THE EFFECTS OF DURATION *IN VITRO* ON COLLAGEN SYNTHESIS AND GROWTH RATE OF CELLS FROM SUTURES OF DIFFERENT AGES

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TABLE OF CONTENTS

ABSTRACT

The significance of the aging phenomenon in orthodontics is its effect on the remodelling capacity of the fibrous joints, such as the periodontal and sutural articulations. Collagen metabolism is an integral part of this remodelling process particularly during growth and development. It has been shown that the ratio of type III collagen to total newly-synthesized collagen in the suture increases with the rate of sutural bone growth and decreases with age until complete disappearance on cessation of sutural growth (Yue, 1984). The ultimate objective of the present investigation was to determine whether this age-related change in collagen phenotype expression was due to changes in the relative proportions of specific cell sub-populations present in the suture at each age, or due to modulation of individual cell expression.

Continuously-labelled rat interparietal sutures in tissue culture were used to assess the origin and migration of the progenitor population(s). Based on the examination of serial sections from sutures labelled for various times *in vitro*, these precursors were found to originate from the sutural area proper. In isolated situations, mitotic activity was also associated with vascular elements. The migration pattern of progenitors from old and young donors was similar, although older donors exhibited a longer latent period of emigration.

The cumulated effects of *in vivo* and *in vitro* aging on the proliferative and synthetic potentials of rat interparietal suture

populations were assessed at seeding concentrations of 20,000 cells/mL in 35mm ($8cm^2$) culture dishes. This culture condition was found to favour proliferation.

The significance of cell proliferative capacity was related to its synthetic potential. Except for variabilities particularly in the very young sutures, there was a positive correlation between the ratio of newly-synthesized type III collagen to total collagen and cell growth rate. The *in vitro* selection of cell populations which tolerated processing conditions and retained the ability to reproduce and migrate appeared to be the major determinant of the changes in the proportions of collagen phenotypes synthesized.

DEDICATION

TO MY FAMILY

FOR THEIR ENCOURAGEMENT

AND CONTINUAL SUPPORT

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LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

CHAPTER I

LITERATURE REVIEW

LITERATURE REVIEW

OUTLINE

1. **Introduction**

    1.1 Objectives

    1.2 Phenomenon of Aging

2. **Structural Changes in Sutural Connective Tissue with Age**

    2.1 Suture Morphology and Function

    2.2 Cellular Elements

    2.3 Extracellular Products
        2.3.1 Fibrous Components
        2.3.2 Non-fibrous Components

3. **Biochemical and Metabolic Changes in Collagen with Age**

    3.1 Microstructure and Biosynthesis
        3.1.1 Cross-linking
        3.1.2 Degradation
        3.1.3 Collagen Phenotype

    3.2 Distribution and Physiology

    3.3 Collagen Phenotype as a Biological Signal

4. **<u>In Vitro</u> versus <u>In Vivo</u> Aging**

1. INTRODUCTION

1.1 Objectives

The long range objectives of our studies in craniofacial biology are to elucidate the mechanisms which regulate and control remodelling of fibrous joints. Of particular interest in the discipline of orthodontics are the periodontal ligament and the craniofacial sutures. The ease with which these joints can be remodelled forms the basis of orthodontic and facial orthopaedic therapy for malocclusions and skeletal malformations, especially in the young growing patients. With age, this capacity to remodel is limited, but is still present (Brandt et al., 1979).

Studies on the remodelling capacity of craniofacial sutures have been successfully illustrated using the interparietal (Yen et al., 1984; Yue, 1984) and coronal (Meikle et al., 1979) sutures. In an investigation of sutural aberrations, such as craniosynostosis, the interparietal suture would be a logical choice since it is the most common site of natural fusion with age (Shillito and Matson, 1968). However, the differences between the sutural and periodontal joints (for review, see Yue, 1984) should be considered when extrapolating results from studies on the effects of mechanical stresses on the cellular metabolism of the cranial sutures. The major difference between these two fibrous joints is the progressive morphologic changes with increasing sutural age (Moss, 1954; Scott, 1954; Pritchard et al., 1956; Persson, 1973; Kokich, 1976), while the periodontal ligament, once developed,

essentially remains unchanged throughout life (Narayanan and Page, 1983).

While morphologic changes in sutural tissues suggest that changes in cell populations contribute to aging and loss of function as a joint, the nature of these changes in terms of cellular remodelling capacity are poorly understood. It is the purpose of the present investigation to study, at the cellular level, age-related changes in the proliferative and synthetic capacities of these sutural populations. Tissue culture techniques will therefore be utilized to assess the mechanisms which control cellular metabolism.

1.2 Phenomenon of Aging

Aging is a quantitative measurement of the progressive changes which occur in a molecule, cell, tissue or organism with the passage of time (Heikkinen, 1969). Considerable variation in these physiologic parameters can be observed as a function of age (Schneider and Mitsui, 1976). This variation may be due to the use of chronologic age as an indicator for biologic age despite the fact that these two measurements of aging may be quite disparate (Hall, 1976).

The physiologic phenomenon of aging is based on four criteria according to Strehler (1977). The first criterion is that of universality; that is, what is considered to be an aging phenomenon must be identifiable, in some degree, in all members of the species in question. As opposed to the aging of skin, for example, osteoarthritis is not considered to be a true aging phenomenon since the latter is not universal (Hough and Webber, 1986).

The second criterion is the concept of intrinsicality, which implies a restriction of aging factors to those of endogenous origin. It is therefore the intrinsic ability of the system to respond, rather than the nature of the external stimulus, that is the age-related factor (Hall, 1976).

The most characteristic feature in the aging process is its progressive nature. Therefore, conditions which occur at a higher incidence above a certain age but which develop rapidly are not strictly defined as true age-related phenomena (Balin and Allen,

1986). The susceptibility to myocardial infarctions, for example, might be higher with age, but it is the underlying changes in the collagen content within the arterial vessels that are truly age-determined (Andreotti et al., 1985).

The last of Strehler's criteria is the concept of deleteriousness, where there is a decreased viability and increased vulnerability to external and internal insults subsequent to a deterioration in function and structure. For example, in the case of man after the age of 30 years, and rat after 400 days, the mortality rate is a logarithmic function of age (Strehler, 1977).

Strehler's criteria for natural aging are general guidelines which allow for the definition of age-related changes during the embryonic stage apart from that during maturation, adulthood and old age. The differences between these stages are defined with the use of histological and biochemical techniques. In the present study, age-related changes of the soft connective tissue elements in the interparietal suture will be investigated by these techniques.

2. STRUCTURAL CHANGES IN SUTURAL CONNECTIVE TISSUE WITH AGE

2.1 Suture morphology and function

Craniofacial sutures have been used as a model for the study of fibrous joints for decades. Nonetheless, there have been many conflicting observations of suture morphology which have not been elucidated even with the use of the improved resolution of the electron microscope (Ten Cate et al., 1977). Many of these inconsistencies have been attributed to the inherent differences in the tissues, the effect of the plane of section (Persson, 1973) and the use of different selective staining techniques (Pritchard et al., 1956).

A description of the "sutural area" (Moss and Young, 1960) includes the fibrous connective tissue and the contiguous bone. The soft connective tissue portion of the suture consists of various cell types, fibres, ground substances and blood vessels (Pritchard et al., 1956; Persson, 1973; Friede, 1975). Occasionally, secondary cartilage may also be present (Pritchard et al., 1956; Moss, 1958; Hall, 1978b; Aaron, 1973) although its postulated role in suture fusion (Moss, 1957) remains obscure (Persson, 1973; Al-Bareedi, 1984). These connective tissue elements will be considered separately.

A uniformity in suture structure was noted by various investigators to be three-layered (Weinmann and Sicher, 1955; Linge, 1972: Melsen, 1975) or four-layered (Scott, 1954; Droschl, 1975). With the use of selective fibre stains, Pritchard et al. (1956) were able to identify five distinct layers of cells and fibres

between the edges of the adjoining bones and two outer uniting layers of adjacent periosteum, regardless of species, age or suture type. Even though different parts of the same suture varied considerably in the process of suture differentiation, all apparently went through similar stages in their development. Therefore, despite profound changes in the sutural elements with aging, the original five-layered structure was always discernible (Pritchard et al., 1956) although it became progressively narrower with age (Scott, 1967). On the other hand, Weinmann and Sicher (1955) described the mature suture as a three-layered structure which was transformed to a completely fibrous one through the gradual disappearance of the central cellular layer. In a study of the age changes in human frontozygomatic sutures, Kokich (1976) also noted that by 20 years, any uniformity in sutural arrangement that might have been present was lost.

Variations in the structural organization within the same suture and between different sutures may also be dependent on the plane of section under study (Miroue and Rosenberg, 1975; Hall, 1976). As a result, one might not necessarily be observing a representative concentration of cells nor orientation of fibres found in the in situ situation.

Changes in the mode of growth in response to function have also been associated with corresponding differences in the organization of suture structure (Persson, 1973). Therefore, the structures of all aging sutures should be essentially the same if they are equally absent of function (Koskinen et al., 1976).

However, sutures (particularly in younger animals) are biologically dynamic and undergo continuous remodelling (Ten Cate, et al., 1977); any histologic observations of the cellular and fibrous layers are but a static description of the functional state of the tissue at that given time (Koskinen et al., 1976). Therefore, variations between different species and sutures (Scott, 1954; Linge, 1972; Persson, 1973; Droschl, 1975) may not be as significant as the ages or maturational stages (Kokich, 1976) and the functional state (Koskinen et al., 1976; Persson, 1976; Ten Cate et al., 1977) of the tissue in question.

Utilizing radioautographic techniques, Persson (1973) clearly demonstrated a variation in the functional organization of sutures. The morphological observation and grain count data in the study by Chiang (1981) also did not support any concept of discrete functional layers.

In a study of human sutures from birth to 18 years, Latham and Burston (1966) concluded that after about 2-3 years of age, cranial sutures in general functioned primarily as sites of bony union, although localized remodelling was a continuous process. Irregularities in sutural morphology reflected this remodelling activity over time (Massler and Schour, 1951; Moss, 1957). With age, the progressive apposition of bone produced bevels and projections into the sutural area. This pattern of interdigitation was retained in mature human sutures (Melsen, 1975) and had become more tortuous (Kokich, 1976) as the numbers and length of these projections increased with advancing age (Washburn, 1947;

Miroue and Rosenberg, 1975).

In early development, bevels and small interdigitations have enabled the suture to accommodate to rapid skull growth by allowing for the sliding of sutural surfaces (Koskinen et al., 1976). However, in adult life, the interlocking of these bony projections initially prevents this adaptive ability (Brandt et al., 1979). Thus, the greater length of time required for resorptive remodelling of these interdigitations is believed to be partially responsible for the slower response to extrinsic forces in adult animals (Miroue and Rosenberg, 1975; Kokich, 1976). Nonetheless, these bony projections have been postulated to play a significant role in increasing both fibrous joint strength and its resistance to tensile or compressive stress with age (Prahl, 1968; Herring, 1972; Melsen, 1975; Kokich, 1976).

If the degree of suture interdigitation is related to the length of time the suture remains patent or active, the sutures which do not undergo synostosis until old age should be more highly serrated (Massler and Schour, 1951), and cause a progressive increase in the rigidity of the entire facial skeleton (Miroue and Rosenberg, 1975). If the resulting immobilization (Giblin and Alley, 1944) and compression of sutures are of an adequate force (Hinrichsen and Storey, 1968), stenosis may also be induced. Even with temporary cyanoacrylate immobilization of the coronal suture in growing rabbits, Foley and Kokich (1980) have been able to demonstrate a partial loss of suture patency given by the formation of a periosteal bone bridge.

The expression of age-related changes such as sutural fusion varies with the species, the suture and the individual. Physiologic obliteration of the cranial sutures usually occurs in great apes after birth but is not necessarily seen before 20 years in man (Bolk, 1915); the exception is the metopic suture which usually closes by the first year of life (Park and Powers, 1920). In an extensive study on the progression of endocranial suture closure with age, Todd and Lyon (1924) have concluded that synostosis of the sagittal and sphenofrontal sutures begins first, at 22 years, is most rapid from 26 to 30 years and then slows down and may not be complete until old age.

The factors responsible for sutural fusion involve more than a proximity of bony edges (Moss and Young, 1960). Except for the midpalatal suture, fusion is not necessarily a part of the life cycle of the circummaxillary sutures (Kokich, 1976). If suture fusion does occur, the circummaxillary sutures fuse after the cranial sutures possibly due to their relationships to the somatic and neural growth periods, respectively (Miroue and Rosenberg, 1975). Pritchard et al. (1956) and Latham (1971) contend that the presence of masticatory forces in the facial skeleton also contributes to the prolonged patency of the facial sutures; therefore, the tendency for partial fusion is a common finding (Miroue and Rosenberg, 1975). In the frontozygomatic suture, for example, the facial surfaces remain patent at all ages, while fusion of the remaining surfaces begins at about 70 years and increases in incidence with age (Kokich, 1976).

Regardless of the foregoing inconsistencies, some degree of suture fusion is universal, intrinsic and progressive in nature. The synostotic process in itself is also deleterious whether the onset is physiologic or age-related, or abnormally premature (David et al., 1982). What is intriguing about the synostotic process is its reflection of the suture's inability to further maintain itself with age. Ironically, the very forces which maintain suture patency appear to eventually produce the array of projections which may restrict movement and ultimately lead to fusion. Once ossification begins, it is no longer a viable articulation and therefore, it can no longer fulfill the functions as an area of growth or a stress absorber. The aged suture assumes a new role of lipid storage, and becomes part of the fat storage system in the body (Miroue and Rosenberg, 1975).

Despite the importance of sutures in normal growth, the mechanism by which a suture maintains its width throughout remodelling is unknown (Droschl, 1975). Shifts in the sutural populations (Gould et al., 1977), and changes in the quantitative and qualitative properties of the existing populations have been postulated (Jensen and Toto, 1968; Miroue and Rosenberg, 1975; Kokich, 1976; Roberts et al., 1981). Pathological disruptions in this mechanism which accelerate the synostotic process, as seen in craniosynostotic syndromes, may mimic the aging phenomenon. However, individuals with generalized premature aging or progeria have not been reported to develop stenosis of fibrous joints, except possibly for tooth ankylosis expressed as delayed tooth eruption

(Wesley et al., 1979).

Needless to say, elucidation of the mechanisms of normal sutural homeostasis with age is the key to the understanding and subsequent treatment of patients with sutural growth aberrations, such as cleidocranial dysostosis and primary craniosynostosis. Tissue homeostasis is defined by the coordinated interaction between the fibrous and non-fibrous matrix with the cells which synthesize this matrix (Amenta et al., 1986). Therefore, although commonly reported, the quantification of the extracellular products alone can only provide one sequelae of aging. If senescence is regarded primarily as an expression of events at the cellular level (Strehler, 1977), the basis for changes that accompany aging will be found in the study of the cells (Bornstein, 1976).

2. STRUCTURAL CHANGES IN SUTURAL CONNECTIVE TISSUE WITH AGE

2.2 Cellular elements

The understanding of the close relationship between the connective tissue cells and their extracellular products is important in any assessment of sutural homeostasis, whether the suture is young or old, or of cranial, facial or periodontal origin. The ability of these fibrous joints to respond and fulfill their morphological, physiological and functional demands rests on the intrinsic activity of the connective tissue cells themselves (Hall, 1976).

The connective tissue of fibrous joints consists of a mixed population of cells with very different proliferative (Leblond et al., 1959; Tonna and Cronkite, 1963; Weiss et al., 1968; Roberts et al., 1981) and synthetic (Gay et al., 1976; Hall, 1978c; Weiss et al., 1986) potentials. Aside from the recruitment of mast cells (Grigorova-Borsos et al., 1988) and histiocytes or macrophages (Hall, 1976) during connective tissue remodelling, the suture is best considered as consisting of two cell populations, the osteocytic and fibrocytic series (Ten Cate et al., 1977) which have the ability to remodel the tissues they form (Ten Cate, 1972; Deporter and Ten Cate, 1973; Ten Cate and Syrbu, 1974; Ten Cate and Deporter, 1975).

The most important and numerous of the connective tissue cells in sutures are those of the fibrocytic series including the fibroblasts, fibrocytes, and fibroclasts. Their function is to remodel the soft connective tissue in the process of synthesizing and

degrading extracellular substances, most of which is collagen (Barbanell et al., 1978; Meikle et al., 1982; Rigby, 1983). Therefore, with the use of biochemical and immunohistochemical assays, fibroblasts are identified on the basis of their synthetic product. However, due to the heterogeneity of collagen type distribution in tissues, it is often difficult to differentiate between fibroblasts solely on their synthetic function; that is, one cell might synthesize more than one collagen type (Gay et al., 1976). Preliminary evidence has also indicated that the capacity of a fibroblast to shift its synthesis to other collagen types may not be a differentiated property, but perhaps a consequence of cell modulation for age and function (Engel et al., 1980; Narayanan and Page, 1983).

The electron microscopic profile for aging cells of the fibrocytic series show ultrastructural features consistent with some cellular activity, although they are not as marked as in growth (Ten Cate et al., 1977). In addition, an immediate renewal of the fibroblast population may be due to the division of these differentiated fibroblasts rather than by cells with a lower degree of differentiation (Everts and Beertsen, 1978). This continued ability to respond is evident following applied extrinsic forces to adult monkey sutures (Brandt et al., 1979), and following in vitro and in vivo applications of force to adult mice (Yue, 1984).

Under normal conditions, the activity of fibroblasts appear to be regulated by a number of factors and physiologic conditions such as, the irreversible steps of cell differentiation (Narayanan and

Page, 1983); environmental ligands derived from plasma (Scher et al., 1979); the presence of calcium ions (Deshmukh and Sawyer, 1977), pyrophosphate (Deshmukh and Sawyer, 1978), and lysosomal enzymes (Deshmukh and Nimni, 1973); cell density (Abe et al., 1979); aging (Praeger, 1986); and its stage in the cell cycle (Ko et al., 1981). In order to investigate the effects of each of these factors on the cells themselves, in vitro techniques utilizing tissue cultures are required. The identification of precursors in stem cell lines are also based on the properties expressed by the cells in culture (Hall, 1978c).

Similar to the cells of the fibrocytic series, the osteocytic population comprises of cells which form (osteoblasts), maintain (osteocytes) and destroy (osteoclasts) their synthetic product (Ten Cate et al., 1977). The most notable ultra-structural characteristic of osteoblasts (Rygh, 1976) as well as fibroblasts (Yee, 1979), orthodontically stimulated or in normal function, is the highly developed rough endoplasmic reticulum system which suggests a high level of matrix synthesis by these cells. Following the application of tensile forces to sutural and periodontal joints, these cell populations are responsible for not only cell proliferation (Young, 1962; Tayler et al., 1968; Baumrind and Buck, 1970; Linge, 1972; Roberts and Jee, 1974; Gould et al., 1977; Meikle et al., 1980; Roberts et al., 1981), but also for the synthesis of proteins (Meikle et al., 1979, 1982; Chiang, 1981; Yue, 1984) and sutural bone (Chiang, 1981) as required for remodelling.

Histologically, the osteoblasts and osteoclasts are easily

recognized by their characteristic ultrastructure and location adjacent to the bony margins of the suture. This cellular cambial layer has been shown to exhibit a high level of bone formation activity in the developing suture (Scott, 1954; Pritchard et al., 1956; Prahl, 1968; Linge, 1972; Droschl, 1975; Friede, 1975; Chiang, 1981), especially around the tips of the interlocking bony projections (Koskinen et al., 1976).

No clear distinction can be made between "maturing" osteoblasts and "young" osteocytes (Aaron, 1973). However, in young sutures, osteoblasts as well as osteocytes possess significant amounts of cytoplasmic organelles indicative of marked secretory activity (Ten Cate et al., 1977). On the other hand, in the mature suture, relative inactivity is depicted by a corresponding diminution in these intracellular structures (Young, 1962; Strehler, 1977; Ten Cate et al., 1977).

Unlike the cells in the osteocytic series, the fibrocytic analogues are often indistinguishable from one another by normal histochemical procedures (Messier and Leblond, 1960; Engel et al., 1980) and have the same morphology whether in vivo or in vitro (Engel et al., 1978; Narayanan and Page, 1983). Only under electron microscopy can the presence of banded collagen-containing phagolysosomes separate the fibroclast from its synthetic counterpart. In any event, the formation and destruction of collagen can be undertaken by the same cell (Ten Cate et al., 1976; Hurum et al., 1982). The presence of compartmental intracellular collagen in connective tissue cells undergoing mitosis infers a

resorptive function before mitosis (Everts and Beertsen, 1978). These complications of terminology and inconsistencies in the descriptions of the sutural elements reiterate the futility of pinpointing the exact location of the proliferative and synthetic cells in the suture. However, there seems to be some consensus concerning the existence of a middle fibrovascular layer, rich in blood vessels and mesenchymal cells, as the site of cell proliferation and fibre rearrangement (Weinmann and Sicher, 1955; Pritchard et al., 1956; Linge, 1972; Droschl, 1975; Ten Cate et al., 1977).

The vasculature has also been observed to traverse from the middle of the suture into the paraosseous layers and marrow spaces in rats (Persson, 1973) and mice (Chiang, 1981). With age, these sinusoidal vessels increasingly dominate the fibrous suture (Persson, 1973) and are accompanied by a decrease in cell number (Kokich, 1976), cell activity (Ten Cate et al., 1977; Bartold et al., 1986) and collagen fibre density (Miroue and Rosenberg, 1975). These findings appear to reflect the diminishing effect of tensile forces on the cellular elements with age (Jensen and Toto, 1968).

It is important to note that changes in suture morphology with increasing age or decreasing function do not necessarily indicate an inability of the sutural cells themselves to act. The measurement of DNA synthesis with tritiated-thymidine has been used to demonstrate marked changes in cellular populations with increasing age in connective tissues (Hall, 1976). However, the diminution in labelling with age (Albini et al., 1988) has been

postulated to represent either a loss in the capacity of aged cell populations to divide (Hall, 1976; Hayflick, 1984; Praeger, 1986; Albini et al., 1988), a decrease in the availability of progenitor cells (Jensen and Toto, 1968; Soukupova et al., 1970), or a quiescent state which could be stimulated to activity upon need (Weiss et al., 1968; Ten Cate et al., 1977; Chiang, 1981; Yue, 1984).

Studies of cells aged in vivo (natural aging) and in vitro (in a cell culture system) have been utilized to elucidate the characteristics of cellular aging noted above (Hayflick, 1984). However, for a more complete assessment of the number and types of active cells in a tissue as well as their changes with age, it is also necessary to measure their synthetic function. Therefore, morphologically homogenous fibroblasts may be separated into functionally different subpopulations based on the type of collagen each produced (Engel et al., 1980). As a result, the viability and efficiency of cells may be reflected as qualitative and quantitative changes in the extracellular milieu.

2. STRUCTURAL CHANGES IN SUTURAL CONNECTIVE TISSUE WITH AGE

2.3 Extracellular Products

2.3.1 Fibres

The various forms of connective tissue are all characterized by the production of the intercellular or extracellular matrix which consists of both fibrous and amorphous substances; however, the collagen fibres are the main components of the matrix (Kivirikko and Risteli, 1976; Bornstein and Traub, 1979). The relative proportions of cells to matrix determine the suitability of the tissue for its specialized function (Hall, 1976). Although the significance of this cell-matrix interaction to tissue integrity can not be fully appreciated in histological sections, it is important to identify the structural changes as an indication of overall sutural activity. The microstructural and biochemical changes in collagen with age will be discussed in a separate section.

At the light microscopic level, the fibre patterns of the sutural connective tissue vary within a given tissue as a reflection of local demands (Persson, 1973; Koskinen et al., 1976). This finding suggests that different parts of the same suture may experience mechanical stress of different direction and magnitude (Nanda, 1978). The collagen fibre pattern of truly aged sutures will therefore be a reflection of the absence of function.

In general, with advancing age, large collagen fibre bundles (Sharpey's fibres) became more prominent (Young, 1962; Ten Cate et al., 1977) and more regularly aligned (Kokich, 1976). Irregular

spaces tended to prevail between the fibre bundles and subsequently contributed to the decrease in fibre density also characteristic of aging (Miroue and Rosenberg, 1975; Kokich, 1976). Fibre orientation, however, continued to reflect local functional stresses (Koskinen et al., 1976) and therefore, sutures did not lose their orientation until the cessation of function or following the initiation of fusion (Miroue and Rosenberg, 1975).

Histological age changes in the collagen fibres have also been postulated in the development of senile elastosis (Bentley, 1979). This apparent increase in elastin fibres may only represent collagen surfaces which have a greater affinity for elastin stains due to partial proteolytic degradation with age (Hall, 1976). However, recent biochemical assays on human and bovine skin have revealed elution patterns suggestive of an accumulation of mature dermal elastin with age (Yamauchi et al., 1988). Similar findings in sutural connective tissue have not been reported.

2.3.2 Non-fibrous components

The extracellular matrix consists of glycoproteins, proteoglycans and glycosaminoglycans that are secreted by cells and assembled locally into an organized network (Hay, 1981). Although it is present in relatively small proportions compared to its fibrous counterparts, this structural component is considered to maintain a wide variety of cellular and tissue functions (Comper and Laurent, 1978). Alterations in its proportions due to aging will undoubtedly

influence the overall performance of the suture as a stress-absorbing structure (Miroue and Rosenberg, 1975).

Given the apparent functions of ground substance in the hydration and sieving of the extracellular molecules (Laurent, 1966), the integrity of this amorphous matrix is essential for the proper transport of metabolites. A proteoglycan carrier system, for example, has been reported to transport tropocollagen molecules to the remodelling fibres (Hay, 1978, 1981), while the glycosaminoglycan side chains function by determining the time, location (Gross, 1969) and dimension of the deposited collagen fibrils (Parry et al., 1982). In addition, the sulfated glycosaminoglycans have been shown to be involved in the orientation of the fibrous network (Toole and Lowther, 1968; Breen et al., 1972).

The presence of glycosaminoglycans gives the proteoglycans a highly anionic charge, making them capable of forming complexes by way of cross-links with many components of the extracellular matrix (Comper and Laurent, 1978; Bartold and Page, 1985). This ground substance-to-collagen microenvironment has been implicated in the initiation of a periodontal ligament response to orthodontic forces. Roberts et al. (1981) have postulated that with periodontal ligament displacement, the negatively charged ground substance migrates toward the positively charged collagen molecules in the widened zones. Despite some buffering by ions in the extracellular fluid, the tendency toward a net negative field or high ground substance-to-collagen ratio results in the initiation of osteogenesis

(Roberts et al., 1981).

Alterations in the non-fibrous matrix that occur with aging also have direct effects on the appearance and function of tissues (Bornstein, 1976; Bartold et al., 1986). In vivo studies of ground substance in human dermis have indicated that the synthetic properties of the resident matrix-producing fibroblasts may vary during aging (Breen et al., 1972; Fleischmajer et al., 1972). Subsequent studies using in vitro aging of cultured fibroblasts have confirmed decreases in [^{35}S]-sulfate incorporation into newly-synthesized sulfated macromolecules (Vogel and Kendell, 1980; Vogel et al., 1981). These latter studies also reported a slower intra-cellular processing and rate of release of proteoglycans by cells aged in vitro. An age-related decline in the synthesis of sulfated glycosaminoglycans has also been reported by Schachtschabel and Wever (1978).

Due to the close functional relationship between the cell and its matrix, age changes in matrix composition and organization will have profound effects on the control of fibroblast proliferation (Wever et al., 1980; Sluke et al., 1981) and cytodifferentiation (Silbert, 1978). By the same token, the in vitro selection of slower proliferating fibroblasts from older donors is related to increases in specific components, such as heparin sulfate (Bartold et al., 1986). This accumulation of heparin sulphate on the cell layer is attributed to age-related alterations in the cell membrane rather than to an elevated production (Matuoka and Mitsui, 1981). Such changes in the relative proportions of the different ground

substance components may have important consequences on its degree of hydration and susceptibility to enzymatic digestion, and may in turn influence the properties of the adjacent fibrous structures (Bornstein, 1976).

3. BIOCHEMICAL AND METABOLIC CHANGES IN COLLAGEN WITH AGE

3.1 Microstructure and Biosynthesis

Although collagen is not "alive", and therefore cannot age in the same sense as cells and organs, it does undergo many changes as a function of time (Reiser et al., 1987). Such changes can ultimately have a profound effect on tissue homeostasis since collagen is the principal structural protein of fibrous joints (Barbanell et al., 1978) and the main extracellular component in connective tissue (Kivirikko and Risteli, 1976; Bornstein and Traub, 1979). Its integrity is therefore essential for the normal development and function of the sutural structure.

From early fetal development to postnatal maturity, the average diameter of collagen fibrils has been reported to increase (Luder et al., 1988), although this finding is not universal for all fibres. The larger diameter fibres (type I collagen) remain relatively constant with age, while the smaller diameter fibres (type III collagen) tend to slightly increase. Depending on the type of tissue, age and species, the collagen fibril diameter may also be related to the type and amount of glycosaminoglycans in the surrounding matrix as well as to the functional status of the tissue involved (Luder et al., 1988).

As the collagen fibrils aggregate to give rise to collagen fibres (Barbanell et al., 1978; Prockop et al., 1979a, 1979b), intra-molecular cross-links form between the N-terminal regions of alpha-chains to contribute to the stability of collagen molecules

within fibrils (Minor, 1980). However, for the development of tensile strength, the collagen fibrils require inter-molecular cross-linkages (Tanzer, 1973; Refer to Appendix J for a review of collagen biosynthesis.).

3.1.1 Cross-linking

Differences in collagen cross-link patterns have been observed in different tissues of the same animal and in the same tissue during embryonic development, maturation, and aging (Bornstein and Traub, 1979). As the tropocollagen molecule matures, the non-covalent and hydrogen bonds (Bailey et al., 1974) which initially hold the molecules together are augmented by the introduction of various covalent linkages. The formation of these cross-links in collagen only occur extracellularly (Rigby, 1983), following its assembly into fibrillar form (Tanzer, 1973; Siegel, 1979).

When the tropocollagen molecules are arranged in native fibrils, the terminal non-helical extension peptides function as the sites where hydroxylysine-derived intra- and inter-molecular cross-links are formed. As the cross-link-containing extension peptides are not in the triple-helical conformation, they are susceptible to proteolytic degradation under conditions in which the triple-helical body of the molecule is left intact (Hay, 1981). This degradative activity represents the basis for limited pepsin digestion which effectively extracts insoluble collagen from tissues (Miller,

1972).

