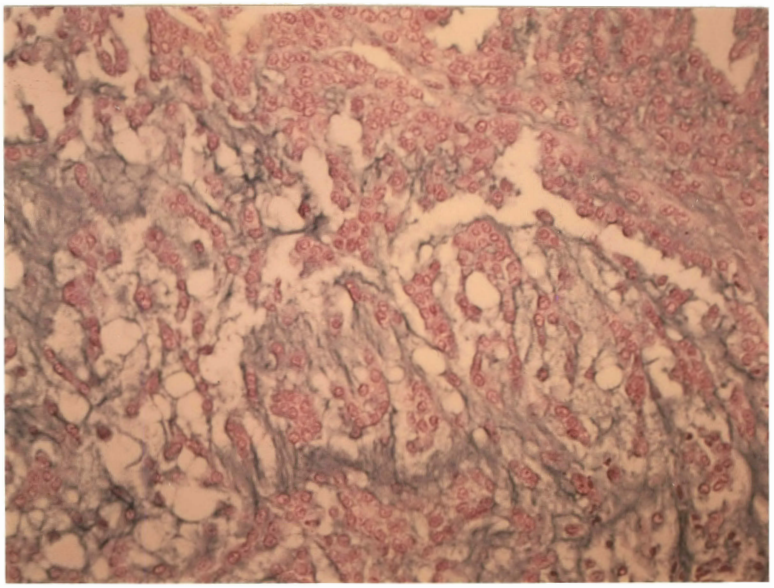

Figure 1. Case 202418-3. Ductal Carcinoma with Abundant Intercellular Mucin (blue) Associated with Neoplastic Epithelium. (AB-NFR x 200)

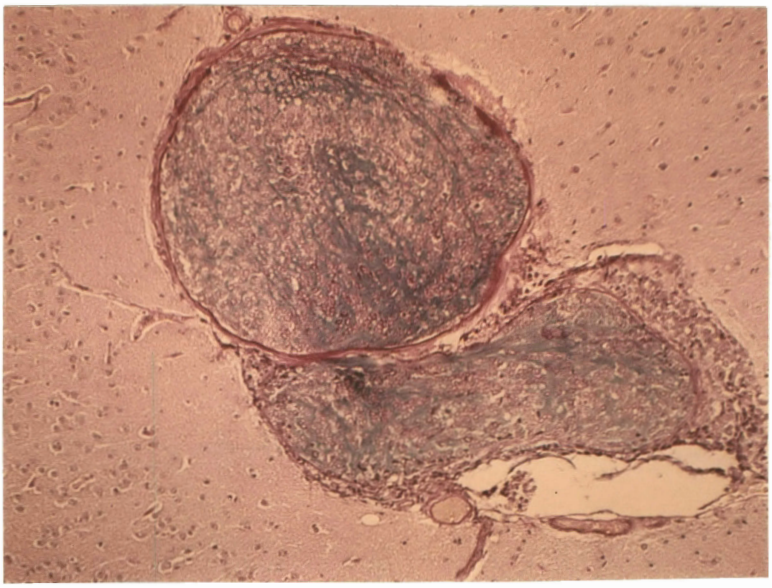

Figure 2. Case 202418-3. Metastic Ductal Carcinoma. Compare the Intercellular Mucin with Figure 1. (AB-PAS x 75)

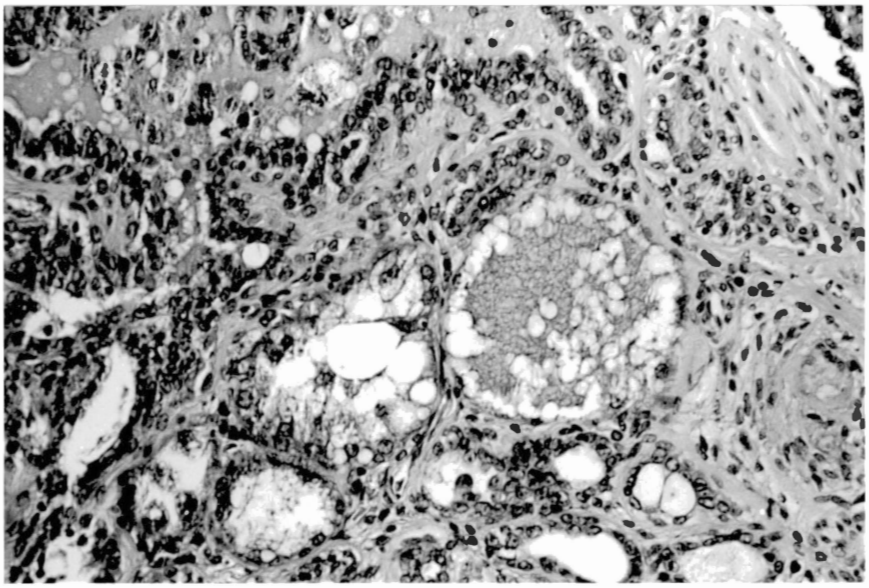

Figure 3. Case 202070. Distended Ducts Containing Varible Amounts of Secretory Material. (H&E x 200)

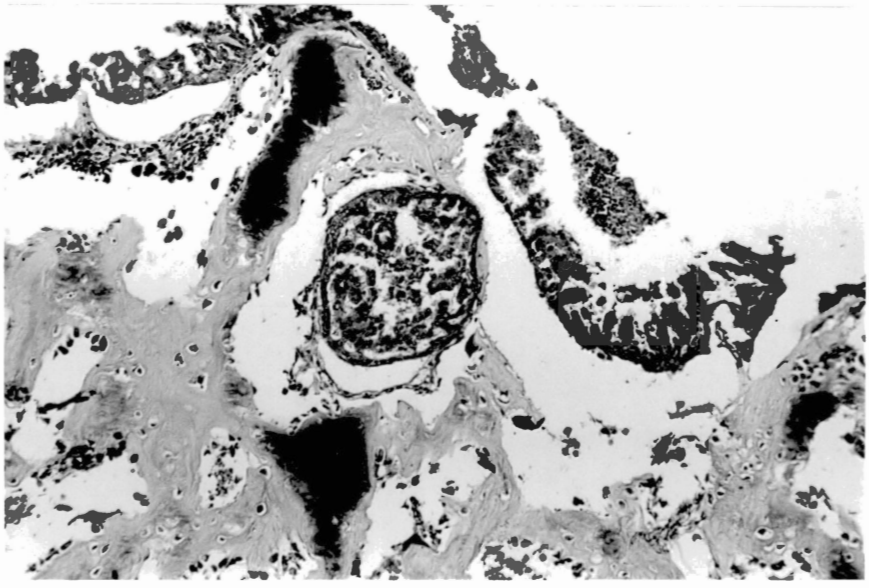

Figure 4. Case 200125. Ductal Carcinoma with Osteoid Appearing to Encase the Neoplastic Epithelium. (H&E x 75)

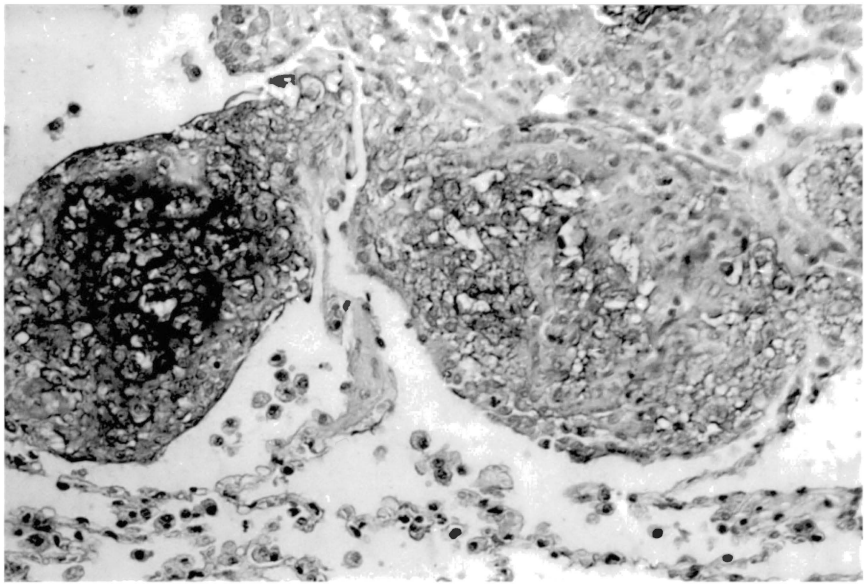

Figure 5. Case 202418-3. Lung Metastasis of a Ductal Carcinoma with Epithelial and Mesenchymal Growth Patterns. (H&E x 200)

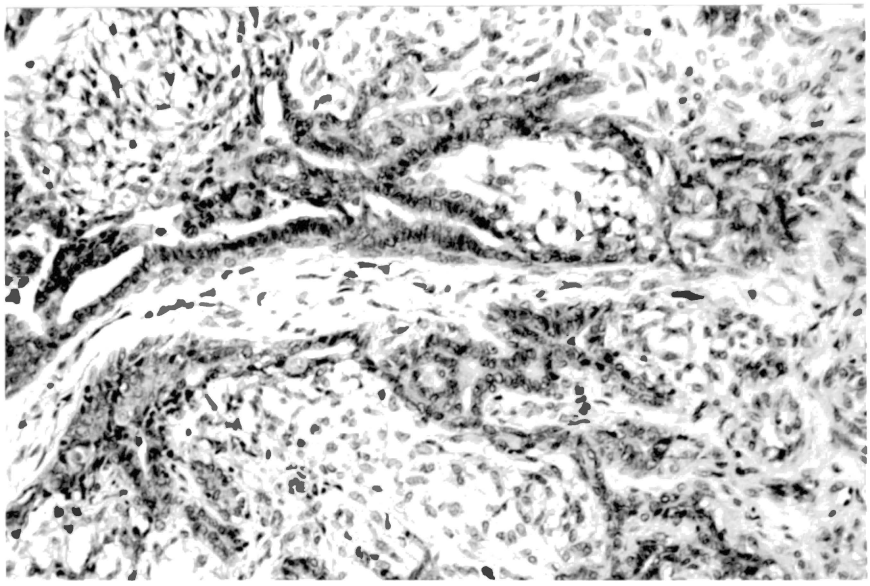

Figure 6. Case 201106. Adenoma with Pre- and Pseudocartilage Changes. (H&E x 200)

were common to both tumors (Figure 7). Papillomas appeared to be associated with the ductal and alveolar components. In a number of these tumors one could readily find support for a postulation of a transition from early vacuolated precartilage mucin accumulation near the glandular basement membranes (Figures 8 and 9) to a precartilage and pseudocartilage matrix (Figures 10 and 11) which often obliterated adjacent ductal systems (Figure 12). Finally mature hyaline cartilage evolved and occasionally matured to the point of mineralization (Figure 13). In this transition basement membranes became unidentifiable as did much of the pleomorphic cell population infiltrated with the accumulating mucinous matrix.

Intraductal carcinomas may be difficult to distinguish from ductal carcinomas and in this study some had many of the features common to both tumors and were usually designated as ductal carcinomas. However, others displayed the typical intraductal growth pattern and lacked the cartilaginous changes observed in the ductal carcinomas, adenomas and papillomas. These "purer" expanding neoplastic masses often contained intercellular vacuolations which gave the appearance of secretory activity (Figure 14). The central portion of these tumor masses was often undergoing progressive necrosis and had accumulations of necrotic, proteinaceous material.

The lobular carcinomas had the characteristic expanding, and only centrally infiltrating neoplastic

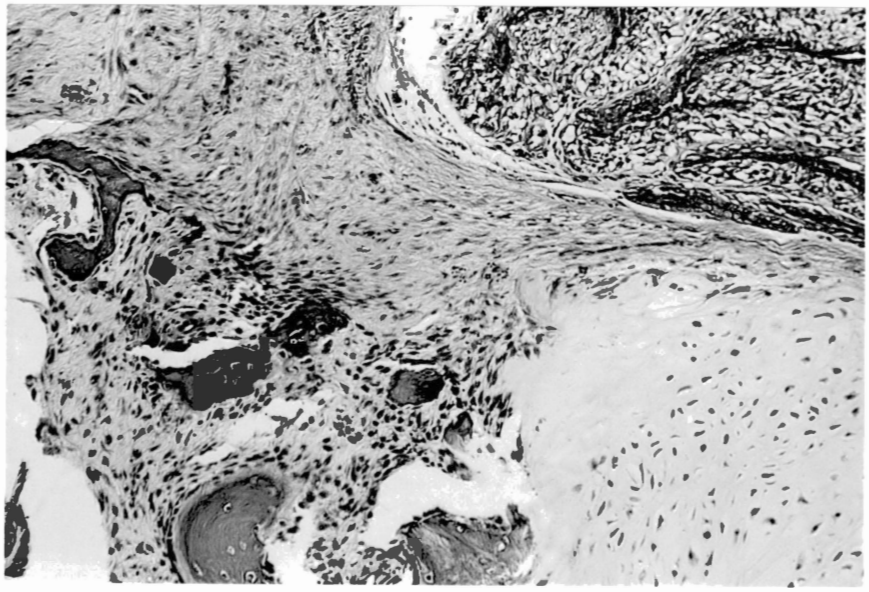

Figure 7. Case 200608. Mammary Tumor with Heterotopic Cartilage and Bone and Fibrous Proliferations. (H&E x 75)

