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Cartilage Matrix Calcification as a Determinant of Perivascular Cell Populations in Cartilage Canals of Developing Epiphysis: Identification of Cell Types Using Morphological and Cytochemical Criteria

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CARTILAGE MATRIX CALCIFICATION AS A DETERMINANT OF PERIVASCULAR CELL POPULATIONS IN CARTILAGE CANALS OF DEVELOPING EPIPHYSIS: IDENTIFICATION OF CELL TYPES USING MORPHOLOGICAL AND CYTOCHEMICAL CRITERIA

by

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DEDICATION

To "my guys"

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VITA

The author, Ada Asbury Cole, was born on October 6, 1944, in Hazard, Kentucky, to Early and Dorothy Caldwell Asbury. She graduated from Hazard High School in June, 1962. From September, 1962 to June, 1966, she attended Berea College in Berea, Kentucky, where she worked for two years as a teaching assistant in the Department of Biology and received a Bachelor of Arts degree with a major in Biology. In September, 1966, she entered The University of Tennessee Graduate School where she was granted a Ford Foundation Fellowship for 1966-67 and a Teaching Assistantship in the Department of Zoology and Entomology for 1967-68. Under the direction of Dr. J. Gordon Carlson, she completed the research requirements for the degree of Master of Arts in College Teaching with a major in Biology and received the degree in December, 1968. In September, 1967, she was married to Madison Brooks Cole, Jr., a fellow graduate student. Their son, Madison Brooks Cole, III, was born in October, 1969.

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PUBLICATIONS

Manuscripts

Cole, A.A. and F.H. Wezeman (1985) Perivascular cells in cartilage canals of the developing mouse epiphysis. Am. J. Anat. 174:119-129.

Cole, A.A. (1968) Autoradiographic detection of protein synthesis in grasshopper neuroblasts. Master's Thesis, University Microfilms, Inc. Ann Arbor, MI. pp. 1-24.

Cole, A.A. and F.H. Wezeman (1986) Morphometric analysis of cartilage canals in the developing mouse epiphysis. Acta Anat. (In Press).

Cole, A.A. and L.M. Walters (1986) Tartrate-resistant acid phosphatase in bone and cartilage following decalcification and cold-embedding in plastic. J. Histochem. Cytochem. (In Press).

Cole, A.A. and F.H. Wezeman (1986) Cytochemical localization of tartrate-resistant acid phosphatase, alkaline phosphatase and nonspecific esterase in perivascular cells of cartilage canals in the developing mouse epiphysis. Submitted to Am. J. Anat.

Abstracts

Cole, A.A. and F.H. Wezeman (1986) Cytochemical localization of tartrate-resistant acid phosphatase (TRAP) in chondroclasts of cartilage canals. Anat. Rec. 214:23A-24A.

Cole, A.A. and F.H. Wezeman (1985) Perivascular cells in cartilage canals of the developing mouse epipysis. Anat. Rec. 211:43A.

Cole, A.A. (1984) Morphological characteristics of perivascular cells in cartilage canals of the developing mouse epiphysis. Midwest Soc. Elect. Microsc. 14:13-14.

Walters, L.M. and A.A. cole (1986) Tartrate-resistant acid phosphatase is present in rat growth plate. J. Cell Biol. (In Press).

Cole, A.A. (1968) Autoradiographic study of protein synthesis in the grasshopper neuroblast. Proc. Tenn. Acad. Sci. 40:54.

Cole, M.B., Jr., A.A. Cole and J.G. Carlson (1968) Action of puromycin dihydrochloride on protein synthesis and mitosis. Assoc.

Southeastern Biol. Bull. 15:35.

Stephens, R.E., M.B. Cole, Jr., A.A. Cole and J.G. Carlson (1968) Parasitization of Chorotophaga viridifasciata eggs by larvae of Scelio bisulcus. Assoc. Southeastern Biol. Bull. 15:55.

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LIST OF ABBREVIATIONS

ATPase	adenosine triphosphatase
CM	calcified matrix
$CaCl_2$	calcium chloride
C	cartilage
CC	chondroclast
^oC	degrees Centigrade
EDTA	ethylenediaminetetracetic acid
Fig	figure
FITC	fluorescein isothiocyanate
GMA	glycol methacrylate
GAG	glycosaminoglycan
gm	gram
hr	hour
kD	kilodalton
kv	kilovolt
$MgCl_2$	magnesium chloride
ul	microliter
um	micron
mg	milligram
ml	milliliter
mm	millimeter
mM	millimolar
mOsm	milliosmoles
min	minute
M	molar
Mol Wt	molecular weight
nm	nanometer
P	perichondrium
PBS	phosphate buffered saline
OsO_4	osmium tetroxide
SLS^4	segment long spacing
S.D.	standard deviation
S.E.	standard error
TRAP	tartrate-resistant acid phosphatase
TSAP	tartrate-sensitive acid phosphatase
vol	volume

CHAPTER I

INTRODUCTION

Cartilage canals are thought to form by the degradative activity of perivascular cells. These cells accompany blood vessels in canals which initially form and elongate in an uncalcified matrix. Calcification begins in the matrix near the tips of canals and further expansion occurs through calcified matrix. If canals form by the degradative activity of perivascular cells, then there may be two or more different populations of cells which are responsible for degradation. Characteristics of the degradative cells may depend, in part, on the type of matrix being degraded. Macrophages have been proposed as the degradative cell responsible for initial canal formation in uncalcified matrix (Andersen and Matthiessen, 1966), while chondroclasts have been identified adjacent to calcified matrix (Kugler et al., 1979). However, the presence of macrophages and chondroclasts has not been reported in other studies on canal cell populations (Lutfi, 1970b; Stockwell, 1971; Knese, 1980).

Inconsistencies in characterization could have resulted from the fact that no single study has characterized perivascular cells during initial stages of canal formation through uncalcified matrix to later stages through calcified matrix. Because cartilage canals provide a unique mammalian model for studying perivascular cell morphology and

function in relation to uncalcified and calcified matrix, the purpose of this study is to identify those perivascular cells having characteristics of degradative cells during the entire sequence of canal development. Cartilage canal development provides a model for 1) documenting morphological differences among perivascular cells during the lengthening of canals through differentiating matrix, 2) identifying perivascular cells with degradative characteristics, and 3) establishing a model for the study of disorders of cartilage growth and differentiation which are defined, in part, by increased or decreased vascularity and of cartilage pathologies involving matrix degradation, such as rheumatoid arthritis. Ultrastructural, cytochemical and immunohistochemical techniques can be used in the study of cartilage canal development to identify perivascular cells having degradative characteristics.

CHAPTER II

REVIEW OF LITERATURE

A. SKELETAL DEVELOPMENT

During mammalian embryonic development, primordia of the appendicular skeleton initially form as cartilage anlagen which are even tually replaced by bone and marrow except at articular surfaces. The process of cartilage replacement by bone is called endochondral ossification and involves blood vessel penetration of the cartilage matrix, matrix calcification and subsequent bone formation in the centers of ossification. The long bones of the appendicular skeleton consist of a shaft or diaphsis and ends or epiphyses; during skeletal development centers of ossification form in both the diaphysis and the epiphyses. In the central portion of the diaphysis, chondrocytes hypertrophy, matrix calcifies, and a collar of bone forms prior to blood vessel penetration (Fig. 1). Capillaries accompanied by osteogenic cells invade the cartilage anlagen from the periosteal surface of the bony collar to form the diaphyseal center of ossification. Hemato poietic stem cells, stromal cells and osteoclast precursors populate the center to form marrow elements, osteogenic cells and degradative cells, respectively. Circumferential growth of the diaphysis results from deposition of new bone along the periosteal sur-

face while longitudinal growth of the diaphysis is a result of chondrocyte division and matrix synthesis within the epiphyseal growth plate.

In the epiphyses of long bones, the sequence of events leading to the formation of the epiphyseal center differs from the sequence leading to the formation of the diaphyseal center (Fig. 1). Blood vessels penetrate the cartilage prior to chondrocyte hypertrophy and matrix calcification (Gray and Gardner, 1969; Agrawal et al., 1984); no bony collar forms surrounding the epiphysis. Increases in epiphyseal size result from both appositional and interstitial growth resulting from chondrocyte division and matrix synthesis within the epiphysis (Hinchliffe and Johnson, 1983).

B. CARTILAGE CANALS

Blood vessels enter the uncalcified matrix in channels called cartilage canals which have been reported in amphibians, reptiles, cartilaginous sharks, birds, and mammals including man (Haines, 1933; Hurrell, 1934; Gray and Gardner, 1950; Lutfi, 1970b; Hoenig and Walsh, 1982). Blood vessels are accompanied by cells described primarily as mesenchymal (Lutfi, 1970b) or polymorphic cells (Stockwell, 1971). Canals begin at the perichondrial surface as shallow bud-like depressions and lengthen through uncalcified matrix containing resting chondrocytes (Lutfi, 1970b; Moss-Salentijn, 1975). As the depressions

enlarge, capillary glomeruli occupy the depressions (Lutfi, 1970b; Moss-Salentijn, 1975; Wilsman and Van Sickle, 1972). Gradually the buds elongate to become canal-like. The canals are continuous with the perichondrium, and the blood vessels are continuations of perichondrial vessels (Hurrell, 1934; Brookes, 1958; Haraldsson, 1962; Spira et al., 1963; Levene, 1964; Moss-Salentijn, 1975). During subsequent development of the epiphyseal center, chondrocytes hypertrophy, and the matrix calcifies adajacent to the tips of canals (Wilsman and Van Sickle, 1970; Agrawal et al., 1984). Further elongation of canals then occurs through calcified matrix. Expansion of the terminal portion of the canal results in the actual formation of the epiphyseal center. Certain canals persist to form the vascular supply for the epiphyseal center (Brookes, 1971); other canals become either filled with fibrous material and disappear or are obliterated by expansion of cartilage matrix (Spira et al., 1963; Lutfi, 1970b; Moss-Salentijn, 1976; Hunt et al., 1979).

Providing a vascular supply for the epiphyseal center of ossification is not the only role of the cartilage canals. Canals are thought to function in providing nutrients to large blocks of cartilage (Reviewed by Stockwell, 1979) since canals form in cartilages which do not normally calcify, such as tracheal, laryngeal, or costal cartilages as well as in cartilages that calcify but do not contain an epiphyseal center, such as the distal epiphysis of metatarsals (Haines, 1933). The period between initial canal formation and the

onset of ossification is highly variable; in some joints the period may be a few days (Kugler et al., 1979) or several years (Haraldsson, 1962). Canal formation appears to be related to the size of the cartilage rather than the age and has been reported to begin only when cartilages reach a critical volume of 0.5 to 2.0 mm^3. The mean distance between canals remained approximately 1.4 mm during the first seven days of neonatal growth in canine pups (Wilsman and Van Sickle, 1972). Each canal appeared to nourish tissue in 0.7 mm radius. At birth canals approached 0.67 mm of the articular surface; during the first week, the distance increased to 1.04 mm. This increased distance was coincident with changes in activity of the pups which resulted in increased circulation of synovial fluid providing additional nutritional supply to the cartilage. Canal capillary endothelium is located immediately adjacent to matrix with no intervening basement membrane; the attenuated endothelium contains fenestrations with diaphragms providing morphological evidence for a nutritional function for the canals (Stockwell, 1971). Chondrocytes within cartilage receive nourishment by diffusion from blood vessels like most tissues in the body. Cartilage is unique in that the blood vessels lie at the periphery or in the perichondrium, and chondrocytes survive in blocks of tissue where maximum diffusion distance in adults can be millimeters or centimeters. In adult costal cartilage the number of chondro cytes per mm^2 of perichondrial surface is approximately 27,000. This may represent the number of chondrocytes in a block of tissue which

can be adequately nourished by perichondrial blood vessels. However, in infant costal cartilage the number of cells per mm^3 of perichondrium is much greater at 72,000. Canals normally develop in this cellular cartilage where metabolic demands are higher than in adult cartilage. Stockwell (1979) calculated that about 3×10^5 chondrocytes are nourished from 1 mm^2 of canal surface based on cell density, canal diameter and canal density within the epiphyseal cartilage.

Other proposed canal functions include providing stem cells for not only the epiphyseal center but also for chondrogenesis. Canals are also thought to provide an access route for cells seeding the epiphyseal center (Kugler et al., 1979). Stem cells for osteogenesis, such as osteoblast precursors, have access from the perichondrium; cells of hematopoietic origin have access through canal blood vessels. These cells include not only bone marrow cells but also osteoclast precursor cells. A role for the canals in chondrogenesis has also been described (Lutfi, 1970a); as the cartilaginous epiphyses rapidly increase in size by interstitial growth, chondrocytes are thought to be released from the matrix adjacent to the canal during early canal development and later to reenter the matrix near the growth plate to provide stem cells for the resting zone of the growth plate.

C. MODES OF CANAL FORMATION

Although over the past fifty years the presence, distribution,

function and relationship of the canals to the epiphyseal center of ossification have been the subject of numerous studies, the manner in which canals form is still uncertain. Two hypotheses have been proposed to explain the development of cartilage canals within cartilage matrix (Fig. 2). The first hypothesis proposes that canals form by active invasion of matrix by degradative cells (Stump, 1925), while the second proposes that canals form passively as blood vessels from the surrounding perichondrium become engulfed or included in the epiphysis as cartilage matrix is synthesized around the vessels during appositional growth (Haines, 1933; 1937). Although the hypothesis of formation by invasion is more widely accepted, evidence to support the hypothesis is primarily morphological and includes: 1) A band of matrix immediately surrounding the canal is often weakly and orthochromatically stained with metachromatic dyes in comparison to strongly stained matrix at a distance from the canal; the decrease in staining may represent a decrease in proteoglycan components and has been in- terpreted as an area undergoing degradation. Chondrocytes present within the weakly stained band appear to be released from the matrix and enter the canal (Lutfi, 1970a; 1970b; Moss-Salentijn, 1975). 2) The pattern of initial canal formation is species-specific. Levene (1964) compared the pattern of cartilage canals in the distal tibia of sheep, goat, rabbit, cat, rat and man prior to the development of the epiphyseal center of ossification. In each species he found that canals initially penetrated the epiphyseal cartilage

from specific sites along the perichondrium resulting in a constant canal pattern. While the pattern was consistent within animals of the same species, the pattern varied between species. Following initial formation, the canals branched repeatedly, and the pattern of subsequent branching varied between animals within the same species. Levene concluded that the species-specific pattern and branching of the canals could not be simply explained as a result of passive inclusion of perichondrial vessels by the growth of the cartilage around them. 3) The distance between terminal portions of canals remained constant as the epiphyses increased in size; if canals are passively included, the distance would be expected to increase. Wilsman and Van Sickle (1972) reported that the distance between the terminal portions of the canals, deep in the epiphysis, remained constant at a mean distance of 1.4 mm in dogs from one to seven days old. During the first seven postnatal days the epiphysis increased rapidly in size. The distance between the canals would be expected to remain constant only if the canals lengthened by matrix degradation. 4) Canals form and elongate in the human spheno-occipital synchondrosis during a period when there is no overall enlargement of the cartilage as a whole (Moss-Salentijn, 1975).

The hypothesis of invasion has been open to question since there is now evidence that uncalcified cartilage matrix contains low molecular weight proteinase inhibitors (Horton et al., 1978), for serine, cysteine and metallo-proteinases (Killackey et al., 1983) as well as

endothelial proliferation inhibitors (Sorgente and Dorey, 1980). These inhibitors provide uncalcified cartilage with resistance to penetration by blood vessels; blood vessels were able to penetrate cartilage matrix from the chick chorioallantoic membrane only after extraction of the matrix to remove inhibitors (Eisenstein et al., 1973; Sorgente et al., 1975; Eisenstein et al., 1975). The extract from the matrix also reduces tumor neovascularization in the avascular rabbit cornea (Langer et al., 1976). Kuettner and Pauli (1983) suggested that if invasion by matrix degradation is occurring then some mechanism must be acting to overcome these inhibitors.

D. DEGRADATIVE CELLS

If cartilage canals form by invasion, there are three possible candidates for the degradative cell: 1) vascular endothelial cells, 2) perivascular cells and 3) chondrocytes in the matrix surrounding the canal. Hurrell (1934) noted the close association between endothelial cells and matrix and postulated that endothelial cells might be responsible for matrix degradation. The penetration of connective tissue requires secretion of proteinases by endothelial cells (Moscatelli et al., 1981), and in culture endothelial cells can only invade cartilage matrix following extraction to remove inhibitors (Kuettner and Pauli, 1983).

Among the perivascular cells reported within the canals are

macrophages and chondroclasts, and their role in canal formation has been proposed (Andersen and Matthiessen, 1966; Kugler et al., 1979). Macrophages have been reported at a number of *in vivo* sites of cartilage matrix degradation during normal development (Schenk et al., 1967; Silvestrini et al., 1979; Sorrell and Weiss, 1980; 1982) and at sites of pathological matrix degradation, such as rheumatoid arthritis (Kobayashi and Ziff, 1975). Macrophages have also been implicated in cartilage resorption following implantation of devitalized cartilage (Ksiazek and Thyberg, 1983) and in resorption of cartilage produced by transplanted epiphyseal chondrocytes (Thyberg and Moskalewski, 1979). *In vitro* studies using activated peritoneal macrophages have shown that macrophages secrete enzymes capable of degrading extracellular matrices (Jones and Werb, 1980; Werb et al., 1980). Chondroclasts have been identified in canals which terminate in calcified matrix (Kugler et al., 1979). The term *chondroclast* has been used to identify large, multinucleated cells with ruffled borders and clear zones. The distinction between chondroclasts which contact calcified cartilage and osteoclasts which contact calcified bone was suggested by Knese (1972). These two cells appear to be morphologically and functionally identical and, in the metaphysis of growing bone where bone surrounds a core of calcified cartilage, the same cells appear to be degrading both matrices simultaneously (Takagi et al., 1982). The term *chondroclast* has been reserved for multinucleated cells located in regions where only calcified cartilage is present, such as in the

cartilage canal prior to bone formation (Moss-Salentijn, 1976; Kugler et al., 1979). The structure of the chondro/osteoclast and its function in calcified matrix resorption has been well documented. (See Holtrop and King, 1977, for review.) The presence of neither chondroclasts nor macrophages has been reported consistently in other canal studies (Lutfi, 1970b, Stockwell, 1971; Knese, 1980). Additionally, while the chondroclast has only been reported to degrade matrix, the macrophage has been shown not only to be capable of degrading matrix but also to be capable of regulating synthetic cell functions in members of the fibroblastic cell line; macrophages produce substances which are both chemotaxic and mitogenic for some fibroblasts, osteoblasts and chondroblasts and which have activity similar to the activity credited to interleukins (Rifas et al., 1984; Takemura and Werb, 1984). The presence of macrophages in the canal is not conclusive evidence that matrix degradation is occurring. The inconsistent characterization of perivascular cells could have resulted from the fact that no single study has characterized perivascular cells during initial stages of canal formation in uncalcified matrix through later stages of development in calcified matrix.

The third candidate for degradative cell is the chondrocyte. Both Lutfi (1970a) and Moss-Salentijn (1975) proposed that the nonhypertrophic chondrocytes along the margins of the canals appeared to free themselves from their matrix and enter the canal. Release of chondrocytes from their matrix has also been reported in the proximal

fragment of Meckel's cartilage where matrix is resorbed without calcifying (Melcher, 1972). Evidence that chondrocytes are capable of degrading their matrix following stimulation by retinol, macrophage derived factors or mononuclear cell factors comes from a number of organ and cell culture studies (Dingle et al., 1975; Deshmukh-Padka et al., 1978; Phadke et al., 1979; Dingle and Dingle, 1980; Jubb and Fell, 1980; Jasin and Dingle, 1981; Hembry et al., 1986).

E. CANAL DEVELOPMENTAL STAGES

Canal development can be divided into three stages based on chondrocyte morphology and matrix calcification: 1) initially canals develop in a rapidly growing cartilage with proliferating chondrocytes, 2) secondly, canals enter matrix containing hypertrophic chondrocytes and 3) finally, canals terminate in calcified matrix. The progressive changes in epiphyseal cartilage may be analogous to those changes which occur in the proliferative, hypertrophic and resorptive zones of the growth plate. In the proliferative zone chondrocytes are rapidly dividing (Shimomura et al., 1973) and synthesizing matrix composed primarily of type II collagen and proteoglycan (Revel and Hay, 1963; 1964; Godman and Lane, 1964). The synthetic function of the proliferative chondrocyte is reflected morphologically by extensive rough endoplasmic reticulum, a large perinuclear Golgi and masses of glycogen (Holtrop, 1972a). In the zone of hypertrophy, chondro-

cytes increase in volume as interritorial matrix decreases in volume (Brighton et al., 1973; Brighton et al., 1982; Buckwalter et al., 1986). Whether this cell is truly hypertrophic, i.e. increases in volume resulting from an increase in cellular organelles, or whether this cell is swollen due to fluid accumulation or both is still unclear (Buckwalter et al., 1986). Conflicting morphometric results vary depending on histological methods employed to preserve the cell and matrix (Holtrop, 1972b; Hunziker et al., 1983; 1984). Despite the morphological appearance of a swollen and dying cell, there is strong biochemical and immunohistochemical evidence that the hypertrophic chondrocyte is capable of altering matrix components, including proteoglycan, phospholipid, calcium and phosphorus content (Reviewed by Ali, 1983). Some matrix components have been identified exclusively in the hypertrophic matrix, such as type X collagen (Kielty et al., 1985; Schmid and Linsemayer, 1985), unique proteoglycan monomers (Carrino et al., 1985) and an electron dense material preserved following the addition of potassium ferrocyanide during processing (Farnum and Wilsman, 1983). These alterations in matrix components are thought to be involved in matrix calcification which is initiated in the hypertrophic matrix. Calcified matrix is found in the longitudinal septa, separating the columns of hypertrophic chondrocytes, while the transverse septa, separating hypertrophic cells within the column, remain uncalcified (Schenk et al., 1967; Ali, 1983). Within the zone of resorption blood vessels and perivascular cells contact the cartilage

matrix. Schenk et al. (1967) proposed that the calcified and uncalcified matrix contacted by the perivascular cells were degraded by two different populations of cells: multinucleated chondroclasts which remove the calcified matrix and mononucleated cells similar to macrophages which resorb the uncalcified matrix. These populations are crowded into a relative small area making identificacation of the various degradative populations difficult. During canal development perivascular cells come into contact with three stages of differentiating cartilage matrix. If the matrix components differ at each stage, then those cells responsible for matrix degradation may be different for each stage of canal development. A study of perivascular cells present adjacent to the matrix during the three stages of canal development may provide insight into the different types of cells capable of degrading cartilage matrix.

F. CLINICAL REVELANCE

The mode of canal formation and the relationship of canals to the development of the epiphyseal center may be of clinical revelance since the presence of the epiphyseal center is used as a diagnostic tool for assessing the maturity of neonates (Kuhns and Poznanski, 1980). Failure of epiphyseal center to develop or retardation of development is seen in several skeletal dystrophies such as achondroplasia, Carpenter's syndrome, chondrodysplasia puntata, diastrophic

dysplasia, spondylepiphyseal dysplasia, and hypothyroidism (Hensinger and Jones, 1981). The apparent influence of hormones on canal proliferation is demonstrated by the fact that a lack of thyroxine results in increased cartilage vascularization (Ogden, 1979). Premature appearance of the center can follow hyperemia associated with fracture, infection, or hypothyroidism, resulting in increased regional blood supply (Hensinger and Jones, 1981).

G. PERIVASCULAR CELL CHARACTERIZATION

Cartilage canals provide a unique mammalian model for studying perivascular cell morphology and function in relation to differentiating matrix. The purpose of this study is to characterize those perivascular cells with characteristics of degradative cells during the entire sequence of canal development. Characteristics of degradative cells include:

1) contact with matrix,
2) synthesis and secretion of matrix-degrading enzymes,
3) extracellular fragmentation of matrix components, and
4) uptake of matrix fragments into vacuoles and phagosomes for intracellular digestion of the fragments Reviewed by Gross, 1981; Stockwell, 1983).

Degradative cells may form a close contact between the cell surface and matrix which permits a segregated resorptive zone to be

established. A well-known resorptive zone is the specialized area of the osteoclast known as the ruffled border. The ruffled border is entirely surrounded by an clear zone rich in contractile proteins (Holtrop and King, 1977). The clear zone is where the plasma membrane is closely opposed to matrix and mediates the attachment of the osteoclast to its substratum. This clear zone is called the "sealing zone" as it is thought to seal off a compartment delimited by the ruffled border and the bone undergoing resorption. In other cells, the resorbing zone is not as morphologically distinct as that of the osteoclast. Other cells extend cytoplasmic projections into matrix so that the cell surface with membrane-bound enzymes comes into close contact with matrix components where initial degradation occurs (Rose and Robertson, 1977; Roberts and Dean, 1986). Cells, such as osteosarcoma cells or endothelial cells, appear unable to establish close contact with the matrix or to degrade the cartilage matrix unless inhibitors have been removed from the matrix (Kuettner and Pauli, 1981; 1983).

The second step in matrix degradation involves the synthesis and secretion of matrix degrading enzymes. Enzymes are initially synthesized in the rough endoplasmic reticulum. Enzymes which are active at neutral or alkaline pH's are packaged in secretory granules and released soon after synthesis. They are generally endopeptidases which are released in a latent form and combine with their substrates in the extracellular spaces at or around neutral pH after their activation (Vaes, 1985). Enzymes that are active at an acidic pH are segregated

into primary lysosomes; over 50 acid hydrolases have been identified including phosphatases, nucleases, glycosidases, proteases, peptidases, sulfatases and lipases (Bainton, 1981). Lysosomal enzymes may be exposed to substrate by two different mechanisms: extracellularly with the release of enzymes or intracellularly following fusion with phagosomes.

A number of studies has shown that isolated cells placed in culture are capable of degrading matrix through the extracellular release of enzymes (Hauser and Vaes, 1978; Werb et al., 1980; Golds et al., 1983). Since lysosomal enzymes have an acidic pH optima, it has been proposed that the resorptive zones into which the enzymes are released are acidified. Baron et al (1985) demonstrated that the resorptive zones of the osteoclast are actively acidified and that the highly convoluted plasma membrane of the osteoclast facing this zone contains a 100 kD integral membrane protein present in lysosomal membranes. This 100 kD lysosomal membrane protein cross-reacts with a proton-pump ATPase from pig gastric mucosae raising the possibility that it plays a role in acidification of both intracellular organelles and extracellular compartments and thereby creates a suitable microenvironment for lysosomal enzyme activity.

The third step in matrix degradation is fragmentation of the large matrix molecules outside the cell. Proteoglycan degradation is thought to precede collagen degradation and to enhance collagenolysis by exposing collagen fibers previously embedded in the large proteo-

glycan domains (Mullins and Rohrlich, 1983). Cartilage proteoglycan exists as an aggregate of proteoglycan monomers attached by link protein to a long hyaluronic acid backbone. The proteoglycan monomer is composed of a core protein with glycosaminoglycan (GAG) side chains primarily consisting of chondroitin sulfate and keratan sulfate. Degradation of proteoglycan requires both endopeptidases and endoglycosidases. A number of enzymes have been identified which are capable of degrading proteoglycan including Cathepsin B and D, both lysosomal enzymes, as well as enzymes active at neutral pH (Hauser and Vaes, 1978; Golds et al., 1983). The second major component of cartilage matrix is type II collagen which consists of three identical a chains. Collagen is cleaved by vertebrate collagenases into fragments of 1/4 and 3/4 lengths of the whole molecule. The cleavage site for mammalian collagneases is about 1/4 the distance from the carboxyl terminus of the helical region in type II collagen. Further degradation of the fragments is achieved by other proteolytic enzymes.

The fourth step involves uptake and further digestion of fragments into phagolysosomes or secondary lysosomes. Fragments are endocytosed into coated vesicles which then fuse with primary lysosomes forming the secondary lysosomes where digestion of the fragments is completed by lysosomal enzymes. Not only collagen fragments but also proteoglycan molecules have been identified in phagolysosomes (Takagi et al., 1982).

Ultrastructural, cytochemical, and immunohistochemical tech-

niques can be applied to various aspects of the degradative cell characterization in order to identify perivascular cells which may function in cartilage canal formation.

1) Morphological characteristics of cells in contact with matrix similarities to cells found at other sites of matrix degradation. An ultrastructural criterion used to identify degradative cells is their close contact with the extracellular matrix. At sites of uncalcified matrix degradation, cells which contacted the matrix had ultrastructural characteristics of fibroblasts and macrophages (Schenk et al., 1967; Kobayashi and Ziff, 1975; Yajima, 1976; Silvestrini et al., 1979; Sorrell and Weiss, 1980). At sites of calcified matrix resorption, multinucleated chondroclasts had been identified (Anderson and Parker, 1966; Schenk et al., 1967; Crissman and Low, 1974; Savostin-Asling and Asling, 1975; Howlett, 1980). The ultrastructure of the cells present adjacent to matrix at the three stages of canal development will be characterized and compared to the ultrastructure of cells found at other sites of cartilage degradation.

2) Enzyme histochemistry. Cytochemical characterization of degradative cells involves the intracellular localization of enzymes associated with matrix degradation, such as acid and alkaline phosphatase, and enzymes characteristic of certain cell types, such as tartrate-resistant acid phosphatase (TRAP) for chondroclasts and non-specific esterase and tartrate-sensitive acid phosphatase (TSAP) for macrophages. Cytochemical localization of acid phosphatase is used to

identify lysosomes (Bainton, 1981) which are organelles containing acid hydrolases capable of degrading both proteoglycan and collagen fragments (Baici, 1980). Alkaline phosphatase activity has been associated with granules containing collagenase (Robertson et al., 1972). Alkaline and acid phosphatases have been cytochemically localized in cells identified as matrix-degrading cells (Deporter and Ten Cate, 1973; Yajima, 1976; Sorrell and Weiss, 1982). Acid phosphatase activity which is inhibited by the presence of tartrate has been localized in macrophages; however, TRAP is associated predominately with osteo/-chondroclasts (Hammarstrom et al., 1971; Minkin, 1982). Nonspecific esterase is an enzyme characteristic of mononuclear phagocytes, including macrophages (Van Furth et al., 1979; Dannenberg and Suga, 1981).

Within the canals, TRAP is expected to be predominately present in chondroclasts of deep canals. Mononucleated cells may occasionally contain stained granules. Nonspecific esterase and TSAP are expected to be localized primarily in the vacuolated macrophages. Alkaline phosphatase reaction product is expected to be present in the fibroblastic cells.

3) Alteration of matrix adjacent to the canal. With toluidine blue O and safranin O, the uncalcified matrix surrounding the canals stains weakly and orthochromatically, while matrix at a distance from the canals stains strongly and metachromatically. The intensity of these stains has been shown to be proportional to the proteoglycan

content of the matrix (Poole, 1970a; Rosenberg, 1971). The decreased staining should represent decreased proteoglycan content of matrix adjacent to the canals which may have resulted from the removal of proteoglycan components accompanying matrix degradation.

Proteoglycans are complex macromolecules that contain a core protein to which GAG chains are covalently bound. In cartilage the primary GAG chains are chondroitin sulfate and keratan sulfate. Age-related changes in the relative concentration of keratan sulfate and chondroitin sulfate have been reported (Hardingham et al., 1976; Sweet et al., 1979; Murate and Bjelle, 1980; Thonar and Sweet, 1981). In embryonic and immature cartilages, chondroitin sulfates are the predominate GAG present, and keratan sulfate is present in lower concentrations. With increasing age the concentration of keratan sulfate increases. Proteoglycan monomers (core protein with attached GAGs) associate with hyaluronic acid to form proteoglycan aggregates. The core protein contains a hyaluronic acid-binding region which contains the active site that binds to hyaluronic acid through a noncovalent highly specific interaction.

Monoclonal antibodies against chondroitinase ABCor chondroitinase ACII-digested, cartilage proteoglycan have been developed (Caterson et al., 1983; Couchman et al., 1984). These antibodies recognize the oligosaccharide GAG chains remaining attached to the core protein following digestion and can be used for the localization of unsulfated chondroitin sulfate (chondroitin), chondroitin-6-sulfate, chondroitin-

4-sulfate or dermatan sulfate. Immunohistochemical localization of GAGs in the epiphysis of the mouse can be used to identify the matrix components which are present within epiphyseal cartilage matrix but are absent from the matrix immediately surrounding the canals. Matrix components will be localized according the procedures described by Caterson et al. (1982) and Couchman et al. (1984).

4) Rate of canal growth compared to epiphyseal growth. If canals form by inclusion or matrix synthesis around perichondrial blood vessels, then canal area should increase proportionately as the epiphyseal area increases. The ratio of canal area to unit epiphyseal area should remain constant. However, if canals form by matrix degradation, increases in canal area should exceed increases in epiphyseal area as canals increase in size. A morphometric study will be conducted to measure changes in canal area compared to changes in epiphyseal area to provide evidence for canal formation by invasion.

Although none of the ultrastructural, cytochemical, immunohistochemical or morphometric techniques used in this study can provide absolute proof that canals form by matrix degradation, the data accumulated from the study should offer substantial support for the hypothesis of canal formation by invasion.

Figure 1 Diagram showing differences between the development of the diaphyseal and epiphyseal centers in mammalian long bone. A. Diaphyseal development. 1) Cartilage model forms. 2) Chondrocytes hypertrophy within the diaphysis, matrix calcifies around the hypertrophic chondrocytes and a bony collar forms. 3) Blood vessels penetrate the bony collar and calcified cartilage matrix. 4) The diaphyseal center expands to establish a marrow cavity and growth plates. B. Epiphyseal center development. 1) Blood vessels penetrate from the perichondrium into the uncalci- fied matrix. 2) Chondrocytes hypertrophy at the tips of the canals. 3) Matrix near the tips of canals becomes calcified. 4) The calcified cartilage is eroded to form the epiphyseal center.