The most important inter-molecular bond is initiated by a reaction of the enzyme lysyl oxidase (Balazs, 1977). This step involves the oxidative deamination of the free amino groups of the lysyl or hydroxylysyl residues into aldehydes (allysine and hydroxyallysine, respectively) (Bornstein and Traub, 1979; Siegel, 1979). These cross-link precursors define two condensation pathways, one based on the allysine precursors which predominate in soft connective tissue (skin) and, the other on hydroxyallysine in the mineralized connective tissue (bone, dentin and cartilage) (Barbanell et al., 1978; Bentley, 1979). Tendon, in contrast, contains both precursors (Eyre et al., 1984).

The formation of the collagen cross-links is accomplished through spontaneous condensation reactions between aldehyde-containing precursors, or between an aldehyde-containing precursor and a side chain, a lysyl or hydroxylysyl residue (Gay and Miller, 1978). These difunctional or Schiff-base cross-links are borohydride-reducible aldimines and keto-amines condensed from the allysine and hydroxyallysine precursors, respectively (Tanzer, 1967; Gallop et al., 1972; Davison, 1978). With normal maturation, the aldimine cross-link can be further stabilized by its conversion into the keto form of the same molecule by an Amadori rearrangement (Eyre et al., 1984). The keto-amine compound is more stable than its aldimine precursor (Bentley, 1979) since it is virtually complete for cross-links between the amino acid residues. Robins, Shimokami and Bailey (1973) have shown that only in tissues actively

synthesizing collagen are the reducible cross-links found in substantial quantity. The greatest increase in these cross-linkages is related to rapidly growing tissues (Verzar, 1964) and those with high turnover rates (Davison, 1978; Eyre et al., 1988). With maturity, the number of reducible cross-links decreases steeply, and virtually disappears in aged cartilage (Eyre et al., 1984), skin (Robins et al., 1973) and dental pulp (Nielsen et al., 1983), although significant levels continue to persist in bone collagen into adult life (Eyre et al., 1984).

In parallel with the decrease in these reducible precursors, there is a progressive replacement by mature non-reducible compounds (Bailey and Shimokomaki, 1971; Vopin et al., 1977; Allain et al., 1978; Bentley, 1979) whether tissues are aged in vivo or in vitro (Robins et al., 1973; Barnard et al., 1987; Yamauchi, 1988). Hydroxypyridinium residues from collagen fractionation assays have been identified as the stable cross-link in collagen of tendon (Reiser et al., 1987) and bone and cartilage (Eyre et al., 1984, 1988). On the other hand, histidino-hydroxylysinonorleucine (HHL) has recently been reported as the definitive cross-link for skin (Yamauchi et al., 1988); however, the HHL peptides have only been found to derive from type I collagen fibrils (Mechanic et al., 1987). An unidentified "Compound M" (Barnard et al., 1987), which is similar in molecular weight to the HHL peptide, has also been characterized as a major cross-link of mature collagen; it differs from HHL peptides in its distribution in bone and tendon as well as skin.

The increasing concentrations of HHL peptides, "Compound M" and hydroxypyridinium residues in aging tissues demonstrate true age-related cross-links. The mechanisms by which these changes occur have been suggested to require increases in oxygen concentration to hasten the transformation of collagen to more stable forms (Rigby, 1983). In vivo, this stabilization has been postulated to involve either a reduction (Mechanic et al., 1971) or oxidative (Bailey et al., 1977; Zs.-Nagy, 1986) process, although direct evidence to support the former hypothesis is lacking (Bailey and Peach, 1971; Robins et al., 1973). By the same token, the oxidation of the reducible cross-links does not appear to be sufficient to account for all changes in mechanical and thermal stability (Rigby, 1983). However, the age-dependent increase in cross-links between collagen and the non-fibrous extracellular matrix, such as glycoproteins, might account for this added stability (Balazs, 1977).

The decrease in the reducible cross-links with age has also been postulated to be due to a decrease in lysyl oxidase activity with age (Anttinen et al., 1973; Reiser et al., 1987). Such a mechanism might explain the deficiency of cross-links noted in collagen that is newly-synthesized by older animals.

Regardless of the mechanism, the decrease in solubility or extractability and increase in resistance to proteolysis characteristic of aged collagen (Bjorksten, 1958; Bailey et al., 1974; Fujimoto, 1984) support the concept of progressive cross-linking throughout life (Verzar, 1964). The cross-links which have been transformed

over time are likely represented in the more stable, non-reducible form of collagen, and therefore would be expected to be prominent in collagens of long-lived animals such as humans (Reiser et al., 1987). The increases in the age-related rate of collagen cross-linking in those with diabetes point out the complications of aging in collagen-rich tissues without proportional increases in degradation (Monnier et al., 1988).

3.1.2 Degradation

Previous studies have suggested that the cross-linking of collagen may not only be important for optimum function but also may be a principal mechanism regulating the rate of in vivo catabolism (Harris and Farrell, 1972; Yamauchi et al., 1988).

Although total body content of collagen may be maintained throughout life (Lovell et al., 1987), collagen itself can undergo rapid synthesis and degradation as required by the environmental conditions. In rapidly remodelling tissues like the growing suture and periodontal ligament, the breakdown of collagen is as essential as its synthesis.

Collagen can be degraded by several different mechanisms. Intracellularly, 20-40% of newly-synthesized procollagen has been shown to be degraded shortly after translation (Bienkowski et al., 1978). This latter process is separate from degradation by way of phagocytosis and extracellular collagenases (Rennard et al., 1982) since the relative amount of degraded collagen is independent of

the addition of serum to the cultures and the use of extracellular proteolytic inhibitors, respectively (Bienkowski et al., 1978).

Although the degradation of de novo collagen may seem wasteful, this process functions to modulate the quantity and quality of the collagen being secreted from the cell (Rennard et al., 1982). Abnormal molecules due to synthesis errors are recognized and destroyed (Goldberg and St. John, 1976). Collagen chain ratios specific for collagen types, or type-specific degradation of collagen to control the final ratio of interstitial collagens, especially types I and III (Bienkowski et al., 1978) are also regulated by intracellular degradation. Although the process itself is age-invariant, age-related shifts in collagen phenotype may be largely controlled by this mechanism. While the possible mechanisms and functions of intracellular degradation are still being defined, it is clear that there is a significant difference between the number of collagen molecules synthesized by fibroblasts and the amount of intact collagen chains actually produced by these cells (Breul et al., 1980; Rennard et al., 1982).

Unlike the other methods of degradation, this process acts only on newly-synthesized collagen (Nimni et al., 1967; Bienkowski et al., 1978). As a result, the level of collagen metabolism can be measured by assaying hydroxyproline in the urine (Prockop et al., 1979a). Since hydroxyproline is found almost exclusively in collagen molecules, the presence of labelled hydroxyproline in the urine in peptide form indicates hydrolytic degradation of intact newly-synthesized collagen. The rate at which hydroxyproline

peptides appear in the urine decreases in old age (Nimni et al., 1967; Hall, 1976). This finding is paralleled by the simultaneous decreases in the rate of collagen synthesis (Martin et al., 1970; Lovell et al., 1987) and degradation (Lindstedt and Prockop, 1961) in the body. However, similar decreases in collagen metabolism with age is not seen in the periodontium (Narayanan and Page, 1983).

In the extracellular space, collagenases are the only physiologic enzymes capable of attacking the collagen triple helix (Harris and Krane, 1974; Weiss, 1976; Bornstein and Sage, 1980). Collagenase exists in virtually all mammalian tissues and is considered to be the primary means of destroying extracellular mature collagen fibrils (Bienkowski et al., 1978). This collagenolytic enzyme attacks collagen molecules within the fibril to disrupt the triple helix structure (Sakai and Gross, 1967). At body temperature and while dispersed in the extracellular fluid, the soluble collagen fragments become susceptible to further degradation by collagenase and other proteases (McCroskery et al., 1975). In addition, the formation of free ends of collagen following extracellular digestion prepares the protein for phagocytosis (Garant, 1976). However, the factors which contribute to collagen stability (such as cross-linking) increase its resistance to proteolysis (Harris and Krane, 1974) despite the continued production of collagenase into old age (Hall, 1976).

The identification of collagenase in diseased tissue, such as hypertrophic synovium, explains the reason for the extensive degradation of joint collagen in the elderly, although this finding

does not specifically confirm an age-related synthesis of the enzyme (Hall, 1976). During in vitro aging, discrepancies with respect to fetal collagenase activity have also been studied (Bauer et al., 1985). In contrast to the in vivo situation, there is an apparent decrease in the synthesis of collagenase in adult cultured cells. This latter finding has been attributed to the lack of in vitro expression of the enzyme rather than a true age-related effect. With successive population doublings in tissue culture, Houck and coworkers (1971) have also demonstrated that cells aged extensively in vitro lose the ability to induce collagenolytic activity.

In contrast to the mechanism of intracellular degradation discussed previously, the function of the collagenases in the control of collagen phenotype distribution is in question. With particular reference to collagen type I and III, some investigators (McCroskery et al., 1975; Miller et al., 1976b) have not found collagenase to selectively degrade collagen types, whereas others have found the contrary, especially during the inflammatory process (Horwitz et al., 1977).

In the acute phase of inflammation, the presence of polymorphonuclear leukocyte collagenase appears to selectively degrade type I collagen (Horwitz et al., 1977) which subsequently increases the relative proportion of the co-existing type III collagen present in the inflamed tissue. This specificity is lost by the time the later stages of inflammation are reached; therefore, the transient increase in the relative type III collagen synthesis is likely a physiologic component of the repair process in fibrous

tissues (Minor, 1980).

Trypsin has been shown to favour type III collagen by cleaving the molecule near the collagenase site (Miller et al., 1976a). However, this unusual chemical lability is not considered physiologic since trypsin is not found in the extracellular matrix in vivo. Nonetheless, type III may be susceptible to other proteases in addition to true collagenase (Miller et al., 1976a). The relative proportion of type III to type I collagen therefore reflects an equilibrium between synthesis and degradation that is controlled by the functional and physiological demands. These findings suggest that the changes in the relative proportions of collagen types I and III during inflammation and healing (type III early, type I late) (Bailey et al., 1975; Miller, 1976; Gay et al., 1978) may be controlled in part, by the collagenases present in the tissues (Horwitz et al., 1977). With advancing age, these events begin later, proceed more slowly, and often do not reach the same level of wound healing (Eaglstein, 1986). Nonetheless, these changes are only qualitative since the healing capacity of the aged still exceeds what is needed.

The only other major mechanism of collagen degradation is that of phagocytosis (Ten Cate and Deporter, 1975). Fibroblasts have been shown to phagocytose and subsequently degrade collagen within phagolysosomes intracellularly in a number of anatomical sites, including periodontal ligament of mice (Deporter and Ten Cate, 1973; Beertsen, 1987), rats (Garant, 1976) and monkeys (Svoboda et al., 1979); gingiva of mice (Ten Cate and Syrbu, 1974)

and man (Yajima and Rose, 1977); and cranial sutures in mice (Ten Cate et al., 1976) over a wide range of ages. Collagen fibre profiles have also been found in mitotic cells in the periodontal ligament (Beertsen and Everts, 1977).

Molecules that cannot be fully degraded by the collagenases may be removed by phagocytosis, although cleavage of collagen into fragments may not necessarily precede its ingestion (Svoboda et al., 1979). In addition, further digestion by lysosomes once phagocytosed is not always immediate (Ten Cate and Syrbu, 1974).

The activity of collagen phagocytosis appears to be dependent on the rate of collagen turnover (Beertsen, 1987). Under conditions of hypofunction, the net loss of collagen fibrils from the extracellular space is thought to be the result of a two-fold increase in its ingestion (Beertsen, 1987). In studies on periodontal ligament remodelling, fibroblasts can be "switched on" to assume a phagocytic function as required during "physiologic" tooth movement (Ten Cate et al., 1976); this may be a significant observation since it suggests the possibility of extrinsic control of collagen metabolism and subsequently, collagen phenotypic expression.

3.1.3 Collagen Phenotype

In most tissues, the structure and function of the extracellular matrix depends upon the amount, type and distribution of collagen (Bienkowski et al., 1978). However, the functional requirements for collagen in connective tissues can not

be fulfilled by a single type of molecule. The collagens represent at least eleven genetically distinct structural proteins designated as type I, I trimer, II, III, IV, V, VI, VII, VIII, IX, X, and XI (Bornstein and Sage, 1980; Cheah, 1985). It is the variation in length, primary structure and the presence, extent and location of both non-collagenous domains and interchain disulfide-bonding which form the basis for categorizing the different collagen types (Cheah, 1985).

The determination of the structural uniqueness of the alpha-chains which make up these various collagen types can be made on the basis of cyanogen bromide and protease peptide maps. These methods are quite sensitive and have been used for detection of new collagen types, determination of the relative ratio of types within a given tissue, and of the purity of selected types (Bornstein and Sage, 1980).

Individual polypeptide chains are separated by cleavage at either the methionyl residues with cyanogen bromide, or at the protease-specific sites along the collagen molecule, followed by one- or two-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bonner and Laskey, 1974; Dunbar, 1987). Each individual polypeptide chain is separated relative to its molecular weight and net charge. Particular significance is given to the delayed addition of Beta-mercaptoethanol to reduce the disulfide bonds present in type III collagen (Chung and Miller, 1974). This method was first introduced by Sykes and coworkers (1976) to separate the $alpha_1$(III) chains from the $alpha_1$(I) chains

during electrophoresis and results in typical "peptide maps" that are unique for each chain type (Bornstein and Traub, 1979; Dunbar, 1987).

Collagen types have also been separated on the basis of size by chromatography on ion exchange cellulose columns (Miller, 1976). Other types of one-dimensional peptide mapping include the use of protease digestion to characterize structural relationships (Sage and Bornstein, 1979; Sage et al., 1979), and segment-long-spacing (SLS) crystalline formation (Hay, 1981; Dunbar, 1987) to compare molecular weights and detect non-collagenous sequences in both procollagen and collagen (Anesey et al., 1975; Bornstein and Sage, 1980).

Very few, if any, of the collagen types have a unique tissue distribution and it is likely that as methods of detection become more sensitive, a broad distribution range will be found for each of the collagens (Bornstein and Sage, 1980). However, quantitative differences in collagen phenotype clearly exist, as certain tissues almost exclusively contain one collagen type (Kefalides and Denduchis, 1969; Miller, 1971), while others appear markedly heterogeneous (Toole et al., 1972; Trelstad, 1974). These observations have therefore raised some interesting questions regarding genetic control of type synthesis and structure-function relationships among the different collagens (Bornstein and Sage, 1980). Age-related changes in collagen polymorphism subsequently reflect the tissue's ability to adapt by modifying its collagen type distribution.

3. BIOCHEMICAL AND METABOLIC CHANGES IN COLLAGEN WITH AGING

3.2 Distribution and Physiology

Type I collagen, which constitutes approximately 90% of human body collagen (Rigby, 1983), is found in soft and hard connective tissues including skin, tendon, bone, periodontal ligament, dentin, fascia, placenta, and blood vessels (Miller, 1976a; Bornstein and Sage, 1980). This widespread distribution pattern of type I collagen fibres indicates that they play a major role as supporting elements in tissues, and that several connective tissue cell types are capable of synthesizing the type I molecule (Gay and Miller, 1978).

The primary structure of type I is essentially the same in all tissues, but the collagen fibres may differ in mechanical properties due to specific post-translational chemical modifications (Barbanell et al., 1978). For example, slightly higher hydroxylysine content (Stoltz et al., 1973) and subsequently more cross-link formation in bone allows for a closer packing of its collagen fibrils, and therefore providing bone its additional tensile strength and stability (Barbanell et al., 1978).

Type I commonly co-exists with other fibrillar collagens, types II or III, and together make up the interstitial collagens (Cheah, 1985). The essential difference between these various types is in the amino acid sequence of the three polypeptide chains. In the case of the type I collagen molecule, the triple helix consists of two identical chains, [$alpha_1$(I)] and a third chain, [$alpha_2$(I)], which has a different amino acid sequence.

Type I trimer is represented by the molecular formula [alpha$_1$(I)]$_3$ and is detected particularly in tissues maintained under adverse *in vitro* conditions. In organ cultures of mouse periodontium explants exposed to low oxygen tension (100-120 mm Hg), abnormally high alpha$_1$(I) to alpha$_2$(I) chain ratios (6:1) suggest the synthesis of large amounts of type I trimer (Yen & Melcher, 1978). This collagen type can also be extracted from *in vitro* assays of normal skin (Uitto, 1979), rat incisor odontoblasts (Munksgaard *et al.*, 1978), and intact or reconstituted mouse molar organs (Hata and Slavkin, 1978); however, it is more prevalent in diseased tissues such as, cloned and aged chondrocytes (Mayne *et al.*, 1976; Minor, 1980), osteoarthrotic cartilage (Nimni, 1974) and diseased gingiva (Narayanan *et al.*, 1980). The synthesis of type I trimer by cultured fibroblasts persists through many cell generations, indicating a genetically stable cell population (Narayanan and Page, 1983).

In vivo, the trimer can also be found in chick embryo tendons and calvaria (Jiminez *et al.*, 1977) and diseased gingiva (Narayanan and Page, 1983), but at much smaller quantities; this may be due to a lower synthesis rate by the cells *in vivo* or a greater susceptibility to degradation. In any case, the presence of the type I trimer in healthy tissue (detected *in vivo*) might indicate the expression of a normal synthetic product of a small population of cells residing in the tissue (Narayanan and Page, 1983).

The cartilage-specific type II collagen consists of three alpha$_1$ type II chains, designated [alpha$_1$(II)]$_3$ (Miller, 1972;

Bornstein and Sage, 1980; Minor, 1980). This $alpha_1$(II) chain significantly differs from the $alpha_1$(I) in amino acid composition and sequence, although the two chains are similar in molecular weight (Cheah, 1985) and in chromatographic behaviour (Bornstein and Traub, 1979). Type II collagen characteristically contains 22 hydroxylysine residues of which 50% are glycosylated, accounting in part for the much thinner fibrils than those in tendon or bone (Barbanell et al., 1978).

Due to the presence of type II collagen in such high proportions in cartilage, it may be assumed that the elaboration and maintenance of this tissue depends at least in part on its synthesis and deposition (Miller, 1976a). However, developmental anomalies affecting cartilage may not necessarily involve qualitative alterations in type II synthesis (Rhodes and Elmer, 1975; Stanescu and Maroteaux, 1975). In any event, the studies demonstrating the lability of the chondrocyte phenotype for collagen synthesis have profound implications with respect to the pathogenesis of rheumatoid diseases such as osteoarthrosis (Gay and Miller, 1978).

Type III collagen has a triple helix composed of three identical chains giving the molecular formula $[alpha_1(III)]_3$. This collagen is distinguished from type I and II by the presence of interchain disulfide bonds near the carboxyl termini of the $alpha_1$(III) chains (Bornstein and Traub, 1979). Type III was found initially in newborn human skin and, though its insolubility made it difficult to characterize, it was differentiated from other collagen types by the amino acid composition of several cyanogen bromide

peptides (Epstein, 1974). Except under specific circumstances, such as during fetal life (Epstein, 1974; Chung and Miller, 1974; Miller, 1976), type I and type III usually co-exist in tissues, with type I predominating (Epstein and Munderloch, 1975). The proportion of types I and III procollagens synthesized by fibroblasts vary with age (Yue, 1984; Ramshaw, 1986; Merkel et al., 1988); with tissue type, such as gingiva, periodontal ligament and skin (Butler et al., 1975; Sodek and Limeback, 1979; Rigby, 1983); with inflammation or altered metabolic states, like diseased gingiva (Narayanan et al., 1980; Narayanan and Page, 1983); in the presence of granulation tissue during wound healing (Miller, 1976; Gay et al., 1978; Merkel et al., 1988); and, with sutural remodelling in response to mechanical forces (Chiang, 1981; Yue, 1984).

The ratio of type I to type III is of general importance to tissue function, because the relative proportion of these two collagen types appears to have a major influence in its mechanical properties (Horwitz, 1977). For example, significant proportions of type III collagen are found in distensible tissues (skin, placenta, aorta, spleen, lung), while in rigid tissues (bone, tendon), there is only type I (Epstein and Munderloch, 1975). With age, there is a shift in the distribution of these collagen types to cause an increase in the type I/III ratio (Epstein, 1974); this is particularly seen in human skin as skin compliance concomitantly decreases with age. However, more recent studies (Ramshaw, 1986; Lovell et al., 1987) analyzing the different layers of human dermis separately, have discovered that the superficial papillary layer maintains a

significant level of type III even in very old age; in contrast, the underlying deep reticular layer continues to exhibit a rise in the I/III ratio with age. As mentioned previously, such changes in collagen phenotype distribution may be attributed to preferential intracellular degradation of the collagen types.

The association of high proportions of type III collagen with rapid collagen synthesis requirements is also apparent. Although the rate of synthesis is inversely related to donor age (Pinnell et al., 1987), the ability to induce type III collagen synthesis reflects the tissues' adaptability to functional and physiological demands.

A delicate network of reticular type III collagen fibres has also been found to co-exist with both type IV collagen (Bornstein and Sage, 1980) and type V collagen (Roll et al., 1980) to act as a support for the basement membranes (Duance et al., 1977). Evidence for this particular function of type III collagen is given by the clinical findings of extreme fragility of skin, vessels, and intestinal walls in individuals affected with Ehlers-Danlos Type IV syndrome (Pope et al., 1975); but, the reasons for the selective inhibition of type III collagen synthesis in these fibroblasts are still under investigation. On the other hand, fibroblasts from patients with other collagen diseases such as osteogenesis imperfecta (Penttinen et al., 1975; Sauk et al., 1980) and Marfan syndrome (Muller et al., 1975), appear to synthesize abnormally high quantities of type III. These latter observations may not necessarily signify alterations in the synthesis of type III collagen, but possibly a selective diminution in type I collagen synthesis (Miller, 1976a; Lovell et al.,

1987). With natural aging of dermal collagen, Lovell et al. (1987) have reported decreases in type III until 65 years, beyond which the level of type III increases again. Since the total collagen content was unchanged with age, the findings were also believed to be due to a selective decrease in the synthesis of type I.

Type IV collagen designated as $[alpha_1(IV)]_3$, has a non-fibrillar structure and is found in basement membranes (Bornstein and Sage, 1980). Unlike the interstitial collagens which are synthesized by mesenchymal cells such as osteoblasts, chondroblasts and fibroblasts, basement membrane proteins are secreted by adjoining cell linings (Minor et al., 1976a, 1976b). The continuous secretion of type IV collagen with age has been demonstrated immunohistochemically and morphometrically by the progressive thickening of the basement membranes of the renal glomeruli, renal tubules (Karttunen et al., 1986), seminiferous tubules and muscle capillaries (Xi et al., 1982). Basement membranes from adults have been reported to contain more hydroxyproline and hydroxylysine than those isolated from younger individuals, indicating an increased proportion of collagen in the basement membrane with age (Deyl et al., 1978; Langeveld et al., 1981; De Bats et al., 1982; Karttunen et al., 1986). The mechanisms responsible for basement membrane collagen accumulation have not been well-defined, although in rats, decreases in catabolism (Heck et al., 1981), synthesis (Hasslacher et al., 1984), and turnover of basement membrane components (Romen et al., 1980) with age have been reported. Given this accumulation, more extensive

cross-linking of collagen can be expected and is reflected by the increasing amounts of hydroxyproline remaining in the residue after enzymatic digestion (Karttunen et al., 1986).

Aside from the type and quantity, the molecular composition of collagen expressed can occasionally vary in different tissues (Niyibizi et al., 1984). For type V collagen, three different chain associations have been characterized: $[[\text{alpha}_1(V)]_2, \text{alpha}_2(V)]$, $[\text{alpha}_1(V)]_3$ and $[\text{alpha}_1(V), \text{alpha}_2(V), \text{alpha}_3(V)]$ (Cheah, 1985); the latter composition represents B, A, and C chains from the old nomenclature, respectively (Bornstein and Sage, 1980). Type V collagen is characteristically distributed in the fetal membranes (Burgeson et al., 1976), in the pericellular connective tissue (Madri and Furthmayr, 1979) and near basement membranes (Roll et al., 1980). As a minor component, type V also co-exists with the interstitial collagens in most connective tissues (Rhodes and Miller, 1978), although its quantity and distribution may be affected by pathological states (Narayanan et al., 1983).

The remaining types of collagens are not well characterized and are usually present in small amounts (Cheah, 1985). The major tissue distribution for type VI is in the intima of blood vessels (Furthmayr et al., 1983); type VII is in chorioamniotic membranes (Bentz et al., 1983); type VIII is in endothelium (Sage et al., 1983); and, type IX (Van der Rest et al., 1985), type X (Kielty et al., 1985) and type XI (Cheah, 1985) are found in cartilage.

While structure-function relationships of the different collagens remain to be defined, it is believed that the coordinate

expression of the collagen family is important to the overall development and differentiation of the body tissues (Cheah, 1985). Therefore, collagen is not only the main structural protein in connective tissue, but has been demonstrated histologically (Ten Cate et al., 1976; Engel et al., 1980) and biochemically (Sodek and Limeback, 1979; Hurum et al., 1982) to be metabolically active as well. Changes in collagen metabolism, in response to physiological and functional demands in tissues are reflected in the distribution of the interstitial collagens, especially for type I and III. Thus, quantitative differences in collagen phenotype distribution not only exist in local anatomic sites and appear related to normal tissue function, but modulated synthesis of the different collagens may also be crucial for the normal progression of dynamic processes such as development and tissue repair (Bornstein and Sage, 1980). Failure of the mechanisms controlling collagen metabolism and distribution often results in various disease states.

3.3 Collagen Phenotype as a Biological Signal

Several aspects of collagen metabolism which may be subject to age-related changes have been considered. The factors of important physiological consequences include collagen cross-linking, modulation of collagen type synthesis, and degradative functions. Although all matrix structural macromolecules, by definition, function extracellularly with certain important steps in collagen biogenesis occurring after its secretion, it seems likely that those age-related processes in connective tissues which are of significance in the pathogenesis of the senescent state will operate primarily at the level of the connective tissue cell. Therefore, the determination of cellular activity based on cellular metabolic behaviour would be an important parameter.

Since collagen is the principal structural protein of fibrous joints, its integrity and appropriate catabolism is essential for the normal development and function of this connective tissue entity. In particular, changes in the collagen phenotype ratio of type III/type I+III can serve as an indicator for changes in cellular activity and its regulation of collagen synthesis. It is well-known that the collagen which is laid down in the tissues is continuously metabolized to permit remodelling. These observations demonstrate that different forms of collagen are synthesized throughout life. The investigation of collagen phenotype ratios is therefore crucial to the understanding of fibrous joint remodelling during growth, development, aging and when subjected to therapeutic manipulation.

4. IN VITRO VERSUS IN VIVO AGING

Based on Strehler's four criteria for aging, differentiations can be made between the effects due to a true aging process and effects due to changes in experimental conditions under which the tissue is studied. The significance of in vivo versus in vitro environments with respect to aging should therefore be defined.

The ability to study aging at the cellular and biochemical level has provided tremendous insights into the basic mechanisms involved in this phenomenon. Most of the information on the relative rates of metabolism of type I and type III collagens has been obtained from in vitro studies (Sodek and Limeback, 1979). Therefore, it is important to assess whether the in vitro truly reflects the in vivo situation because if it does, then in vitro studies become valuable adjuncts to aging studies since the environment of the tissues can be manipulated.

With respect to collagen, the manipulation of oxygen tension and ascorbic acid concentrations can have marked effects on collagen aging. Increases in oxygen concentration, for example, have been shown to age young collagen fibres, as assessed on the basis of their increased mechanical and thermal stabilities (Mitchell and Rigby, 1975). This stability reaches a maximum level, similar to that obtained with in vivo aging, but can not be improved upon with further oxygen treatment. The finding that the same mechanical strength of native collagen can be attained with in vitro aging indicates similar stabilizing processes (Danielsen, 1987).

Ascorbic acid must also be present in sufficient concentrations in the culture medium to allow for uninhibited collagen synthesis (Pinnell et al., 1987). As noted previously, this component is essential to the stability of the newly-synthesized tissues (Levene et al., 1972; Ten Cate et al., 1976; Murad et al., 1981); however, once the cross-links have been formed and the collagen has been stabilized, as in older donor tissues, the level of ascorbate is not as critical (Rigby, 1983). The presence of high levels of ascorbic acid will also promote in vitro aging, but the effect will similarly become less obvious the longer the tissue remains in culture (Paz and Gallop, 1975; Rigby, 1983). Apparently, the degree of stimulation by ascorbic acid is similar in all cell strains from fetal to adult origin but its affect on the rate of collagen synthesis is inversely related to donor age (Pinnell et al., 1987).

In the investigation of the macromolecular aspects of aging, the intercellular substances are analyzed for clues to the mechanisms of cell degeneration and cell loss from the tissues of aging subjects, as well as from replicated cell populations in tissue culture. From the original studies of Hayflick and Moorhead (1961), the demonstration of a limited replicative capacity in fibroblast cultures has provided a useful model for the study of cellular aging (Muggleton-Harris and Defuria, 1985) since cultured normal human and animal cells have also been observed to have a finite ability to replicate and function (Cristofalo, 1972; Hayflick, 1984). However, with respect to the cells' functional capacity, some investigators

have expressed doubts that this *in vitro* model reflects age-related changes *in vivo* (Harrison, 1973; Kohn, 1975; Schneider and Mitsui, 1976).

Decreases in proliferative activity with old age are not necessarily indicative of a uniform loss of replicative ability (Praeger, 1986). With extensive *in vitro* aging, there is ultimately a preferential shift toward the non- or slowly-proliferating pool of cells (Martin *et al.*, 1974; Matsumara *et al.*, 1979; Pieraggi *et al.*, 1984; Mollenhauer and Bayreuther, 1986). This decrease in the proliferative activity has been attributed to the accumulation of non-fibrous extracellular substances, such as heparin sulfate on the cell layer (Bartold *et al.*, 1986). Changes in the relative proportions of other ground substance components, such as dermatan sulfate and chondroitin sulfate, have also been considered to reflect metabolic changes associated with aging (Matuoka and Mitsui, 1981). However, the shift or cell selection with *in vitro* aging is an expression of not only cellular aging, but the ability of the cultured cells to withstand the *in vitro* conditions. Therefore, with the continuous selection of the most proliferative and viable cell populations with *in vitro* aging (Martin *et al.*, 1974), the cells cultured after several subcultures may be expected to differ from those of earlier passages, as well as from donors aged *in vivo* (Schneider and Mitsui, 1976).

In terms of synthetic capacity, fibroblasts from both proliferating and non-proliferating pools (early and late passages, respectively) appear to synthesize collagen at the same rate overall

(Breul et al., 1980), although the amount of collagen synthesized decreases with donor age (Kivirikko and Risteli, 1976; Johnson et al., 1986). However, depending on the proliferative and synthetic potentials of these cells selected in vitro, the relative proportions of type I and III collagens will change (Mollenhauer and Bayreuther, 1986). Compared to the cell cultures of healthy donors, cultures from young progeric patients exhibited a proliferative capacity similar to old donors and late passage cultures (Praeger, 1986); nonetheless, normal amounts of both type I and III collagen may be extracted from the soft connective tissue of progeric patients (Bruckner-Tuderman et al., 1987).