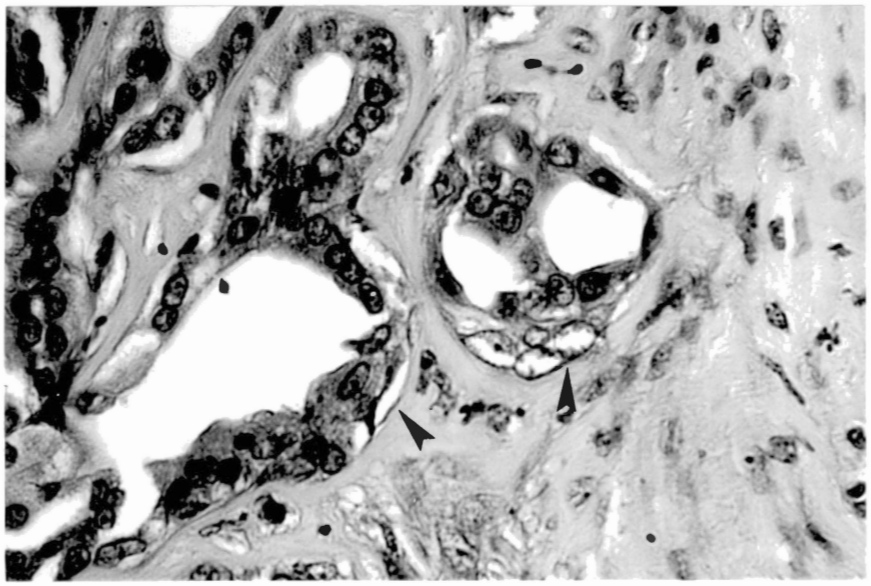

Figure 8. Case 204989. Precartilage changes. Note the Vacuolation Near the Glandular Basement Membrane (Arrows). (H&E x 480)

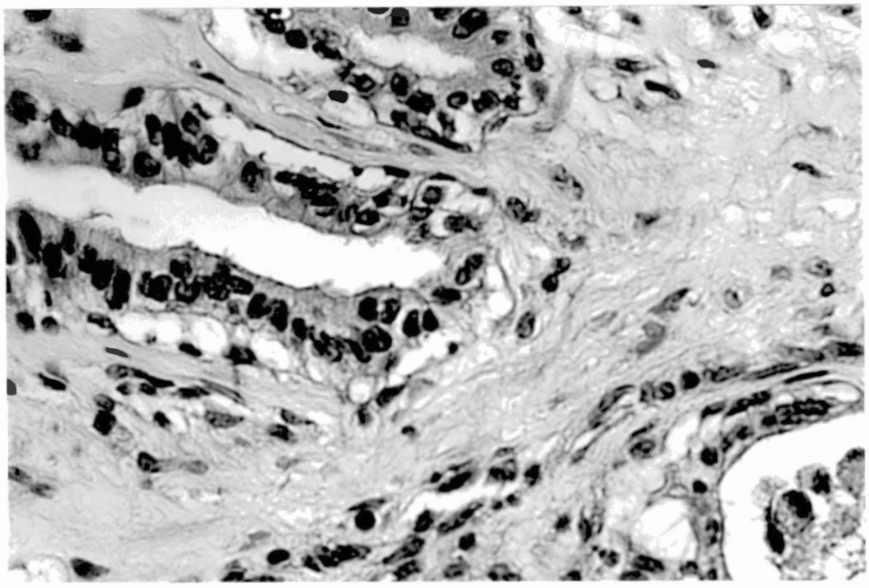

Figure 9. Case 204989. Precartilage Changes. Which are More Advanced than Figure 8. (H&E x 480)

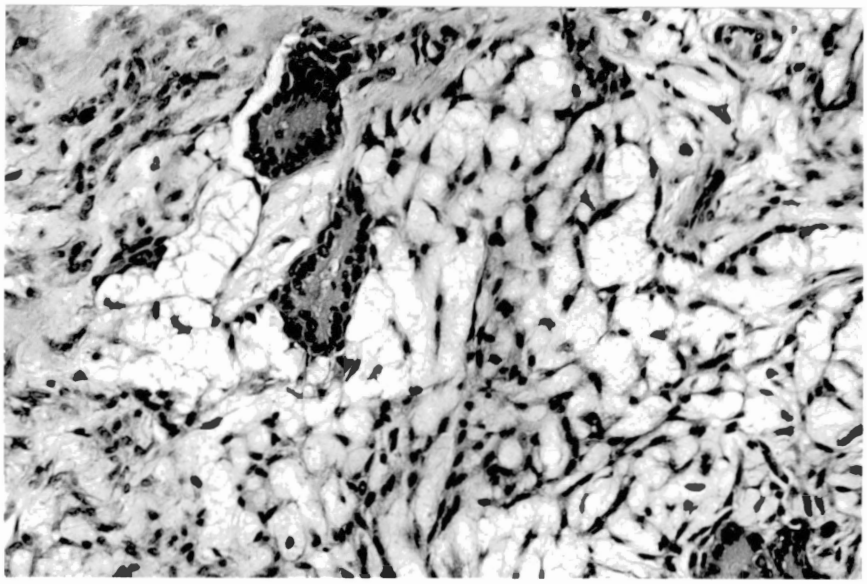

Figure 10. Case 204989. Pseudocartilage. Note the Loose Vacuolated Matrix and Abundant Spindle-shaped Cells. (H&E x 200)

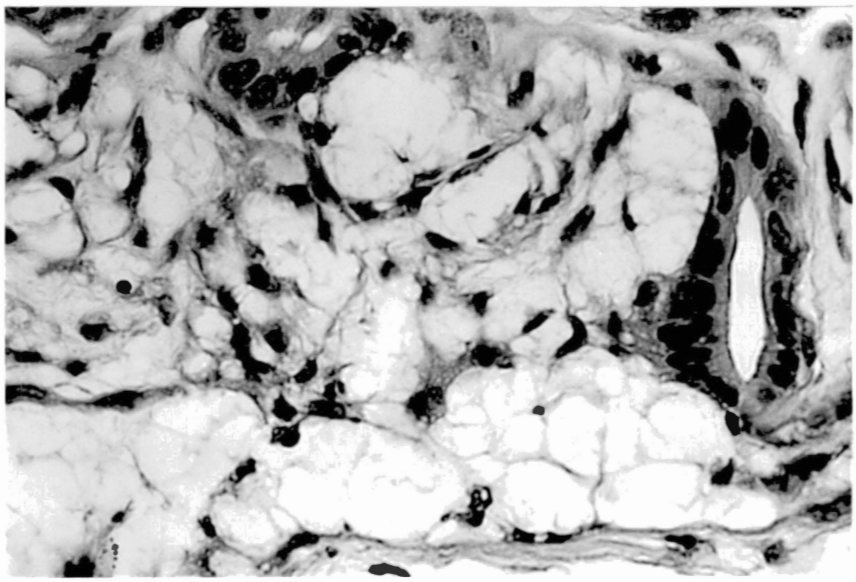

Figure 11. Case 204989. Pseudocartilage. A Higher Magnification of Figure 10. Note the Wispy Contents of the Vacuoles. (H&E x 480)

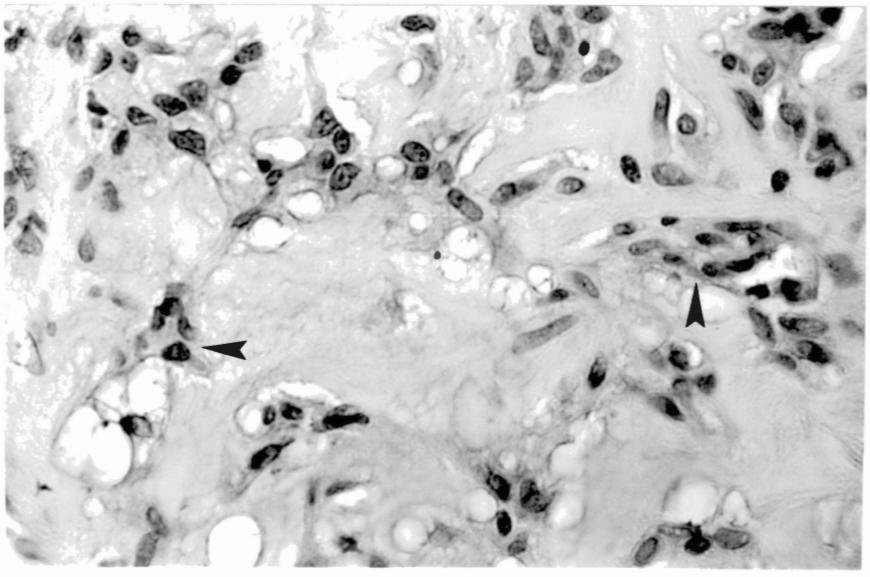

Figure 12. Case 204989. Pseudocartilage. Note the More Condensed Matrix Which Appears to Obliterate Adjacent Ductal Systems (Arrows). (H&E x 480)

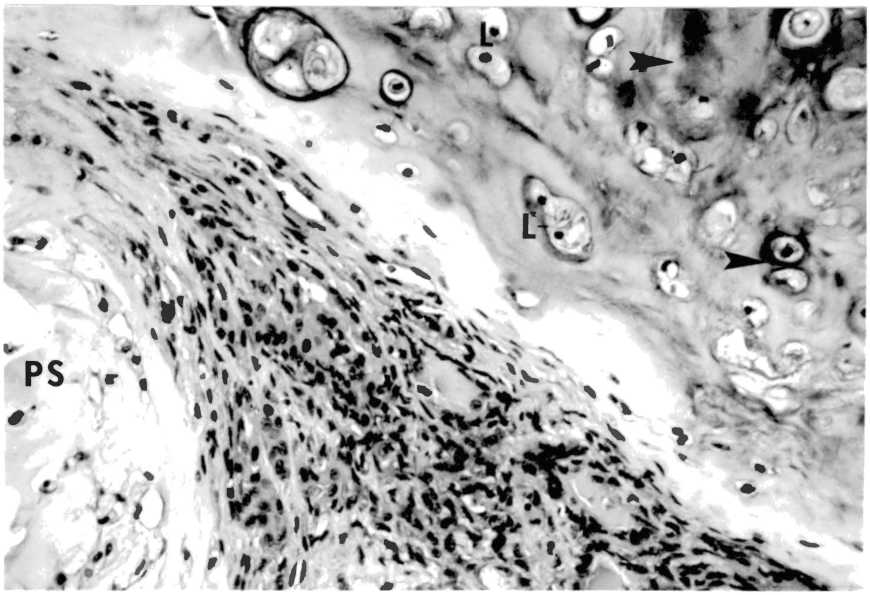

Figure 13. Case 205860. Mammary Tumor with Pseudocartilage (PS) and Mature Cartilage with Lacunae (L) and Mineralization (Arrows) (H&E x 200)

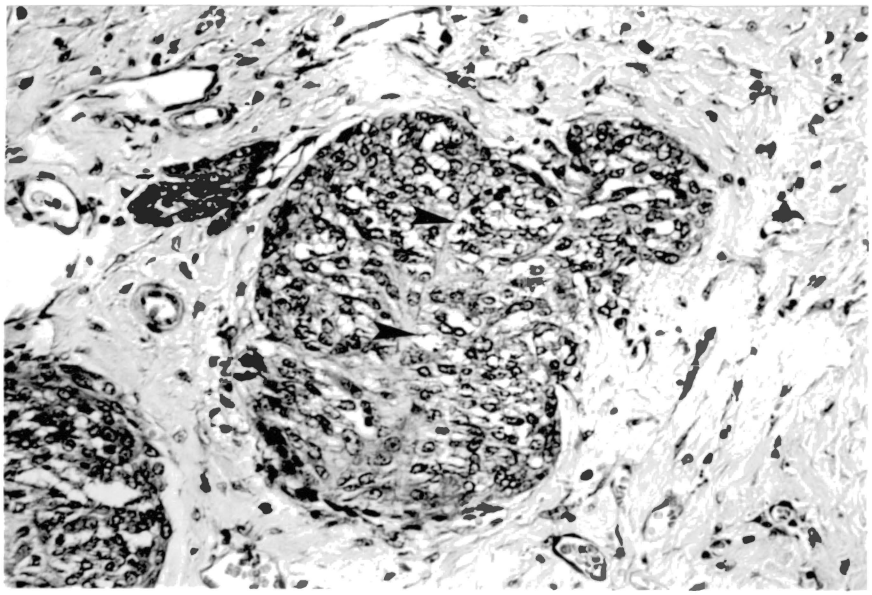

Figure 14. Case AS-13. Intraductal Carcinoma. Note the Intercellular Vacuolations (Arrows) giving the Appearance of Secretory Activity. (H&E x 200)

epithelium (Figure 15). Some areas had accumulations of intercellular secretory-like material. The stroma was loose, edematous and contained primitive mesenchymal-like cells but lacked the cartilaginous changes noted in other tumors.

Electron Microscopic

The electron microscopic findings considered pertinent to this study were limited to two cases: A ductal papilloma with cartilage metaplasia and an early ductal carcinoma with precartilage changes. The early cartilagenous changes were studied in an attempt to identify the possible cell(s) producing the abundant acid mucopolysaccharides.

The most consistent findings were numerous assorted basal lamina irregularities (thickenings, duplications, breaks and absences) (Figure 16) and an increase in the number of varible sized intercellular spaces between the epithelial cells (Figure 17). Vacuolated areas staining positive for acid mucopolysaccharides at the light microscopic level, appeared as intercellular spaces containing sparse, finely granular, and moderately to faintly electron dense material (Figure 18).