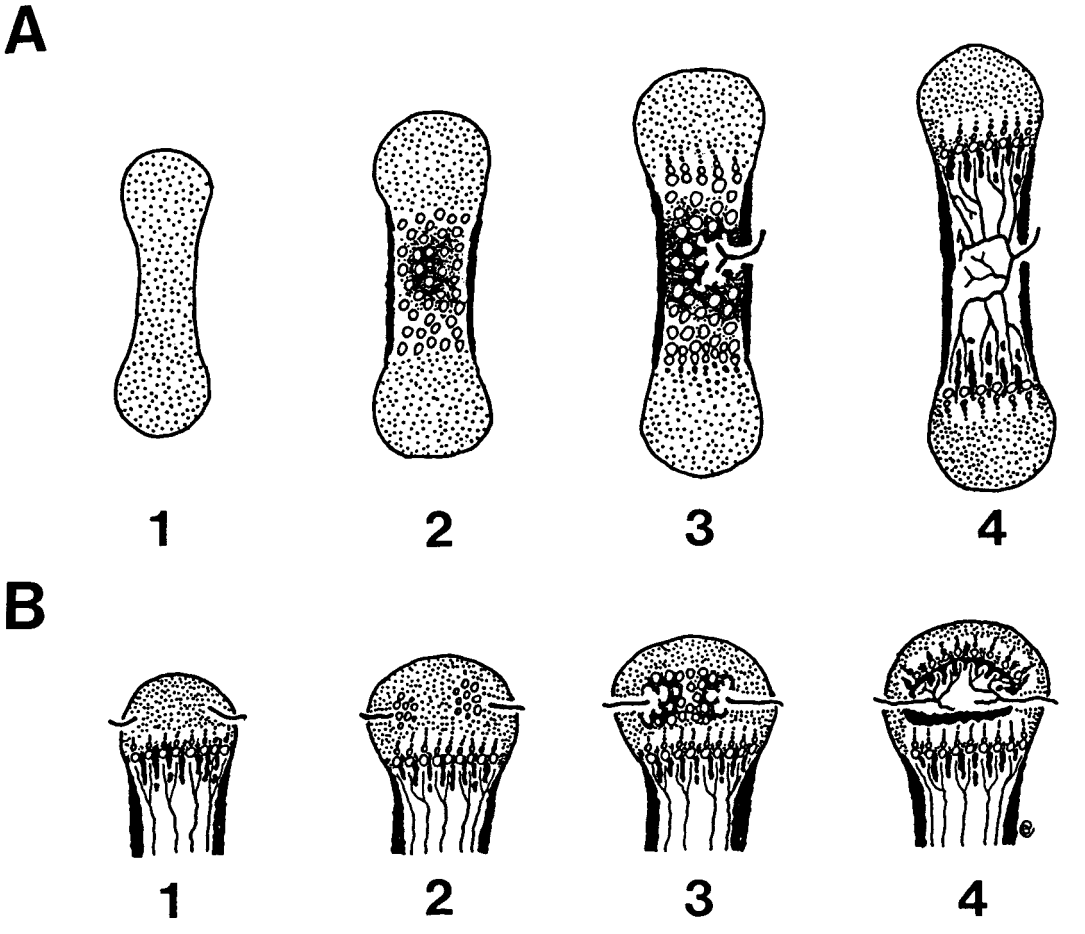
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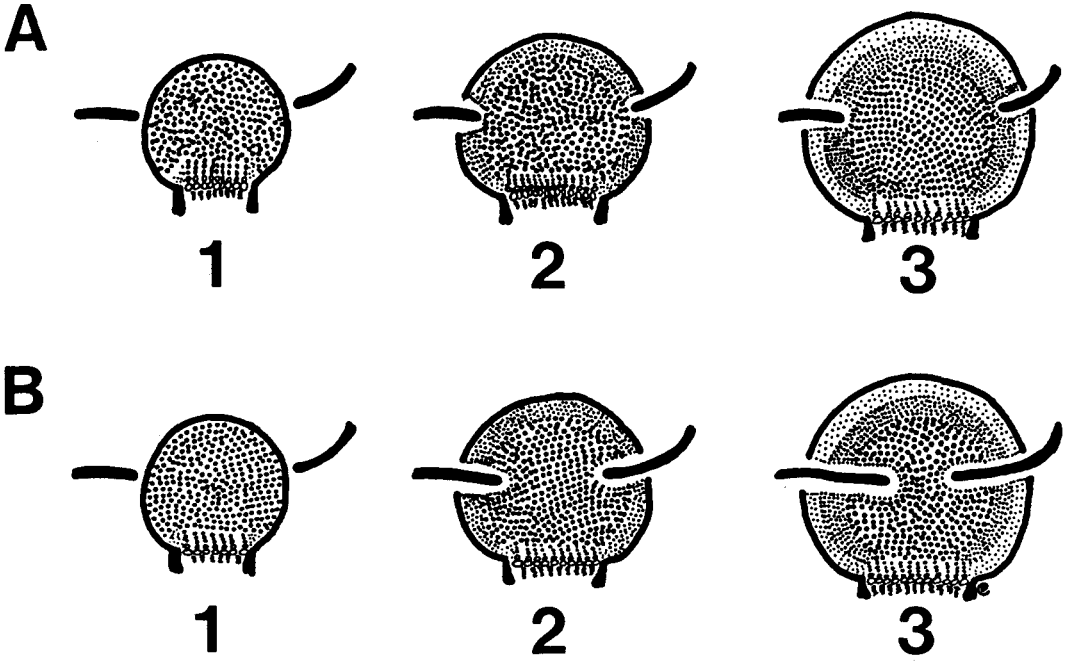
Figure 2 Diagram illustrating the two hypotheses of canal formation. A. Canal formation by inclusion. 1) Blood vessels are initially present in perichondrium. 2) Blood vessels become included in the cartilage matrix as the epiphysis enlarges by appositional growth. 3) Continued epiphyseal growth results in further elongation of the canals. B. Canal formation by invasion. 1) Blood vessels are initially present in perichondrium. 2) and 3) Canals elongate faster than the cartilage volume increases.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CHAPTER III

SPECIFIC AIMS

The specific aims of this investigation are:

1) To establish an animal model based upon the localization of cartilage canals at ages during which canals are associated with differentiating matrix including both uncalcified and calcified matrix.

2) To morphologically characterize perivascular cells located immediately adjacent to uncalcified and calcified matrix using electron microscopic techniques.

3) To stereologically analyze the volume density of cartilage canals to determine whether increases in area of the canal exceeds increases in epiphyseal area during canal development.

4) To functionally characterize perivascular cells adjacent to the matrix using cytochemical localization of hydrolytic enzymes that may be associated with matrix degradation and that are indicators of cells that degrade matrix.

5) To immunohistochemically label the epiphyseal cartilage matrix with antibodies against glycosaminoglycan molecules to determine regional canal matrix content of glycosaminoglycans.

The significance of this work is threefold. First, it will document morphological differences among perivascular cells during the

lengthening of canals through calcifying matrix. Morphological differences may reflect the inductive properties of the matrix on cellular differentiation and on the differentiation of degradative cell characteristics. Secondly, it will identify perivascular cells with degradative characteristics adjacent to the matrix which will add support to the hypothesis that canal formation results from matrix degradation rather than from inclusion as matrix is synthesized around perichondrial blood vessels. Thirdly, it will establish a model for the possible study of disorders of cartilage growth and differentiation, such as achondroplasia and other skeletal dysplasias which are defined, in part, by either decreased or increased vascularization during growth, and for the study of cartilage pathologies involving matrix degradation such as rheumatoid arthritis.

CHAPTER IV

MORPHOMETRIC ANALYSIS OF CARTILAGE CANALS IN THE DEVELOPING MOUSE EPIPHYSIS

ABSTRACT

Cartilage canal development in the distal femoral epiphysis of 5 - 7 day old mice can be divided into three stages as previously described (Cole and Wezeman, 1985). Using this model, a morphometric analysis of canal volume density at the three stages of development was performed and provided evidence that canal formation significantly exceeds epiphyseal growth. These data are consistent with initial canal formation by invasion rather than by inclusion.

INTRODUCTION

Cartilage canals, containing blood vessels and perivascular cells, are formed in epiphyseal cartilage prior to matrix calcification (Gray and Gardner, 1969; Wilsman and Van Sickle, 1970; Agrawal et al., 1984). Two methods of canal formation have been described. Stump (1925) was credited with the hypothesis of canal formation by invasion proposing that canals form by degradation of the cartilage matrix. Haines (1933, 1937) challenged this hypothesis and proposed that, during rapid appositional growth, blood vessels from the perichondrium became included within the cartilage matrix. Haines (1933, 1937) concluded that canals formed by inclusion as cartilage matrix was rapidly synthesized around the vessels.

Some experimental evidence has supported initial canal formation by degradation of uncalcified cartilage matrix (Levene, 1964; Wilsman and Van Sickle, 1972). However, canal formation by inclusion has not been excluded (reviewed by Kuettner and Pauli, 1983) since uncalcified cartilage matrix has been shown to contain proteinase inhibitors (Horton et al., 1978) as well as inhibitors of endothelial cell proliferation (Sorgente and Dorey, 1980). These low molecular weight cartilage-derived inhibitors provide resistance to cellular invasion of cartilage (Eisenstein et al., 1973; Eisenstein et al., 1975; Sorgente et al., 1975).

Canal formation by inclusion is dependent on appositional growth

(Haines, 1937). If canals form by inclusion during appositional growth, increase in canal length should be proportionate to the increase in epiphyseal volume due to appositional growth. Conversely, if canals form by invasion, increase in canal length should be significantly greater than the increase in epiphyseal volume. Cartilage canals in the mouse provide an experimental model for a morphometric analysis of changes in canal length relative to changes in epiphyseal volume. Mouse canals are relatively short and unbranched unlike the longer, highly-branched canals present in larger mammals (Levene, 1964). The entire length of a canal can be seen in a single cross-section through the epiphysis. Also in the mouse, three stages of canal development are distinguishable based on characteristics of chondrocytes and matrix surrounding the canal (Cole and Wezeman, 1985). Cartilage canals in the mouse provide a model for the comparison of canal volume densities at the three stages of development and will provide data to support canal formation by invasion.

MATERIALS AND METHODS

Mice (Swiss) used for this study were obtained from a breeding colony housed in a centrally located, fully accredited animal care facility. Nursing mothers were housed with food and water available ad libitum. Twenty mice at 5 to 7 days of age were weighed, and the distal femoral epiphyses were removed following ether anaesthesia and decapitation. The tissue was fixed overnight in 0.1M cacodylate-buffered (pH 7.4) 2.5% glutaraldehyde at 4^{o}C, dehydrated in ethanol, and embedded in glycol methacrylate. Sections were cut at 3um and stained with 1% aqueous toluidine blue O or 1% aqueous safranin O. Mineralized matrix was histochemically identified using the Von Kossa stain.

Sections of epiphyses containing either superficial, intermediate or deep canals (Cole and Wezeman, 1985) were viewed with a microprojector at a magnification of 49X. Camera lucida drawings of sections were used to identify the stage of canal development and plane of section through the canal. Because of variations in epiphyseal areas through the cross-sections of the epiphyses, the widest portion of each epiphysis was selected for measurement. Those canals which arose from intercondylar fossae were chosen for canal measurements since these canals were consistently located in the widest portion of the epiphysis. Canal areas and epiphyseal areas were measured using a digitizing tablet interfaced with a computerized image analyzer. Canal volume densities were calculated from a minimum of 24 canals per

stage of canal development. The data were analyzed using tests for one-way independent analysis of variance and least significant differences.

RESULTS

Camera lucida tracings of cross-sectional areas of epiphyses are shown in Figure 1. Canal outlines have been superimposed on the epiphyseal areas to indicate canal distribution along the medial and lateral condyles and the intercondylar fossae. In mice between 5 and 7 days of age, three stages of canal development could be distinguished. In 5 day old mice (mean weight $\pm$ S.D. = 2.88 $\pm$ 0.24 gm), canals were first evident along the medial condyle as short, bud-like invaginations from the perichondrium. This initial stage of development was classified as the superficial canal (Fig. 2). These canals were surrounded by matrix containing non-hypertrophic chondrocytes. In serial sections through the entire epiphysis, there was no histochemical evidence of mineralization.

In 5 day old mice (mean weight $\pm$ S.D. = 3.82 $\pm$ 0.11 gm), chondrocyte hypertrophy was evident. However, mineralized matrix was still histochemically undetectable. Canals terminated in matrix containing hypertrophic chondrocytes and were classified as intermediate canals (Fig. 3) . In 6 to 7 day old mice (mean weight $\pm$ S.D. = 4.76 $\pm$ 0.18 gm), the matrix surrounding the tips of the canals was stained positively with Von Kossa. Matrix adjacent to the canals was most uniformly stained, and the amount of stained matrix decreased with increasing distance from the canal. Canals which terminated in mineralized matrix were classified as deep canals (Fig. 4).

Measurements of canal length and width are presented in Table I, and canal and epiphyseal area measurements and canal volume densities are presented in Tables II and III. Canals more than doubled in length during development; canals also increased in width by more than one and one-half times. Increases in epiphyseal area were significantly different in mice at all three different weights and ages, while superficial canal area was significantly different from both intermediate and deep canal areas. There was no significant difference between intermediate and deep canal areas. Finally, volume density of superficial canals was significantly different from the volume density of intermediate and deep canals, but the volume density of intermediate canal was not significantly different from the volume density of deep canals.

TABLE 1. CANAL MEASUREMENTS

CANAL STAGE	LENGTH (um) Mean ± S.E.	WIDTH (um) Mean ± S.E.
Superficial	102 ± 4.2	101 ± 3.9
Intermediate	186 ± 5.1	130 ± 6.9
Deep	279 ± 26.4	178 ± 12.6

TABLE 2. AREA MEASUREMENTS

CANAL AREA (um^2) X 10^4 Mean ± S.E.	% INCREASE	EPIPHYSEAL AREA (um^2) X 10^4 Mean ± S.E.	% INCREASE
Superficial stage			
0.47 ± 0.05[a]		132.62 ± 2.26[b]	
	710		133.5
Intermediate stage			
3.35 ± 0.35		177.12 ± 4.85[b]	
	153		126.5
Deep stage			
5.14 ± 0.54		224.20 ± 4.38[b]	

[a] Superficial canal area was significantly different from intermediate and deep canal areas, P = 0.0005

[b] Epiphyseal areas were significantly different, P = 0.0005

TABLE 3. CANAL VOLUME DENSITY

	VOLUME DENSITY X 10^2 Mean ± S.E.
Superficial stage	0.35 ± 0.03[a]
Intermediate stage	1.91 ± 0.20
Deep stage	2.40 ± 0.20

$$\text{VOLUME DENSITY} = \frac{\text{CANAL AREA}}{\text{EPHIPHYSEAL AREA}}$$

[a] Significantly different from volume density of intermediate and deep canals at P = 0.0005.

DISCUSSION

Cartilage canals in the distal femoral epiphysis of the mouse are similar to those canals described in other animals (Moss-Salentijn, 1975; Lutfi, 1970b). Canal development can be divided into three stages based on morphological and histochemical criteria (Cole and Wezeman, 1985). Morphometric analysis of the volume density of canals at the three stages of development should provide data to distinguish canal formation by inclusion from canal formation by invasion.

Canal formation in the distal femoral epiphysis of the mouse occurred over a short time period of three days compared to the longer periods of weeks or months in other mammals (Levene, 1964). During this period of rapid growth, epiphyseal area measurements nearly doubled, while canal area measurements increased by a factor of 10. The percent increase between epiphyseal areas at the three stages of canal development remained constant, indicating that epiphyseal growth was increasing at a constant rate in mice during weight increases from 2.88 ± 0.24 gm to 4.76 ± 0.18 gm. This growth was sufficient to result in significant differences between epiphyseal areas. However, the canal growth rate was not equal between the developmental stages. The percent increase in canal area between superficial and intermediate canals was greater than the percent increase between intermediate

and deep canals. The results indicate that canal growth, in both length and width, was initially rapid but slowed considerably during later development as the canal approached the site of the epiphyseal center of ossification. A comparison of percent increase in canal area between superficial and intermediate canals with epiphyseal areas at the same developmental stages indicated that increases in canal areas were not proportionate to increases in epiphyseal areas. This disproportion was also reflected in the differences between canal volume densities. The volume density of intermediate canals was more than five times greater than the volume density of the superficial canals again indicating a significantly greater growth rate for the canal compared to the epiphysis. These data support the hypothesis of canal formation by invasion.

The hypothesis of canal formation by inclusion cannot explain increases in canal width, the significant differences between growth rates of the canals and the epiphyses, and the differences in canal volume densities. Canal formation by inclusion is dependent on appositional growth of cartilage through mitotic division of subperichondrial chondrocytes and deposition of matrix at the periphery of the cartilage. Appositional growth could account for increased canal length as new matrix is synthesized around the canal near its origin along the perichondrium. Increases in canal length could occur during interstitial growth as well. Interstitial matrix synthesis by chondrocytes along the walls of the canal could result in an overall in-

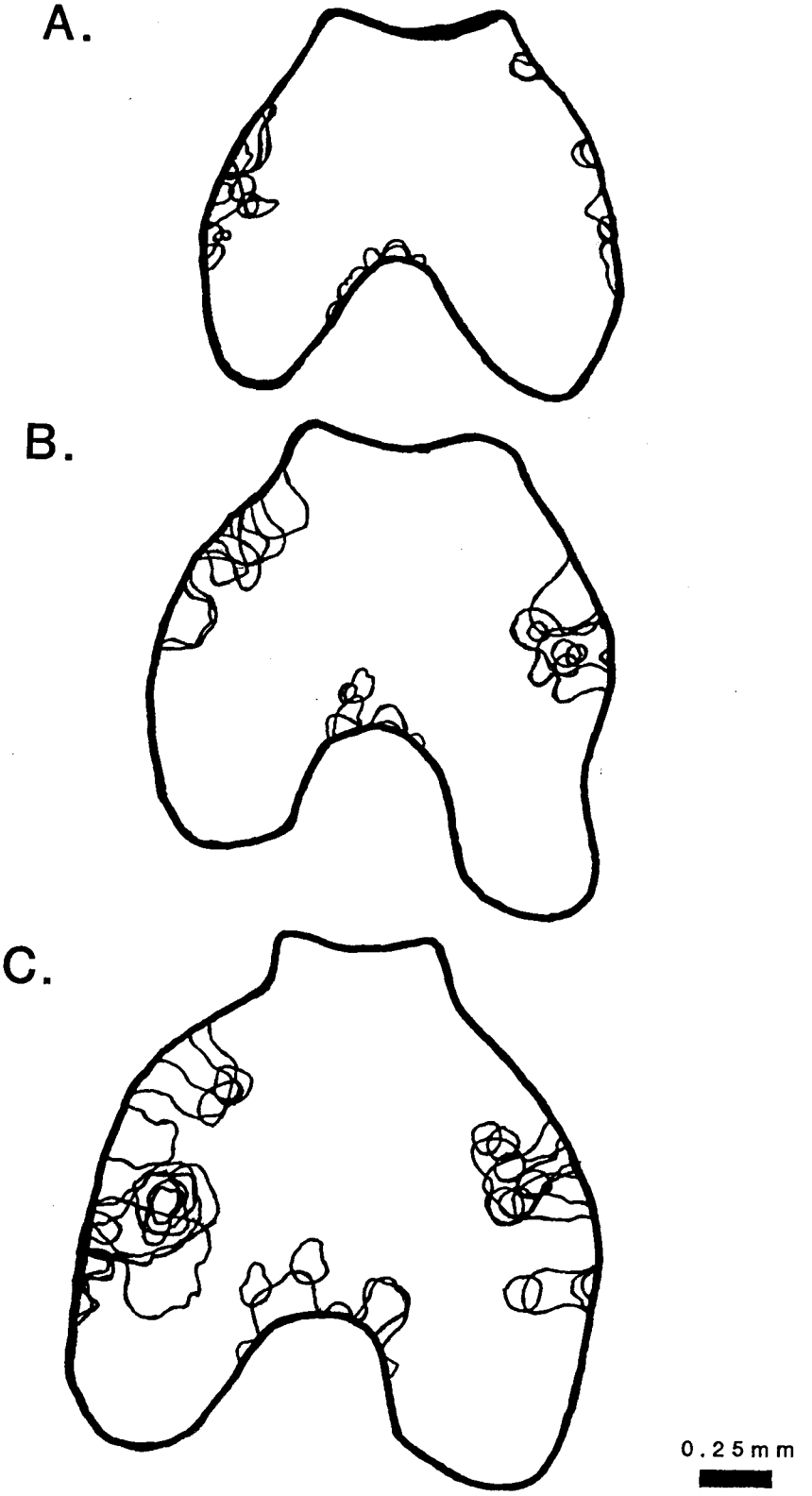
crease in canal length. However, neither appositional nor interstitial growth could account for increases in canal width. Conversely, interstitial growth might result in an overall decrease in canal width. Increases in canal width could only be accomplished through matrix degradation. Furthermore, the unequal growth rate between the canal and epiphysis indicates that canal growth is independent of epiphyseal growth and must occur by a mechanism other than interstitial or appositional growth. The growth rate of canals formed by inclusion would be dependent on the epiphyseal growth rate, and the two rates should not differ significantly. In addition, the significant difference between the volume densities of the superficial and intermediate canals, expressed as the percent epiphyseal area occupied by canals, would not be expected if canals formed by inclusion.

These data also support results from other studies which demonstrated canal formation by invasion by comparing growth of the epiphysis to canal growth. Levene (1964) compared the pattern of canal development in several mammals and provided morphological evidence that the appositional growth of the epiphysis exceeded the increase in canal length. Unequal growth between canals and cartilage areas was also noted by Moss-Salentijn (1975) in the human spheno-occipital synchondrosis. Wilsman and Van Sickle (1972) measured the distance between terminations of canals in dogs between 1 and 7 days of age. The distance between the ends of the canals remained constant during this rapid period of epiphyseal growth. Wilsman and Van Sickle (1972)

speculated that the distance could only remain constant if the canals elongated by matrix erosion.

The cellular mechanism responsible for matrix degradation during initial canal development through uncalcified matrix is unknown. In intermediate and deep canals which terminated in matrix containing hypertrophic chondrocytes and in calcified matrix respectively, cells with degradative characteristics were present (Kugler et al., 1979; Cole and Wezeman, 1985). In the superficial canals morphological evidence of matrix degradation was less clear. Vacuolated macrophages have been identified among the cells within canals (Andersen and Matthiessen, 1966; Cole and Wezeman, 1985; Rodriguez et al., 1985). Although vacuoles in those cells contained SLS fragments suggesting phagocytosis, the macrophages were rarely seen in direct contact with the matrix making their role as the primary degradative cell questionable (Cole and Wezeman, 1985). Evidence of the cellular mechanism for initial canal formation in uncalcified matrix is still unknown and is the focus of current investigations.

Figure 1 A composite tracing of cross sections through the distal femoral epiphyses. A. Epiphyses from 5 day old mice weighing 2.4-3.1 gm. Only superficial canals were present. B. Epiphyses from 5 day old mice weighing 3.1-3.7 gm. Intermediate canals were present. C. Epiphyses from 6-7 day old mice weighing 4.2-5.6 gm. Deep canals were present. Original magnification X49; bar = 250 um.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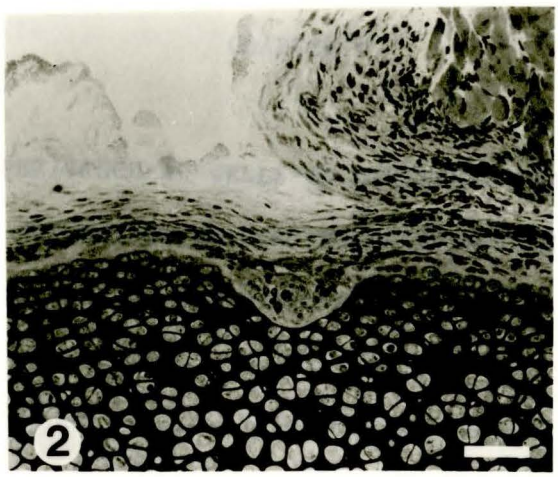
Figures 2-4 Low power photomicrographs of the three stages of canal development. (2) Superficial canal. Perivascular cells and capillaries within the canal abut onto cartilage matrix which stained weakly and othrochromatically with toluidine blue. (3) Intermediate canals which have developed adjacent to one another. At the tips of the intermediate canals, perivascular cells and capillaries occupy opened lacunae (arrows). Toluidine blue. (4) Deep canal. Mineralized matrix was black following Von Kossa staining. This canal may have increased in width by the removal of matrix between two canals such as those seen in (b). Von Kossa stain; counter-stained with Safranin O. Original magnification = X51.2; bar = 50 um.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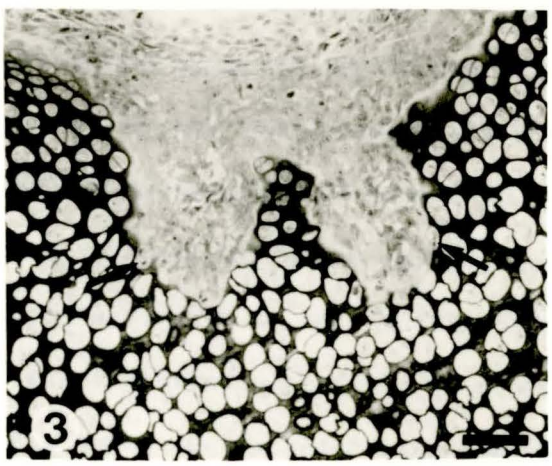
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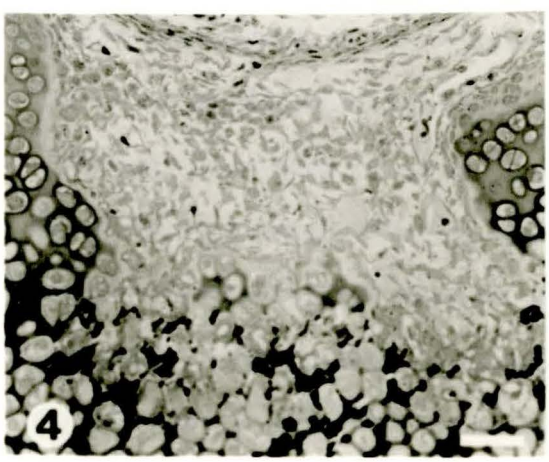
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CHAPTER V

PERIVASCULAR CELLS IN CARTILAGE CANALS OF THE DEVELOPING MOUSE EPIPHYSIS

ABSTRACT

Morphological variability among perivascular cells adjacent to cartilage matrix during the elongation of canals through both uncalcified and calcified matrix has not been reported. Cartilage canals were located in distal femoral epiphyses of 5 to 7 day old mice and identified as vascular channels arising from perichondrial surfaces along the condyles and intercondylar fossae. Three stages of canal development were identified based on the length of canals and on characteristics of chondrocytes and matrix surrounding the canals. Superficial canals terminated in uncalcified matrix of resting cartilage; intermediate canals terminated in matrix containing hypertrophic chondrocytes; deep canals terminated in calcified matrix. The ultrastructural morphology of perivascular cells in contact with the matrix varied in the three stages. Cells resembling fibroblasts and vacuolated macrophages were present adjacent to the uncalcified matrix in superficial canals. At the tips of intermediate canals, cells resembling fibroblasts were larger, contained numerous lysosomes and phagolysosomes, and were in intimate contact with the matrix. At the tips of deep canals, chondroclasts with ruffled borders and clear zones contacted the calcified matrix. The results indicate that 1) mouse epiphyses provide a suitable model for studying cartilage canal perivascular cells, 2) calcification of cartilage matrix occurs along the course of the canal, and 3) the morphology of perivascular cells in

contact with the matrix may be determined, in part, by matrix calcification.

INTRODUCTION

Cartilage canals form in epiphyseal cartilage preceding the development of the secondary center of ossification. The canals contain 1) blood vessels which will eventually become incorporated into the developing ossific center (Brookes, 1971; Ogden, 1979) and 2) connective tissue cells generally described as mesenchymal or polymorphic cells (Lutfi, 1970b; Stockwell, 1971). Initially the canals develop at the perichondrial surface and lengthen through uncalcified resting cartilage. Calcification begins in the matrix adjacent to the canals, and further elongation and expansion of the canals occurs through calcified matrix (Gray and Gardner, 1969; Wilsman and Van Sickle, 1970). As the canal expands through the calcified matrix, the secondary center of ossification is established with the deposition of bone by osteoblasts (Kugler et al., 1979).

Various functions have been described for the pleuropotential cells that occupy the extravascular compartment of the canal. These perivascular cells, accompanying blood vessels in the canals, can differentiate into chondrocytes which increase the epiphysis by interstitial growth (Lutfi, 1970a), into preosteoblasts and osteoblasts to populate the secondary center of ossification (Kugler et al., 1979), or into the degradative cells that lengthen the canals. The focus of this study is the morphology of perivascular cells that contact the

matrix surrounding the canal and which have characteristics of degradative cells.

In a light microscopic study, Andersen and Matthiessen (1966) identified macrophages at the tips of canals and proposed that they had a role in the degradation of uncalcified matrix. Macrophages have not, however, been reported at the tips of elongating canals in other studies (Lutfi, 1970b; Stockwell, 1971; Wilsman and Van Sickle, 1972). Chondroclasts were identified (Kugler et al., 1979) along the calcified matrix, and a role for them in cartilage matrix resorption was proposed. In other studies, however, chondroclasts have not been observed (Knese, 1980). Inconsistencies in the morphological characterization of perivascular cells could have resulted from the fact that no single ultrastructural study has identified perivascular cells adjacent to the matrix during the entire sequence of canal development, through both uncalcified and calcified matrix.

At sites of uncalcified matrix degradation, cells with morphological characteristics of both macrophages and fibroblasts have been identified (Schenk et al., 1967; Kobayashi and Ziff, 1975; Yajima, 1976; Silvestrini et al., 1979; Sorrell and Weiss, 1980; 1982). On the other hand, both mononucleated and multinucleated cells have been identified at sites of calcified-cartilage matrix degradation. Chondroclasts were reported adjacent to calcified matrix (Schenk et al., 1967; Crissman and Low, 1974; Savostin-Asling and Asling, 1975; Howlett, 1980). Additionally, mononucleated phagocytes and macrophages

have been identified at sites of calcified matrix degradation both *in vivo* and *in vitro* (Anderson and Parker, 1966; Dorey and Bick, 1977; Kahn et al., 1978; Rifkin et al., 1979; Holtrop et al., 1982).

The purpose of this study is to identify morphologically the perivascular cells immediately adjacent to the matrix surrounding elongating cartilage canals in the developing epiphysis of the distal femur. The morphology of perivascular cells in three stages of canal development will be characterized with respect to the presence or absence of matrix calcification.

MATERIALS AND METHODS

Animals

Newborn mice (Swiss) were obtained from a breeding colony housed in a centrally located, fully accredited animal care facility. Following breeding, females were individually caged with food and water available *ad libitum*. Neonates were weighed and sacrificed by decapitation with a minimum of handling. All animals used were 5 to 7 days of age and weighed between 2.9 and 5.3 gm.

Localization of cartilage canals

The distal femoral epiphysis of 5 to 7 day old animals was chosen as the model for the localization of cartilage canals. Mice were sacrificed 2 to 4 days prior to the age at which ossification has been reported. The earliest age at which an ossification center was detected in the distal femoral epiphysis of albino mice was at 7 to 9 postnatal days (Johnson, 1933).

Distal femoral epiphyses were dissected free of adjacent tissues and fixed overnight in 0.1M cacodylate-buffered 2.5% glutaraldehyde (pH 7.4) at 4°C. The tissue was rinsed in buffer, dehydrated through alcohol and glycol methacrylate (GMA) and embedded in GMA following the procedure of Cole (1982). Serial sections were cut at 2 um and stained with 1% toluidine blue or 1% safranin O. Calcified matrix was histochemically identified using the Von Kossa stain.

Ultrastructural morphology

Central wedges of epiphyseal cartilage, including the intercondylar area and free of the growth plate and lateral and medial portions of the epiphysis, were fixed overnight at 4°C in 0.1M cacodylate-buffered 2.5% glutaraldehyde with $CaCl_2$ (pH 7.4), rinsed in buffer, post-fixed in 1% buffered OsO_4 for 2 hrs at 22°C, dehydrated and embedded in Epon. Thick sections were cut and stained with 1% toluidine blue until canals were located. Thin sections were cut into a collecting trough filled with 0.1M cacodylate buffer (pH 7.4) to prevent decalcification during sectioning (Boothroyd, 1964; Thorogood and Gray, 1975; Bishop and Warshawsky, 1982). The sections were collected onto single hole grids and stained for 5 min each with 5% aqueous uranyl acetate and lead citrate. The sections were placed on Formvar-coated single-hole grids and viewed with a Hitachi H-600 electron microscopy at an accelerating voltage of 75 kv.

RESULTS

Light microscopy

Cartilage canals containing sinusoidal capillaries and perivascular cells began at perichondrial surfaces of the condyles and the intercondylar fossae in distal femoral epiphyses, often near the attachment of ligaments. Branching was infrequent and was observed only in canals arising from intercondylar fossae. Canals, originating in juxtaposition at perichondrial surfaces, often fused deeper within the cartilage, resulting in widened canals. Canals were identified as superficial, intermediate, or deep, based on the length of the canal and the characteristics of chondrocytes and matrix in which it terminated. Superficial canals were short, budlike invaginations, approximately 100 um long and 100 um wide, surrounded by uncalcified matrix of resting cartilage. In smaller 5 day old mice, only superficial canals were present; in the entire epiphyses only resting cartilage was observed. In larger 5 day old mice intermediate length canals, approximately 180 um long and 130 um wide, terminated in matrix containing hypertrophic chondrocytes; calcified matrix was histochemically undetectable. In 6 to 7 day old mice, deep canals, approximately 255 um long and 130 um wide, terminated in matrix that was histochemically positive for mineral. In animals older than 7 days, epiphyseal centers were evident as the tips of the canals within the

cartilage matrix became expanded and bulblike, and cells resembling osteoblasts were present adjacent to uncalcified bone matrix. Animals chosen for the study were 5-7 days old and contained canals that were not expanded into the epiphyseal center and in which no bone formation was evident.