In monkey periodontal ligament, increasing passage numbers have been reported to decrease the amount of type III collagen produced (Limeback and Sodek, 1979). This finding may be due to the selection of a population of cells which proliferate, but are more friable and inherently synthesize less type III. In contrast, type III collagen production has been found to increase with in vitro aging of chick tendon fibroblasts (Herrmann et al., 1980) and of mass fibroblast cultures of rat skin and lung (Mollenhauer and Bayreuther, 1986).

These above findings indicate that there are obviously differences in collagen metabolism for species and tissue type as well as for in vivo and in vitro aging. Many of the differences in cell behaviour between cultured cells and their counterparts in vivo stem from the dissociation of cells from a three-dimensional geometry and their propagation onto a two-dimensional substrate

(Freshney, 1983). As a result, specific cell interactions are lost since only the cell populations more adaptable to culture conditions survive. The assumption is that these cells are derived from a progenitor population that can be recruited for their capacity to divide, regardless of age. If there is any relationship between *in vivo* and *in vitro* cells, it most probably exists between proliferating cell cultures and those which proliferate *in vivo* (Cristofalo, 1972). As long as the differentiated properties of the cells are maintained *in vitro*, they can be used as indicators of cellular function under controlled physicochemical environments (Breul *et al.*, 1980); in this manner, cultured cells can be studied and related to the functional cells in the tissue.

CHAPTER II

STATEMENT OF THE PROBLEM

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An understanding of the controlling mechanisms which regulate fibrous joint remodelling will facilitate more efficient and expedient clinical manipulations. The adaptations of these joints to the stresses invoked by clinical forces on normal growth and development can be assessed by the relative metabolism of different collagens. Due to the significance of collagen composition in connective tissues during normal function, collagen metabolism has been considered to be an appropriate parameter in the study of cellular activity in remodelling joints.

This investigation selected male Sprague-Dawley rats as the experimental model since these animals have previously been used for studies on craniosynostosis, as well as, the effects of mechanical stresses on cellular metabolism. The interparietal suture has been selected because it exhibits natural loss of suture patency with age (Shillito and Matson, 1968), and for its convenient accessibility for surgical and orthopaedic manipulations. The capacity of interparietal sutures to respond with in vivo aging (Chiang, 1981; Yue, 1984) demonstrates its suitability as a model for remodelling studies. In addition, the larger quantity of tissue and the rat suture's more robust growth characteristics compared to mice render the rat model more attractive for biochemical analyses of the tissue when maintained in vitro.

As a preliminary step to the study of cellular metabolism in vitro, it was necessary to define the cells under study in the tissue

culture system. The first experiment (Chapter III) was done to demonstrate the origin and migration of the cells which have been assumed to populate the cell culture.

The second group of experiments (Chapter IV) consist of pilot studies necessary for the determination of the culture parameters used in the subsequent age study (Chapter V). The viability of the cells in primary and secondary culture will be assessed on the basis of their proliferative potential.

Finally, the last experiment (Chapter V) will ascertain the ratio of newly-synthesized type III collagen to total of type I and type III collagen *in vitro* in cell populations cultured from the rat interparietal suture at different stages of normal growth and development. The ultimate objective is to determine whether changes in the proportions of collagen phenotypes synthesized are due to changes in relative proportions of specific cell sub-populations present in the suture at each age, or due to modulation of individual cell expression. This study will also establish baseline data for future studies on mechanical stress of sutural cell populations utilizing comparable tissue culture conditions. A comparison between collagen synthesis and proliferative capacity will be discussed. In addition, the suitability of this *in vitro* system as a model for *in vivo* aging will be determined.

CHAPTER III

PROGENITOR CELL ORIGIN AND MIGRATION IN RAT INTERPARIETAL SUTURE *IN VITRO*

SUMMARY

The origin, proliferation, and migration of the progenitors populating cell cultures were characterized for rat interparietal sutures at ages 7 days, 2 weeks and 4 weeks. Tritiated-thymidine was added at the initiation of the tissue culture and continued over a period of 6 hours, 24 hours, 3 days, or 6 days for each of the selected animal ages. The interparietal sutural tissues were processed for histologic and radioautographic examination. Based on the location of the radio-labelled nuclei, the progenitor cells were found to originate from the sutural area proper, but subsequently divided and migrated toward the tissue culture plate with *in vitro* aging. As an *in vitro* expression of natural aging, the "latent period of emigration" of the explants in culture increased with donor age. However, taking into consideration this latent period, the proliferative and migratory activities of both old and young donors did not appear to significantly differ. These findings indicated that the progenitors which populated the cell culture system possessed a similar innate capacity to regenerate as a function of environmental conditions, but which was independent of age.

INTRODUCTION

The significance of defining the "sutural area" (Moss and Young, 1960) is two-fold. First of all, it emphasizes the continuity and interrelationship between the connective tissue layer(s) and the contiguous bone (Scott, 1954); and secondly, it attempts to infer some knowledge about the origin of the progenitor population that repopulates the area during growth and remodelling. When explants of interparietal sutures are placed in culture medium, the progenitor cells which proliferate and migrate from this tissue also presumably represent a sampling of cells which would have participated in the remodelling process *in vivo*.

Given the nature of the cell culture system employed, proliferation and migration of cells from the explant are necessary. However, age-related declines in fibroblast migration *in vitro* have been documented in rats (Soukupova *et al.*, 1970) and man (Schneider and Mitsui, 1976). In addition, known decreases in cellularity have been reported to occur with normal aging (Praeger, 1986). A two-fold difference in explant cellularity, for example, could lead to the need for an older donor culture to undergo one additional cell population doubling to reach confluency (Schneider and Mitsui, 1976). Whether this additional doubling is significant to *in vitro* aging or to the resulting phenotypic expression such as synthetic patterns of ratios of collagen types III/I *in vitro* is unknown.

In an age study that involves the proliferative and synthetic

functions of cell monolayers, it is obviously important that the origin of culture progenitors be determined. Therefore, it is convenient to postulate the existence of a progenitor population (Hall, 1978a; Melcher, 1986) which replaces the aging differentiated cells by undergoing mitosis (Leblond et al., 1959; Ten Cate et al., 1976; Everts and Beertsen, 1978). One of the daughter cells is thought to differentiate into a functional cell while the other remains in an undifferentiated state, retaining the capacity to divide once appropriately stimulated (Melcher, 1986). The presence of progenitors in externally stimulated (Weiss et al., 1968; Roberts and Jee, 1974) and untreated (Messier and Leblond, 1960; Jensen and Toto, 1968) periodontal ligament and cranial sutures (Chiang, 1981; Ten Cate et al., 1977) in adult animals has been well established.

Based on studies of continually erupting molars of adult rats (Jensen and Toto, 1968), progenitor cells have also been shown to retain the ability to differentiate along various cell lines to repopulate the periodontium and allow for repair. However, as a manifestation of aging, the number of connective tissue cells in the premitotic phase decreases with a concomitant decrease in mitosis (Hall, 1976); therefore, the rate of repair is expected to be slower (Jensen and Toto, 1968) or less responsive initially (Brandt et al., 1979). Nonetheless, even in old animals treated with irradiation (Skougaard and Carsten, 1978), the sutural connective tissues have been shown to recover their ability to metabolize collagen.

Based on these previous studies, there does not appear to be a

generalized age-associated loss of stem cell proliferative ability. Reported alterations in the progenitor numbers with age result from comparing growing animals with older ones, from changes in cellular environment, or from stimuli to growth or cell turnover elsewhere in the body (Kohn, 1975). These studies do not focus on the intrinsic capacity for cell division during remodelling.

Aside from the proliferative requirement, the sutural population must also differentiate as a function of externally-imposed demands, regardless of age (Jensen and Toto, 1968; Roberts and Jee, 1974; Weiss et al., 1986). The differentiated cells subsequently respond by the synthesis and degradation of extra-cellular components until the structural microenvironment satisfies the functional stresses experienced. The dynamics of sutural morphology should therefore reflect the metabolic activities of the cellular components (Koskinen et al., 1976). However, the lineage from which these progenitors are derived remains obscure (Hall, 1978c). It is not known whether the different population of functional cells arise from a single progenitor or whether each population of functional cells is generated by its own progenitor population (Hall, 1978a; Melcher, 1986).

One of the problems encountered in lineage determination is our inability to differentiate the degree of multi-potency based on progenitor cell morphology (Roberts, 1975). In general, the primitive stem cells are universally small cells with close-faced nuclei and minimal cytoplasm (Roberts et al., 1981; Melcher, 1986). However, depending on the degree of commitment of the progenitors along

their cell line, these so-called progenitor or precursor cells may also exhibit some of the characteristics of their more differentiated counterparts (Everts and Beertsen, 1978; Gould et al., 1980).

Using radioautographic techniques, the progenitors are identified solely by their labelling index for tritiated-thymidine (Leblond et al., 1959); as a result, any cell, regardless of apparent degree of cytodifferentiation, that retains the ability to undergo mitosis for remodelling or repair is considered a progenitor (Tonna and Cronkite, 1963; Roberts and Jee, 1974; Roberts, 1975). Yet, these cytodifferentiated progenitors (usually found in wounding) may only be a sub-population of the true mesenchymal prototype which exhibits the uniform cytological characteristics (Gould et al., 1980). Efficient remodelling or repair ultimately depends on the recruitment, proliferation and differentiation of such fibro- and osteo-progenitors (Roberts et al., 1981). The slower rate of response with age reflects a general diminished level of these cellular activities (Brandt et al., 1979).

In the case of the osteoprogenitors, the osteoclasts are believed to be recruited primarily from the macrophage/monocyte series; therefore, the association of mitotic cells (presumably progenitor cells) with blood vessels regardless of age has been a common finding (Linge, 1972). On the other hand, the osteoblasts appear to be produced by the proliferation and differentiation of local cell populations (Roberts et al., 1981). In sutures demonstrating continued remodelling growth, osteocytes in bone

matrix of periosteal or endosteal origin are believed to be released from the resorptive surfaces to rejoin the progenitor cell population (Young, 1963); however, convincing supportive evidence is lacking (Persson, 1973).

With respect to the fibrocytic series, a local rather than hematogenous origin of the fibroblast precursors has been favoured (Ross et al., 1970; Gould et al., 1980). By way of its vascular network (Roberts, 1975), a lateral migration of labelled cells has been demonstrated using tritiated-thymidine precursors in normally aged sutures (Persson, 1973) and wounded periodontal ligament (Gould et al., 1977); at the same time, the amount of label per cell decreases as the cells divide (Leblond et al., 1959; MacDonald, 1959). These findings support the concept of a distinct paravascular population of progenitor cells, some of whose progeny migrate and divide again (Gould et al., 1977).

It is the purpose of the present study to utilize tritiated-thymidine to characterize the origin, proliferation and migration of the progenitor population that is being studied in the tissue culture of the rat interparietal suture. Before any conclusions can be made concerning the remodelling capacity of these fibrous connective tissue joints, the origin of the cells under study must be identified as those from the sutural area proper.

MATERIALS AND METHODS

Dissection and Preparation of In Vitro Cultures

Four Sprague Dawley rats, inbred in our facility, were randomly selected for each of the following age groups: 7 days, 2 weeks, and 4 weeks. The animals were killed by cervical dislocation following ether anesthesia (Mallinckrodt Inc., Paris, Kentucky). The calvaria were exposed with a midsagittal dissection through the scalp. The interparietal sutures were excised following the removal of the overlying periosteum.

The sutural tissues were immediately transported to a laminar flow hood (Nuaire, Minnesota) in a growth medium. The growth medium consisted of Dulbecco's minimum essential medium (Gibco/BRL, Burlington, Ont.), 400 units per mL penicillin G (Gibco Laboratories, Grand Island, NY), 0.56mg per mL streptomycin sulphate (Gibco, Grand Island, NY), 0.2mg per mL ascorbic acid (Sigma Chemical Co., St. Louis, MO), and 2.2mg per mL sodium bicarbonate (Fisher Laboratory Chemical, Fair Lawn, NJ), pH 7.4. The medium was sterilized using a Millipore 0.22 μm filter (Millipore Corp., Mississauga, Ont.). Sterile, 10% fetal bovine serum (Flow Laboratories Inc., Mississauga, Ont.) was added.

The dura and any adherent soft tissue were stripped from the bony surfaces and the remaining blood was removed with 2 washes in sterile growth medium. Individual sutures were trimmed into 1.5mm wide strips and placed in prepared 35mm ($8cm^2$) tissue culture dishes (Falcon, Oxnard, CA). These dishes were incubated

with a coating of 100% fetal bovine serum for 1 hour prior to the addition of the explants to facilitate their adhesion to the dish. The fetal bovine serum was decanted prior to the addition of the suture and the 2mL of growth medium. 10μL/mL fungizone (Flow Laboratories, Inc., Mississauga, Ont.) was also added to each dish separately.

To isolate the location of the progenitor cells over time, all dishes were continuously labelled with 5μCi/mL [^{3}H]-thymidine ([specific gravity 84.4Ci/mmol] DuPont Canada Inc. (NEN Products), Lachine, Que.) since the initiation of the culture. The same concentrations of ^{3}H-thymidine was replaced in the medium every 2.5 days with feedings. This latter procedure was therefore only required for the cultures maintained in vitro for 3 to 6 days.

The dishes were incubated (National incubator model 4200, Portland, Oregon) in a humidified atmosphere of 5% CO_2 at 37 degrees C. and the cells were monitored with a phase contrast microscope (Nikon, Japan). After 6 hours, one suture from each of the 3 age groups was placed in Bouin's solution for fixation. The remaining cultures were similarly terminated at the end of 24 hours, 3 days and 6 days.

Preparation for Histology and Radioautography

The sutures were fixed in Bouin's solution for 24 hours, dehydrated in 70% alcohol for 48 hours, and decalcified in 12% EDTA (ph 7.2) for 3 weeks. After embedding in paraffin, 5μm serial sections were cut and mounted on glass slides (Corning Glass

Works, Corning, NY) with an albumin fixative. The slides were then placed in a 60 degree C. oven for a minimum of 6 hours (to permanently fix the sections onto the glass slides), and allowed to cool to room temperature. Every fourth slide was stained with haematoxylin and eosin and examined under the photomicroscope (Zeiss, West Germany). Adjacent unstained slides from representative sections were processed for radioautography.

Five slides from each age and time period were divided into 5 separate slide boxes, so that a representative slide from each group was processed separately. Using the technique of Kopriwa and Leblond (1962), the slides were dewaxed and dipped in Kodak NTB2 emulsion (Kodak Canada Inc., Brampton, Ont.). Exposure was made in the dark at 4 degrees C. for 28 days. The radioautographs were developed with Dektol (Kodak Canada Inc., Brampton, Ont.) fixed with rapid Fixer (Kodak Canada Inc., Brampton, Ont.) and stained through the emulsion with haematoxylin and eosin.

Photomicrographs of the interparietal suture were taken of each radioautograph with 35mm Panatomic-X film (Kodak Canada Inc., Brampton, Ont.), and developed using microdol (Kodak Canada Inc., Brampton, Ont.). Black and white prints, enlarged 6.5X the original magnification, were made on Kodak polycontrast rapid II RC paper, developed with Kodak Dektol, and fixed with Kodak Fixer.

RESULTS

In all cases, the progenitor cells have originated from the sutural area proper with comparatively minimal labelling in the bony cavities. Labelled cells often appear to lie adjacent to the bony edges as they migrate out to the culture plate surface. In only two instances (Figure III-24 and Figure III-36), examination of serial sections (not shown) suggest that the mitotic cells may be paravascular. Otherwise, labelled cells do not appear to be associated with any vascular elements, since blood vessels are not prominent features in any of these sutures. This is particularly the case in young sutures. Most of the lucent areas noted in the 4 week specimens are probably artifacts subsequent to histological preparation. These areas have no evidence of endothelial linings as would be seen in vascular structures.

There is definite evidence of an age-related latent period which is defined as the time necessary for the first cells to emigrate from tissue explants in vitro. Although it is subject to inter-animal variabilities (Macieiro-Coelho and Azzarone, 1982), the present results indicate a progressive increase in the latent period with donor age.

Associated with this slower migration time, in cultures from 2 week and 4 week donors, is the initially fewer number of labelled cells in the entire suture (Figure III-13 and Figure III-25). In contrast, a large amount of mitotic activity in the sutural connective tissue area was obviously present within the first 6

hours of culture in the 7 day old animal (Figure III-1). However, with in vitro aging, there was a progressive increase in the proportion of cells labelled in the 2 and 4 week old sutures (with the exception of the 4 week sample in its third day in culture [Figure III-31]). By 6 days, the proliferative and migratory activities of the progenitors in all sutures were clearly evident (Figures III-10; III-22; III-34).

With in vitro aging, the labelling pattern became more heterogeneous; that is, with the migration of progenitors out to the culture plate surface, labelling was noted not only within the suture but along one of its periosteal surfaces as well. This pattern was evident in 7 day old sutures by 24 hours in vitro (Figure III-4), but not in the 2 week and 4 week sutures until 3 days (Figure III-19) and 6 days (Figure III-34), respectively. It is important to note that although the periosteum could not be easily stripped from the bony surface in the 7 day and 2 week old animals (without causing excessive surgical trauma), the labelling of this soft connective tissue was only evident at later periods in vitro which would probably suggest cell migration of the labelled progenitors. Arrowheads noted on the radioautographs represent the asymmetric direction of this migration.

TABLE III-1

Radiographic Presentation of Cross-sections of Rat Interparietal Sutures

Figure No. III-*	Donor Age (In vivo Age)	Culture Period (In Vitro Age)
1-3	7 days	6 hours
4-6		24 hours
7-9		3 days
10-12		6 days
13-15	2 weeks	6 hours
16-18		24 hours
19-21		3 days
22-24		6 days
25-27	4 weeks	6 hours
28-30		24 hours
31-33		3 days
34-36		6 days

* Three radioautographs consisting of low, medium and high original magnifications have been presented for each culture period.

Low magnification: X300
Medium magnification: X800
High magnification: X2000

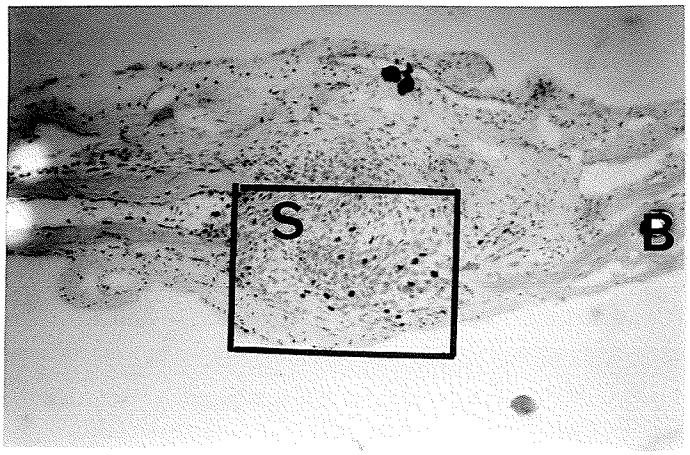
LEGEND: B = bone

S = sutural soft tissue

Figure III-1: Radioautograph of cross-section of rat interparietal suture. Donor age is 7 days; culture period is 6 hours. Note abundance of tritiated-thymidine label confined to sutural area. Vascular elements not evident in the magnifications in the following figures. Haematoxylin and eosin. X300

Figure III-2: Magnification of area delineated in Figure III-1. Arrowhead indicates direction of cellular migration. Note asymmetric location of labelled cells. X800

Figure III-3: Magnification of Figure III-1. Note labelled cells adjacent to bony margin as well as in suture proper. X2000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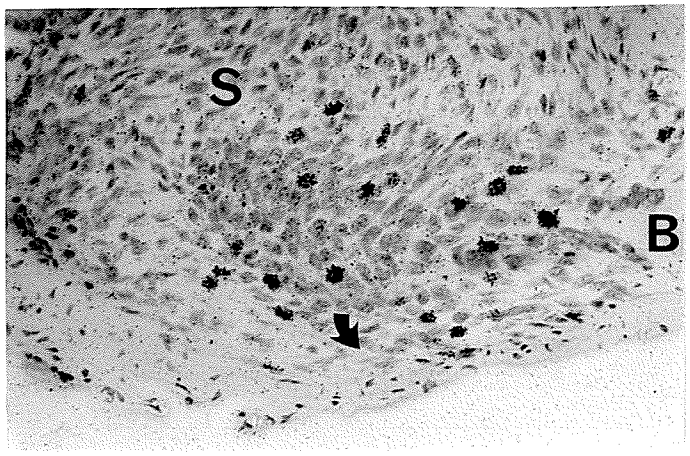
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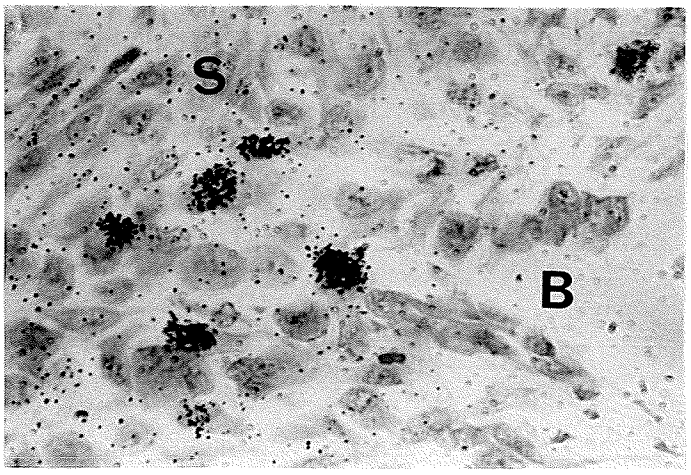
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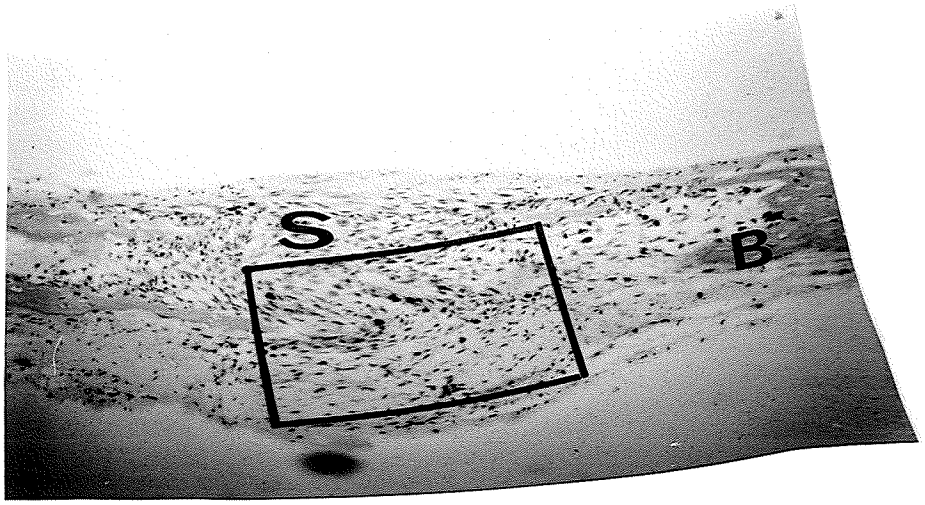
Figure III-4: Radioautograph of cross-section of rat interparietal suture. Donor age is 7 days; culture period is 24 hours. Haematoxylin and eosin. X300

Figure III-5: Magnification of area delineated in Figure III-4. Black arrowhead indicates direction of cellular migration. Two open arrows point to cells which have migrated just outside suture area proper. Compare with Figure III-2. X800

Figure III-6: Magnification of Figure III-4. Note comparable level of mitotic activity to Figure III-3. Mitotic cells adjacent to bone as well as in suture proper. X2000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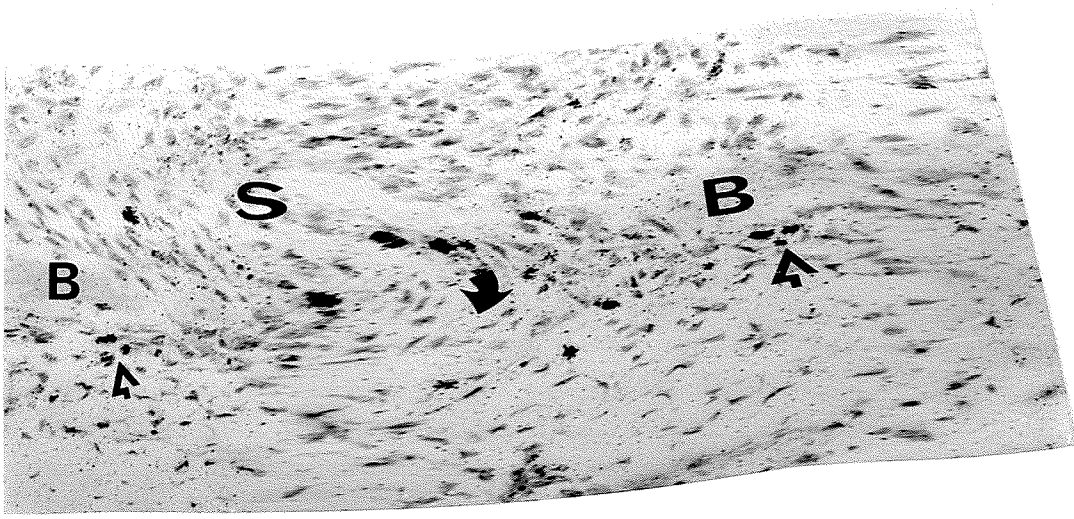
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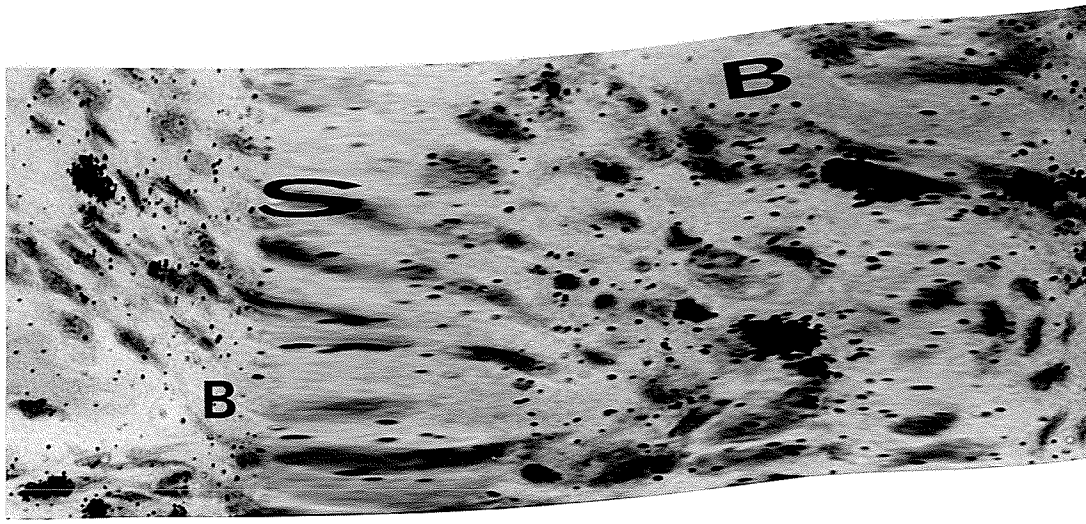
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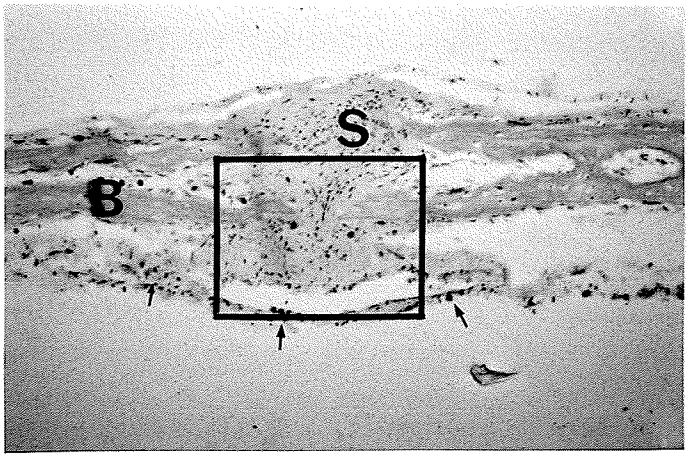
Figure III-7: Radioautograph of cross-section of rat interparietal suture. Donor age is 7 days; culture period is 3 days. Arrows point to widespread migration of cells adjacent to tissue culture plate surface. Haematoxylin and eosin. X300

Figure III-8: Magnification of area delineated in Figure 7. Arrow indicates direction of cellular migration. X800

