PHENOTYPIC CHARACTERIZATION OF CARTILAGE CELLS DURING ENDOCHONDRAL OSSIFICATION (AN AVIAN GROWTH PLATE MODEL)

BY

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ABSTRACT

This study applies the countercurrent centrifugal elutriation method for the separation of growth plate chondrocytes. This method is capable of isolating cells which have districtive biochemical and physical characteristics of maturation. Using chicken as the experimental animal, the high cell yield achieved (approximately 100 x 10^6 chondrocytes per gram of cartilage), makes this animal a good primary model for studying endochondral ossification. Increase in cellular volume, together with specific phenotypic changes is often associated with the process of cell maturation during ossification. For the different subpopulations of chondrocytes, biochemical differences were present in DNA synthesis, proteoglycan synthesis as well as activities of hyaluronidase, alkaline and acid phosphatase.

By using the countercurrent centrifugal elutriation method coupled with flow cytometry cell cycle analysis, relative homogeneous subpopulations of chondroyctes, i.e., resting, proliferative and hypertrophic chondrocytes can now be identified and used for subsequent studies.

Chondrocytes with size smaller than 10 um and low DNA activity are regarded as resting chondrocytes. Chondrocytes with mean cell diameter ranging between 10 to 10.65 um, showing the highest DNA activity, are referred to as proliferative chondrocytes. Chondrocytes with relatively high DNA activity and cell diameter larger than 10.65 um are the hypertrophic chondrocytes. The sulphate incorporation rate and hyaluronidase activity are very high in both proliferative and hypertrophic subpopulations when

compared to resting subpopulation. It indicates that whenever chondrogenesis is onset, the surrounding cartilage matrix is being actively synthesized and broken down. Thus, a dynamic model of cartilage matrix has been indirectly demonstrated. Alkaline and acid phosphatase activities continue to increase when chondrocyte enlarge and reach a peak at the hypertrophic subpopulation. Since these enzymes are thought to be related to calcification, the increase in activities seem to be quite consistent with one of the speculated physiological functions of hypertrophic chondrocytes. As well, there is strong evidence suggesting that hypertrophic chondrocytes are physiologically active rather than degenerating.

In the present study, eight commonly used growth factors have been tested for their effects on acid and alkaline phosphatase activities in chick growth plate and articular chondrocytes. Besides testing the cellular enzyme activities of these two enzymes, the excretory enzymes in the culture medium were also tested. For the growth plate chondrocyte, both cellular and excretory alkaline phosphatase activities were suppressed by a number of growth factors, such as PTH, D_3, FGF-a, FGF-b and TGF-β. Their cellular acid phosphatase activity were stimulated by PTH, FGF-a and FGF-b.

For articular chondrocyte, PTH, FGF-a and FGF-b suppress the cellular alkaline phosphatase activity. At the same time, inhibition of cellular acid phosphatase activity by FGF-b was also noted. In summary, FGF-b was found to be the most potent effector on acid and alkaline phosphatases in growth plate and articular chondrocytes.

Since special autocrine function of acid phosphatase in osteoblast has recently been discovered, its activities in chick growth plate and articular chondrocytes were characterized in detail in this study. The optimal activity of acid phosphatase in both types of chondrocyte is obtained at pH 4. The Km for the enzyme in both types of chondrocytes were almost identical, about 0.2 mg/ml substrate. The Vmax for growth plate chondrocyte was 322.95 umol/10^6 cell/hr which was larger than that for articular chondrocytes, 261.97 umol/10^6 cell/hr. It seems that the growth plate chondrocytes have slightly more enzyme molecules per cell than articular chondrocytes.

About 90% acid phosphatase activity in articular chondrocyte was inhibited by 50 mM tartrate. However, in chick growth plate chondrocyte 30% of acid phosphatase activity still remained even when 100 mM of tartrate inhibitor was present. This implied that both tartrate sensitive and tartrate resistant acid phosphatase isoenzymes were presented within chick growth plate chondrocytes. The activities of tartrate sensitive and tartrate resistant isoenzymes performed same distribution pattern in chondrocytes of different size as the total acid phosphatase.

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TABLE OF CONTENTS

CHAPTER FOUR - METHODOLOGIES

CHAPTER FIVE - RESULTS

CHAPTER ONE - INTRODUCTION

Cartilage is a specialized dense connective tissue. It is avascular and contains no nervous tissue. The cartilage is made up of chondrocytes embedded in a matrix which consists largely of water, collagen fibrils and proteoglycan macromolecules. These macromolecules form a fiber-reinforced gel. In all mammals, cartilage acts as temporary skeleton in the embryo and serves as a basic structure on which bones develop. In adults the main functions of cartilage are to provide support for soft tissues as well as gliding areas for joints. Moreover, it plays an important role in callus formation during fracture repairing.

Skeleton formation, fracture-healing and bone degeneration, the three main fields of research in Orthopaedics, are directly related to the development of cartilage. The characteristics of cartilage are determined by the different amounts of proteoglycan and the ratio of the different types of collagen in the matrix. As all the matrix substances are secreted by the cartilage producing cells, the chondrocytes; it would be quite valuable to establish an in vitro model to study the differentiation of chondrocytes, which in terms would help us to understand more fully the process and control of bone development, fracture-healing and bone degeneration. Once the process of progressive cartilage differentiation is understood, it is expected that more will be known about the regressive (degenerative) processes. This kind of information is important in the study of degenerative joint diseases such as

osteoarthritis. A better understanding of the cartilage differentiation process is also essential for the improvement in the treatment of fractures and other orthopaedic diseases.

During the last decade, most of the laboratory work on the cartilage research has been focused on the composition of the extracellular matrix and the molecular structure of its components (Delbruck, 1986), and very little advance has been made in the area of cartilage diseases. It seems logical to put more emphasis on the understanding of biochemical characteristics of the chondrocytes in normal cartilage, and on their responses to endogenous and exogenous factors which might disturb the dynamic equilibrium of the cartilage structures.

Most of the embryonic cartilaginous skeleton is eventually replaced by bone in a process called endochondral ossification in the growth plate cartilage. Since complex biochemical processes occur in a small anatomical area (endochondral ossification center) in a short period of time, in order to study these processes, it is necessary to prepare homogeneous samples of resting, proliferating and hypertrophic chondrocytes. Most investigators have attempted to prepare pure samples of the different zones of growth-plate cartilage by microdissection. Wuthier (1969) scraped off sequential layers of calf growth plate. Kuhlman (1965) took microtome sections of dog and rabbit growth plates. The scraping technique presented problems in reproducibility and identification of layers, while microtome method required preliminary freezing. Another technique, micropuncture, allowed precise localization of the cells to be analyzed, but the potential number of analyses that could be performed was limited. Ray (Ray, Ehrlich and Mankin, 1982) then tried to use

Ficoll density Gradient to separate chondrocytes with different cell density, but this was not very successful.

Most of the previous investigations used a population of mixed stages of chondrocytes (Boyan and Shaffer, 1982; Boyan, Schwartz, Carnes and Ramirez, 1988). We put forward a hypothesis that cartilage cells at different stages of differentiation have different metabolic activities and respond differently to various kinds of hormones and stimulators. An in vitro model using chick growth plate chondrocytes developed in this study is able to identify and separate the cartilage cells in different stages of differentiation by applying countercurrent centrifugal elutriation method (CCE). Using CCE, it is possible to obtain pure chondrocyte populations for more precise investigations.

In addition, acid phosphatase, with its special autocrine function in rat osteoblasts recently discovered in the Orthopaedics Research Center, University of Rochester, has also been characterized in the present study using chick chondrocyte as a model system.

CHAPTER TWO - STRUCTURE OF CARTILAGE

2.1 Characteristics of Cartilage

Cartilage is a specialized dense connective tissue consisting of cells, and an extracellular collagen fiber network embedded in a hydrophilic matrix composed chiefly of chondroitin sulfate (Turek, 1984). The matrix, which is synthesized and secreted by chondrocytes, contains type II collagen and chondroitin sulfate proteoglycan, and gives cartilage its characteristic stiffness and resiliency. Due to the fact that cartilage is an avascular tissue, the chondrocytes receive only oxygen and nutrients both of which can diffuse readily through the matrix. In vivo, chondrocytes reside in small lacunas within the cartilage matrix, where they tend to have a round shape.

2.2 Types of Cartilage

There exists three varieties of cartilage in vivo, namely hyaline cartilage, fibrocartilage and elastic cartilage. They are distinguishable from each other on the basis of the amount of extracellular matrix and the relative proportions of elastic and collagen fibers embedded in it. In hyaline cartilage, the matrix appears to be homogeneous and consists principally of collagen fibers arranged in a loose meshwork. This type of cartilage is the most common variety and is found on the ventral surface of ribs, on the joint surfaces of bones, and in the tracheal rings. Like hyaline cartilage, the matrix

of fibrocartilage consists largely of collagen fibers, but these fibers form many thick bundles. This type of tissue, which can be viewed as a transitional form between cartilage and dense connective tissue, is found at the site of attachments of tendons to bones, where it gradually merges into the dense connective tissue of the tendons. Elastic cartilage, on the other hand, differs from the other types in that it contains many elastic fibers in its matrix. It occurs in the external ear, Eustachian tube, and epiglottis.

2.3 Matrix

Cartilage matrix consisting largely of water, collagen fibrils and proteoglycans (protein-carbohydrate complexes), forms a fiber-reinforced gel. A minute fraction of the matrix lies within the lacuna which contains finely-textured pericellular matrix distinct from the coarsely-fibrous matrix elsewhere. The physico-chemical properties of the matrix and its constituents determine the mechanical properties of cartilage.

2.3.1 Collagen

On average, 50% of the dry weight of cartilage is collagen although the proportion of collagen to other constituents decreases with age. It has been shown by electron microscopy that collagen fibrils appear as a characteristic cross banding with a periodicity of 67 nm. The width of the fibrils varies from

30 to 150 nm. In general they are narrower where they lie close to the cell or to certain boundaries of the tissue.

The precursor molecules of the collagen fibrils in cartilage are synthesized and secreted by the chondrocytes but fibrogenesis occurs extracellularly. Collagen fibrils are cross-linked aggregates of tropocollagen molecules with a high tensile strength (15-30 kg/mm^{-2}) which by weight for weight is equivalent to mild steel. Each tropocollagen molecule, 300nm long and 1.5 nm wide, contains three peptide a-chains. Each a-chain is coiled in a left-handed helix and the three chains are twisted together in a right- handed helix (like a rope). The tropocollagen molecules aggregate in bundles to form fibrils of various widths. Strength and stability is conferred on the fibrils by hydrogen bonding and covalent cross-linkages.

In hyaline cartilage, most of the collagen is of the characteristic type II variety which contains more carbohydrate than that of skin and bone where the fibrils are much thicker. It is generally accepted that the superficial area of articular cartilage contain type I collagen. The collagen of fibro-cartilage is either exclusively type O (i.e. same as in the meniscus of the knee joint) or a mixture of type O and type II (i.e. same as in the intervertebral disc). Type I collagen consists of three chains, two similar chains designated a_2I. Type II collagen contains three identical a_1II chains. The so-called 'minor' (1a, 2a, 3a, M) collagens and some type III collagens have been detected in immature cartilage and elsewhere (Duance, Xhimokomaki and Bailey, 1982); these are located close to the cells and may have a different function from the main type of fibril.

The importance of the type of collagen in articular cartilage has not been established. Cartilage containing Type II collagen may possess enhanced resistance to tear and may also be the only adequate substitute material for damaged articular cartilage. It is possible that the natural repair mechanism of damaged articular cartilage in joints is by the formation of fibrocartilage, containing Type I collagen predominantly.

In vitro studies of chick embryo morphogenesis showed that chondrocytes at various developmental stages synthesize different types of collagens (Floyd, Zaleske, Shciller, Trahan and Mankin, 1987). The committed mesenchymal cells, (i.e. precursor of chondrocytes), found first in the limb bud and then in the perichondrium, produce type I collagen and fibronectin and possibly minimal amount of type II collagen. Proliferating chondrocytes produce large amounts of type II collagen and cartilage- specific proteoglycans. The hypertrophic chondrocytes localized in the hypertrophic calcifying cartilage region are characterized by their ability to synthesize type X in addition to type II collagen.

2.3.2 Proteoglycan

Proteoglycans are large hydrophilic and negatively charged macromolecules (mol. wt 1-2 x 10^6 daltons) consisting of a linear protein core to which are attached 50-100 side chains of glycosaminoglycans, chondroitin sulfate and keratan sulfate. These side chains are linear carbohydrate polymers with large numbers of carboxyl and sulfate groups. This highly fixed negative charge attracts large number of positively charged counter-ions (such

as Ca^{2+}) and results in a high osmolarity, which largely accounts for the retention of water in cartilage.

The predominant glycosaminoglycan is chondroitin sulfate, which consists of a repeating disaccharide unit of N-acetyl galactosamine and glucuronic acid. Each side chain, on average, contains 25-30 repeating units. Keratan sulfate consists of a repeating disaccharide unit of N-acetyl glucosamine and galactose, and the chains ares shorter (about 13 repeating units) than in chondroitin sulfate. Two isomeric forms of chondroitin sulfate occur, chondroitin 4-sulfate and chondroitin 6-sulfate, but the latter predominates in adult cartilage. However, the position of sulfate groups and the degree of sulphation can vary along the chain. The significance of the position of the sulfate groups remains to be determined. In chondroitin 6-sulfate, the sulfate groups project further from the chain than that of in chondroitin 5-sulfate and may therefore interact with collagen and other proteins to a greater extent.

In cartilage, the majority of the proteoglycans form aggregates with hyaluronic acid, an non-sulfated glycosaminoglycan 102 um long. About 30-50 proteoglycans attach, at intervals of 25-50 nm, along the hyaluronic acid molecule. The proteoglycan-hyaluronate aggregate is very large (molecular weight approximately 50×10^6 daltons) and is about 1 um diameter when fully hydrated. In normal cartilage the aggregates are compressed to about one fifth of their potential volume and have a tendency to swell.

It is important to note that, in articular cartilage, the content of proteoglycan varies from joint to joint and among individuals. An increase in

the proteoglycan content is observed from the surface to deeper layers of the articular cartilage and the distribution of different glycosaminoglycans also varies in relationship to depth and with proximity to the cells. In addition, an increase in the proportions of chondroitin 6-sulfate occurs with age, as does that of keratan sulfate has been reported (Mankin and Lippiello, 1970).

2.4 Diffusion of Solutes in Cartilage

The proteoglycans and collagen content of the matrix regulate passage of solutes into and through cartilage. Firstly, since diffusion is most rapid in water, solutes must pass more slowly through cartilage-which is never more than 90% water. Secondly, the macromolecules obstruct the passage of solutes, by imposing an increasingly restricted and tortuous pathway of diffusion with increasing size of the solute and by imparting a frictional retardation on the velocity of the solute. Thirdly, the negative charges on the proteoglycans attract cations but repel anions and the magnitude of this effect depends also on the tonicity of the external solution.

Thus, uncharged nutrient molecules and metabolites of small molecular size, such as glucose,diffuse into and through cartilage comparatively easily. Large molecules, such as the t-globulins, are almost completely excluded from cartilage; although very long and narrow molecules, such as hyaluronic acid, may diffuse with anomalous rapidity through the tissue. Anionic metabolites, such as sulfate, can diffuse into cartilage; although less easily than cations such as Ca^{2+}

2.5 Chondrocytes

The size of chondrocyte varies from about 10um diameter in articular cartilage to about 30um diameter in the hypertrophic zone of the growth plate. Although chondrocytes often lie in groups, cell to cell contacts among chondrocytes rarely if ever, occur in adult cartilage. The chondrocyte surface has the usual antigens and receptors but it is shielded from cytotoxic antibodies by the matrix, which is just barely permeable to high molecular weight proteins. The cells lie in spheroidal lacunae, although the cell itself has a scalloped contour with many projecting cell processes about 1-2 um long. The tips of the constantly moving processes may become detached to form vesicles in the matrix near the lacunas rim. As in many specialized cells, the chondrocyte nuclei tend to be small and densely basophilic. Aging nuclei often assume bizarre irregular and lobulated shapes.

During embryonic development, cartilage differentiates from mesenchyme, and the mesenchyme is usually of mesodermal origin. However, mesenchyme may also develop from ectoderm, as in the cartilage derived from cranial neural crest (mesectoderm). Chondrogenic mesenchyme from the above mentioned source can undergo chondrogenesis in tissue culture.

It is generally accepted that chondrocytes of adult articular cartilage do not undergo mitotic division. This can be shown by uptake experiment using tritiated thymidine. That uptake could not be detected in mature articular cartilage, whereas immature cartilage showed two zones of division. The first

zone was in the superficial layer whereas the second was near the osseous center. It has been suggested that the division occur in the superficial zone contributed to growth of the articular surface and that of the deep zone contributed to the ossification center.

Nevertheless, cell division has been demonstrated in adult cartilage when the surface has become damaged either in early osteoarthritis, chondromalacia. Uptake of tritiated thymidine by cell clusters of chondrocytes has also been shown under experimental injury conditions.

In summary, the cartilage matrix, which is synthesized and secreted by chondrocytes, is highly complex in nature. There is constant interaction between the cells and matrix. Due to the rigid nature of the matrix, one can easily see the difficulties in isolating chondrocytes from cartilage. This is one of the reasons why chondrocyte research has been relatively slow in the past few decades.

After describing the components of cartilage, the physiological changes within cartilage, such as endochondral ossification, hormone responses in cartilage and degradative enzyme system will be reviewed in the next chapter. In addition, the evidence for the role of degradative enzymes in osteoarthritis will also be discussed.

CHAPTER THREE - PHYSIOLOGICAL CHANGES WITHIN CARTILAGE

3.1 Endochondral Ossification

A major function of cartilage is to serve as a structural component of the embryonic skeleton. The cartilage matrix, which is resilient enough to permit cell growth yet strong enough to provide support, makes the tissue ideally suited for this function.

In the center of embryonic cartilaginous center, the chondrocytes enlarge and become arranged radically. Lime salts are deposited in the matrix. This calcified cartilage disintegrates and is destroyed by invoking vascular tissue from the perichondrium. At the same time the invasive budlike mass differentiate into osteoblasts thus initiating bone formation. This spongy bone formation continues to replace the cartilage, by extending proximally and distally, resulting in longitudinal growth of the bone. Most of the embryonic cartilaginous skeleton is eventually replaced by bone and marrow in such a process called endochondral ossification, but some cartilage persists into adult life.