The surface of the matrix along the margin of canals was irregular and often exhibited cup-shaped depressions. Some canal cells occupying the depressions exhibited mitotic figures. A band of matrix abutting the canal was weakly and orthochromatically stained with either toluidine blue or safranin O and extended into the matrix to a depth of 4-15 um. An abrupt transition was evident between the weakly stained orthochromatic matrix of this band and the strongly stained metachromatic matrix of the remainder of the epiphyseal cartilage. Chondrocytes were frequently present within this weakly stained band of matrix.

Electron microscopy

In the superficial canals, perivascular cells adjacent to uncalcified matrix were uniformly dispersed around the margins of the canals, and no clustering of cells was apparent in the deepest portions or tips of the canals (Fig. 1). No morphological differences were observed between cells at the tips of canals and along the lateral walls of canals. Cells most commonly found adjacent to the matrix were mononucleated with fibroblastic features (Fig. 2) including dilated rough endoplasmic reticulum, one or two well-defined Golgi

zones, fat droplets, and coated vesicles. Membrane-bound dense bodies resembling lysosomes and phagolysosomes were present in varying numbers within these cells. Cilia were evident in some of these cells. From the surface of cells adjacent to the matrix, short, thin cytoplasmic projections often were present but did not contact the matrix, although these cells often occupied depressions in the matrix. Other cells, only occasionally found adjacent to the matrix, resembled vacuolated macrophages (Fig. 3), having nuclei that often appeared indented. The cytoplasm contained scant rough endoplasmic reticulum, abundant polyribosomes, and coated vesicles. Dense bodies resembling lysosomes were infrequent in these cells. The numerous large, cytoplasmic vacuoles sometimes contained cross-banded, SLS fragments of collagen (Fig. 5). Where vacuolated cells abutted the matrix, numerous collagen fibers were exposed and appeared to contact the cell surface. Although vacuolated cells were occasionally adjacent to the matrix, they were more often separated from the matrix by cells with fibroblastic features, giving the appearance of two perivascular cell layers lining the matrix (Fig. 4). Points of contact between the surfaces of the two cell types were often observed.

Endothelial cells were only rarely found in contact with the matrix surrounding superficial canals (Fig. 6). In those rare instances when endothelial cells were found immediately adjacent to the matrix, the cytoplasm was attenuated and contained numerous pinocytotic vesicles (Figs. 6a and b). The areas of attenuated endothelium

were often penetrated by fenestrae closed by diaphragms. No basement membrane was seen between the matrix and endothelial cells. Extravasated red blood cells and monocytes were occasionally found within the canals.

In intermediate canals, the tips of the canals were distinctly different from the lateral walls (Fig. 7). At the tips of intermediate canals, both the size and number of cells in contact with the matrix increased. These larger cells (Fig. 8) were elongated with their long axes perpendicular to the matrix and were densely packed at the tips of canals, giving an epithelioid appearance to the clustered cells. Occasionally the arrangement of cells was disrupted as the perivascular cells and capillaries entered an opened chondrocyte lacuna. Perivascular cells were typified by abundant rough endoplasmic reticulum and by other morphological characteristics of those cells with fibroblastic features in superficial canals. Numerous dense bodies resembling lysosomes and phagolysosomes were present within the cells and were localized predominantly in cytoplasm adjacent to the matrix. Nuclei in some cells were indented or irregularly shaped. These fibroblastic cells were found in intimate contact with the matrix; cytoplasmic projections extended from the cell surface into the matrix and were closely associated with collagen fibers (Fig. 9). An electron-dense material was often present between the cell and the matrix. Interspersed among the enlarged fibroblastic cells were capillaries whose endothelium often contacted the matrix. The morpholo-

gical features of the endothelium of capillaries in intermediate canals were identical to those of capillaries in superficial canals. Along the lateral walls of intermediate canals, the cells adjacent to the matrix had fibroblastic features of the cells in superficial canals except that the cytoplasm contained more lysosomes and phagolysosomes, and the cells were elongated with their long axes in contact with the matrix. Although vacuolated cells were present within intermediate canals, they were not located adjacent to the matrix or the fibroblastic cells.

At the tips of deep canals, both perivascular cells and capillary endothelium contacted the calcified matrix (Fig. 10). There was also close contact between the perivascular cells and capillary endothelium, resulting in a densely packed appearance to the canals. Perivascular cells were randomly arranged among the capillaries, unlike the epithelioid arrangement at the tips of intermediate canals. The margins of canals were irregular and consisted primarily of opened lacunae. Perivascular cells having fibroblastic features were not present, having been replaced primarily by cells having chondroclastic features. The chondroclasts were large, multinucleated cells that contacted the calcified matrix with ruffled borders surrounded by clear zones (Fig. 11). Large cytoplasmic vacuoles were present in the cells between the ruffled borders and areas of cytoplasm containing numerous mitochondria.

DISCUSSION

Cartilage canals are necessary for the vascularization of the secondary center of ossification in growing long bones, and cartilage canal development should therefore precede the formation of the center (Wilsman and Van Sickle, 1970; Kugler et al., 1979). To date, cartilage canals have not been reported in mice. However, ossification centers had previously been reported in mice (Johnson, 1933), and we hypothesized that cartilage canals should be present in the neonatal mouse. Cartilage canals were observed in mouse distal femoral epiphyses with characteristics similar to those of canals described in different species by other investigators. These principal characteristics were 1) canals containing sinusoidal capillaries and perivascular cells and penetrating resting cartilage (Haines, 1933; Gardner and Gray, 1970); 2) canals entering the femur from the intercondylar fossa and the collateral aspects of the condyles (Haines, 1933) near the attachment of ligaments (Gray and Gardner, 1950); 3) the boundaries of the canals staining weakly and indistinctly, canal cells often occupying depressions in the matrix lining the canals (Lutfi, 1970b; Moss-Salentijn, 1975); and 4) as canals elongated, chondrocytes surrounding the tips of canals undergoing hypertrophy, and the matrix immediately adjacent to the tips of canals calcifying (Wilsman and Van Sickle, 1970; Agrawal et al., 1984).

Cartilage canals in the mouse have two features that make them a

suitable model for the study of perivascular cells in relationship to matrix calcification. First, the majority of the canals were short and infrequently branched, unlike the extensively branched, long canals reported in other animals (Levene, 1964). In cross sections of the epiphyses, the majority of canals could be observed throughout their entire length. Second, the period of time between the initial penetration of canals into the uncalcified matrix and the onset of matrix calcification was 2 days, unlike the much longer periods of weeks or years in other animals (Haraldsson, 1962; Lutfi, 1970b; Kugler et al., 1979). This short period permitted the sampling of fewer time points for the identification of all stages of canal development.

To facilitate the description of differences among perivascular cells in cartilage canals with respect to characteristics of the surrounding cartilage, three developmental stages were defined using the terms "superficial", "intermediate", and "deep". The corresponding cartilage characteristics included uncalcified matrix containing resting chondrocytes, uncalcified matrix containing hypertrophic chondrocytes, and calcified matrix, respectively. Stockwell (1971) previously described canals as "superficial" or "deep" but based the description on canal depth from the articular surface. Our nomenclature, on the other hand, relates the extent of canal development to the characteristics of the cartilage in which canals terminate. By comparing these three stages of canal development, differences in the arrangement and morphology of perivascular cells adjacent to the matrix were

readily apparent.

Perivascular cells in the superficial canals were evenly distributed around the margin of the canal so that no distinct tip was apparent. Superficial canal size increased in both length and width during penetration through resting cartilage. In intermediate and deep canals, perivascular cells were clustered at the tips of the canals, forming a distinct cellularity similar to the clustering of cells at tips of canals surrounded by hypertrophic matrix as described by Kugler et al. (1979). In intermediate and deep canals, the length was increased over that of superficial canals, whereas the width did not change appreciably.

Ultrastructural differences were observed among perivascular cells adjacent to the matrix. In both superficial and intermediate canals, perivascular cells having morphological features of fibroblastic cells predominated. However, the fibroblastic cells of intermediate canals differed from those of superficial canals in size, position within the canal, manner in which they contacted the matrix, and cytoplasmic content of dense bodies. In deep canals the fibroblastic cells were not observed, and chondroclasts appeared adjacent to the calcified matrix. Vacuolated macrophages of the superficial canals were never observed adjacent to the matrix at the tips of intermediate or deep canals.

Kugler et al. (1979) attributed the increased numbers of cells at the tips of canals and the presence of chondroclasts to the inductive

effect of hypertrophic matrix. Hypertrophic matrix has been shown to be capable of inducing cell differentiation and proliferation, leading to endochondral ossification (Hall, 1978). In the mouse, changes in the morphology and the number of cells at the tips of canals were apparent when chondrocytes in the matrix surrounding the canal underwent hypertrophy. However, chondroclasts were not observed adjacent to the matrix until the matrix was heavily calcified; the signal for chondroclast differentiation from prechondroclasts and activation appeared more closely associated with matrix calcification than with chondrocyte hypertrophy. Mononucleated chondroclast precursors may enter the perivascular cell compartment from the blood vessels in response to hypertrophy.

Kugler et al. (1979) proposed that chondroclasts were responsible for the lengthening of canals by resorption of the hypertrophic matrix. Our observations indicate that chondroclasts appeared actively engaged in matrix resorption only after calcification of the hypertrophic matrix had occurred. The association of chondroclasts with calcified matrix resorption is well established, and chondroclasts seem to be responsible for increasing canal length through the calcified matrix. Prior to matrix calcification, enlarged fibroblastic cells appeared to be involved in matrix degradation. Their ability to degrade collagenous matrices is well established (Deporter and Ten Cate, 1973; Yajima, 1976).

The identification of cells responsible for canal formation in

the superficial canals was less clear. Andersen and Matthiessen (1966) proposed that macrophages were responsible for lengthening canals through uncalcified cartilage matrix. We observed that, in superficial canals where macrophages were adjacent to the matrix, collagen fibers were exposed and contacted the cells. Vacuoles contained cross-banded fibrous material which resembled collagen, suggesting phagocytic activity. Collagen has been reported in phagolysosomes of other degradative cells including macrophages (Parakkal, 1969; Deporter and Ten Cate, 1973). Macrophages produce enzymes that allow them to degrade and penetrate cartilage matrix (Takemura and Werb, 1984). However, the vacuolated macrophages were only occasionally observed directly adjacent to the matrix, so a role as the primary degradative cells seems unlikely. The vacuolated cells were more often found adjacent to cells with fibroblastic features forming a second cell layer similar to the arrangement reported along the uncalcified matrix in the chick diaphysis. Sorrell and Weiss (1982) interpreted this arrangement of macrophages and fibroblastic cells as indicating that macrophages secrete factor(s) that stimulate enzyme production by fibroblastic cells. With regard to cell-cell and cell-matrix interactions, it is well known that fibroblasts produce elevated levels of trypsin-activatable collagenase upon their stimulation by type I, II, or III collagen (Biswas and Dayer, 1979). Fibroblast contact with cartilage collagen could therefore facilitate their release of collagenase, thus promoting matrix degradation necessary for

canal elongation. The stimulatory effect of collagen is further enhanced by factors arising from monocytes (Dayer et al., 1977; Biswas and Dayer, 1979). Indeed, the inductive effect of proteinases on fibroblast secretion of collagenase (Werb and Aggeler, 1978) suggests that the products of secretion of macrophages as well as the contact by fibroblasts with cartilage collagen may facilitate matrix degradation in the superficial canals.

Similarities between the fibroblastic cells within the canals and chondrocytes surrounding the canal could be interpreted either as a release of chondrocytes from the matrix into the canal by matrix degradation or as an incorporation of fibroblastic cells into the matrix by matrix synthesis. Both Lutfi (1970b) and Knese (1980) described the freeing of chondrocytes from the matrix surrounding the canal. Lutfi (1970a) further proposed that canal cells transform into chondrocytes, providing stem cells for interstitial growth at later stages of epiphyseal development in the chick. Wilsman and Van Sickle (1970) also described a chondrogenic function for the canals. The two early theories of canal formation would also support either interpretation: Stump (1925) proposed that canals form by invasion and matrix degradation, whereas Haines (1933) proposed that canals form by inclusion during rapid synthesis of cartilage matrix around the perichondrial vessels.

Based on ultrastructural appearance alone, a distinction between the two alternatives in the superificial canals cannot be made. How-

ever, an ultrastructural interpretation combined with light-microscopic observations indicates that 1) the canals increased in length and width in the uncalcified cartilage; 2) the margins of canals were irregular with matrix depressions resembling opened lacunae; and 3) the band of matrix surrounding superficial canals was palely stained with metachromatic stains, indicating reduced proteoglycan content (Sorrell and Weiss, 1980), and was more indicative of matrix degradation than matrix synthesis. Based on our combined ultrastructural and light-microscopic observations, it would appear that fibroblastic cells are secreting enzymes responsible for matrix degradation.

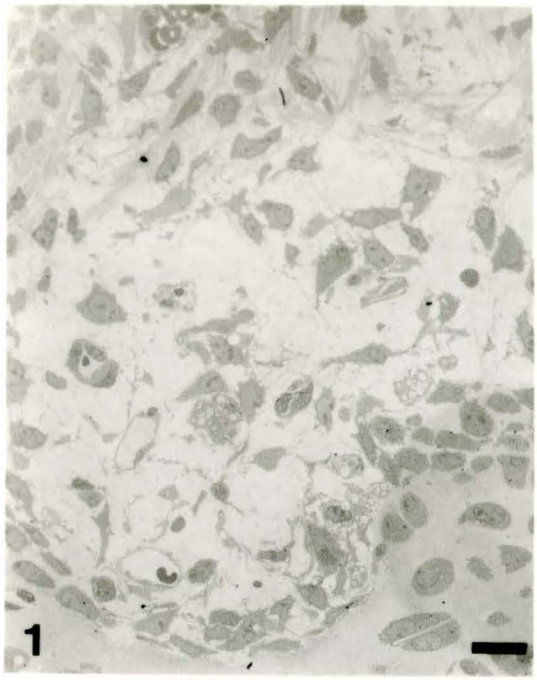
This investigation has reported the presence of cartilage canals in the neonatal mouse and presents a model for the analysis of perivascular cells in canal development. The morphological differences observed among perivascular cells adjacent to the matrix in various stages of canal development appear to be related to differences in matrix components, specifically to matrix calcification. Final determination of the functional characteristics of the various populations of perivascular cells adjacent to the matrix at each stage of canal development awaits the application of histochemical and autoradiographic techniques, which are currently in progress.

Figure 1 Low magnification electron photomicrograph of a superficial canal surrounded by resting cartilage. Cells having fibroblastic features are predominantly present adjacent to the matrix. Vacuolated cells resembling macrophages are also present. Sinusoidal capillaries are seen within the canal. Original magnification = X540; bar = 15 um.

Figure 2 Fibroblastic cells adjacent to the matrix appear morphologically similar to chondrocytes within the matrix. Superficial canal. Original magnification = X2,700; bar = 5 um.

Figure 3 Vacuolated cell resembling a macrophage in contact with the collagenous matrix of a superficial canal. Original magnification = X5,400; bar = 2 um.

Figure 4 Fibroblastic cell in a superficial canal adjacent to the matrix. A vacuolated cell is often seen adjacent to the fibroblastic cell. Original magnification = X2,700; bar = 5 um.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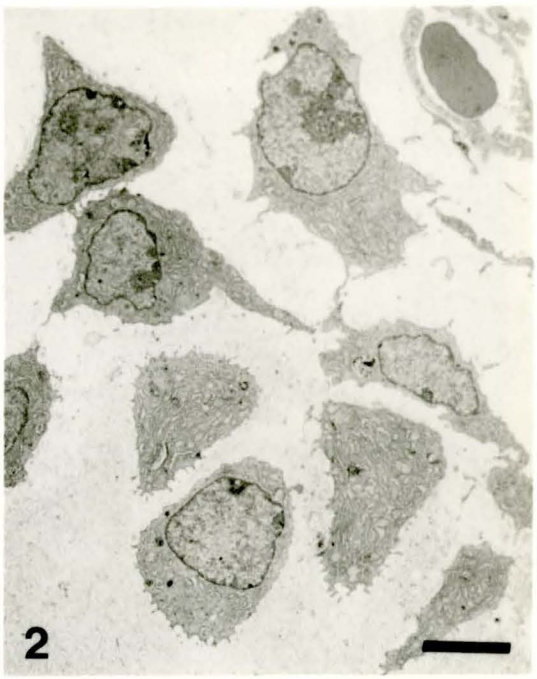
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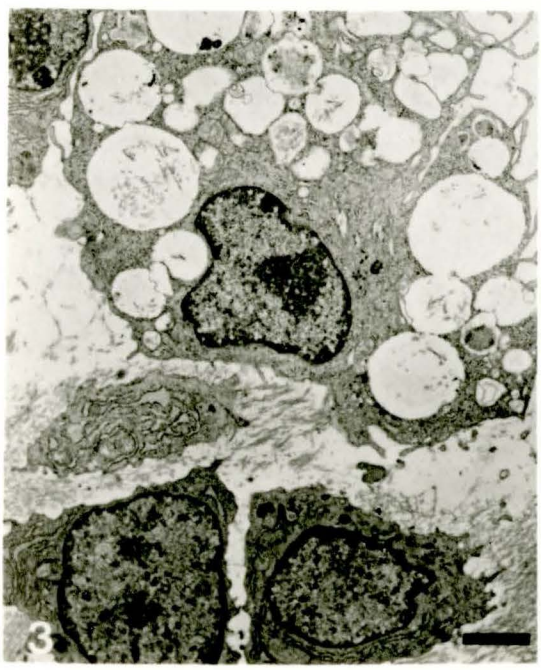
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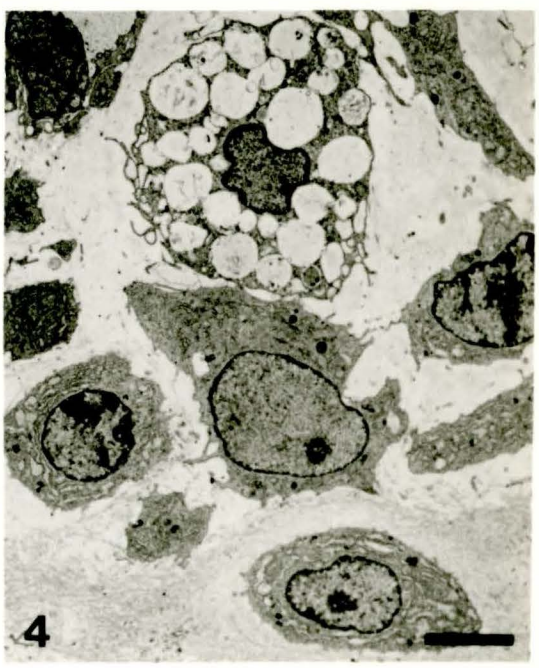
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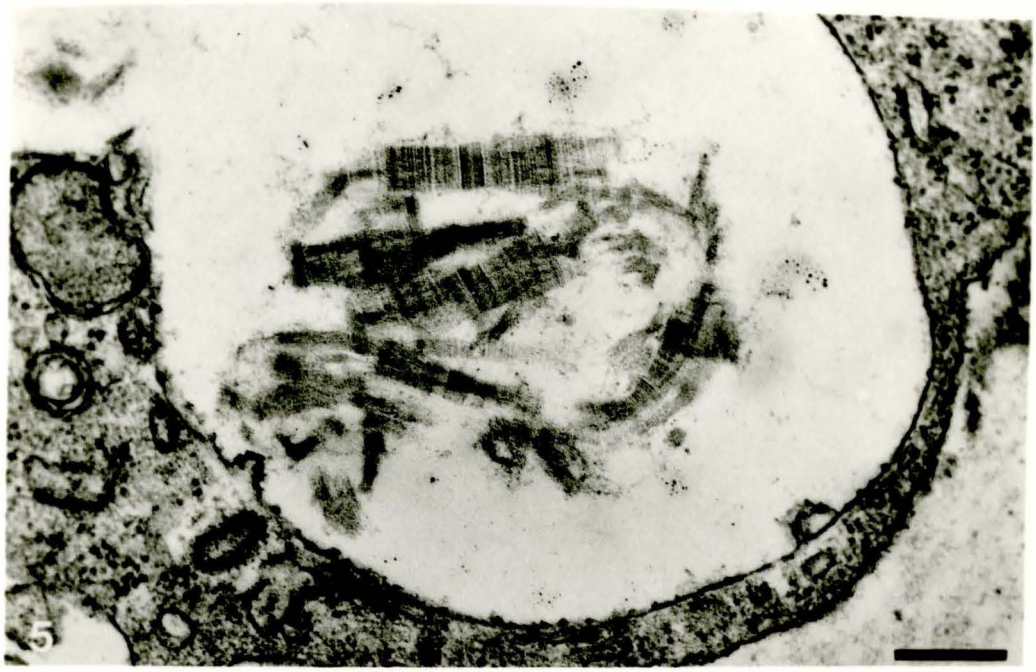
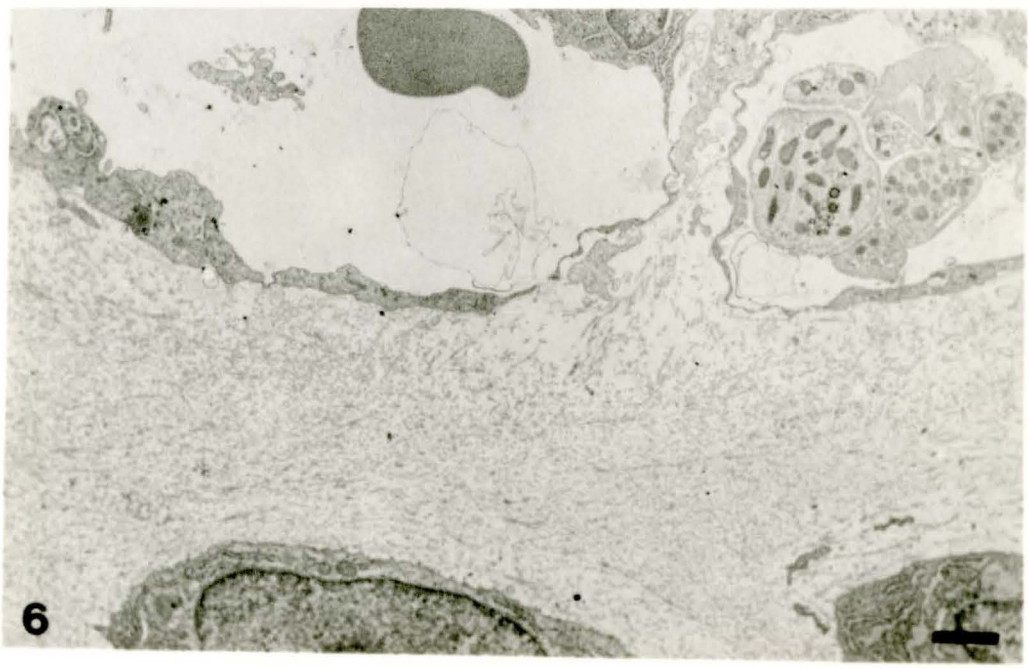
Figure 5 Morphological evidence of SLS fragments of collagen within a macrophage phagolysosome. Superficial canal. Original magnification = X36,000; bar = 0.5 um.

Figure 6 Endothelial contact with matrix in a superficial canal. Original magnification = X5,400; bar = 2 um.

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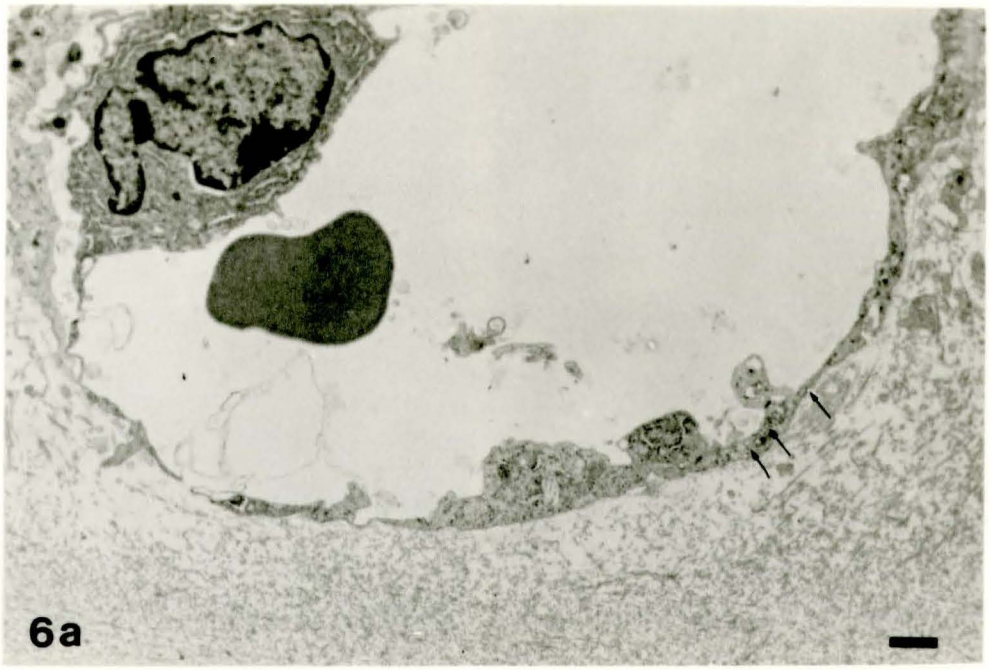
Figures 6a and b Endothelial cells in contact with matrix surrounding intermediate canal. Cells contain pinocytotic vesicles (arrows). Original magnification = X8,400; bar = 10 um.



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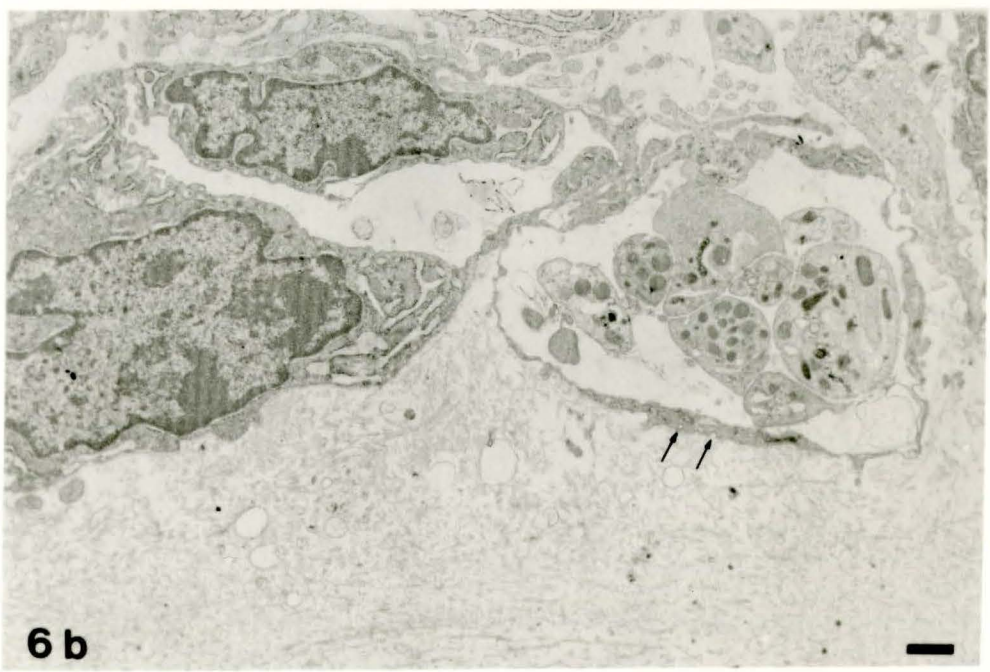
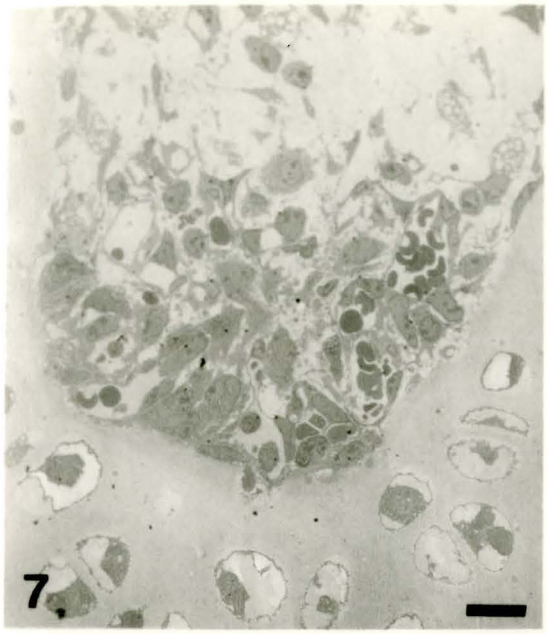


6 b

Figure 7 Low magnification electron photomicrograph of an intermediate canal with hypertrophic chondrocytes surrounding the tip of the canal; enlarged fibroblastic cells and capillary endothelium are found in close contact with the matrix. Original magnification = X540; bar = 15 um.

Figure 8 Enlarged fibroblastic cell in contact with matrix at the tip of an intermediate canal. Original magnification = X2,700; bar = 5 um.

Figure 9 Intimate cell-matrix contact of an enlarged fibroblastic cell at the tip of an intermediate canal. Original magnification = X18,000; bar = 0.5 um.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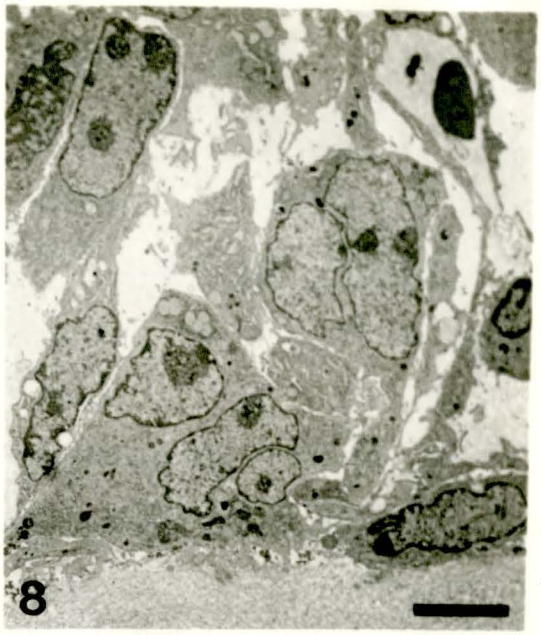
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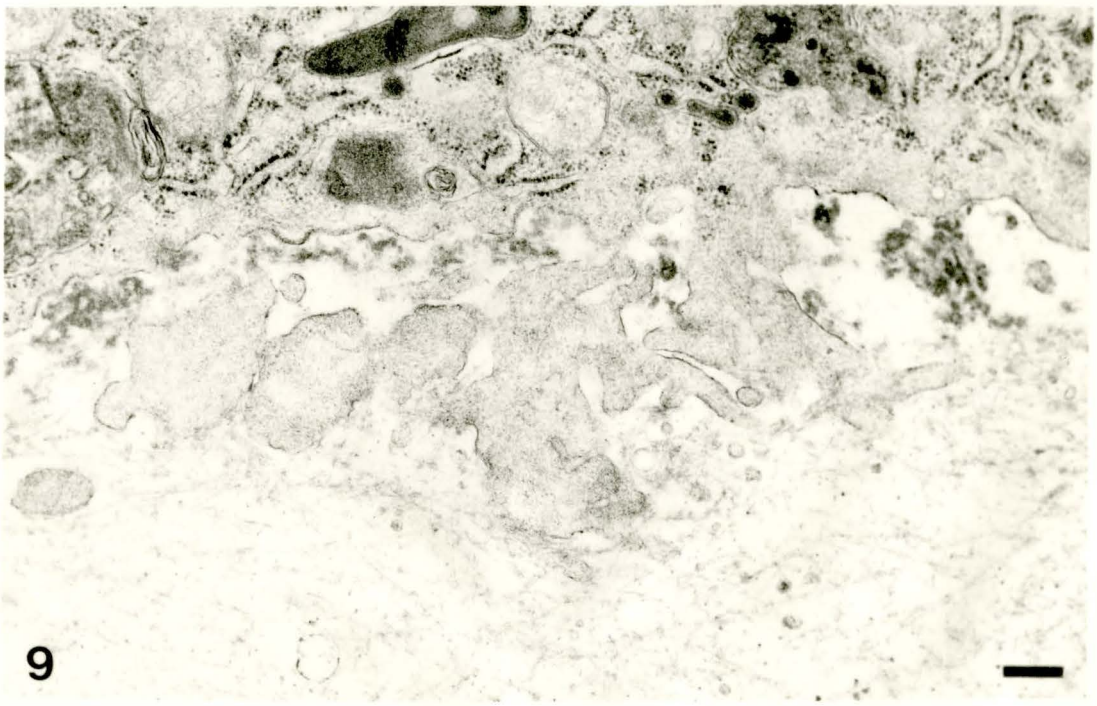
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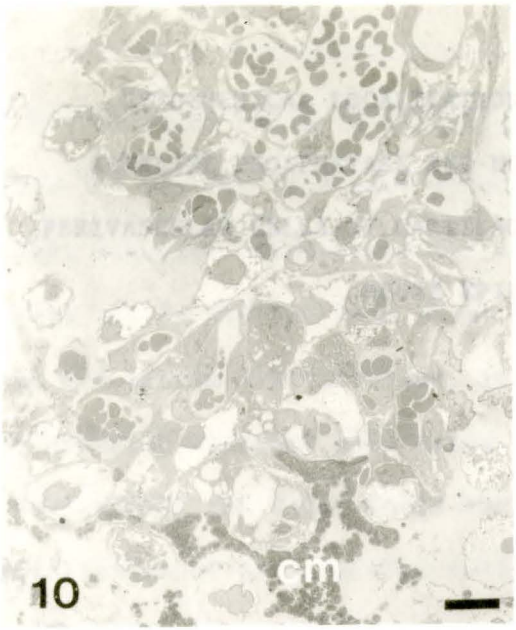
Figure 10 Low magnification electron photomicrograph of a deep canal. Perivascular cells at the tip of the canal contact the calcified matrix (cm). Original magnification = X540; bar = 15 um.