Figure III-9: Magnification of Figure III-7. Note labelled cells located adjacent to bony surfaces. X2000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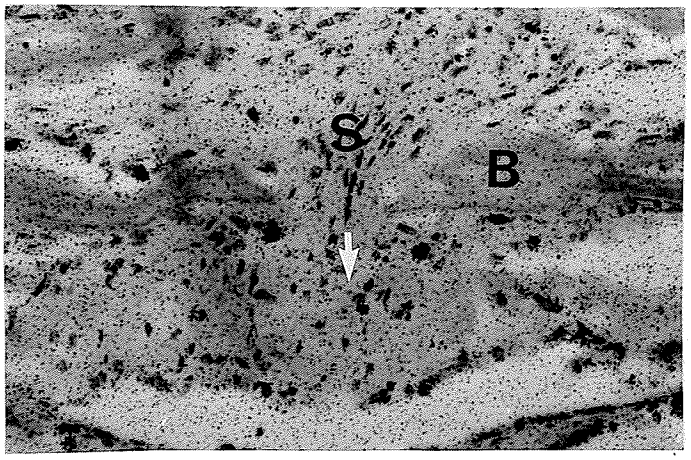
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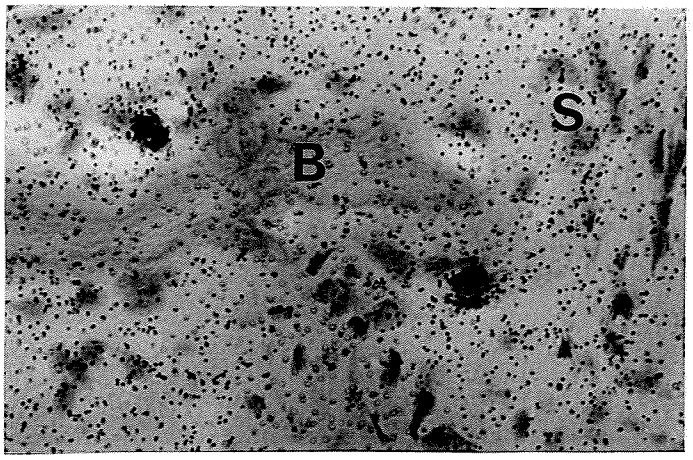
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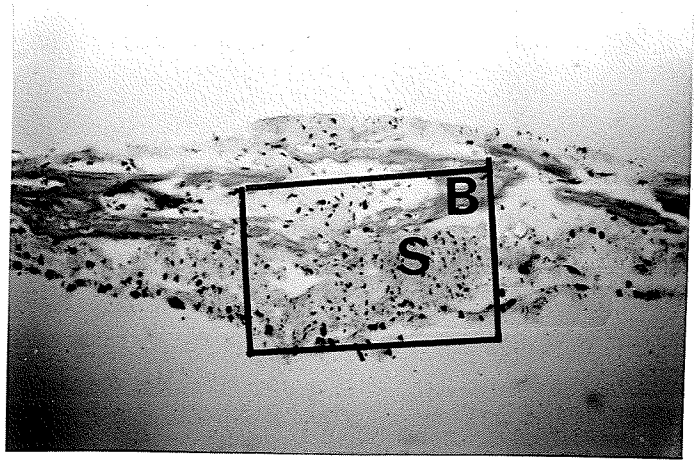
Figure III-10: Radioautograph of cross-section of rat interparietal suture. Donor age is 7 days; culture period is 6 days. Note relatively minimal labelling within the explant compared to earlier culture periods. Clear spaces are artifacts due to histological processing rather than vascular elements. Haematoxylin and eosin. X300

Figure III-11: Magnification of area delineated in Figure III-10. Compare distribution of labelled cells with those of Figure III-8. Note the more peripheral distribution in the present figure. Arrow indicates direction of cellular migration. X800

Figure III-12: Magnification of Figure III-10. Note the location of labelled cells in the suture proper as well as adjacent to the periosteal surface (arrow). X2000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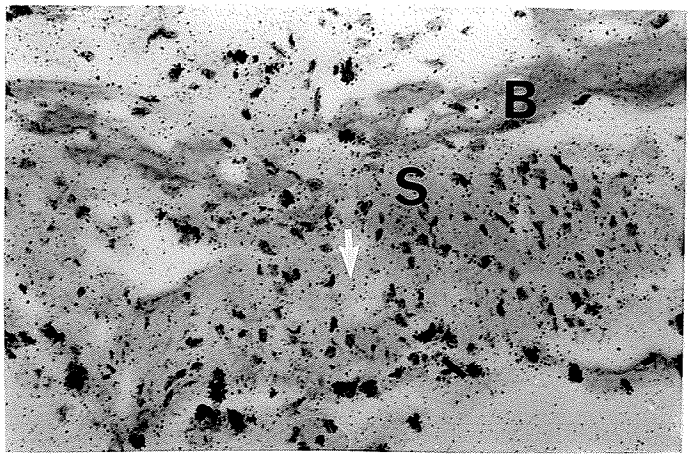
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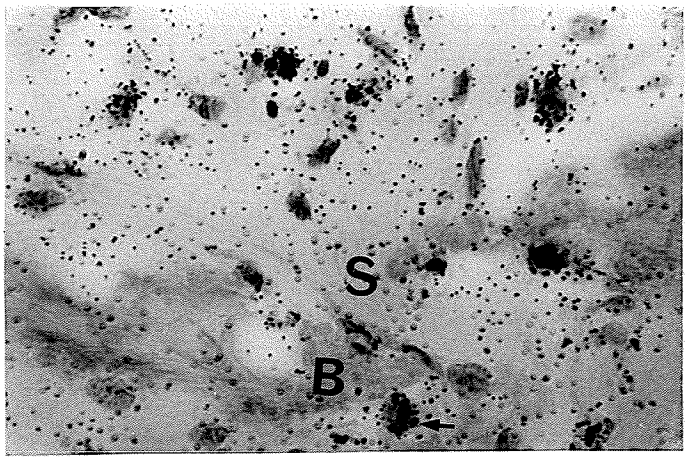
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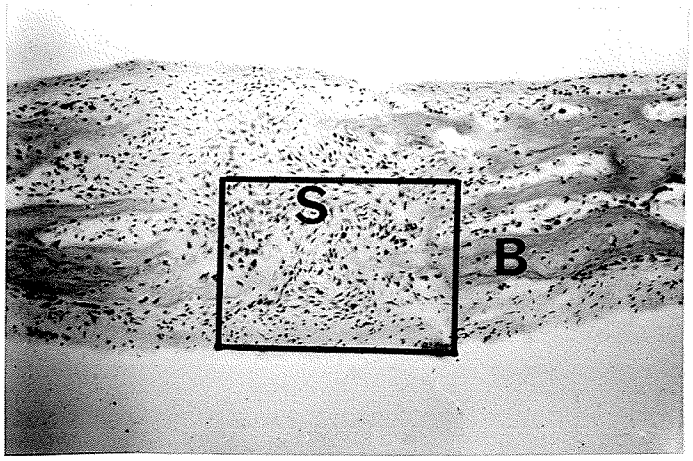
Figure III-13: Radioautograph of cross-section of rat interparietal suture. Donor age is 2 weeks; culture period is 6 hours. Comparatively few labelled nuclei unlike 6 hour culture of 7 day animal (Figure III-1). Haematoxylin and eosin. X300

Figure III-14: Magnification of area delineated in Figure III-13. Arrow indicates direction of cellular migration. White arrows indicate labelled cells. X800

Figure III-15: Magnification of area delineated in Figure III-13. Mitotic cells in this section are not associated with bony surfaces, but lie within suture proper. X2000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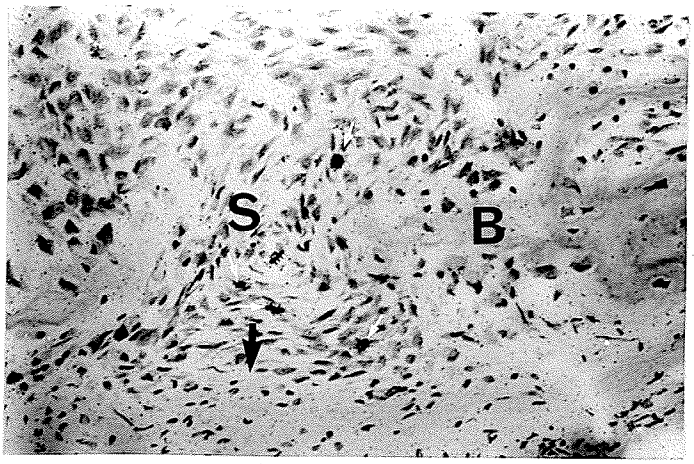
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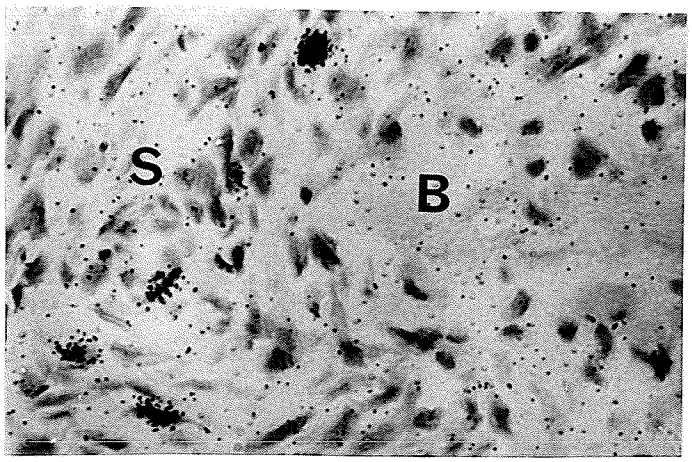
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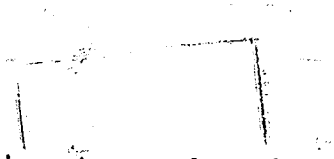
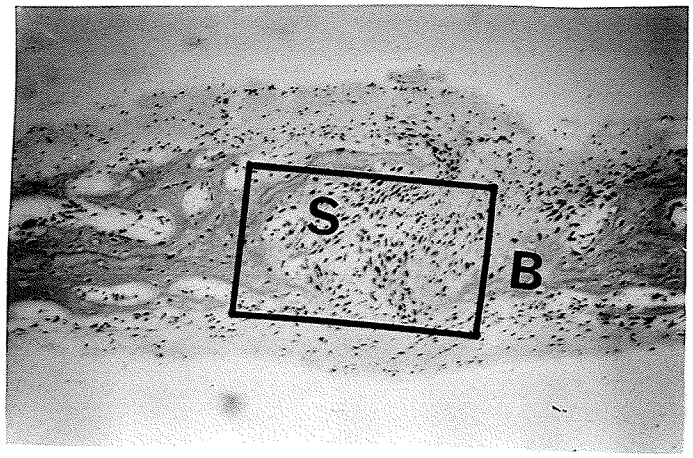


Figure III-16: Radioautograph of cross-section of rat interparietal suture. Donor age is 2 weeks; culture period is 24 hours. Haematoxylin and eosin. X300

Figure III-17: Magnification of area delineated in Figure III-16. Arrow indicates direction of cellular migration which is minimal at this time. X800

Figure III-18: Magnification of Figure III-16. Where radio-labelling is noted, mitotic cells are located in the suture proper. X2000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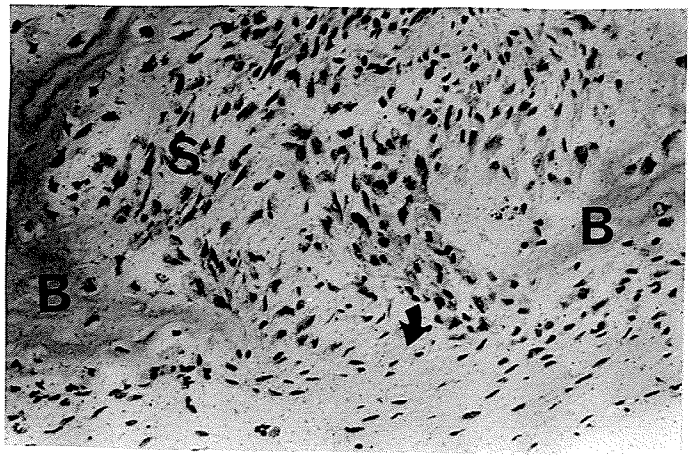
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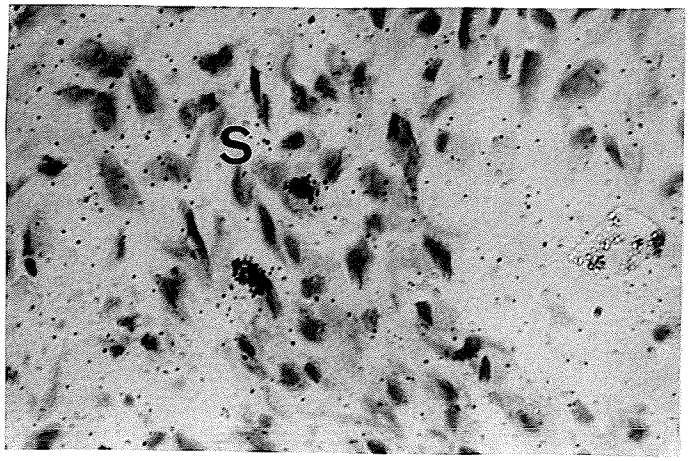
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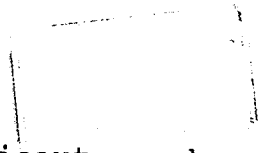
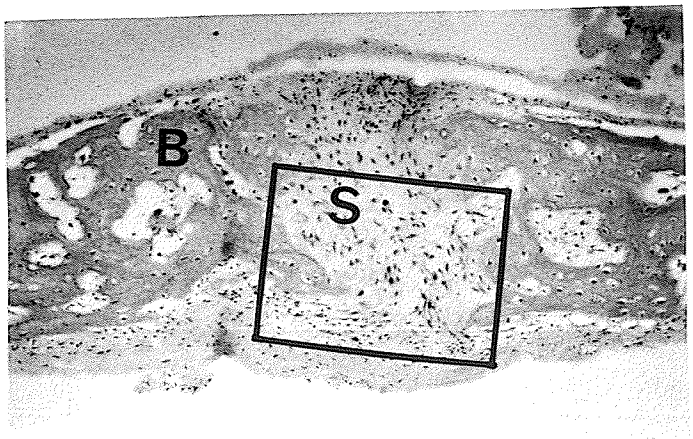


Figure III-19: Radioautograph of cross-section of rat interparietal suture. Donor age is 2 weeks; culture period is 3 days. Haematoxylin and eosin. X300

Figure III-20: Magnification of area delineated in Figure III-19. Closed arrow indicates direction of cellular migration. Open arrow points to cells beginning to migrate beyond suture proper, similar to state of 7 day old animal after only 24 hours in culture (Figure III-5). X800

Figure III-21: Magnification of Figure III-19. Note location of labelled cells in the suture proper. X2000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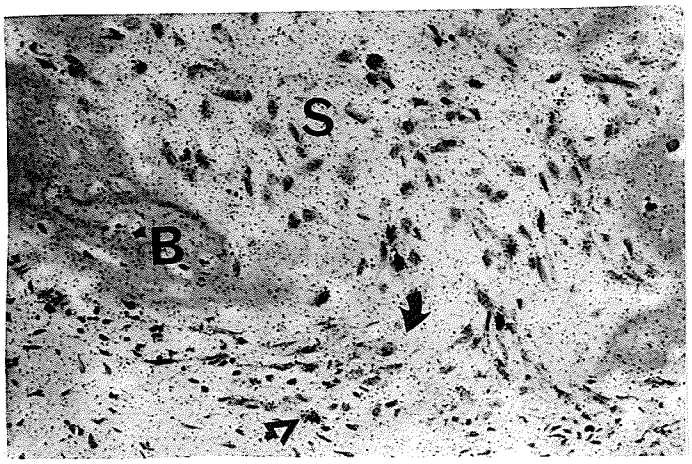
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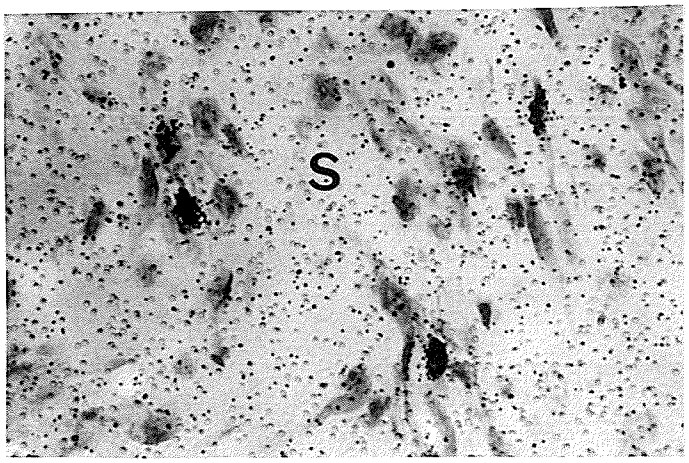
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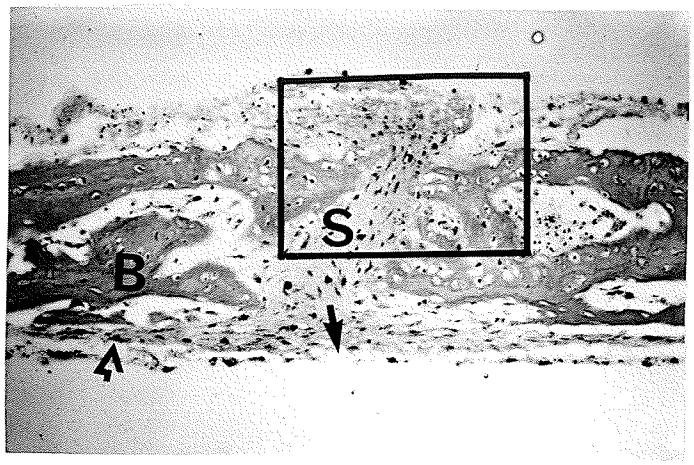
Figure III-22: Radioautograph of cross-section of rat interparietal suture. Donor age is 2 weeks; culture period is 6 days. Closed arrow indicates direction of cellular migration, as given by the presence of labelled cells along the bony surface (open arrow). Haematoxylin and eosin. X300

Figure III-23: Magnification of area delineated in Figure III-22. This is the only case where the labelling is not asymmetric. X800

Figure III-24: Magnification of Figure III-24. Labelling pattern of suture similar to the 7 day old animals aged in vitro for 6 and 24 hours (Figures III-3 and III-6). Arrow points to mitotic cells identified within a clear area in the suture due to histological preparation rather than to a vascular element. X2000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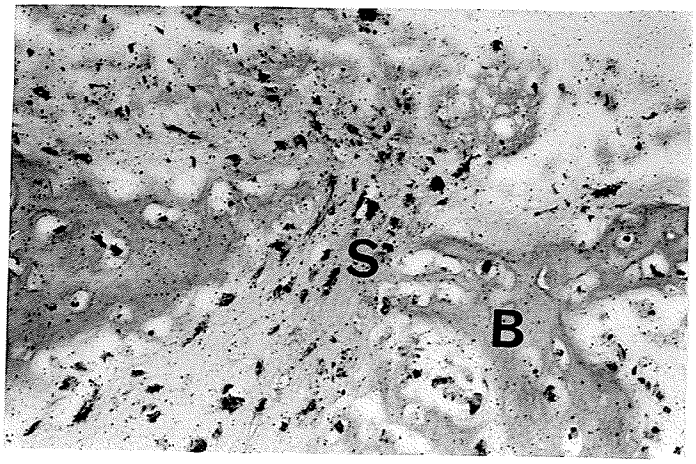
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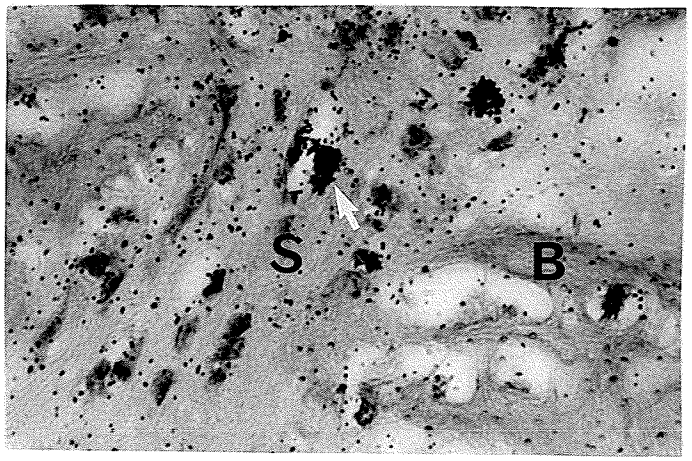
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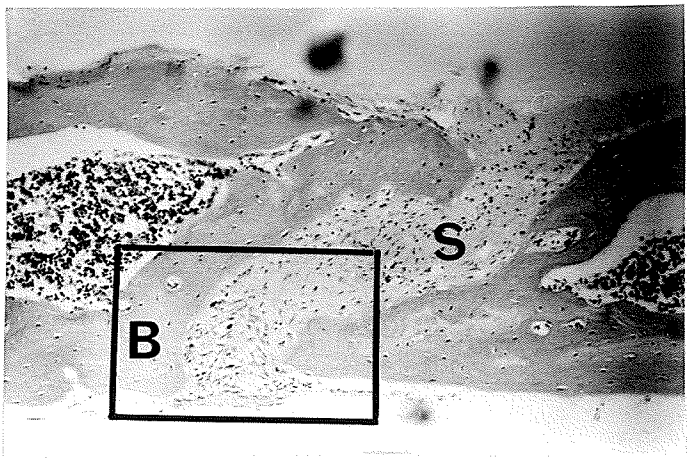
Figure III-25: Radioautograph of cross-section of rat interparietal suture. Donor age is 4 weeks; culture period is 6 hours. Note minimal number of labelled cells in general compared to 7 day and 2 week old animals. Haematoxylin and eosin. X300

Figure III-26: Magnification of area delineated in Figure III-25. Arrow indicates direction of cellular migration. X800

Figure III-27: Magnification of Figure III-25. Note labelled cells adjacent to bony margin as well as in suture proper. X2000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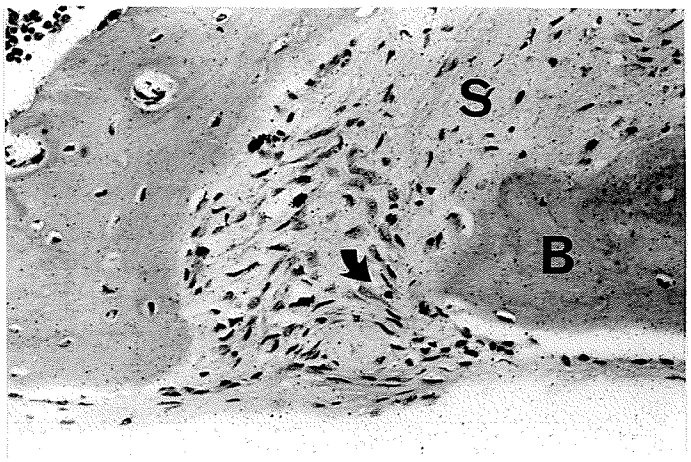
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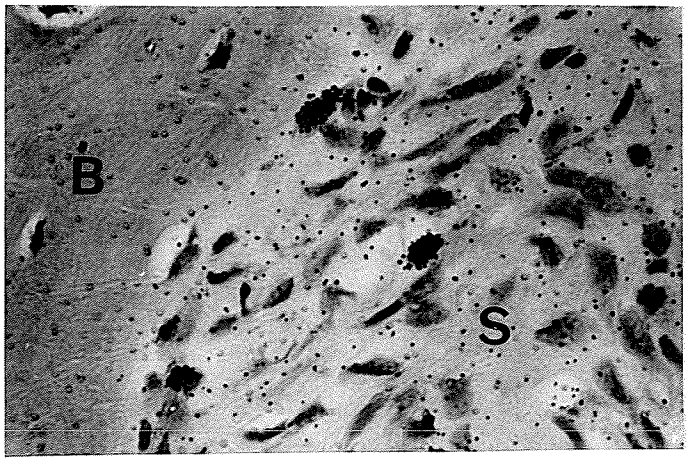
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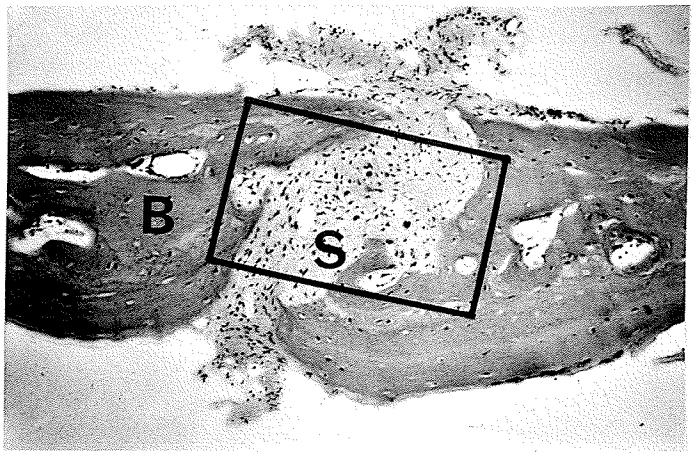
Figure III-28: Radioautograph of cross-section of rat interparietal suture. Donor age is 4 weeks; culture period is 24 hours. Note increase in relative labelling in suture compared to Figure III-25. Haematoxylin and eosin. X300

Figure III-29: Magnification of area delineated in Figure III-28. Mitotic cells present in suture proper. Arrow indicates direction of cellular migration. X800

Figure III-30: Magnification of Figure III-28. Note labelled cells in sutural area proper. X2000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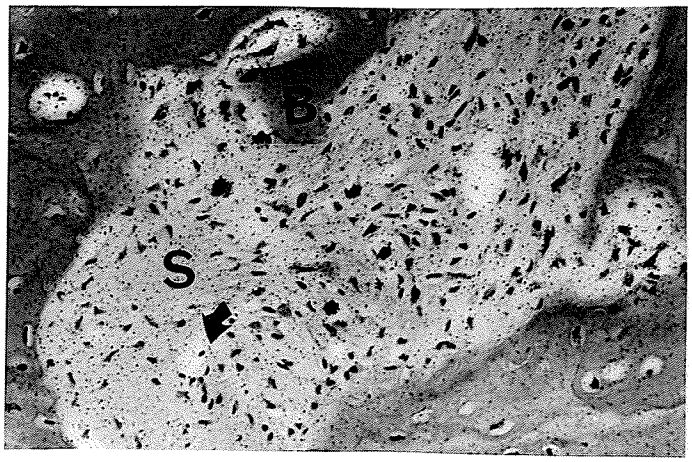
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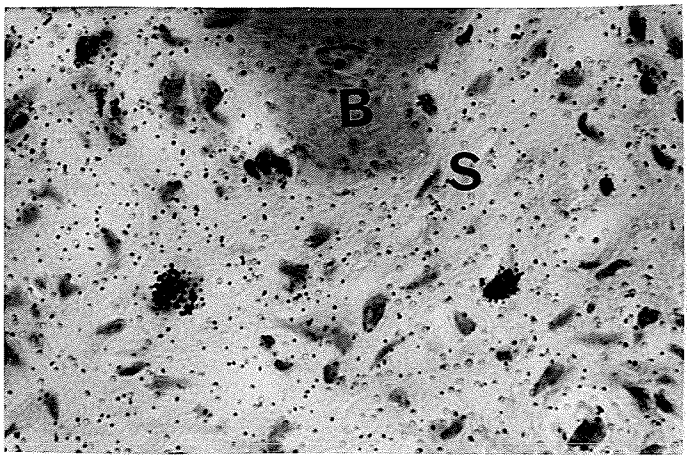
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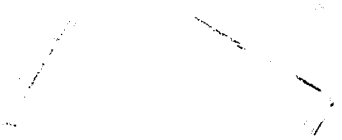
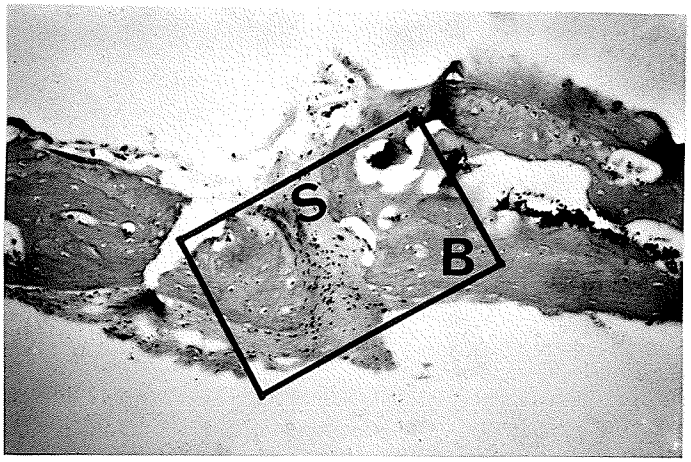


Figure III-31: Radioautograph of cross-section of rat interparietal suture. Donor age is 4 weeks; culture period is 3 days. Haematoxylin and eosin. X300

Figure III-32: Magnification of area delineated in Figure III-31. Due to limited amount of labelling (similar to 4 week suture aged in vitro for 6 hours [Figure III-26]), direction of migration questionable. However, arrows point to possibly migrating mitotic cells. X800

Figure III-33: Magnification of Figure III-31. Arrow pointing to artifact due to histological preparation. X2000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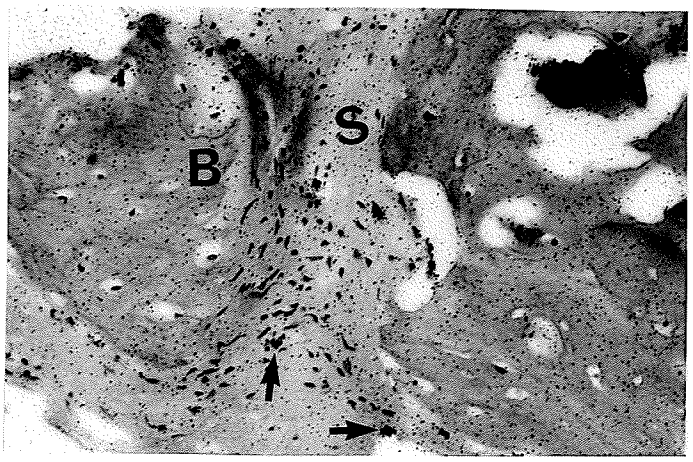
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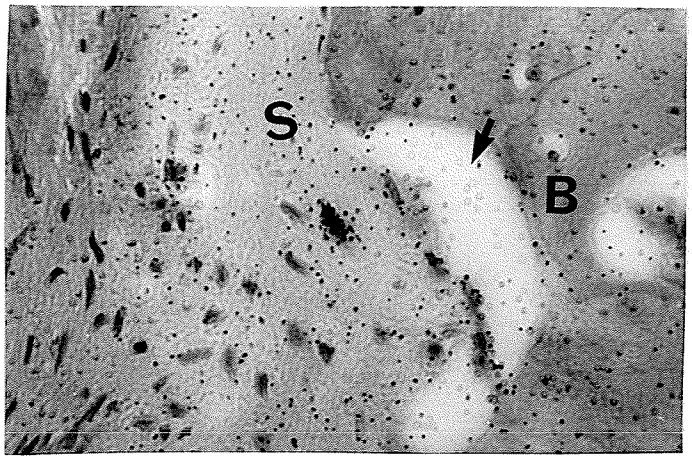
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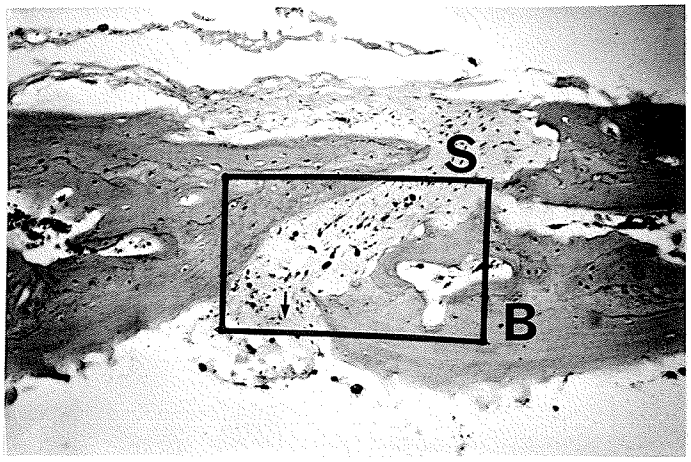
Figure III-34: Radioautograph of cross-section of rat interparietal suture. Donor age is 4 weeks; culture period is 6 days. Note labelled cells in soft connective tissue near bony surface indicating active migration onto the tissue culture plate surface. Arrow indicates direction of asymmetric cellular migration. Haematoxylin and eosin. X300

Figure III-35: Magnification of area delineated in Figure III-34. Greater amount of labelling when compared to 4 week sutures with less *in vitro* aging, but similar to 7 day old animals aged *in vitro* for 6-24 hours. Arrow indicates migrating direction of cells from the sutural area proper. X800

Figure III-36: Magnification of Figure III-34. Arrow points to radiolucent area adjacent to mitotic cells which is an artifact of the histological preparation. X2000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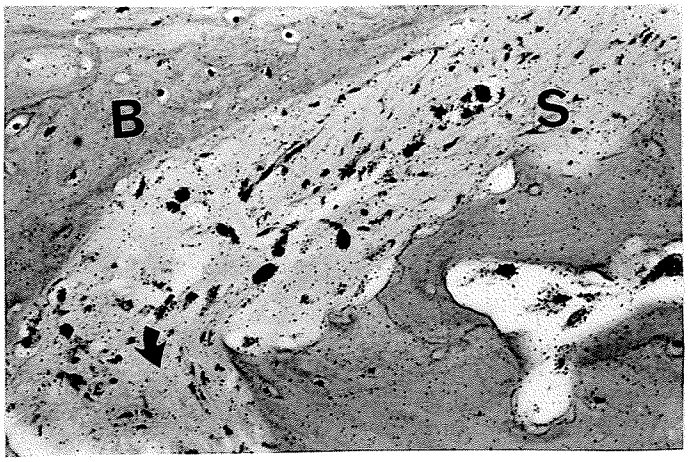
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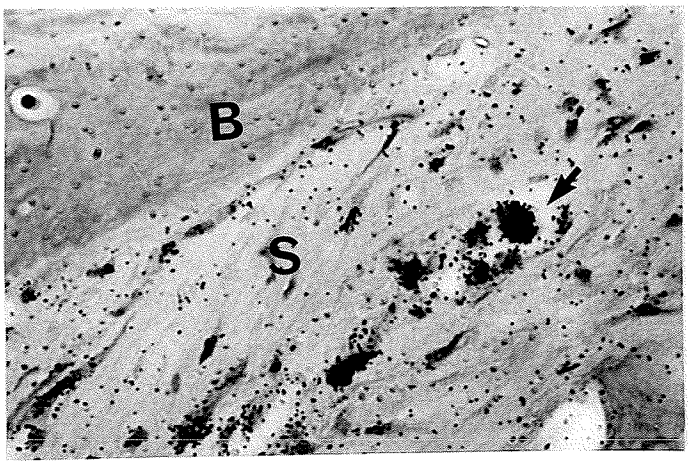
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DISCUSSION

Although the observations made in this study are qualitative, they serve their purpose by illustrating both the site of origin of the tissue culture progenitors as well as their migratory activity.