The endochondral ossification center can be divided into 5 zones as shown in Figure 3.1 (Jungueir and Carneire, 1980): (1) The **resting zone** consisting of hyaline cartilage without morphological changes in the homogeneous cells. (2) In the **proliferative zone**, chondrocytes divide rapidly and form parallel rows of stacked cells along the long axis of the bone. (3)

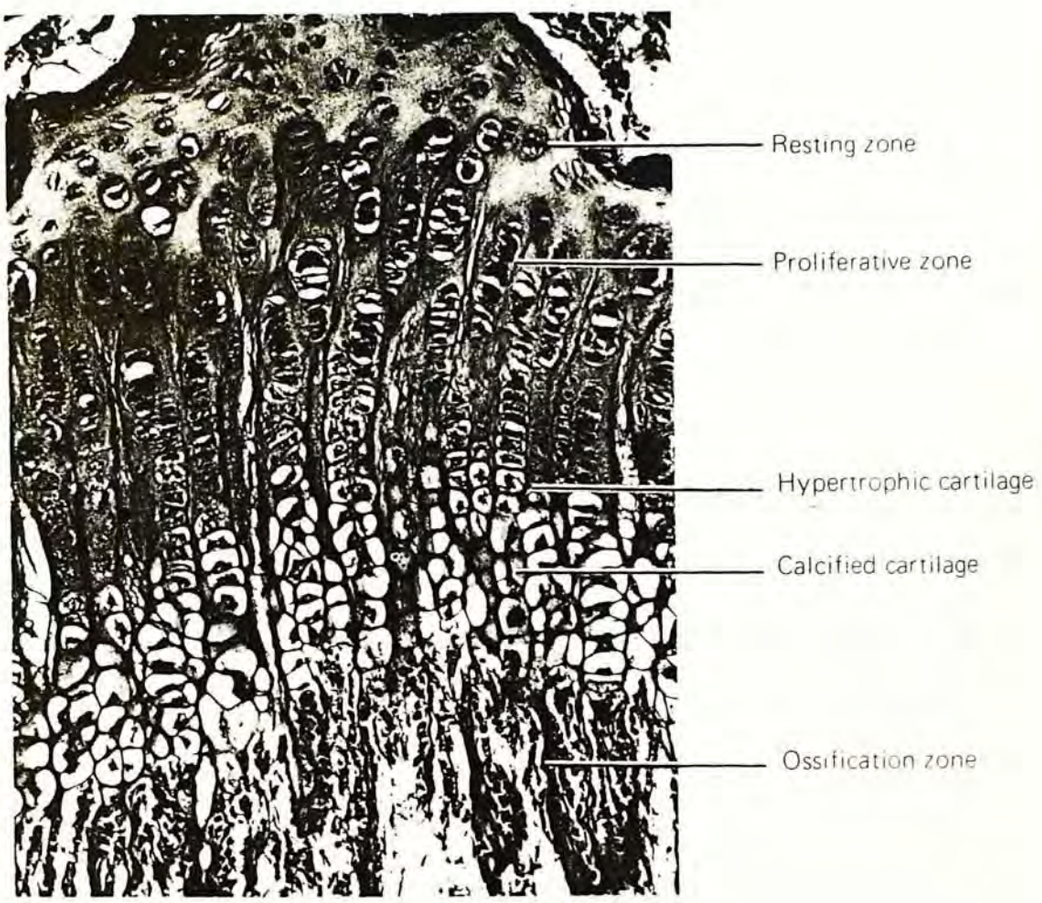

Figure 3.1 Photomicrograph of the epiphyseal plate, showing different zones at the endochondral ossification center. H&E stain, x 100. (from Junqueira L C, Carneiro J: Basic Histology, 3rd ed. Lange Medical Publications, 1980)

The **hypertrophic cartilage zone** containing large chondrocytes whose cytoplasm has accumulated glycogen. The resorbed matrix is reduced to thin septa between the chondrocytes. (4) In the **calcified cartilage zone**, the thin septa of cartilage matrix become calcified by the deposition of hydroxyapatite. (5) In the **ossification zone**, endochondral bone tissue appears. Blood capillaries and undifferentiated cells formed by mitosis of cells originating from the periosteum invade the cavities left by the chondrocytes. The undifferentiated cells developed into osteoblasts, which in turn form a discontinuous layer over the septa of calcified cartilage matrix. Over these septa, the osteoblasts lay down bone matrix.

During endochondral ossification, chondrocytes undergo a programmed sequence of biochemical and morphological changes. In maturing cartilage, zones of chondrocytes can be seen at various stages in the sequence, with progressive stages marked by a change in shape from round to flattened to hypertrophic. Such morphological changes provide us physical parameters for identifying chondrocytes in different stages of maturity. Rooney et al. (1984) have suggested that these shape changes may be influenced by the surrounding matrix. Shinomura (Shinomura, Kimata, Oike, Maedo, Yano and Suzuki, 1984) and Kimata (Kimata, Okayoma, Ochera and Suzuki, 1984) have reported the appearance of a specific proteoglycan (PG-Lb) as the maturing chondrocytes acquire a flattened shape; it is not clear, however, whether the change in the shape of chondrocytes is related to expression of the new molecule or not. On the other hand, hypertrophic chondrocytes produce type X collagen and this specific type of collagen is being accepted as the marker for hypertrophic cartilage (Schmid and

Linsemayer, 1985). Interestingly, chondrocytes derived from cartilage that normally does not undergo hypertrophy can be induced to synthesize type X collagen in vitro (Solurshet et al. 1986).

Recently, the sequence of events which leads to the formation of the ossification center in the distal femoral epiphysis of the CD1 mouse has been demonstrated by Floyed et al (1987). The distal femoral epiphysis in the newborn mouse was found to consist entirely of cartilage, containing no vessels and no hypertrophic chondrocytes. An island of hypertrophic chondrocytes appeared in five day old CD1 mouse. On the sixth day of neonatal mouse, capillary invasion began to occur at the periphery, and by the seventh day, vessels had grown in towards the island of hypertrophic chondrocytes. Finally, on the eighth day, when vascular in-growth had penetrated deep into the island of hypertrophic chondrocytes, mineralization of the matrix was demonstrated by staining with alizarin. Osteoblasts deposited bone on the spicules of calcified cartilage, and in this way the ossification center began to form. These observations suggest that a factor may be synthesized by the hypertrophic chondrocyte. This factor is chemotactic for endothelial cells and being secreted into the extracellular matrix, thus inducing capillaries to grow into the cartilaginous epiphysis toward the hypertrophic chondrocytes. Besides giving a clear demonstration of the endochondral ossification process, Floyed's findings also provide us with some insight about the control of this process.

Formation of the skeleton, fracture-healing, and bone growth are three of the main fields in orthopedic study. All these processes are directly related to the endochondral ossification. In patients who have disorders of bone growth such as metatrophic dwarfism, the number of columns of hypertrophic

cells that are invaded by vessels is dramatically decreased. The control mechanism of osteogenesis is therefore an important field awaiting for future investigation.

3.2 Hormone Responses on Growth in Cartilage

Various hormones promote growth in cartilage. Somatotrophin (growth hormone) stimulates growth in cartilage through intermediary serum growth factors, the somatomedins, resulting in chondrocyte proliferation and enhanced matrix synthesis. Excess production of the somatotrophin in the adult (acromegaly) may cause degenerative joint disease. Thyroxine is necessary for normal levels of chondroitin sulfate synthesis, but somatomedins is also required. It seems that both hormones are working together to promote maturation. Other hormones such as insulin and testosterone indirectly stimulate matrix synthesis and cell growth respectively. Oestrogens, on the other hand, inhibit cell growth and matrix synthesis, producing growth plate closure and antagonizing the effect of the growth hormone, somatotrophin.

Inhibition of proteoglycan and collagen synthesis in cartilage by cortisone has been shown (Turek, 1984). This is possibly triggered by depressing glycolysis - involution of cytoplasmic organelles occurs. The steroid also retards macromolecule degradation by stabilizing the lysosomal membrane as well as by suppressing soft tissue activation of chondrocyte-mediated matrix degradation. Where high rates of synthesis are required, for example, in embryonic growth or replenishment of macromolecules after experimental depletion of cartilage matrix, cortisone can have deleterious effects.

Although the effects of many hormones or mediators on cartilage have been reported, most of the detail mechanisms are still not clear. How the cartilage differentiation process is controlled by hormones is a potential research field.

3.3 Degradative enzymatic system

There appears to be a final common pathway in the pathogenesis of osteoarthritis, regardless of the initiating cause. This involves the increase of degradative enzymes that arise from the cartilage. Both proteoglycan and collagen degrading enzymes, active at a neutral pH, increase in proportion to the severity of the arthritis until a final end-stage state is reached. The increase in enzyme activity may be triggered by the release of a synovial messenger protein similar to that of interleukin-1 (Ollivierre, Gubler, Toule, 1986). It is suggested by studies in an animal model that inhibition of these enzymes could lead to an effective treatment of osteoarthritis.

There are evidences for intrinsic cartilage enzymes. Lucy (Lucy, Dingle and Fell, 1961) first showed that vitamin A, presumably acting by rupturing lysosomal membranes in the cartilage, could lead to destruction of the cartilage since the lysosome is a membrane-bound organelle which contains a series of enzymes with degradative functions. In experiments incubating strips of rabbit ear cartilage, Ali (Ali, Evans, STainthorpe and Lack, 1967) observed that the cartilage could destroy its own matrix by showing the release of hexuronic acid, a proteoglycan degradation product, to be maximal

at pH 5. Since these enzymes showed prime activity at the acid spectrum, they are thus called acid hydrolases. Ali also initiated work with inhibitors on acid hydrolysis in cartilage degradation, it is for this reason, he is probably the first person to use these inhibitor in chemotherapy for arthritis. By demonstrating that 6-amino-hexanoic acid could inhibit the breakdown of the proteoglycanaceous part of the matrix, he suggested that acid cathepsins, especially cathepsin B, were the prime factors for the acid hydrolysis of proteoglycan. He further suggested that the active enzymes were not metalloproteases. However, it was doubtful whether the pH of the cartilage microenvironment ever reached the extremely acidic pH optima of these enzymes. Subsequent investigations carried out by Harris and his co-workers (Harris, Parker, Radin and Krane, 1970) demonstrated for the first time that degradative enzymes could be active at a neutral pH, such as those found in body fluids. They also reported that sixty percent of the proteoglycan could be lost within three days in slices of canine cartilage incubated at a neutral pH.

3.3.1 Evidences for the Role of Cartilage Enzymes in Osteoarthritis

Having demonstrated in previous work that enzymes were found in cartilage with pH optimal at both acid pH as well as neutral pH, and also one of the findings in osteoarthritis reported by Mankin et al (1970) was the loss of part of the proteoglycan portion of the matrix, it seemed logical to speculate that these cartilage enzymes might well be responsible in osteoarthritis. An investigation was then undertaken by Dingle et al (Dingle, Saklatuala,

Hembry, Tyler, Fell and Jubb, 1979) to determine whether these enzymes were increased in human osteoarthritis or not. Acid phosphatase has long been regarded as a classic marker of lysosomal activity (Mankin et al , 1970). Using a histological histochemical grading system, the level of enzyme activity in various specimens obtained at the time of arthroplasty, amputation or fracture specimens were compared as a measure of the severity of arthritis (Ehrlich, et al, 1975). One of the difficulties encountered in this type of comparison is that osteoarthritis is a focal disease, and if one just looks at all the cartilage mixed together from a femoral head, the result obtained from this determination of enzyme activity will give areas of many different degrees of severity. To overcome the problem of inaccuracy, enzyme analyses were performed on pieces of cartilage which weighed as little as 50 ug, and histological analysis was done on immediately adjacent tissue slices. By analyzing samples of osteoarthritis, it was found that as the disease became more severe, the level of enzyme activity rose correspondingly. This suggested strongly that the intrinsic cartilage enzymes were responsible for the eventual destruction. At the same time, Sapolsky and Woessner (1973, 1974, 1976) also made major contributions to this field by measuring cathepsin D activity in cartilage. They found that activity of cathepsin D was higher in osteoarthritis than normal cartilage (Sapolsky, Matsuta, Wosner and Howell, 1978).

The above mentioned direct correlation of enzyme activity and severity of arthritis seems to hold regardless of the initiating factor for the arthritis. This is supported an experimental model utilizing joint instability designed by Telhag and Lindberg (1972). In this model, both the medical collateral and cruciate ligaments were severed, and the medial meniscus was excised. As the

experiment went on, a progressive arthritis would occur, which biochemically and histologically was similar to the human arthritis condition. It was repeatedly found that the measured acid phosphatase activity showed the pattern which correlated proportionally with the severity of arthritis (Ehrlich, Mankin, Jones, Grossman, Crispen and Ancona, 1975).

3.3.2 Neutral protease activity

Evidence accumulated so far substantiates claims that degradative enzymes play an important role in osteoarthritis. However, it remains to explain how these enzymes work in vivo as these enzymes have pH optima in the acid range, and body fluids are well fairly well-buffered at pH 7.4. Woessner (1973) purified cathepsin D and found that it did not digest cartilage at a physiologic pH and in fact was completely inactive above pH 7.2.

Apart from the occurrence of cathepsin D in cartilage, Sapolsky and his co-workers (Sapolsky et al, 1974, 1976) reported on the finding of a neutral protease from cartilage that was distinct from cathepsin D. On the basis of inhibitor studies, they found that the neutral protease was a metal-dependent protease. Neutral protease activity was also reported and suggested to be an endopeptidase (Ehrlich, Mankin, Bigliani, Wright and Crispen, 1977). More recently, Woessner and Selzer (1984) purified protease even further and showed that the neutral enzyme had a molecular weight of about 56,000 and activation of the enzyme is required, i.e. there seems to be a built-in control mechanism, an inhibitor, or a latent form of the enzyme and activation of the enzyme can be achieved by the addition of p-aminophenyl mercuric acetate.

Sapolsky (Sapolsky and Howell, 1982) also reported the occurrence of another enzyme which was much smaller, with a molecular weight of 24,000-27,000. These small, metal-dependent, neutral proteoglycanases seem to be prevalent in all forms of cartilage. Similar enzymes have been described in the growth plate (Ehrlich, Armstrong, Neuman, Davis and Mankin, 1982; Ehrlich, Armstrong and Mankin, 1984).

3.3.3 Neutral Proteases in Osteoarthritis

With the presence of neutral proteases in cartilage, the next task was to examine whether or not the neutral protease activity was also increased in osteoarthritic cartilage, as in the case for acid protease. Sapolsky et al (1978) measured the activity of the metal-dependent neutral protease from ulcerated cartilage, and compared it with normal cartilage. It was found that the levels of neutral protease activity were three to four times higher in the arthritic specimens than normal cartilage specimens. The activity was also measured and compared with the severity of the arthritis. As the disease became worse, the level of neutral proteases increased, except in the very late stage. In the other late stage of osteoarthritis, the cartilage was essentially destroyed and the level of the enzymic activity declined (Lahey, Ehrlich and Mankin, 1979). Martel and Pelletier (1984), studied the release of endogenous proteoglycan from arthritic cartilage of different levels of severity. They showed concrete evidence for the release of more proteoglycan, hence higher enzyme levels, as the disease became more severe. This study was performed under physiologic

conditions so neutral proteases were being measured. Finally, Nojima and his co-workers (Nojima, Mankin and Treadwell, 1984) reported higher degradation when studying isolated chondrocytes from arthritic as opposed to normal cartilage.

3.3.4 Collagenase Activity in Articular Cartilage

The greater part of the matrix, by weight, is the collagen component. Originally, it was thought that the collagen was not degradable, since levels remained relatively constant. However, it becomes evident that there also is increased collagen synthesis (Lappiello, Hall and Mankin, 1977). With the increase of the synthesis, there must be an increase of its degradation. Also, strands of cartilage or collagen fibers are not found in the joint or synovium in most cases of osteoarthritis, suggesting that the collagen is degraded. Collagenase activity in articular cartilage was finally determined, but surprisingly it did not come from the same source as the proteoglycanase, i.e., the collagenase was not lysosomal in nature. Further, there was a regulatory system, with an inhibitor of collagenase activity found in cartilage (Ehrlich, Mankin, Jones, Wright, Crispen and Vigliani, 1977).

Almost no activity in normal articular cartilage was found, but investigation of osteoarthritic cartilage revealed enzyme levels paralleling the severity as determined by on the histological histochemical grading system and the levels of collagenase. Probably even more important, the enzyme was calcium-dependent, just as with the neutral protease. Ethylene-diamine-tetraacetate (EDTA), a chelator of metallic cations, completely abolished the activity.

In summary, since there is direct correlation between certain cartilage degradative enzyme activities and severity of arthritis, it seems logical to speculate that these enzymes might be responsible for the eventual destruction of cartilage in osteoarthritis. However, the factors and conditions for stimulating the secretion of such degradative enzymes are still not clear. There is an urgent need for establishing a study model to investigate these problems. Which is why the major aim of this study is to develop an in vitro model to identify and separate the chondrocytes in different stages of maturation, this in turn would supply us with pure subpopulations of cartilage cells for more precise investigations.

CHAPTER FOUR - METHODOLOGIES

4.1 Isolation of Chick Growth Plate & Articular Chondrocytes

Chondrocytes were isolated from chicks between three to four week. After sacrifice in a CO_2 canister, the leg bones were aseptically dissected and cleaned until free from soft tissue. The cartilaginous tissue of the distal proximal and femoral as well as the distal tibial growth plate or the articular cartilage was dissected separately. The shavings were placed in a modified F-12 medium (magnesium-free, 0.5 mM $CaCl_2$; Sigma) for maintaining the viability of chondrocytes. After rinsing and weighing, the cartilage was subjected to an digestion with 0.1% trypsin (type III; Sigma) for 30 minutes in Modified F-12 medium at 37^{o}C in order to break down the core and link protein in the cartilage matrix. The tissue was subsequently rinsed twice with modified F-12 medium and subjected to 0.1% hyaluronidase (type I-S; Sigma) digestion for 1 hour for cleavaging the proteoglycan aggregate. Overnight digestion in 0.1% collagenase (type IIA; Sigma) in a shaking water bath at 37^{o}C was required to break down the collagen fibrils. One ml of each enzyme solution was used for every 20 mg of cartilage. The cells were then filtered through a 40um mesh, centrifuged for 4 min at 600 x g, and washed twice with modified F-12 medium to get rid of the digested matrix debris. The cells were then rinsed twice in a solution of citrate-buffered saline (125 mM NaCl, 18 mM citric acid, and 10 mM K_2HPO_4, pH 6.0) at 4^{o}C to dissolve and remove

any mineral debris. After a final rinsing in modified F-12 medium, the cells were counted with a hemocytometer. The chondrocytes are then ready for culture and countercurrent elutriation.

4.2 Countercurrent Centrifugal Elutriation

Countercurrent centrifugal elutriation (CCE) method was applied for the separation of isolated growth plate chondrocytes. The separation is based on the differences in size, shape and density of cells among different subpopulations of chondrocytes. (For the principle of CCE, see APPENDIX I).