Figure 11 Chondroclast (cc) with ruffled borders and clear zones actively resorbing calcified matrix (cm). Original magnification = X1,800; bar = 5 um.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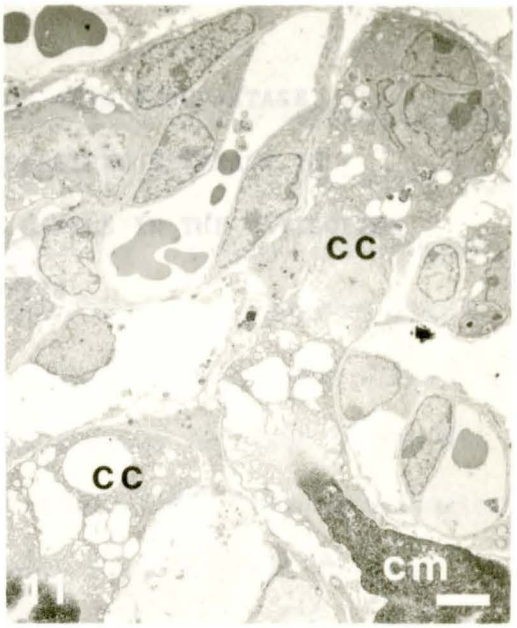
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CHAPTER VI

CYTOCHEMICAL LOCALIZATION OF ACID PHOSPHATASE, ALKALINE PHOSPHATASE AND NONSPECIFIC ESTERASE IN PERIVASCULAR CELLS OF CARTILAGE CANALS IN THE DEVELOPING MOUSE EPIPHYSIS

ABSTRACT

Perivascular cells in cartilage canals of distal femoral epiphyses from 5-7 day old mice were cytochemically characterized using TRAP and TSAP, alkaline phosphatase and nonspecific esterase. Development of canals can be divided into three stages based on chondrocyte morphology and matrix calcification, and at each developmental stage morphologically distinct cell types were present adjacent to the matrix surrounding the canal (Cole and Wezeman, 1985). The cytochemical characteristics of perivascular cells were also distinct for each stage of development. In superficial canals which developed prior to matrix calcification, perivascular cells were negative for both acid phosphatases and alkaline phosphatase and were only weakly positive for nonspecific esterase; in superficial canals which developed in epiphyses following matrix calcification, mononucleated cells-positive for TRAP were present. These TRAP-positive cells in the superficial canals may represent degradative cells which enter the canals or differentiate within the canals in response to inductive factors released from the calcifying cartilage. In intermediate canals, a small number of both mononucleated and multinucleated cells were positive for TRAP, while a larger number of cells contained alkaline phosphatase. The presence of alkaline phosphatase in perivascular cells appeared to be correlated with the presence of alkaline phosphatase in hypertrophic chondrocytes surrounding the canal. Chondroclasts in deep canals

contained TRAP as well. No cells at any developmental stage demonstrated TSAP or a strong nonspecific esterase reaction product. The significance of the distribution of the enzymes within the perivascular cells is discussed relative to their role in matrix degradation.

INTRODUCTION

Cartilage canals are channels which form in epiphyseal cartilage prior to the formation of the secondary center of ossification. Canals contain blood vessels accompanied by perivascular cells which are thought to be responsible for canal formation. In the mouse, canal development can be divided into three stages characterized by differences in chondrocyte morphology and matrix calcification (Cole and Wezeman, 1985). At each developmental stage, morphologically distinct perivascular cells are present adjacent to the matrix surrounding the canal. In the earliest developmental stage, the superficial canal, fibroblastic cells are predominantly found adjacent to the matrix, while vacuolated cells are often found near the fibroblastic cells. Vacuolated cells thought to be macrophages have also been reported in human cartilage canals (Andersen and Matthiessen, 1966; Rodriquez et al., 1984). In the intermediate canal, enlarged fibroblastic cells formed an intimate contact with the matrix, and in the deep canal which terminates in calcified cartilage matrix, chondroclasts were present with characteristics similar to those of chondroclasts as reported in other studies (Moss-Salentijn, 1976; Kugler et al., 1979). Cytochemistry has been used to further characterize canal perivascular cells. Vacuolated cells in cartilage canals of the human were positive for both acid phosphatase and nonspecific esterase (Andersen and Matthiessen, 1966); Rodriquez et al. (1984), however, were unable to

demonstrate the presence of acid phosphatase in these vacuolated cells. In chondroclasts located in canals which terminated in calcified matrix, acid phosphatase has been reported (Kugler et al., 1979). Although fibroblastic cells have been reported in other canal studies (Wilsman and Van Sickle, 1972; Kugler et al., 1979), these cells have not been cytochemically characterized.

At other sites of cartilage matrix degradation, fibroblastic cells, macrophages and osteo/chondroclasts have also been morphologically and cytochemically characterized. In chick diaphysis where matrix is removed prior to calcification, alkaline phosphatase-positive cells resembling fibroblastic cells were predominately found adjacent to the matrix, and a second cell layer was composed of acid phosphatase-positive macrophagic cells (Sorrell and Weiss, 1982). Macrophages can be further distinguished by the presence of TSAP (Seifert, 1984), while TRAP has been reported in osteoclasts (Hammarstrom et al., 1971; 1983). Macrophages and other mononuclear phagocytes are also positive for nonspecific esterase as are osteoclasts although the pH optima in the two cell types may be different (Ries, 1984).

Cytochemical localization of TRAP, TSAP, alkaline phosphatase and nonspecific esterase was used to further characterize perivascular cells in the cartilage canals of the mouse.

MATERIALS AND METHODS

Tissue Processing

The distal femoral epiphyses including growth plate and bone as well as spleen were removed from mice (Swiss) at 5-7 days of age. Liver was obtained from an adult mouse. Tissue was processed according to the cold-embedding procedure of Namba et al. (1983). All procedures were conducted at 4°C except when indicated. Tissue for acid phosphatase and nonspecific esterase localization was fixed in 0.1M cacodylate buffered 2.5% glutaraldehyde with 7% sucrose for 4 hrs. Tissue for alkaline phosphatase localization was fixed in 1% glutaraldehyde, Lillie's buffered formalin (Lillie, 1965), or 100% acetone. The tissue was rinsed in buffer containing 7% sucrose, stored overnight in buffer, dehydrated in acetone or ethanol and infiltrated in Polysciences JB-4 solution A with catalyst overnight and embedded in complete JB-4 media. Prior to dehydration, pieces of bone were decalcified in 10% buffered EDTA for 48 hrs. Serial sections of epiphyses containing all stages of canal development and representative sections of growth plate, bone, liver and spleen were cut at 3um on a Sorvall JB-4 microtome at 22°C and placed on cold slides which had been coated with 2% gelatin. The slides were stored at 4°C for a minimum of 5 days before incubation to allow the sections to firmly adhere to the slides. Some tissue for alkaline phosphatase localization was frozen, and cryostat sections were left unfixed or fixed in 100% acetone.

Macrophages were obtained from peritoneal lavage and prepared as described in Appendix A.

Histochemical Staining

1) Acid Phosphatase

Sections and macrophage smears were incubated for 90 min at 37°C in Burstone's complete media (Pearse, 1968) containing Naphthol AS-BI phosphate (Sigma) as the substrate and Red-violet LB diazonium salt (Sigma) as the capture agent in 0.2M acetate buffer, pH 5.1. Two drops of 10% $MgCl_2$ was added per 25 ml of media. L(+)-tartaric acid (Sigma) was added to some of the media at a concentration of 50 mM. This concentration of tartrate was found to inhibit acid phosphatase in peritoneal macrophages (Cole and Walters, 1986). Control media did not contain substrate.

2) Alkaline Phosphatase

Sections of epiphyses and growth plate were incubated for 90 min at 37°C in Burstone's complete media (Pearse, 1968) containing Naphthol AS-BI phosphate as substrate and Red-violet LB diazonium salt as the capture agent in 0.1M Trizma buffer, pH 8.5. The substrate was omitted from control media.

3) Non-specific Esterase

Macrophage smears and sections of epiphyses, bone and liver were incubated for 60 min to 24 hrs at 37°C in complete media (Pearse, 1975) containing Naphthol AS-D-acetate (Sigma) as substrate and Garnet GBC diazonium salt (Sigma) or Fast Blue B diazonium salt (Sigma) in

0.05M Trizma buffer, pH 6.0, 6.8 or 7.1. The substrate was omitted from control media.

Following incubation the sections were rinsed in running water for 30 min, allowed to air dry and counter-stained, when necessary, with 1% fast green. Coverslips were mounted with Euparol (Gallard-Schlesinger Chem. Mfg. Corp.)

Both acid phosphatase and nonspecific esterase activity could still be localized in sections cut from blocks stored up to 4 months at $4^{o}C$; alkaline phosphatase reaction could not be demonstrated in embedded tissue after 2 months of storage. The enzyme product formed from Naphthol AS-BI phosphate and the Fast Red Violet LB salt and from Naphthol AS-D-acetate and Garnet GBC diazonium salt was stable in sections even after being stored at room temperature for up to 2 years.

RESULTS

Control tissues known to be positive for each particular enzyme were processed identically to the sections of epiphysis containing cartilage canals. Sections of bone and resorptive zone of the growth plate containing osteo/chondroclasts were used as the positive control for TRAP. Macrophages obtained from peritoneal lavage and present within synovial membrane near the cruciate ligaments of the distal femur served as controls for both TSAP and nonspecific esterase. Growth plate served as the positive control for alkaline phosphatase. Negative controls for each tissue were incubated in the absence of substrate; all controls contained no reaction product.

Tartrate-Resistant and Tartrate-Sensitive Acid Phosphatases

Optimal conditions for preserving both morphology and acid phosphatase activity were 4 hr fixation in glutaraldehyde, acetone dehydration and cold-embedding in JB-4 with a 90 min incubation in enzyme media. The reddish, granular reaction product was primarily localized intracellularly. Some extracellular distribution was noted along the bone surface adjacent to osteoclasts and in the hypertrophic zone of the growth plate. Due to a virtual absence of nuclear staining with this procedure, the multinucleated osteoclasts could be readily identified and distinguished from mononucleated cells (Fig. 1). Within osteoclasts, a heavy concentration of reaction product was uniformly distributed throughout the cell.

The presence of TSAP was determined by comparing staining patterns in adjacent serial sections incubated in the presence or absence of tartrate. Those cells which could be identified as positive following incubation in media with no tartrate and negative following incubation in medium with tartrate were considered to be TSAP containing cells, while cells which showed no difference in enzyme concentration were considered to be TRAP-containing cells (Fig. 2).

In the deep canals, multinucleated chondroclasts stained with TRAP were present adjacent to the calcified matrix (Fig. 3). Other TRAP-containing multinucleated cells were observed in the lumen of deep canals at a distance from the matrix. In chondroclasts (Fig. 4) the concentration of TRAP was not as heavy as it was in osteoclasts located along the bone or in the growth plate. Canal chondroclasts exhibited a slightly heavier concentration of granules following formalin fixation; however, despite reduction in reaction product, glutaraldehyde was the preferred fixative for morphological preservation. Hypertrophic chondrocytes in the matrix surrounding the canal also contained a sparce number of TRAP granules scattered throughout their cytoplasm (Fig. 5). These hypertrophic chondrocytes have a cytochemical content and distribution of TRAP similar to that of hypertrophic chondrocytes in the growth plate (Fig. 6). In intermediate canals (Fig. 7), elongated mononucleated and multinucleated cells adjacent to the matrix also demonstrated a heavily concentrated TRAP reaction product (Fig. 8). Superficial canals found in epiphyses prior to

chondrocyte hypertrophy contained no cells positive for TRAP or TSAP. The vacuolated cells present in the canals did not contain a granular reaction product; occasionally, a faint diffuse staining was noted which persisted even following incubation in media containing tartrate. As the epiphyses grew in animals between 5 and 7 days of age, cartilage canals increased in number. In epiphyses which contained intermediate and deep canals, superficial canals could also be found. Unlike the superficial canals present in epiphyses prior to chondrocyte hypertrophy and matrix calcification which contained no acid phosphatase-positive cells, superficial canals which developed following chondrocyte hypertrophy and matrix calcification contained heavily stained mononucleated cells located in the lumen of the canal both at a distance from the matrix and adjacent to the matrix (Fig. 9). Additionally, heavily stained mononucleated cells could be occasionally identified within the fibrous perichondrium (Fig. 10) surrounding the epiphyseal cartilage and in the zone of Ranvier.

Alkaline Phosphatase

Demonstration of alkaline phosphatase activity was inconsistent following formalin or glutaraldehyde fixation, acetone or ethanol dehydration and cold-embedding in JB-4. Using growth plate as a positive control, alkaline phosphatase was localized in 1) unfixed frozen sections, 2) in frozen sections fixed in acetone and 3) in JB-4 embedded sections fixed in acetone. The distribution of alkaline phosphatase which was present in glutaraldehyde-fixed sections was compared

to that in the frozen sections and acetone-fixed embedded sections.

In 6 um frozen sections, the red reaction product was strong throughout the growth plate, bone, epiphyseal hypertrophic chondrocytes and perichondrium. Reaction product was not significantly reduced following 1 min fixation in acetone. In frozen sections, the contents of the canal were poorly preserved, and often canals contained no recognizable cells or blood vessels. Alkaline phosphatase activity was still demonstrable in the 3 um JB-4 embedded sections following fixation in 100% acetone although the amount of reaction product appeared somewhat reduced. In the growth plate (Fig. 11) alkaline phosphatase was not visible until late in the proliferative zone where the lacunar rim and interterritorial matrix were stained. In the hypertrophic zone, chondrocytes contained a few stained granules, and the extracellular product appeared heavier. In the epiphysis the perichondrial reaction product was still present adjacent to areas containing hypertrophic chondrocytes. Reaction product was first evident in hypertrophic chondrocytes located near the canal. Reaction product was absent from cells within the superficial canals (Fig. 12) but was present in perivascular cells of intermediate (Fig. 13) and deep canals (Fig. 14). Within canal cells, alkaline phosphatase was primarily distributed at the periphery of the cell although a few intracellular granules could be recognized (Fig. 13). No alkaline phosphatase appeared to be associated with endothelial cells. Intermediate canals were initially associated with matrix-containing hyper-

trophic chondrocytes that were negative for alkaline phosphatase. In these canals only a few perivascular cells were positive for alkaline phosphatase. As alkaline phosphatase increased in the hypertrophic chondrocytes, the number of alkaline phosphatase-positive canal cells also increased.

Nonspecific Esterase

Nonspecific esterase was demonstrated in macrophages obtained from peritoneal lavage cells and in macrophages present in synovial membrane. In sections of spleen, bone, growth plate and epiphysis, the activity of nonspecific esterase was preserved following 4 hr fixation in 2.5% glutaraldehyde, ethanol dehydration and cold-embedding in JB-4. A 1 hr incubation was sufficient to produce a distinctive reddish-brown intracellular reaction product. The reaction product was primarily intracellular except within the canal where some extracellular product was consistently present. Prolonged incubation resulted in increased, orange, background staining of cells, matrix and plastic by the diazonium salt but did not appreciably increase the amount of cellular reaction product. Increasing the pH of the incubation media from 6.0 to 7.5 also resulted in increased background staining. The staining pattern of nonspecific esterase following incubation with Fast Blue B diazonium salt in place of the Fast Garnet GBC was also tested. With Fast Blue, there was no detectable background staining of the matrix or plastic. The reaction product was not granular but rather formed a uniform lake throughout the positive

cells, and the number of cells which were stained for nonspecific esterase with Fast Blue appeared reduced. Additionally, the reaction product was not as stable within the sections following incubation as was the reaction product formed with Fast Garnet GBC. Some bleeding of the reaction product from cells and decrease in amount of product was noted following storage of slides at room temperature after several weeks.

In the epiphyses and growth plates nonspecific esterase was ubiquitously distributed in canal cells, chondrocytes and perichondrial cells (Fig. 15). These cells, as well as chondrocytes within the growth plate (Fig. 16), contained only a few scattered granules of reaction product unlike the heavily stained macrophages from the synovial membrane (Fig. 17). The vacuolated canal cell contained only a few scattered granules similar to the staining pattern in chondrocytes (Fig. 15). There were no canal cells at any developmental stage which exhibited the same staining pattern as peritoneal or synovial macrophages. Increasing the pH of the incubation media in an attempt to localize nonspecific esterase in osteoclasts and chondroclasts resulted in an increase in the orange background staining of cells, matrix and the plastic. This increase in background staining made a positive identification of reaction product difficult at pH 7.5, and the results were ambigious.

DISCUSSION

Cytochemical localization of TRAP, TSAP, alkaline photphatase and nonspecific esterase was used to characterize perivascular cells within cartilage canals at three developmental stages. Cytochemically distinct populations of cells contact the matrix at each stage. In superficial canals, which developed in 5 day old mice prior to evidence of chondrocyte hypertrophy or matrix calcification, there were no cells stained for TRAP, TSAP or alkaline phosphatase or cells heavily stained for nonspecific esterase. In superficial canals which were located in epiphyses of 6 to 7 day old mice containing intermediate and deep canals, mononucleated cells positive for TRAP were present. Vacuolated cells displayed little or no TSAP, TRAP or nonspecific esterase. In intermediate canals both TRAP- and alkaline phosphatase-positive cells were located adjacent to matrix and within the lumen of the canal at a distance from the matrix. Cells in contact with matrix and positive for TRAP were primarily found at the tips of canals, while cells positive for alkaline phosphatase were distributed along the walls of the canals as well. The presence of alkaline phosphatase has not been previously reported in perivascular cells (Andersen and Matthiessen, 1966; Rodriquez et al., 1985). In the mouse the presence of alkaline phosphatase in these cells appeared closely related to the appearance of alkaline phosphatase in chondrocytes. Smaller hypertrophic chondrocytes were negative for alkaline phosphatase,

while larger hypertrophic chondrocytes were positive. The distribution of alkaline phosphatase in chondrocytes in the epiphysis differs from the distribution in the growth plate where hypertrophic zone as well as proliferative zone chondrocytes contain alkaline phosphatase (Kenrad and Vilmann, 1977). Perivascular cells, located adjacent to matrix containing hypertrophic chondrocytes negative for alkaline phosphatase, were also alkaline phosphatase-negative, while perivascular cells, located adjacent to matrix containing hypertrophic chondrocytes positive for alkaline phosphatase, were also alkaline phosphatase-positive. Deep canals were characterized by the presence of TRAP-positive multinucleated cells in contact with matrix at the tips of canals.

The TRAP-positive chondroclasts were similar to those acid phosphatase-positive cells described by Kugler et al. (1979) in canals of rat and rabbit. Within developing mammalian skeletal tissues, cartilage canals are one of the few locations in which chondroclasts distinct from osteoclasts can be studied. A difference was noted between the concentration of TRAP in chondroclasts within deep canals and osteoclasts located along diaphyseal bone. Chondroclasts contained a lower concentration of TRAP granules than did the osteoclast whose cytoplasm was often so filled with granules that only nuclei were left unstained.

The use of TRAP as a cytochemical marker for osteoclasts was proposed by Minkin (1982) and Hammarstrom et al. (1971) and its pres-

ence in osteoclasts has been described (Doty and Schofield, 1972; Gothlin and Ericsson, 1976). Its presence in cultured multinucleated cells has more recently been used to characterize the cultured cells as osteoclast-like (Ibbotson et al., 1984; Wong, 1984; Roodman et al., 1985; Jilka, 1986; Snipes et al., 1986). The two main isoenzymes which have been biochemically identified in bone are represented by the TRAP and TSAP. Substrates used to characterize enzyme activity were p-nitrophenyl phosphate and sodium B-glycerophosphate respectively (Anderson and Toverud, 1979; 1982). The ultrastructural distribution of reaction product formed with the two substrates has been shown intracellularly in Golgi elements, agranular endoplasmic reticulum, lysosomes and vacuoles and extracellularly between the membrane of the ruffled border and bone (Doty and Schofield, 1972). Biochemical studies of the subcellular distribution of p-nitrophenyl phosphate enzyme activity has shown that in cartilage the enzyme was contained in lysosomes (Arsenis et al., 1971). For light microscopy, a chromogenic reaction product can be formed using a naphthol phosphate as substrate and a diazonium salt as the coupler; TSAP is inhibited during incubation in media containing sodium tartrate (Janckila et al., 1978). In a light microscopic study using frozen section of whole rats, Hammarstrom et al. (1971) described the distribution of TRAP primarily in bone and developing teeth following incubation with concentration of tartrate from 1 to 100 mM. Chappard et al. (1983) reported the presence of TRAP in osteoclasts in plastic-embedded, undecalcified bone

sections and noted TSAP was inhibited in osteoblasts following incubation in 1 mM L(+)-tartaric acid. Subsequently Cole and Walters (1986) (See Appendix A) were able to demonstrate TRAP in both osteoclasts and osteoblasts in decalcified, plastic-embedded bone sections following incubation in media containing 50 mM L(+)-tartaric acid. Cole and Walters (1986) used peritoneal lavage macrophages as control cells to determine the appropriate concentration of tartrate which inhibited TSAP. In the rat and mouse, chondroclasts contained a heavy concentration of granular reaction product, while osteoblasts contained a sparce concentration of granules.

In this study the cytochemical distribution of TRAP has been extended to cartilage of the developing mouse epiphysis and growth plate where TRAP is present in chondroclasts and chondrocytes. The presence of TRAP in cartilage has been reported but its distribution was not described (Doty and Schofield, 1972). In chondroclasts, TRAP appears to be related to that cell's function in degrading calcified cartilage matrix. Ultrastructurally chondroclasts within the canals contacted the matrix with a ruffled border surrounded by clear zones (Cole and Wezeman, 1985). Lysosomal enzymes secreted by osteoclasts into the resorbing compartment between ruffled border and bone are thought to be active in degrading the organic components of bone (Doty and Schofield, 1972; Gothlin and Ericsson, 1976). The compartment appears to be acidified and is similar to the secondary lysosome, and the membrane of the ruffled border contains a 100-kD protein compris-

ing epitopes related to those present in the limiting membrane of secondary lysosomes (Barone et al., 1985). This compartment appears to provide conditions suitable for dissolving the mineral component of bone as well as providing an optimal pH for acid hydrolase activity.

Cells positive for TRAP were also present adjacent to the uncalcified matrix near the tips of superficial and intermediate canals. In the rabbit, multinucleated chondroclasts were reported close to the tips of certain canals, and their role in uncalcified matrix degradation was discussed (Moss-Salentijn, 1976). Enlarged multinucleated cells were also observed in the intermediate canals of the mouse; however, this cell did not exhibit ruffled borders or clear zones and was not described as a chondroclast (Cole and Wezeman, 1985). Cytoplasmic extensions from the surface of this cell extended into the matrix; electron dense material was localized between the cell's surface and the matrix, and the cell's cytoplasm contained a large number of lysosomes and phagolysosomes located within the cytoplasm closest to the matrix. Based on these morphological characteristics, we proposed that this cell functions in matrix degradation. These cells which formed a close contact with hypertrophic matrix at the tips of intermediate canals were similar to the TRAP-positive cells within the intermediate canal which also contacted the hypertrophic matrix at the tips of canals. The uniform distribution of the granular reaction product within these cells did not correspond to the apparent polarized distribution of dense bodies resembling lysosomes and phagolyso-

somes seen ultrastructurally.

Cells positive for TRAP were only found in superficial canals which developed in epiphyses in which calcified matrix was present. The majority of these cells were located in the lumen of the canal at a distance from the matrix, whereas only a few TRAP-positive cells were found adjacent to the matrix generally near the tips of canals. Ultrastructurally, the only cells observed in close contact with the matrix surrounding the superficial canal were the vacuolated cells, and this contact was only rarely seen. The predominant cell type resembled chondrocytes in matrix surrounding the canal, contained an extensive rough endoplasmic reticulum and was never observed directly in contact with matrix. We proposed (Cole and Wezeman, 1985) that the cells characterized by the presence of abundant rough endoplasmic reticulum belonged to the fibroblastic family of cells which includes fibroblasts, chondroblasts, chondrocytes and osteoblasts, while the vacuolated cell appeared to be macrophage-like.

Cytochemical characterization of the morphologically distinctive vacuolated cells have been inconsistent. Andersen and Matthiessen (1966) identified acid phosphatase-positive vacuolated cells at a number of sites of human fetal skeletal development including cartilage canals. They identified these cells as histiocytes based on their strong staining reaction for both acid phosphatase and nonspecific esterase and noted that the arrival of these cells was closely timed with the vascularization of the skeletal tissue suggesting that

histiocytes had a vascular origin. Andersen and Matthiessen (1966) also noted that these cells were more highly vacuolated in tissues containing high concentrations of acid mucosaccharides. In a more recent study Rodriquez et al. (1985) failed to localize acid phosphatase in vacuolated cells within human cartilage canals. They proposed that these cells were degenerated macrophages based on their irregular electron dense nuclei and cytoplasm which contained dilated rough endoplasmic reticulum. They suggested that the vacuoles represented dilations of endoplasmic reticulum rather than phagocytic vesicles. In the mouse the vacuolated cells were not only negative for acid phosphatase but also for nonspecific esterase with the same substrate used by Andersen and Matthiessen (1966). This lack of reaction places doubt on the identity of the cell as a macrophage. Macrophages characteristically contain high concentrations of both these enzymes (Dannenberg and Suga, 1981) and are highly phagocytic cells. In the mouse Cole and Wezeman (1985) also suggested these cells were phagocytic macrophages. Within the canals of the mouse the vacuolated cells were sometimes in direct contact with collagen fibers of the matrix surrounding the canal, and SLS collagen was frequently observed in vacuoles along with other electron dense material. In the mouse these vacuolated cells appear to be involved in phagocytosis, but they do not have the cytochemical profile of macrophages or other mononuclear phagocytes.

The presence of TRAP could be related to cartilage matrix de-

gradation. It is selectively localized in perivascular canal cells thought to be degradative cells and in chondrocytes located in matrix which will eventually be degraded such as the growth plate. Cells positive for TRAP were also scattered along the epiphyseal perichondrium and in the zone of Ranvier. In the rapidly growing femur, both the epiphysis and zone of Ranvier are areas where matrix is being degraded during modelling.

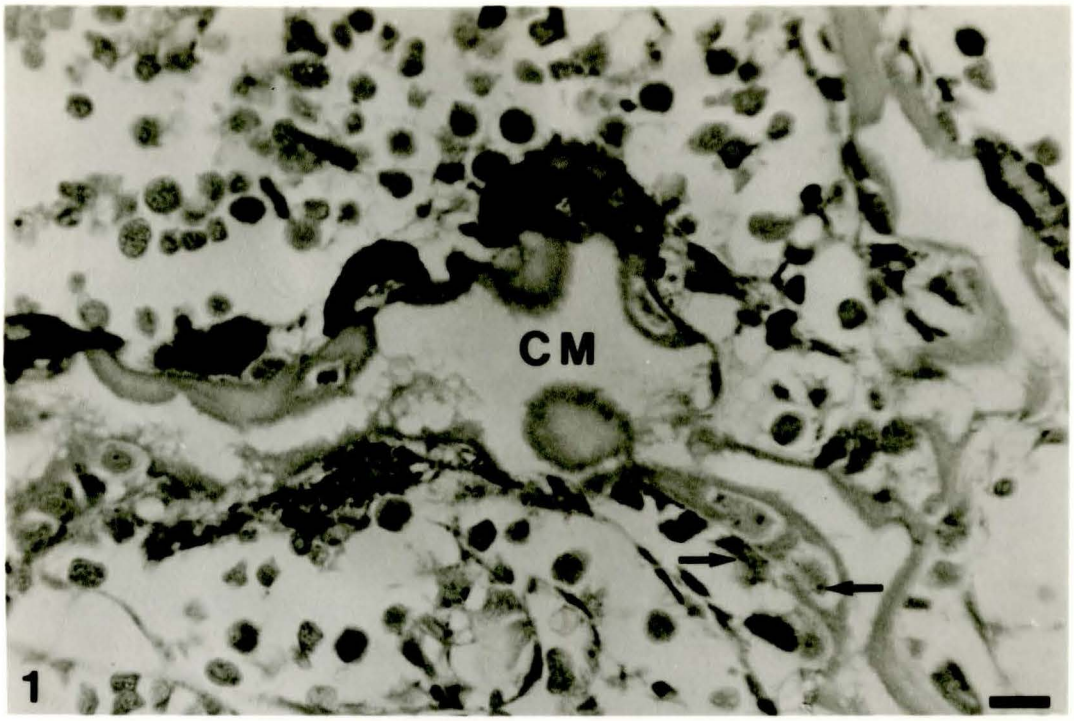
Alkaline phosphatase distribution was limited to perivascular cells in intermediate and deep canals. In cartilage alkaline phosphatase activity has primarily been associated with matrix calcification (Salomon, 1974; Wuthier and Register, 1985); however, alkaline phosphatase has also been localized in fibroblastic cells involved in uncalcified cartilage matrix degradation. In the chick diaphysis, fibroblastic cells were alkaline phosphatase-positive along the uncalcified matrix which was being degraded (Sorrell and Weiss, 1982). Alkaline phosphatase has also been localized in the developing periodontium of the mouse; the presence of the enzyme on the cell membrane in close contact with collagen fibrils and within collagen-containing vesicles suggested a relationship between alkaline phosphatase activity and collagen degradation (Ten Cate, 1972; Deporter and Ten Cate, 1973; Ten Cate and Syrbu, 1974; Hirashita et al., 1985). Additionally, alkaline phosphatase activity has been associated with granules containing collagenase (Robertson et al., 1972). The presence of alkaline phosphatase in perivascular cells of late superficial canals

may reflect a requirement for additional or different enzymes to degrade the modified hypertrophic matrix which has been shown to contain type X collagen (Schmid and Linsenmayer, al., 1985) as well as other unique matrix components (Farnum and Wilsman, 1983; Carrino et al., 1985).

Perivascular cells in cartilage of the mouse were cytochemically characterized with TRAP and alkaline phosphatase. The selective distribution of these TRAP- and alkaline phosphatase-positive cells within the canals can be used to further define the three stages of canal development which were originally defined, in part, by cartilage morphology and cytochemistry and, in part, by the ultrastructural differences in perivascular cells adjacent to the matrix surrounding canals. The presence of these enzymes appears to be related to the cells' function in degrading the differentiating cartilage matrix except for the early superficial canal. If matrix degradation is occurring in the uncalcified matrix prior to chondrocyte hypertrophy, then enzymes other than lysosomal hydrolases or alkaline phosphatase must be employed. Demonstration of other enzymes in the superficial canal will be the topic of future studies.

Figure 1 Section of bone containing osteoclasts stained for TRAP following 4 hrs fixation in glutaraldehyde and decalcification. These heavily stained, multinucleated cells contacted bone and cartilage matrix (CM). Along the surface of bone, osteoblasts and osteocytes were present which contained a few granules of TRAP (arrows). Original magnification = X912; bar = 10 um.

Figure 2 Photomicrographs of synovial membrane surrounding cruciate ligaments located in the intercondylar fossa. a) Mononucleated cells (arrows) present near blood vessels were heavily stained for acid phosphatase following incubation in media containing no tartrate. b) Following incubation in medium containing 50 mM L(+)-tartaric acid, no cells in the synovial membrane contained reaction product. Original magnification = X912; bar = 10 um.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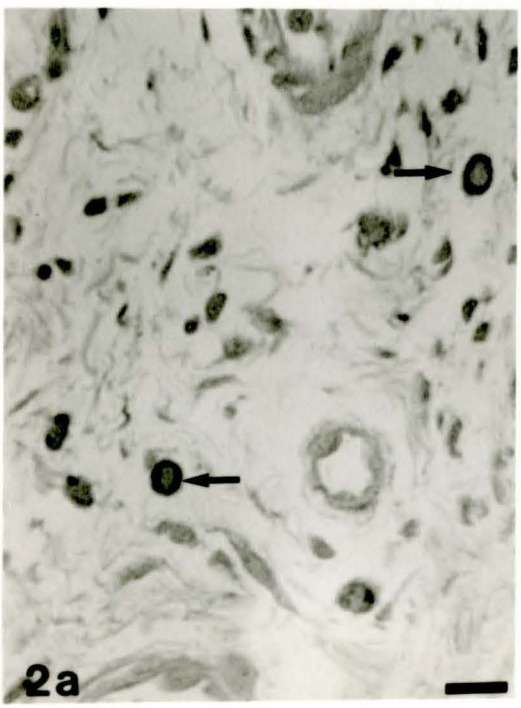
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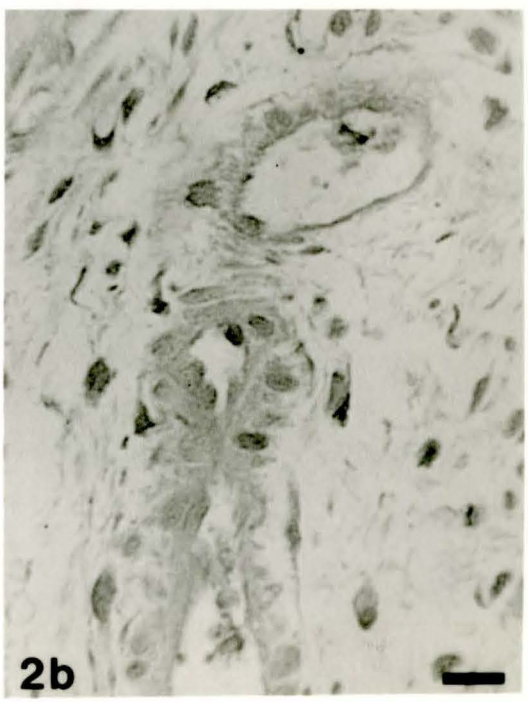
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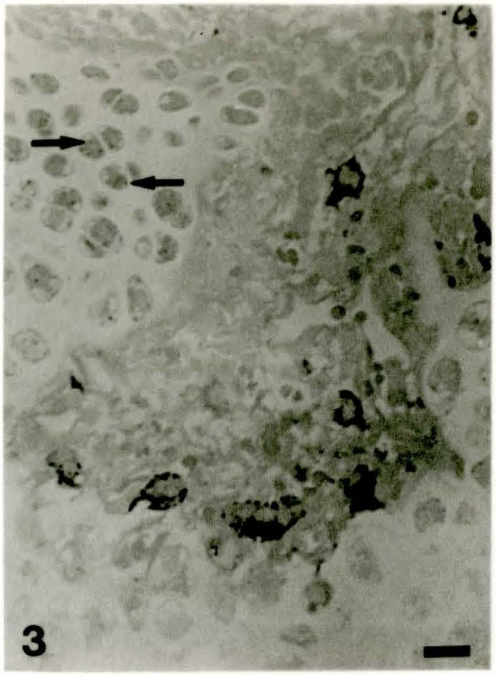
Figure 3 Deep canal containing TRAP-positive chondroclasts adjacent to matrix at the tip of the canal. Chondrocytes near the canal (arrows) were also TRAP-positive although the concentration of granules within the two cell types was different. Original magnification = X365; bar = 20 um.