Isolated cells were difficult to identify with complete certainity and differentiation of pleomorphic cells in some areas was difficult to impossible. In the more normal areas, however, myoepithelial cells could be identified by their spindle shape, basal location adjacent to

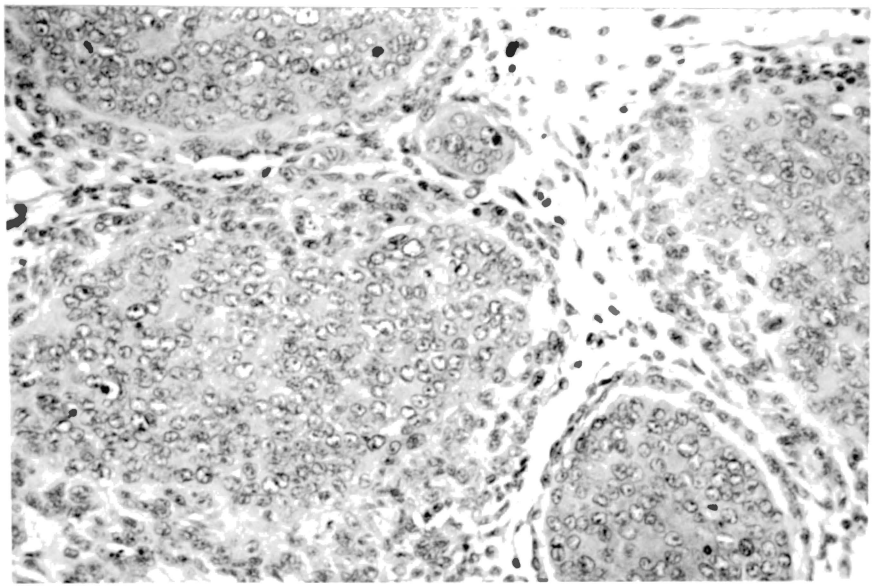

Figure 15. Case 200756. Lobular Carcinoma Note the Loose Edematous Stroma with Primitive Mesenchymal-like Cells. (H&E x 200)

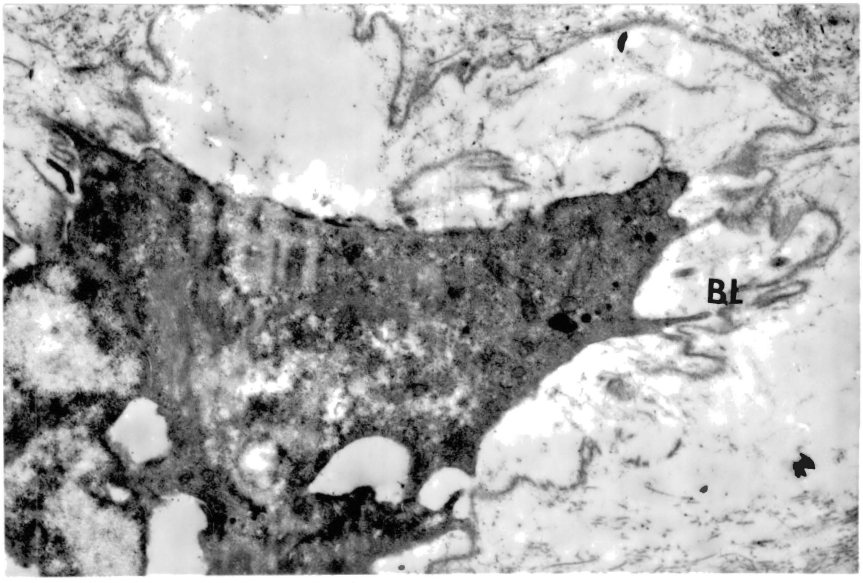

Figure 16. Block 17D. Basal Lamina (BL) Irregularities. (x 11,500)

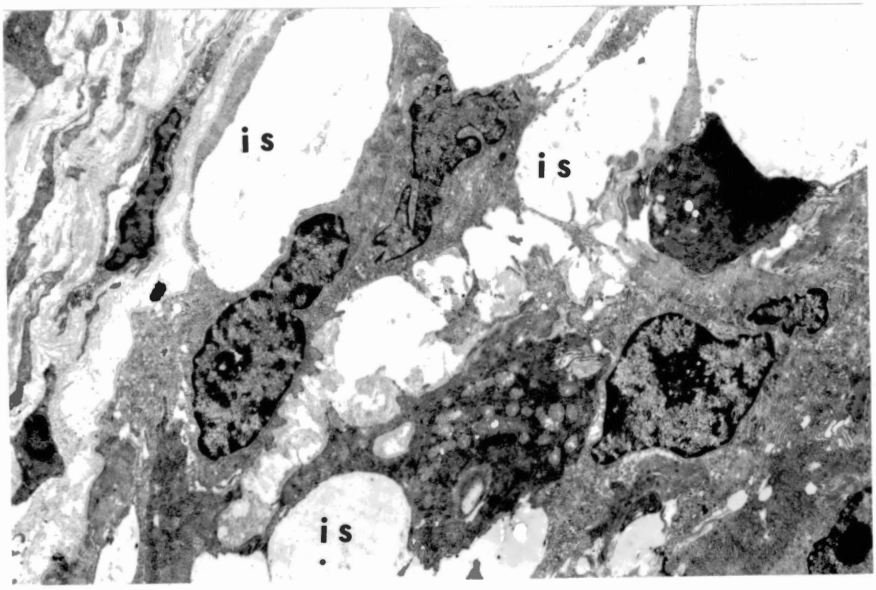

Figure 17. Block 26D. Varible Sized Intercellular Spaces (is). (x 5,000)

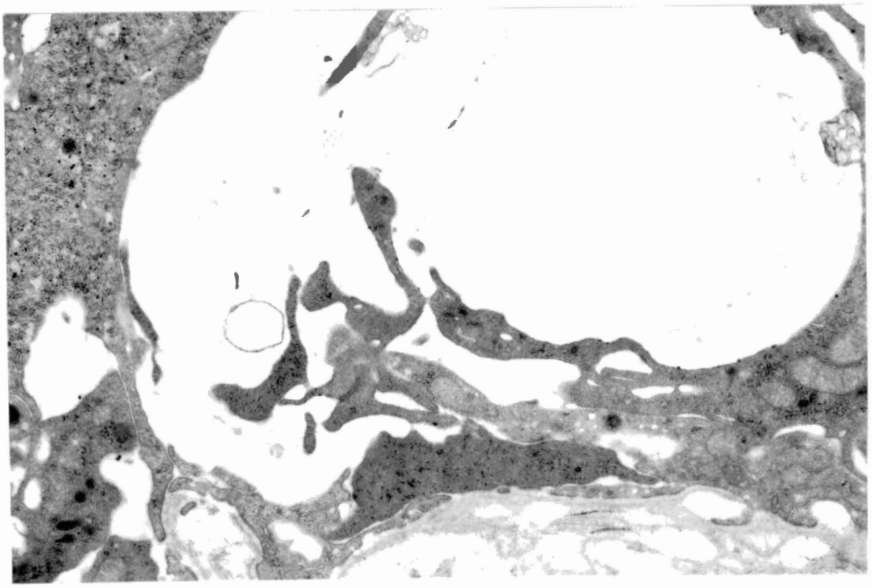

Figure 18. Block 22K. Intercellular Spaces with Sparse, Faintly Electron Dense Material. (x 15,000)

the basal lamina and abundant cytoplasmic microfilaments (Figure 19). Some possessed small vesicles within the cell membrane along the basal lamina. The apical or luminal cells possessed a microvillus surface and various cytoplasmic organelles including granular endoplasmic reticulum and secretory granules typical of epithelium (Figure 20). In some areas an intermediate cell was recognized having scattered microfilaments, cytoplasmic projections and organelles comparable to those of the epithelium. Cell junctions were common between the apical cells but were sparse to lacking between the basal and the intermediate cells (Figure 21). These latter cells often possessed numerous cytoplasmic projections of varying lengths and widths along the plasmalemma (Figure 22).

The adjacent stroma had a very loose irregular structure with scattered clumps of mature collagen (Figure 23). There were relative clear areas containing very fine granular and fibrilar amorphous material as well as areas completely void of any electron densities (Figure 24). The mesenchymal and fibroblastic cells present were spindle shaped with their long axis parallel to the basal lamina of the ducts or ductules. The cytoplasm of these cells contained abundant ribosomes and endoplasmic reticulum which is indicative of secretory activity (Figure 25). The blood vessels found within the stroma were normal except for a duplication of their basal lamina (Figure 26). The significance of this latter finding was not included

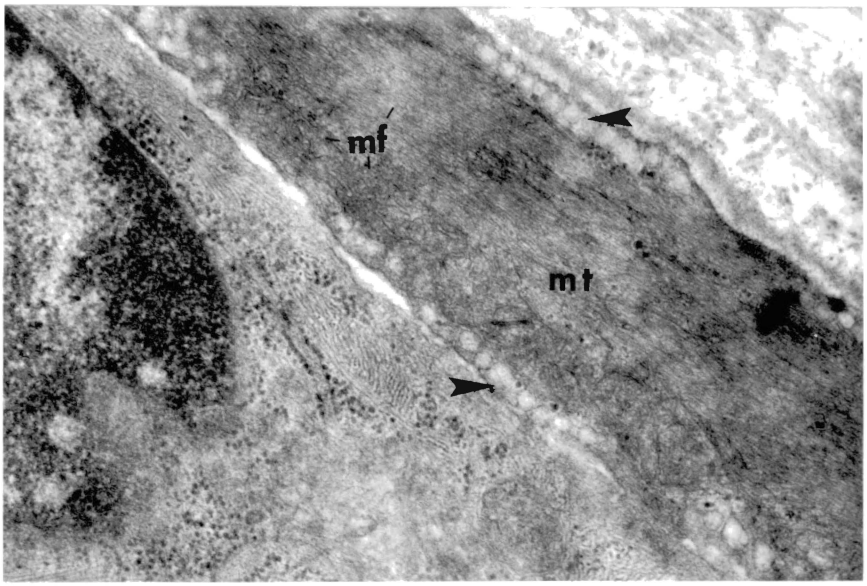

Figure 19. Block 17D. Myoepithelial Cell (mt). Note the Prominent Microfilaments (mf) and Vesicles (Arrows). (x 40,000)

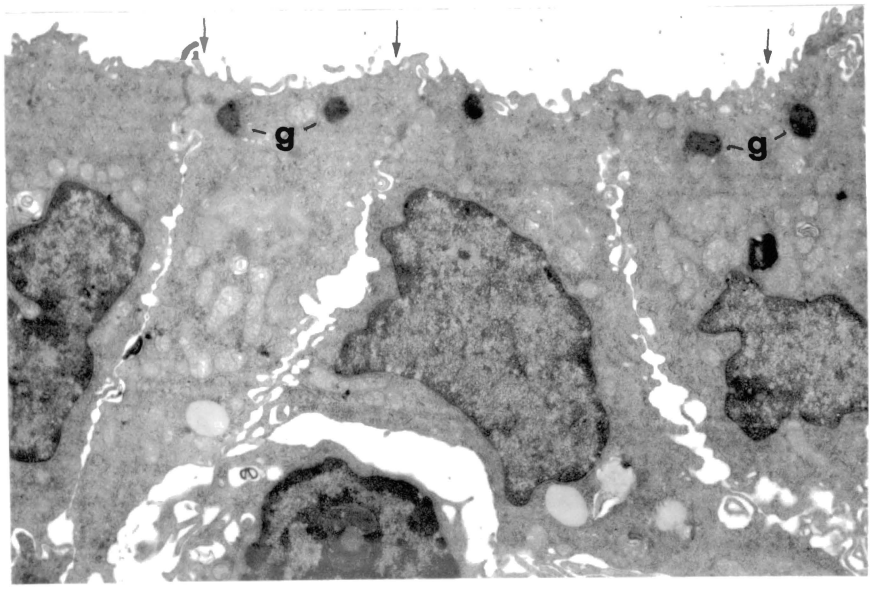

Figure 20. Block 22K. Apical Epithelial Cells. Note the Microvillus Border (Arrows) and Secretory Granules (g). (x 8,000)

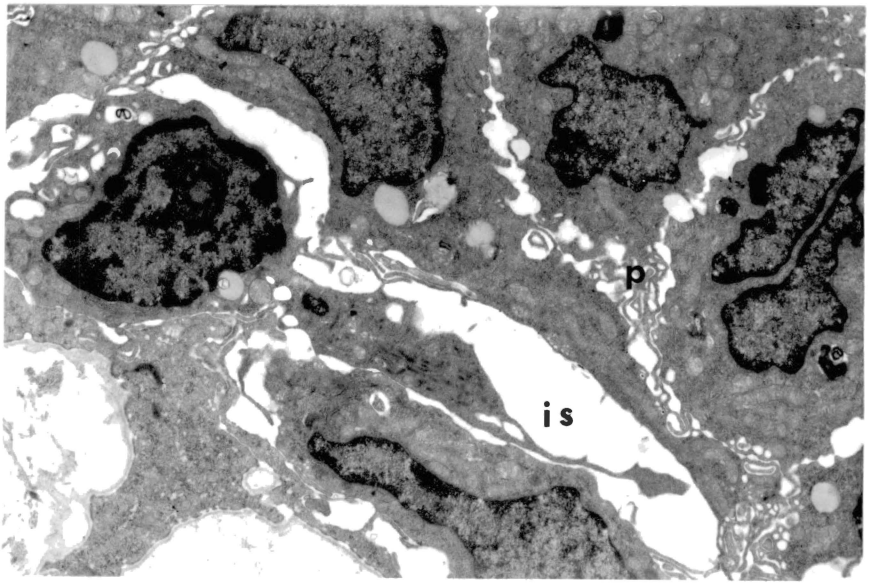

Figure 21. Block 22K. Basal and Intermediate Cells with Increased Intercellular Spacing (is) and Cytoplasmic Projections (p). (x 8,000)