Using an analogous model such as periodontal ligament, previous studies by Gould et al. (1977, 1980) have reported progenitor cells to be paravascular in nature. In the present study, the progenitors were found to be paravascular in only two instances (Figure III-24, Figure III-36) since very few blood vessels were evident in the interparietal sutures examined. Even in the in vivo material presented by Chiang (1981), vascular elements were not prominent features of interparietal sutures. Therefore, it was unlikely that blood vessels were lost in the present study due to the in vitro exposure. However, particularly in the older donors, the present findings may not rule out the possibility that the progenitor cells labelled in the suture area proper had earlier migrated from paravascular areas.

Utilizing a continuous labelling technique, tritiated-thymidine was incorporated in mitotic cells during the S-phase (DNA synthesis) of the cell cycle throughout the culture period. Continuous labelling differs from pulse-labelling where, in the latter technique, the number of silver grains in each cell will decrease by one-half with every cell division (Leblond et al., 1959; MacDonald, 1959) as the culture period increases. However, with the use of either of the above techniques, as the progenitor cells proliferate

from their origin in the sutural area proper and migrate, the labelled cells subsequently increase in number and change in location. This change in location is demonstrated ideally by serially following the mitotic cells over different culture periods or by the cross-sectional study of sutures of different ages as was done in this study.

In the animals aged 7 days, the high proliferative capacity is shown by the immediate labelling of mitotic cells (Figure III-1). With in vitro aging, the labelled cells appear to have relocated asymmetrically from the sutural area proper toward one periosteal surface (Figure III-5; Figure III-7). By the 6th day in culture, the labelled cells from the 7 day old donor sutures have concentrated adjacent to the periosteal surface (Figure III-10). This change in the location of labelled cells has been interpreted as migration of sutural cells toward the charged surface of the tissue culture plate (Freshney, 1983). However, one can also argue that the labelled cells at the bony periphery are resident populations of the periosteum that have failed to divide until late in vitro. This latter conclusion is unlikely given the migration noted in the older animal explants (Figures III-25 and III-34) in the absence of periosteum.

In an attempt to verify the migratory effect, a pulse labelling study may be more informative. With the use of this latter technique, changes in labelling intensity (number of silver grains per cell) subsequent to cell division and migration can be determined simultaneously. However, particularly in the older donor explants, the latent period would still have to be determined in

order that the pulse of isotope will be applied at a time for maximal incorporation.

As a follow-up to the present radioautographic study, an alternative approach to ensure that cells emigrating from sutural explants are not from the periosteal layer would be to predigest the explant enzymatically to remove the "superficial" layer of cells. Both techniques (enzymatic digestion or mechanical stripping of periosteum) are not only relatively traumatic, but present risks of incomplete removal of periosteal cells. However, gross stripping of the outer periosteum might lend to the maintenance of the heterogeneous "internal" sutural population, while enzymatic digestion has the added disadvantage of possibly removing these "internal" cells present *in vivo*.

Where the tissue culture system has met all requirements for cellular attachment and nutrition, it is quite possible that the *in vitro* environment has also recruited very different populations of cells than those found *in vivo*. However, perhaps the strongest source of support for the relationship between *in vivo* and *in vitro* aging is derived from the finding that the latent period in this study has increased with donor age.

The latent period of emigration is significant in that it provides a direct measure of the speed with which cells within the explant can react to an "activation stimulus" presented by the conditions of culture (Lefford, 1964). This inactivity of cell movement with tissue differentiation is evident even after the cessation of active growth (Soukupova *et al.*, 1970) as shown by the

continual increase in the latent period with age. Therefore, if much older animals had been studied in the present investigation, the onset of tritiated-thymidine labelling probably would not have been expected until well past 6 days in culture.

Explanted tissues are expected to behave like a mosaic of active and inactive cells in which the percentage of slowly migrating cells increases with natural aging (Hayflick, 1977). Age-dependent metabolic changes, such as ATP turnover, also correlate with the inability of older cells to be stimulated to migrate in culture (Muggleton-Harris and Defuria, 1985). Despite this slower rate of cell migration (Lefford, 1964), the mitotic index does not appear to vary (Chaytor, 1962). Any decline in the mitotic index with donor age usually represents an intermediate response of the cells to their environment. These findings suggest that the mitotic response is a function of the time during which the emigrated cells are directly exposed to the culture medium (Lefford, 1964). The considerable difference in cell numbers found in the zone of growth depends on the amount of emigration from the explant, which in turn depends on the growth state of the explant (Chaytor, 1962). Therefore, the latent period has been attributed to the progressive decrease in the number of available progenitors as a function of donor age (Soukupova *et al.*, 1970), rather than to a generalized decrease in stem cell proliferative capacity (Kohn, 1975).

In effect, the latent period is an *in vitro* expression of cell cycle inhibition, evident in late passage cells and older donor tissues, where there is an increase in the length of the division

cycle. Macieira-Coelho (1970) demonstrated a decrease in the proportion of cells included in DNA synthesis due to the increase in the G_1 and G_2 resting periods of the cycle with *in vitro* aging. Cells which become G_1 blocked do not synthesize DNA, while cells that are G_2 blocked do not mitose once the DNA is synthesized (Gelfant and Smith, 1972). The longer normal diploid cells spend in these stationary phases, the smaller the number of accrued doublings the population could potentially undergo (Cristofalo, 1972). Nonetheless, most cells are able to become "unblocked" and eventually undergo DNA synthesis and division, although at a slower rate than younger populations (Macieira-Coelho and Azzarone, 1982).

These previous results are compatible with the present study and with those of others (Martin *et al.*, 1970; Schneider and Mitsui, 1976; Albini *et al.*, 1988) whose findings have also demonstrated an overall decrease in the relative growth potential with donor age. It has therefore been postulated that the limitation of the *in vitro* life-span of diploid cell populations is due to a progressive selection of slowly-proliferating cells rather than a uniform decrease in proliferative capacity of all cells. It is possible that cells incapable of division, due to increased cell volume (Bowman *et al.*, 1975), or slowly-dividing are the ones that are aging, while rapidly-dividing cells, even at high passages and old donor ages, are fewer in number but "young" in function (Cristofalo, 1972). This latter hypothesis could provide one explanation for the delayed but positive response of adult sutural joints to external stresses (Brandt

et al., 1979). The relative proportions, transitions and migration of the potentially proliferating cells, which change with environment, physiological conditions and age (Gelfant and Smith, 1972), will therefore influence the remodelling capacity of the fibrous joints.

CONCLUSIONS

1. The progenitor cells populating the tissue culture system originate from the sutural area proper. With only two exceptions, vascular elements are not prominent features of rat interparietal sutures; therefore, these progenitors are not necessarily considered to be paravascular in origin as suggested by Gould et al. (1977, 1980).

2. The emigration of progenitors from the suture proper to tissue culture plates can be illustrated by following continuously-labelled tritiated-thymidine metabolites.

3. The latent period of emigration increases as a function of donor age. For 7-day animals, migration begins within the first 24 hours in tissue culture. In contrast, 2- and 4-week animals do not show definite signs of emigration from the suture proper until 3 days and 6 days in culture, respectively.

4. Once the latent period is taken into an account, the proliferative and migratory activities of both old and young donors do not appear to differ. In addition, the direction of migration is also consistently asymmetric, presumably due to the cells' attraction to the charged surface of the tissue culture plates.

CHAPTER IV

PILOT STUDY #1:
THE EFFECT OF SEEDING CONCENTRATION ON CULTURE ENDPOINT AND CELL GROWTH RATE

PILOT STUDY #2:
CULTURE VIABILITY DEMONSTRATED AS A CELL GROWTH CURVE

SUMMARY

As a preliminary step to an assessment of the effect of *in vivo* and *in vitro* cellular aging on synthetic function, culture conditions must be established for the tissue system under study. Apart from the use of standardized conditions including temperature, pH, oxygen tension and medium components, the cell density from which the cultures may age or proliferate in its log phase of growth was determined. Culture viability was assessed on the basis of cell number and growth rate. In the first pilot study, 20,000 to 90,000 cells/mL seeding concentrations were taken from 7-day, 8-day and 10-day primary cultures of 5-week old rat interparietal sutures. Viable first passage subcultures were obtained for all seeding concentrations although confluency was established earlier for those seeded at higher densities.

Absolute cell numbers at confluency were variable due to the subjective nature of the culture endpoint. However, at $p<0.0001$, a relatively high negative correlation ($r=-0.669$) existed between increasing seeding concentration and decreasing growth rate. The most proliferative cultures were derived from cells harvested after 7 days in primary culture and seeded at 20,000 cells/mL in 35mm ($8cm^2$) culture dishes. Therefore, the second pilot study utilized these latter culture conditions to confirm the proliferative capacity demonstrated in the first pilot. Cell numbers and growth rates of first passage cultures from 5-week old Sprague Dawley rats were assessed daily. Over an eight day period, a logarithmic pattern

of cell growth illustrated that cells cultured under the present conditions favoured proliferation.

INTRODUCTION

In any investigation where tissue culture techniques are used, the working assumption is that basic aspects of growth regulation observed *in vitro* also apply *in vivo*. However, tissue cultures continually select for populations which are best suited to propagate (Martin *et al.*, 1974); therefore, the growth kinetics are subject to important modulations by a variety of *in vitro* environmental factors. Differences in serum (Narayanan and Page, 1977; Ahn *et al.*, 1978) or active ascorbic acid concentrations (Gay *et al.*, 1976), and cell density (Abe *et al.*, 1979; Hassell *et al.*, 1986), for example, can lead to widely disparate experimental results. Therefore, it is the purpose of the present investigation to define the culture conditions which would allow for not only the propagation of the sutural populations, but for the maintenance of the heterogeneity in the subpopulations seen *in vivo*.

Cellular characteristics, such as population density (defined as the number of cells per unit area of culture dish), can be modified by a number of external factors including those that can be found in serum (Holley, 1974; Sodek *et al.*, 1977; Morton and Barnes, 1983). In addition, with cell crowding (Westermark, 1976), decreased amounts of nutrients may be available to the cellular microenvironment (O'Neill *et al.*, 1979). The finding that inhibitory effects of cell crowding may be overcome by adding at least 10% fetal bovine serum to the nutrient medium suggests the significance of the humoral mechanism in cell growth regulation

(Tucker et al., 1981).

The significance of an investigation of tissue cultures with varying population densities is in the effect of cell crowding on proliferation or cell cycle inhibition (Macieira-Coelho and Azzarone, 1982) and collagen synthesis (Abe et al., 1979; Hassell et al., 1986). Inadequate spreading of the "anchorage dependent" fibroblasts due to poor adhesion or overcrowding will inhibit both cell proliferation (Bartholomew et al., 1976; Ahn et al., 1978; Fisher and Solursh, 1979) and synthetic activity (Chen, 1981; Holderbaum and Ehrhart, 1984). Collagen synthesis rates have been reported to be not only higher in preconfluent cells (Holderbaum and Ehrhart, 1984), but the percentage of collagen synthesis is also elevated by low-density cultures (Hassell et al., 1986).

As for cell cycle inhibition during cell crowding, there is a constant decline in the rate of entrance into the S-phase of DNA synthesis (Macieira-Coelho and Azzarone, 1982) with increasing cell concentration. Similar results have also been demonstrated with a cell population which has approached the middle of its lifespan and inoculated at a low seeding concentrations. In contrast, by decreasing the inoculum at early passages, the rate of entrance into the S-phase and the fraction of rapidly-dividing cells are increased (Macieira-Coelho and Azzarone, 1982). The in vitro selection of more actively-dividing cells in low density cultures have also been reported to maximize collagen synthesis (Peterkofsky, 1972; Ko et al., 1981).

In the in vitro age study to follow (Chapter V), changes in

the biochemical parameters of confluent cell cultures will be assessed after first passage. An environment in which the cells favour proliferation as well as synthesis is therefore necessary. In addition, attempts need to be made to mimic the in vivo situation and decrease the tendency for extensive cell selection. Subsequently, the length of the in vitro aging period in both primary and secondary cultures should be kept to a minimum.

The objectives of the following pilot studies are to determine (1) the minimal seeding concentration required to subculture with reliability, and (2) the minimal time an explant must be maintained in primary culture to provide sufficient cell numbers to seed three-35mm tissue culture plates (as required by the subsequent age study [Chapter V]). Under these above conditions, the cells following subculture should demonstrate a characteristic growth cycle consisting of the lag, exponential and stationary phases (Freshney, 1983).

PILOT STUDY #1

MATERIAL AND METHODS

Preparation of Cultures

1.Primary Cultures

Nine 5-week old Sprague Dawley rats, inbred in our facility, were randomly selected and divided into three groups to determine the *in vitro* growth response in each of the following time periods: 7 days, 8 days and 10 days. The animals were sacrificed by cervical dislocation following ether anesthesia (Mallinckrodt Inc., Paris, Kentucky). The calvaria were exposed with a midsagittal dissection through the scalp, the overlying periosteum was removed and the interparietal sutures were excised.

The sutural tissues were immediately transported to a laminar flow hood (Nuaire, Minnesota) in a growth medium. The growth medium consisted of Dulbecco's minimum essential medium (Gibco/BRL, Burlington, Ont.), 400 units per mL penicillin G (Gibco Laboratories, Grand Island, NY), 0.56mg per mL streptomycin sulphate (Gibco, Grand Island, NY), 0.2mg per mL ascorbic acid (Sigma Chemical Co., St. Louis, MO), and 2.2mg per mL sodium bicarbonate (Fisher Laboratory Chemical, Fair Lawn, NJ), at pH 7.4. The medium was sterilized using a Millipore 0.22 μm filter (Millipore Corp., Mississauga, Ont.). Sterile, 10% fetal bovine serum (Flow Laboratories Inc., Mississauga, Ont.) was added.

The dura and any adherent soft tissue were stripped from the

bony surfaces and the remaining blood was removed with 2 washes in sterile growth medium. Individual sutures were trimmed and cut into 1.5mm x 1.5mm segments and placed in prepared 60mm tissue culture dishes (Falcon, Oxnard, CA). These dishes were incubated with a coating of 100% fetal bovine serum for 1 hour prior to the addition of the explants to facilitate their adhesion to the dish. The fetal bovine serum was decanted prior to the addition of the suture segments and 4mL of growth medium. 10µL/mL fungizone (Flow Laboratories, Inc., Mississauga, Ont.) was also added to each plate separately. The tissue culture plates were then incubated (National incubator model 4200, Portland, Oregon) in a humidified atmosphere of 5% CO_2 at 37 degrees C.. The growth medium and fungizone were replaced every 2.5 days until the end of the designated time period. The health of the cells was monitored with a phase contrast microscope (Nikon, Japan).

2. Subculturing Technique and Secondary Cultures

The cells that emigrated from the explants onto the surface of the culture dishes were subcultured. The first group of primary cultures were subcultured after 7 days. The following subculturing technique was repeated for the remaining explants following the 8th day or 10th day <u>in vitro</u>. The dishes were washed 3 times in Hanks Buffered Saline followed by 3mL of trypsin (Sigma Chemical Co., St. Louis, MO) at 4 degrees C. for 60 seconds. The excess trypsin was decanted and the dishes were incubated for 3-5 minutes in a humidified atmosphere of 5% CO_2 at 37 degrees C.. Following cell

detachment from the dish, as determined by their rounded appearance, the cells were suspended in 1mL of growth medium with a sterile pasteur pipette. The number of cells present in this suspension was calculated from a 100µL sample volume using a Coulter counter (Coulter Electronics Inc., Hialeah, Florida). The Coulter values were repeated twice, adjusted for background, and extrapolated to determine cell number in the remaining suspension (Appendix A).

The cell suspensions of the explants cultured for each time period were pooled together to seed 9 - 35mm tissue culture plates (Falcon, Oxnard, CA) under various seeding concentrations (Appendix A). From the 7-day cultures, 3 plates were seeded at each of the following concentrations: 20,000, 30,000 and 40,000 cells/mL growth medium. Similarly, the cell suspension from the 8- and 10-day cultures were seeded at 35,000, 50,000, 60,000 and 70,000, 80,000, 90,000 cells per dish, respectively.

The appropriate volume of growth medium was subsequently added to the cell suspension to make up a 2 mL volume per dish. 10µL/mL fungizone was added before the dishes were incubated in a humidified atmosphere of 5% CO_2 at 37 degrees C.. The growth medium was replaced every 2.5 days and the cells were monitored daily with the phase contrast microscope for confluence.

Once confluent, the cell count was determined using the Coulter counter. Cell growth rate was calculated from the total cell number less the seeding concentrations, and as a function of the time (in days) required to reach confluency.

PILOT STUDY #1

RESULTS

Accepting the inter-animal variability present in all animal models, these results indicated that after 7, 8 and 10 days in primary culture, the average number of cells generated from 5-week rat interparietal sutures were about 250,000, 400,000 and 540,000 cells, respectively. Therefore, after deducting 10% for the 100uL sample volume used to determine the cell count, the number of cells available for subculturing would be approximately 225,000 after 7 days in primary culture, for example. Since the original objective was to obtain a sufficient number of cells from the primary culture to adequately seed a minimum of 3 - 35mm tissue culture dishes, these 7-day primary cultures were able to provide enough cells to seed 3 dishes at a maximum concentration of 40,000 cells/mL (or 80,000 cells/dish).

Similarly, following 8 days in primary culture, the maximum seeding concentration was at 60,000 cells/mL. In order to obtain 3 tissue culture dishes seeded at 70,000 to 90,000 cells/mL, the explants were required to remain in primary culture for 10 days.

Subcultures derived from 7-day old primary cultures and seeded at 20,000 cells/mL generated high cell numbers, averaging 770,000 cells, but required 7 days to reach confluency (Appendix A). Comparable counts were also found with subcultures seeded at 30,000 cells/mL; however, the variabilities in both cell number and

time to reach confluency were much greater. More consistent results were evident with subcultures seeded at 35,000, 50,000 and 60,000 cells/mL, following 8 days in primary culture. Confluency was reached in these latter subcultures between 5-6 days. In comparison, the subcultures derived from the 10 day cultures reached confluency after 3-4 days, but with almost half the cell counts.

Despite the disparity in the cell numbers noted at confluency, the cell growth or proliferative rates were found to decrease with increasing seeding concentration. The primary exceptions were the samples seeded at 40,000 and 60,000 cells/mL which presented with markedly lower and higher proliferative rates, respectively, when compared to the other samples. Nonetheless, the correlation coefficient between cell growth rate and seeding concentration is relatively high ($r=-0.669$) and significant at $p<0.0001$ (Tate, 1989). The greatest contribution to this negative slope is the consistent drop in the cell growth rate at seeding concentrations greater than 70,000 cells/mL. The data from this pilot have been outlined in Table IV-1 and illustrated in Figure IV-1. Note that the y-intercept denoted on the linear regression equation is obviously hypothetical since the cell count must be zero in the absence of seeding.

TABLE IV-1

Pilot #1

Seeding Concentration
versus
Cell Count and Cell Growth Rate

Seeding Concn. (cells/mL)	Average number of cells at confluency (days/1st culture) 7	8	10	Std.Dev.	Growth Rate (#cells/day) Avg.	Std.Dev.
20000	770000			67964	104286	9706
30000	605000			148250	90024	15875
35000		660000		19923	98333	3321
40000	356667			11502	55333	2301
50000		545000		54787	83444	5479
60000		696667		64813	94611	10802
70000			400000	0	65000	0
80000			355000	54787	65000	18262
90000			365000	34864	46250	8716

*Calculation of growth rate accounted for seeding concentration
**Refer to Appendix A for raw data

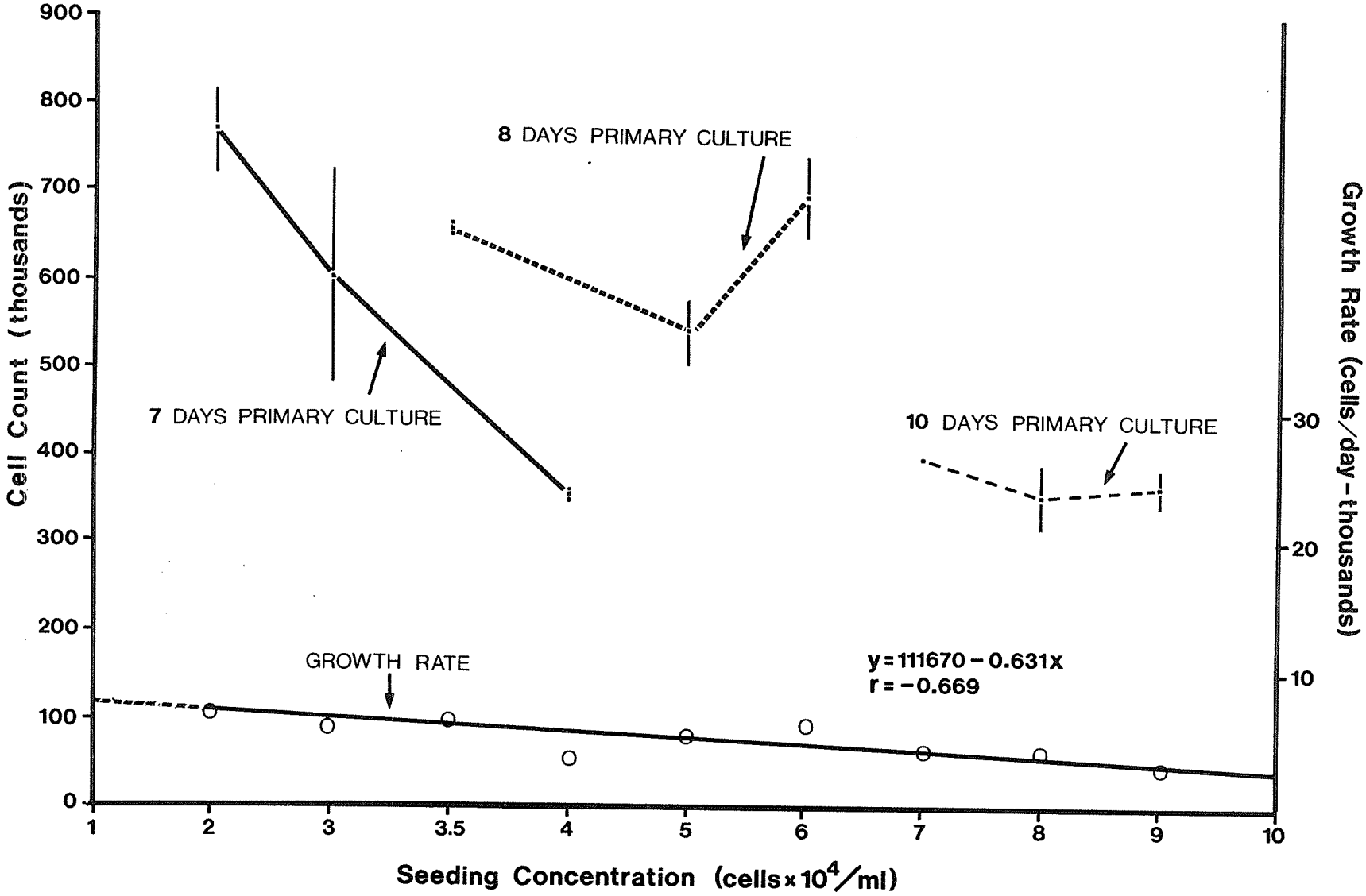

Fig. IV-1: CELL COUNT AND GROWTH RATE vs. SEEDING CONCENTRATIONS

PILOT STUDY #2

MATERIALS AND METHODS

Preparation of Cultures

1. Primary Cultures

Eight 5-week old Sprague Dawley rats, inbred in our facility, were randomly selected and sacrificed by cervical dislocation following ether anesthesia (Mallinckrodt, Inc., Paris, Kentucky). The calvaria were exposed with a midsagittal dissection through the scalp, the overlying periosteum was removed and the interparietal sutures were excised.

The sutural tissues were immediately transported to a laminar flow hood (Nuaire, Minnesota) in growth medium, and were prepared as primary cultures in the manner described in Pilot #1.

2. Subculturing Technique

Primary cultures were prepared for subculturing after 7 days in vitro. The dishes were washed 3 times in Hanks Buffered Saline followed by 3mL of trypsin (Sigma Chemical Co., St. Louis, MO) at 4 degrees C. for 60 seconds. The excess trypsin was decanted and the dishes were incubated for 3-5 minutes in a humidified atmosphere of 5% CO_2 at 37 degrees C.. Cell detachment from the dish was determined by the rounded appearance of the cells. The cells were suspended in 1mL of growth medium (without FBS) with a sterile pasteur pipette. A 100µL sample volume was counted

using a Coulter counter (Coulter Electronics Inc., Hialeah, Florida) to determine the aliquot required to seed each 35mm culture dish at a concentration of 20,000 cells/mL (or 40,000 cells/dish).

3. Secondary Cultures

The appropriate volumes of fungizone and growth media were added to the 35mm dishes to make up a 2mL volume. The secondary cultures were then incubated in a humidified atmosphere of 5% CO_2 at 37 degrees C. and monitored with the phase contrast microscope. The nutrient medium was replaced every 2.5 days.

To isolate the time period required to obtain confluency under these primary and secondary culture conditions, a minimum of 3 dishes were counted (each three times) on day 1 to day 8 following subculture. Cultures from different animals were randomly chosen and terminated at each of the eight time periods. Cell counts were converted from Coulter readings, less background and seeding concentration, to determine the rate of cell division (growth rate).

PILOT STUDY #2

RESULTS

As shown in Table V-2 and Figure V-2, average cell numbers increased progressively until about the 5th day in culture when cell division had levelled off and confluency was noted. The average cell count at this point was about 550,000 cells. Variabilities in cell number were evident especially in the exponential phase of the cell growth curve; however, this was expected due to inter-animal variability.

As evident by the low cell counts at day 1, approximately 50% of the cells were lost due to trypsinization. In this pilot, 2 dishes were relatively acellular, probably a result of an excessive exposure to trypsin, and therefore were discarded. As noted by the growth curve, the cells appeared to have required a lag time of 2 days to recover before they were able to enter into a reproductive cycle (Appendix C). The doubling time was also extrapolated from the growth curve to be 29.25 hours. The sigmoid appearance of this curve for 5-week Sprague Dawley rats indicated that the cells reproduced at their maximum rate between 4-5 days after subculture. This was also evident in the assessment of cell growth rate which progressively increased in the log phase, but followed by a decrease during confluency.