Figure 4 Higher magnification of two chondroclasts present in Figure 3. The cells form a close contact with the matrix (arrows). Original magnification = X912; bar = 10 um.

Figure 5 Higher magnification of chondrocytes containing a few granules of TRAP (arrows). These chondrocytes were located in cartilage between two deep canals. Original magnification = X912; bar = 10 um.

Figure 6 Chondrocytes in proliferative and hypertrophic zones of the distal femoral growth plate contained a few granules of TRAP reaction product (arrows) similar to the TRAP content of chondrocytes in Figures 3 and 5. Original magnification = X912; bar = 10 um.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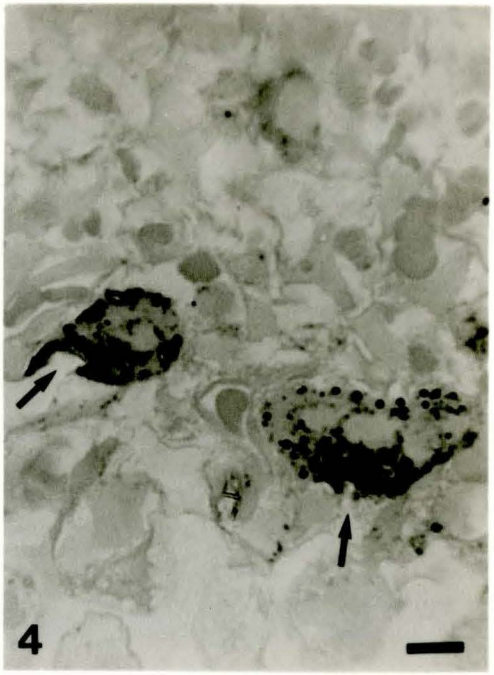
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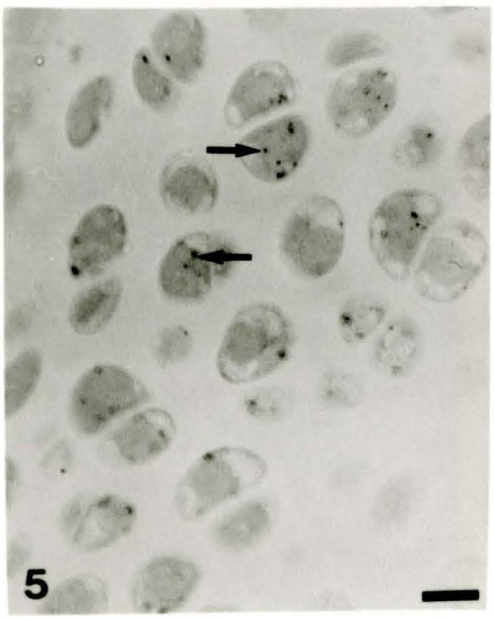
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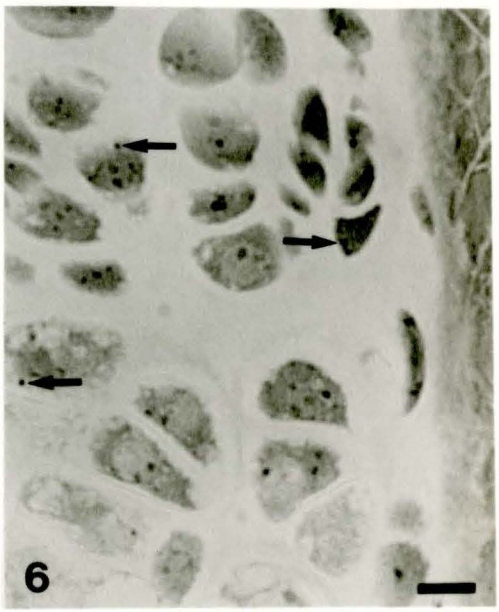
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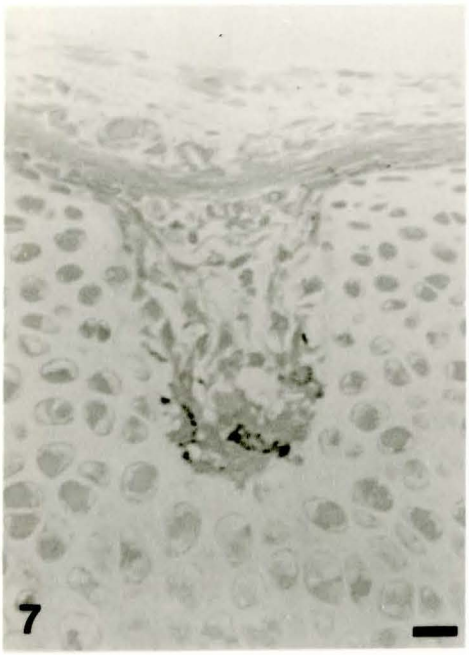
Figure 7 Intermediate canal containing TRAP-positive cells. Chondrocytes surrounding the canal were negative for TRAP. Original magnification = X365; bar = 20 um.

Figure 8 Higher magnification of the tip of the intermediate canal shown in Figure 7. Original magnification = X912; bar = 10 um.

Figure 9 Cell located near the tip of superficial canal is positive for TRAP. Vacuolated cells (arrows) in the canal were negative for both TSAP and TRAP. Original magnification = X912; bar = 10 um.

Figure 10 Perichondrial cell positive for TRAP is representative of a population of TRAP-positive cells scattered throughout the epiphyseal perichondrium. Original magnification = X912; bar = 10 um.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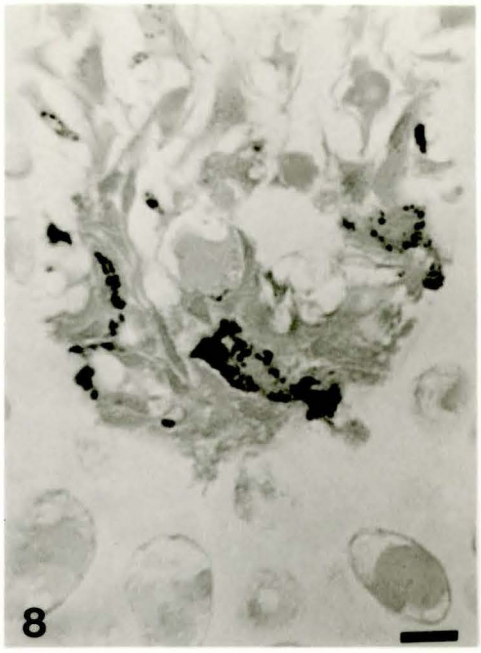
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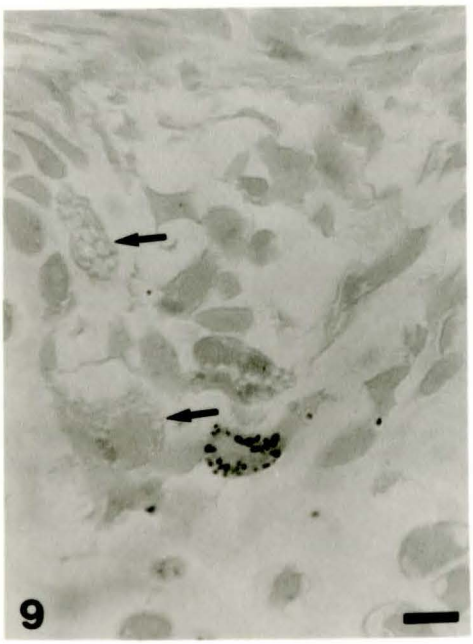
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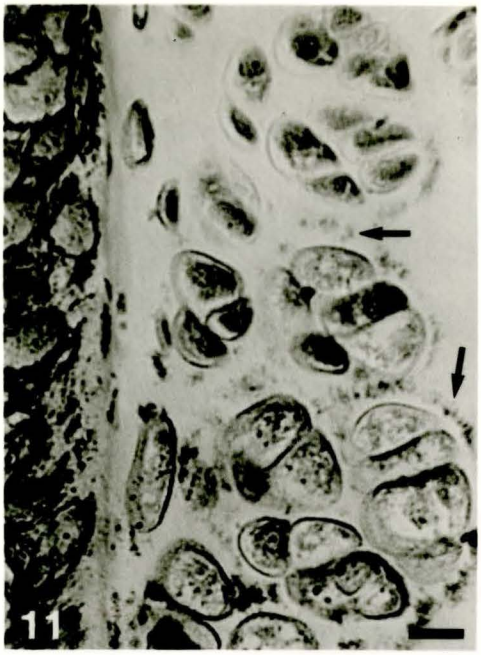
Figure 11 Alkaline phosphatase-positive chondrocytes and perichondrial cells surrounding the growth plate. The reaction product was localized within the cells and along the cell membrane. Extracellular distribution was also noted (arrows). Original magnification = X912; bar = 10 um.

Figure 12 Alkaline phosphatase was absent from perivascular cells in superficial canals. Original magnification = X912; bar = 10 um.

Figure 13 Alkaline phosphatase-positive cells present in a portion of an intermediate canal. Perivascular cells immediately adjacent to the matrix as well as cells at a distance from the matrix were positive for alkaline phosphatase (arrows). Hypertrophic chondrocytes near the canal were also positive (arrowhead). Original magnification = X912; bar = 10 um.

Figure 14 Photomicrograph of the tip of a deep canal containing chondroclasts which were unstained for alkaline phosphatase (arrows). Original magnification = X912; bar = 10 um.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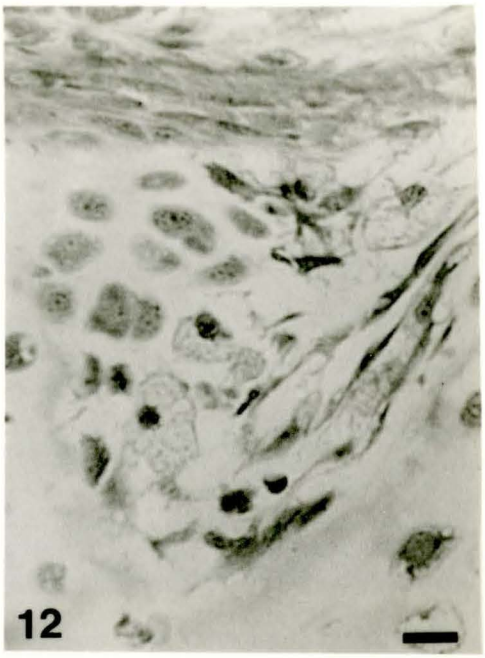
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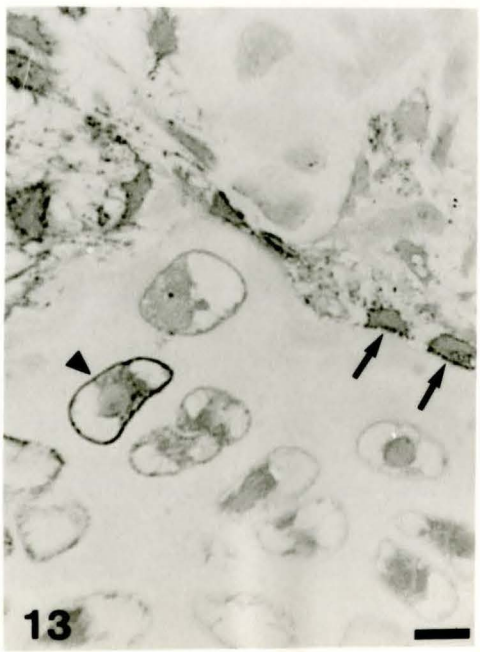
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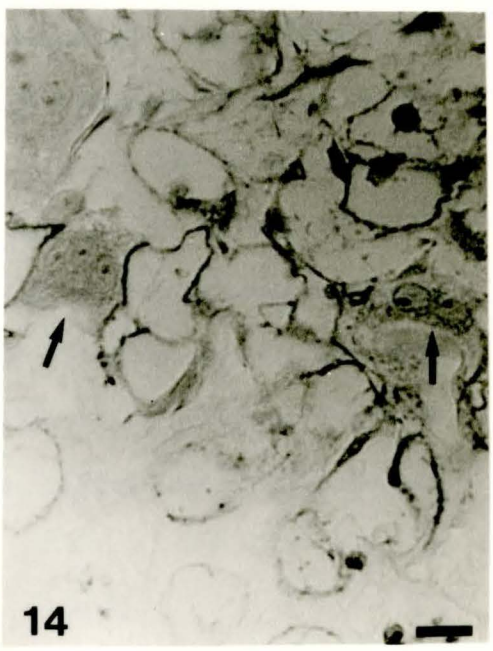
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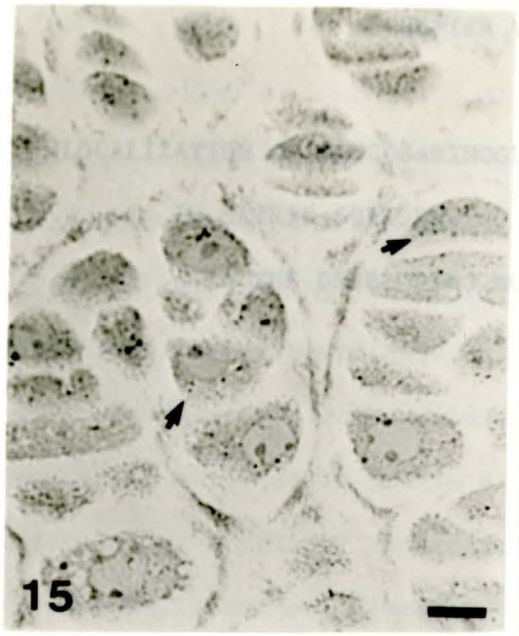
Figure 15 Nonspecific esterase in the growth plate incubated for 1 hr in complete medium at pH 6.0. Cells and matrix were stained with Fast Garnet GBC following incubation, and no counterstain was used. Nonspecific esterase is present as distinct intracellular granules (arrows). Original magnification = X912; bar = 10 um.

Figure 16 A few granules of nonspecific esterase reaction product (arrows) was present in canal cells, perichondrial cells and chondrocytes. No heavily stained cells were present within the entire cartilaginous epiphysis. Original magnification = X912; bar = 10 um.

Figure 17 Macrophages (arrows) in the synovial membrane were heavily stained for nonspecific esterase following incubation for 1 hr at pH 6.0. These cells were found in the same sections used for the localization of nonspecific esterase in perivascular canal cells. Original magnification = X912; bar = 10 um.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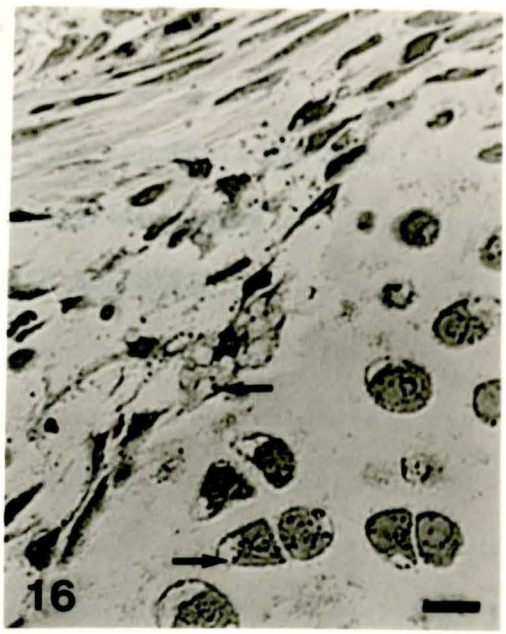
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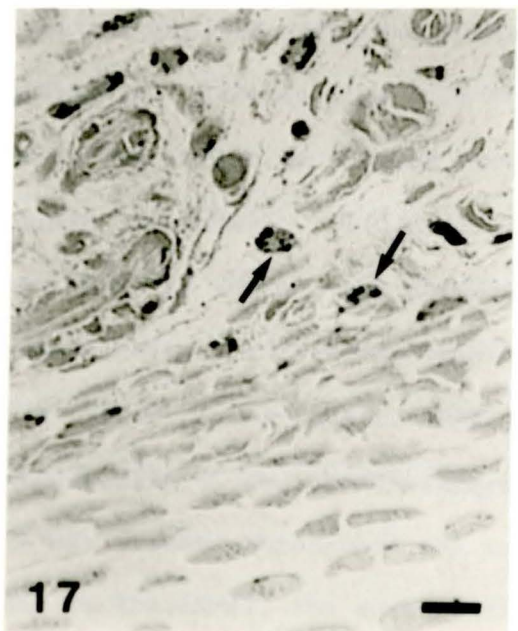
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CHAPTER VII

LOCALIZATION OF GLYCOSAMINOGLYCANS AND PROTEOGLYCANS IN MATRIX SURROUNDING CARTILAGE CANALS IN THE DEVELOPING MOUSE EPIPHYSIS

ABSTRACT

Cartilage canals are thought to form by the degradative activity of perivascular cells, and a weakly and non-metachromatically stained band of matrix surrounding the canal has been presented as evidence for matrix degradation (Lutfi, 1971b; Moss-Salentijn, 1975). This study was undertaken to further characterize matrix immediately surrounding canals compared to matrix at a distance from canals in the distal femoral epiphysis of neonatal mice. The distribution of specific glycosaminoglycans and proteoglycan monomers within the epiphyseal cartilage was studied using immunohistochemical and ultrastructural histochemistry. Results with both techniques indicate that matrix components are absent or reduced within the band of matrix immediately surrounding the canals. With monoclonal antibodies to chondroitin sulfates, keratan sulfate and dermatan sulfate, immunoreactivity for only chondroitin-6- and chondroitin-4-sulfates was consistently detectable within the epiphysis. While chondrocytes were labeled with both chondroitin-6- and chondroitin-4-sulfates, only chondroitin-6-sulfate was present throughout the epiphyseal matrix. Immediately adjacent to the canal, the chondroitin-6-sulfate immunofluorescence in the matrix was diminished or absent. Following fixation and staining of the matrix with ruthenium red, differences in the concentration of proteoglycan monomers were also detectable in matrix immediately adjacent to the canal and in matrix at a distance from the canal. A re-

gion of decreased proteoglycan content correlated to the band of weakly and non-metachromatically stained matrix and to the matrix with reduced or absent immunofluorescence with chondroitin-6-sulfate. This band of altered matrix surrounding the canal may represent the extent of activity of matrix degradaing enzymes which have been released from perivascular cells active in canal formation.

INTRODUCTION

Cartilage canals containing blood vessels and perivascular cells are present in the distal femoral epiphysis of 5-7 day old mice (Cole and Wezeman, 1985). The canals initially form in uncalcified matrix. At the tips of the growing canals chondrocytes hypertrophy, and the matrix calcifies. The matrix immediately surrounding the canals in the mouse and other animals stains weakly and orthochromatically with toluidine blue O or safranin O, in contrast to matrix at a distance from the canals which stains strongly and metachromatically (Lutfi, 1970b; Moss-Salentijn, 1975). This non-metachromatic band extends into the uncalcified matrix surrounding the canal for varying distances up to 20 um and is absent from calcified matrix. A metachromatically stained matrix is a characteristic feature of the matrix secreted by chondrocytes. Detection of metachromasia in matrix is one of the early events in chondrogenesis (Urist, 1983), while loss of metachromasia results when cartilage is cultured with lysosomal enzymes (Poole, 1970b) or with cells capable of releasing matrix-degrading enzymes (Hamerman et al., 1967). A similar band of altered staining has been reported at cartilage matrix-cell interfaces where uncalcified cartilage matrix resorption is occurring, such as the cartilage-marrow interface in the diaphysis and growth plate of chick long bones (Silverstrini et al., 1979; Howlett, 1980; Sorrell and Weiss, 1980; Howlett et al., 1984; Yamaguchi et al., 1985) and the cartilage-pannus

junction of rheumatoid joints (Bromley and Woolley, 1984). The intensity and metachromasia of toluidine blue and safranin has been shown to be proportional to the sulfated GAG or proteoglycan content of the matrix (Godman and Lane, 1964; Poole, 1970a; Rosenberg, 1971), and therefore, the decreased staining surrounding the canal should represent decreased proteoglycan content or more specifically decreased content of sulfated glycosaminoglycan.

Canals are thought to form by the degradative activity of perivascular cells (Andersen and Matthiessen, 1966; Kugler, 1979; Cole and Wezeman, 1985). If the matrix-degrading enzymes are being secreted by perivascular cells lining the margin of the canal, the non-metachromatic band may represent the extracellular extent of the activity of proteoglycan degrading enzymes. Proteoglycan degradation precedes collagen degradation and enhances collagenolysis by exposing fibers previously embedded in the large proteoglycan domains (Harris et al., 1975).

The distribution of glycosaminoglycans (GAG) and proteoglycan in the matrix surrounding the canals was studied using two different techniques. First, epiphyseal cartilage was stained immunohistochemically with monoclonal antibodies for GAGs, including chondroitin, chondroitin-4-sulfate, chondroitin-6-sulfate, keratan sulfate and dermatan sulfate (Caterson et al., 1982; 1983; 1985; Couchman et al., 1984). Secondly, proteoglycan was cytochemically localized following fixation and staining with ruthenium red (Hunziker et al., 1982).

MATERIALS AND METHODS

Immunohistochemistry

Epiphyses were removed from 6-7 day old mice following decapitation. Some epiphyses were left unfixed and frozen for cryostat sectioning, while others were fixed in 0.5% phosphate-buffered paraformaldehyde for 1 hr at 4°C, rinsed overnight in buffer with 7% sucrose, dehydrated in ethanol and xylene and embedded in paraffin. Sections were cut at 6 um.

Some sections were digested with chondroitinase ABC or chondroitinase ACII (Sigma) for 1 hr in a moist chamber at room temperature. Chondroitinase ABC and ACII were diluted 1:10 with 0.1% bovine serum albumin in 0.1M Tris buffer (pH 7.3). Slides were rinsed in phosphate buffered saline (PBS), and primary antibody (1:10 dilution with PBS) was added to the sections. Incubation with primary antibody (See below) was for 1 hr at 37°C. Slides were rinsed for 5 min and incubated for 45 min in secondary antibody (See below) (1:50 dilution with PBS). Sections were then rinsed, cover-slipped with PBS/glycerol (1:1 vol/vol), viewed and photographed with a Leitz Ortholux microscope equipped with phase and epifluorescence optics using specific filters for visualizing fluorescein. Photomicrographs of the same fields were taken with fluorescence and phase optics. The length of exposure for control photomicrographs was timed identically to length of exposure for positive sections.

Primary antibodies were raised in mouse and purified from ascites fluid. Primary antibodies used were 5-D-4, 1-B-5, 3-B-3, and 9-A-2 and have been characterized by Caterson et al (1985) (Kindly provided by Dr. Jon Daniel). Following chondroitinase ABC digestion, 1-B-5 labels chondroitin; and 3-B-3 labels chondroitin-6-sulfate. Antibody 9-A-2 labels chondroitin-4-sulfate following chondroitinase ABC digestion or dermatan sulfate following chondroitinase ACII digestion. Antibody 5-D-4 labels keratan sulfate and requires no enzyme digestion to expose antigenic sites. (Kindly provided by Dr. Jon Daniel). The secondary antibody for keratan sulfate, dermatan sulfate, chondroitin and chondroitin-4-sulfate was FITC-labelled goat anti-mouse IgG (Miles), while secondary antibody for chondroitin-6-sulfate was FITC-labelled anti-mouse IgM (Miles). (Kindly provided by Dr. Jon Daniel).

Control sections 1) incubated in the absence of antibody following enzyme digestion, 2) not exposed to enzyme prior to antibody incubation, or 3) preincubated with mouse serum or hybridoma supernatant.

Electron Microscopy

Mice (Swiss), 5-7 days old, were sacrificed by decapitation. Knees were removed, disarticulated and immediately placed in 0.1 M cacodylate buffer (pH 7.4) at 0-4^{o} C. Distal femoral epiphyses were dissected free of soft tissues and the growth plate. Epiphyses were either processed for routine electron microscopy or fixed and stained with ruthenium red.

Routine electron microscopy: Tissue blocks were fixed in 2.5% glutaraldehyde buffered with 0.1 M cacodylate (pH 7.4) for 24 hrs at $4^{o}C$, rinsed in cacodylate buffer, post-fixed in 1% osmium tetroxide buffered with 0.1 M cacodylate (pH 7.4) for 2 hrs at room temperature, dehydrated in ethanol and propylene oxide and embedded in Epon.

Ruthenium red fixation and staining: Epiphyses were processed according the the technique described by Hunziker et al. (1982) except that ruthenium red was used in place of ruthenium hexamine trichloride. Osmolarity of all solutions was adjusted to 330 $\pm$ 20 mOsm with NaCl and pH was adjusted to 7.4. Ruthenium red $(Ru(NH_3)_4)OHCl \times 2 H_2O$, Mol. Wt. 293.14, Electron Microscopy Sciences) at a concentration of 0.7% was added to the glutaraldehyde fixative, buffer and osmium fixative. Epiphyses were fixed at room temperature for 2 hr in 2% glutaraldehyde in 0.05 M cacodylate buffer, rinsed in 3 changes of 0.05 M cacodylate buffer, postfixed for 2 hr at room temperature in 1% osmium tetroxide buffered with 0.05 M cacodylate, rinsed in 3 changes of buffer and stored overnight at room temperature in buffer. The tissue was dehydrated in ethanol and propylene oxide and embedded in Epon.

Blocks were trimmed, and thick sections were cut until canals were located. Epiphyseal cartilage in the thick sections was uniformly stained with ruthenium red, and no additional staining was necessary to visualize the tissue for light microscopy. Thin sections were cut with a diamond knife and floated onto 0.1 M cacodylate buffer (pH

7.4) to prevent decalcification of the sections (Boothroyd, 1964; Thorogood and Gray, 1975; Bishop and Warshawsky, 1982). The sections were collected onto single-hole grids, stained for 5 min each with 8% uranyl acetate and lead citrate and viewed with a Hitachi H-600 electron microscope at an accelerating voltage of 75 kv.

RESULTS

Epiphyseal cartilage matrix stains intensely with a number of metachromatic stains except for a weakly stained, non-metachromatic band of matrix immediately surrounding cartilage canals. This band of matrix is particularly evident in thick sections cut from blocks of cartilage processed for routine electron microscopy and stained with toluidine blue (Fig. 1). Cells are often present within this band of weakly stained matrix.

Immunofluorescence staining techniques were used to further characterize matrix components within this band of matrix surrounding the canals. Sections of epiphyses containing canals were incubated with monoclonal antibodies to chondroitin-6-sulfate, chondroitin-4-sulfate, chondroitin, keratan sulfate and dermatan sulfate. Of all the antibodies tested only immunoreactivity with monoclonal antibodies for chondroitin-6- and chondroitin-4-sulfates were consistently detectable in the epiphyses. A faint, diffuse fluoresence was present in tissue sections of paraformaldahyde-fixed, paraffin-embedded cartilage. A nonspecific autofluorescence was also noted in unfixed, frozen sections. The faint immunofluorescence was not photographically detectable on film following film exposures timed identically to film exposures used for either the chondroitin-6-sulfate or the chondroitin-4-sulfate immunofluorescence. Immunofluorescence was also undetectable in control sections incubated in the absence of primary anti-

body following enzyme digestion or in control sections which were not exposed to enzymes prior to antibody incubation. There was no difference in immunoreactivity present in the cartilage which had been preincubated with either mouse serum or hybridoma supernatant.

Chondroitin-6-sulfate immunoreactivity (Fig. 2) following incubation with the monoclonal antibody, 3-B-3, was present in the majority of chondrocytes present throughout the epiphysis. Some chondrocytes did not contain any detectable reactivity. In the immunoreactive chondrocytes, chondroitin-6-sulfate was either uniformly distributed throughout the cell or was limited to the periphery of the cell's cytoplasm or in the pericellular matrix. Immunoreactivity within the matrix was observed either as a diffuse or punctate immunofluorescence. In some areas of matrix immediately surrounding the canal, the immunofluorescence was either diminished or absent. There was strong immunoreactivity immediately surrounding canal cells, while immunofluoresence between the canal cells was weaker. None of the canal cells appeared to contain immunoreactive label.

Chondroitin-4-sulfate immunoreactivity, following incubation with monoclonal antibody, 9-A-2, (Fig. 3) was primarly confined to the pericellular matrix surrounding chondrocytes, and little, if any, label was present in the matrix. Canal cells were outlined with immunofluorescence.

In epiphyses processed with ruthenium red (Fig. 4), the cartilage matrix appeared more electron dense than cartilage matrix pro-

cessed for routine electron microscopy. The increased density appeared to be due to the presence of a larger number of electron dense granules within the matrix. These spherical- or polygonal-shaped granules were approximately 10-40 nm in diameter and could often be seen in close association with collagen. The size and distribution of these granules within the cartilage matrix are characteristic for the appearance of proteoglycan monomer following fixation and staining with ruthenium red (Hascall, 1980a; 1980b). Chondrocytes completely filled their lacunae (Fig. 4a), and no artifactual space was evident between the chondrocyte plasmalemma and the lacunar wall. These are criteria used by Hunziker et al. (1982) to indicate improved preservation of matrix components and chondrocyte morphology.

Perivascular cells were often observed in close contact with the matrix surrounding canals (Figs. 4a and b). Adjacent to these perivascular cells, at least three different gradations in the concentration of proteoglycan granules could be observed (Fig. 4c). Proteoglycan granules were present in the highest concentration in matrix farthest from the perivascular cells and were arranged so that the matrix had a honeycomb-like appearance. Proteoglycan granules were distributed along linear structures resembling collagen; individual collagen fibers were not detectable because of the heavy concentration of granules attached to them. An intermediate concentration of proteoglycan granules was present in matrix closer to the canal. In this matrix fewer proteoglycan granules were present, collagen fibers were more

exposed, and the matrix no longer had a honeycomb-like appearance. This band of matrix varied in width from 4 to 8 um. A third gradation in the concentration of proteoglycan granules was sometimes present closest to the perivascular cells and in a narrow band of matrix immediately adjacent to the canal. This matrix contained even fewer proteoglycan granules and collagen fibers than the matrix with an intermediate concentration of proteoglycan granules. The concentration of matrix components was so reduced that the components were separated by large electron lucent spaces. Membrane-bound vacuoles, similar in size to vacuoles present in perivascular cells, were often present within this matrix. Within the cells these vacuoles resembled phagolysosomes. Membrane-bound dense bodies resembling lysosomes were present in these perivascular cells and were concentrated in the cytoplasm nearest the matrix (Fig. 4c).

DISCUSSION

The content of GAG and proteoglycan observed in the matrix immediately surrounding cartilage canals was different from the content of GAG and proteoglycan in the matrix at a distance from the canal. Immunohistochemical staining for chondroitin-6-sulfate, the primary GAG detectable in neonatal mouse cartilage, was either decreased or absent in matrix surrounding the canal. Following fixation and staining of the matrix with ruthenium red, the matrix immediately surrounding the canal contained a reduced amount of proteoglycan granules. These results are consistent with the staining pattern observed with metachromatic dyes.

The monoclonal antibodies used in this study have been extensively characterized (Caterson et al., 1985). The antibodies were produced by hybridoma cell lines and raised against determinants present in cartilage proteoglycans, specifically, oligosaccharide stubs attached to proteoglycan core protein after chondroitinase digestion. Chondroitinase digestion results in removal of delta-unsaturated disaccharide units containing either 4-sulfated, 6-sulfated or unsulfated N-acetylgalactosamine moieties (Saito et al., 1968). Following digestion the remaining proteoglycan protein core contains short oligosaccharide chains consisting of linkage sugars covalently bound to protein and one or two disaccharide units adjacent to a terminal nonreducing delta-unsaturated hexuronic acid residue.