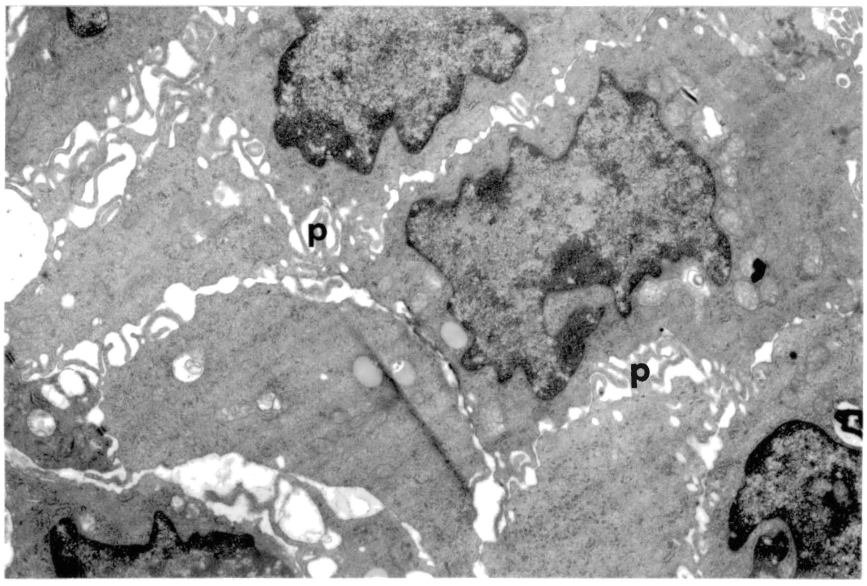

Figure 22. Block 22K. Basal and Intermediate Cells with Abundant Cytoplasmic Projections (p) of Varible Lengths and Widths. (x 9,000)

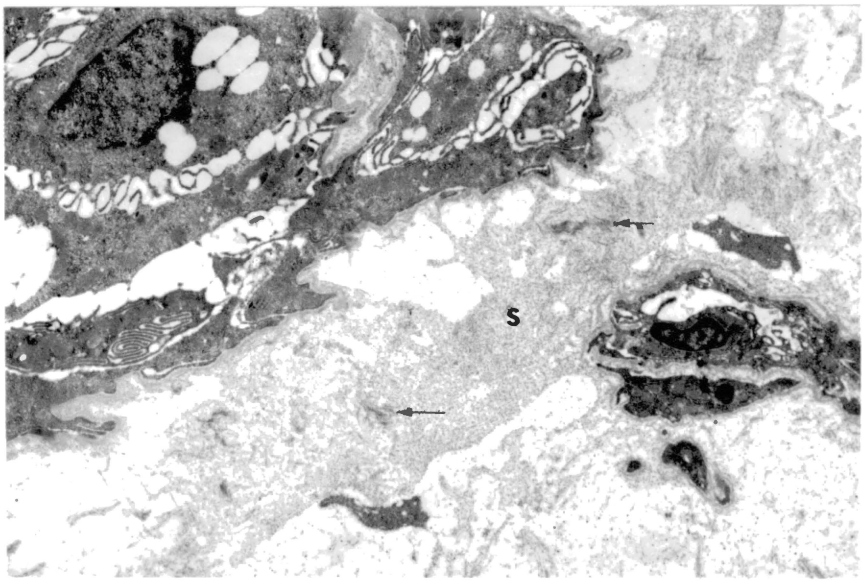

Figure 23. Block 26E. A Loose Irregular Stroma (s) with Scattered Clumps of Collagen (Arrows). (x 8,000)

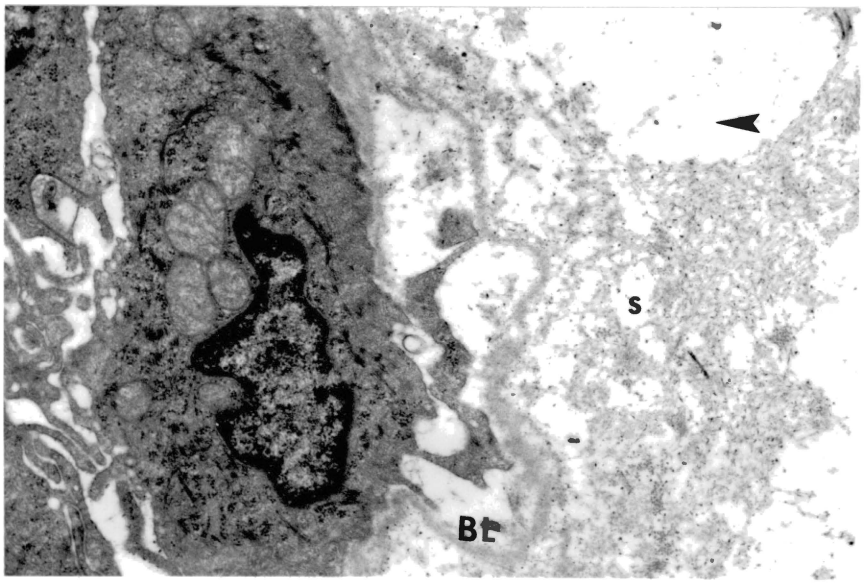

Figure 24. Block 22K. A Loose Irregular Stroma (s). Note the Empty Spaces (Arrow) and Basal Lamina (BL) Irregularities. (x 16,000)

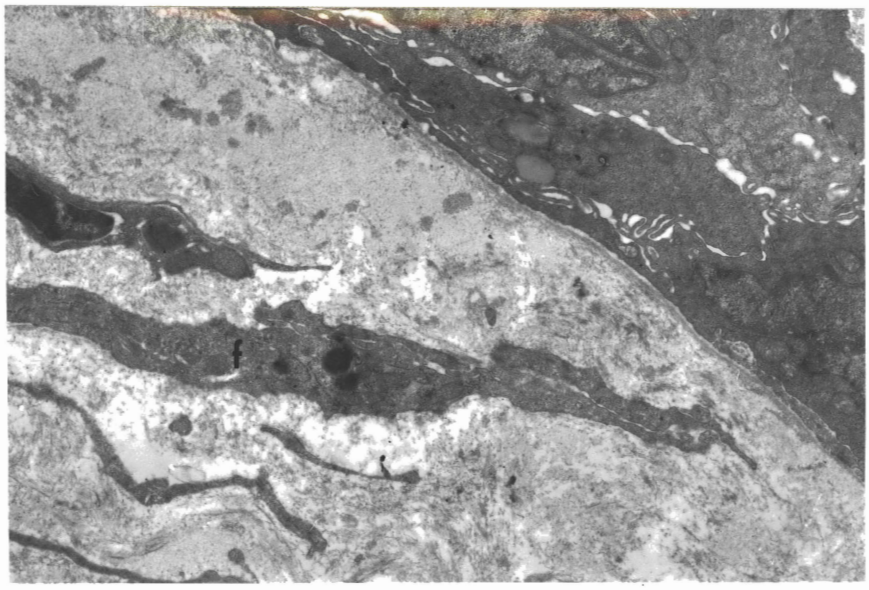

Figure 25. Block 22E. Fibroblastic Cells (f) with Evidence of Secretory Activity. (x 9,000)

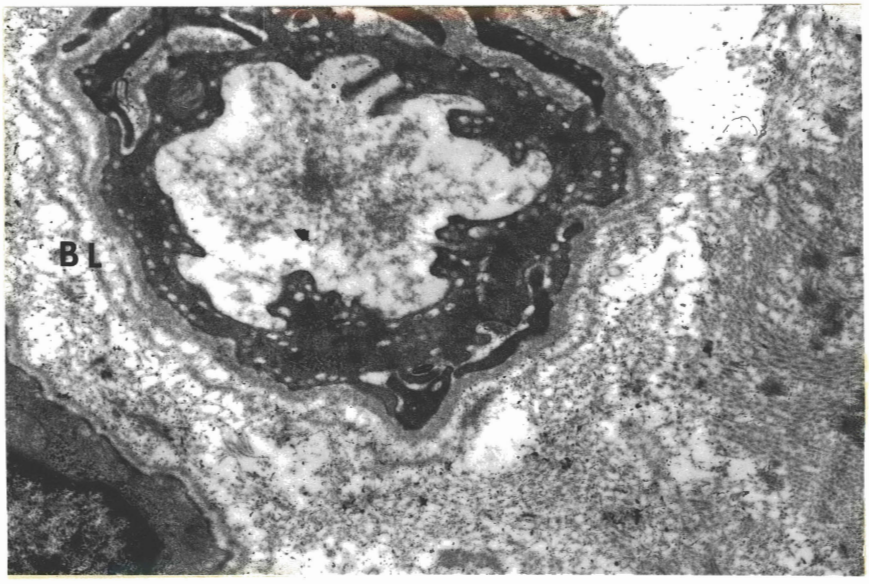

Figure 26. Block 22K. Blood Vessel with Duplication of the Basal Lamina (BL). (x 16,000)

within the scope of this study.

Stain Reactions

The acid mucopolysaccharides were initially identified as a group by their blue staining in the AB-PAS procedure. This positive reaction with the Alcian blue distinguished them from the PAS positive, periodate reactive, red staining neutral mucosubstances identified as non-ionic homoglycans and glycoproteins. The PAS reactions were primarily confined to the luminal material previously described. Occasionally this material would stain blue to purple indicating a mixture of acid and neutral substances (Figure 27). A PAS positive reactivity also was found around some cells within the pseudocartilage and cartilage matrix (Figure 28) as well as within a varible number of macrophages.

In most cases the more abundant acid mucopolysaccharides were found in areas identifiable as pre- and pseudocartilage and cartilage. Its morphological appearance ranged from an amphorous finely granular material to a more course granular and fibrilar substance (Figure 29 and 30). There were less but more uniform amounts of acid mucopolysaccharides found in close association with fibrous elements of the connective tissue stroma. Varible amounts of acid mucopolysacchardies were identified in metastatic sites and in most cases appeared to be confined to the intercellular spaces or supporting connective tissue

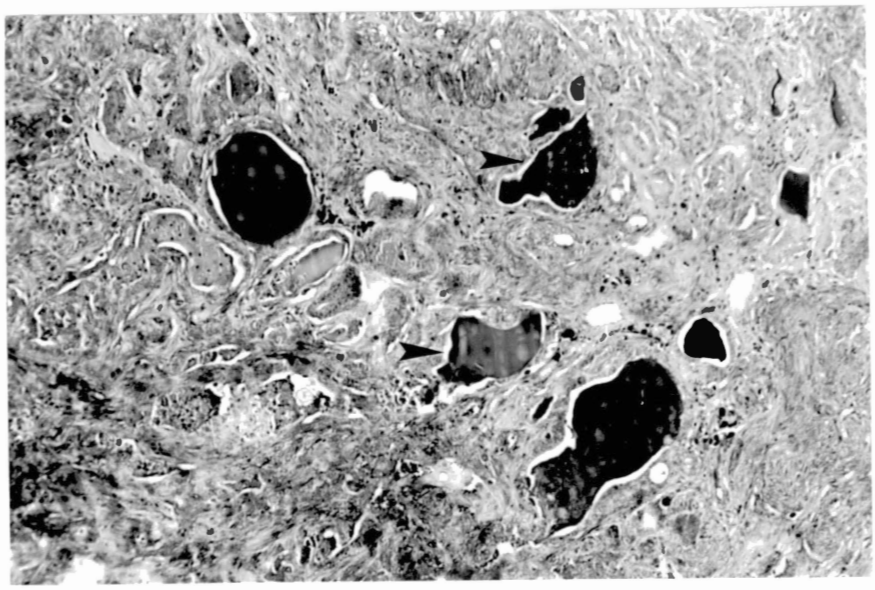

Figure 27. Case 205238. Varible Staining Luminal Material (Arrows). (AB-PAS x 75)

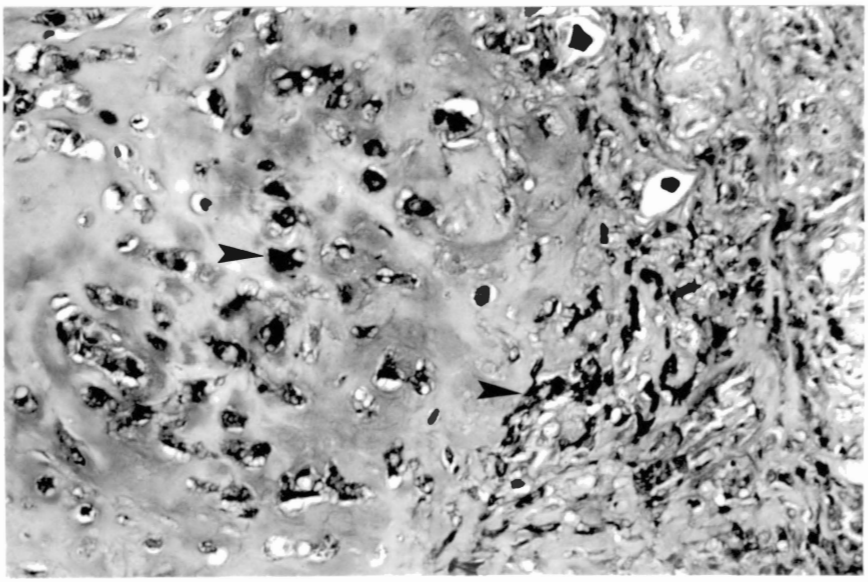

Figure 28. Case 205860. PAS Positive Activity (Arrows) Around Cells Within the Pseudocartilage and Cartilage Matrix. (AB-PAS x 200)