A Beckman JE-6 rotor (Beckman, Palo Alto, California) was thoroughly equilibrated with modified F-12 medium containing 5% FBS before elutriation. The cells were exposed briefly to DNase (Sigma), to prevent cell aggregation. About 200 x 10^6 cells were loaded onto the rotor in 20 ml of modified F-12 medium containing 5% FBS at 4^oC. Loading was performed at a constant rotor speed of 3,250 rpm (1,060 x g). After the cells being loaded and equilibrated in the separation chamber, elutriation was performed at different rotor speeds between 3,130 and 1900 rpm. Slight changes from the established method of O'Keefe et al (1989) in the rotor speeds were made in order to obtain better separation of the chondrocytes (see Table 4.1). The fluid flow was maintained at a rate of 20 ml/min throughout the procedure. Cells were collected for 4 min with 50 ml sterile centrifuge tubes (Corning) at each rotor speed.

4.3 Size Determination of Chondrocytes

The Coulter Counter-Channelyzer system (Coulter Electronics, Hialeah , Florida) was used to determine the size distribution of the separated cells in each tube. The median volume of the cell population was determined from the median channel number of the volume distribution, using a calibration constant derived form latex spheres of uniform size. Standard deviations for the mean volumes and diameters of the elutriation fractions were determined by analyzing the distribution curves from the Coulter Counter.

Table 4.1 Rotor speed settings for countercurrent centrifugal elutriation

< Rotor Speed: rpm >	
O'keefe's Method	Lee's Method
3130	3130
3030	3030
2930	2930
2860	2860
	2790
2730	2720
2660	2650
2600	2600
	2550
2520	2500
2440	2450
	2400
2360	2370
	2340
	2310
2280	2280
2260	2250
	2200
2120	2150
	2100
2040	2050
1960	2000
1900	1900
Number of steps: 16	23

The cell suspension of individual tube then can be pooled into several fractions as described in the result section (5.2). The cell size of the pooled fractions were determined again, after which the chondrocytes were ready culture or flow cytometry cell cycle analysis.

4.4 Chondrocyte Cell Culture

Chondrocytes were plated in 2-cm^2 24-well culture plates (Corning) at a density of 1 x 10^5 cell/cm^2 in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) containing 5% fetal bovine serum (FBS; Gibco), 50 mg/ml ascorbate (Sigma). Assay of cell cultures were performed after 16 hr in culture.

4.5 Flow Cytometry Cell Cycle Analysis of Elutriated Chondrocytes

1.5 x 10^6 chondrocytes from different elutriated fractions were directly fixed with 75% (v/v) alcohol for 24 hours. The cells were spun at 1000 rpm for 10 min and the supernatant was discarded. Since propidium iodide is specific for both RNA and DNA, 1 ml of 1mg/ml RNase in PBS was added and the resulted cell resuspension was incubated for 30 min at room temperature in order to remove all the RNA within the cells. After spinning, the cells were stained with propidium iodide (10ug/ml, 1ml/1 x 10^6 cells). Cell aggregates were removed by vortexing and filtering through 35um nylon mesh before

injection of cells into the EPICS Profile Flow Cytometer (Coulter Electronics, USA) for total DNA determination of individual cell (for detailed principle see APPENDIX II). For each sample, 2 x 10^4 cells were examined in order to determine the percentage of cells in different stages of cell cycle such as G_0-G_1 phase, S phase and G_2-M phase. The result was then being analyzed by applying the latest statistic software installed at the workstation of the cytometer.

4.6 Thymidine Incorporation Assay on Elutriated Chondrocytes

Thymidine is one of the critical building blocks of DNA synthesis. In this assay, incorporation of radioactive thymidine was used as the primary indication of DNA synthesizing ability (Puzas and Felter, 1988). Cultured cells were labeled with 5 uCi/ml [^{3}H]-thymidine (40Ci/mM) (New England Nuclear) in the presence of 5uM unlabeled thymidine as carrier in DMEM containing 5% FBS for 4 hr at 37^{o}C,. The medium with excess thymidine was aspirated and the cells were washed with 1 ml 0.15 N NaCl. The cells were then lysed with 0.25ml 0.25N NaOH. After 20 min, attached cells were scraped out with a cell scraper. The solution with cells was transferred to a 5 ml plastic tube. Another 0.25ml NaOH was used to rinse the well of the culture plate and then put into the same tube also. The alkalinity was neutralized by adding 0.5ml of 0.25 N HCl. 1ml of Hepes-Mg-Ca with 2.5 mg/ml BSA, which acted as protein carrier, was also added to the tube. DNA was precipitated with 0.5 ml of 10N perchloric acid at 4^o for 20 min. Precipitate was spun at 13,000 rpm at 4^{o}C for 30 min. After aspirating the supernatant, 0.5 ml of 0.25N NaOH was added to resuspend the pellet. The resuspension was transferred to counting

vials and 4 ml of Ecoscint, a scintillation fluid, was added. The radioactivity of the acid-insoluble DNA content was measured by a Beckman liquid scintillation spectrometer. Standards of the radiolabeled medium were prepared for direct calculation of the incorporation of thymidine into DNA.

4.7 Sulfate Incorporation Assay on Elutriated Chondrocytes

Sulfate containing glycosaminoglycans, such as chondroitin sulfate and keratan sulfate, are the predominant macromolecules in cartilage matrix proteoglycan. Thus, the incorporation rate of radioactive sulfate into cellular and extracellular macromolecules is a good reflection of proteoglycan synthesis (O'Keefe et al, 1989). In this assay, cell cultures were exposed to 12 uCi/ml of $^{35}SO_4^{=}$ sodium salt in DMEM, which contained 0.814 mM carrier sulfate, for 4 hr at 37^{o}C. After incubation, the medium was transferred into small plastic tube. Cells were lysed by adding 0.25 ml 0.25 N NaOH to each well for 20 min and then scraped out with cell scrapper. Cells in NaOH were transferred to the same plastic tube. Another 0.25 ml NaOH was used to rinse the well of culture plate and transferred to the tube also. 0.75 ml of 0.15N NaCl was added. The solution was dialyzed against phosphate buffered saline (1:19 in water) with 12,000 - 14,000 MW dialysis tubing (Spectra) in order to remove unincorporated radiolabeled sulfate of small molecular size. Phosphate buffered saline (PBS) were changed twice a day. After 3 days, 0.75 ml of sample inside the dialysis tubing was placed into a scintillation vial and 4

ml of Ecoscint added. The radioactivity of $^{35}SO_4^{=}$ incorporated macromolecules were quantified by a Beckman scintillation spectrometry.

4.8 Hyaluronidase Assay on Elutriated Chondrocytes

Hyaluronidase being one of the degradative enzymes, its activities in chondrocytes of various elutriated fractions are determined to show the degradation rate of cartilage matrix. It is a endohexosaminidases and catalyze the degradation of hyaluronic acid with the liberation of acetylglucosamine terminal groups which can be measured in a colorimetric assay (Bergmeyer, 1984).

In the assay, the anhydro-sugar is first formed from N-acetyl-glucosamine in alkaline solution. This is then converted in acid solution to the furan derivative, which reacts with 4-dimethylaminobenzaldehyde to form a coloured complex. The amount of acetylglucosamine liberated per unit time is a measure of the hyaluronidase activity.

For the first step in the assay, acetate buffer (50 mM, pH 4.0) containing NaCl (0.15 M) and hyaluronic (1 g/l) acid solution were used after preincubation at 37^{o}C for 15 min. Culture medium was removed. Assay was carried out in culture plate after the cells froze and thawed three times with dry ice. The reaction was started when 0.2 ml acetate buffer and 0.8 ml hyaluronate solution were added into the culture well. After incubating for 20 hr at 37^{o}C, 0.5 ml reaction solution was transferred into a tube containing 1 ml tetraborate solution, then heated for 3 min in boiling water-bath and followed by cooling under running tape water. 3ml of dimethylaminobenzaldehyde

reagent (Sigma) was added. Mixture vortexed and incubated for 20 min. in 37^{o}C water-bath. Absorbance then read at 585 nm immediately.

4.9 Alkaline Phosphatase Assay on Elutriated Chondrocytes

Alkaline phosphatase is an important maturational marker and its activity increases in the hypertrophic zone of the growth plate. In this assay, the enzyme activity was measured using a well established colorimetric method with p-nitrophenyl phosphate as the substrate (O'Keefe, 1989). Activity of alkaline phosphatase was measured directly in the culture wells containing approximately 1 x 10^5 cells/well after the isolated chondrocytes have settled for 16 hours. The culture medium was aspirated from the wells, the cells were then rinsed with 150 mM NaCl solution and supernatant again aspirated. 1 ml of reaction buffer containing 0.25 M 2-methyl-2-amino propanol, 1 mM MgCl, and 2.5 mg/ml p-nitrophenyl phosphate (Sigma) at pH 10.3 was then added to the wells at 37^{o}C to initial the reaction. After the reaction was stopped by adding 0.5 ml 0.3M trisodium phosphate (pH 12.3, Sigma), the absorbance at 410 nm was measured using 0.15 M NaOH as blank.. The activity of acid phosphatase was calculated from standard curve prepared by using p-nitrophenol standards.

4.10 Acid Phosphatase Assay

4.10.1 Total Acid Phosphatase Assay on Elutriated Chondrocytes

Acid phosphatase activity was measured with a method modified from that of alkaline phosphatase assay (see section 4.9) directly in the culture wells when the chondrocytes had been settled for 16 hours. After aspirating the medium and washing with 150 mM NaCl solution, 0.5 ml 180mM citrate buffer (pH 4, Sigma) was added. Then 0.5 ml of 6 mg/ml p-nitrophenyl phosphate (Sigma) was added and initiating the reaction. After two hour of incubation at 37^{o}C, the reaction was stopped by adding 0.5 ml 0.48M trisodium phosphate (pH 12.3 Sigma), and the absorbance at 410 nm was measured using 0.15 M NaOH as blank. The activity of acid phosphatase was calculated from standard curve prepared by using p-nitrophenol standards.

4.10.2 Optimal pH Determination of Phosphatase in Isolated Chondrocytes

The phosphatase activities in cultured articular and growth plate chondrocytes at different pH were determined with modified acid phosphatase assay method. After aspirating the medium and washing with 150 mM NaCl solution, 0.5 ml buffer (180mM citrate buffers, pH 2, 3, 3.6, 4.2, 4.8, 5.6; 50 mM PIPES buffers, pH 6, 7; 150mM AMP buffer, pH 8,9,10.10.2; Sigma) was added. Then 0.5 ml of 6 mg/ml p-nitrophenly phosphate (Sigma) was added

and reaction started. After two hours of incubation at 37^{o}C, the reaction was stopped by adding 0.5 ml 0.48M trisodium phosphate (pH 12.3 Sigma), the absorbance at 410 nm was measured using 0.15 M NaOH as blank. The activity of acid phosphatase was calculated from standard curve prepared by using p-nitrophenol standards.

4.10.3 Enzyme Kinetics of Acid Phosphatase of Isolated Growth Plate and Articular Chondrocytes

Enzyme kinetics curve was determined by testing acid phosphatase activity with different substrate concentrations. Assay method was modified from section 3.2.10.1. After aspirating the medium and washing the cells with 150 mM NaCl solution, 1 ml 180 mM citrate buffer (pH 4, Sigma) was added. Then 0.5 ml of p-nitrophenly phosphate (0.75, 1.5, 3, 6, 12, 24 mg/ml; Sigma) was added to start the reaction. After two hours of incubation at 37^{o}C, the reaction was stopped by adding 0.5 ml 0.48M trisodium phosphate (pH 12.3 Sigma), and absorbance at 410 nm was measured immediately with 0.15M NaOH as blank solution. The activity of acid phosphatase was calculated from standard curve prepared by using p-nitrophenol standards. The kinetics curve was obtained by plotting reaction velocity against velocity over substrate concentration.

4.10.4 Tartrate Inhibition Effect on Acid Phosphatase of Growth Plate and Articular Chondrocytes

Tartrate is a competitive inhibitor of acid phosphatase. On the basis of sensitivity to tartrate inhibition. two types of acid phosphatase isoenzymes can be identified, i.e. tartrate sensitive acid phosphatase (TSAP) and tartrate resistant acid phosphatase (TRAP). TSAP has been detected in osteoblasts, osteocytes and osteoclasts, whereas TRAP is present only in osteoclasts. In this assay, activity of acid phosphatase isoenzymes were measured directly in the culture wells after the isolated chondrocytes had been settled for 16 hours. After aspirating the medium and washing with 150 mM NaCl solution, 0.5 ml 180mM citrate buffer (pH 4, Sigma) was added. 0.5 ml potassium tartrate (18.75, 37.5, 75, 150, 300 mM; Sigma) 0.5 ml H_2O was used as control. After 2 hours of incubation at 37^oC reaction was stopped by adding 0.5 ml 0.48M trisodium phosphate (pH 12.3 Sigma), and absorbance at 410 nm was measured immediately using 0.15M NaOH as blank. The activity of acid phosphatase was calculated from standard curve prepared by using p-nitrophenol standards.

4.10.5 Distribution of Acid Phosphatase Isoenzymes Among Chondrocytes of Different Size

Activity of acid phosphatase isoenzymes were measured directly in the culture wells when the chondrocytes from different elutriated fractions had been settled for 16 hours. After aspirating the medium and the cells washing with 150 mM NaCl solution, 0.5 ml 180mM citrate buffer (pH 4, Sigma) was

added. 0.5 ml 300 mM potassium tartrate (Sigma) 0.5 ml H_2O was used as control. Then 0.5 ml of 6 mg/ml p-nitrophenol phosphate (Sigma) was added and the reaction started. After two hours of incubation at 37°C, the reaction was stopped by adding 0.5 ml 0.48M trisodium phosphate (pH 12.3 Sigma), and the absorbance at 410 nm was measured immediately with 0.15M NaOH as blank. The activity of acid phosphatase was calculated from standard curve prepared by using p-nitrophenol standards.

4.11 Hormonal Effects on Acid and Alkaline Phosphatase Activity in Growth Plate and Articular Chondrocytes

In order to have a better insight about the influence of various hormones on the activities of acid and alkaline phosphatase in chick growth plate and articular chondrocytes, eight commonly used growth factors have been tested. Besides testing the cellular enzyme activity of these two enzymes, the excretory enzyme in the culture medium was also tested.

In the assay, after chondrocytes released from the growth plate or articular cartilage they were transferred to culture wells with DMEM containing 5% FBS and allowed to settle for 16 hours, medium was then exchanged with freshly prepared DMEM with 2 % BSA and different growth factors. The concentration of each growth factors used were listed in the next page:

Growth Factors	Concentration
TGF-β	0.1, 0.3, 1, 3, 10 ng/ml
FGF-a	1, 3, 10, 30, 100 ng/ml
FGF-b	1, 3, 10, 30, 100 ng/ml
IGF-I	1, 3, 10, 30, 100 ng/ml
EGF	0.01, 0.1, 1, 10, 25 ng/ml
GH	0.075, 0.75, 7.5, 75, 750 ug/ml
PTH	10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} M
D3	10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} M

After 24 hours incubation, cellular alkaline and acid phosphatases were measured by the assay methods described in section 4.9 and 4.10 respectively. Level of secreted alkaline phosphatase was measured by transferring 0.5ml medium from the culture into a test tube. Enzyme reaction was initiated by adding 0.5 ml 0.5 M 2-methyl-2-amino propanol, 2 mM MgCl, and 5 mg/ml p-nitrophenyl phosphate (Sigma) at pH 10.3 to the test tube. After 5 min incubation at 37^{o}C, 0.5ml of 0.48 M trisodium phosphate, pH 12.3 was added to stop the reaction. The absorbance at 410 nm was measured immediately. The activity of alkaline phosphatase was calculated from standard curve prepared by using p-nitrophenol standards.

Level of secreted acid phosphatase was measured by transferring 0.5ml medium from the culture into a test tube. 0.5 ml 180mM citrate buffer (pH 4, Sigma) and 0.25 ml 600 mM potassium tartrate (Sigma) were added, whereas 0.5 ml H_2O was used as control. 0.5 ml of 6 mg/ml p-nitrophenol phosphate

(Sigma) was then added to start the reaction. After two hours of incubation at 37^{o}C, the reaction was stopped by 0.5 ml 0.48M trisodium phosphate (Sigma). The absorbance at 410 nm was measured immediately. The activity of acid phosphatase was determined in the same way as that for secreted alkaline phosphatase.

CHAPTER FIVE - RESULTS

5.1 Morphology of the Isolated Chick Chondrocytes

After digesting the cartilage specimen with trypsin, hyaluronidase and collagenase in the exact sequence as described in the methodologies section (4.1), spherical chondrocytes were released as shown in Figure 5.1. Then the cells were either cultured in 24 wells culture plate or separated into subpopulations by countercurrent centrifugal elutriation. The cell would remain spherical for the first 24 hour cultivation and then some of them become flattened as shown in Figure 5.2a & 5.2b.