TABLE IV-2

Pilot #2

Growth Curve for Subcultures
of 5 Week Rat Interparietal
Sutures

Seeding concentration: 20,000 cells/mL

Days in subculture	Cell Count		Growth rate (#cells/day)
	Avg.	Std. Dev.	
0	40000		0
1	19460	3611	-20540
2	100427	38703	30213
3	201770	35573	53923
4	363657	123332	80914
5	527847	113416	97569
6	557469	65737	86245
7	579080	98430	77011
8	489090	43657	56136

*Growth rate accounts for seeding concentration
**Refer to Appendix B for raw data

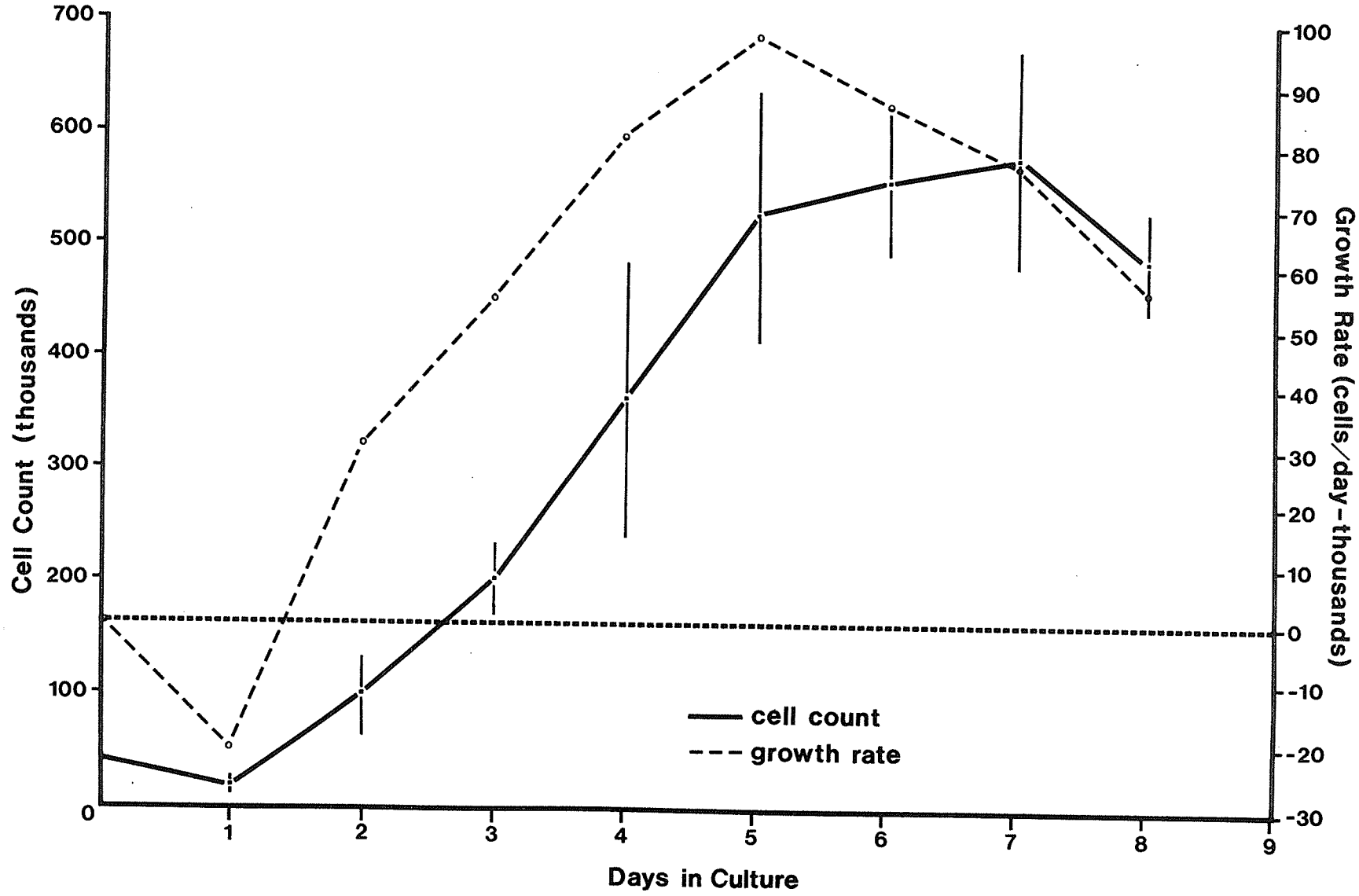

Fig. IV-2 : CELL COUNT AND GROWTH RATE vs. CULTURE PERIOD

DISCUSSION

It is apparent from the results of these pilots that the seeding concentration at 20,000 cells/mL in 35mm ($8cm^2$) culture plates will not only stimulate proliferative activity and cell growth rate, but will minimize the in vitro aging of explants in primary culture. Therefore, explants only need to remain in culture for a maximum of 7 days. Given the fact that these results represent 5-week old animals, it is expected that the proliferative capacity of younger and older sutural populations harvested in the subsequent age study (Chapter V) will vary accordingly. Nonetheless, these cell populations should similarly exhibit the characteristics of the growth curve while in culture.

The disparity in cell numbers at confluency is given by the subjective nature of this assessment of culture endpoint. In particular, higher cell densities were noted in the first pilot under seemingly similar culture conditions. However, the cultures in the first pilot were harvested from pooled populations of cells, whereas the activities of the subcultures in the second pilot represented individual animals. The main purpose for pooling the cells was to limit some of this inter-animal variability. Although this technique appeared to have been relatively successful for the cultures seeded from the 8- and 10-day primary cultures, a great deal of variability in cell numbers at confluency was noted between those seeded at 20,000, 30,000 and 40,000 cells/mL.

If the growth rates of the subcultures in the first pilot can

be compared to those in the second pilot, the cultures seeded at 40,000 cells/mL appear to have been terminated prematurely in the log phase of the growth cycle. In comparison, confluent cultures seeded at just higher and lower concentrations demonstrated high cell yields and growth rates, but after slightly longer subculture periods. Therefore, it might be assumed that these particular results at 40,000 cells/mL seeding concentration are not representative of the cells' proliferative potential at this culture density. Despite this variability, the correlation between cell growth rate and seeding concentration is relatively high.

The significance of the seeding concentration is in the determination of conditions under which both primary and secondary cultures must be placed. In an attempt to overcome the factors related to extended in vitro conditions, the environment which provides the optimal conditions for cells to display their true potential for survival, proliferation and function must be selected.

The viability of the cell cultures at seeding concentrations of 20,000 cells/mL is illustrated by the cell growth curve (Figure IV-2). One of the most significant features of this growth curve is the initial drop in cell number due to the subculturing procedure. The ability to repopulate the tissue culture plate therefore is largely dependent on the selection of fibroblast populations which are trypsin-resistant. Subsequently, the rate at which confluency is reached will depend on the seeding concentration as well as the average growth rate of the selected populations.

As indicated by the exponential rise in cell number in Figure

IV-2, cell proliferation has been stimulated by low density cultures. Similar findings have also been reported by Bartholomew and coworkers (1976), Fisher and Solursh (1979), and Macieira-Coelho and Azzarone (1982). The length of the log phase depends on the seeding density, the growth rate of the cells, and the density at which cell proliferation is inhibited by cell-cell interactions (Freshney, 1983). Therefore, increasing the seeding concentration will minimize *in vitro* aging or the time required to reach confluency. In effect, the highly-seeded cultures are likely placed at the end of the log phase in the cells' growth cycle, under conditions in which inhibitions due to cell crowding become apparent.

At confluency, the cells enter a "plateau" or stationary phase, which does not imply a complete cessation of cell proliferation, but represents a steady state where cell division is balanced by cell loss (Freshney, 1983). However, as evident in Figure IV-2, the cell growth rate begins to fall during this steady state. It is important to emphasize that these growth rates are average figures and describe the net result of a wide range of division rates, including zero, within the culture (Freshney, 1983). The significance of obtaining cultures with high cell growth rates is its reflection of fibroblast subpopulations which also have the potential to synthesize collagen maximally. This latter concern will be evident in the subsequent study on the effect of aging on the synthetic and proliferative capacity of rat interparietal sutures (Chapter V).

CONCLUSIONS

1. Cell cultures can be reliably seeded at concentrations of 20,000 cells/mL in 35mm ($8cm^2$) culture dishes.

2. For 5-week old rat interparietal sutures, primary cultures maintained *in vitro* for 7 days will supply a sufficient number of cells to seed three - 35mm tissue culture plates. Based on these results and with those of Chapter III, the sutural populations of younger and older donors will be expected to take less and greater than 7 days, respectively, to emigrate in primary culture.

3. Cultures seeded at 20,000 cells per mL have been shown to progress through a characteristic cell growth cycle toward confluency. Extrapolations from this graph indicate a lag time of 2 days and a doubling time of about 29.25 hours.

4. In general, seeding concentrations between 20,000 to 60,000 cells/mL will produce cultures which may proliferate at high growth rates. Higher seeding densities appear to minimize the time required to reach confluency, but at slower rates of cell division.

CHAPTER V

THE EFFECTS OF DURATION *IN VITRO* ON COLLAGEN SYNTHESIS AND GROWTH RATE OF CELLS FROM SUTURES OF DIFFERENT AGES

SUMMARY

The aging phenomenon represents a coordinated expression of a number of changes in cellular function over time. The effect of *in vivo* and *in vitro* aging on cell growth rate and collagen metabolism have been studied.

The ratio of type III to the total of type I and type III collagen in cell cultures from male rat interparietal sutural tissues was determined for selected ages from newborn to adulthood (20 weeks). Cells, proliferated and migrated from these suture explants, were grown out in Dulbecco's minimum essential medium plus 10-15% fetal bovine serum and antibiotics at 37 degrees celsius in a humidified atmosphere. Each primary culture was subcultured once into three 35mm plastic tissue culture plates and grown to confluency. [^{14}C]-glycine was added to label the newly-synthesized collagen in one plate, while the other plates were counted to determine cell growth rate. Collagen extractions from the radio-labelled cultures were processed as separate cell and medium layer samples. The alpha-chains were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis using a delayed reduction step. Fluorograms prepared from the dried gels were scanned densitometrically.

The ratios of type III collagen alpha-chains to the total of type I and III alpha-chains in the cell layer at different donor ages were assessed under *in vitro* conditions on the basis of three growth phases; these included ages newborn to 21 days (phase 1),

22 to 56 days (phase 2), and 57 to 84 days (phase 3). Using a one way analysis of variance, phase 3 was found to be significantly different from phases 1 and 2 at $p<0.0001$ and $p<0.0012$, respectively.

Compared to the level of type III collagen to total newly-synthesized collagen *in vivo*, much higher ratios were produced under cell culture conditions. These findings indicated that type III collagen synthesis was stimulated in cell cultures from explants of non-growing interparietal sutures. However, taking into consideration the Bonferroni correction for multiple comparisons using the t-distribution, significantly different collagen ratios were only found at particular donor ages (7-10 days, 35 days, and 77 days). Nonetheless, the two-way analysis of variance, comparing the effects of *in vivo* and *in vitro* aging, indicated significantly higher proportions of type III to total newly-synthesized collagen *in vitro* for phases 1 (9.49%), 2 (13.23%) and 3 (7.32%), at $p<0.0001$.

Associated with the stimulation of type III collagen synthesis *in vitro* was the parallel increase in cell growth rate of the fibroblast populations in cell culture. Changes in the ratio of collagen phenotypes were therefore due only in part to donor age. The *in vitro* selection of cell populations which have tolerated trypsinization and retained the ability to reproduce and migrate appears to be the major determinant of the changes in the proportions of collagen phenotypes synthesized.

INTRODUCTION

Aging is a natural but ultimately degenerative process that can be characterized in a number of ways. As a reflection of both maturity and true senile decay (Bentley, 1979), the significance of the aging phenomenon in orthodontics is in its effect on the remodelling capacity of the fibrous joints, such as the periodontal and sutural articulations. With the use of histological and biochemical techniques, age-related changes in these fibrous joints can be studied at the cellular level.

Cellular aging is commonly defined on the basis of changes in the cells' proliferative and synthetic capacities. For the most part, investigators are in agreement with respect to the maintenance of proliferative potential in aging donor populations (Martin et al., 1970; Hayflick, 1984; Albini et al., 1988). However, numerous studies in the literature on age-related effects on collagen synthesis are replete with conflicting results. Differences in cell or tissue type, species and biochemical assays plus inherent biases specific to certain in vitro conditions have all contributed to these variabilities. In addition, aging in itself is a progressive phenomenon, consisting of several phases (Strehler, 1977). Age-related changes that have been reported during the embryonic stage may differ from that during maturation, adulthood and old age.

The expression of senescence at the cellular level is given by the finite capacity of normal diploid cell populations to replicate

(Martin et al., 1970; Hayflick, 1977; Albini et al., 1988). Although the finite limit of this life span is rarely reached in vivo, it can be demonstrated with extensive aging in vitro. Apparent decreases in growth potential with increasing age are therefore considered to be due to the expression of cell populations in the middle of their lifespan, rather than to the uniform loss of proliferative capacity (Hayflick, 1984). Therefore, the recruitment of progenitor cells from older donors in cell culture will undergo a latency period, but the proliferative potential of these cells, particularly in early passages, may parallel those derived from younger donors (Cristofalo, 1972).

Although cellular aging has been considered to be an intrinsic cell property (Praeger, 1986), the phenomenon has also been attributed to functional losses due to progressive and cumulative damage to cytoplasmic organelles (Hayflick, 1984). In fact, many functional changes are expressed with in vitro aging, such as decreases in collagen synthesis, well before they lose their capacity to replicate (Houck et al., 1971; Hayflick, 1984). Subtle changes indicated by the ratio of collagen types III to I are therefore likely parameters involved in the expression of aging.

The identification of changes in phenotypic expression as a function of in vivo and in vitro cellular aging probably illustrates shifts in the resident population of the culture system. When explants of interparietal sutures are placed in culture medium, cells which migrate and proliferate from this tissue presumably represent a sampling of cells which would have participated in the remodelling process in vivo. However, it is evident that at any

given time, different populations of cells in different cell cycles reside in tissue culture. With *in vitro* aging, there is a continual selection for the more vigorous stem cells which are capable of giving rise to the largest number of progeny (Martin *et al.*, 1974). Therefore, the predominant cell phenotype and functional characteristics may be altered by selecting against a subpopulation of cells which is present in primary culture and *in vivo* (Murphy and Daniel, 1987). Subsequently, cell populations existing early in the cell line may not be present in later passages; such shifts in the cell population are evident with respect to concomitant changes in growth potential and by implication, various biochemical and physiologic parameters (Martin *et al.*, 1974). Due to this inherent tendency for cell selection particularly with repeated subculturing, the present age study has been designed to assess collagen synthesis following only one passage.

Despite the importance of determining the cell populations which are influenced by aging, the regulatory mechanisms controlling the cells' collagen metabolism are equally significant. These mechanisms are undoubtedly complex, since the progeny of a single cell appear to have the ability to synthesize and modify, by numerous post-translational steps, both types I and III collagens (Kivirikko and Risteli, 1976; Bornstein and Traub, 1979). It has also been demonstrated that fibroblasts, in human skin (Gay *et al.*, 1976) and gingiva (Engel *et al.*, 1980) for example, may synthesize both collagen types I and III simultaneously. Using immunohistochemical techniques, compartmentalization of the different collagens and

respective precursors have been visually apparent (Gay et al., 1976).

The finding that one cell type synthesizes more than one collagen phenotype raises questions concerning the level of regulatory control of phenotypic expression. The means by which collagen metabolism can be manipulated with remodelling and aging is subsequently of interest. At the molecular level, the expression of genes for the $alpha_1$(III), $alpha_1$(I), and $alpha_2$(I) chains are coordinately controlled (Miskulin et al., 1986) despite the fact that these genes are located on different chromosomes (Emmanuel et al., 1985). Age-related effects on the collagen phenotype must subsequently result from the manipulation of the expression of these genes.

It can not always be assumed that both collagen types I and III are synthesized by the same cell in every tissue. Although collagen fibre types co-exist in many tissues (Epstein and Munderloch, 1975; Engel et al., 1980; Shekhonin et al., 1985; Ramshaw, 1986; Keene et al., 1987; Merkel et al., 1988), separate collagen types I and III fibres are also located in anatomically distinct areas and are therefore presumed to have been synthesized by different subpopulations (Nowack et al., 1976).

The finding that different fibroblast subpopulations may be responsible for the synthesis of different collagen types supports the concept of heterogeneity in sutural populations. Cells surrounding explants in primary cultures in gingival and periodontal tissues, for example, are naturally heterogeneous with respect to protein expression (Connor et al., 1983); these cells have been

differentiated with immunofluorescence microscopy. Subsequent to an increasing number of population doublings with *in vitro* aging, the culture population tends to become more homogeneous as given by the synthesis of limited phenotypes. However, this theory of heterogeneity can not disregard the fact that selected cells might have been able to synthesize more than one phenotype, but under certain environmental conditions express only one phenotype.

The relative expression of a given collagen type may be a function of the maturational or differentiative state of that cell (Engel *et al.*, 1980). Therefore, fibroblasts which are morphologically homogeneous can be functionally different subpopulations. True phenotypic differences amongst fibroblasts of the same origin have been detected by comparing the synthetic activity of cloned populations (Connor *et al.*, 1983). As a result, the behaviour of mass cultures has been actively studied as an *in vitro* expression of cellular aging (Hayflick, 1984).

In a study by Mollenhauer and Bayreuther (1986) in skin and lung tissues, age-related changes were examined in primary explant, mass and clonal cultures, on the basis of the relative selection of three specific fibroblast subpopulations. The cell types, designated FI, FII, and FIII, differed in their morphological and proliferative properties, and were distinguished from each other by the amount and type of collagen they synthesized in culture. The FI cells were highly proliferative and were considered to be the precursors of FII and FIII cells. The FIII cells, on the other hand, possessed the least proliferative potential but apparently synthesized the

largest amount of type I and type III collagens, particularly type III. The relative abundance of these three cell types apparently determined the phenotypic expression of the cells in cultures. With advancing age, the increased predominance of FIII cells was consistent with the increased level of collagen synthesis in these cultures, although the expected increase in the type III/I ratio was insignificant.

This hypothesis of heterogeneity in fibroblast subpopulations is an interesting concept because it allows for an explanation of age-related shifts in collagen synthesis. This shift may be due to an *in vitro* expression of an otherwise dormant cell population residing in the soft connective tissue *in vivo*. The finding that type III collagen is favoured with *in vitro* aging (Mollenhauer and Bayreuther, 1986) contrasts the *in vivo* situation since this collagen phenotype has been shown to be particularly prevalent in young tissues *in vivo* (Chung and Miller, 1974; Epstein, 1974). One exception *in vivo* is in the skin of the elderly where relative amounts of type III collagen have been found to increase (Lovell *et al.*, 1987).

It is important to note that the synthetic function of cells aged *in vitro* may not necessarily reflect cells aged *in vivo* (Schneider and Mitsui, 1976). Therefore, the indiscriminate use of early and late passage cells to represent young and old donor populations may subsequently promote variabilities in the proportion of collagen type III to type I synthesized.

The significance of type III collagen is in its role in rapid

connective tissue remodelling (Duncan et al., 1984), wound healing (Gay et al., 1978), inflammation (Weiss et al., 1975), bearing of physical stress (Yue, 1984), and in normal growth and development of connective tissues (Chung and Miller, 1974; Butler et al., 1975; Yue, 1984). The relative changes in the presence of type III collagen in these situations therefore reflect the type of tissue, the age of the donor, and the conditions under which it is measured (Limeback et al., 1978; Limeback and Sodek, 1979).

Increases in the collagen type III/I+III ratio noted with in vivo and in vitro aging have been attributed to increases in type III synthesis (Limeback and Sodek, 1979), decreases in type I synthesis (Lovell et al., 1987), or influences of intracellular (Rosenbloom et al, 1984) and extracellular (Narayanan et al, 1988) collagen degradation. Yue (1984) has also shown that the ratio of type III collagen to total newly-synthesized collagen increases with the rate of sutural bone growth and decreases with age until complete disappearance on cessation of sutural growth.

The aim of the present study is to determine whether these changes in the proportions of collagen phenotypes synthesized are due to changes in the cell sub-populations present in sutures at each age, or due to individual cell expression. Change in collagen phenotype may therefore serve as an indicator for evaluating factors which regulate this cellular function during growth, aging and remodelling. With the use of the cell culture system, the relative effects of in vitro aging on the proliferative and synthetic capacities of cells from sutures of different ages will be assessed.

MATERIALS AND METHODS

Preparation of Cultures

1. Primary Cultures

Four Sprague Dawley rats, inbred in our facility, were randomly selected for each of the following age groups: newborn, 1 day, 2, 3, 4, 5, 6, 7, 10 days and 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 16, 20 weeks. The animals were killed by cervical dislocation following ether anesthesia (Mallinckrodt Inc., Paris, Kentucky). The calvaria were exposed with a midsagittal dissection through the scalp. The interparietal sutures were excised surgically following the removal of the overlying periosteum.

The sutural tissues were immediately transported to a laminar flow hood (Nuaire, Minnesota) in a growth medium. The growth medium consisted of Dulbecco's minimum essential medium (Gibco/BRL, Burlington, Ont.), 400 units per mL penicillin G, 0.56mg per mL streptomycin sulphate (Gibco, Grand Island, NY), 0.2mg per mL ascorbic acid (Sigma Chemical Co., St. Louis, MO), and 2.2mg per mL sodium bicarbonate (Fisher Laboratory Chemical, Fair Lawn, NJ), pH 7.4. The medium was sterilized using a Millipore 0.22 μm filter (Millipore Corp., Mississauga, Ont.). Sterile, 15% fetal bovine serum (Flow Laboratories Inc., Mississauga, Ont.) was added to cultures of newborn to 10 day old animals, while 10% was added to cultures of 2-20 week old animals.

The dura and any adherent soft tissue were stripped from the

bony surfaces and the remaining blood was removed with 2 washes in sterile growth medium. Individual sutures were trimmed and cut into 1.5mm x 1.5mm segments and placed in prepared 60mm tissue culture plates (Falcon, Oxnard, CA). These dishes were incubated with a coating of 100% fetal bovine serum for 1 hour prior to the addition of the explants to facilitate their adhesion to the dish. The fetal bovine serum was decanted prior to the addition of the suture segments and 4mL of growth medium. 10µL/mL fungizone (Flow Laboratories, Inc., Mississauga, Ont.) was also added to each dish separately. The dishes were then incubated (National incubator model 4200, Portland, Oregon) in a humidified atmosphere of 5% CO_2 at 37 degrees C.. The growth medium and fungizone was replaced every 2.5 days. The health of the cells was monitored with a phase contrast microscope (Nikon, Japan).

2. Subculturing Technique

Primary cultures were ready to be subcultured after 5-9 days (for the newborn to 5 week animals inclusive) or 10-14 days (for the 6-20 week animals). These dishes were washed 3 times in Hanks Buffered Saline followed by 3mL of trypsin (Sigma Chemical Co., St. Louis, MO) at 4 degrees C. for 60 seconds. The excess trypsin was decanted and the dishes were incubated for a minimum of 1 minute (for the animals 10 days and younger) and a maximum of 5 minutes (for the older animals) in a humidified atmosphere of 5% CO_2 at 37 degrees C.. Cell detachment from the dish was determined by the rounded appearance of the cells when viewed under the phase

contrast microscope. The cells were suspended in 1mL of growth medium (without FBS) with a sterile pasteur pipette. The number of cells present in this suspension available for subculturing was calculated from a 100µL sample volume using Coulter counter values (Coulter Electronics Inc., Hialeah, Florida).

3. Secondary Cultures

The appropriate aliquot of the primary culture suspension required to seed at a concentration of 20,000 cells/mL medium was calculated and added to three-35mm tissue culture plates (Falcon, Oxnard, CA). Two mL of medium, with 10% FBS (for donor ages newborn to 10 days) or 15% FBS (for donor ages 2-20 weeks), and 10µL/mL fungizone were added to each dish, and incubated in a humidified atmosphere of 5% CO_2 at 37 degrees C.. The growth medium was replaced every 2.5 days and the cells were monitored with the phase contrast microscope for confluence.

Radioautography

When confluency was reached, one of the three subcultured dishes was prepared for the labelling of newly-synthesized collagen. In total, a minimum of three radio-labelled samples were obtained for each animal age. Fresh growth medium and 10µCi/mL [^{14}C]-glycine ([Specific Activity 53mCi/mmol] ICN Biomedicals Canada Ltd., Montreal, Que.) were added, and incubation was continued for 6 hours. To terminate the culture, the medium was first removed with a pipette into a glass test tube for pepsin

extraction. The cell layer was harvested with two 1 mL washes of 0.5M acetic acid. The cell samples were sonicated for about 30 seconds or until the cells were dispersed. The acetic cell extracts and medium samples were frozen in -80 degree C. in preparation for biochemical analysis.

Rate of Cell Division (Cell Growth Rate)

The remaining subculture plates were used to determine cell number at confluency. The time required to reach this phase was noted in days since the initiation of the subculture. Using the same procedure for cell counting in the subculturing technique, the cell layer was washed with Hanks Buffered Saline and trypsinized. 100μL aliquots were counted three times using the Coulter counter; the average value was used to determine cell number to the nearest hundred. The calculation of cell growth rate accounted for the initial seeding concentration and was expressed as the number of daughter cells produced per day.

Pepsin extraction of Radio-labelled Collagen

The frozen samples were lyophilized and subjected to limited pepsin digestion for collagen solubilization. Each sample was digested twice in a pepsin solution (0.1mg/mL in 0.5 N acetic acid; Sigma Chemical Co., St. Louis, MO) at 16 degrees C. for a total of 20 hours. The cell and medium samples were then dialyzed for 24 hours and 5 days, respectively, against 1% acetic acid at 4 degrees C.. 100μL of each sample was placed in mini-scintillation vials with

5mL of scintillation cocktail (Aquasol, New England Nuclear, Boston, Mass.) and counted for [^{14}C]-glycine in a liquid scintillation counter (Nuclear Chicago Isocap 300, Searle Instrumentation, Toronto, Ont.). The remaining sample was frozen and lyophilized in preparation for gel electrophoresis.

Collagen Separation and Quantification

Collagen alpha-chains and procollagens were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 20cm slab gel. The procedure used was a modified method previously described by Laemmli (1970) and utilized a 7.5% cross-linked separating gel, a 2.5% stacking gel, and tris/glycine buffers (Bio-Rad Laboratories, Richmond, California). Type I and type III collagen alpha-chains were separated using the interrupted electrophoresis method of Sykes et al. (1976).

Freeze-dried samples were dissolved in 70 µL of reservoir buffer containing 2M urea, 2% sodium dodecyl sulphate, and 0.1% bromophenol blue, and were heated at 60 degrees C. for 30 minutes to denature the collagen. Samples were introduced to the sample wells and electrophoresis was performed for 1 hour at 130 volts under non-reducing conditions. Electrophoresis was stopped and samples were then reduced by the addition of 20% Beta-mercaptoethanol (Sigma Chemical Co., St. Louis, MO) to the sample wells to allow type III alpha-chains to penetrate the gel. Reduction was carried out for 60 minutes, then electrophoresis was resumed at 24 mA/gel until the tracking dye reached the base of

the gel.

For fluorographic visualization of separated radio-labelled collagen bands, gels were dehydrated with two washes of dimethyl sulfoxide (Fisher Scientific, Fair Lawn, NJ) and impregnated with 2,5-diphenyloxazole / dimethy sulfoxide (Dupont-New England Nuclear, Boston, Mass.) as described by Bonner and Laskey (1974). The gels were placed on filter paper (Bio-rad Laboratories, Richmond, CA), dried on a slab drier (Bio-Rad, Richmond, CA) and exposed to Kodak XRP-1 x-ray films (Kodak Canada, Inc., Toronto, Ont.) at -80 degrees C. for varying periods of time as indicated by the scintillation counts of the samples.

Individual sample tracks from the developed fluorographs were scanned at 550 nm with a spectrophotometer (Bio-Rad Model 620 Video Densitometer, Matsushita Electric Industrial Co. Ltd, Japan). The proportions of type III alpha-chains relative to the total of type I and type III alpha-chains were calculated from the integrator (Bio-Rad Model 3392A integrator, Richmond, CA) readings. The values obtained for these collagen ratios were subjected to statistical analysis using a one analysis of variance with the Bonferroni correction for multiple comparisons.

RESULTS

The effect of age on the ratios of type III collagen to the total of type I and III (III/I+III) in the cell layer of rat interparietal suture subcultures has been illustrated in Figure V-1. As previously noted, these collagen phenotype ratios have been calculated from densitometer readings of fluorograms. It is interesting to note that due to either the in vitro conditions during collagen synthesis or to the biochemical assays, many of the collagen type III fluorographs have separated as two distinguishable bands rather than as one thick band (Appendix H). Since the two thinner bands were located within the area of the single band evident in pure collagen type III fluorographs (Yen and Suga, 1989) both peaks scanned were included in the calculations. Although this separation was not a consistent finding in all cases, it was evident in most of the cell layer samples (Appendix D), while rarer in those of the medium layer (Appendix E).

The corresponding ratios of the collagens secreted into the medium layer fluctuate tremendously from the cell layer data. The raw data and the graph of collagen type III/I+III in the medium layer have been presented in Appendix E.

The relationship between donor age and the average rate of cell growth (or cell division) has been illustrated in Figure V-2. Variabilities in the proliferative capacity of subcultured cells from sutures of different ages were given by the differences in the rate at which confluency was reached. Subcultures from older donors

generally required a longer culture period (Appendix G). Cells migrating from older donor explants also required between 10-14 days before an adequate number of cells were available for subculturing at 20,000 cells per mL; this was compared to the younger animals (under 5 weeks of age) which required as little as 5 days in primary culture.

The relationship between the collagen phenotype ratio and cell growth rate has been depicted in Figure V-3. These changes have been assessed as three separate growth phases: phases 1, 2 and 3 represent the ages from newborn to 21 days, 22 to 56 days, and 57 to 84 days, respectively. The period after 84 days will be described separately.

Apart from the initial drop in the level of collagen type III/I+III from newborn to 1 day in the first phase, there was a consistent increase in this ratio to a maximum of 26.78% by 7 days in tissue culture. In contrast, the cell growth rate exhibited a large increase at 1 day in tissue culture. With the transfer of young tissue from an *in vivo* to an *in vitro* environment, and previous findings which have indicated the lack of rapid growth in the first three days of life (Massler and Schour, 1951; Yue, 1984), this large difference between growth rates from newborn to 3 days is inexplicable.

By the end of the first phase, at 21 days, there was a significant drop in both the ratio of III/I+III (12.67%) and cell growth rate, followed by a parallel rise in both parameters to peak at 35 days. In the third phase, the cell growth rate progressively

fell to its lowest level; similarly, corresponding collagen phenotype ratios dropped in a step-wise fashion. The particular exception to this pattern was at 77 days of age when the collagen type III/I+III ratio was high at 11.46%, but the cell growth rate was at its lowest level (6,287 cells/day).

In a comparison between these three growth phases with respect to collagen synthesis, the one-way analysis of variance indicated that phases 1 and 2 were not different ($p=0.6376$), while phases 1 and 3 ($p=0.0001$) as well as 2 and 3 ($p=0.0012$) were statistically different. The Bonferroni correction ($0.05/3=0.017$) was used to account for the greater chance of statistical error when multiple comparisons were made; therefore, statistically significant differences at 95% confidence must have a probability value greater than 0.017 (Tate, 1989).

After 84 days, the level of collagen type III/I+III continued to rise; this finding was unexpected since growth should be insignificant by this time (Massler and Schour, 1951). However, the values for animals aged 112 days and 140 days continued to indicate a positive correlation between the collagen type III/I+III and cell growth rate.