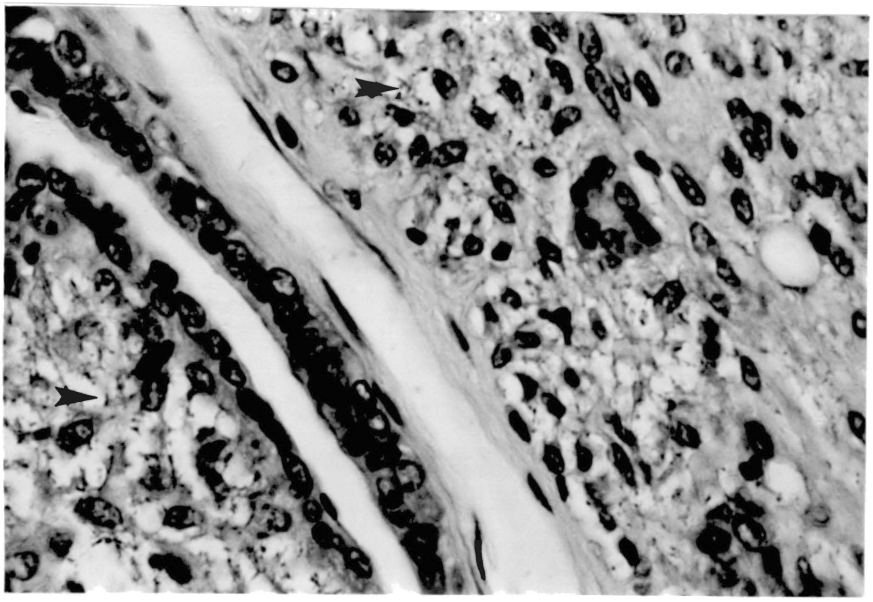

Figure 29. Case 202070. Coarse Granular Acid Mucopolysaccharide (Arrows) in Vacuolated Precartilage. (H&E x 480)

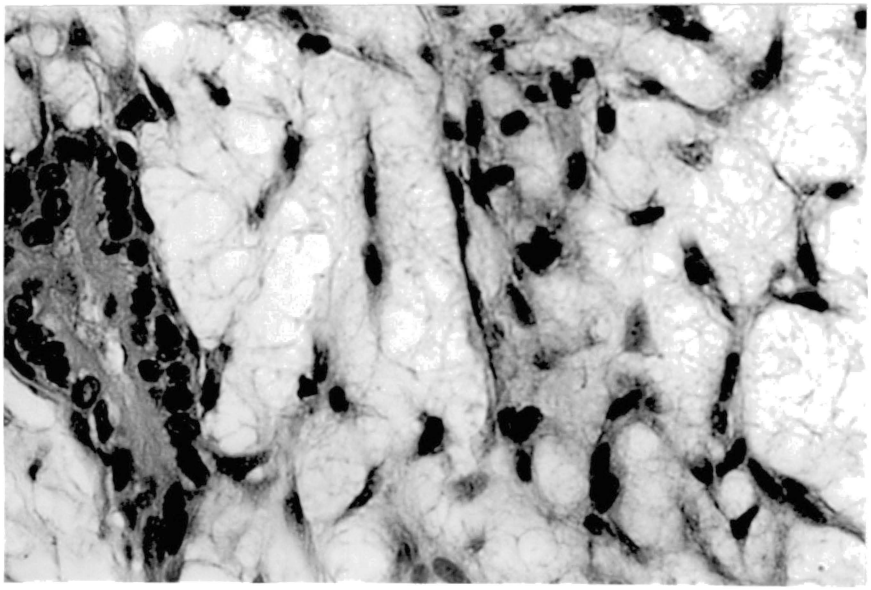

Figure 30. Case 204989. Fibrilar Appearance Associated with a Pseudocartilage Matrix. (H&E x 480)

stroma (Figure 31). In one case even the brain metastasis had some accompanying acid mucopolysaccharide but considerably less than the other sites (Figure 32).

The hexachrome stain with its Alcian blue component verified the localization and morphology of the acid mucopolysaccharides as well as aided in the differentiation of various other tissue components such as collagen and elastic fibers, osteoid and in some cases normal myoepithelial cells (Figure 33). In most cases however a myoepithelial cell component was impossible to identify within the tumor.

Once the acid mucopolysaccharides were localized, subsequent staining reactions provided additional information as to their individual identity. When compared to the general AB (2.5) staining, the positive blue staining of AB (1.0) was confined primarily to the more readily identifiable connective tissue stroma and mature cartilage and indicated this acid mucopolysaccharide to have a high degree of sulfation (Figure 34 and 35). This reaction is typical of the sulfated mucosubstances such as the chondroitin sulfates, keratosulfate, heparin and sulfated sialomucins. In general the more condensed stroma and the more differentiated cartilage stained more intense at the lower pH. In contrast the vacuolated pre- and pseudocartilage areas which were positive with AB (2.5) had a reduced staining reaction or failed to stain at all with AB (1.0) (Figure 36 and 37).

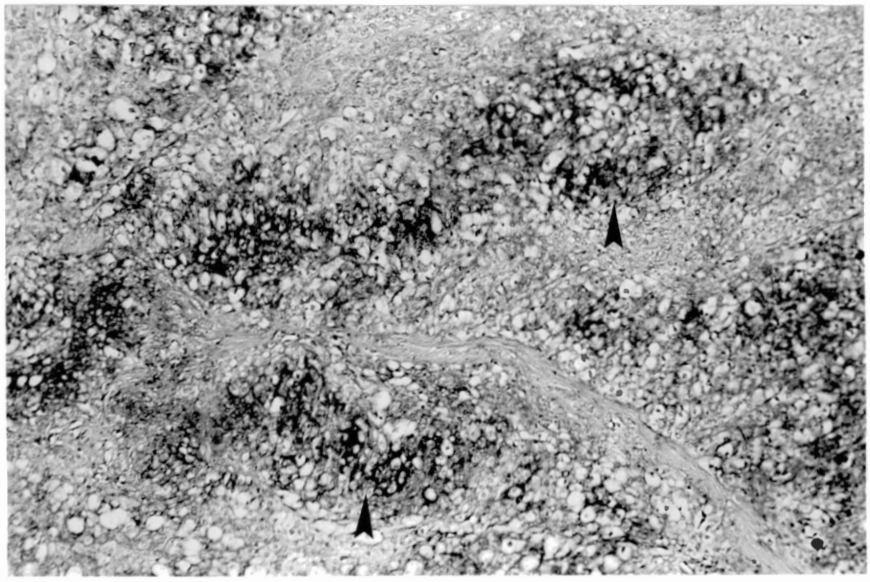

Figure 31. Case 202418-3. Spleen Metastasis with Varible Amounts of Acid Mucopolysaccharide (Arrows). (AB-NFR x 200)

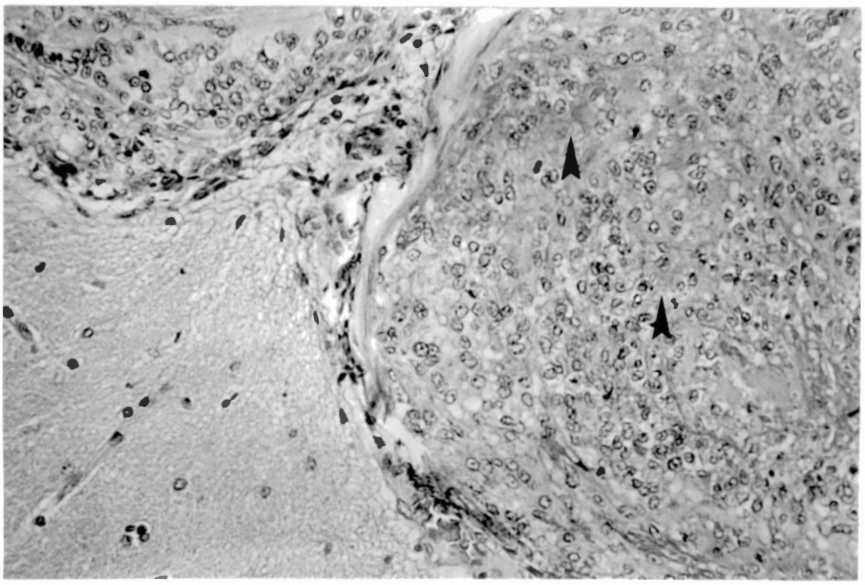

Figure 32. Case 202418-3. Brain Metastasis with Varible Amounts of Acid Mucopolysaccharides (Arrows) (AB-NFR x 200)

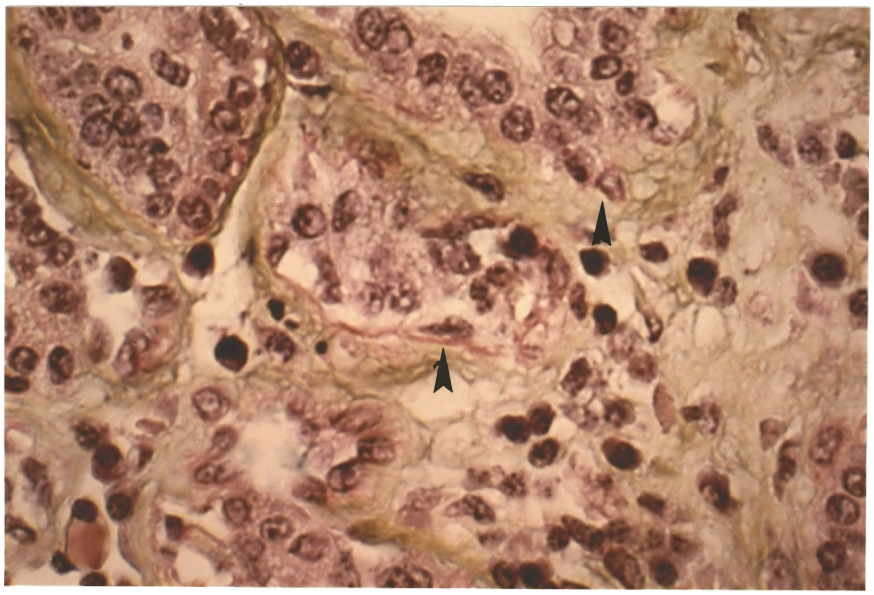

Figure 33. Case 202070. Ductal Elements with Normal Appearing Hyoepithelial Cells (Arrows). (Hexachrome x 480)

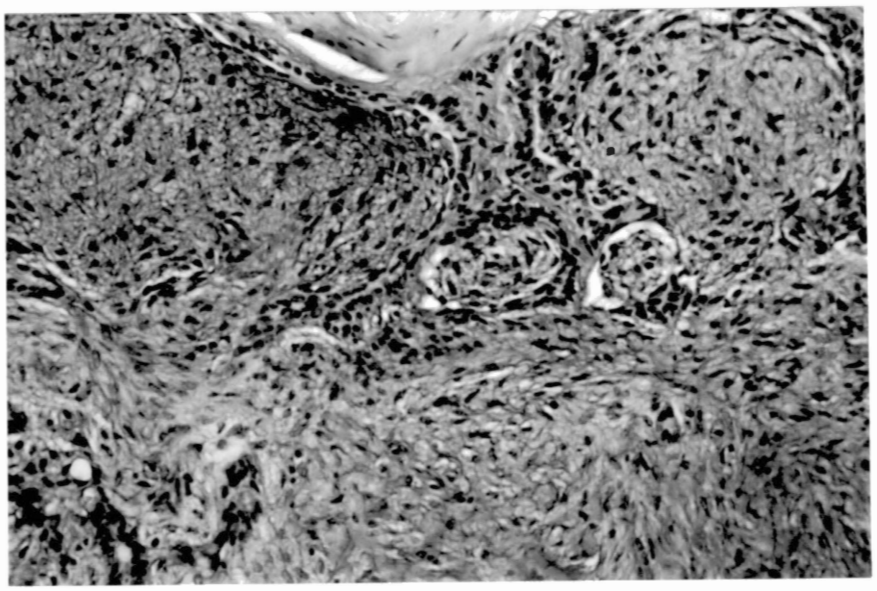

Figure 34. Case 700-76. Pre- and Pseudocartilage Stained with Alcian Blue, pH 2.5. (AB-NFR x 200)

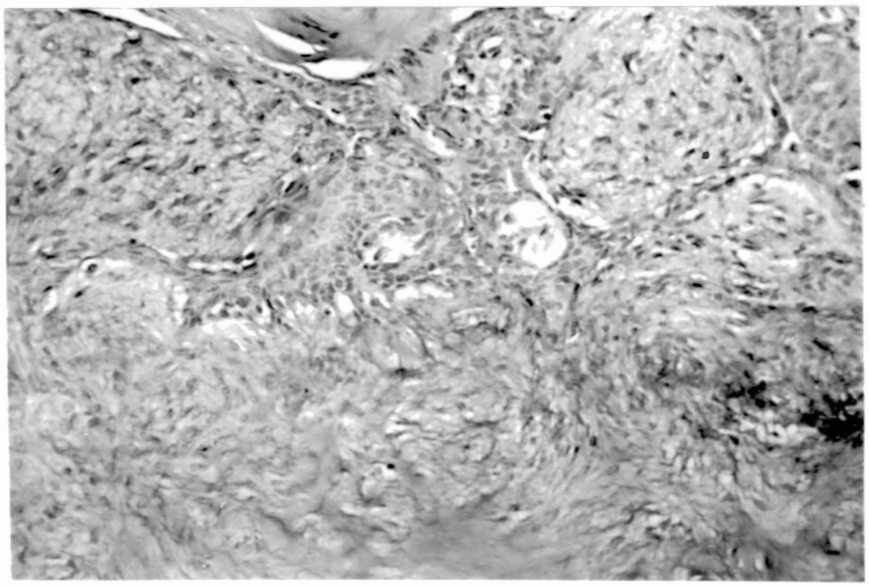

Figure 35. Case 700-76. Same Area as Figure 34 Stained with Alcian Blue, pH 1.0. (AB-NFR x 200)