The selective distribution of monoclonal antibodies 5-D-4, 9-A-2, 1-B-5 and 3-B-3 have been reported in cartilage and in a number of other tissues. The monoclonal antibody, 5-D-4, recognizes keratan sulfate, while 9-A-2, 1-B-5 and 3-B-3 can be used to distinguish between different sites of sulfation on galactosamine moieties of repeating disaccharide units. The monoclonal antibody, 5-D-4, is an IgG, raised against human articular cartilage; this antibody recognizes a determinant in keratan sulfate present in both proteoglycan aggregate and monomer isolated from cartilages from a number of species (human, monkey, cow, sheep, chicken and shark) (Caterson et al., 1983). This antibody has been used to detect proteoglycan synthesis in cultured chick chondrocytes (Vertel et al., 1985). Antibody 9-A-2 is an IgG, raised against bovine nasal cartilage proteoglycan-protein core generated by chondroitinase digestion of chondroitin sulfate-proteoglycan. This antibody recognizes chondroitin-4-sulfated proteoglycan and stains cartilage and the tunica media and adventitia of the aorta (Couchman et al., 1984). Chondroitin-6-sulfate is recognized by antibody 3-B-3 which is an IgM produced against the chondroitinase ABC-digested proteoglycan-core protein from Swarm rat chondrosarcoma and has been used to localized chondroitin-6-sulfate in cartilage, in the tunica intima and media of aorta and in the basement membrane of kidney, blood vessel endothelium, muscle and peripheral nerve (Couchman et al., 1984); this antibody also has slight activity toward unsulfated chondroitin. Antibody 1-B-5 was produced against proteoglycan-

core protein from Swarm rat chondrosarcoma and is an IgG which recognizes unsulfated chondroitin sulfate. The distribution of this GAG appears to be virtually restricted to cartilage (Caterson et al., 1985).

Within the neonatal mouse epiphysis, immunoreactivity was detectable with only two of the monoclonal antibodies, 3-B-3 and 9-A-2, representing the distribution of chondroitin-6- and chondroitin-4-sulfates respectively. The presence of only chondroitin-6- and chondroitin-4-sulfates within the mouse epiphysis may be due to the age of this cartilage. Age-related differences in the distribution of GAGS have been noted in a number of animals. Chondroitin-6- and chondroitin-4-sulfates have both been shown to be present in articular and growth plate cartilages taken from embryonic, young and adult animals, but the relative proportions of the two GAGs differed with respect to age and distribution in the two cartilages (Vasan and Lash, 1978; Mourao and Dietrich, 1979). Chondroitin-6-sulfate was present in relatively higher amounts in the articular surfaces whereas chondroitin-4-sulfate was the main chondroitin present in growth cartilages. Based on these results, Mourao and Dietrich (1979) suggested that chondroitin-4-sulfate seems to be related to the ossification process, whereas chondroitin-6-sulfate appeared to be related to the maintenance of articular surfaces. Chondroitin-6-sulfate is present in relatively higher concentrations in embryonic and young animals, as the tissue increases in age proportionally more chondroitin-4-sulfate

is present. While the relative concentrations of the two GAGS cannot be determined using immunohistochemical techniques, the distribution of both chondroitin-6- and chondroitin-4-sulfates appears to be uniform within the entire epiphysis; no differences in distribution was noted between the articular surface and the center of the epiphysis which is destined for calcification. In the 5-7 day old mouse the epiphyseal center has not yet formed and the distinction between articular cartilage and growth plate cartilage is not as evident as it is in epiphyseal cartilages which contain the center; perhaps at this age differences in the distribution of the two chondroitin-sulfates have not yet developed. Keratan sulfate synthesis begins during bovine and chick fetal development and increases proportionally with age (Vasan and Lash, 1978; Thonar and Sweet, 1981). The presence of keratan sulfate is, in part, responsible for increasing cartilage stiffness and resistance to deformation necessary when the animal begins weigh-bearing on the articular cartilage. The lack of an immunohistochemically detectable amount of keratan sulfate in the neonatal mouse may reflect the lack of weight-bearing on the articular surfaces of the knee at this age.

The reduced or absent matrix staining with chondroitin-6-sulfate adjacent to the canal is consistent with the staining pattern of the matrix with metachromatic dyes and with ruthenium red. Ruthenium red is a fixative and stain for proteoglycan (Luft, 1971; Thyberg et al., 1973); proteoglycan monomers are present in the matrix in the form of

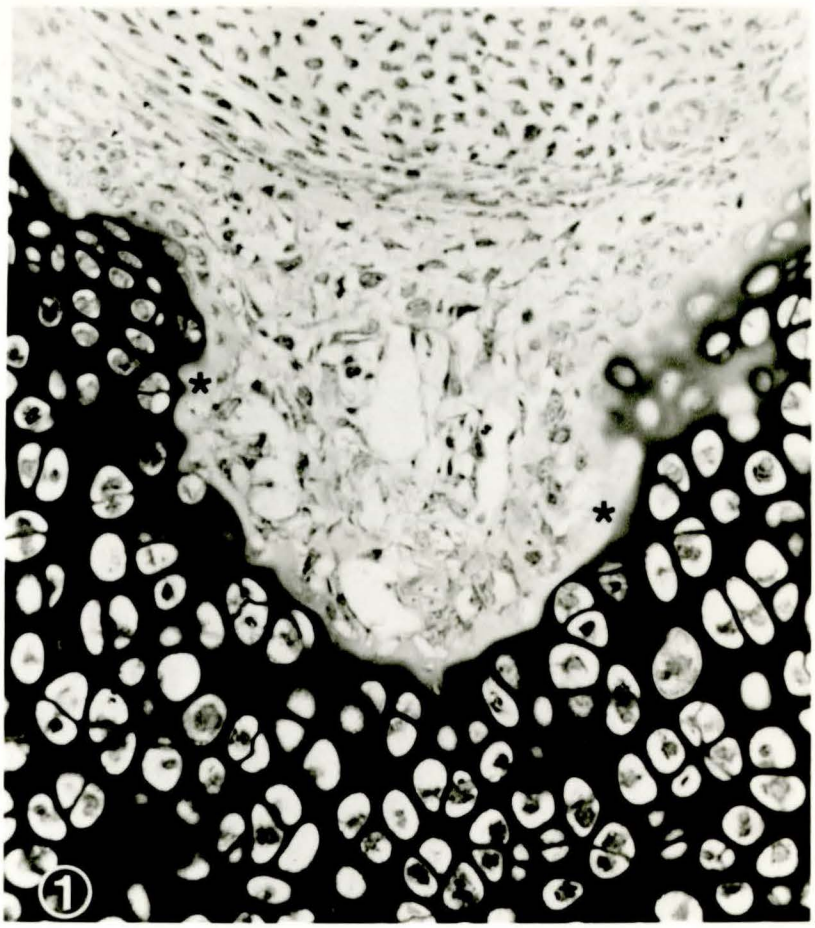
granules (Hascall, 1980a; 1980b). Hunziker et al. (1982; 1983) reported a technique using ruthenium hexamine trichloride in place of ruthenium red which also included adjustment of osmolarity in all solutions to 330 mOsm, room temperature fixation and reduced buffer concentration to achieve improved chondrocyte preservation. Ruthenium hexamine trichloride has a higher density positive charge than ruthenium red and was expected to penetrate the negatively charged matrix better. Penetration of ruthenium red into cartilage had been reported to be limited to the periphery of the tissue (Luft, 1971; Thyberg et al., 1973). The procedure employed in our laboratory included all of reported technique modifications (Hunziker et al., 1982) except that ruthenium red was used in place of ruthenium hexamine trichloride. Sections of mouse epiphyseal cartilage fixed with ruthenium red were uniformly stained indicating good penetration, and preservation of matrix components and chondrocyte morphology with ruthenium red was similar to that reported with ruthenium hexamine trichloride. Luft (1971) reported that most commercially prepared ruthenium red contains varying amounts of contaminants, such as ruthenium brown, ruthenium violet and ruthenium hexamine trichloride. The possibility that the ruthenium red used in this experiment contained a high concentration of ruthenium hexamine trichloride as a contaminant cannot be ruled out, nor can the possibility that the other technical modifications, including osmolarity adjustment and room temperature fixation might have improved the penetration of a more pure ruthenium red prepara-

tion.

Differences in the relative concentrations of proteoglycan granules in the matrix surrounding the canal and in matrix at a distance from the canal may reflect the extent of the extracellular activity of enzymes released from perivascular cells. Perivascular cells closely associated with the matrix surrounding the canal contained dense bodies resembling lysosomes. The dense bodies appeared concentrated in the cytoplasm nearest the matrix. Vacuoles resembling phagolysosomes were also present in the cytoplasm near the lysosomes. The presence of lysosomes and phagososomes is characteristic of degradative cells and suggests that these cells are active in degrading the cartilage matrix surrounding the canals. If lysosomal enzymes are being released by these perivascular cells, the enzymes may be active within the matrix only to a depth indicated by the decreased proteoglycan content. Proteoglycan degradation is thought to precede collagen degradation; the removal of the large proteoglycan allows collagen fibers to be exposed to degradative enzymes (Harris et al., 1975; Mullins and Rohrlich, 1983).

This study provides evidence that chondroitin-6-sulfate proteoglycan and proteoglycan monomers are present in reduced amounts in the matrix surrounding cartilage canals. Combined with other ultrastructural, cytochemical and morphometric evidence, this study adds support to the hypothesis of canal formation by the degradative activity of perivascular cells.

Figure 1 Photomicrograph of cartilage canal present within cartilage which has been processed for routine electron microscopy and stained with toluidine blue. The matrix immediately surrounding the canal is weakly and non-metachromatically stained (astericks). Original magnification = X520.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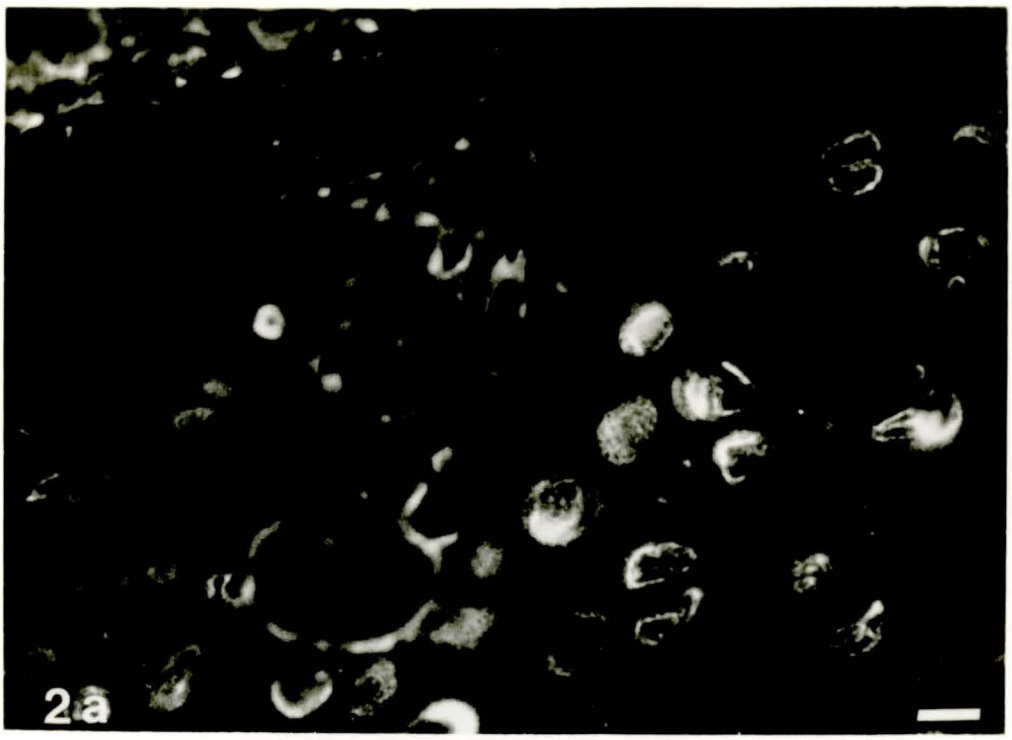
①

Figure 2a Immunofluorescent localization of chondroitin-6-sulfate with monoclonal antibody 3-B-3 in a section of epiphysis containing a cartilage canal. Immunofluorescence was present following digestion of the section with chondroitinase ABC and incubation with hybridoma supernatant. Original magnification = X614; bar = 15 um.

Figure 2b Phase contrast photomicrograph of the same field shown in Fig. 2a. Original magnification = X614; bar = 15 um.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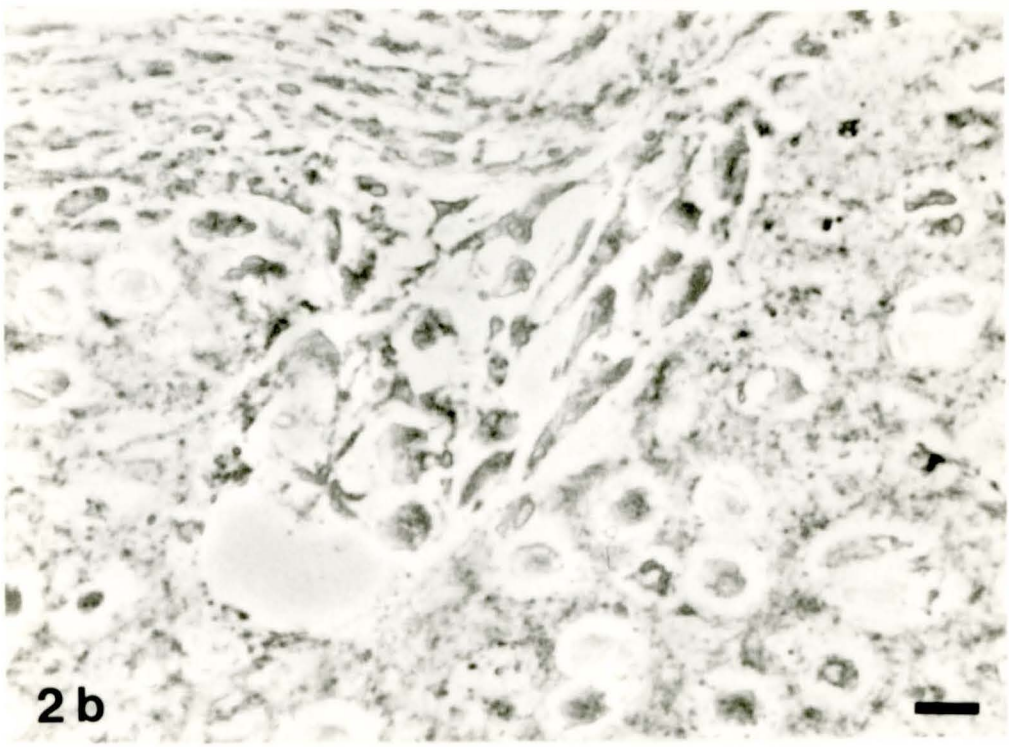
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Figure 2c Control section for antibody 3-B-3 which was not chondroitinase ABC-digested prior to incubation with the primary antibody. The film exposure for this photomicrograph was timed identically to the exposure used in Fig. 2a. Original magnification = X614; bar = 15 um.

Figure 2d Phase contrast photomicrograph of the same field shown in Fig. 2c. Original magnification = X614; bar = 15 um.


2 c

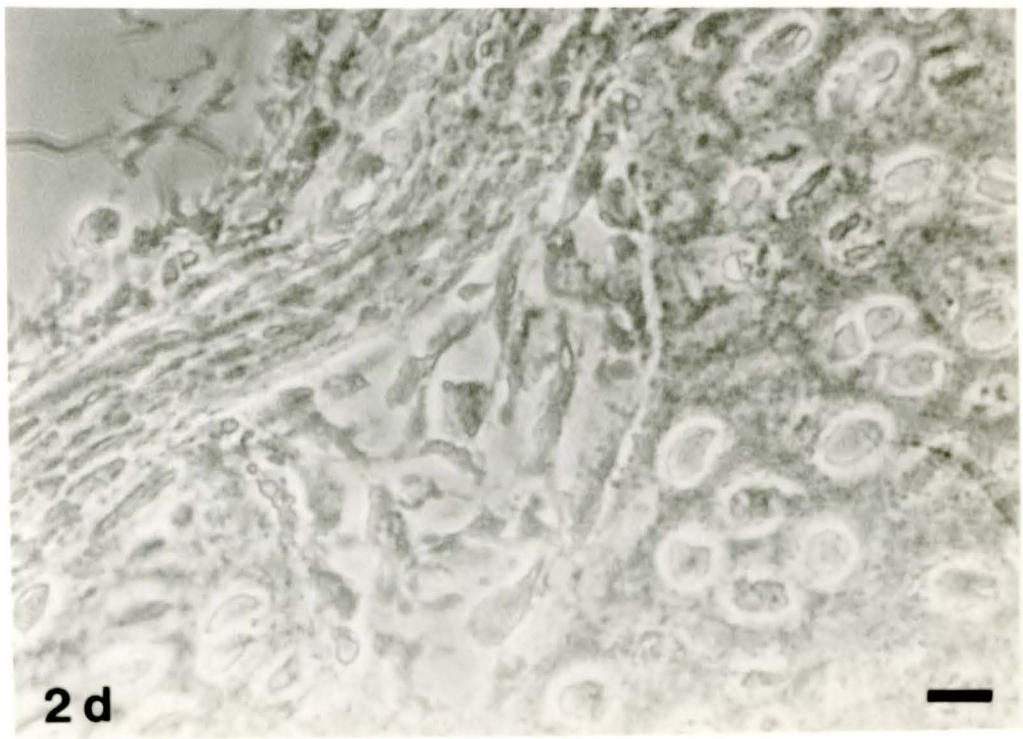

2 d

Figure 3a Immunofluorescent localization of chondroitin-4-sulfate with monoclonal antibody 9-A-2 in a section of epiphysis containing a cartilage canal. Immunofluorescence was present following digestion of the section with chondroitinase ABC and incubation with hybridoma supernatant. Original magnification = X614; bar = 15 um.

Figure 3b Phase contrast photomicrograph of the same field shown in Fig. 3a. Original magnification = X614; bar = 15 um.


3 a

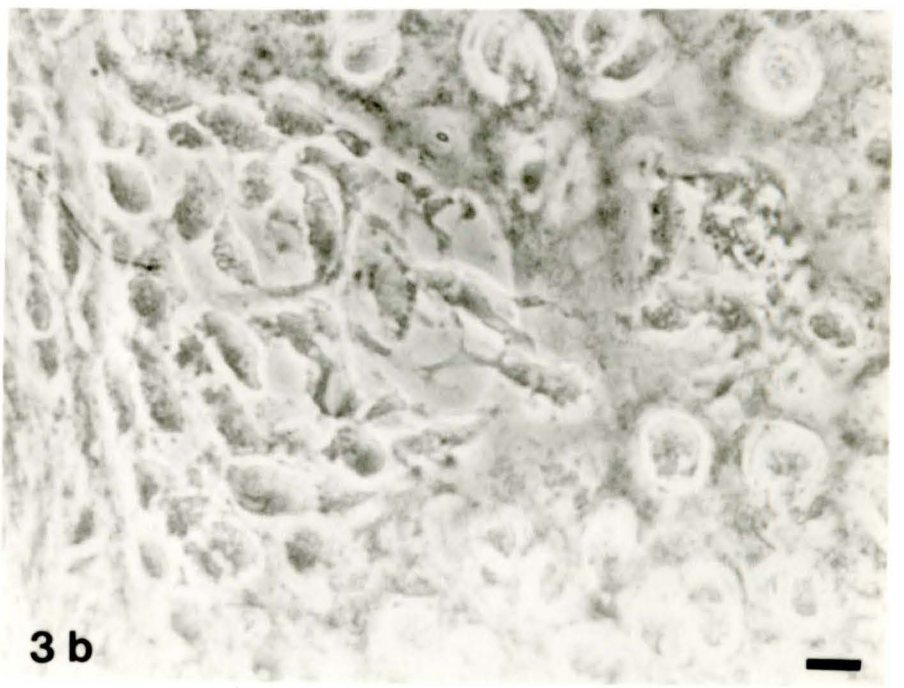
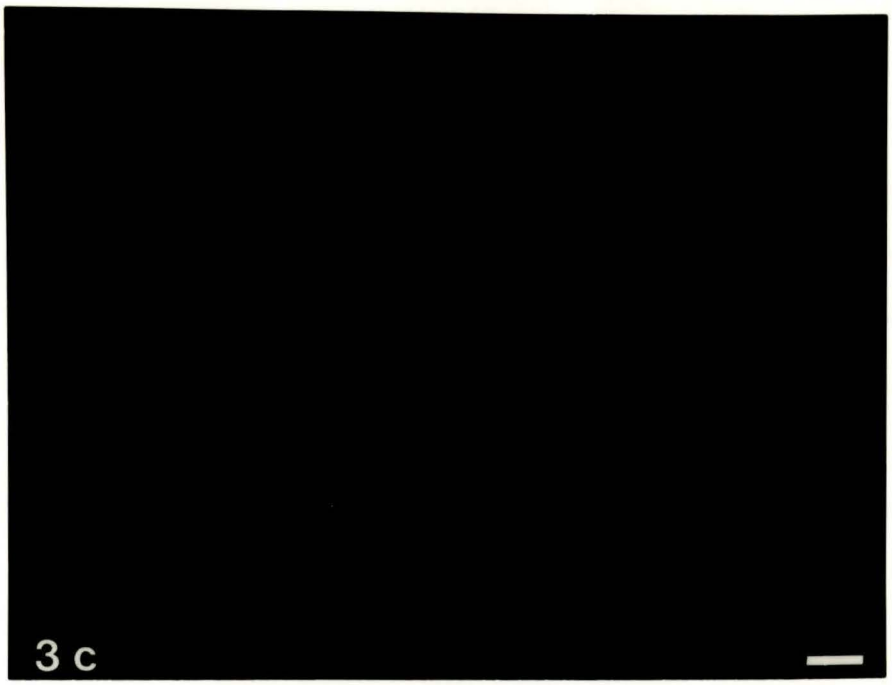

3 b

Figure 3c Control section for antibody 9-A-2 which was not exposed to chondroitinase ABC digestion prior to incubation with the primary antibody. The film exposure for this photomicrograph was timed identically to the exposure used in Fig. 3a. Original magnification = X614; bar = 15 um.

Figure 3d Phase contrast photomicrograph of the same field shown in Fig. 3c. Original magnification = X614; bar = 15 um.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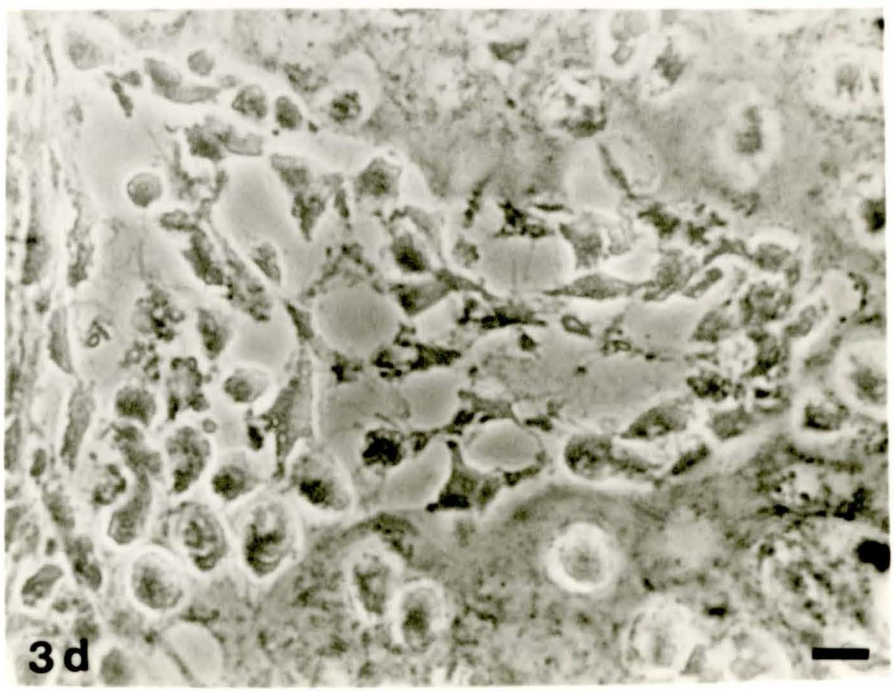
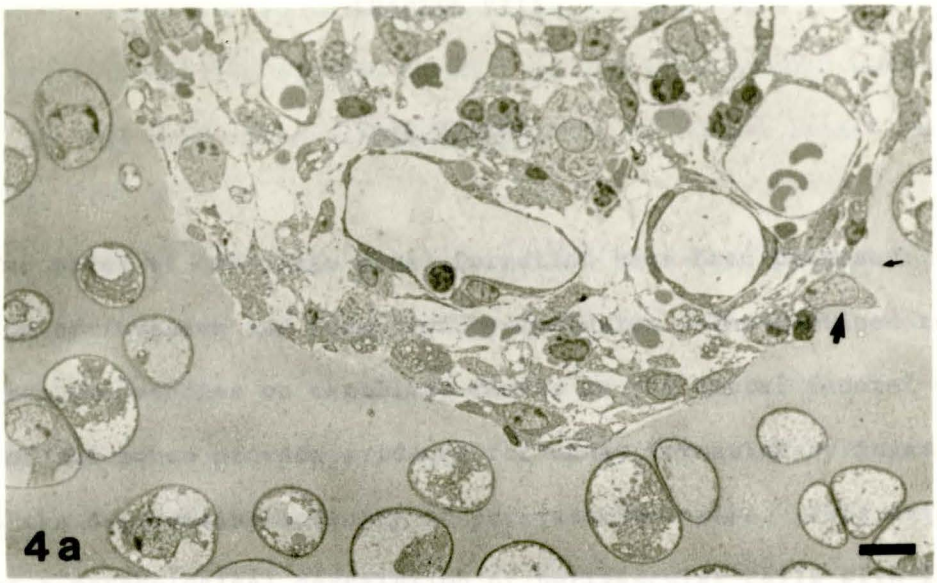

3d

Figure 4 Electron photomicrographs of cartilage canal and matrix following fixation and staining with ruthenium red. (a) Low power photomicrograph showing the interface between the canal and cartilage matrix. Hypertrophic chondrocytes within the cartilage completely fill their lacunae and do not appear to have collapsed as do hypertrophic chondrocytes following routine processing for electron microscopy. A number of perivascular cells are in close contact with matrix; some cells appear to be present within indentations within the matrix (large arrow). Differences in electron densities of the matrix are evident even at this low magnification (small arrow). Original magnification = X960; bar = 10 um. (b) Higher magnification of the pervascular cell seen in (a). A portion of this cell appears to be embedded in the matrix forming a deep indentation. A close association between the surface of the cell and the matrix is apparent near the canal lumen. The surface of the cell embedded in the matrix does not closely contact matrix, and matrix components appear to have been removed. Original magnification = X3600; bar = 0.5 um. (c) Photomicrograph taken from section adjacent to the one shown in Figs. 4a and 4b. Membrane-bound dense bodies resembling lysosomes (small arrows) are present within the cytoplasm of the cell near the matrix. Arrows (large) indicate a transition in the concentration of proteoglycan granules. The heaviest concentration of proteoglycan granules is seen at the top of the photomi-

crograph. In matrix between the arrows and the cell, an area of intermediate concentration of proteoglycan granules is apparent, while matrix at the bottom of the photomicrograph demonstrates the area of least proteoglycan granule concentration. Original magnification = X8400; bar = 1.5 um.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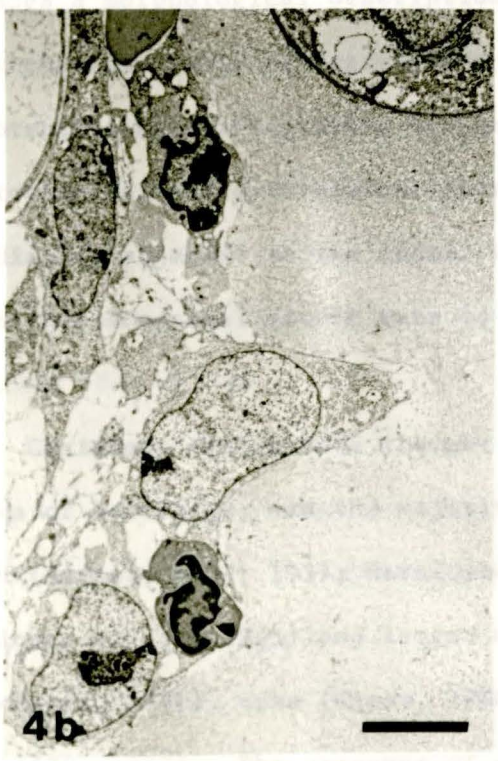
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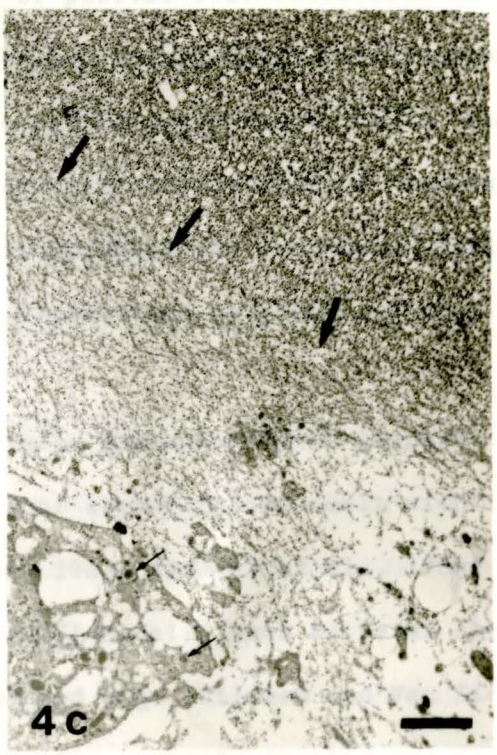

4c

CHAPTER VIII

DISCUSSION

Two modes of cartilage canal formation have been proposed: formation by invasion and formation by inclusion. The combined results from the studies on cartilage canals in the distal femoral epiphysis of the mouse provide evidence for canal formation by invasion through the degradative activity of perivascular cells. This evidence includes a morphological description of perivascular cells with features characteristic of degradative cells, cytochemical localization of potential matrix-degrading enzymes within perivascular cells, immunocytochemical and cytochemical characterization of altered matrix immediately adjacent to the canal, and morphometric data which indicates that the canal growth rate is greater than epiphyseal growth rate.

Cartilage canals were thought to be present only in larger blocks of cartilage, and the majority of these studies dealt with human (Haines, 1933; 1937; Haraldsson, 1962; Agrawal et al., 1984; Rodriquez et al., 1985) and larger animal models, such as fetal sheep (Stockwell, 1971), cows (Knese, 1980) and dogs (Wilsman and Van Sickle, 1970; 1972). The presence of cartilage canals in mice was first reported based on results from this study. The mouse was chosen as the animal model for this study because the mouse is a relatively

inexpensive laboratory animal and litter sizes are large (10-20 pups per litter). These two factors permit the use of a greater number of samples for the study than would be practical if larger animal models were used.

In the distal femoral epiphysis of the mouse, canal development appears to be related not only to the age of the mice but also to the weight of the animal. No canals were observed in pups less than 5 days old or in 5 day old pups from litters with an average weight less than 2.5 gm. Canals were initially present in 5 day old mice weighing approximately 3.0 gm. In animals between 5 and 7 days of age and weighing 3 to 5 gm, canals increased in number and in both length and width and were distributed along the condyles and intercondylar fossae often near the attachment of ligaments similar to the distribution described in humans (Haines, 1933; Gardner and Gray, 1970). Epiphyseal center development was first noted in larger 7 day old mice weighing more than 5.0 gm.

At the time of initial canal formation the epiphysis was entirely cartilaginous. Neither chondrocyte hypertrophy nor matrix calcification was evident. As canals increased in size, hypertrophic chondrocytes were first observed near the tips of canals. With further increases in canal size, matrix adjacent to the terminal portions of the canal became calcified. Changes in chondrocyte morphology and matrix calcification were used to define three stages of canal development (Fig. 1): superficial, intermediate and deep. Superficial

canals were found in uncalcified matrix containing nonhypertrophic chondrocytes and represent the earliest developmental stage. These canals were the only type of canal present in smaller 5 day old pups. The second stage was the intermediate canal which terminated in uncalcified matrix containing hypertrophic chondrocytes. The last developmental stage, the deep canal, terminated in calcified matrix and was first evident in pups 6 to 7 day old. In these larger pups the number of canals had increased, and all developmental stages could be found within a single epiphysis.

The close association between the canals and initial chondrocyte hypertrophy followed by matrix calcification confirms the results reported in only a few other studies (Lutfi, 1970b; Wilsman and Van Sickle, 1970; Agrawal et al., 1984). In the majority of earlier studies a close relationship between the canal and foci of calcification had not been observed, and matrix calcification was thought to begin in the avascular areas between terminal portions of canals (Haines, 1933; Haraldsson, 1962). Canal development, chondrocyte hypertrophy and matrix calcification begin in the lateral condyle of the distal femoral epiphysis prior to their development in the medial condyle. This lateral to medial sequence of development corresponds to the pattern of epiphyseal center development. The center initially forms as two separate centers (Johnson, 1933) which eventually fuse to form the final large single center of ossification.

An ultrastructural study was undertaken to morphologically char-

acterize perivascular cells located immediately adjacent to uncalcified and calcified matrix. Since perivascular cells were hypothesized to be the matrix degrading cells within the canals, the study primarily focused on those cells which were located adjacent to the matrix surrounding the canal. At each stage of canal development, morphologically distinctive cells with characteristics of degradative cells were present adjacent to the matrix (Fig. 2). In the superficial canal, mononucleated cells with an extensive rough endoplasmic reticulum were the predominate cell type found adjacent to the matrix. These cells did not contain an abundance of dense bodies resembling lysosomes nor did they form an intimate contact with the matrix. The second cell type encountered within the superficial canal was a mononucleated cell containing numerous large vacuoles. This cell was occasionally found intimately in contact with collagen fibers of the matrix, and SLS collagen was present within some vacuoles. In the intermediate canal, an enlarged cell which contained an extensive rough endoplasmic reticulum and was occasionally multinucleated was found in intimate contact with the matrix. Cellular projections from the basal surface of the cell in contact with the matrix extended into the matrix and electron dense material was present between the cells and the matrix. The cytoplasm of this cell closest to the matrix contained numerous lysosomes and phagolysosomes. In the deep canal, multinucleated chondroclasts were present which contacted the calcified matrix with ruffled borders and clear zones.