4.2 Countercurrent Centrifugal Elutriation

Using the elutriation protocol published by O'keefe (1989), the mean cell volume and mean cell diameter of the elutriated cells obtained in each collection tube were determined by Coulter channelizer. The cell population obtained after countercurrent centrifugal elutriation had one single peak with the mean volume about 450 um^3(mean diameter about 9.5 um). When the new elutriation protocol, described in the methodologies

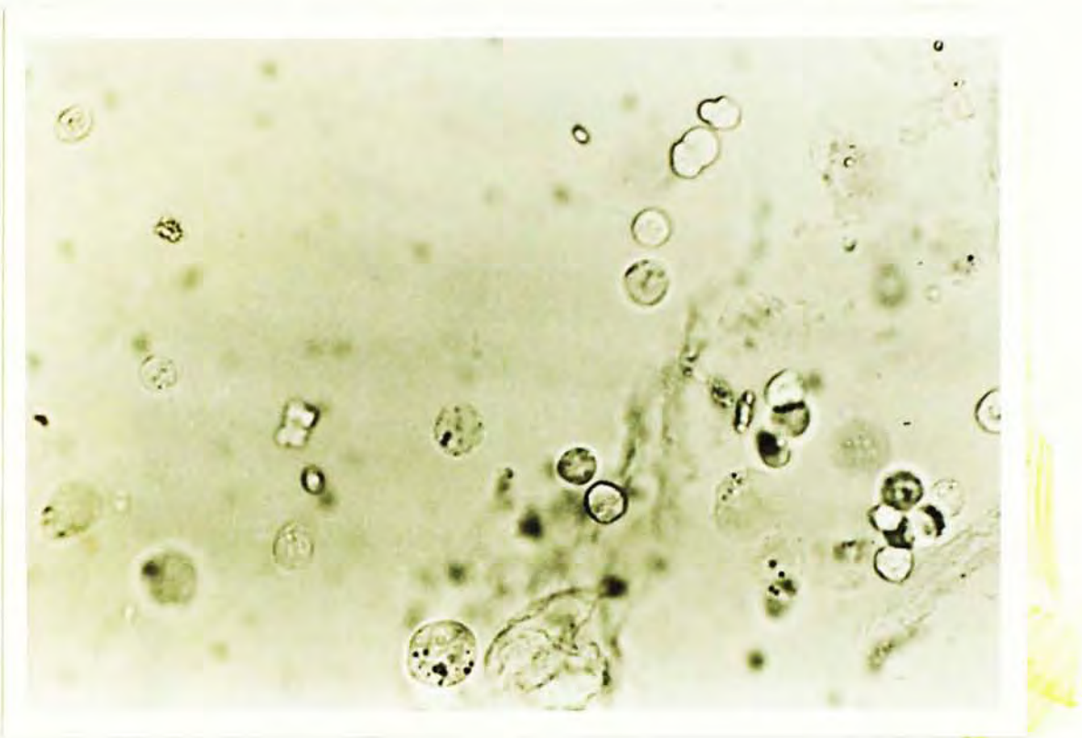

Figure 5.1 Chondrocytes released from chick growth plate after enzyme digestion. They are heterogeneous in terms of cell size. (phase-contrast microscopy, x 100)

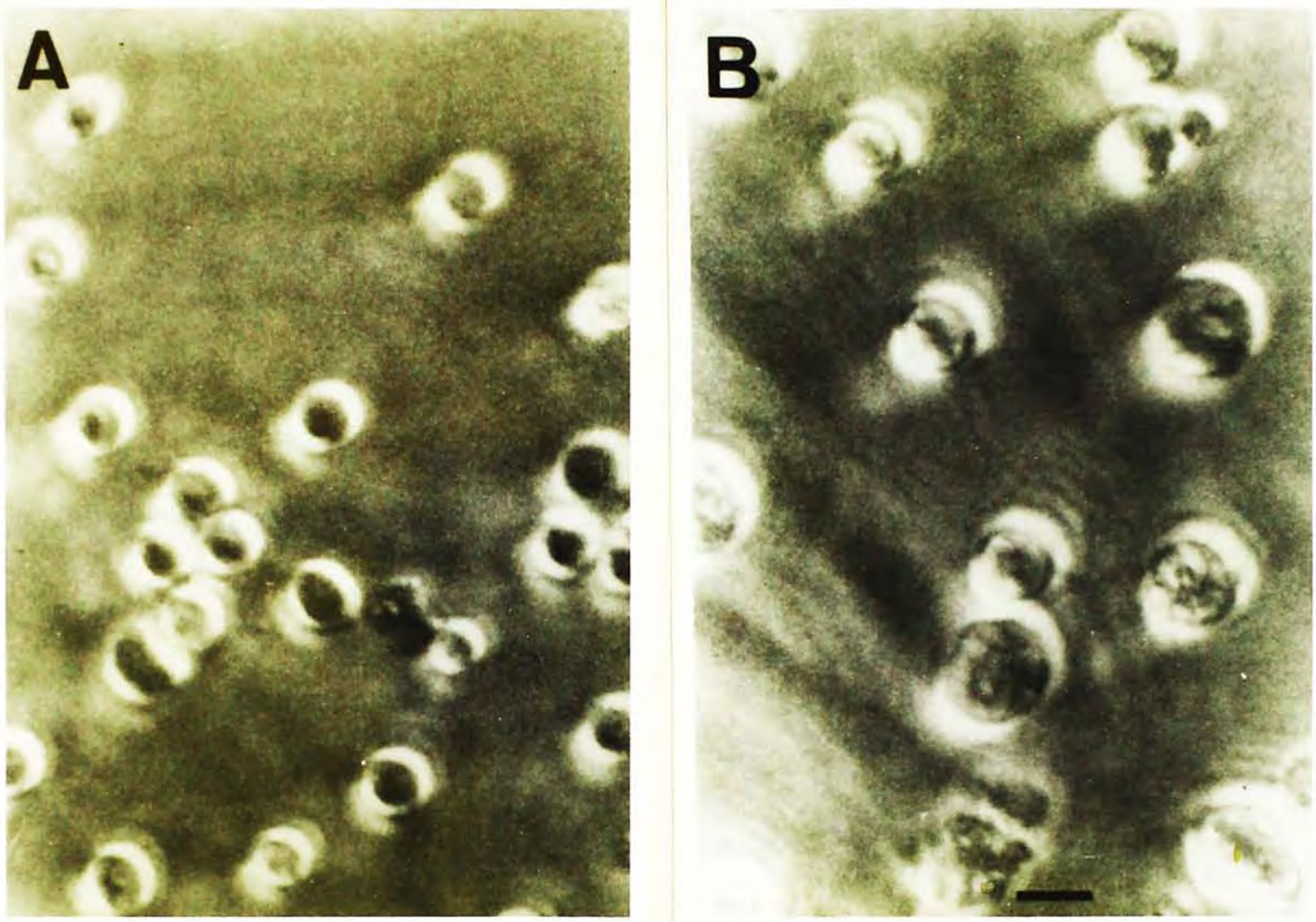

Figure 5.2 Cultured chondrocytes separated by elutriation. Photographs of first (A) and eighth (B) fractions of elutriated chondrocytes after 24 hours in monolayer culture. The cells were plated at subconfluence in Dulbecco modified Eagle medium containing 5 % fetal bovine serum (phase-contrast microscopy, x 132).

section (4.3) was adopted, a distinctive subpopulation of chondrocytes with larger size (mean volume about 680 um^3 and mean diameter about 11 um), was obtained (Figure 5.3a & 5.3b). Obviously, this could be the hypertrophic chondrocyte subpopulation, whereas the large subopulation of smaller size cells might contain the resting and proliferative chondrocytes. In order to distinguish the proliferative chondrocytes from the resting chondrocytes, thymidine incorporation assay was used to measure their different DNA synthesizing activities. Figure 5.3a & 5.3b also show how the cells in each collection tube were pooled into different fractions for subsequent investigations. The mean cell diameters and mean cell volume of chondrocytes in each pooled fractions were determined using Coulter Counter-Channelyzer System, and the results are shown in Table 4.1.

Table 4.1 Size Differences of Chondrocytes in Various Elutriated Fractions

Fraction no.	Mean diameter (um)	Mean volume (um^3)
F1	8.374	307
F2	8.880	366
F3	9.334	425
F4	9.668	473
F5	10.050	532
F6	10.48	603
F7	11.240	745
F8	11.860	875

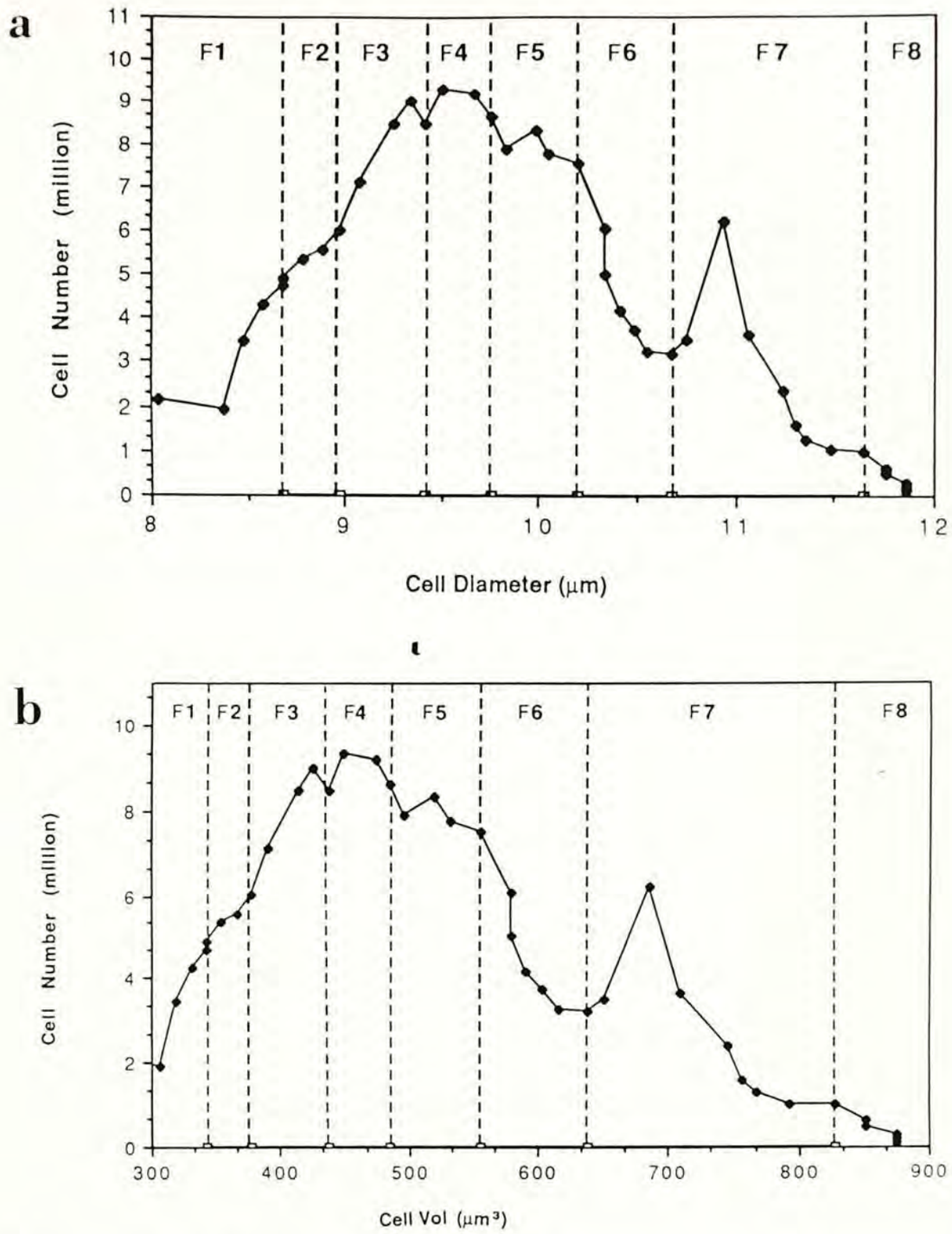

Figure 5.3 Size distribution pattern of elutriated chondrocytes (a. cell diameter; b. cell volume). A distinct peak of subpopulation with mean diameter of 11 um and mean volume of 745 um^3 was observed. The mean diameter and volume of the main growth plate population is about 9.5 um and 470 um^3 respectively. The dotted lines show how the cells in each collection tube were pooled into different fractions for subsequent investigations.

5.3 Thymidine Incorporation Assay on Elutriated Chondrocytes

Having allowed the chondrocytes settled for 16 hours in the culture wells, the DNA synthesis activity of each fraction of chondrocyte was determined by using tritium labeled thymidine incorporation assay. The result obtained was presented in Figure 5.4. There was a tendency for the increase of thymidine incorporation with the increased cell size. The highest thymidine incorporation rate was detected in fraction F6, indicating that proliferative chondrocyte subpopulation have a mean cell volume just smaller than the hypertrophic subpopulation.

5.4 Flow Cytometer Cell Cycle Analysis of Elutriated Chondrocytes

Since prolong cultivation and incubation are required for the thymidine incorporation assay, one could argue that the isolated chondrocytes may have gone through in vitro differentiation. A more direct and accurate method is thus needed to confirm the result of the thymidine incorporation assay. Flow cytometer cell cycle analysis (FCCCA) is a method well suited for this purpose.

Chondrocyte from different elutriated fractions were fixed in 75% alcohol directly. After removing the RNA content by treating with RNase, the DNA was stained with propidium iodide. The total DNA content of individual cells were then measured by flow cytometry. The percentages of cell in

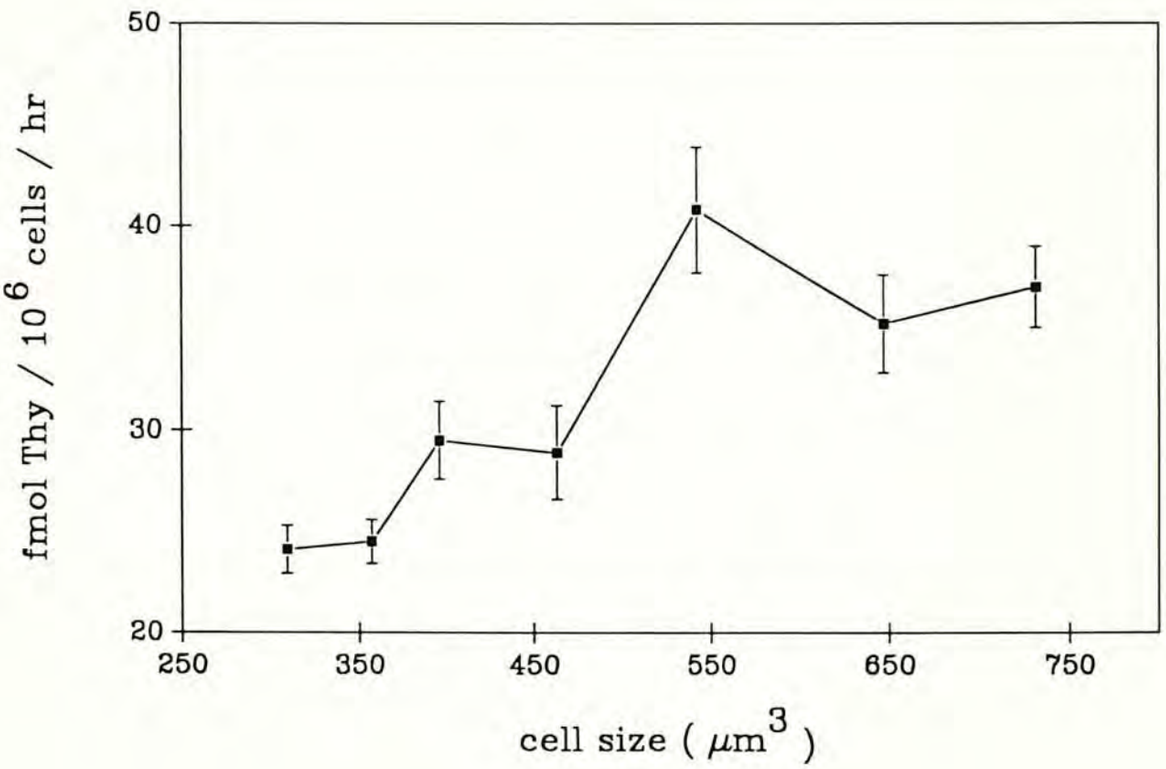

Figure 5.4 Thymidine incorporation rates of chondrocytes in various fractions after 16 hours of cultivation. An increase in uptake of [^{3}H]-thymidine was observed with increase in cell size. A distinct peak was present in fraction F6.

different cell cycle stages were analyzed by the data analysis program of the cytometry work station. G_0-G_1 referred to the cell cycle stage that the DNA content remained at 2N. S phase cell had DNA content more than 2N. G_2-M phase cell referred to the cells just before mitosis and contained double the normal DNA. The higher the percentage of G_2-M cell, the more active the cells in proliferation.

Figure 5.5 demonstrated the percentage of G_2-M cells in relation to cell size. Cell population with diameter smaller than 9.5 um contains less than 2 % G_2M phase cell. Generally, this is accepted to be representing the resting population. When the cell size became larger, the percentage of G_2M cell increased and the peak fraction which contained about 10 % G_2M phase cell, was located at the fraction F6 with a mean cell diameter about 10.5 um. This could be regarded as the proliferative subpopulation of chondrocyte and this finding is consistent with that of the thymidine incorporation assay. Then the G_2M cell percentage reduced while the cell size further increased. Due to the insufficient number of cells obtained after elutriation, cell fractions larger than 11.4 um diameter had not been examined.

By using the CCE method coupled with FCCCA, relative homogeneous subpopulations of chondrocyte, i.e., resting, proliferative and hypertrophic chondrocytes can now be identified and used for subsequent characterization assays.

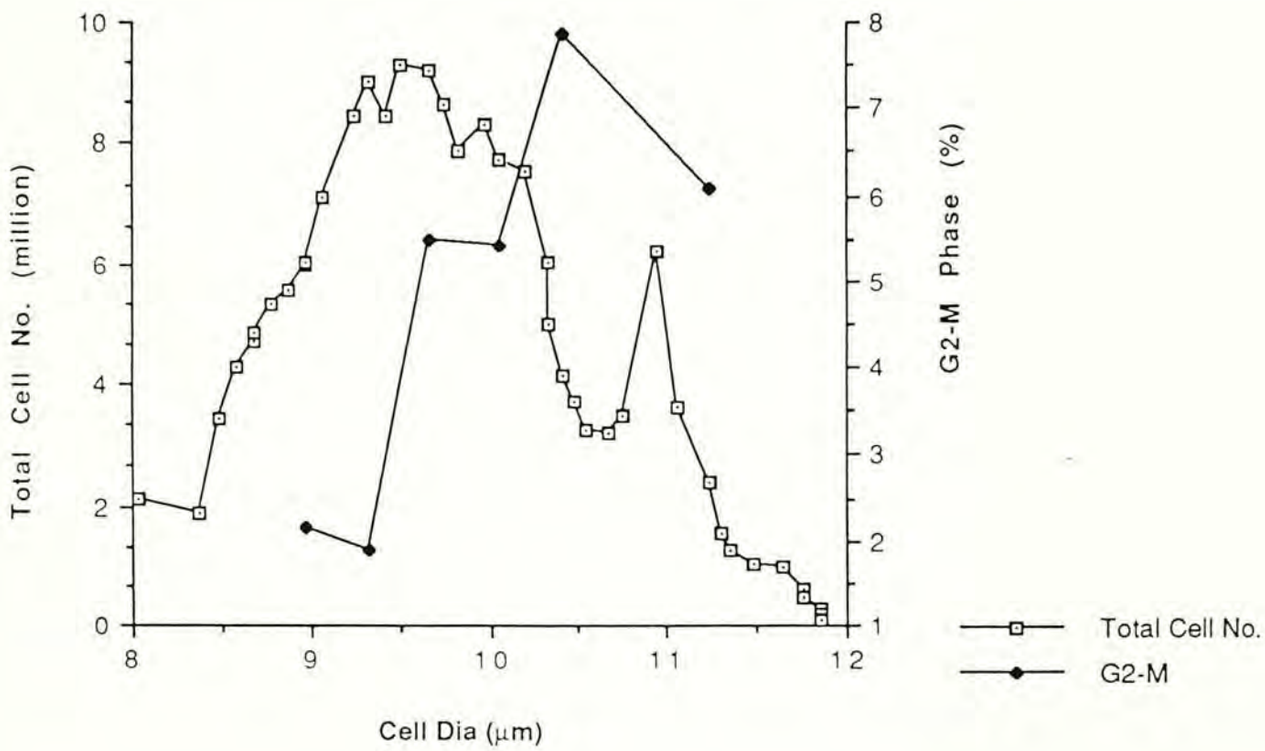

Figure 5.5 Percentages of G_2M phase cells, determined by flow cytometry, present in various elutriated fractions of chondrocytes. Cell population with diameter smaller than 9.5 um contains less than 2 % G_2M phase cell. Generally, this is accepted to be representing the resting population. The maximum percentage of G_2M phase was found at fraction F6, with cell diameter 10.5 um. i.e., about 10 %. This fraction could be regarded as the proliferative subpopulation of chondrocytes.