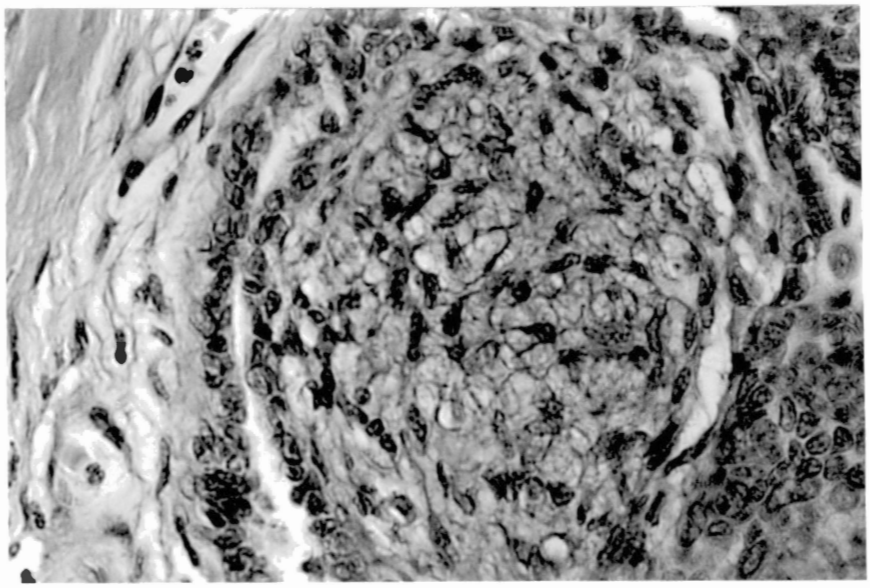

Figure 36. Case 201106. Pre- and Pseudocartilage Stained with Alcian Blue, pH 2.5. (AB-NFR x 480)

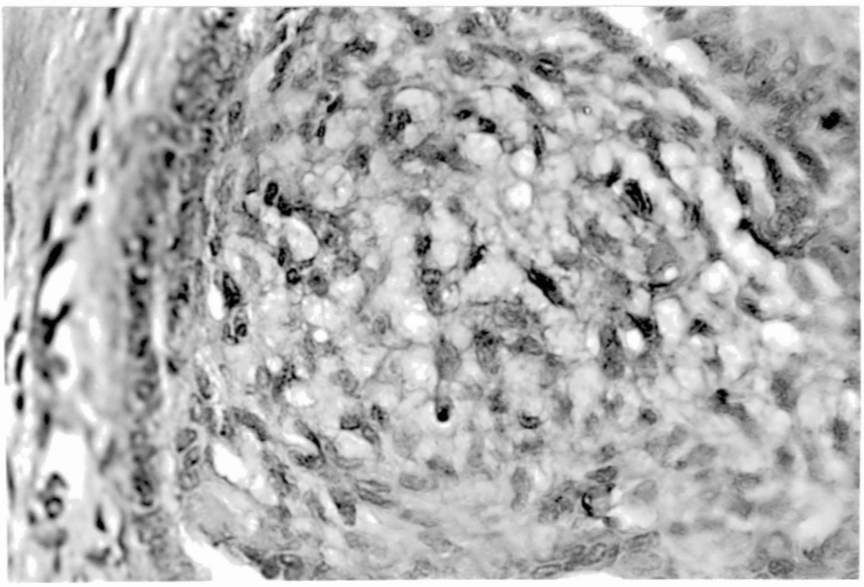

Figure 37. Case 201106. Same Area as Figure 36 Stained with Alcian Blue, pH 1.0. (AB-NFR x 480)

The aldehyde fuchsin-alcian blue (AF-AB) stain procedure provided a single slide contrast which supported the findings of the Alcian blue staining at pH 1.0 and pH 2.5. The AB (1.0) positive acid mucopolysaccharides stained the purple of the aldehyde fuchsin while all others stained the blue of the Alcian blue.

Enzyme Digestion Procedures

Diastase

The PAS positive reaction observed in the AB-PAS procedure was found consistently around cells within some of the pseudocartilage matrix and within the lacunae of the more differentiated cartilage. In all cases the positive reactions in these areas were eliminated by prior diastase digestion indicating these substances to be non-ionic homoglycans or more specifically glycogen (Figure 38 and 39). The PAS positive luminal material was more varible. All the material which stained positive with PAS alone had very little of its staining eliminated by diastase. However, very little PAS reactivity occurred in this material when pretreated with phenylhydrazine (Figure 40 and 41). The general but moderate PAS positive reactivity observed in the stroma was not affected by diastase but was completely blocked by phenylhydrazine.

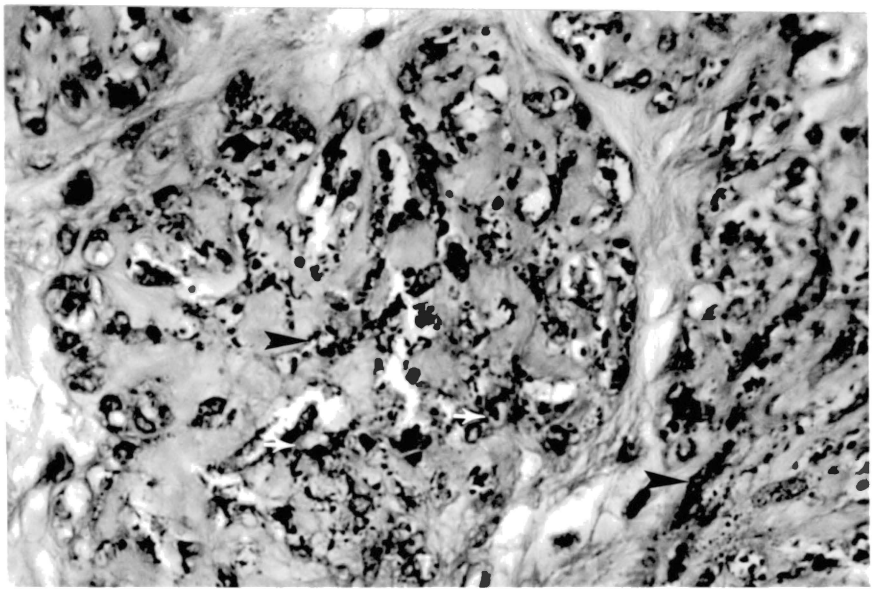

Figure 38. Case 202070. Pre- and Pseudocartilage with Abundant Glycogen (Arrows). (PAS x 480)

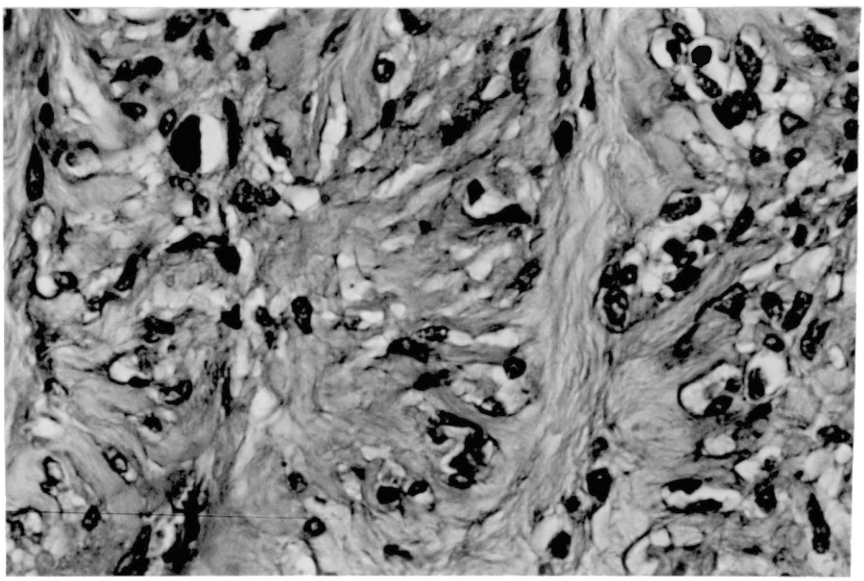

Figure 39. Case 202070. Same Area as Figure 38 Digested with Diastase. (PAS x 480)

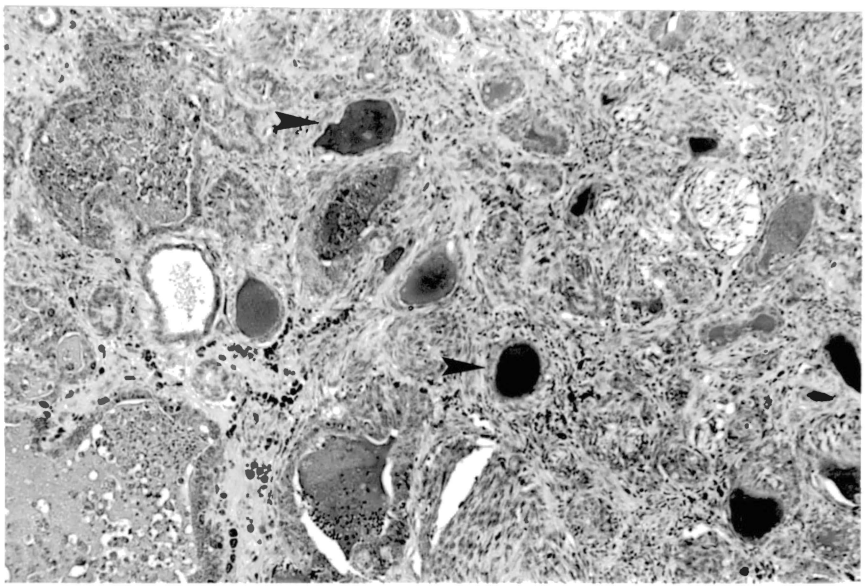

Figure 40. Case 205238. PAS Activity (Arrows) Remaining after Diastase Digestion (PAS x 75)

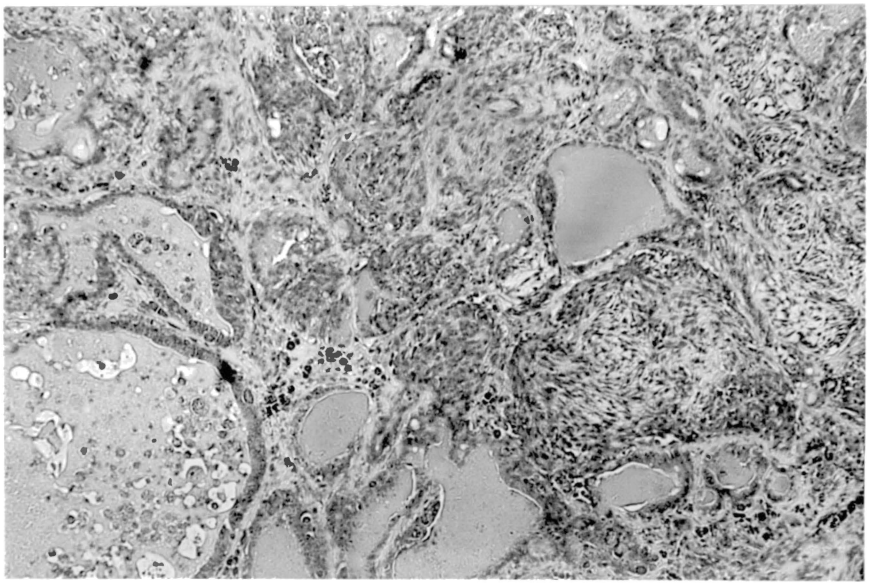

Figure 41. Case 205238. Same Area as Figure 40 Pretreated with Phenylhydrazine. Note the Loss of PAS Activity. (PAPS x 75)

Hyaluronidase

In all cases the acid mucopolysaccharides found in the pre- and pseudocartilage and the cartilage matrix showed reduction to complete loss of Alcian blue staining following hyaluronidase digestion (Figure 42 and 43). This therefore indicates these acid mucopolysaccharides to be hyaluronic acid or chondroitin-4 and chondroitin-6-sulfate or a mixture thereof. The loose, vacuolated pre-cartilage areas appeared to be the most reactive to the enzyme as there was complete loss of staining (Figure 44). Since these same areas are void of sulfated mucopolysaccharides (negative with AB 1.0), the acid mucopolysaccharide present is identified as hyaluronic acid. While the more compact, fibrous and mature cartilaginous areas showed a reduction in staining not all the Alcian blue reaction was lost. These latter areas correspond to those previously described as staining positive with AB (1.0). The hyaluronidase however had no appreciable effect on the Alcian blue staining in the normal connective tissue stroma.