Of the three possible candidates for degradative cells within the canal - perivascular cells, chondrocytes and endothelial cells - only the endothelial cells can be eliminated with any degree of certainty. In the superficial canal, endothelial cells rarely contact the matrix; in the intermediate and deep canal endothelial cells contact the matrix often, but in the area of contact the endothelial cell is attenuated and contains fenestrae covered by diaphragms. The morphological appearance of the endothelium suggests a nutritive function rather than a degradative function. The morphological evidence would support either perivascular cells or chondrocytes as degradative cells at some stages of canal development. Perivascular cells with characteristics of macrophages and chondroclasts have been suggested as the degradative cells in other studies (Andersen and Matthiessen, 1966; Kugler et al., 1979). In the superficial canals of the mouse, cells resembling the vacuolated macrophage appeared to be a degradative cell but was only occasionally adjacent to the matrix. This cell appeared to be involved in the degradation of the collagenous component of the matrix. In deep canals, chondroclasts appeared to be the primary degradative cells and was in contact with the calcified spicules of cartilage matrix. The identity of the cell in the intermediate canal is unknown, but this perivascular cell has the morphological characteristics of a degradative cell. The cell in both the intermediate and superficial canal with extensive rough endoplasmic reticulum had features characteristic of chondrocytes except that the cell was not

surrounded by cartilage matrix. There were often cells close to the margin of the canal which appeared to be chondrocytes being released from the canal. The possibility cannot be eliminated at this point that chondrocytes are being stimulated to produce matrix degrading enzymes which enable them to be released from their lacunae perhaps from some factors released by the vacuolated cell. The possibility also exists that the released chondrocyte and the vacuolated cell are the same cell and that the vacuolation develops as the released chondrocyte degrades cartilage matrix.

Morphological differences between the cells adjacent to the matrix may be attributed to differences in the composition of the matrix surrounding the canal at each developmental stage. As the matrix differentiates from an uncalcified to a calcified matrix, a number of changes have been reported to occur in the components of the matrix. Not only is a different type of collagen present (Kielty et al., 1985; Schmid and Linsenmayer, 1985) but also different proteoglycans are present (Carrino et al., 1985) in hypertrophic matrix. Content of phospholipids, calcium, phosphates and enzymes also change prior to matrix calcification (Ali, 1983). The morphological differences in cells adjacent to the matrix surrounding the canal may reflect the requirement for different cellular mechanisms in order to degrade matrix. The appearance of the chondroclast only when matrix is calcified appears to reflect the ability of that cell to degrade the calcified matrix with the ruffled border which has been shown to

contain epitopes to lysosomal membranes (Baron et al., 1985); additionally, the resorption zone formed by the osteoclast has been shown to contain lysosomal enzymes, be acidified to permit optimal activity of the lysosomal enzymes and to contain both collagenous and proteoglycan matrix fragments. The close association between the plasmalemmata of perivascular cells and the matrix in the intermediate canal is more characteristic of cells which are degrading an uncalcified matrix. A similar association has been reported between cells and matrix in the chick diaphysis where uncalcified matrix is being resorbed (Sorrell and Weiss, 1980). In the superficial canal few cells were observed in close association with the matrix. Although the vacuolated cells were occasionally observed in close contact with collagenous components of the matrix and their vacuoles contained SLS collagen, these cells were never observed sending cytoplasmic projections into the matrix. This may reflect the composition of nonhypertrophic matrix which contains inhibitors to cellular invasion. Kuettner and Pauli (1983) reported that invasive cells such as osteosarcoma or endothelial cells did not form a close assiciation with nonhypertrophic matrix until the matrix had been extracted with guanidine-HCl to remove the inhibitors.

The method of matrix degradation in the uncalcified, nonhypertrophic matrix is unclear. Not only do the perivascular cells not form an intimate contact with the matrix but they also do not appear to contain lysosomal enzymes. Perivascular cells in superficial ca-

nals which form prior to chondrocyte hypertrophy did not contain any cytochemically detectable TSAP or TRAP. If these cells are degrading the matrix, then enzymes other than acid hydrolases are being synthesized. A number of neutral proteinases have been described which are capable of degrading both the proteoglycan and the collagenous components of the cartilage matrix (Hauser and Vaes, 1978; Morales and Kuettner, 1982; Golds et al., 1983). These cells may be producing enzymes active at a neutral pH; these enzymes would not have been detectable with either the acid or alkaline phosphatase cytochemistry used in this study.

Lysosomal enzymes do appear to be involved in the degradation of matrix following chondrocyte hypertrophy within the epiphysis. TRAP was present within perivascular cells found in superficial canals which develop after chondrocyte hypertrophy, in intermediate canals and in deep canals. Perivascular cells within the intermediate canals also contained alkaline phosphatase. Although alkaline phosphatase has primarily been associated with matrix calcification, it has also been associated with matrix degradation in noncalcifying systems (Deporter and Ten Cate, 1973; Yajima, 1976; Sorrell and Weiss, 1982).

Alterations in matrix adjacent to the canal would also appear to support an extracellular release of enzymes. Both chondroitin-6-sulfate and proteoglycan monomers are present in a reduced amount or absent from a band of matrix surrounding the canal. This band corresponds to the weakly and orthochromatically stained band observed with

light microscopy following staining with toluidine blue. Poole (1970a) showed that the metachromasia resulting from toluidine blue staining was proportional to the amount of hexuronic acid within the cartilage matrix; he also found that the ability to stain the matrix was lost when cartilage possessed approximately 42% or less of its normal hexuronic acid content. Within the band of matrix observed following matrix fixation and staining with ruthenium red, proteoglycan granules are still present, but their concentration may be reduced sufficiently to result in lack of metachromasia.

An alternative technique which has been used to study alterations in matrix components is light and electron microscopic autoradiography. Radioisotopes, ^{3}H-proline and $^{35}SO_4$, are incorporated into collagen and proteoglycan respectively and were originally used to study cartilage matrix synthesis (Godman and Lane, 1964). Within a few minutes following injection of the isotope into an animal, its presence can be detected autoradiographically within the rough endoplasmic reticulum, Golgi and secretory vesicles of chondrocytes. After a longer time period the radiolabel is present within the matrix. Sorrell and Weiss (1980) and Yamaguchi et al. (1985) both used autoradiographic techniques for a kinetic study to demonstrate that matrix was not being synthesized along the palely stained band of matrix in the chick diaphysis; however, with this technique they were unable to provide positive evidence that matrix was being degraded. While autoradiography is useful in demonstrating matrix synthesis

because of the short time between introduction of the isotope and its incorporation into the matrix, it has not been a useful technique to demonstrate degradation. The difficulty in using this technique in degradative systems has been the problem of judging the time at which the matrix is originally synthesized since the period between synthesis and degradation may be weeks or months. Additionally, during this period matrix turnover normally occurs and the radiolabel may be partially or completely removed.

In the mouse, the junction between the walls of the canal and the surrounding cartilage matrix was weakly and orthochromatically stained. Additionally, the junction appeared irregular in outline often with cells resembling chondrocytes occupying the irregularities which appeared to be opened chondrocyte lacunae. This weakly stained band of cartilage matrix surrounding the canal has been reported in other animals (Lutfi, 1970b; Moss-Salentijn, 1975); however, the boundary has also been described as darkly stained and containing collagen fibers (Stockwell, 1971; Kugler et al., 1979). This fibrous boundary of the canal along with elongated cells lining the boundary has been presented as evidence that the canals are simply extensions of the perichondrium into the underlying matrix. In the mouse, canals are morphologically different from the perichondrium at all stages of canal development. The walls of canals do not contain the heavy collagenous extracellular matrix present in the perichondrium. Additionally, the blood vessels of the canal are large sinusoidal capillaries.

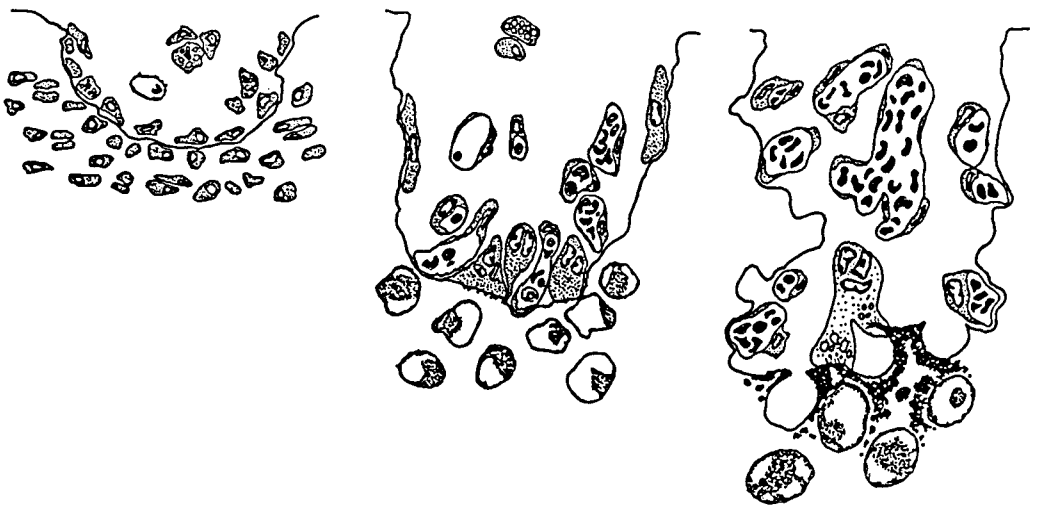
The differences in the appearance of the canal boundary may represent different stages in canal development. When the boundaries are palely stained, the canals may have initially formed; in canals which persist in cartilages over longer periods of time (weeks or years), the boundaries of the canals may become collagenous. In the mouse, canal development occurs in a relatively short time period of two days.

Canal formation by matrix degradation is also supported by morphometric studies. Measurements of epiphyseal area and canal area at three developmental stages indicate that increases in canal area are greater than increases in epiphyseal area. The volume density of intermediate and deep canals are significantly greater than the volume density of superficial canals suggesting that the growth rate of the canal is not directly proportional to the growth rate of the epiphysis. If canal formation were occurring by synthesis, canal growth would be dependent on epiphyseal growth.

This study has provided the preliminary data which can be used to establish cartilage canals in the mouse as a model for studies of uncalcified matrix degradation and for studies of calcified cartilage matrix degradation separate from bone matrix degradation. Cartilage canal development provides one of the few mammalian models for the study of uncalcified matrix degradation. Further characterization of the chondrocytes and perivascular canal cells along with characterization of factors which stimulate or depress their activity may provide insight into disease states which affect cartilage growth and func-

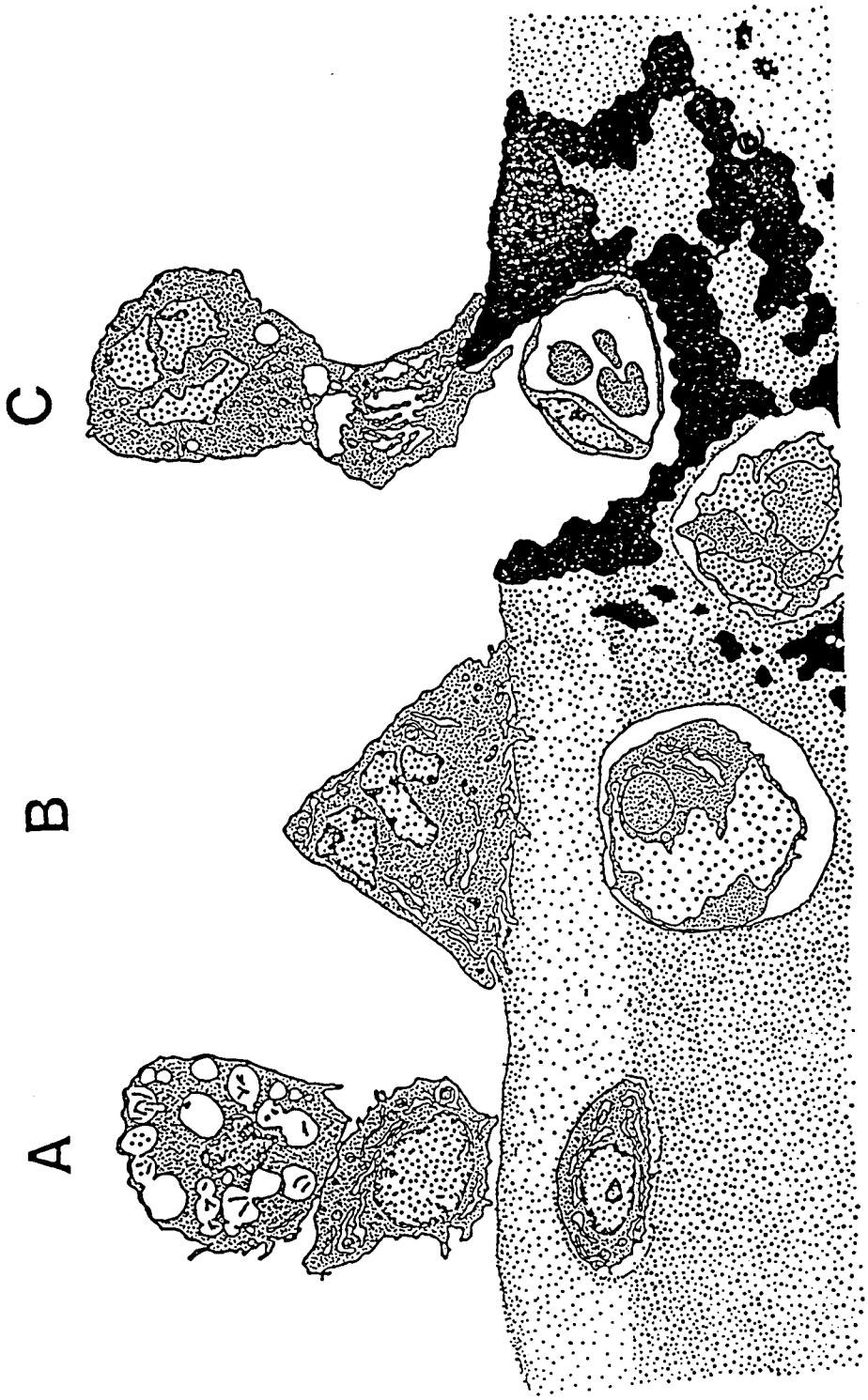
tion. These disease states include rheumatoid arthritis and a number of skeletal dystrophies in which epiphyseal center development is retarded or absent. As with most scientific studies, the results presented here raise a number of questions about canal formation, some of which are difficult or impossible to answer using an in vivo model. These questions include: Will cartilage canals form in the absence of blood vessels and/or the mechanical forces present in the knee? Once formed, will canals continue to grow in the absence of blood vessels? Will hormones, such as growth hormone or thyroxine, increase or decrease the number of canals and the rate of canal growth as has been reported in other animals (Ogden, 1979)? Are morphologically and cytochemically distinct chondroclast precursor cells present in the canals prior to chondrocyte hypertrophy and matrix calcification? Experiments used to answer these and other questions about cartilage matrix degradation may be designed around the cartilage canal model using in vitro methods.

Figure 1 Diagram depicting characteristic features used to define the three stages of canal development.



SUPERFICIAL CANAL INTERMEDIATE CANAL DEEP CANAL

Figure 2 Diagram showing morphological differences among perivascular cells immediately adjacent to the matrix in the three stages of canal development.


A
B
C

CHAPTER IX

SUMMARY AND CONCLUSIONS

Perivascular cells in cartilage canals are thought to be responsible for canal formation. In this study, canal development in epiphyses of neonatal mice was investigated and divided into three stages. Superficial canals were surrounded by nonhypertrophic matrix; intermediate canals terminated in uncalcified, hypertrophic matrix; deep canals terminated in calcified matrix. During development canals increased in length and width and canal growth rate exceeded epiphyseal growth rate suggesting that canals form by matrix degradation. At each developmental stage ultrastructurally distinct perivascular cells with characteristics of degradative cells were present. In the superficial canals vacuolated macrophage-like cells contacted the collagenous component of the matrix and vacuoles contained collagen fragments. In intermediate canals fibroblastic cells extended cytoplasmic projections into the matrix, while chondroclasts were present in deep canals. Cells positive for tartrate-resistant acid phosphatase, an enzyme associated with bone and cartilage matrix degradation, were present in canals, and cells positive for alkaline phosphatase lined the hypertrophic matrix. No cells were present with enzymes characteristic of macrophages. Matrix surrounding canals contained decreased chondroitin-6-sulfate and proteoglycan. Differences in mor-

phology and enzyme cytochemistry among perivascular cells at the three stages of development suggest that different mechanisms are used to degrade the differentiating matrix.

In conclusion, this study of cartilage canal development has demonstrated that

1. Cartilage canals are present in the distal femoral epiphysis of the mouse prior to the development of the epiphyseal center.
2. Canal development can be divided into three stages characterized by differentiating epiphyseal cartilage.
3. Canal formation by matrix degradation is supported by differences in growth rates of the canal and epiphyses.
4. At each stage of development, the cartilage matrix appears to be degraded by morphologically different perivascular cells.
5. Perivascular cells contain tartrate-resistant acid phosphatase and alkaline phosphatase, enzymes which have been associated with cartilage matrix degradation.
6. An extracellular release of matrix degrading enzymes is suggested by a decrease in GAG and proteoglycan matrix components immediately surrounding the canal.

LITERATURE CITED

Agrawal, P., P. R. Atre and D. S. Kulkarni 1984 The role of cartilage canals in the ossification of the talus. Acta Anat. 119:238-240.

Ali, S. Y. 1983 Calcification of cartilage. In: Cartilage: Structure, Function and Biochemistry. Vol. I. B.K. Hall, ed. Academic Press, New York, pp. 343-378.

Andersen, H., and M. E. Matthiessen 1966 The histiocyte in human foetal tissues. Its morphology, cytochemistry, origin, function, and fate. Z. Zellforschung. Mikrosk. Anat. 72:193-211.

Anderson, C. E., and J. Parker 1966 Invasion and resorption in endochondral ossification. J. Bone Joint Surg. 48-A:899-914.

Anderson, T. R., and S. U. Toverud 1979 Purification and partial characterization of two acid phosphatases from rat bone. Calcif. Tissue Int. 27:219-226.

Anderson, T. R., and S. U. Toverud 1982 Effect of Fe++ and ascorbic acid on acid phosphatases from rat bone. Calcif. Tissue Int. 34:54-58.

Arsenis, C., R. Eisenstein, L. W. Soble and K. E. Kuettner 1971 Enzyme activities in chick embryonic cartilage: Their subcellular distribution in isolated chick chondrocytes. J. Cell Biol. 49:459-467.

Baici, A. 1980 Structure and breakdown of proteoglycans, collagen and elastin. In: Proteinases and Tumor Invasion. P. Strauli, A.J. Barratt and A. Baici, eds. Raven Press, New York, pp. 17-47.

Bainton, D. F. 1981 The discovery of lysosomes. J. Cell Biol. 91:66s-76s.

Baron, R., L. Neff, D. Louvard and P. J. Courtoy 1985 Cell-mediated extracellular acidification and bone resorption: evidence for a low pH in resorbing lacunae and localization of a 100-kD lysosomal membrane protein at the osteoclast ruffled border. J. Cell Biol. 101: 2210-2222.

Baron, R., L. Neff, P. Tran Van, J. R. Nefussi and A. Vignery 1986 Kinetic and cytochemical identification of osteoclast precursors and their differentiation into multinucleated osteoclasts. Am. J. Path. 122:363-378.

Bishop, M. A., and H. Warshawsky 1982 Electron microscopic studies on the potential loss of crystallites from routinely processed sections of young enamel in the rat incisor. Anat. Rec. 202:177-186.

Biswas, C., and J. M. Dayer 1979 Stimulation of collagenase production by collagen in mammalian cell cultures. Cell 18:1035-1041.

Boothroyd, B. 1964 The problem of demineralisation in thin sections of fully calcified bone. J. Cell Biol. 20:165-173.

Brighton, C. T., Y. Sugioka and R. M. Hunt 1973 Cytoplasmic structures of epiphyseal plate chondrocytes. Quantitative evaluation using electron micrographs of rat costochondral junctions with special reference to the fate of hypertrophic cells. J. Bone Joint Surg. 55-A: 771-784.

Brighton, C. T., Y. Sugioka and R. M. Hunt 1982 Quantitative zonal analysis of cytoplasmic structures of growth plate chondrocytes in vivo and in vitro. J. Bone Joint Surg. 65-A:1336-1349.

Bromley, M., and D. E. Woolley 1984 Histopathology of the rheumatoid lesion. Identification of cell types at sites of cartilage erosion. Arthritis Rheum. 27:857-863.

Brookes, M. 1958 The vascularization of long bones in the human foetus. J. Anat. 92:261-267.

Brookes, M. 1971 The Blood Supply of Bone. An Approach to Bone Biology. Appleton-Century-Crofts, New York.

Buckwalter, J. A., D. Mower, R. Ungar, J. Schaeffer and B. Ginsberg 1986 Morphometric analysis of chondrocyte hypertrophy. J. Bone Joint Surg. 68-A:243-255.

Carrino, D. A., M. Weitzhandler and A. I. Caplan 1985 Proteoglycans synthesized during the cartilage to bone transition. In: The Chemistry and Biology of Mineralized Tissues. W.T. Butler, ed. Ebsco Media, Inc., Birmingham, pp. 197-208.

Caterson, B., J. R. Baker, J. E. Christner and J. R. Couchman 1982 Immunological methods for the detection and determination of connective tissue proteoglycans. J. Invest. Dermatol. 79:45s-50s.

Caterson, B., J. E. Christner and J. R. Baker 1983 Identification of a monoclonal antibody that specifically recognizes corneal and skeletal keratan sulfate. J. Biol. Chem. 258:8848-8854.

Caterson, B., J. E. Christner, J. R. Baker and J. R. Couchman 1985 Production and characterization of monoclonal antibodies directed against connective tissue proteoglycans. Fed. Proc. 44:386-393.

Chappard, D., C. Alexandre and G. Riffat 1983 Histochemical identification of osteoclasts. Review of current methods and reappraisal of a simple procedure for routine diagnosis on undecalcified human iliac bone biopsies. Bas. Appl. Histochem. 27:75-85.

Cole, A. A., and L. M. Walters 1986 Tartrate-resistant acid phosphatase in bone and cartilage following decalcification and cold-embedding in plastic. J. Histochem. Cytochem. (In Press) :.

Cole, A. A., and F. H. Wezeman 1985 Perivascular cells in cartilage canals of the developing mouse epiphysis. Am. J. Anat. 174:119-129.

Cole, M. B., Jr 1982 Glycol methacrylate embedding of bone and cartilage for light microscopic staining. J. Microsc. 127:139-148.

Couchman, J. R., B. Caterson, J. E. Christner and J. R. Baker 1984 Mapping by monoclonal antibody detection of glycosaminoglycans in connective tissues. Nature 307:650-652.

Crissman, R. S., and F. N. Low 1974 A study of fine structural changes in the cartilage-to-bone transition within the developing chick vertebra. Am. J. Anat. 140:451-470.

Dannenberg, A. M., Jr, and M. Suga 1981 Histochemical stains for macrophages in cell smears and tissue sections: B-galactosidase, acid phosphatase, nonspecific esterase, succinic dehydrogenase, and cytochrome oxidase. In: Methods for Studying Mononuclear Phagocytes. D.O. Adams, P.J. Edelson and H. Koren, eds. Academic Press, New York, pp. 375-396.

Dayer, J. M., R. G. G. Russell and S. M. Krane 1977 Collagenase production by rheumatoid synovial cells: Stimulation by a human lymphocyte factor. Science 195:181-183.

Deporter, D. A., and A. R. Ten Cate 1973 Fine structural localization of acid and alkaline phosphatase in collagen-containing vesicles of fibroblasts. J. Anat. 114:457-461.

Deshmukh-Phadke, K., M. Lawrence and S. Nanda 1978 Synthesis of collagenase and neutral proteases by articular chondrocytes: Stimulation by a macrophage-derived factor. Biochem. Biophys. Res. Comm. 85:490-496.

Dingle, J. T., and T. T. Dingle 1980 The site of cartilage matrix degradation. Biochem. J. 190:431-438.

Dingle, J. T., P. Horsfield, H. B. Fell and M. E. J. Barratt 1975 Breakdown of proteoglycan and collagen induced in pig articular cartilage in organ culture. Ann. Rheum. Dis. 34:303-311.

Dorey, C. K., and K. L. Bick 1977 Ultrastructural analysis of glycosaminoglycan hydrolysis in the rat peridontal ligament. Calcif. Tissue Res. 24:135-141.

Doty, S. B., and B. H. Schofield 1972 Electron microscopic localization of hydrolytic enzymes in osteoclasts. Histochem. J. 4:245-258.

Eisenstein, R., K. E. Kuettner, C. Neapolitan, L. W. Soble and N. Sorgente 1975 The resistance of certain tissues to invasion. III. Cartilage extracts inhibit the growth of fibroblasts and endothelial cells in culture. Am. J. Pathol. 81:337-348.

Eisenstein, R., N. Sorgente, L. W. Noble, A. Miller and K. E. Kuettner 1973 The resistance of certain tissues to invasion. Penetrability of explanted tissues by vascularized mesenchyme. Am. J. Pathol. 73:765-774.

Farnum, C. E., and N. J. Wilsman 1983 Pericellular matrix of growth plate chondrocytes: A study using postfixation with osmium-ferrocyanide. J. Histochem. Cytochem. 31:765-775.

Gardner, E., and D. J. Gray 1970 The prenatal development of the human femur. Am. J. Anat. 129:121-140.

Godman, G. C., and N. Lane 1964 On the site of sulfation in the chondrocyte. J. Cell Biol. 21:353-366.

Golds, E. E., V. Santer, J. Killackey and P. J. Roughley 1983 Mononuclear cell factors stimulate the concomitant secretion of distinct latent proteoglycan, gelatin and collagen degrading enzymes from human skin fibroblasts and synovial cells. J. Rheumatol. 10:861-871.

Gothlin, G., and J. L. E. Ericsson 1976 The osteoclast. Review of ultrastructure, origin, structure-function relationship. Clin. Orthopaed. Rel. Res. 120:201-231.

Gray, D. J., and E. Gardner 1950 Prenatal development of the human knee and superior tibiofibular joints. Am. J. Anat. 86:235-287.

Gray, D. J., and E. Gardner 1969 The prenatal development of the human humerus. Am. J. Anat. 124:431-445.

Gross, J. 1981 An essay on biological degradation of collagen. In: Cell Biology of Extracellular Matrix. E.D. Hay, ed. Plenum Press, New York, pp. 217-297.

Haines, R. W. 1933 Cartilage canals. J. Anat. 68:45-64.

Haines, R. W. 1937 Growth of cartilage canals in the patella. J. Anat. 71:471-479.

Hall, B. K. 1978 Developmental and Cellular Skeletal Biology. Academic Press, New York.

Hamerman, D., R. Janis and C. Smith 1967 Cartilage matrix depletion by rheumatoid synovial cells in tissue culture. J. Exp. Med. 126:1005-1017.

Hammarstrom, L. E., T. R. Anderson, S. C. Marks Jr and S. U. Toverud 1983 Inhibition by dithionite and reactivation by iron of the tartrate-resistant acid phosphatase in bone of osteopetrotic (ia) rats. J. Histochem. Cytochem. 31:1167-1174.

Hammarstrom, L. E., J. S. Hanker and S. U. Toverud 1971 Cellular differences in acid phosphatase isoenzymes in bone and teeth. Clin. Orthopaed. Rel. Res. 78:151-167.

Haraldsson, S. 1962 The vascular pattern of a growing and fullgrown human epiphysis. Acta Anat. 48:156-167.

Hardingham, T. E., R. J. F. Ewins and H. Muir 1976 Cartilage proteoglycans. Structure and heterogeneity of the protein core and the effects of specific protein modifications on the binding to hyaluronate. Biochem. J. 157:127-143.

Harris, E. D., C. S. Faulkner II and F. E. Brown 1975 Collagenolytic systems in rheumatoid arthritis. Clin. Orthopaed. Rel. Res. 110:303-316.

Hascall, G. K. 1980a Ultrastructure of the chondrocytes and extracellular matrix of the Swarm rat chondrosarcoma. Anat. Rec. 198: 135-146.

Hascall, G. K. 1980b Cartilage proteoglycans: Comparison of sectioned and spread whole molecules. J. Ultrastruc. Res. 70:369-375.

Hauser, P., and G. Vaes 1978 Degradation of cartilage proteoglycan by a neutral proteinase secreted by rabbit bone-marrow macrophages in culture. Biochem. J. 172:275-284.

Hembry, R. M., G. Murphy, T. E. Cawston, J. T. Dingle and J. J. Reynolds 1986 Characterization of a specific antiserum for mammalian collagenase from several species: Immunolocalization of collagenase in rabbit chondrocytes and uterus. J. Cell Sci. 81:105-123.

Hensinger, R. N., and E. T. Jones 1981 Neonatal Orthopaedics. Grune & Stratton, New York.

Hinchliffe, J. R., and D. R. Johnson 1983 Growth of cartilage. In: Cartilage: Development, Differentiation, and Growth. Vol. II. B.K. Hall, ed. Acadmemic Press, New York, pp. 255-295.

Hirashita, A., K. Noda, K. Kaida, Y. Nakamura and Y. Kuwabara 1985 Phatocytosis of collagen by fibroblasts incident to experimental tooth movement. Arch. Histol. Jpn. 48:149-158.

Hoenig, J. M., and A. H. Walsh 1982 The occurrence of cartilage canals in shark vertebrae. Can. J. Zool. 60:483-485.

Holt, S. J. 1959 Factors governing the validity of staining methods for enzymes, and their bearing upon the Gomori acid phosphatase technique. Exp. Cell Res. 7:1-27.

Holtrop, M. E. 1972a The ultrastructure of the epiphyseal plate. I. The flattened chondrocyte. Calcif. Tissue Res. 9:131-139.

Holtrop, M. E. 1972b The ultrastructure of the epiphyseal plate. II. The hypertrophic chondrocyte. Calcif. Tissue Res. 9:140-151.

Holtrop, M. E., K. A. Cox and J. Glowacki 1982 Cells of the mononuclear phagocytic system resorb implanted bone matrix: a histologic and ultrastructural study. Calcif. Tissue Int. 34:488-494.

Holtrop, M. E., and G. J. King 1977 The ultrastructure of the osteoclast and its functional implications. Clin. Orthopaed. Rel. Res. 123:177-196.

Horton, J. E., F. H. Wezeman and K. E. Kuettner 1978 Inhibition of bone resorption in vitro by a cartilage-derived anticollagenase factor. Science 199:1342-1345.

Howlett, C. R. 1980 The fine structure of the proximal growth plate and metaphysis of the avian tibia: endochondral osteogenesis. J. Anat. 130:745-768.

Howlett, C. R., M. Dickson and A. K. Sheridan 1984 The fine structure of the proximal growth plate of the avian tibia: vascular supply. J. Anat. 139:115-132.

Hunt, C. D., D. A. Ollerich and F. H. Nielsen 1979 Morphology of the perforating cartilage canals in the proximal tibial growth plate of the chick. Anat. Rec. 194:143-157.

Hunziker, E. B., W. Herrmann and R. K. Schenk 1982 Improved cartilage fixation by ruthenium hexammine trichloride (RHT). J. Ultrastruc. Res. 81:1-12.

Hunziker, E. B., W. Herrmann and R. K. Schenk 1983 Ruthenium hexammine trichloride (RHT)-mediated interaction between plasmalemmal components and pericellular matrix proteoglycans is responsible for the preservation of chondrocytic plasma membranes in situ during cartilage fixation. J. Histochem. Cytochem. 31:717-727.

Hunziker, E. B., W. Herrmann, R. K. Schenk, M. Mueller and H. Moor 1984 Cartilage ultrastructure after high pressure freezing, freeze substitution, and low temperature embedding. I. Chondrocyte ultrastructure - implications for the theories of mineralization and vascular invasion. J. Cell Biol. 98:267-276.

Hurrell, D. J. 1934 The vascularisation of cartilage. J. Anat. 69:49-61.

Ibbotson, K. J., G. D. Roodman, L. M. McManus and G. R. Mundy 1984 Identification and characterization of osteoclast-like cells and their progenitors in cultures of feline marrow mononuclear cells. J. Cell Biol. 99:471-480.

Janckila, A. J., C. Y. Li, K. W. Lam and L. T. Yam 1978 The cytochemistry of tartrate-resistant acid phosphatase. Am. J. Clin. Pathol. 70:45-55.

Jasin, H. E., and J. T. Dingle 1981 Human mononuclear cell factors mediate cartilage matrix degradation through chondrocyte activation. J. Clin. Invest. 68:571-581.

Jilka, R. L. 1986 Parathyroid hormone-stimulated development of osteoclasts in cultures of cells from neonatal murine calvaria. Bone 7:29-40.

Johnson, M. L. 1933 The time and order of appearance of ossification centers in the albino mouse. Am. J. Anat. 52:241-271.

Jones, P. A., and Z. Werb 1980 Degradation of connective tissue matrices by macrophages. II. Influence of matrix composition on proteolysis of glycoproteins, elastin and collagen by macrophages in culture. J. Exp. Med. 152:1527-1536.

Jubb, R. W., and H. B. Fell 1980 The breakdown of collagen by chondrocytes. J. Pathol. 130:159-167.

Kahn, A. J., C. C. Stewart and S. L. Teitelbaum 1978 Contact-mediated bone resorption by human monocytes in vitro. Science 199:988-990.

Kenrad, B., and H. Vilmann 1977 Phosphomonoesterases in growth cartilages of the rat. Cell Tissue Res. 181:349-359.