5.5 Sulfate Incorporation Assay on Elutriated Chondrocytes

Figure 5.6 presented the sulfate incorporation rate in various elutriated fractions of chondrocyte. For the cells with volume less than 340 um^3, sulfate incorporation rate was low, about 1 $nmol/10^6$ cells/hr. The rate was raised when the cell size increased. It remained at a high level, about 1.5 $nmol/10^6$ cells/hr, for the cells equal to or larger than 10.4 um in diameter.

5.6 Hyaluronidase Assay on Elutriated Chondrocytes

Figure 5.7 showed the hyaluronidase activity of different elutriated fractions of chondrocyte. The enzyme activity was 2.6 $nmol/10^6$ cells/hr with some fluctuation for cells of up to 600 um^3. For cells larger than 600 um^3 in volume, the activity rised and then remained at a higher level, about 3.8 $nmol/10^6$ cells/hr. The results obtained in this assay was similar to that of the sulfate incorporation assay. The implication on this findings will be discussed in section 6.2 of next chapter.

5.7 Alkaline Phosphatase Assay on Elutriated Chondrocytes

From figure 5.8, it was clear that alkaline phosphatase activity increased from 2.5 to 4 $mmol/10^6$ cells/hr while the size of chondrocytes increased from 300 to 870 um^3 in volume. Result obtained showed a trend of the larger the cells, the higher the alkaline phosphatase activity.

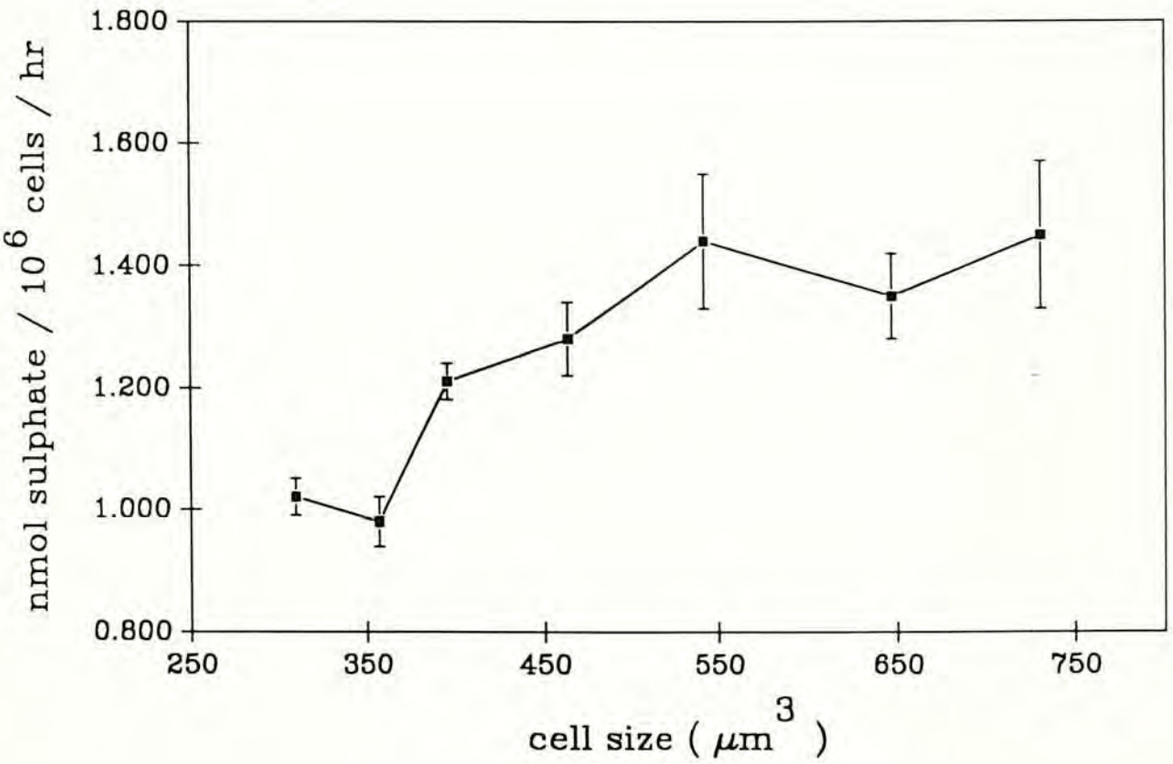

Figure 5.6 Sulfate incorporation rate of chondrocytes in various elutriated fractions after 16 hours of cultivation. For cells with diameter less than 9.5 um, sulfate incorporation rate was about 1 nmol/10^6 cells/hr. The rate rised when the cell size increased. It remained at a high level, about 1.5 nmol/10^6 cells/hr, for the cells equal to or larger than 530 um^3 in volume.

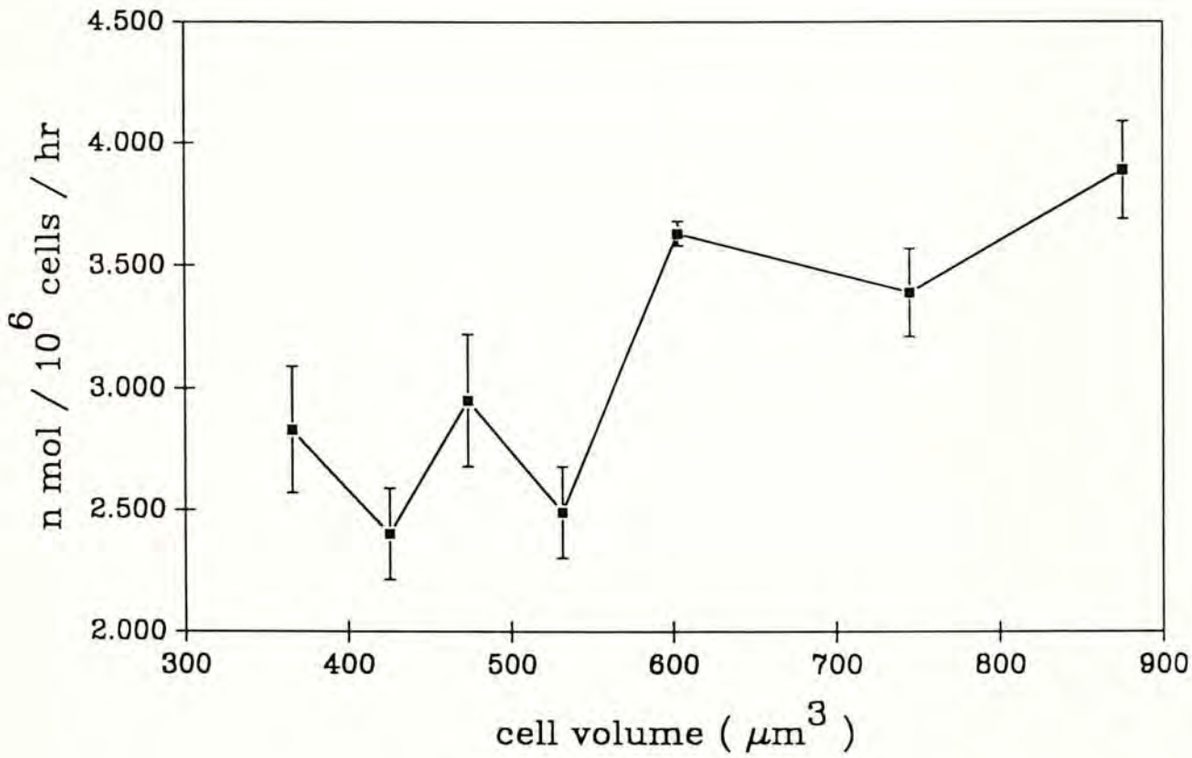

Figure 5.7 Hyaluronidase activities of chondrocytes in various elutriated fractions after 16 hours of cultivation. The enzyme activity was kept at low level of about 2.6 nmol/10^6 cells/hr, with fluctuation. For the cell larger than 600 um^3 in volume, it remained at a higher level of about 3.6 nmol/10^6 cells/hr.

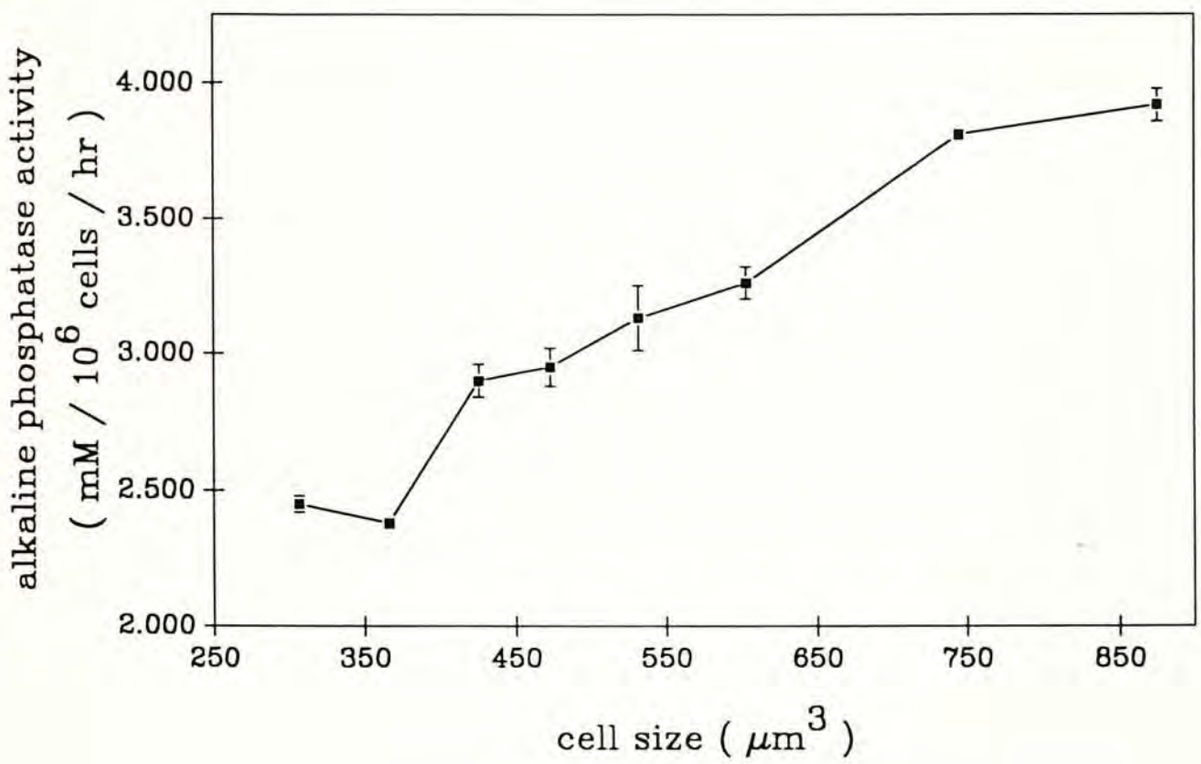

Figure 5.8 Alkaline phosphatase activities of chondrocytes in various elutriated fractions after 16 hours of cultivation. Enzyme activities increased from 2.5 to 4 mmol/10^6 cells/hr while the size of chondrocytes increased from 300 to 870 um^3 in volume.

5.8 Acid Phosphatase Assay

As acid phosphatase in isolated chondrocytes have not been investigated in vitro before, this enzyme in chick growth plate and articular chondrocyte has been examined more extensively in the present study,

5.8.1 pH Curve of Phosphatase of Isolated Chondrocytes

Figure 5.9 showed the optimal pH of acid phosphatase in both chick growth plate and articular chondrocytes to be at 4. Moreover, a high activity of alkaline phosphatase could be observed in growth plate chondrocyte, but not in the articular chondrocytes.

5.8.2 Enzyme Kinetics of Acid Phosphatase of Isolated Growth Plate and Articular Chondrocytes

After determining the optimal pH for chick chondrocyte acid phosphatase and in order to get more information about the properties of this enzyme, the enzyme kinetics was studied by measuring the enzyme activity at different substrate concentrations. Figure 5.10a & 5.10b showed the Eadie-Hofstee plot of kinetic curve of acid phosphatase in chick growth plate and articular chondrocytes respectively. The slope of the curve representing the Km and the Y intersection representing the Vmax. The Km for the enzyme in

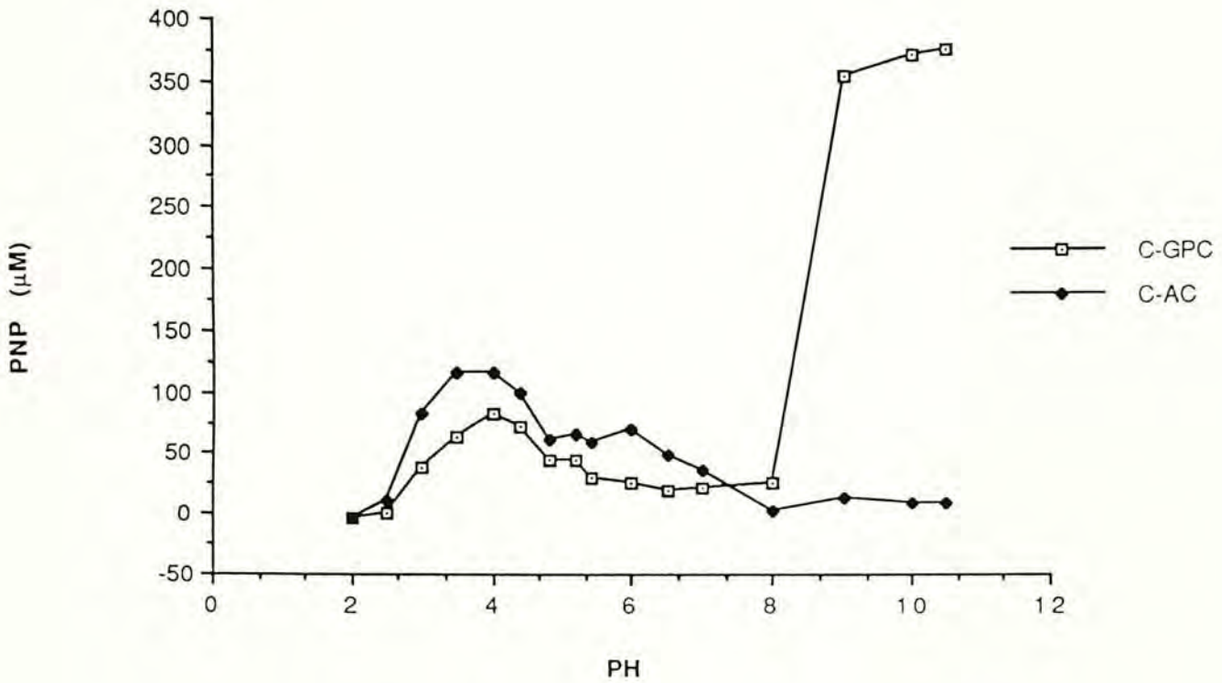

Figure 5.9 The pH curve for phosphatase activity on chick growth plate (C-GPC) and articular chondrocytes (C-AC). Acid phosphatase of both types of chondrocytes has its pH optimal at 4. A high activity of alkaline phosphatase could be observed in growth plate chondrocytes, but not in the articular chondrocytes.

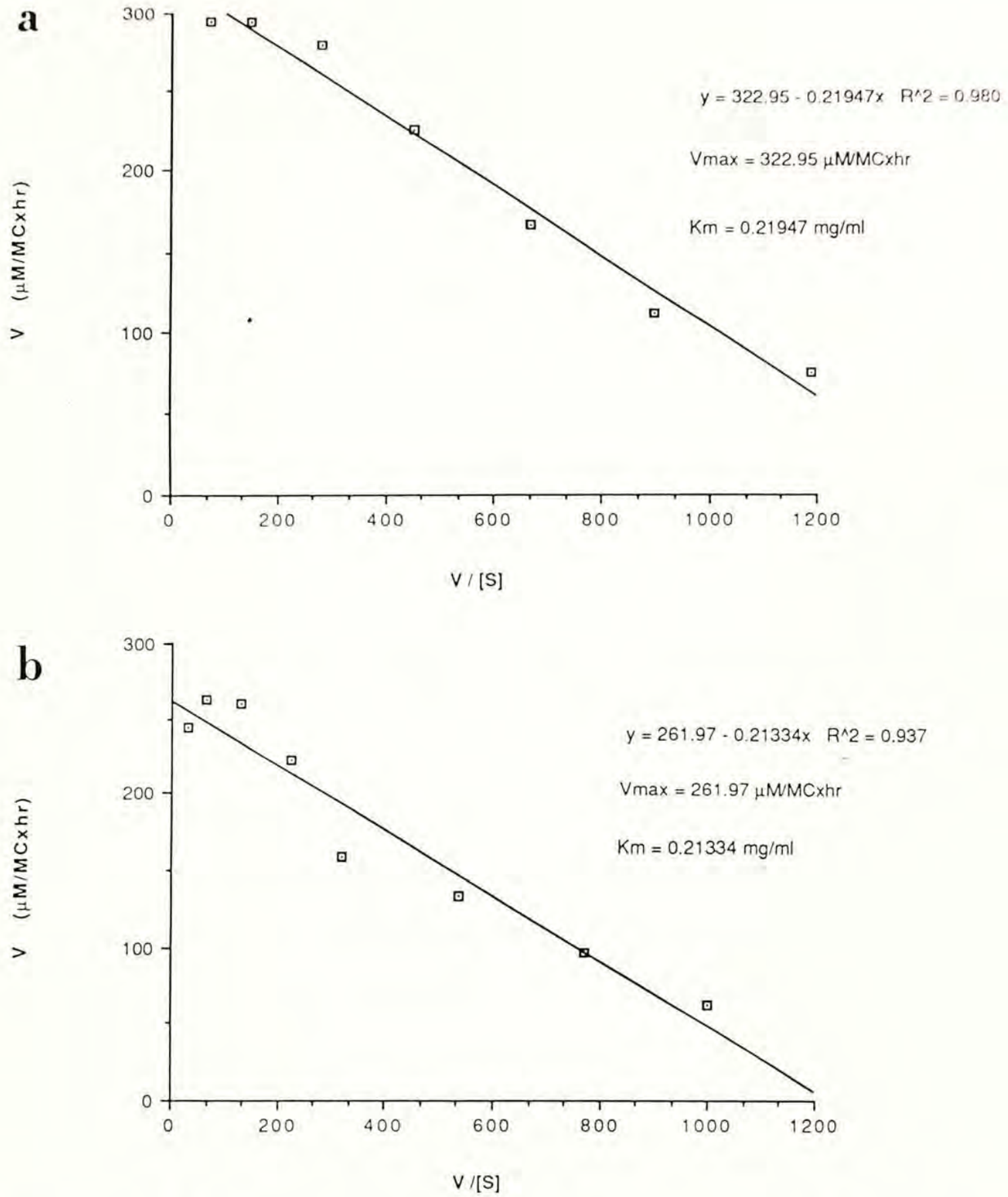

Figure 5.10 Eadie-Hofstee plot of kinetic curve of acid phosphatase in a) chick growth plate and b) articular chondrocytes. The Km for the enzymes in both types of chondrocytes were found to be almost identical, about 0.2 mg/ml. The Vmax for growth plate chondrocyte being 322.95 umol/10^6 cells/hr was larger than that for articular chondrocytes, which was 261.97 umol/10^6 cells/hr. The correlation coefficients of both curves were 0.98 and 0.937 respectively, which are considered to be quite satisfactory.

both types of chondrocytes were found to be almost identical, about 0.2 mg/ml substrate. It indicates that the acid phosphatase in both tissues may have similar properties. The Vmax for growth plate chondrocyte was 322.95 umol/10^6 cells/hr which was larger than that for articular chondrocytes, which was 261.97 umol/10^6 cells/hr. It seems that the growth plate chondrocytes may have slightly more enzyme molecules per cell than that of articular chondrocytes. The correlation coefficients of both curves were 0.98 and 0.937 respectively, which is considered to be quite satisfactory.