Neuraminidase

The Alcian blue staining of the acid mucopolysaccharides in all the tumors studied was not affected in any manner by prior digestion with neuraminidase. This was true of the luminal material in the ducts as well as the matrix of the

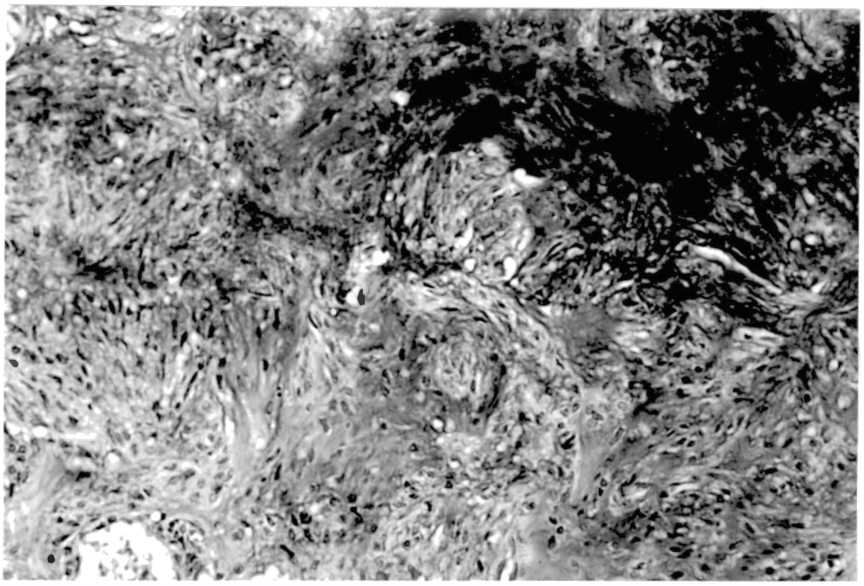

Figure 42. Case 205238. Abundant Acid Mucopolysaccharides. Compare with Figure 43. (AB-NFR x 200)

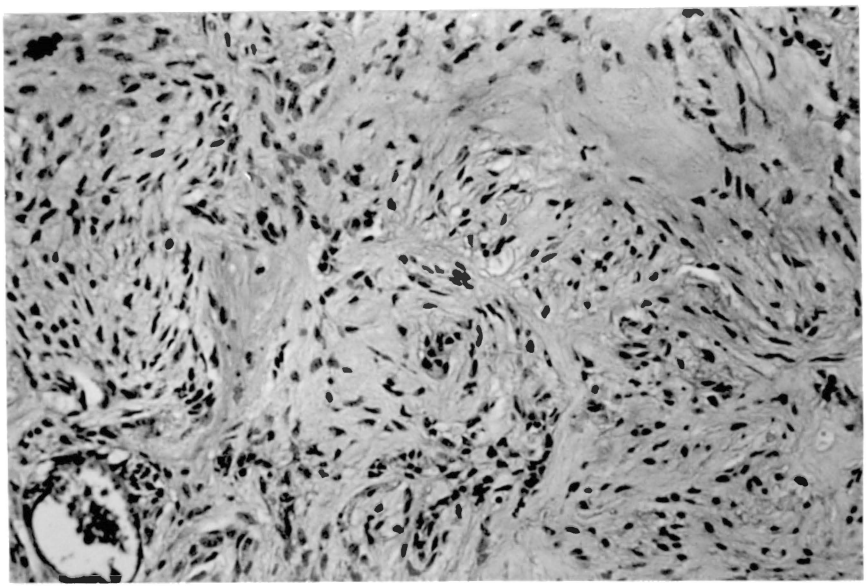

Figure 43. Case 205238. Same Area as Figure 42 Digested with Hyaluronidase. (AB-NFR x 200)

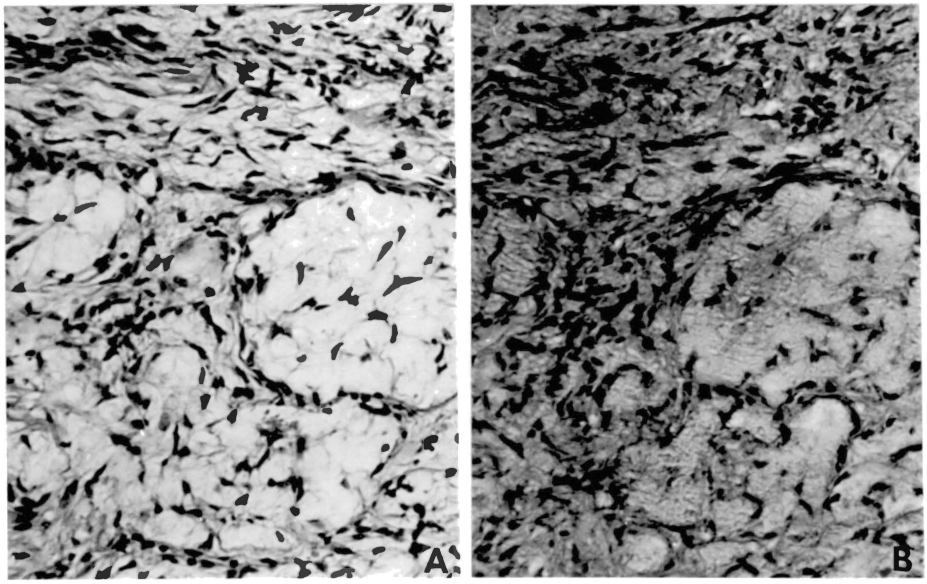

Figure 44. Case 201179. Pre- and Pseudocartilage with (A) and Without (B) Hyaluronidase Digestion. (AB-NFR x 200)

pre- and pseudocartilage and mature cartilage (Figure 45 and 46). This would indicate therefore that there were no appreciable amounts of sialomucin with the acid mucopolysaccharides studied. In some cases these results were verified by use of the acid hydrolysis procedure in place of neuraminidase digestion.

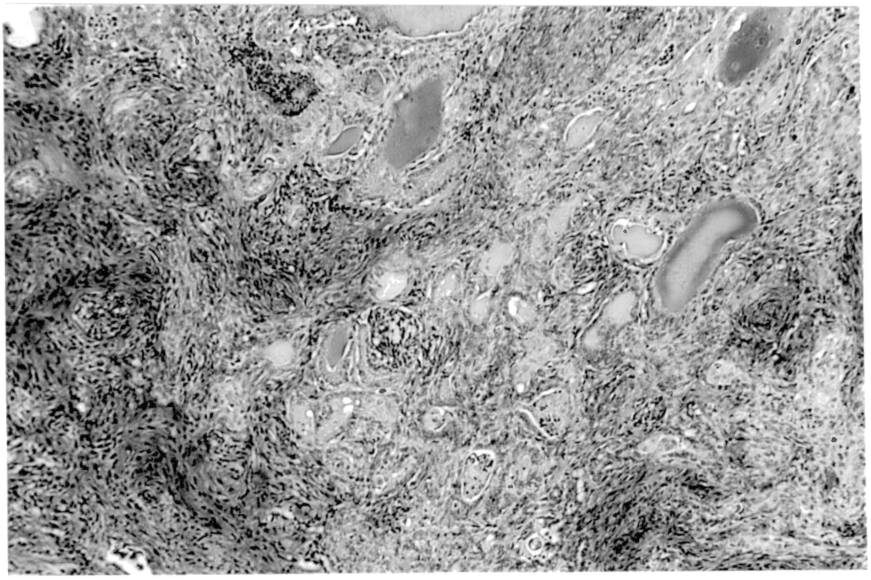

Figure 45. Case 205238. Alcian Blue Staining Without Neuraminidase Digestion. (AB-NFR x 75)

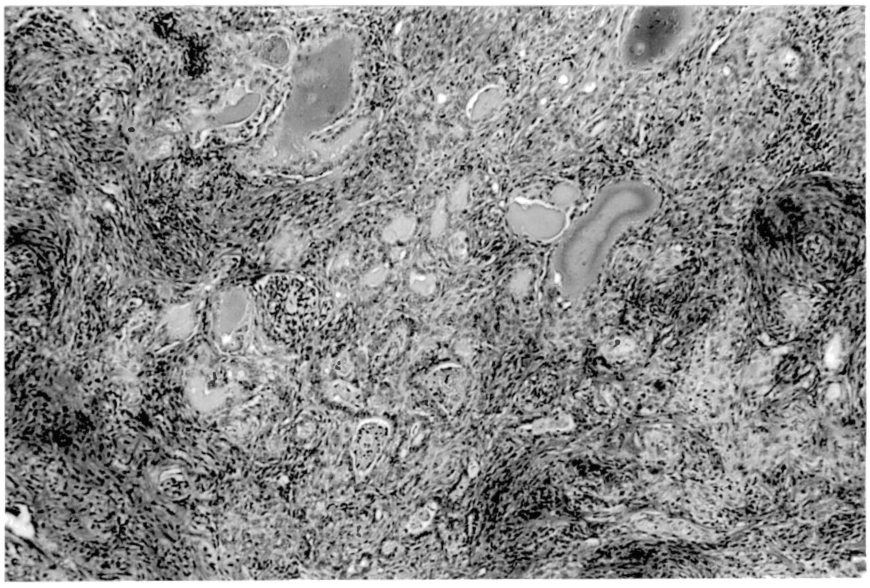

Figure 46. Case 205238. Same Area as Figure 45 Digested with Neuraminidase. (AB-NFR x 75)

CHAPTER V

DISCUSSION AND CONCLUSION

Discussion

Cartilage formation in a developing mammal is a rather complex but orderly process, the details of which need not be reviewed here in their entirity. It is generally accepted, however, that the primitive mesenchymal cell and its more differentiated counterparts (fibroblasts and chondroblasts) are responsible for secreting both the collagen and the ground substance which make up the matrix of cartilage. It has been reported (39) that the ground substance of the more primitive connective tissues including cartilage is composed primarily of hyaluronic acid. In maturation and aging there is a progressive loss of hyaluronic acid and an increase in the chondroitin sulfates. As the aging progresses there is a decrease in the amount of chondroitin sulfate, an increase in the keratosulfate fraction along with a possible increase in a neutral mucopolysaccharide-glycogen. It has been reported that glycogen is found very often in close association with not only the mature chondrocyte but in the developing chondroblasts as well (21). The

presence of this neutral polysaccharide is not fully understood but may relate in some manner to the metabolism and the secretory activity of these cells.

Electron microscopic studies of normal cartilage development have shown that as the mesenchymal cell differentiates into chondroblasts there is marked acquisition of an extensive endoplasmic reticulum, enlargement and concentration of the Golgi apparatus and concentration of membrane bound cytoplasmic inclusions. These modifications are indicative of matrix secretion (21). Evidence has been presented which indicates that both the fibrilar (collagen) and ground substance components of the matrix are produced within the same cell by a similar mechanism (9, 22, 29).

The concept of heterotopic cartilage arising by metaplasia of connective tissue is neither new nor startling. On the contrary when such cartilage is found in tumors, it is a logical assumption that this tissue of specific embryological derivation can transform under certain conditions and still maintain its genetic affinity. The question arises as to whether connective tissue metaplasia is the means of heterotopic cartilage formation in canine mammary tumors. The presence of specific connective tissue mucopolysaccharides as demonstrated in this study provides considerable evidence to support this concept.

Theories of myoepithelial cell involvement in the formation of various components in the so called "mixed" tumors have received considerable attention in recent years. A number of investigators have attempted to show that the myoepithelium secretes components of the cartilage matrix or differentiates into cartilage producing cells (49, 57). These investigators as well as others have experienced much difficulty in identifying the myoepithelial cell. This is especially true when the cells are involved in a neoplastic process. This difficulty therefore casts some doubts on the proposed theories.

In the electron microscopic studies of myoepithelium, the presence of abundant cytoplasmic microfilaments is used as the major identifying characteristic. However, as these cells undergo changes, the amount and the morphology of these cytoplasmic organelles varies considerably (19, 48, 52). Also similar microfilaments have been reported in a variety of cells including the ductal epithelium and cells with major epithelial characteristics (19, 24, 28, 50, 52, 72). To avoid this uncertainity in identification, some investigators advocate the use of histochemical methods for demonstrating alkaline phosphatase and adenosine triphosphatase activity in myoepithelial cells (34, 44, 45, 63, 64, 73). However, others have questioned the specificity of these methods by pointing out that the activity of these enzymes may be demon-

strated along the plasma membrane of any cell engaged in a transport phenomena in relation to that membrane (15). Ahmed (2) went as far as to demonstrate by electron microscopic histochemistry such enzyme activity in tumor cells showing obvious epithelial differentiation as well as mucin production.

The difficulties in the identification of myoepithelium demonstrates how little is actually known about the cell. Although much is known of its major function and physiology, the exact derivation of the cell is still a subject of controversy. The work of Hamperl (27) and the myoepithelial location on the epithelial side of the basement membrane indicates an ectodermal origin. Also the identification of intermediate cells with both epithelial and myoepithelial characteristics, plus the seemly absence of demonstratable myoepithelium in the "purer" ductal epithelial tumors support the concept that the myoepithelium is truly a modified and possible reserve epithelial cell.

It is rather surprising that with the vast knowledge of mucopolysaccharides available, more histochemical work such as presented in this study has not been reported. Extensive research in the area of cartilage development and in such disease processes as mucopolysaccharidoses has contributed significantly not only to the identification of mucopolysaccharides but also to the knowledge of their physical and chemical characteristics and their

biosynthesis.

According to the classification system outline in Culling (11), the acid mucopolysaccharides (anionic heteroglycans) are found only in connective tissues. These are identified as hyaluronic acid, chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, heparin and keratosulfate. Keratosulfate has only been found in the aorta, cornea and aged cartilage. Heparin likewise occurs only in mast cells and the intima of arteries. Because of these limited sources, the presence of these two acid mucopolysaccharides were not of serious consideration in this study. Dermatan sulfate is found in the skin and connective tissues as well as related areas, but can be separated from the chondroitin sulfates and hyaluronic acid (as can keratosulfate and heparin) by its stability to hyaluronidase.