Kielty, C. M., A. P. L. Kwan, D. F. Holmes, S. L. Schor and M. E. Grant 1985 Type X collagen, a product of hypertrophic chondrocytes. Biochem. J. 27:545-554.

Killackey, J. J., P. J. Roughley and J. S. Mort 1983 Proteinase inhibitors of human articular cartilage. Collagen Rel. Res. 3:419-430.

Knese, K. H. 1972 Osteoklasten, chondroklasten, mineraloklasten, kollagenoklasten. Acta Anat. 83:275-288.

Knese, K. H. 1979 Stutzgewebe und Skelletsystem. In: Handb. Mikrosk. Anat. Menschen. Vol. II. Springer-Verlag, Berlin, pp. 1-937.

Knese, K. H. 1980 Die Knorpelgefasse und der Knochenkern. Der metamorphosierende Knorpel: II Mitteilung. Gegenbaurs Morphol. Jahrb. 126:86-101.

Kobayashi, I., and M. Ziff 1975 Electron microscopic studies of the cartilage-pannus junction in rheumatoid arthritis. Arthritis Rheum. 18:475-483.

Ksiazek, T., and J. Thyberg 1983 Electron microscopic studies on resorption of xenogenic cartilage implants. Virchows Arch. (Pathol. Anat.) 399:79-88.

Kuettner, K. E., and B. U. Pauli 1981 Resistance of cartilage to invasion. In: Bone Metastasis. L. Weiss, and H.A. Gilbert, eds. G.H. Hall Med. Pub., Boston, pp. 131-165.

Kuettner, K. E., and B. U. Pauli 1983 Vascularity of cartilage. In: Cartilage: Structure, Function and Biochemistry, Vol. I. B.K. Hall, ed. Academic Press, New York, pp. 281-312.

Kugler, J. H., A. Tomlinson, A. Wagstaff and S. M. Ward 1979 The role of cartilage canals in the formation of secondary centres of ossification. J. Anat. 129:493-506.

Kuhns, L. R., and A. K. Poznanski 1980 Radiological assessment of maturity and size of the newborn infant. CRC Critical Reviews in Diagnostic Imaging 12:245-308.

Langer, R., H. Brem, K. Falterman, M. Klein and J. Folkman 1976 Isolation of a cartilage factor that inhibits tumor neovascularization. Science 193:70-72.

Levene, C. 1964 The patterns of cartilage canals. J. Anat. 98:515-538.

Lillie, R. D. 1965 Histopathologic Technic and Practical Histochemistry. McGraw-Hill Book Co., New York.

Luft, J. H. 1971 Ruthenium red and violet. I. Chemistry, purification, methods of use for electron microscopy and mechanism of action. Anat. Rec. 171:347-368.

Lutfi, A. M. 1970a Study of cell multiplication in the cartilaginous upper end of the tibia of the domestic fowl by tritiated thymidine autoradiography. Acta Anat. 76:454-463.

Lutfi, A. M. 1970b Mode of growth, fate and functions of cartilage canals. J. Anat. 106:135-145.

McArthur, W., A. M. Yaari and I. M. Shapiro 1980 Bone solubilization by mononuclear cells. Lab. Invest. 42:450-456.

Melcher, A. H. 1972 Role of chondrocytes and hydrocortisone in resorption of proximal fragment of Meckel's cartilage: An in vitro and in vivo study. Anat. Rec. 172:21-36.

Minkin, C. 1982 Bone acid phosphatase: tartrate-resistant acid phosphatase as a marker of osteoclast function. Calcif. Tissue Int. 34:285-290.

Morales, T. I., and K. E. Kuettner 1982 The properties of the neutral peoteinase released by primary chondrocyte cultures and its action on proteoglycan aggregate. Biochim. Biophys. Acta 705:92-101.

Moscatelli, D., J. L. Gross and D. B. Rifkin 1981 Angiogenic factors stimulate plasminogen activator and collagenase production by capillary endothelial cells. J. Cell Biol. 91:201.

Moss-Salentijn, L. 1975 Cartilage canals in the human spheno-occipital synchondrosis during fetal life. Acta Anat. 92:595-606.

Moss-Salentijn, L. 1976 The epiphyseal vasculature of growth plates. A developmental study in the rabbit. Ph.D. Thesis. Univ. of Utrecht.

Mourao, P. A. S., and C. P. Dietrich 1979 Chondroitin sulfates of the epiphysial cartilages of different mammals. Comp. Biochem. Physiol. 62B:115-117.

Mullins, D. E., and S. T. Rohrlich 1983 The role of proteinases in cellular invasiveness. Biochim. Biophys. Acta 695:177-214.

Murate, K., and A. O. Bjelle 1980 Constitutional variations of acidic glycosaminoglycans in normal and arthritic bovine articular cartilage proteoglycans at different ages. Connective Tissue Res. 7:143-156.

Namba, M., A. M. Dannenberg Jr and F. Tanaka 1983 Improvement in the histochemical demonstration of acid phosphatase, B-galactosidase and nonspecific esterase in glycol methacrylate tissue sections by cold temperature embedding. Stain Technol. 58:207-213.

Ogden, J. A. 1979 The development and growth of the musculoskeletal system. In: The Scientific Basis of Orthopaedics. J.A. Albright, and R.A. Brand, eds. Appleton-Century-Crofts, New York, pp. 41-103.

Parakkal, P. F. 1969 Involvement of macrophages in collagen resorption. J. Cell Biol. 41:345-354.

Pearse, A. G. E. 1968 Histochemistry. Theoretical and Applied. Vol. I. 3rd Edition. Little, Brown and Co., Boston.

Pearse, A. G. E. 1975 Histochemistry. Theoretical and Applied. Vol. II. 3rd Edition. Brown and Co., Boston.

Phadke, K., S. Nanda and K. Lee 1979 Release of proteases from cartilage cells as a result of activation by a macrophage factor - effects of some anti-inflammatory drugs. Biochem. Pharmacol. 28:3671-3673.

Poole, A. R. 1970a The relationship between toluidine blue staining and hexuronic acid content of cartilage matrix. Histochem. J. 2:425-430.

Poole, A. R. 1970b The degradation of cartilage matrix by a lysosomal preparation, isolated from a malignant tumour, and its inhibition by an antiserum to this preparation. Histochem. J. 2:431-439.

Revel, J. P., and E. D. Hay 1963 An autoradiographic and electron microscopic study of collagen synthesis in differentiating cartilage. Z. Zellforschung. Mikrosk. Anat. 61:110-144.

Revel, J. P., and E. D. Hay 1964 Light and electron microscopic study of mucopolysaccharides in developing amphibian and mammalian cartilage. Anat. Rec. 148:326.

Ries, W. L. 1984 Osteogenic periosteum esterase activity: A comparative morphological and cytochemical study of bone cells in situ on rat proximal tibiae and in smears. J. Histochem. Cytochem. 32:55-62.

Rifas, L., V. Shen, K. Mitchell and W. A. Peck 1984 Macrophage-derived growth factor for osteoblast-like cells and chondrocytes. Proc. Natl. Acad. Sci. 81:4558-4562.

Rifkin, B. R., R. L. Baker and S. J. Coleman 1979 An ultrastructural study of macrophage-mediated resorption of calcified tissue. Cell Tissue Res. 202:125-132.

Roberts, C. R., and R. T. Dean 1986 Degradation of cartilage by macrophages in culture: Evidence for the involvement of an enzyme which is associated with the cell surface. Connective Tissue Res. 14: 199-212.

Robertson, P. B., R. B. Ryel, R. E. Taylor, K. W. Shyu and H. M. Fullmer 1972 Collagenase: localization in polymorphonuclear leukocyte granules in the rabbit. Science 177:64-65.

Rodriquez, J. I., E. Delgado and R. Paniagua 1985 Multivacuolated cells in human cartilage canals. Acta Anat. 124:54-57.

Roodman, G. D., K. J. Ibbotson, B. R. MacDonald, T. J. Kuehl and G. R. Mundy 1985 1,25-dihydroxyvitamin D3 causes formation of multinucleated cells with several osteoclast characteristics in cultures of primate marrow. Proc. Natl. Acad. Sci. 82:8213-8217.

Rose, G. G., and P. B. Robertson 1977 Collagenolysis by human gingival fibroblast cell lines. J. Dent. Res. 56:416-424.

Rosenberg, L. 1971 Chemical basis for the histological use of safranin O in the study of articular cartilage. J. Bone Joint Surg. 53A:69-82.

Saito, H., T. Yamagata and S. Suzuki 1968 Enzymatic methods for the determination of small quantities of isomeric chondroitin sulfates. J. Biol. Chem. 243:1536-1542.

Salomon, C. D. 1974 A fine structural study on the extracellular activity of alkaline phosphatase and its role in calcification. Calc. Tissue Res. 15:201-212.

Savostin-Asling, I., and C. W. Asling 1975 Transmission and scanning electron microscope studies of calcified cartilage resorption. Anat. Rec. 183:373-392.

Schenk, R. K., D. Spiro and J. Wiener 1967 Cartilage resorption in the tibial epiphyseal plate of growing rats. J. Cell Biol. 34:275-291.

Schmid, T. M., and T. F. Linsenmayer 1985 Immunohistochemical localization of short chain cartilage collagen (Type X) in avian tissues. J. Cell Biology. 100:598-605.

Schneider, G. B., K. Abe and J. J. Goggin 1981 Phagocytosis and acid phosphatase activity of the phagocytic cells in the ia (Osteopetrotic) rat. J. Reticuloendothelial Soc. 30:391.

Seifert, M. F. 1984 The biology of macrophages in osteopetrosis. Clin. Orthopaed. Rel. Res. 182:270-277.

Shimomura, Y., F. H. Wezeman and R. D. Ray 1973 The growth cartilage plate of the rat rib: cellular differentition. Clin. Orthopaed. Rel. Res. 90:246-254.

Silvestrini, G., M. E. Ricordi and E. Bonucci 1979 Resorption of uncalcified cartilage in the diaphysis of the chick embryo tibia. Cell Tissue Res. 196:221-235.

Snipes, R. G., K. W. Lam, R. C. Dodd, T. K. Gray and M. S. Cohen 1986 Acid phosphatase activity in mononuclear phagocytes and the U937 cell line: Monocyte-derived macrophages express tartrate-resistant acid phosphatase. Blood 67:729-734.

Sorgente, N., and C. K. Dorey 1980 Inhibition of endothelial cell growth by a factor from cartilage. Exp. Cell Res. 128:63-71.

Sorgente, N., K. E. Kuettner, L. W. Soble and R. Eisenstein 1975 The resistance of certain tissues to invasion. II. Evidence for extractable factors in cartilage which inhibit invasion by vascularized mesenchyme. Lab. Invest. 32:217-222.

Sorrell, J. M., and L. Weiss 1980 A light and electron microscopic study of the region of cartilage resorption in the embryonic chick femur. Anat. Rec. 198:513-530.

Sorrell, J. M., and L. Weiss 1982 The cellular organization of fibroblastic cells and macrophages at regions of uncalcified cartilage resorption in the embryonic chick femur as revealed by alkaline and acid phosphatase histochemistry. Anat. Rec. 202:491-499.

Spira, E., I. Farin and H. Karplus 1963 The capillary conducting channels in the distal epiphyses of radius and ulna of the rabbit. J. Anat. 97:255-258.

Stockwell, R. A. 1971 The ultrastructure of cartilage canals and the surrounding cartilage in the sheep fetus. J. Anat. 109:397-410.

Stockwell, R. A. 1979 Biology of Cartilage Cells. Cambridge Univ. Press, Cambridge, pp. 124-136.

Stockwell, R. A. 1983 Metabolism of cartilage. In: Cartilage: Structure, Function, and Biochemistry. Vol. I. B.K. Hall, ed. Academic Press, New York, pp. 253-280.

Stump, C. W. 1925 The histiogenesis of bone. J. Anat. 59:136-154.

Sweet, M. B. E., E. J. M. A. Thonar and J. Marsh 1979 Age-related changes in proteoglycan structure. Arch. Biochem. Biophys. 198:438-448.

Takagi, M., R. T. Parmley, Y. Toda and F. R. Denys 1982 Extracellular and intracellular digestion of complex carbohydrates by osteoclasts. Lab. Invest. 46:288-297.

Takemura, R., and Z. Werb 1984 Secretory products of macrophages and their physiological functions. Am. J. Physiol. 246:C1-C9.

Teitelbaum, S. L., C. C. Stewart and A. J. Kahn 1979 Rodent peritoneal macrophages as bone resorbing cells. Calcif. Tiss. Int. 27:255-261.

Ten Cate, A. R. 1972 Morphological studies of fibrocytes in connective tissue undergoing rapid remodelling. J. Anat. 112:401-414.

Ten Cate, A. R., and S. Syrbu 1974 A relationship between alkaline phosphatase activity and the phagocytosis and degradation of collagen by the fibroblast. J. Anat. 117:351-359.

Thonar, E. J. M. A., and M. B. E. Sweet 1981 Maturation-related changes in proteoglycans of fetal articular cartilage. Arch. Biochem. Biophys. 208:535-547.

Thorogood, P. V., and J. C. Gray 1975 Demineralization of bone matrix: Observation from electron microscope and electron-probe analysis. Calcif. Tissue Int. 19:17-26.

Thyberg, J., S. Lohmander and U. Friberg 1973 Electron microscopic demonstration of proteoglycans in Guinea pig epiphyseal cartilage. J. Ultrastruc. Res. 45:407-427.

Thyberg, J., and S. Moskalewski 1979 Bone formation in cartilage produced by transplanted epiphyseal chondrocytes. Calcif. Tissue Res. 204:77-94.

Urist, M. R. 1983 The origin of cartilage: Investigations in quest of chondrogenic DNA. In: Cartilage: Development, Differentiation, and Growth. Vol. II. B.K. Hall, ed. Academic Press, New York, pp. 1-85.

Vaes, G. 1985 Macrophage secretory products and connective tissue remodeling: role of macrophage enzymes and of "matrix regulatory monokines". In: Developments in Cell Biology. 1. Secretory Processes. R.T. Dean, and P. Stahl, eds. Butterworths, London, pp. 99-117.

Van Furth, R., J. A. Raeburn and T. L. Van Zwet 1979 Characteristics of human mononuclear phagocytes. Blood 54:485-500.

Vasan, N. S., and J. W. Lash 1978 Proteoglycan heterogeneity in embryonic chick articular and epiphyseal cartilages. Connective Tissue Res. 6:191-199.

Vertel, B. M., L. L. Barkman and J. J. Morrell 1985 Intracellular features of type II procollagen and chondroitin sulfate proteoglycan synthesis in chondrocytes. J. Cellular Biochem. 27:215-229.

Walters, L. M., and G. B. Schneider 1986 A comparison of the morphological and histochemical properties of the osteoclast, macrophage polykaryon, and bone-induced multinucleated cell. J. Cell. Biochem., Supplement 10B :117.

Werb, Z., and J. Aggeler 1978 Proteases induce secretion of collagenase and plasminogen activator by fibroblasts. Proc. Natl. Acad. Sci. 75: 1839-1843.

Werb, Z., D. F. Bainton and P. A. Jones 1980 Degradation of connective tissue matrices by macrophages. III. Morphological and biochemical studies on extracellular, pericellular and intracellular events in matrix proteolysis by macrophages in culture. J. Exp. Med. 152:1537-1553.

Wergedal, J. E. 1970 Characterization of bone acid phosphatase activity. Proc. Soc. Exp. Biol. Med. 134:244-247.

Wilsman, N. J., and C. D. Van Sickle 1972 Cartilage canals, their morphology and distribution. Anat. Rec. 173:79-94.

Wilsman, N. J., and D. C. Van Sickle 1970 The relationship of cartilage canals to the initial osteogenesis of secondary centers of ossification. Anat. Rec. 168:381-392.

Wong, G. L. 1984 Paracrine interactions in bone-secreted products of osteoblasts permit osteoclasts to respond to parathyroid hormone. J. Biol. Chem. 259:4019-4022.

Wuthier, R. E., and T. C. Register 1985 Role of alkaline phosphatase, a polyfunctional enzyme in mineralizing tissues. In: The Chemistry and Biology of Mineralized Tissues. W.T. Butler, ed. Ebsco Media, Inc., Birmingham, pp. 113-124.

Yajima, T. 1976 Ultrastructural and cytochemical studies on the remodelling of the tracheal cartilage. Arch. Histol. Jpn. 39:79-97.

Yamaguchi, A., M. Yamanouchi and S. Yoshiki 1985 Osteoblastic and osteoclastic differentiation of mononuclear cells facing the resorbing surface of uncalcified cartilage in the tibia of embryonic chick. Cell Tissue Res. 240:425-431.

APPENDIX A

This Appendix represents data obtained through the combined laboratory effort by the author and another graduate student, Linda M. Walters. This document will be part of both dissertations. Because of this, the Graduate College ruled that the data was to be placed in an Appendix separate from the remainder of the dissertation which is original for each student.

APPENDIX A: TARTRATE-RESISTANT ACID PHOSPHATASE IN BONE AND CARTILAGE FOLLOWING DECALCIFICATION AND COLD-EMBEDDING IN PLASTIC

ABSTRACT

Tartrate-resistant acid phosphatase (TRAP) has been proposed as a cytochemical marker for osteoclasts. We have developed an improved technique for the localization of TRAP in rat and mouse bone and cartilage. This procedure employs JB-4 plastic as the embedding medium, permits decalcification, and results in improved morphology compared with frozen sections. Peritoneal lavage cells were used to determine the appropriate isomer and concentration of tartrate necessary for inhibition of tartrate-sensitive acid phosphatase. Following incubation in medium containing 50 mM L(+)-tartaric acid, osteoclasts and chondroclasts were heavily stained with reaction product. On the basis of their relative sensitivity to tartrate inhibition, three populations of mononuclear cells could also be distinguished. These three populations may represent: (1) heavily stained osteoclast/chondroclast precursors, (2) sparcely stained osteoblast-like cells lining the bone surface, and (3) unstained cells of monocyte-macrophage lineage. Our results are consistent with the use of TRAP as a histochemical marker for study of the osteoclast.

INTRODUCTION

On the basis of sensitivity to tartrate inhibition, two types of acid phosphatase can be identified in bone. Both forms have been biochemically characterized with respect to substrate specificity, pH optima, and have been shown to be functionally responsive to osteotropic hormones in vitro (Wergedal, 1970; Anderson and Toverud, 1982; Minkin, 1982; Ibbotson et al., 1984). The tartrate-sensitive acid phosphatase has been cytochemically localized in osteoblasts, osteocytes, and osteoclasts, while the tartrate-resistant acid phosphatase (TRAP) is present in osteoclasts (Minkin, 1982; Hammarstrom et al., 1983). Osteoclasts have been identified primarily by morphological criteria as large multinucleated cells which contact calcified bone matrix and exhibit ruffled borders surrounded by clear zones (Gothlin and Ericsson, 1976). Tartrate-resistant acid phosphatase as a histochemical and biochemical marker for the osteoclast would provide an additional experimental tool for studying osteoclastic differentiation and function. Baron et al. (1986) employed TRAP as such a marker for the cytochemical identification of proposed osteoclast precursors at both the light and electron microscopic levels.

Various investigators have used different techniques to localize TRAP within tissues making comparison of the results of

the studies difficult. Unfixed, frozen sections have been used to localize bone TRAP in vivo (Minkin, 1982; Hammarstrom, et al, 1983). These methods demonstrate enzyme product, but lack of fixation results in a loss of morphological detail. Chappard et al. (1983) describe a TRAP procedure which includes cold-embedding in a mixture of glycol and methyl methacrylates. This technique requires sectioning of undecalcified bone and the purification of methacrylates. We have incorporated glutaraldehyde fixation into a histochemical procedure based on the cold-embedding method of Namba et al. (1983) which utilizes commercially available JB-4 (Polysciences, Inc.) as the embedding medium eliminating the need for purification of methacrylate. In addition, our procedure permits the use of EDTA decalcified bone and cartilage for ease in sectioning. The description of this technique includes an evaluation of two isomers of tartaric acid, L(+)- and D(-)-, as effective inhibitors of tartrate-sensitive acid phosphatases. This evaluation was accomplished through the use of peritoneal lavage cells, the majority of which are macrophages known to contain tartrate-sensitive acid phosphatase (Schneider et al., 1981; Seifert, 1984). Rodent peritoneal macrophages appear to be capable of bone resorption in vitro and have been repeatedly investigated as a possible cell source of osteoclast precursors (Teitelbaum et al., 1979; McArthur et al., 1980). The use of peritoneal lavage

cells, therefore, provides not only a means to evaluate the effective inhibition of tartrate-sensitive acid phosphatase, but also a means to compare the acid phosphatase characteristics of these proposed osteoclast precursors with those of in situ osteoclasts. Our procedure is an attempt to provide a convenient and reproducible method for TRAP localization within skeletal tissues.

MATERIALS AND METHODS

All animals used for this study were obtained from breeding colonies housed in a centrally located, fully accredited animal care facility.

Fixation and Embedding. Except where indicated, all of the following procedures were conducted at 4°C (Namba et al., 1983). Proximal tibiae from 2 week old rats and proximal femurs including growth plates from 1 week old mice were removed and dissected free of adherent soft tissue. Tissues were fixed for 2 or 4 hrs in either 3.7% formalin in phosphate buffer (pH 7.4) (Lillie, 1965) with 7% sucrose or in 2.5% cacodylate-buffered glutaradehyde (pH 7.4) containing 7% sucrose, rinsed 3 times, and stored overnight (17-19 hr) in the appropriate buffer. Both bones and growth plates were decalcified for 48 hrs in 10% EDTA in Tris buffer, pH 7.4 (Pearse, 1968). Undecalcified tissue was used as a control. The formalin-fixed tissue was dehydrated in 50, 75 and 95% (2 changes) acetone for 15 min each; the glutaraldehyde-fixed tissue was dehydrated in either 50, 75, and 95% (2 changes) ethanol or acetone for 15 min each. The tissue was infiltrated overnight in JB-4 solution A with catalyst (Polysciences, Inc., Warrington, PA) and embedded in complete JB-4 medium in BEEM or gelatin capsules. During embedding, the capsule trays were placed on cracked ice to

reduce the high temperature which accompanies JB-4 polymerization at room temperature. The blocks were allowed to polymerize overnight at 4°C. Sections (3 um) were cut dry at 25°C on a Dupont Sorvall JB-4 microtome with glass knives and placed on ice-cooled slides which had been alcohol-cleaned and gelatin-subbed. The sections were allowed to air-dry at 4°C for 5-7 days.

Preparation of Peritoneal lavage cells. Cells obtained by peritoneal lavages from adult mice and 4 week old rats were used as a control to determine the appropriate isomer and concentration of tartrate for inhibition of tartrate-sensitive acid phosphatase. The abdominal cavity was injected with 10 ml of 0.9% saline and massaged for 2 min. The peritoneal fluid was removed and centrifuged. The cell pellets were resuspended in 0.5 ml of 0.9% saline. The cells were smeared onto gelatin-coated glass slides, air-dried at 4°C, fixed in 3.7% formalin fumes for 5 min, and stored at 4°C.

Histochemical Staining. Burstone's complete medium for acid phosphatase (Pearse, 1968) was prepared by dissolving 4 mg naphthol AS-BI phosphate substrate (Sigma, St. Louis, MO) in 0.25 ml of N,N-dimethyl formamide followed by the addition of 25 ml of 0.2M acetate buffer (pH 5.0), 35 mg of either Fast Red Violet LB or Fast Garnet GBC diazonium salt (Sigma) as the coupling agent, and 2 drops (60ul) of 10% $MgCl_2$. The media was then filtered into

acid-cleaned Coplin jars. As a control, the substrate was omitted. In addition, sodium tartrate (Mallinckrodt, Paris, KY), L(+)-tartaric acid, disodium salt (Sigma), or D(-)-tartaric acid (Sigma) at concentrations of 1, 32.5, 50, or 100 mM were added to individual Coplin jars containing 25 ml of filtered, complete media in media prewarmed to 37°C. Both tissue sections and peritoneal lavage preparations were allowed to come to room temperature and incubated for 30 min to 3 hr. Following incubation, the slides were washed for 30 min in running water, allowed to air-dry at 25°C, counter-stained with 1% aqueous Fast Green FCF (Fisher Scientific Co., Chicago, IL, C.I. 42053) for approximately 1 min. Cover slips were mounted with Euparol (Gallard-Schlesinger Chem. Mfg. Corp., Carle Place, N.Y.), and preparations were examined for the presence or absence of reaction product. For each isomer and concentration of tartrate, the percentage of stained peritoneal lavage cells per 1000 cells was calculated.

RESULTS

The red-maroon acid phosphatase reaction product was granular and confined to the cell cytoplasm in all tissues, except for some diffuse staining of the bone matrix adjacent to osteoclasts. No difference in reaction product or intensity was observed between 2 or 4 hr fixation or between phosphatate-buffered formalin or cacodylate-buffered glutaraldehyde. The 4 hr glutaraldehyde fixation demonstrated superior morphological preservation and is the preferred fixative. Using cacodylate-buffered glutaraldehyde also eliminates possible artifactual staining due to the presence of phosphate in the buffer. Decalcification of bone and growth plate for 48 hrs at $4^{\circ}C$ did not decrease the reaction product. When Fast Garnet GBC was used as the capture agent, the tissue non-specifically stained yellowish-orange, and a heavy, red precipitate often covered the sections. With Fast Red Violet LB, there was no non-specific staining of the tissue and very little precipitate adhered to sections incubated up to 90 min. Rat bone, incubated for 45 min, gave a strong reaction product; in mouse bone and growth plate a 90 min incubation was required to give the same intensity. No difference in reaction product was noted in sections from tissue blocks stored up to four months; however, after six months of storage, decreased reaction product was noted.

An attempt was made to define the inhibitory concentration of each isomeric form of tartrate by determining the concentration at which reaction product was absent from peritoneal lavage cells. At 50 mM L(+)-tartaric acid, no reaction product was seen in peritoneal lavage cells (Table I) or in a population of bone marrow mononuclear cells located adjacent to blood vessels. Reaction product localized within tissue sections incubated with 50 mM L(+)-tartaric acid reflected the presence of TRAP in those tissues. Multinucleated cells immediately adjacent to bone or calcified cartilage matrix contained a heavy concentrated reaction product uniformly distributed throughout the cytoplasm (Fig. 1). The majority of mononuclear cells along the bone surface contained a few granules of reaction product which was not inhibited even at concentrations as high as 100 mM L(+)-tartaric acid (Fig. 2). In addition, small populations of mononuclear cells within the bone marrow and perichondrium (Fig. 3) exhibited a staining pattern similiar to that of the multinucleated cells (Fig. 1).

TABLE 1. PERITONEAL LAVAGE CELLS STAINED WITH TARTRATE-RESISTANT ACID PHOSPHATASE

INCUBATION MEDIA	% STAINED CELLS*
Control	0.00
Complete Media	87.60
with 1.0 mM Sodium Tartrate	74.77
32.5 mM	1.20
50.0 mM	0.20
with 1.0 mM L(+)-tartaric acid	48.70
32.5 mM	1.00
50.0 mM	0.00
with 1.0 mM D(-)-tartaric acid	80.96
32.5 mM	80.22
50.0 mM	82.70

*Determined from samples of 1000 cells

DISCUSSION

This study describes a technique for TRAP localization in bone and cartilage based on a modification of the acid phosphatase technique of Namba et al. (1983). The recommended technique is: 1) fixation in 2.5% cacodylate-buffered glutaraldehyde, 2) decalcification in 10% buffered EDTA, 3) dehydration in acetone, 4) embedding in JB-4 medium, and 5) incubation in medium containing Naphthol AS-BI phosphate as substrate, Fast Red Violet as capture agent and L(+)-tartaric acid (50 mM) to inhibit tartrate-sensitive acid phosphatase. This method results in an easily distinguished granular TRAP reaction product within the cells which remained stable over a period of months. The enzyme preservation in this technique may be due to cold polymerization in JB-4 (Namba et al. 1983), decalcification with cold buffered EDTA (Pearse, 1968) or the inclusion of sucrose in the solutions used in tissue processing. The addition of sucrose has been shown to prevent osmotic damage to cells during processing (Pearse, 1968) and is recommended for enzyme preservation (Holt, 1959).

In frozen sections of bone, Hammarstrom et al. (1983) described an intense TRAP reaction in osteoclasts and a weak TRAP reaction in osteoblasts following incubation with 100 mM sodium tartrate. Chappard et al. (1983), using glycol and methyl methycrylate embedded sections and 1 mM L(+)-tartaric acid, locali-

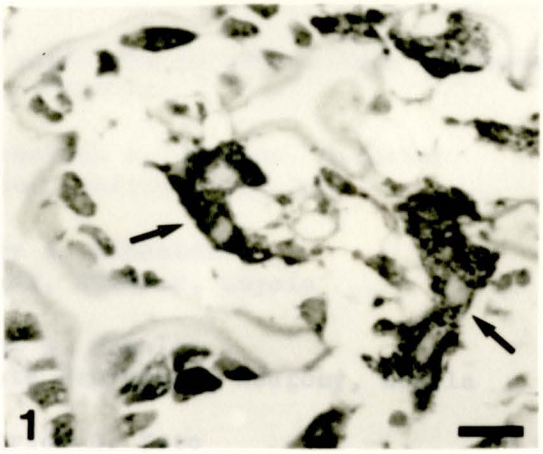
zed TRAP in osteoclasts but not in osteoblasts. Baron et al. (1986), employing an alveolar bone model to study osteoclastic lineage, identified TRAP positive osteoclasts and mononuclear cells with 10 mM sodium tartrate inhibition. The authors describe the TRAP containing mononuclear cells as members of the mononuclear phagocyte system and probable osteoclast precursors. Our study using plastic embedded sections and 50 mM L(+)-tartaric acid also demonstrated the intense localization of TRAP in osteoclasts. Our results differ from those of Chappard et al. (1983) in that mononuclear cells lining bone also contained granules of reaction product although in decreased amounts as compared to that found in the osteoclasts. We were able to distinguish populations of mononuclear cells present in the bone marrow and perichondrium exhibiting the same concentration of reaction product as found in osteoclasts. These cells may represent osteoclast/chondroclast precursors. A third type of mononuclear cell can be identified based on complete inhibition by tartrate. These tartrate-sensitive cells, like the peritoneal lavage macrophages, may represent members of the monocyte-macrophage lineage. Walters and Schneider (1986) examined the TRAP-staining characteristics of the cell populations recruited to subcutaneously implanted bone and suture. The tissue from the implant studies was processed through gluteraldehyde fixation, EDTA decalcification and JB-4 embedding procedures iden-

tical to those used in processing the bone and cartilage sections of the present study. They found that the macrophage populations surrounding the implants displayed similar acid phosphatase inhibition with 50 mM L(+)-tartaric acid. Peritoneal lavage cells, tissue macrophages and some bone marrow mononuclear cells, therefore, exhibit similar patterns of tartrate inhibiton with 50 mM L(+)-tartaric acid while the osteoclast does not. With our technique, then, we are able to distinguish the osteoclast from some members of the mononuclear phagocyte system. Our findings, therefore, differ from those of Baron et al. (1986) who were unable to distinguish between members of the mononuclear phagocytic system and osteoclasts. This difference could possibly be explained by their use of 10 mM tartrate rather than our higher 50 mM concentration. On the basis of these observations, our studies are consistent with the use of TRAP as a histochemical marker for the study of osteoclastic differentiation and lineage.

Figure 1. Section of 2 week old rat tibia, counterstained with Fast Green, demonstrating osteoclasts (arrows) stained intensely with granular TRAP reaction product. Original magnification X160; bar = 11.3 um.

Figure 2. Trabecular bone (rat) with calcified cartilage core (CC) lined by mononuclear cells containing a few intensely stained TRAP granules (arrows). Note the heavily stained osteoclastic profile at the bottom of the field. Counterstained with Fast Green. Original magnification X160; bar = 10.4 um.

Figure 3. Heavily staining mononuclear cells (arrows) located in mouse perichondrium (P) adjacent to cartilage matrix (C). Chondrocytes are negative for reaction product. Counterstained with Fast Green. Original magnification X160; bar = 13.8 um.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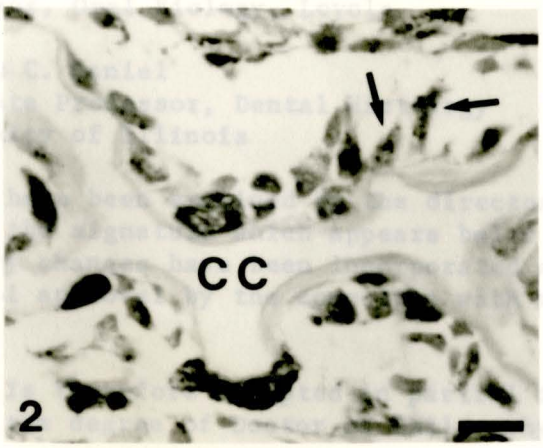
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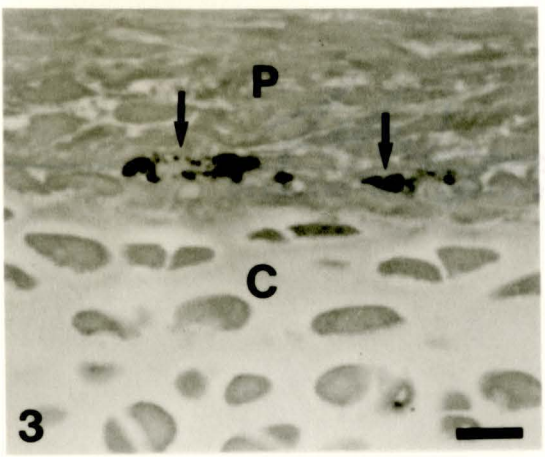
P
C
3

APPROVAL SHEET

The dissertation submitted by Ada A. Cole has been read and approved by the following committee:

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and the dissertation is now given final approval by the committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

9-12-86
Date

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