5.8.3 Tartrate Inhibition Effect on Acid Phosphatase of Growth Plate and Articular Chondrocytes

When potassium tartrate, a competitive inhibitor of acid phosphatase, was added before the assay, the enzyme activity was suppressed as shown in Figure 5.11. 90% enzyme activity in articular chondrocyte was inhibited by 50mM tartrate. However, in chick growth plate chondrocyte 30% of acid phosphatase still remained even at the condition that 100 mM of tartrate was present. This finding suggested the presence of tartrate sensitive and tartrate resistant acid phosphatase isoenzymes within chick growth plate chondrocytes.

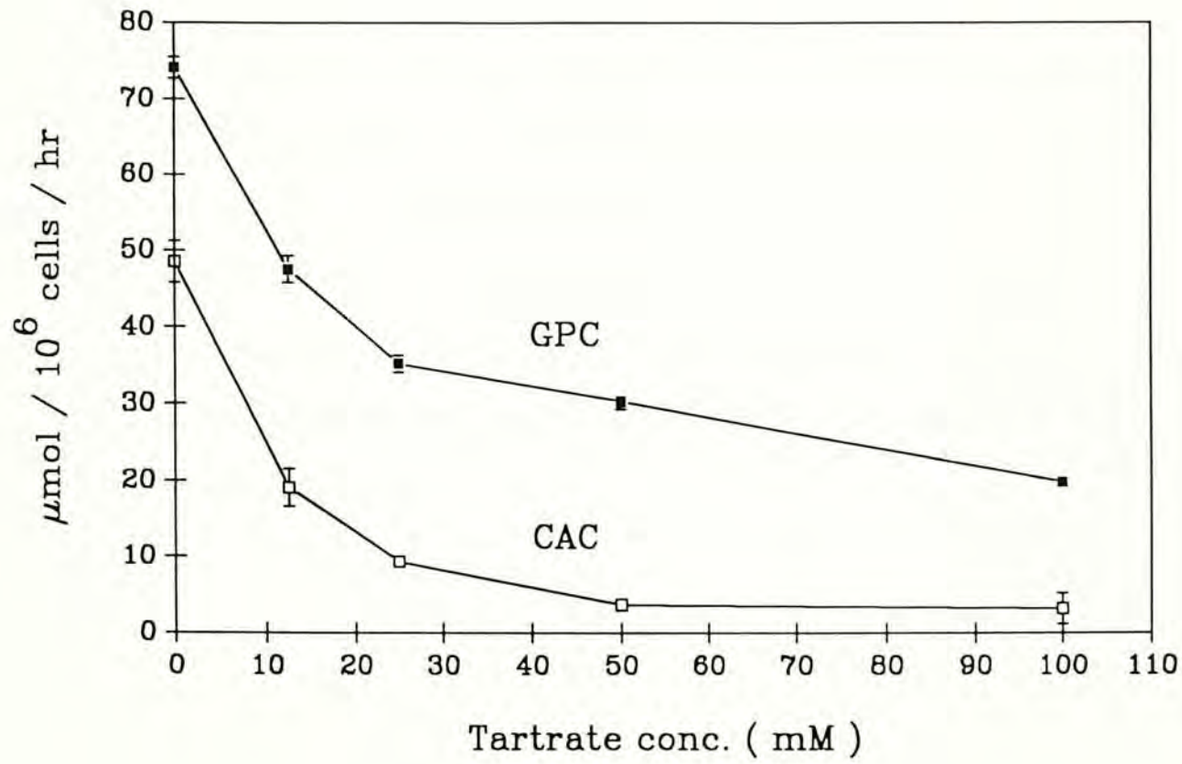

Figure 5.11 Tartrate effect on acid phosphatase activity of chick chondrocytes. 90% enzyme activity in articular chondrocyte was inhibited by 50 mM tartrate. However, in chick growth plate chondrocyte 30% of acid phosphatase still remained even at the condition that 100 mM of tartrate was present. This finding suggested the presence of tartrate sensitive and tartrate resistant acid phosphatase isoenzymes within chick growth plate chondrocytes.

5.8.4 Distribution of Acid Phosphatase Isoenzymes Among Chondrocytes of Different Size

The activities of total acid phosphatase as well as the amount of tartrate sensitive and tartrate resistant isoenzymes were investigated in each elutriated fraction of chick growth plate chondrocytes. The results were illustrated in Figure 5.12. The total acid phosphatase activity increased from 25 to 150 umol/10^6 cells/hr as cell size increases. When the cell diameter exceeded 11.4 um, the enzyme activity was dramatically reduced to a very low level of 20 umol/10^6 cells/hr. This might be due to the large number of dead cells present in the last elutriated fraction. The activities of tartrate sensitive and tartrate resistant isoenzymes performed with the same distribution pattern as the total acid phosphatase.

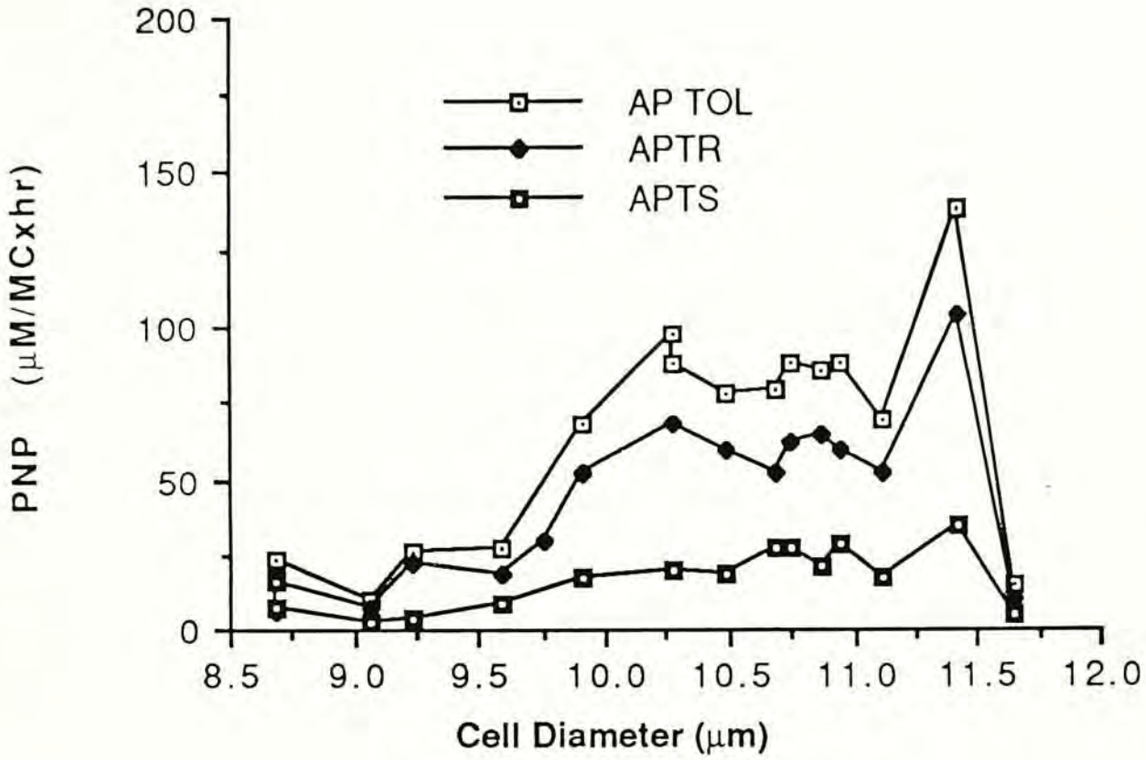

Figure 5.12 Activities of total (AP TOL), tartrate resistant (APTR) and tartrate sensitive (APTS) acid phosphatases of elutriated chondrocytes in different size. The total acid phosphatase activity increased with fluctuation from 25 to 150 umol/10^6 cells/hr as cell size increases from 8.7 um to 11.4 um in diameter. The dramatic reduction of the enzyme active for the cells with diameter exceeded 11.4 um was due to large number of dead cells. The activities of tartrate sensitive and tartrate resistant isoenzymes performed the same distribution pattern as that of the total acid phosphatase.

5.9 Hormonal Effects on Acid Phosphatase and Alkaline Phosphatase Activities in Growth Plate and Articular Chondrocytes

Eight commonly used growth factors were used here to test for their effects on acid phosphatase and alkaline phosphatase activities in chick growth plate and articular chondrocytes. The eight growth factors are listed as follow:

PTH : Parathyroid Hormone

D3 : Vitamin D_3

FGFa : Fibroblast Growth Factor - acidic

FGFb : Fibroblast Growth Factor - basic

GH : Growth Hormone

IGF-I: Insulin like Growth Factor - type I

EGF : Epidermal Growth Factor

TGF-β: Transforming Growth Factor - B type

Besides the cellular enzyme activities, the excretory enzyme activities in the medium were also determined. Table 4.2 and Table 4.3 summarized the findings of the hormonal effect. For the growth plate chondrocytes, both cellular and excretory alkaline phosphatase activities were suppressed to different extents by a number of growth factors, such as PTH, D_3, FGF-a, FGF-b and TGF-β. Their cellular acid phosphatase activity were stimulated by PTH, FGF-a and FGF-b.

For articular chondrocytes, FGF-a and FGF-b suppressed the cellular alkaline phosphatase activity. FGF-b could also inhibit the cellular acid phosphatase activity. From these studies, one may draw the conclusion that FGF-b was the most potent effector on acid and alkaline phosphatases in growth plate and articular chondrocytes.

Table 5.2 Hormonal Effects on Phosphatase Activity of Chick Growth Plate Chondrocyte

	Cellular				Excretory			
	ALP	Tol AP	TRAP	TSAP	ALP	Tol AP	TRAP	TSAP
PTH	---	++++	+	++++++++	----	0	0	0
D3	----	0	0	0	----	0	0	0
FGFa	-----	++++			--	0		
FGFb	---	++	0	+++++++	---	--	--	0
GH	0	0	0	0	0	0	0	0
IGF-I	0	0	0	--	-	0	0	0
EGF	0	0	0	0	0	0	0	0
TGF-B	--	0	0	0	--	0	0	0

Remark:
'+' = 10% stimulation
'-' = 10% inhibition
'0' = no significant changes
ALP = Alkaline Phosphatase
Tol AP = Total Acid Phosphatase

Table 5.3 Hormonal Effects on Phosphatase Activities of Chick Articular Chondrocyte

	Cellular				Excretory			
	ALP	Tol AP	TRAP	TSAP	ALP	Tol AP	TRAP	TSAP
PTH	0	--	0	--	0	0	0	0
D3	0	0	0	0	0	0	0	0
FBFa	---	0	0	0	0	+	0	0
FGFb	---	---	0	------	-	-	0	0
GH	0	0	0	0	0	0	0	0
IGF-I	0	0	0	0	0	0	0	0
EGF	0	0	0	0	0	0	0	0
TGF-B	0	0	0	0	0	0	0	0

Remark:
'+' = 10% stimulation
'-' = 10% inhibition
'0' = no significant changes
ALP = Alkaline Phosphatase
Tol AP = Total Acid Phosphatase

CHAPTER SIX - DISCUSSION

6.1 Identification of Chondrocyte Subpopulations

In the present study, an approximate yield of growth-plate chondrocytes of 100 million cells per every gram of cartilage (from two chicks) was achieved. This can be considerd a very good high yield of chondrocytes and such a high cell yield makes it possible to use chicken as a good primary model for studying endochondral ossification. In addition, results obtained from the present investigation demonstrated that growth plate chondrocytes could be effectively separated according to their size by countercurrent centrifugal elutriation. Data presented here suggested that the process of maturation occurred during endochondral ossification is marked by large increases in cellular volume which are associated with specific phenotypic changes. Furthermore, biochemical differences, including DNA synthesis, proteoglycan synthesis, as well as activities of hyaluronidase, alkaline phosphatase and acid phosphatase, were observed among the separated fractions of chondrocytes,

On the basis of the findings obtained in the present study, it is fair to said that an effective application of the technique of countercurrent centrifugal elutriation for the separation of growth plate chondrocytes has been perfected. The method described here is also capable of reproducibly and isolating accurately large numbers of cells which have well defined and

distinct biochemical and physical properties of maturation. As shown in Figures 5.3a & 5.3b, there is a distinct peak of subpopulation with mean diameter of 11 um,(690 um^3 in volume), while the mean diameter of cells of the main growth plate population is about 9.5 um (450 um^3). This small subpopulation of larger size cells might represent the hypertrophic chondrocyte subpopulation, whereas the large subpopulation of smaller size cells might contain both the resting chondrocytes and proliferative chondrocytes. Evidence to support such claims derived from studies of these subpopulation of cells by flow cytometry (for detail see below). The overlapping of these large and small subpopulations suggests that the hypertrophy of chondrocytes occurred in a progressive manner. As far as the sizes of cells of these two subpopulations are concerned, the size of the smallest released cells is about 300 um^3, whereas the largest was about 900 um^3, showing a three folds increase in size.

It has been shown in a recent in situ study (O'Keefe, Crabb and Puzas, 1989) of chondrocytes, that the increase in cellular volume during maturation from the resting and proliferative zones to the lowest portion of the hypertrophic zone of the mammalian growth plate was approximately five to nine folds (Buckwalter, Mower, Ungar, Schaeffer and Ginsberg, 1986; Hunziker, Schenk and Cruz-orive, 1987). Calibrated photomicrographs of sections of the chick growth plate have also been measured in order to estimate cellular volume of growth plate chondrocytes in situ. It was found that the diameter and the height of the flattened proliferating cells averaged

15.7 $\pm$ 0.3 um and 4.6 $\pm$ 0.2 um, respectively, resulting in an estimated cellular volume of 600 $\pm$ 0.3 um^3 in situ. The average diameter of hypertrophic chondrocytes in situ was 18.5 $\pm$ 0.3 um, resulting in an estimated volume of 3,300 $\pm$ 160 um^3. Thus, the relative increase (5.5 times) in the size of chondrocytes in the growth plate of chicks is close agreement with findings reported previously in mammalian tissue.

Although a similar increasing pattern of cellular volume occurs in the chick growth plate in the present study, there is a discrepancy between the magnitude of 5.5 fold increase in volume occurred in situ and a 3 folds increase in vitro in cellular size. As indicated in the "Summary" (see Table 5.1), the mean cellular volumes of the isolated proliferating and hypertrophic chondrocytes are smaller than the corresponding cellular volumes in situ. This suggests that chondrocytes may undergo alterations in cellular volume during or after isolation, possibly as a result of osmotic changes or due to the loss of interactions between the cells and the matrix (O'Keefe et al, 1989). Since the elutriated cells are pooled into fractions, it is possible that the smallest cells are pooled with slightly larger cells, and the largest cells are pooled with slightly smaller cells, thus decreasing the mean difference between the smallest and the largest cellular fractions. As demonstrated in the elutriation profiles (Figure 5.3a & 5.3b), there is, in fact, more than a fivefold difference between the largest cells in the last fraction and the smallest cells in the first fraction. To the best of my knowledge, there has been up to now, no

information available in literature indicating that the absolute or relative volumes of isolated chondrocytes are the same as those that are measured in situ.

Alkaline phosphatase is an important maturational marker and its activity increases in the hypertrophic zone of the growth plate (Kuhlman, 1965; Lewinson, Toister and Silbermann, 1982). Although its role is not fully understood, alkaline phosphatase is essential for calcification of the matrix (Fallon, Whyte and Teitelbaum, 1980). Extensive investigations carried out by others suggest that alkaline phosphatase plays an important role in both bone formation (Wlodarski and Reddi, 1986; Farley and Baylink, 1986) and extracellular mineralization (Fauran-Clavel and Oustrin, 1986; Register, McLean, Low and Wuthier, 1986). However, the mechanism by which the enzyme promotes calcification in mineralizing tissues, i.e., bone and cartilage, is poorly understood. Recently, de Bernard et al. (1987) provided evidence that cartilage-derived alkaline phosphatase is a glycoprotein that binds calcium ions with high affinity. This enzyme is membrane-bound and present in matrix vesicles (Grant, Sussman and Balian, 1985). In the present study, elutriated chondrocytes exhibited activity of alkaline phosphatase differentially, i.e., the largest cells had the highest levels of this enzymes, as expected for hypertrophic chondrocytes. The increase the alkaline phosphatase activity was about 5 times and the increase was expected from large cells with larger surface area of the greater plasma membrane. As for the corresponding increase in alkaline phosphatase to the cell size, the increase cannot be accounted by the greater surface area of the plasma membrane in the larger

cells. This is because the increase in volume of 2.5 times corresponds only to an increase in cellular surface area of only 1.8 folds. The differences in alkaline phosphatase are therefore not due solely to increase total area of plasma membrane in large cells, but representing a specific biochemical change associated with cellular hypertrophy. Thus, the content of this enzyme per unit of membrane area increases as chondrocytes mature in the growth plate; the present finding is consistent with earlier observations (Grant et al, 1985).

O'keefe (1989) conducted experiment on collagen typing for the elutriated chondrocyte fractions. He found that the synthesis of collagen increased with cellular volume, reaching a maximum in the fraction with mean cell volume of 463 um^3, and was maintained at a high level in the largest cells. Type X collagen was found to be present in the forms of polypeptids having molecular weight of sixty and seventy kilodaltons. Although type II collagen was present in all of the fractions of chondrocytes, the proportion of synthesis of type X collagen increased with the mean cellular volume. The peak of type X collagen was in the last fraction. The synthesis of type I collagen by the largest chondrocytes , on the other hand suggested the presence of both a-1 and a-2 collagen chains in the last fraction, although type II collagen remained the predominant structural collagen that was synthesized. This provided strong evidence to confirm that the fractions of chondrocyte with mean diameter larger than 10.65 um were hypertrophic chondrocytes.