TABLE V-1

% Type III Collagen / Cell Growth Rate
versus Age

AGE days	% Type III (in cell layer) Avg.	Std.Dev.	Growth rate (#cells/day) Avg.	Std.Dev.
0	11.56	3.16	20132	4609
1	7.47	1.12	51333	15616
2	11.16	1.93	25632	22340
3	11.81	1.45	35463	9356
4	14.47	2.09	49944	12786
5	16.75	2.24	51611	6233
6	20.90	6.23	56120	26797
7	26.78	0.37	41667	1521
10	26.23	1.24	45933	3317
14	21.67	2.76	30847	13688
21	12.67	3.34	25444	1829
28	14.85	2.42	32892	11664
35	18.99	0.94	43148	6756
42	17.97	0.33	35815	13693
49	13.24	3.94	29926	7601
56	13.66	3.24	16364	4325
63	8.48	1.85	12781	5967
70	9.64	0.77	10373	2951
77	11.46	0.88	6288	3000
84	4.81	4.19	11317	7399
112	9.55	0.00	23455	0
140	19.47	3.06	34417	588

*Refer to Appendix D for cell layer raw data
**Refer to Appendix G for cell growth raw data

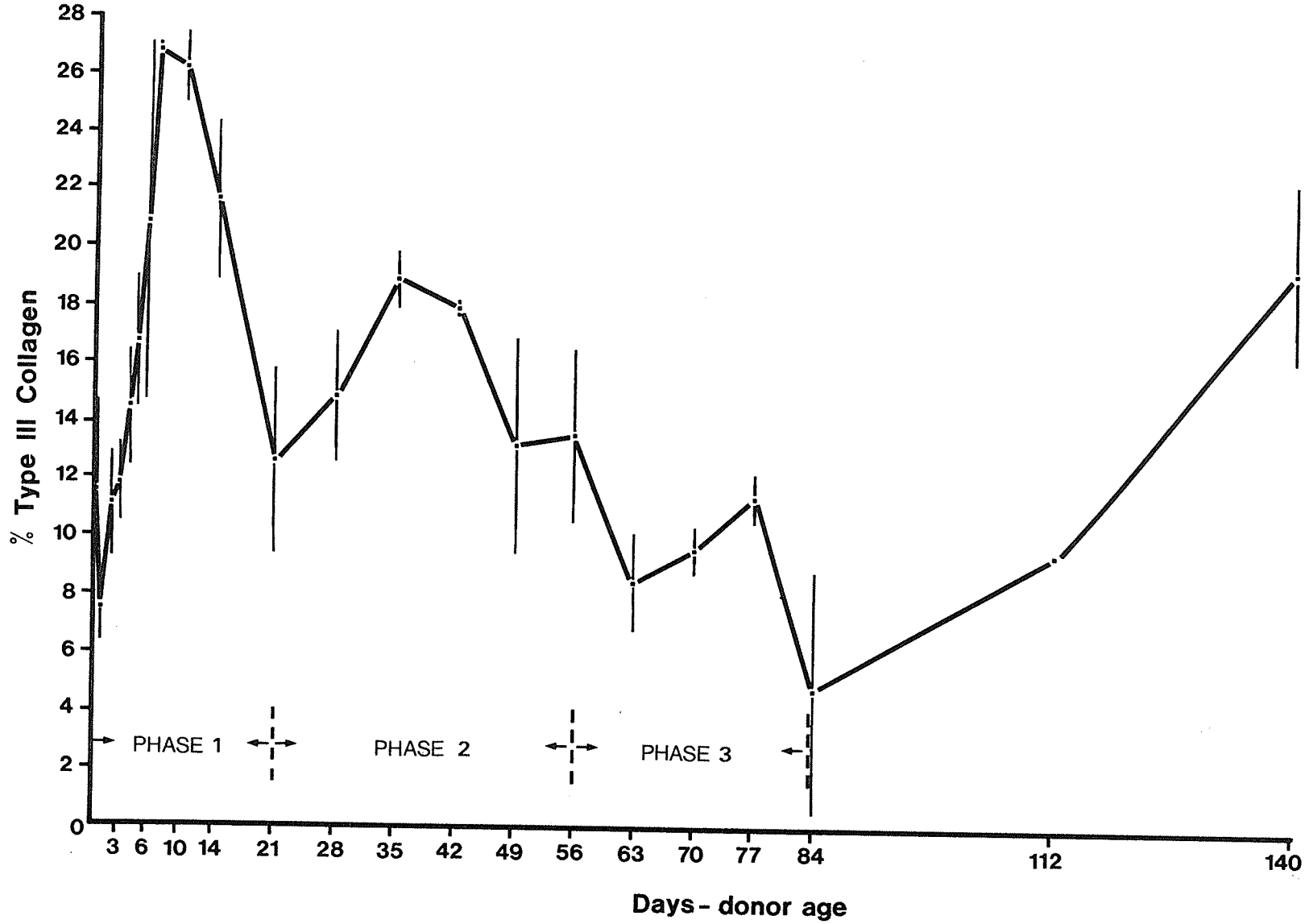

Fig. V–1: % COLLAGEN TYPE III vs. DONOR AGE

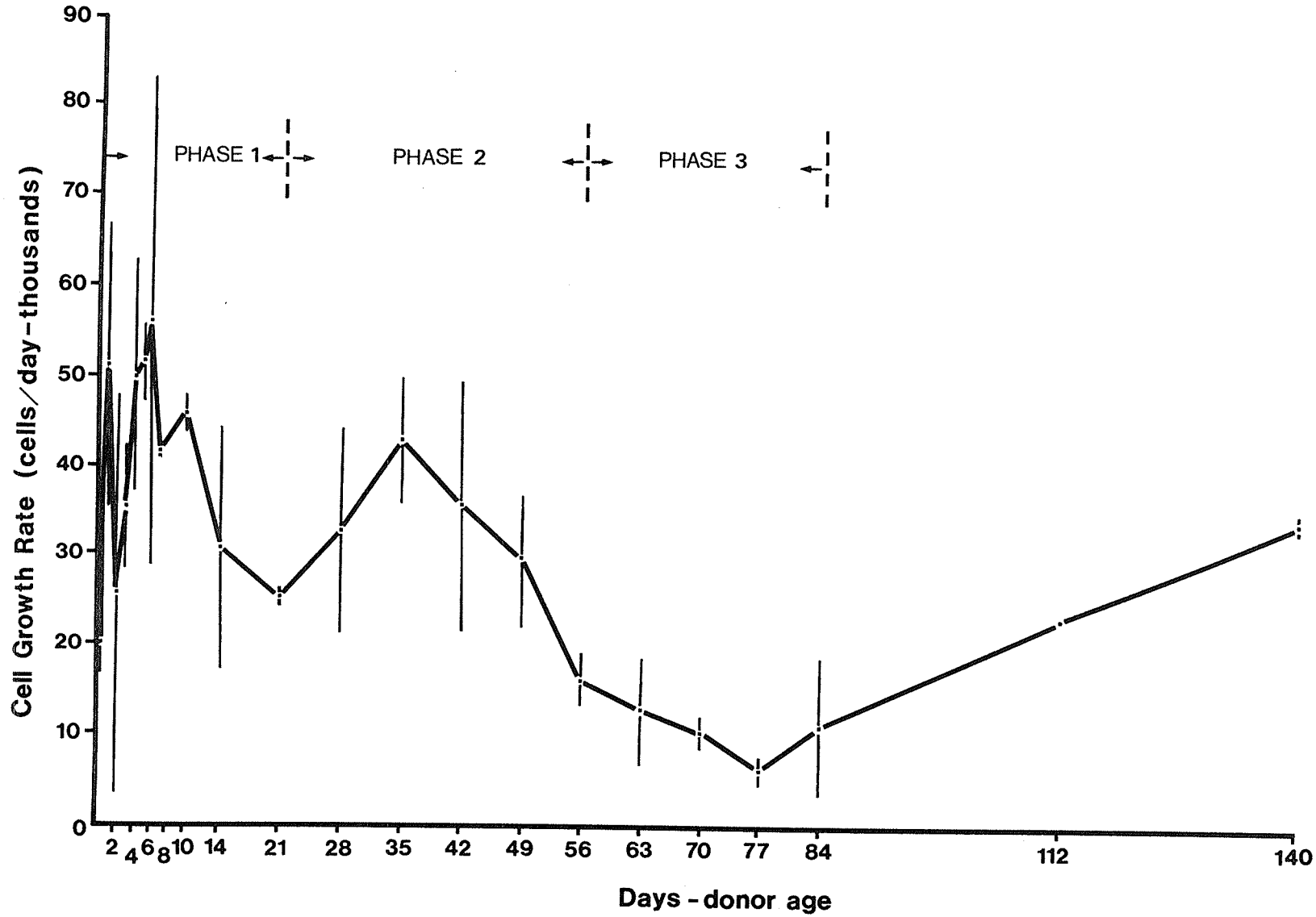

Fig. V-2: CELL GROWTH vs. DONOR AGE

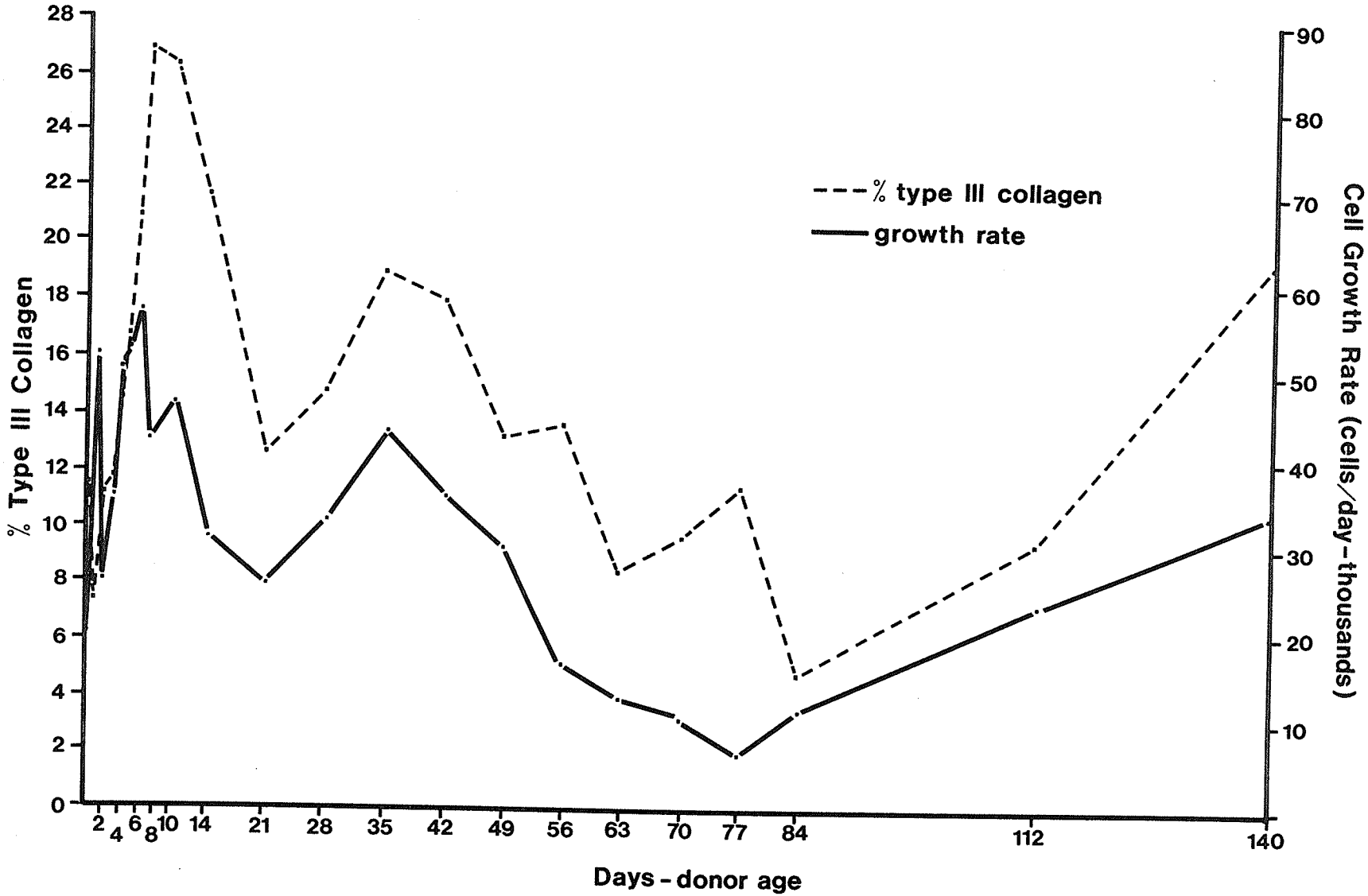

Fig. V-3: CELL GROWTH RATE AND % TYPE III COLLAGEN vs. DONOR AGE

DISCUSSION

The cumulated effects of *in vivo* plus *in vitro* aging have been shown by comparing donor age with the proliferative and synthetic capacities of cells cultured from the respective explants. The cell populations which have survived under the present tissue culture conditions have expressed an ability to not only proliferate but synthesize high proportions of type III to total newly-synthesized collagen, despite donor age.

As evident in the data presented and illustrated in Table V-1 and Figure V-1, there is a fair amount of inter-animal variability in the proportion of type III collagen produced *in vitro* for a given donor age. However, this variability has been somewhat minimized with the inclusion of both peaks in the densitometer scans of type III collagen. The cause for this separation is of interest since it has been found primarily in the cell layer extracts. The problem of excess pepsin digestion has been considered, as adverse effects of pepsin on type III collagen have been reported (Mayne *et al.*, 1978).

Variabilities in the ratio of collagen type III/I+III may also be due to a disproportionate degradation of type I collagen, particularly if an excess amount of type I trimer has been synthesized. However, abnormal culture conditions which might have promoted the synthesis of type I trimer, at $alpha_1(I):alpha_2(I)$ ratios greater than 6:1 (Yen and Melcher, 1978), were not evident. With the odd exception, the $alpha_1(I):alpha_2(I)$ ratios in the cell layer data in the present study generally ranged between 1:1 and 1:3

(Appendix D), compared to the medium layer data in which the ratios were commonly between 1:1 and 2:1 (Appendix E). However, a number of *in vitro* conditions could have selected for different subpopulations and subsequently for collagen phenotype. In addition, each of these factors may be individually influenced by donor age.

First of all, comparisons of cellular functions *in vitro* are only meaningful if the cultures are in good health and processed under identical conditions. In this study, standardization of culture conditions has been achieved by regulating temperature, pH and oxygen tension. However, an additional 5% of fetal bovine serum (FBS) was added to the medium of young cultures (under age 10 days) due to the relatively slow growth rate observed when only 10% concentrations were used.

It is evident that serum contains substances that can both stimulate (Gospodarowicz, 1974) and inhibit cellular proliferation (Harrington and Godman, 1980). However, significant changes related to serum are of concern primarily when serum concentrations are at the extremes (O'Neill *et al.*, 1979); both serum deprivation (<0.5%) and excess (>30%) will inhibit proliferation, while concentrations between 0.5 to 30% will stimulate DNA synthesis (Tucker *et al.*, 1981). As evident in the literature, concentrations at 10% were most often used (Hurych *et al.*, 1974; Schneider and Mitsui, 1976; Breul *et al.*, 1980; Kirchhofer *et al.*, 1986), however, the addition of 15% (Sodek *et al.*, 1977; Limeback and Sodek, 1979) and 20% serum (Manner, 1971) were not

uncommon. Although the optimal serum concentration might differ for different tissues, no significant difference in growth rate was generally noted between 10 to 30% (Tucker et al., 1981). In the present investigation, the total of 15% FBS appeared to have encouraged proliferation of the young sutural populations; similar observations were not evident when 15% FBS was added to cultures of older donors.

It is important to note that with increasing serum concentration, confluent cells aged in vitro have also been found to synthesize more collagen type I relative to type III and therefore, reducing the collagen type III/I ratio (Narayanan and Page, 1977). The maximum stimulation of type III and I collagens appears to occur with the addition of 10% and 20% serum, respectively. Whether this effect is peculiar to gingival cells aged in vitro is still in question, since the inability of serum to modify the synthetic activities of cells from various other sources must be taken into consideration (Narayanan and Page, 1977). However, given the high proportions of type III to total newly-synthesized collagen reported in this study, the effect of the additional proportion of fetal bovine serum to type III synthesis is probably insignificant. This finding is in agreement with other in vitro studies in which collagen synthesis was not found to differ in the presence of serum (Peterkofsky, 1972; Kirchhofer et al., 1986). The discrepancy of findings with respect to serum may be caused by the much longer labelling period used (24 hours) in the experiments by Narayanan and Page (1977), which might indicate the greater need

for serum factors over long culture periods. In the present study, labelling with [^{14}C]-glycine was extended for only 6 hours.

Variabilities in the collagen type III/I+III ratio, particularly in the medium layer (Appendix E), may also be due to a lack of active ascorbic acid in the medium. Although the presence of ascorbic acid crystals in the nutrient media may have indicated its saturation in solution, the ascorbic acid may have been rapidly decomposed in tissue culture (Peterkofsky, 1972). In order to maintain maximal or uninhibited synthesis of type III collagen in its more stable native form, it may be necessary to replenish the medium with ascorbic acid more frequently (Gay et al., 1976). A lack of active ascorbic acid could also affect the secretion of collagen into the medium layer (Jimenez et al., 1973; Murad et al., 1981); and as a result, an excessive accumulation of collagen precursors would remain in the cells (Gay et al., 1976).

Due to the use of limited pepsin digestion in the present investigation, it is not considered crucial that the collagen be converted to its native form. Treatment with SDS-PAGE results in the separation of both procollagen and collagen into their appropriate alpha-chains (Layman et al., 1971). In addition, the extracellular extraction of type III procollagen is a normal feature due to its limited conversion in vivo and in vitro (Gay et al., 1976; Nowack et al., 1976; Limeback and Sodek, 1979). Therefore, variabilities in the phenotypic ratio due to a lack of active ascorbic acid, for example, would more likely result from selective collagenase digestion in culture or pepsin digestion in processing of

either type I or III collagen, rather than from discrepancies in synthesis.

Selective digestion of type III collagen has been reported especially if temperatures higher than 15 degrees celsius have been used (Mayne et al., 1978). Nonetheless, pepsin digestion has been carried out at 16 degrees celsius in this study due to the findings of Limeback and Sodek (1979) who have refuted the significance of both temperature and incubation time on type III degradation. Others (Lovell et al., 1987), on the other hand, have reported that type I and not type III has been more susceptible to pepsin digestion with age. They have suggested that apparent increases in type III collagen with age may only be a result of in vitro processing and may not necessarily reflect the in vivo situation.

The effect of in vitro processing on the collagen phenotype is also evident with respect to the use of trypsin in the subculturing procedure. Due to the relative cytotoxicity of trypsin (Freshney, 1983), the procedure lends to the selection of more trypsin-resistant populations. The accumulated effects of this in vitro influence, with those previously mentioned, all define the environment in which the cell populations in the tissue culture system must reside.

The effect of in vitro selection is particularly evident in the ratio of type III/I+III for donor ages 112 and 140 days. The relative increase in proportions of type III collagen found is unusual since sutural growth is insignificant by age 70 days in vivo (Massler and Schour, 1951). However, this increase in vitro appears to be due to

a stimulus toward proliferation as given by the associated rise in cell growth rate.

As illustrated in Figure V-3, there appears to be a positive relationship between collagen phenotype ratio and the cells' proliferative function. However, under conditions of stable environment, fibroblast populations have been reported to rigidly control their collagen production (Breul et al., 1980). These mass cultures produced collagen during periods of cell proliferation at the same average rate per cell as when the cells were quiescent. On the other hand, mass cultures undergoing logarithmic growth have also been reported to synthesize collagen maximally just prior to confluency (Mollenhauer and Bayreuther, 1986). These assessments of the proliferative and synthetic capacities of cell cultures are expressions of average potentials of all cells residing in the culture. Therefore, the averages include rapidly proliferating cells with relatively low synthetic potentials, as well as, slowly proliferating cells with high synthetic potentials (Mollenhauer and Bayreuther, 1986).

Depending on the synthetic capacity of the cells which have been selected in a culture during in vitro aging, the absolute amount of collagen synthesized, its rate of synthesis, as well as its phenotype are subsequently determined (Mollenhauer and Bayreuther, 1986). Age-related shifts in collagen biosynthesis and proliferative potential, apparent in Figure V-3, could therefore be associated with progressive changes in the relative abundance of the selected cell types. Cultures with large amounts of type III

collagen may be indicative of an initial selection for the proliferative FI population, described by Kontermann and Bayreuther (1979) and Mollenhauer and Bayreuther (1986). A specific proportion of the daughter cells from this progenitor population must subsequently differentiate into the collagen-producing FII or FIII cell, while the remaining daughters continue to propagate.

The relative proportion of the proliferating population committed to collagen synthesis is questionable. Studies with synchronous cultures have suggested that the relative commitment of a population depends on the cell cycle (Ko et al., 1981). The proliferating cultures consistently produce more collagen and at a more rapid rate than non-proliferating or confluent cultures (Manner, 1971). This discrepancy in synthetic capacity is a reflection of the higher cell density conditions at confluency (Hassell et al., 1986). Although the absolute amount of protein synthesis is much lower in confluent cultures, the relative amount of collagen synthesized is higher due to the greater repression of non-collagenous protein synthesis (Manner, 1971).

The particular mechanism responsible for the stimulation of cell proliferation and collagen synthesis in vitro is unclear. The low seeding density utilized in the present study has probably contributed to both proliferative (Ahn et al., 1978; Fisher and Solursh, 1979) and synthetic (Abe et al., 1979; Hassell et al., 1986) potentials, regardless of donor age. In addition, the ability of anchorage-dependent cells, such as those in the sutural population,

to synthesize their appropriate matrix while proliferating will determine the cells' ability to migrate following division (Ben-Ze'ev et al., 1980; Freshney, 1983). By the same token, cellular migration will decrease the effect of inhibitions due to cell density and further encourage both proliferation and collagen synthesis (Peterkofsky, 1972; Ko et al., 1981).

Underlying the study of cells derived in cell culture is the assumption that these cells have properties or potentials that are peculiar to the tissue in vivo (Marmary et al., 1976). However, this assumption has been in question since the function of cells aged in vitro may not necessarily reflect cells aged in vivo (Schneider and Mitsui, 1976). Nonetheless, tissue culture studies are required for the elucidation of the age-related mechanisms which control collagen biosynthesis at the cellular level. Changes in proportions of collagen types synthesized due to aging under culture conditions should therefore be compared to collagen synthesis in vivo.

The effect of donor age on collagen synthesis has been quantitated using in vivo as well as in vitro labelling with [^{14}C]-glycine (Yue, 1984). Using a 4-hour organ culture system, Yue (1984) has shown that the metabolic behaviour of sutures in this in vitro system is comparable with the sutural behaviour in vivo. The ratios of type III collagen to total newly-synthesized collagen in mouse interparietal sutures have also been shown to parallel the rate of in vivo sutural growth in rats. This similarity exists despite the longer life span of the rat which might have been expected to mature over a slightly different time period (Hayflick, 1984).

Therefore, the phenotypic ratios under tissue culture conditions in the present investigation of rat interparietal sutures have been compared to the results from Yue's age study in mouse interparietal sutures.

The relative amount of type III collagen newly-synthesized by cells under tissue culture conditions (in vivo aging and in vitro aging with in vitro labelling) is much greater than under organ culture conditions (in vivo aging with in vitro labelling) (Table V-2; Figure V-4). Using the t-distribution, individual comparisons of the phenotype ratios for different donor ages have been found to be statistically different by $p<0.05$ except for newborns, 1 day and 3 day ratios. However, given the fact that this statistical analysis has required the comparison of 20 sets of results, the Bonferroni correction ($0.05/20=0.0025$) at a 95% confidence level has decreased the probability of what is considered statistically significant to those comparisons where $p<0.0025$ (Tate, 1989). Subsequently, there is more than a 5% chance that the ratios at ages 4, 5, 21, 28, 49, 56, and 63 days are also not statistically different. From these results, it can not be implied that the collagen phenotype ratio in vitro simulates the in vivo situation. The lack of statistical significance is due primarily to the large variances between samples in each age; therefore, a larger number of samples is required to determine whether these values are truly different. Future investigations are required to focus on the mechanisms regulating collagen synthesis of interparietal sutures at the ages during peak synthesis; that is, 7-10 days, 35 days and 77 days.

TABLE V-2

IN VIVO AND IN VITRO
Type III/I+III Collagen Ratios
versus Age

AGE Days	% Type III in tissue culture (Avg.)	(Std.Dev.)	% Type III in organ culture (Avg.)	(Std.Dev.)	p value
0	11.56	3.17	6.54	2.95	*0.0513
1	7.47	1.12	6.57	2.04	*0.5068
2	11.16	1.93	6.06	0.79	0.0004
3	11.81	1.45	8.13	3.36	*0.1196
4	14.47	2.09	9.94	3.49	*0.05
5	16.75	2.24	8.22	3.01	*0.0036
6	20.90	6.23	8.31	2.49	0.0013
7	26.78	0.37	6.53	1.42	0.0001
8			5.83	1.35	
9			5.04	1.22	
10	26.23	1.24	5.77	3.14	0.0001
14	21.67	2.76	4.43	0.9	0.0005
21	12.67	3.34	4.39	0.69	*0.0132
28	14.85	2.42	2.48	1.07	*0.0124
35	18.99	0.94	2.66	1.64	0.0001
42	17.97	0.33	2.77	0.9	0.0001
49	13.24	3.94	2.47	1.36	*0.0381
56	13.66	3.24	2.23	1.01	*0.0235
63	8.48	1.85	3.72	0.59	*0.0133
70	9.64	0.77	2.19	2.28	0.0003
77	11.46	0.88	0.72	1.12	0.0001
84	4.81	4.19	0	0	**
112	9.55	0	0	0	**
140	19.47	3.06	0	0	**

*Not significant with Bonferroni correction ($p<0.0025$)

**No variance due to insignificant values in the organ culture samples. Note graphs drawn to one standard deviation.

***Refer to Appendices D and F for raw data

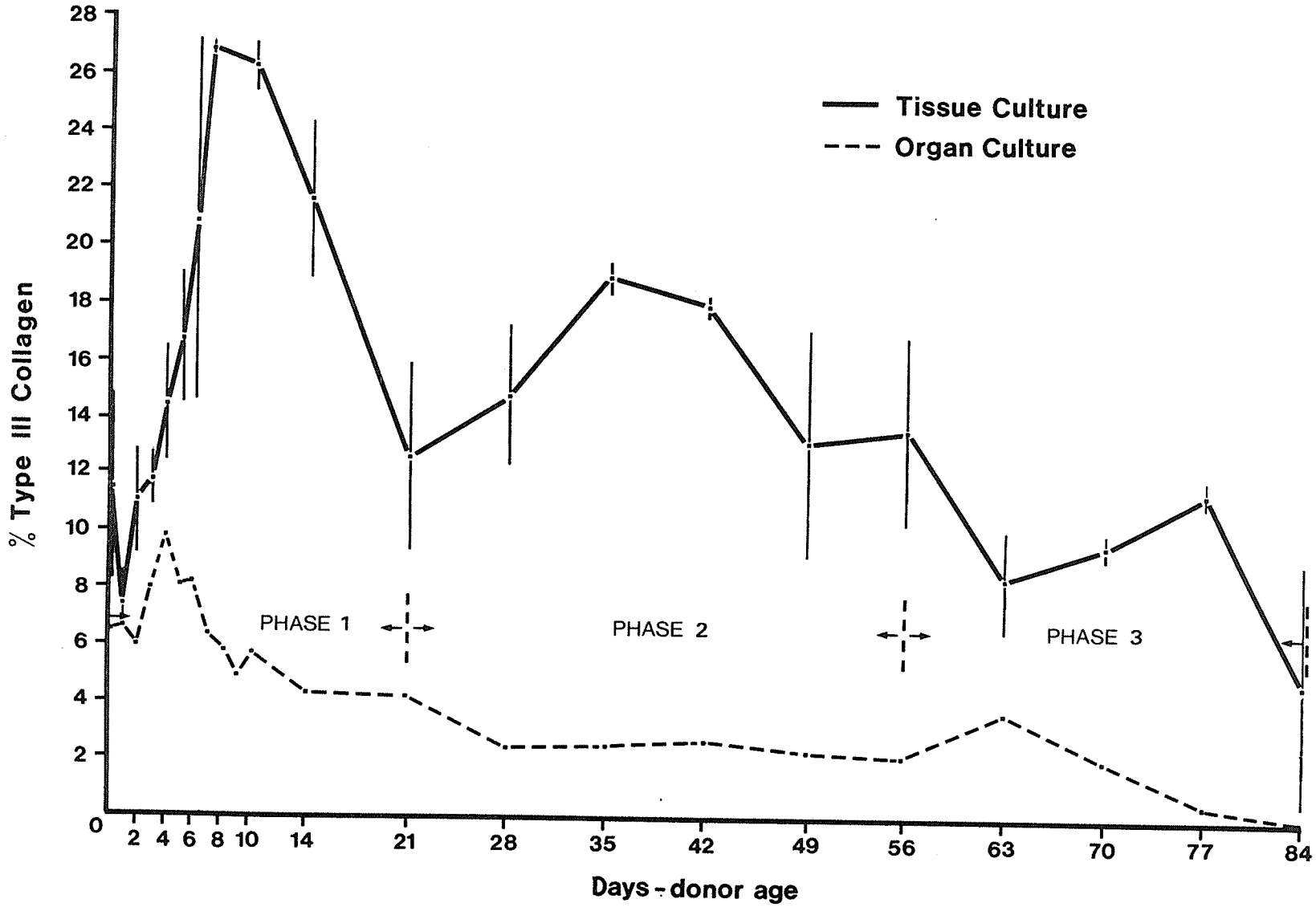

Fig. V-4: % TYPE III COLLAGEN vs. DONOR AGE

A more representative comparison of the in vivo versus in vitro expression of collagen phenotype, as a function of donor age, may be made on the basis of the 3 growth phases defined for Figure V-3 (that is, phase 1: newborn to 21 days, phase 2: 22-56 days, and phase 3: 57-84 days).

In phase 1, the in vivo and in vitro collagen ratios produced show similar initial decreases followed by a peak or maximal expression of relative amounts of type III collagen. The pattern of fluctuations within this phase also appears to be similar although, in vitro, the peak expression is later and higher in value. Hourly differences between the chronologic and biologic ages in young donors are crucial, and may have contributed to the discrepancies noted in the age of peak synthesis. On the other hand, the tremendous increase in type III/I+III values in cell culture may be due to the selection of specific type III collagen producers, regardless of donor age.