Glycoproteins are a group of naturally occurring polysaccharides found in epithelial mucins. With the exception of the serum glycoproteins and the blood group substances most are products of endodermal derived tissues such as the salivary glands and the intestinal epithelium. However, Spicer et al. (69) have found that the luminal secretions of human breast tumors are composed primarily of the glycoprotein sialomucin. Also two groups of glycoproteins have reactive side groups which give them histochemical properties similar to acid mucopolysaccharides. Sialomucin with its sialic acid possesses a reactive

carboxyl group and sulfated sialomucin has a carboxyl and sulfate group. The former is found in a number of locations but the latter has been only demonstrated in colonic mucins.

It is essential that in any study of mucopolysaccharides the glycoproteins and acid mucopolysaccharides be differentiated. Besides the procedures outlined in the standard histochemical texts (11, 55, 71, 77), a large number of individual investigations support the reliability of the methods in common use (20, 58, 60, 61, 65, 68). The major differentiating characteristic besides certain limited localizations is the presence or absence of certain histochemical reactions. All glycoproteins possess 1:2 glycol groups which make them potentially PAS positive. Sialomucins have a terminal sialic acid group which make these glycoproteins potentially labile to neuraminidase digestion or acid hydrolysis. All acid mucopolysaccharides by contrast are PAS negative and stable to neuraminidase and acid hydrolysis. All acid mucopolysaccharide have uronic acids in their chemical structure and according to Dorfman (13) this component has not been demonstrated conclusively in any epithelial mucin. Also, certain acid mucopolysaccharides are labile to hyaluronidase where in contrast epithelial mucins are never digested by this enzyme (11, 12, 55). These latter findings are supported by Sorvani in his study of epithelial mucins of the genital system (67).

Various aspects of this study are supported in part by other investigations. In a study of salivary gland mucins, Leppi and Spicer (35) found only a mixture of glycoproteins. There was no evidence that the epithelium of these glands produced any detectable amounts of acid mucopolysaccharides. Quintarelli and Robinson (59) in their study of the glycosaminoglycans of salivary gland tumors reported only neutral glycoproteins in the epithelial secretions. Acid glycosaminoglycans were confined to the myxomatous and chondroid areas. They also reported finding areas of finely vacuolated basophilic ground substance which appeared to transform to a more homogenous densely arranged ground substance with lucunae. These latter areas correspond well to the precartilage and pseudocartilage described within the present study. Quintarelli and Robinson also state they found no evidence to suggest either a transformation from epithelial mucin to connective tissue mucin, or metaplasia of epithelium to connective tissue.

In human breast tumors, Takeuchi et al. (70) described acid glycosaminoglycans of connective tissue origin and suggested the individual amounts of each varied with the different tumor types. Spicer et al. (69) in a study of epithelial mucins in the normal and diseased breast found sialomucins but no acid mucopolysaccharides. The luminal secretions described in the present study were mainly neutral glycoproteins. In some ducts however,

acid mucopolysaccharides were demonstrated either alone or in a mixture with the glycoproteins. In all cases where luminal acid mucopolysaccharides were identified the basement membrane was disrupted and abundant stromal acid mucopolysaccharides could be found adjacent to these ducts. Except for possible species variation, no reason could be found for the lack of identifiable sialomucin in any of the tumors studied.

The mechanism for the production of the excessive amounts of acid mucopolysaccharides in the tumors studied could not be determined. The same can be said of identifying a specific cell of origin. However, a number of possible theories should be considered. The probability of a connective tissue cell origin has been discussed and need not be repeated. Although the electron microscopic findings of basal lamina irregularities and increased numbers of varible sized intercellular spaces have been reported in numerous investigations (19, 52), they need to be emphasized here as support for the theory that neoplastic epithelium when in contact with stromal elements in some manner stimulates the connective tissue production of acid mucopolysaccharides (41, 70). The regularity of these lesions also supports the epithelial-stromal junction (ESJ) theory as proposed by Ozzello (51, 52, 53). This theory proposes that the ESJ is "a morphophysiological unit and actively participates in the genesis of mammary dysplasia by losing its control of the transport of materials

to and from the epithelium". This theory has considerable merit when the major functions of the acid mucopolysaccharides are considered. These functions include regulation of small molecular substances across cell membranes which influences the metabolism of both connective tissue and epithelium. Besides serving as intercellular cements and structural elements, they also protect tissues (such as cartilage) from mineralization. When the ESJ theory is considered in connection with acid mucopolysaccharide production, the effects of estrogenic hormones must be taken in consideration as well. It is known that estrogens stimulate mucopolysaccharide synthesis in certain target organs (8, 19, 52, 53). The mammary gland is a major target organ and with each heat period, estrogens produce prominent cyclic changes in the mammary glands of the intact bitch. These effects are not limited to the glandular epithelium but are reported in the connective tissue stroma as well (54).

The fibrous, osseous and bone changes in the "mixed" tumors have not received the attention given the cartilage. Although some investigators have suggested these elements are formed by epithelial and/or myoepithelial metaplasia (5, 42, 57), it is increasingly being postulated that they arise by metaplasia of connective tissue possibly stimulated by neoplastic epithelial changes (41, 70). Some controversy exists as to whether these mesenchymal changes represent neoplasia or not. The results of this study supports

the connective tissue origin of the elements and suggests they are not neoplastic but rather heterotopic or heteroplastic. When areas of metastasis contain mesenchymal elements similar to those in the primary site, they are always in company of neoplastic epithelium. It is conceivable that if the neoplastic epithelium stimulates mesenchymal changes in the mammary gland, they could possess the same potential in metastatic sites as well.

The acid mucopolysaccharides associated with the fibrous, osseous and bone changes were not in abundance and those present had histochemical properties comparable to those of the chondroitin sulfates and dermatan sulfate. When compared to the ground substance, the fibrilar (collagen) ratio appeared much higher than what was found in the cartilage formation. When bone was being formed, there appeared to be a condensation of the collagen and a decrease in the amount of acid mucopolysaccharides demonstrated. This decrease in acid mucopolysaccharide may then allow mineralization of the osteoid matrix and stimulate differentiation of the fibroblasts into osteoblasts and osteoclasts, thereby producing true heterotopic bone.

Conclusions

The histological study presented demonstrates that the hyaline cartilage found in the canine mammary tumors has its beginnings as intercellular accumulations of hydrophillic

material around the basement membranes of ducts, ductules and alveoli. This precartilage material is transformed into a pseudocartilage matrix with a progressive loss of water and accumulation of a fibrilar component. Pseudocartilage is transformed into mature hyaline cartilage by further condensation of the matrix and differentiation of the forming cells into chondroblasts within lacunae. The surrounding ductal elements and alveoli are crowded out by the expanding matrix.

The histochemical studies demonstrated the ground substance of the matrix to be composed primarily of the acid mucopolysaccharides, hyaluronic acid, chondroitin-4- and chondroitin-6-sulfate. The high content of hyaluronic acid in the precartilage areas and its subsequent decrease in transitional areas supports the transitionary process described. The acid mucopolysaccharides demonstrated are reported to be produced only by mesenchymal cells. This evidence therefore supports the mesenchymal origin of the pseudocartilage and mature hyaline cartilage found in canine mammary tumors.

The electron microscopy demonstrated connective tissue stromal changes that supported secretory activity of its cellular components. The looseness of the stroma, the basal lamina irregularities, the lack of basal cell junctions and the increased intercellular spaces supports the accumulation of the acid mucopolysaccharide and its origin around the basement membrane.

No conclusive evidence could be found in this study or the literature to support a transition of epithelium or myoepithelium into cells with the mesenchymal properties described. Neither was there any evidence found which indicated that the epithelial secretion was capable of transforming into mucin with mesenchymal properties. It is therefore concluded that the acid mucopolysaccharides demonstrated in the canine mammary tumors studied are produced by cells derived from the mesoderm.

The results of the present study indicates further research is needed in two general areas. More detailed studies are needed in identifying the exact derivation of the cell or cells producing the acid mucopolysaccharides and the factors which stimulate the process. This may be accomplished in part by further in-depth histochemical studies of the individual acid mucopolysaccharides and establishment of better methods for distinguishing the carboxylated and sulfated groups, the uronic acid components and the mechanisms involved in their formation.

The second area of research indicated is further study of the morphophysiological aspects of the epithelial-stromal junction complex as suggested by Ozzello (51, 52, 53). Since studies of single aspects of the complex canine mammary tumors do not always provide the answers sought, a broader approach then is indicated. It is therefore proposed that a study is needed on the hormonal influences on the ESJ complex, first in the cyclic changes of the bitch,

and then how these findings compare to a similar study in specific mammary tumors.

CHAPTER VI

SUMMARY

This study was designed to identify and determine the origin of the intercellular acid mucopolysaccharides found in various epithelial tumors of the canine mammary gland. The predominate acid mucopolysaccharides were Alcian blue positive, hyaluronidase labile, and identified as hyaluronic acid, chondroitin-4- and chondroitin-6-sulfate. The amounts of these acid mucopolysaccharides varied in the transitional process from precartilage to pseudocartilage to mature hyaline cartilage. The limited electron microscopic studies indicated numerous basal lamina irregularities, increased intercellular spacing and a loss of basally located cell junctions. There was evidence of connective tissue cell secretory activity and stromal changes indicative of accumulation of excessive ground substance.

The results indicated that the acid mucopolysaccharides which contribute to the cartilage and cartilage-like material found in these tumors were of a mesenchymal nature. The exact derivation of the cells involved could not be conclusively demonstrated but there was no indication of involvement of either the epithelium and myoepi-

thelium or their products. It was therefore concluded that the cells producing the acid mucopolysaccharides are mesodermal in origin.

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APPENDIX

HISTOCHEMICAL METHODS EMPLOYED IN THE IDENTIFICATION OF ACID MUCOPOLYSACCHARIDES IN CANINE MAMMARY TUMORS*

Method	Chemical Reaction Involved	Histochemical Results
Alcian blue at controlled pH values	Probably formation of Alcian blue complexes with carboxyls and some sulfates. Extinction values indicate degree of acidity of the polyanion.	At pH 2.5 all acidic mucins stain blue, at pH 1.0 only the strong sulfated mucins stain blue selectively.
Periodic acid Schiff	Oxidation of vicinal hydroxyls to dialedhydes by periodate and formation of colored complexes with Schiff's reagent.	All polysaccharides (PS) and mucosubstances (MS) containing hexoses and deoxyhexoses with vic-glycol groups, i.e., periodate reactive polymers color magenta to red.
Alcian blue-Periodic acid Schiff	Addition of results by single methods	Neutral PS and MS color magenta; Alcian blue-reactive, periodate unreactive acid MS stain blue. Alcian blue and periodate reactive MS color purple-blue.
Periodic acid-Phenylhydrazine-Schiff	Phenylhydrazine selectively blocks periodate-engendered dialdehydes in neutral PS and MS, leaving unblocked dialdehydes in periodate reactive and MS available to subsequent Schiff staining.	Periodate reactive acidic MS stained red presumably are those in which acid groups are proximal to vic-glycols.

Aldehyde Fuchsin-Alcian blue pH 2.5	Formation of salt complexes between cationic staining entity and sulfate and carboxyl groups. Replacement through mass action of the first dye by second on sites where former has low affinity.	Sulfated MS color purple or blue-purple; all other non-sulfated acidic MS color blue.
Diastase	Hydrolyzes and removes glycogen.	Loss of PAS reactivity in sites containing glycogen.
Neuraminidase digestion following treatment with potassium hydroxide in ethanol	Removes the neuraminic acid (sialic acid) from MS. The KOH enhances the reaction.	Sialomucins lose their basophilia or affinity for Alcian blue.
Hyaluronidase digestion	The $\beta 1 \rightarrow 4$ bonds are split, liberating non-reducing glucuronyl groups.	Hyaluronic acid, chondroitin-4 and chondroitin-6 sulfates lose their basophilia affinity to Alcian blue.

*Taken in part and modified from Leppi and Spicer (35).

VITA

Thomas Earl Palmer

Candidate for the Degree of

Doctor of Philosophy

Thesis: THE DEMONSTRATION AND IDENTIFICATION OF ACID MUCOPOLYSACCHARIDES IN CANINE MAMMARY TUMORS: A HISTOLOGICAL AND HISTOCHEMICAL STUDY

Major Field: Veterinary Pathology

Biographical:

Personal Data: Born in Villa Ridge, Illinois, February 25, 1942, the son of Mr. and Mrs. Earl Palmer.

Education: Graduated from Mounds Township High School, Mounds, Illinois, in May, 1960; attended the University of Illinois, 1960-61, and McKendree College, Lebanon, Illinois, 1961-65; received Bachelor of Arts degree in Biology from Southern Illinois University, Edwardsville, Illinois, in 1969; received Master of Science (Research) in Anatomy from Saint Louis University in 1973; completed requirements for the Doctor of Philosophy degree at Oklahoma State University in July, 1978.

Professional Experience: Teaching assistant, McKendree College, 1963-64; Laboratory technician and research assistant for Ralston Purina Company, 1965-72; Laboratory Manager for Department of Pathology, College of Veterinary Medicine, Oklahoma State University, 1972-78. Cytochemist, prosector and assistant in the National Institute of Health, Cancer Institute Registry of Canine and Feline Neoplasms (Dr. A. W. Monlux, Principal Investigator), 1972-78.

Honorary and Professional Societies: Sigma Zeta National Science Society, National Society for Histotechnology, Oklahoma Society of Histotechnologists.