Incorporation of [^{3}H]-thymidine has been examined in short-term cultures of elutriated chondrocytes. The rate of thymidine incorporation varied with mean cellular volume and was the lowest in the smallest cells. The incorporation of thymidine peaked in the F6 fraction and then declined slightly in the last few final fractions. The higher the rate of thymidine incorporation, the more active the DNA synthesis suggesting the greater ability in cell proliferation. As a result, this F6 fraction of elutriated chondrocytes, with mean cell volume just smaller than the hypertrophic chondrocytes, represent the proliferative chondrocyte subpopulation.

Cultured chondrocytes maintained their initial phenotypic characteristics presented in the freshly separated cells. Previous investigations demonstrated the ability of cultured chondrocytes in maintaining their specific characteristics (Prins, Lipman and Sokoloffk, 1982). Monolayer cultures, established after 16 hours of cultivation in vitro, on the other hand demonstrate only the chondrocytes phenotype both morphologically and biochemically, even after growth to confluence (O'Keefe et al, 1988). This finding confirms results of earlier investigations that the chondrocytes from different regions of the growth plate maintains a specific cellular subphenotype (Skantze, Brinkerhoff, Collier, 1985; Wier and Scott, 1986), and such finding bear important implications as it permits the study of a subpopulation of chondrocytes in culture. By using a particular phenotypic subpopulation of chondrocytes, specific interventions or stimuli can be

evaluated with special reference to their ability in promoting or inhibiting maturation. Thus, the culture of chondrocytes in vitro after they have been separated by elutriation may well be a powerful tool for the investigation of factors which control endochondral ossification.

However, it is interesting that hypertrophic chondrocytes do have certain amount of cell division ability. Recent histomorphometric studies have demonstrated that individual hypertrophic cells are responsible for the production and maintenance of increased areas of matrix (Hunziker et al, 1987). This suggests an increase metabolic activity by these cells and is supported by findings of increased numbers of mitochondria and secretory organells on a subcellular basis (Buckwalter et al, 1986). In the present study, the rates of synthesis of the components of collagen and proteoglycan matrix have been directly measured. Hypertrophic chondrocytes were found to be the most metabolically active cells and thus synthesized the largest amount of collagen and proteoglycan matrix. Therefore, the larger size of these cells is not secondary due to cellular swelling which represents the preceding of cell death, but probably the results of an active process.

As shown in Figure 5.4, hypertrophic chondrocytes incorporated [^{3}H]-thymidine at a faster rate than those smaller cells found in the earlier elutriation fractions. It is a well documented fact that isolated cells in culture may proliferate in vitro, but they do not undergo cellular division in situ. For

example, osteoblasts, myoblasts, and articular chondrocytes do not normally divide in situ, but they proliferate in cell culture (Puzas and Brand, 1986; Devlin and Konigsberg, 1983; Prins et al, 1982). Previous work by Gibson and his co-workers (Gibson and Flint, 1985) demonstrated that hypertrophic chondrocytes proliferate in culture and maintain synthesis of type X collagen. This is in contrast to in situ studies by Kember and Walker and by others demonstrating that only proliferating chondrocytes undergo synthesis of DNA in situ (Walker and Kember, 1972).

A possible explanation for the increased uptake of [^{3}H]-thymidine in hypertrophic chondrocytes is that the terminally differentiated chondrocytes from the deep hypertrophic zone may not survive the procedures of isolation and separation. Thus, the cultures and fractions may not be a true reflection of the metabolic activity of all the hypertrophic chondrocytes. However, it appears in either incidence that, although hypertrophic chondrocytes do not undergo synthesis of DNA in situ, and they do proliferate in monolayer cultures.

Although cultured chondrocytes in our system can be maintained spherical in shape for the first 24 hours, and it is believed that spherical chondrocytes will express their physiological phenotypes, previous studies with embryonic chondrocytes indicated that chondrocytes could undergo maturational changes in monolayer culture (Gibson et al, 1985). Since a 4 hours incubation period is required for the thymidine incorporation assay and the assay was performed after a period up to 16 hours to allow chondrocytes to

settle down, one could argue that the isolated chondrocytes may have gone through differentiation or dedifferentiation in vitro. In order to clarify the above mentioned argument, a more direct and accurate method is needed to measure the DNA activities in individual fractions of elutriated chondrocytes, and the flow cytometer cell cycle analysis method is well suited for this purpose.

Prior to applying flow cytometer cell cycle analysis, chondrocytes obtained from different elutriated fractions were fixed in 75% alcohol directly, thus any in vitro differentiation was avoided. Data presented here suggested very strongly that the cell population with diameter smaller than 9.5 um and with less than 2% of G_2M phase cell represents the resting population. As the cell size becomes larger, the percentage of G_2M cell increases, and the maximum amount (i.e. 10 %) of G_2M phase cell is detected at fraction F6. This is followed by a decrease in the G2M cell percentage. However, an increase in the cell size has been observed. This is consistent with findings obtained from the [^{3}H]-thymidine incorporation assay discussed above. Furthermore, it confirmed that the cell population of fraction F6 with a mean cell volume of 630 um^3 did represent the in vitro proliferative chondrocyte subpopulation. Thus, one may concludes that proliferative chondrocytes are larger than resting chondrocytes. Using countercurrent centrifugal elutriation method and coupled with flow cytometry cell analysis, relative homogeneous subpopulations of chondrocytes, i.e. resting, proliferative and hypertrophic chondrocytes can now be identified and use for subsequent studies.

6.2 Characterization of Chondrocyte Subpopulations

In order to characterize the proteoglycan synthesis ability of the chondrocyte subpopulations isolated from elutriation, sulfate incorporation assay was carried out. As mentioned in the review section (for details, see 2.1.2), sulfate containing glycosaminoglycans, such as chondroitin sulfate and keratan sulfate, are the predominant macromolecules in cartilage matrix proteoglycan. Thus, the incorporation rate of radioactive sulfate into cellular and extracellular macromolecules is a good reflection of proteoglycan synthesis.

Data obtained here showed that the synthesis of proteoglycan was greatest in the larger chondrocytes. In addition, the results of these experiments indicated clearly that for chondrocytes which have a mean volume less than 473 um^3 proteoglycan synthesizing rate was low, the rate of synthesis was raised when the cell size increased. The rate was then maintained at a high level for the cell size equal to or larger than 603 um^3 in diameter. On the basis of the data obtained from these experiments, it seems that hypertrophic phenotype are metabolically and synthetically active even after they have been isolated and plated in culture plate as primary monolayer cultures.

Having performed experiments to examine the synthetic process of proteoglycans, it seems logical to investigate the degradative aspect of the cartilage matrix. Hyaluronidase (HAase), EC 3.2.1.35, a lysosomal enzyme

with endoglucuronidase activity and capable of breaking down hyaluronic acid (HA), appears to be very good enzymes marker for this purpose (Kulyk and Kosher, 1987). Since glycosaminoglycan hyaluronate is synthesized by chondrocytes and has both an important role in forming proteoglycan aggregates in the extracellular matrix of cartilage, and possibly, a regulatory role in controlling the extent of proteoglycan synthesis by the cells, the regulation of hyaluronate turnover may therefore be of some importance in the maintenance of normal cartilage function (Bansal and Mason, 1986). It has generally been assumed that the turnover of extracellular HA occurred at the onset of chondrogenesis is controlled by the appearance or increase in activity of the enzyme hyaluronidase. Toole (1972) reported that HAase activity was first become detectable in whole limb buds at about the time when metachromatic cartilage matrix is forming in proximal regions of the limb. A recent proposed model (Oster, Murray and Maini, 1985) of limb chondrogenesis suggested that the onset of the crucial condensation process of proteoglycan, which is regulated by the appearance of a secreted HAase, degrades extracellular HA thus allows close juxtaposition of the cells.

It is rather surprising to discover here that the hyaluronidase activities of different elutriated fractions of chondrocytes (Figure 5.7) have a pattern similar to that of proteoglycan synthesis (Figure 5.6). The enzyme activity remained at a low level in resting subpopulation whereas a higher level of hyaluronidase activity was observed in both the proliferative and hypertrophic subpopulation. The increase in activity of such enzyme marker indicates that

the chondrogenesis starts when the chondrocytes have been triggered to proliferate and chondrogenesis continues during the hypertrophy of the cells.

On the other hand, the high proteoglycan synthetic rate and degradative rate occurred in these subpopulations during initial cell division and cell enlargement seems to be logical because modification is needed in the surrounding matrix to provide lacunae with different sizes to accommodate chondrocytes of different differentiation states. This also implies that interaction with the surrounding rigid matrix is probably one of the major events involved in chondrocyte differentiation. It also suggests that during chondrogenesis the matrix is in a dynamic equilibrium between synthesis and degradation while it is relatively quiet or stable in the resting zone.

6.3 Characterization of Acid Phosphatase in Chick Chondrocytes

Acid phosphatase is an orthophosphoric-monoester phosphohydrolase with an (low pH) acid optimum (EC 3.1.3.2). It hydrolyzes a number of phosphomonesters and phosphoproteins but phosphodiesters are not hydrolyzed by this enzyme (Stadtman, 1961), and non-specific acid phosphatase activity is widely distributed throughout living cells. In human and animal tissues, acid phosphatase occurs in the lysosomes of human and animal tissues. Extra-lysosomal acid phosphatase are also shown to be present in many cells, and because of its clinical importance, acid phosphatase secreted

by the human prostate gland has attracted most attention. Although acid phosphatase has been found in many human tissues, particularly in calcifying human cartilage, its role in the process of calcification, however, is not yet understood (Vetter, Helbing, Heinze, Gammert and Landolt, 1985).

In addition, isoenzymes of acid phosphatase have been demonstrated in a variety of cells (Barka 1961; Moore and Angeletti 1961; Li et al. 1970; Lin and Fishman 1972). On the basis of sensitivity to tartrate inhibition, two types of acid phosphatase isoenzymes can be identified in bone i.e. tartrate sensitive and tartrate resistant acid phosphatase. 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; Minkin, 1982; Ibbosoj, Roodman, McManus and Mundy, 1984). Tartrate-sensitive acid phosphatase (TSAP) has been cytochemically localized in osteoblasts, osteocytes and osteoclasts, whereas tartrate-resistant acid phosphatase (TRAP) is present only in osteoclasts (Hammarstrom, Anderson, Marks and Toverud, 1983). As a result, tartrate-resistant acid phosphatase as a histochemical and biochemical markers for the osteoclast would be an additional experimental tool to study osteoclastic differentiation and function (Cole and Walters, 1987). Osteoclasts contain large amounts of acid phosphatase and, since the latter enzyme (TRAP) is secreted during resorption in vitro, acid phosphatase has also been proposed as a good marker for the study of bone resorption (Minkin, 1982; Lau, Onishi, Wergedal, Singer and Baylink, 1987).

Acid phosphatase activity can also be found in all areas of spetal cartilage of healthy adults. In comparative studies of acid phosphatase of

normal and acromegalic cartilage, it was found that the acid phosphatase activity is significantly increased in the superpremaxillary and posterior area of nasal spetal in acromegaly (Vetter et al, 1985). In epiphyseal cartilage the enzyme can only be detected in zones of calcification (Ali, Sajdera and Anderson, 1970). Recently, acid phosphatase has been demonstrated histochemically at the matrix of hypertrophic zone of the endochondral ossification center of Meckel's cartilage in embryonic rats. (Gosta, Zellin, Magnusson and Mangs, 1988). Since its role in chondrocyte metabolism has not yet been fully understood, a series of experiments had been conducted here to characterize the acid phosphatase in both chick growth plate chondrocytes and articular chondrocytes.

The optimal activity of acid phosphatase in both types of chick chondrocyte examined here is found at pH 4. After determining the optimal condition of the enzyme, enzyme kinetics of acid phosphatase in chick growth plate and articular chondrocyte were also investigated. From the Eadie-Hofstee plot presented in Figures 5.10a & 5.10b, the Km for the enzyme present in both types of chondrocytes were found to be almost identical, i.e. about 0.2 mg/ml. The Vmax for growth plate chondrocyte was 322 umol/10^6 cells/hr which was higher than that for articular chondrocytes, i.e. 261 umol/10^6 cells/hr. Km is a constant which is characteristic for any given enzyme for a specific set of conditions such as pH and temperature. It also refers to the concentration of substrate at which half the active sites are filled. The point to note here is that Km is a measure of the strength of the enzyme-

substrate complex, with high Km represents weak binding and vice versa. This means that the acid phosphatases in both types of chondrocyte process similar enzyme-substrate strength.

Vmax, on the other hand, is the maximum rate that the enzyme is "saturated" with its substrate and it means that the enzyme cannot function any faster. When Km is constant, the higher the Vmax for a enzyme the more enzyme molecules. If this is the case, it seems that the growth plate chondrocytes may have slightly more enzyme molecules per cell than those of articular chondrocytes. This may be due to the presence of higher amount of enzyme in the larger hypertrophic subpopulation in the growth plate chondrocytes population.

As shown in Figure 5.11, a 90% enzyme activity in articular chondrocyte was inhibited by 50 mM tartrate. However, in chick growth plate chondrocytes, as much as 30% of acid phosphatase still remained even at the condition that 100 mM of tartrate inhibitor was added, suggesting that both tartrate sensitive and tartrate resistant acid phosphatase isoenzymes were presented in chick growth plate chondrocytes.

As for the total acid phosphatase activity, it increased from 25 to 150 umol/10^6 cells/hr in accordance with the increase of cell size. In addition, a similar increase pattern was also observed for alkaline phosphatase even though the relationship between these two types of phosphatase and their physiological functions are still not fully understood. When the cell diameter excess 11.4 um, the enzyme activity was dramatically reduced to a very low level, i.e. 20 umol/10^6 cells/hr. This might be due to the large number of dead

cell present in the last elutriated fraction. The activities of tartrate sensitive and tartrate resistant isoenzymes showed similar distribution pattern as the total acid phosphatase.

Table 6.1 summarizes the phenotypic characterization of resting, proliferative and hypertrophic chondrocyte subpopulations. Chondrocytes with size smaller than 10 um are regarded as resting chondrocyte since both the [^{3}H]-thymidine incorporation rate and the percentage of G_2M phase cell are extremely low. Fraction F6, with mean cell diameter ranging between 10 to 10.65 um, showing the highest DNA activities, is referred as proliferative chondrocytes. The cell population larger than 10.65 um in diameter is the hypertrophic chondrocyte which maintained relatively high DNA contents. The sulfate incorporation rate and hyaluronidase activity are very high in both proliferative and hypertrophic subpopulations when compared to the resting subpopulation. It indicates that whenever chondrogenesis is onset, the surrounding cartilage matrix is being actively synthesized and broken down. Thus, a dynamic model of cartilage matrix has been indirectly demonstrated. Alkaline and acid phosphatase activities continue to increase when chondrocytes enlarge and reach a peak at the hypertrophic subpopulation (Figures 5.8 & 5.12). Although the real functions of these enzymes are still not very clear, it is possible that they are related to calcification. It seems to be quite consistent with one of the speculated functions of hypertrophic chondrocytes. At least, there is a strong direct evidence to confirm that hypertrophic chondrocytes are physiologically active rather than degenerating.

Table 6.1 Summary of Characteristics of Different Chondrocyte Subpopulations

	Resting	Proliferative	Hypertrophic
Size			
(dia.: um)	< 10	10 - 10.65	> 10.65
(vol.: um^3)	< 530	530 - 620	> 620
Thymidine ($fmol/10^6$ cells/hr)	25	41	35
G2-M (%)	2	8	6
Sulfate ($nmol/10^6$ cells/hr)	1.2	1.5	1.3
Hyaluronidase ($nmol/10^6$ cells/hr)	2.6	3.6	3.9
Alk.Phosphatase ($mmol/10^6$ cells/hr)	3	3.25	4
Acid Phosphatase ($umol/10^6$ cells/hr)	25	75	140

In the present study, eight commonly used growth factors have been tested for their effects on acid phosphatase and alkaline phosphatase activities in chick growth plate and articular chondrocytes. Besides testing the cellular enzyme activities of these two enzymes, the excretory enzyme in the culture medium were also tested. For the growth plate chondrocyte, both cellular and excretory alkaline phosphatase activities were suppressed by a number of growth factors, such as PTH, D_3, FGF-a, FGF-b and TGF-β. Their cellular acid phosphatase activity were stimulated by PTH, FGF-a and FGF-b.

For articular chondrocyte, FGF-a and FGF-b suppress the cellular alkaline phosphatase activity. At the same time, inhibition of cellular acid phosphatase activity by PTH and FGF-b was also noted. In summary, FGF-b was found to be the most potent effector on acid and alkaline phosphatases in growth plate and articular chondrocytes.

The above findings are only a preliminary screening of the effects of certain growth factors. The exact molecular control mechanisms of those effectors on acid and alkaline phosphatase production, especially the FGF, awaits further investigation.

Elutriation appears to have a tremendous potential for the investigations of factors involved in the process of endochondral ossification. Furthermore, this technique may also have important applications for the study of calcification of cartilage in other areas, such as cartilaginous tumors, fracture callus, and heterotopic ossification, where less organized cellular arrangement makes biochemical investigations difficult.

At present, endochondral ossification is only partially understood and a lot can be done in this area. For example, hormonal stimulation of resting chondrocyte to proliferate seems to be one of the projects which bear clinical significance. On the other hand, projects for testing other degradative enzyme systems, such as collagenase and cathepsin may also provide better understanding about degenerative joint diseases. Nutrient uptake by different chondrocyte subpopulations will be an interesting area for investigation. Cartilage is a avascular tissue and the soluble nutrient supply for the chondrocyte is by simple diffusion. Hypertrophic chondrocytes are located just adjacent to the blood supply whereas the resting chondrocytes are located distantly form the nutrient source. The metabolism and the differentiation of chondrocytes being related to the availability of certain critical nutrient should also form an important aspect for future investigations. Furthermore, it is quite valuable to develop some new functional differentiation markers in order to obtain a clear molecular backgrounds about the chondrocyte differentiation process.

One of the direct follow-up of this study is to use the relatively homogeneous chondrocyte subpopulations obtained from elutriation to develop specific monoclonal antibodies against specific cell surface markers for various stages of avian chondrocyte differentiation. It will be quite useful for setting up a new identification method for chondrocyte subpopulations.

Establishing another mammal model is substantially important for comparing with the findings in avian model and aiming at a more complete understanding of human endochondral ossification and bone growth. Thus, I have spent much time and effort in attempting to develop a mammalian

model using rabbit costal cartilage. However, there are many problems to be overcome before sufficient amount of pure chondrocyte subpopulations can be isolated for extensive investigations. The following is a brief discussion of my findings.

For the cell releasing method, ribs were taken from a 9 day old rabbit and all connective tissue was removed with aseptic technique. Resting cartilage and the hypertrophic zone cartilage were then dissected under a dissecting microscope, the hypertrophic zone cartilage being the part immediately adjacent to the osteochondral junction and the resting cartilage referring to the section 5mm from the above mentioned junction. The cartilage were then digested with hyaluronidase (350 unit / gram of tissue) for 45 min at $37^{o}C$ followed by trypsin digestion for 45 min (0.25%, 500 ul / gram of tissue). Finally, tissue was digested with collagenase (1%, 500ul/ gram of tissue). Cell suspension was then filtered through a loosely packed glass wool filter column in order to remove the digestion debris. The cells were then ready for culture or other investigations.

The sequence of enzyme digestion for hyaluronidase and trypsin was reversed from that for the chick chondrocytes. This was found to be quite critical since the digestion sequences for these two types of cells are not interchangeable. The duration of digestion and concentration of enzyme are also critical for cell viability.

One of the problems encountered when working with the rabbit chondrocyte model is the low cell yield obtained from hypertrophic cartilage (10×10^{6} cell/animal). In order to obtain sufficient chondrocytes for proper

investigation, large number of animals would have to be sacrificed thus making it an impractical experimental model unless extensive man power and resources are available.

On the positive side, the rabbit costal cartilage can provide a very pure resting chondrocyte subpopulation because of the homogenicity of the resting cartilage confirmed histomorphologically before hand. A pure subpopulation of chondrocytes can provide a better basis for characterizations.

Finally, when the different populations of cells released from the resting zone and hypertrophic cartilage were analyzed with flow cytometer, the cell size of rabbit costal hypertrophic chondrocytes was found to be much larger than the resting chondrocytes with cell diameters of 10 um and 35 um respectively. This 3.5 fold increase in cell diameter between resting and hypertrophic chondrocytes is greater than that of chick growth plate chondrocytes, which is about 0.16. Although the countercurrent centrifugal elutriation method has not yet been applied to separate the rabbit costal chondrocytes into their various stages of maturation, it is anticipated that separation can be achieved. In my opinion, bovine or porcine costal cartilage would be ideal animals for trying to initial establishment of a mammal in vitro model for studying endochondral ossification since they may provide higher cell yield.

LIST OF FIGURES

Figure 5.12 Activities of total (AP TOL), tartrate resistant (APTR) and tartrate sensitive (APTS) acid phosphatases of elutriated chondrocytes in different size. The total acid phosphatase activity increased with fluctuation from 25 to 150 umol/10^6 cells/hr as cell size increases from 8.7 um to 11.4 um in diameter. The dramatic reduction of the enzyme active for the cells with diameter exceeded 11.4 um was due to large number of dead cells. The activities of tartrate sensitive and tartrate resistant isoenzymes performed the same distribution pattern as that of the total acid phosphatase.
[p 58]

Figure A.1 Countercurrent centrifugal elutriation apparatus. The cells travel from the cell-insertion site into a JE-6 rotor, where they obtain an equilibrium position in the separation chamber that is based on the outwardly directed centrifugal force and the inwardly directed flow of fluid. Once the cells have reached an equilibrium, the speed of the rotor is decreased, and the cells nearest the outlet of the separation chamber are elutriated from the rotor and are collected. (from O'Keefe R J, Crabb I D, Puzas E J and Rosier R N, Countercurrent Centrifugal Elutriation. J Bone & Joint Surgery, 71-A;607-620)
[p 96]

Figure A.2 Schematic representation of a flow cytometer: 1-flow cell; 2-sheath; 3-laser beam; 4-sensing system; 5-computer; 6-deflection plates; 7-droplet collection. (from Macey M G: Flow cytometry: principles and clinical applications. Medical Laboratory Sciences. 45;165-173, 1988.)
[p 99]

Figure A.3 The parameters of flow cytometric analysis: 1-forward angle light scatter=size; 2-90^0 light scatter=granularity; 3-green or red=fluorescence. (from Macey M G: Flow cytometry: principles and clinical applications. Medical Laboratory Sciences. 45;165-173, 1988.)
[p 100]

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APPENDIX I - PRINCIPLE OF COUNTERCURRENT CENTRIFUGAL ELUTRIATION

Countercurrent centrifugal elutriation is a cell-separation technique that has been generally available since the early 1970's and has been used extensively to separate populations of hematological cells (Keng, 1981; Eerkow, 1984; ; Lord, 1984). During elutriation, cells attain a position of equilibrium within the rotor of a centrifuge. This position depends on the outwardly directed centrifugal force and on the inwardly directed forces of flow and buoyancy of the fluid; it is a complex function of the sizes, shapes, and densities of the cells. The fluid flows constantly through the elutriation rotor, entering at the bottom of the separation chamber and exiting at the top. After reaching equilibrium, cells are elutriated by either decreasing the speed of the rotor or increasing the rate of flow of the fluid, shown as Figure A.I. Subpopulations of cells can be sequentially collected by successively decreasing the speed of the rotor.

Elutriation separates cells with better resolution than does conventional separation by sedimentation velocity or by density gradient. Elutriation avoids the harsh conditions that are associated with the exposure of cells to chemical gradients, as the separation is performed in culture medium. This technique should have great applicability for the separation of epiphyseal chondrocytes, given the large change in cellular size that occurs in the growth plate.

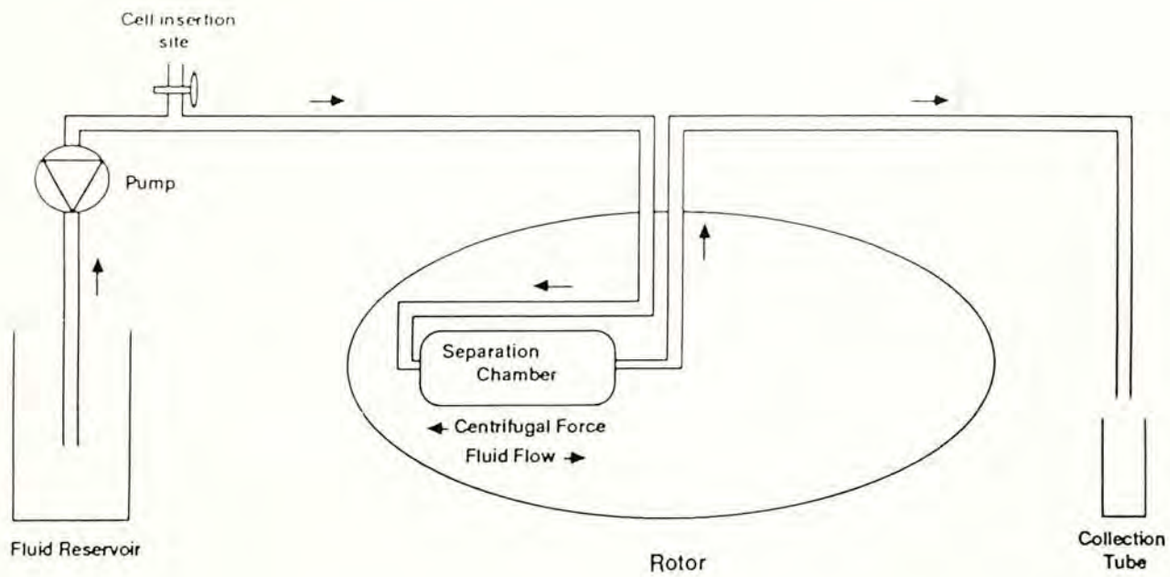

Figure A.1 Countercurrent centrifugal elutriation apparatus. The cells travel from the cell-insertion site into a JE-6 rotor, where they obtain an equilibrium position in the separation chamber that is based on the outwardly directed centrifugal force and the inwardly directed flow of fluid. Once the cells have reached an equilibrium, the speed of the rotor is decreased, and the cells nearest the outlet of the separation chamber are elutriated from the rotor and are collected. (from O'Keefe R J, Crabb I D, Puzas E J and Rosier R N, Countercurrent Centrifugal Elutriation. J Bone & Joint Surgery, 71-A;607-620)

In the present study, countercurrent centrifugal elutriation was used to separate growth-plate chondrocytes. The separated cell fractions were examined for differences in the sizes and biochemical characteristics of the cells.

APPENDIX II - PRINCIPLE OF FLOW CYTOMETRY

Flow Cytometry is a system for sensing cells or particles as they move in a liquid stream through a laser beam past a sensing area, and the relative light scattering and color discriminated fluorescence of the microscopic particles is measured as shown in Figure A.II.1. Analysis and differentiation of the cells is based on size and granularity, and whether or not the cells is tagged with a fluorescent marker, Figure A.II.2. As the cells passes through the laser beam, light is scattered in all directions, and that scattered in a forward direction is proportional to the size of the cell. Light may enter the cell and be reflected and refracted by the nuclear and granular contents of the cell, thus light scatter at 90^{o} is proportional to the granularity of cell. The cells may be labeled with fluorescein isothiocyanate-conjugated (FITC) monoclonal antibodies or stained with fluorescent nuclear dyes (Stohr, Vogt-Schaden and Knobloch, 1978). The former allows identification of different cell types, and the latter allows analysis of the DNA content of the cell.

Flow cytometers are generally multi-parameter, recording several measurements on each cell, therefore it is possible to identify a cell population within a heterogeneous population, This is one of the most useful features of flow cytometers, and makes them preferable to other instruments such as spectrofluorimeters, in which measurements are base on analysis of the entire population.

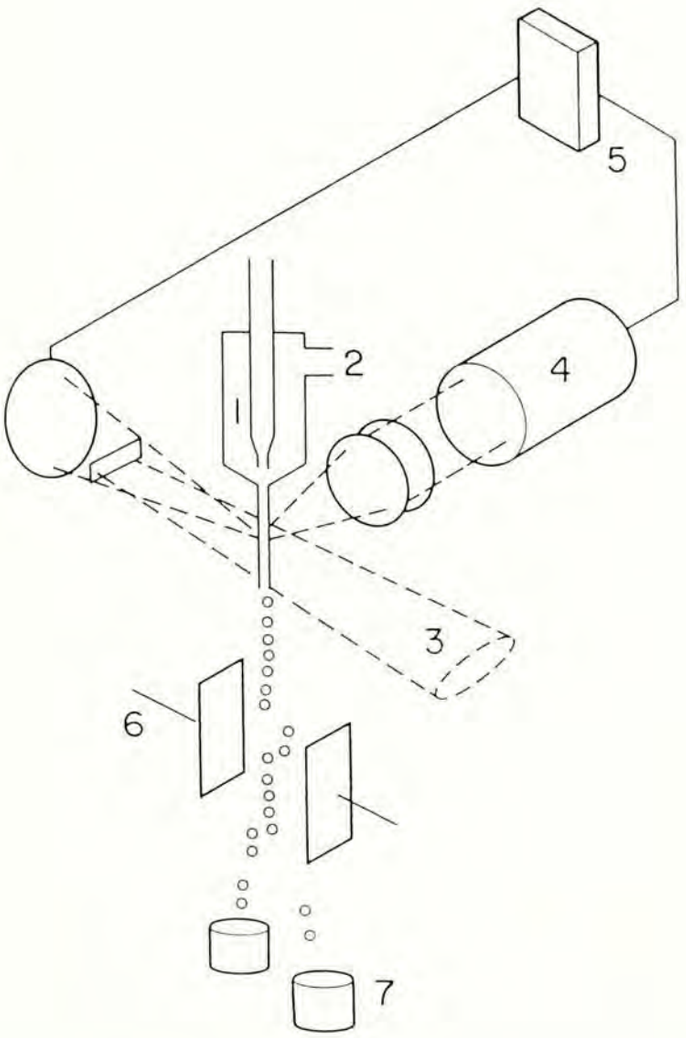

Figure A.2 Schematic representation of a flow cytometer: 1-flow cell; 2-sheath; 3-laser beam; 4-sensing system; 5-computer; 6-deflection plates; 7-droplet collection. (from Macey M G: Flow cytometry: principles and clinical applications. Medical Laboratory Sciences. 45;165-173, 1988.)

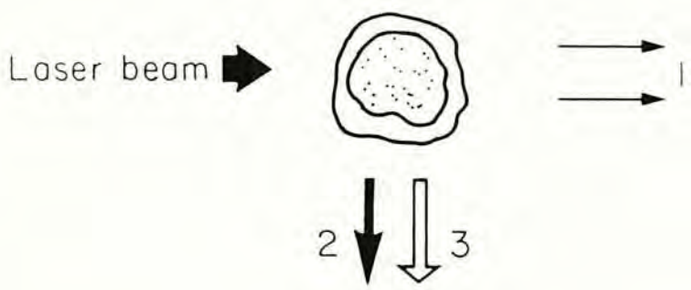

Figure A.3 The parameters of flow cytometric analysis: 1-forward angle light scatter = size; 2-90^{0} light scatter = granularity; 3-green or red = fluorescence. (from Macey M G: Flow cytometry: principles and clinical applications. Medical Laboratory Sciences. 45;165-173, 1988.)

A laser source in the range 50 mW - 5 W output power is used for most fluorescence and light scatter measurements. Lasers have the advantage of producing an intense beam of monochromatic light which can be tuned to several different wavelengths. Argon laser produce light between the wavelengths 351 and 528 nm, and krypton lasers produce light between 350 and 799 nm, Cells suspended in isotonic fluid are transported through a sensing system. Most instruments utilize a lamina/sheath flow technique (Crosland,1953) to confine the cells to the center of the flow stream, and this also reduces blockage due to clumping. Cells enter the flow chamber under pressure through a needle which is surrounded by sheath fluid. The sheath fluid in the sample chamber creates a hydrodynamic focusing effect and draws the sample fluid into the stream. Accurate and precise positioning of the sample fluid within the sheath fluid is critical to efficient operation of the flow cytometer (Shapiro, 1985). This alignment is at present performed manually, but no doubt more advance technology in the future will provide electronically controlled alignment.

Fluorescence is excited as cells traverse the laser excitation beam, and this fluorescence is collected by optics placed at 90^{o} to the incident beam. A barrier filter blocks laser excitation illumination, while a dichroic mirror and appropriate filters are used to select the required wavelengths of fluorescence for measurement. The photons of light falling upon the detectors are converted and amplified by photomultiplier tubes to an electrical impulse, and this signal is processed by an analog-to-digital converter which changes the analog impulse to a digital signal. The quantity and intensity of the

fluorescence are recorded by the computer system and displayed on a visual display unit as a frequency distribution, which may be double or single parameter. Dual parameter histograms of forward angle light scatter and 90^{o} light scatter allow identification of the different cell types within the preparation, based on size and granularity. Single parameter histograms usually convey information regarding the intensity of a given fluorescence intensity, so that weakly fluorescent cells are distinguished from those which are strongly fluorescent.

The most important application of the flow cytometer is perhaps its ability to separate and collect a sub-population of cells, identified by multi-parameter analysis. This form of sorting of cells is accomplished as the cells exit from the sample chamber in a liquid jet. Savart (1933) showed that when a small jet of fluid was vibrated at the correct frequency the stream could be broken into a series of uniform droplets. In the flow cytometer the sheath stream is broken into a series of uniform droplets by vibrating the sample chamber with a piezoelectric crystal at high frequency. Cells flowing through the flow cytometer are isolated in these tiny droplets. When the computer detects a cell that satisfies the parameters determined by the operator for sorting , an electrical charge is applied to the droplet (Tomson, 1967). The polarity of the charge (positive or negative) is determined by the sorting criteria. As the charged droplet passes through an electrostatic field it is deflected to the right or left, carrying the sorted cell. Extremely pure population of cells may be sorted at a relatively rapid speed.

APPENDIX III REAGENTS FOR EXPERIMENTS

A Cell Isolation

1. Modified F-12 medium (Mg-free, 0.5mM $CaCl_2$, pH 7.4)
   One packet of specially ordered Magnesium and calcium free Ham-F12 medium (Sigma) was dissolved into 1 liter of millipored deionized distilled water, milli Q water.

2. Trypsin (type III, 0.1% w/v, Sigma)

3. Hyaluronidase (type I-S, 0.1% w/v, Sigma)

4. Collagenase (type A, 0.1% w/v, Sigma)

5. Citrate-buffered saline (125 mM NaCl, 18 mM citric acid, 10 mM K_2HPO_4, pH 6.0)

B Culture Medium

Dulbeceo's Modified Eagle's Medium (DMEM, Gibco) 5 % (v/v)fetal bovine serum (Gibco), 50 mg/ml ascorbate (Sigma)

C Flow Cytometry Cell Cycle Analysis

1. RNase in phosphate buffered saline (PBS) 1 mg/ml

2. Propidium iodide (10 ug/ml)

3. [^{3}H-] thymidine, 40 Ci/mM, New England Nuclear, in the presence of 5 uM thymidine in DMEM

4. NaCl, 0.15N

5. NaOH, 0.25N

6. HCl, 0.25N

7. Hepes-Mg-Ca with 2.5 mg/ml BSA

8. Perchloric acid, 10N

D Alkaline Phosphatase Assay

1. AMP buffer (0.5 M 2-amino2-methyl propanol; 2mM $MgCl_2$; pH 10.15)

   Dissolve 0.2033 g $MgCl_2.6H_2O$ in about 400 ml dd water; add 23.85 ml AMP and adjust pH to 10.15; and make it up to 500 ml using a volumetric flask and store in brown reagent bottles at room temperature.

E pH Curve for phosphatase

1. 1,4-piperazinediethanesulfonic acid (PIPES) 100 mM

   3.78 g 1,4-piperazinediethanesulfonic acid disodium salt (Sigma) was dissolved into 100ml dd water. pH of 50ml solution was adjusted to 6 and the other was adjusted to 7 with 4 N NaOH.

F Hyaluronidase Assay

1. Acetate buffer (acetate, 50 mM/l, pH 4.0; containing NaCl, 150 mM/l)

   a. dilute 5.78 ml acetic acid to 1000 ml with water (100 mM/l)

   b. dissolve 13.6 g $CH_3COONa.3H_2O$ in water and make up to 100 mM/l); add 41.0 ml solution (a) to 9 ml solution (b) and, if necessary adjust the pH of the mixture to pH 4.0. Add 0.875 g NaCl and dilute to 100 ml with buffer.

2. Hyaluronate (1 g/l)

   dissolve 50 mg hyaluronic acid in 50 ml acetate buffer (1), The compound is not readily soluble; it is best to prepare the solution the day before use.

3. N-Acetylglucosamine standard solution

   dissolve 10 mg N-acetylglucosamine in 10 ml acetate buffer (1); dilute a) 1 ml to 50 ml with acetate buffer (1) (20 ug/ml) and b) 1 ml to 100 ml (10 ug/ml).

4. Tetraborate (0.8 M; pH 9.1)

dissolve 24.44 g potassium tetraborate ($K_2B_4O_7.4H_2O$) in water and make up to 100 ml. Adjust to pH 9.1 with KOH, 5 mol/l.

5. Dimethylanimobenzaldehyde (1% w/v)

dissolve 10 g 4-dimethylaminobenzaldehyde in 100 ml acetic acid (contain 12.5% v/v HCl, 10 M). Just before use dilute with 9 volume of acetic acid.

Remarks:

1. All solutions were prepared in deionized, distilled water.

2. Samples were collected and prepared just before the assay: take up in buffer (1). They can be stored for one week at 0 - 4°C