The finding that the type III/I+III collagen ratios peak at young ages (7 days in vitro, 4 days in vivo) may also be a function of the number of progenitor cells readily available for sutural growth and development. Since the differentiated collagen-producers appear to synthesize collagen at a constant rate regardless of the growth status (logarithmic or stationary) of the culture as a whole (Kontermann and Bayreuther, 1979), the increase in the ratio of III/I+III in the young may be related to the rapid and consistent addition of an absolute number of these differentiated cells. In older donors, given the relative inactivity of sutural growth

(Massler and Schour, 1951), fewer progenitors are available for repopulation. However, once proliferative activity is stimulated with in vitro aging, the synthetic function of cells from older donors will follow (Figure V-3).

In phase 2, the in vivo and in vitro collagen ratios both peak in the middle of the phase, although the tissue culture data show definite prominence. By phase 3, the relative amount of newly-synthesized type III collagen in vivo eventually decreases and becomes virtually non-detectable by donors aged 84 days; the small peak at 63 days of age in the in vivo study is unusual and may not necessarily be representative of this age group. In contrast, the relative amount of type III collagen synthesized under tissue culture conditions for the respective ages in this last phase maintains relatively high levels.

Using a two-way analysis of variance, the in vivo and in vitro collagen phenotype ratios may be compared on the basis of the three growth phases. Although the general patterns within each phase may appear similar, they differ quantitatively due to the non-uniform increases in the in vitro collagen ratios for each donor age. At $p<0.0001$, the two-way analysis of variance indicates significantly higher type III proportions relative to total newly-synthesized collagen in vitro at phases 1 (9.49%), 2 (13.23%), and 3 (7.32%) (Appendix I; Tate, 1989).

Using the one-way analysis of variance to analyze each of these two studies, the phases which are statistically different are phase 3 in tissue culture (as previously described) and phase 1 in

organ culture. Therefore, in organ culture, the mean values of phase 1 are distinguished from the other two phases such that comparisons between phases 1 and 2 or 1 and 3 are significantly different ($p<0.0001$). The significance of the one-way analysis is the finding that *in vivo*, high but fluctuating levels of type III/I+III ratios are found only early in life in conjunction with rapid sutural growth (Massler and Schour, 1951). After 21 days, the minimal amount of growth remaining is illustrated by the presence of low but relatively constant levels of type III/I+III.

Under tissue culture conditions, the high level of type III/I+III found in phase 2 indicates an enhanced expression of fibroblast proliferation and collagen synthesis compared to the *in vivo* situation. Despite insignificant findings in organ culture (or *in vivo*) for donors 84 days and older, this stimulation of relative collagen synthesis is particularly evident in tissue culture. Therefore, increases in the proportion of collagen type III synthesized in this age study are not necessarily a reflection of the heterogeneity that exists in the sutural population *in vivo*.

Although general age-related effects with respect to the slower initial proliferative potentials of older donors have been demonstrated, progenitor cells from non-growing tissue have the potential to undergo remodelling in a manner comparable to mesenchymal cells from growing tissue. With these progenitor cells in both young and old donors, their expression can be manipulated by local environmental stimuli. In the present tissue culture system, the selection of the cell populations which have tolerated

trypsinization and retained the ability to reproduce and migrate appears to represent the populations responsible for the changes in the proportions of collagen phenotypes synthesized.

CONCLUSIONS

1. Type III collagen synthesis persists in cell cultures from explants of non-growing interparietal sutures.

2. Cell cultures may be stimulated under tissue culture conditions to proliferate, regardless of donor age.

3. High proportions of type III collagen are positively correlated with the proliferative capacity of the cells in culture. Therefore, the ratio of collagen type III to total newly-synthesized collagen tends to parallel cell growth rate.

4. Based on the one-way analysis of variance, the mean difference in the collagen phenotype ratios in phase 3 (57-84 days) under cell culture conditions is significantly lower than in phases 1 and 2 at $p=0.0001$ and $p=0.0012$, respectively.

5. The two-way analysis of variance, comparing the effects of *in vivo* versus *in vitro* aging, indicates significantly higher proportions of collagen type III to total collagen synthesized *in vitro* for growth phases 1 (9.49%), 2 (13.23%), and 3 (7.32%) at $p<0.0001$.

6. Early subcultures of sutural cell populations are capable of demonstrating general age shifts in collagen phenotypic expression; however, due to the effects of *in vitro* aging, this capability is not sensitive enough to be used as the sole test for factors regulating phenotypic expression.

7. The selection of the cell populations which have tolerated *in vitro* conditions and retained the ability to reproduce and migrate appears to represent the populations responsible for the changes in the proportions of collagen phenotypes synthesized.

CHAPTER VI

SUMMARY AND FUTURE CONSIDERATIONS

SUMMARY AND FUTURE CONSIDERATIONS

It has been shown that the ratio of type III collagen to total newly-synthesized collagen increases with the rate of sutural bone growth and decreases with age until complete disappearance on cessation of sutural growth (Yue, 1984). The ultimate objective of the present investigation was to determine whether this age-related shift in collagen phenotype expression was due to changes in relative proportions of specific cell sub-populations present in the suture at each age, or due to modulation of individual cell expression. Cell culture studies were therefore required to examine the collagen synthesis pattern for cell populations at specific ages.

The assessment of collagen phenotypic expression with age began with the characterization of the cell populations which developed from sutural explant culture. The culture was assumed to be populated by progenitors which had retained the ability to proliferate and migrate. In vivo, these cells were presumed to be the same cells which would proliferate during remodelling. In the present investigation, the precursor cells were shown to originate from the sutural area proper. In two cases, the mitotic activity was also associated with vascular elements.

With increasing donor age, the emigration of progenitors from the sutural area exhibited an increased period of latency. This period was overcome with in vitro aging. As a result, migratory activity in both young and old donors appeared to be similar. The

direction of the resulting migration was also consistently asymmetric.

The effects of *in vivo* and *in vitro* cellular aging on the synthetic and proliferative activities of rat interparietal suture cell populations were assessed at seeding concentrations of 20,000 cells/mL in 35mm ($8cm^2$) culture dishes. Low cell density cultures derived from five-week rat interparietal sutures were used to illustrate a characteristic cell growth cycle over a period of 8 days in subculture; this culture condition was found to favour proliferation. In contrast, higher seeding densities minimized the time required to reach confluency, but was also associated with less proliferative cultures.

The significance of cell proliferative capacity was related to its synthetic potential. In a study comparing donor age with the cellular activities of *in vitro*-aged sutural cell populations, there was a parallel stimulation of collagen synthesis with the cell growth rate of tissue culture populations. Except for variabilities particularly in the very young sutures, this relationship was evident regardless of donor age.

As expected, maximal increases in both proliferative and synthetic capacities were found between 7-10 days of age during active sutural growth *in vivo*. Peak levels of cellular activity were also noted at 35 days and interestingly at 140 days. Within the animal ages studied in this investigation, changes in the proportions of collagen phenotypes synthesized were therefore due only in part to donor age. The *in vitro* selection of cell populations which

tolerated processing conditions and retained the ability to reproduce and migrate appeared to be the major determinant of the changes in the proportions of collagen phenotypes synthesized.

The results presented in these studies will serve as baseline data for comparison with future investigations. Studies in progress in this laboratory are using the present model system to characterize the synthetic function of sutural and periodontal cell populations under mechanical stress. The effect of seeding density on the synthetic capacity will also be assessed since seeding concentrations used will be slightly higher. In addition, the present model system will be equally suitable for the isolation of the cell populations contributing to sutural aberrations, such as craniosynostosis.

With respect to investigations utilizing rat sutural populations, animals aged 35 days are of particular interest. Although in vivo, both sutural growth and the ratio of newly-synthesized collagen type III to total collagen are minimal at this age, the inherent capacity of sutural populations to proliferate, migrate and synthesize collagen can be demonstrated in tissue culture. Therefore, with the future isolation of these populations using immunohistochemical and cloning techniques, some of the mechanisms controlling the shifts in proliferative and synthetic expression may be elucidated. Subsequently, the effects of various suggested biologic mediators, such as, cyclic-AMP, prostaglandins, parathyroid hormone, calcitonin and vitamin D metabolites, on collagen metabolism or sutural bone remodelling may be studied.

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BIBLIOGRAPHY

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APPENDICES

APPENDIX A

PILOT #1: Phase 1
Raw Data

Time in Primary Culture (days)	Coulter Counts (less background)	Average	Total Cells per plate (calculated)	# Cells Available for subculture	Maximum # cells (pooled) Average
7	1743				
	1700				
	1673				
		1705.3	238742	214868	
	1612				
	1599				
	1590				
		1600.3	224047	201643	667461
	2024				
	1994				
	1957				
		1991.7	278833	250950	
8	3201				
	3190				
	3189				
		3193.3	447067	402361	
	2646				
	2635				
	2616				
		2632.3	358527	331675	1075287
	2714				
	2710				
	2701				
		2708.3	379167	341251	
10	4370				
	4355				
	4351				
		4358.7	610213	549192	
	3880				
	3871				
	3852				
		3867.7	541473	487326	1455637
	3349				
	3331				
	3299				
		3326.3	465687	419119	

APPENDIX A

Pilot #1: Phase 2
Raw Data

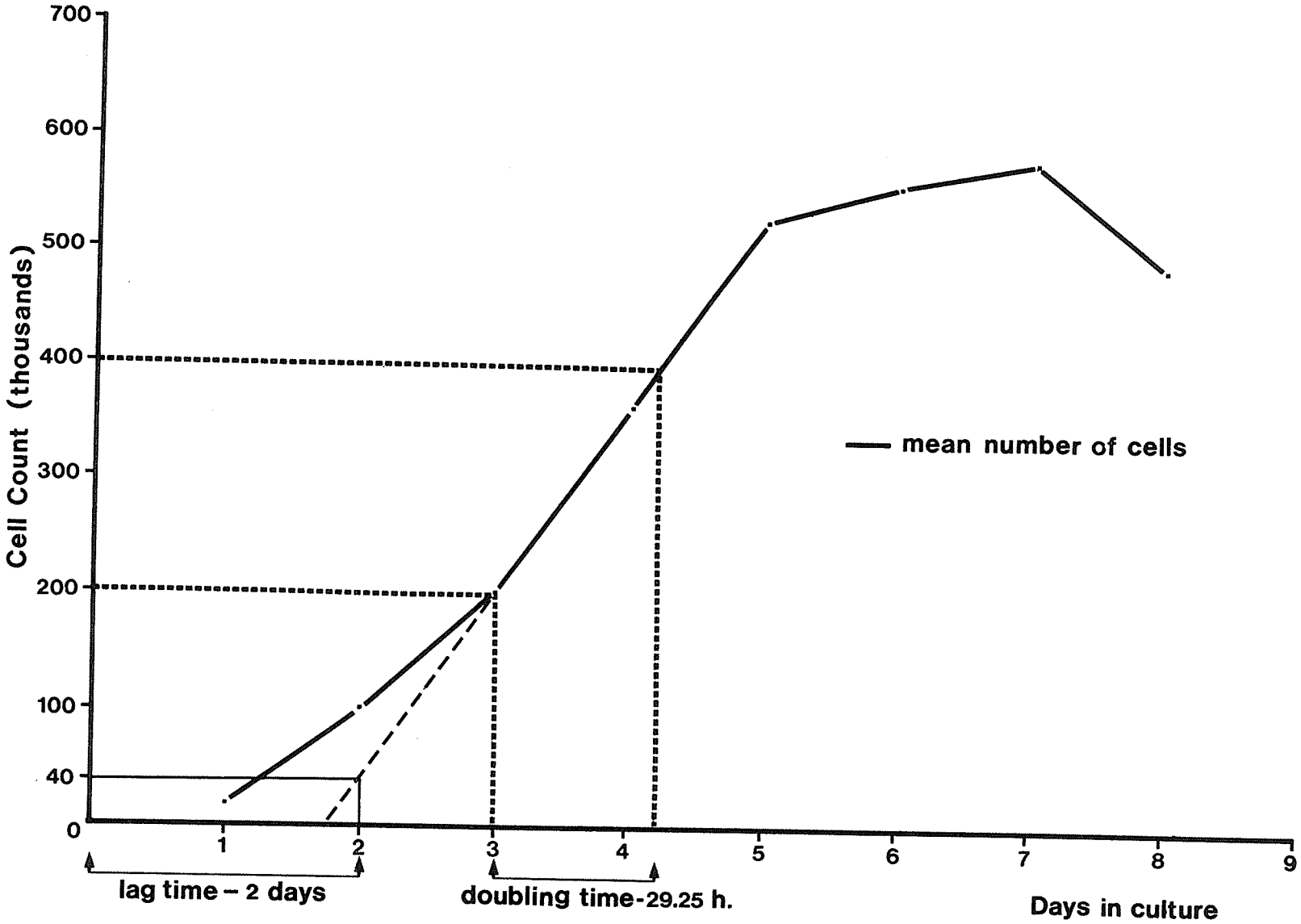
Seeding Concn. (#cells/mL)	Number of cells at confluency (days in 1st culture) 7	8	10	Avg.	Std.Dev.	#cells - seeding conc.	Days to Confluency	Growth rate (cells/day)	Avg.	Std.Dev.
20000	830000					790000	7	112857		
	825000					785000	7	112143		
	730000					690000	7	98571		
	695000			770000	67964	655000	7	93571	104286	9706
30000	770000					710000	7	101429		
	470000					410000	5	82000		
	690000					630000	6	105000		
	490000			605000	148250	430000	6	71667	90024	15875
35000		640000				570000	6	95000		
		680000				610000	6	101667		
		660000		660000	19923	590000	6	98333	98333	3321
40000	350000					270000	5	54000		
	350000					270000	5	54000		
	370000			356667	11502	290000	5	58000	55333	2301
50000		600000				500000	6	83333		
		490000				390000	5	78000		
		545000		545000	54787	445000	5	89000	83444	5479
60000		760000				640000	6	106667		
		700000				580000	6	96667		
		630000		696667	64813	510000	6	85000	96111	10802
70000			400000			260000	4	65000		
			400000	400000	0	260000	4	65000	65000	0
80000			300000			140000	3	46667		
			410000			250000	3	83333		
			355000	355000	54787	195000	3	65000	65000	18262
90000			330000			150000	4	37500		
			400000			220000	4	55000		
			365000	365000	34864	185000	4	46250	46250	8716

APPENDIX B

Pilot #2
Raw Data

Cells at Confluency / Cell Growth Rate
v.s. Time in days

Days in subculture	#cells at confluency	Avg	Std.Dev.	#cells less seeding concn.	Growth rate (cells/day)	Avg.	Std.Dev.
0	(40000)	40000		0			
1	23590			-16410	-16410		
	16800			-23200	-23200		
	17990	19460	3611	-22010	-22010	-20540	3611
2	91000			51000	25500		
	143127			103127	51564		
	67153	100427	38703	27153	13577	30213	19352
3	160670			120670	40223		
	219430			179430	59810		
	225210	201770	35573	185210	61737	53923	11857
4	335720			295720	73930		
	499050			459050	114763		
	256200	363657	123332	216200	54050	80914	30833
5	426670			386670	77334		
	505730			465730	93146		
	651140	527847	113416	611140	122228	97569	22683
6	533820			493820	82303		
	489390			449390	74898		
	546187			506187	84365		
	666680			626680	104447		
	551270	557469	65737	511270	85212	86245	10957
7	563780			523780	74826		
	456470			416470	59496		
	694260			654260	93466		
	601810	579080	98430	561810	80259	77011	14061
8	458220			418220	52278		
	519960	489090	43657	479960	59995	56136	5457



Appendix C: CALCULATION OF LAG AND DOUBLING TIMES

APPENDIX D

Cell Layer Raw Data

AGE DAYS	CODE	FILM No.	Densitometer readings III Peak1	III Peak2	TYPEIII (total)	ALPHA1	ALPHA2	III/I+III %	Avg.III %	Std.III %
0	A-C37	5	3.243	2.585	5.828	25.566	7.936	14.818		
0	C-C84	8	0.000	3.040	3.040	16.123	7.558	11.377		
0	F-C82	8	0.000	2.807	2.807	22.510	7.805	8.475	11.557	3.163
1	A-C38	5		6.464	6.464	49.028	21.225	8.426		
1	C-C50	6	0.000	5.555	5.555	57.716	25.806	6.236		
1	G-C52	6		6.256	6.256	56.025	18.338	7.760	7.474	1.117
2	A-C39	5		6.215	6.215	27.196	17.934	12.104		
2	C-C54	6		8.863	8.863	45.123	19.215	12.108		
2	E-C59	7		9.878	9.878	44.801	26.662	12.144		
2	G-C40	5		4.832	4.832	37.898	15.722	8.267	11.156	1.927
3	A-C41	5	0.000	6.465	6.465	37.207	14.632	11.088		
3	C-C42	5	1.301	6.160	7.461	33.694	14.156	13.489		
3	E-C56	6	3.553	4.184	7.737	46.023	17.463	10.863	11.814	1.449
4	C-C57	6	0.000	10.362	10.362	49.300	20.926	12.858		
4	E-C44	5	1.929	8.449	10.378	39.298	15.051	16.033		
4	G-C45	5	2.732	4.907	7.639	27.568	11.047	16.515		
4	A-C43	6	3.022	4.637	7.659	37.280	16.381	12.490	14.474	2.094
5	E-C48	5	2.050	10.468	12.518	53.061	22.772	14.168		
5	A-C46	6	0.000	12.103	12.103	39.382	15.988	17.938		
5	C-C47	6	0.000	15.247	15.247	47.516	21.306	18.136	16.747	2.227
6	E-C76	8	0.000	21.200	21.200	42.642	25.674	23.683		
	A-C72	10	4.189	4.371	8.560	40.524	17.362	12.883		
	C-C74	10	2.746	7.020	9.766	37.626	12.110	16.413		
	G-C77	8	0.000	21.635	21.635	45.731	26.980	22.932		
	A-C23	A4	2.778	1.760	4.538	8.168	3.179	28.568	20.896	6.226
7	E-C26	3	9.281	11.629	20.910	38.715	18.582	26.737		
	C-C29	A4	11.097	16.077	27.174	50.734	22.091	27.174		
	A-C24	3	3.255	5.566	8.821	13.615	10.925	26.441	26.784	0.367
10	A-C11	A2	2.306	7.932	10.238	19.411	8.293	26.983		
	C-C17	3	0.000	6.364	6.364	13.638	5.657	24.802		
	E-C18	3	2.214	2.235	4.962	7.445	6.030	26.913	26.233	1.235
14	A-C27	3	2.727	17.841	20.568	38.529	26.741	23.961		
	E-C31	3	5.035	12.930	17.965	41.771	20.309	22.444		
	G-C36	5	1.882	8.298	10.180	31.542	13.016	18.598	21.668	2.754
21	C-C32	3	1.771	7.929	9.700	31.179	19.056	16.184		
	E-C33	3	2.786	4.101	6.887	42.775	21.922	9.621		
	G-C34	3	1.978	3.598	5.576	27.313	12.812	12.201	12.669	3.294
28	A-C12	A2	9.629	4.068	13.697	52.807	32.301	13.863		
	C-C1	A2	4.866	6.134	11.000	31.841	19.631	17.608		
	G-C2	A2	4.064	5.340	9.404	37.562	24.971	13.073	14.848	2.413
35	A-C78	10	4.385	5.229	9.614	31.301	10.542	18.684		
	C-C28	3	5.686	10.018	15.704	39.988	22.632	20.050		
	E-C19	A4	1.549	11.286	12.835	38.178	19.351	18.241	18.991	0.939

APPENDIX D con't

Cell Layer Raw Data

AGE DAYS	CODE	FILM No.	Densitometer readings III Peak1	III Peak2	TypeIII (total)	ALPHA1	ALPHA2	III/I+III %	Avg.III %	Std.III %
42	A-C3	2	5.254	7.250	12.504	36.061	19.572	18.351		
	E-C4	2	2.090	5.056	7.146	21.525	11.408	17.830		
	G-C5	2	3.086	6.275	9.361	29.831	13.614	17.727	17.969	0.333
49	G-C128	12	2.369	4.002	6.371	24.404	9.592	15.783		
	A-C126	12	0.000	6.507	6.507	53.919	14.300	8.708		
	C-C127	12	0.000	7.872	7.872	7.836	35.946	15.240	13.243	3.922
56	C-C20	3	4.986	5.138	10.124	55.946	27.953	10.768		
	E-C25	3	4.130	6.380	10.510	46.264	23.717	13.057		
	A-C6	2	3.310	5.246	8.556	30.617	10.674	17.165	13.663	3.228
63	A-C60	7	0.000	7.496	7.496	53.016	17.201	9.646		
	C-C61	7	1.562	3.467	5.029	55.744	18.546	6.340		
	G-C62	7	3.179	4.029	7.208	52.988	16.130	9.444	8.477	1.845
70	A-C63	7	0.000	8.243	8.243	58.065	26.650	8.867		
	C-C64	7	2.821	2.997	5.818	43.415	12.292	9.456		
	E-C65	A7	2.010	3.605	5.615	40.525	12.791	9.528		
	C-C35	A4	0.000	5.604	5.604	30.737	16.032	10.700	9.638	0.768
77	C-C69	A7	0.000	3.576	3.576	22.844	7.409	10.571		
	E-C66	A7	4.911	6.593	11.504	72.640	15.856	11.504		
	G-C67	A7	2.124	2.597	4.721	24.372	9.228	12.320	11.465	0.871
84	A-C68	A7		5.218	5.218	45.318	17.545	7.664		
	E-C70	A7	0.000	0.000	0.000	19.601	17.687	0.000		
	G-C71	A7	0.000	2.634	2.634	27.124	9.131	6.773	4.813	4.175
112	A-C14	A2		6.919	6.919	40.151	25.396	9.548	9.548	
140	A-C15	2	10.284	5.449	15.733	37.700	14.915	23.019		
	C-C7	2	3.988	6.648	10.636	31.611	18.230	17.587		
	E-C16	3	2.388	9.070	11.458	31.752	21.181	17.794	19.467	3.066

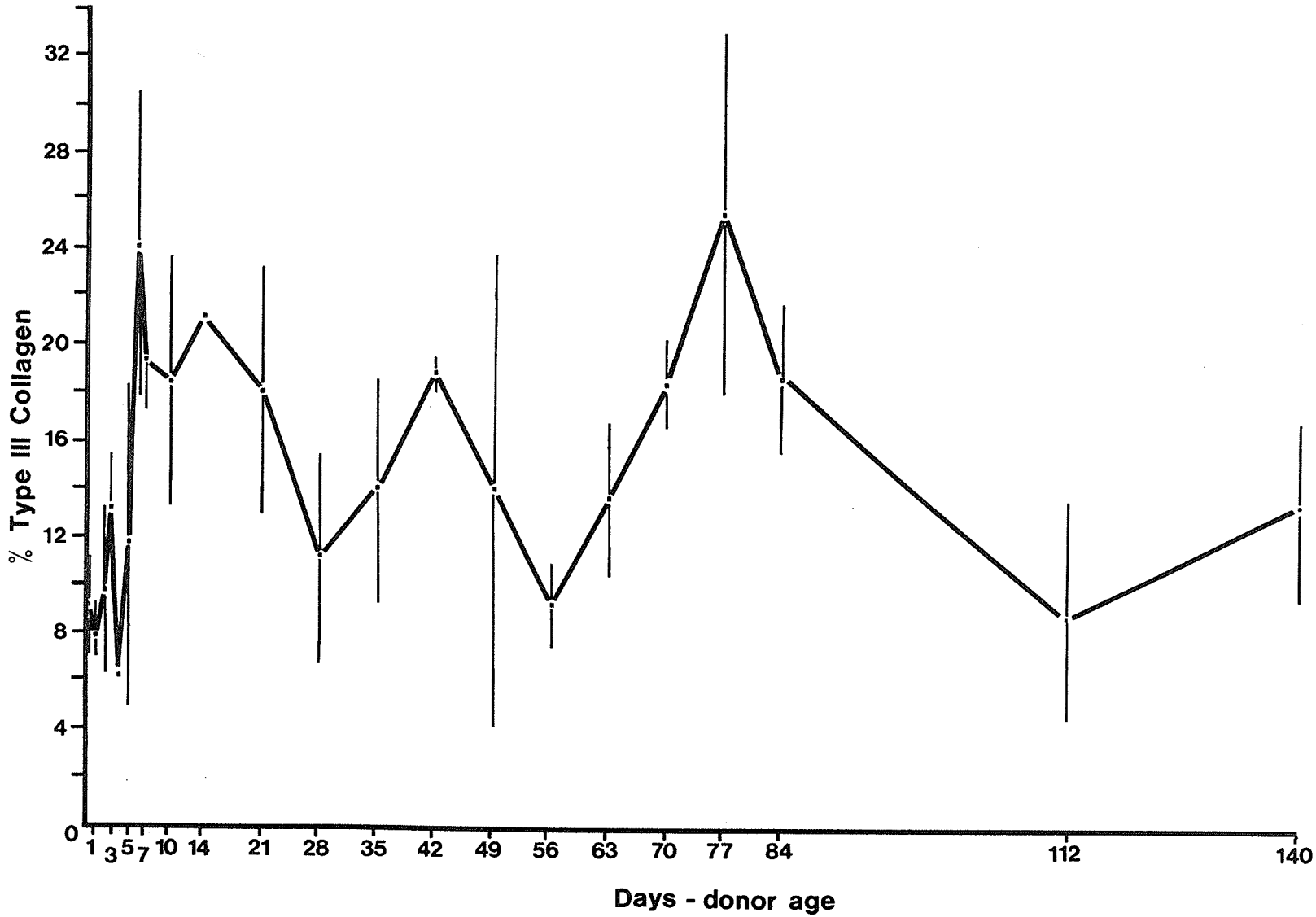
APPENDIX E

Medium Layer Raw Data

AGE DAYS	CODE	FILM No.	Densitometer readings III Peak1	III Peak2	TYPEIII (total)	ALPHA1	ALPHA2	III/I+III %	Avg.III %	Std.III %
0	A-M81	A8	0	10.595	10.595	58.699	30.706	10.595		
	C-M84	A8	6.032	5.491	11.523	43.276	37.524	12.481		
	G-M86	A8	0	6.06	6.06	50.562	21.839	7.724		
	C-M95	A9	0	5.448	5.448	55.226	24.126	6.425		
	E-M96	A9	0	10.251	10.251	52.396	31.402	10.900		
	G-M97	A9	0	5.859	5.859	47.316	34.247	6.702	9.138	2.517
1	A-M98	A9	0	5.531	5.531	51.485	31.507	6.248		
	C-M99	A9	0	7.712	7.712	46.348	34.211	8.737		
	C-M107	A11	1.593	7.653	9.246	58.919	31.127	9.312		
	J-M106	11	0	6.467	6.467	54.255	27.136	7.361	7.914	1.380
2	C-M100	A9	0	5.209	5.209	46.663	33.153	6.126		
	L-M112	A11	0.734	11.795	12.529	47.092	35.734	13.139		
	J-M108	11	0	9.576	9.576	49.647	36.108	10.045	9.770	3.501
3	G-M102	A9	0	8.982	8.982	47.062	35.479	9.814		
	E-M105	10	0	14.174	14.174	43.698	32.37	15.707		
	L-M109	11	0	13.517	13.517	44.728	37.029	14.188	13.236	3.048
4	B-M110	11	0	5.757	5.757	57.3	29.037	6.251	6.251	0.000
5	G-M92	A9	0	6.2	6.2	52.741	28.033	7.129		
	E-M91	A9	0	6.923	6.923	49.543	25.446	8.452		
	A-M90	A9	0	16.601	16.601	39.922	27.634	19.726	11.769	6.897
6	A-M72	A8	0	24.521	24.521	40.174	28.881	26.204		
	C-M74	A8	0	28.034	28.034	39.831	28.54	29.079		
	C-M30	A4	0	6.749	6.749	17.94	8.254	20.487		
	G-M77	A8	0	42.45	42.45	59.06	38.44	30.332		
	E-M76	A8	3.639	9.525	13.164	46.729	30.18	14.615	24.144	6.539
7	E-M26	A4	0	12.939	12.939	29.747	25.286	19.036		
	K-M118	11	0	18.006	18.006	38.947	21.423	22.974		
	J-M117	A11	0	14.458	14.458	42.56	29.669	16.678		
	L-M114	A11	0	15.797	15.797	39.599	28.923	18.735	19.356	2.630
10	A-M11	A2	0	16.189	16.189	51.074	32.737	16.189		
	C-M17	4	0	10.818	10.818	41.819	21.83	14.527		
	E-M18	4	0	24.622	24.622	50.137	25.241	24.622	18.446	5.391
14	A-M27	4	0	18.171	18.171	42.504	24.171	21.416		
	E-M31	4	0	12.82	12.82	30.263	18.048	20.971	21.194	0.315
21	C-M32	4	0	13.98	13.98	35.85	25.079	18.663		
	E-M33	4	0	19.377	19.377	30.624	32.838	23.391		
	E-M33	4	0	6.467	6.467	29.155	23.236	10.987		
	G-M34	4	0	14.755	14.755	47.775	18.092	18.301	18.051	5.122
28	C-M1	A2	0	4.078	4.078	48.251	28.655	5.036		
	A-M12	A2	0	11.664	11.664	40.738	32.617	13.719		
	G-M2	A2	0	13.018	13.018	39.848	27.41	16.217		
	I-M121	11	0	7.577	7.577	50.176	34.524	8.211		
	J-M122	A11	1.07	9.984	11.054	45.744	29.748	12.772	11.191	4.498

APPENDIX E con't
Medium Layer Raw Data

AGE DAYS	CODE	FILM No.	Densitometer readings III Peak1	III Peak2	TypeIII (total)	ALPHA1	ALPHA2	III/I+III %	Avg.III %	Std.III %
35	A-M78	A8	0	14.375	14.375	45.732	35.508	15.034		
	C-M80	A8	0	5.583	5.583	44.604	28.482	7.097		
	C-M28	4	0	9.907	9.907	28.799	21.924	16.340		
	E-M19	A6	0	12.843	12.843	42.628	16.133	17.936	14.102	4.820
42	A-M3	2	3.869	11.008	14.877	43.593	23.859	18.070		
	E-M4	A2	0.824	15.724	16.548	44.935	28.773	18.335		
	G-M5	A2	1.589	14.634	16.223	40.111	23.578	20.301	18.902	1.214
49	A-M8	A2	0	16.137	16.137	34.496	25.044	21.324		
	C-M9	A2	0	1.683	1.683	44.705	21.778	2.469		
	G-M10	A2	0	16.108	16.108	44.587	26.747	18.421	14.071	10.113
56	A-M13	A2	0	6.654	6.654	57.119	36.227	6.654		
	D-M21	4	0	7.362	7.362	41.082	22.986	10.307		
	E-M25	A4	0	2.583	2.583	12.269	6.552	12.068		
	F-M22	4	0	7.555	7.555	50.273	36.45	8.014	9.260	2.404
63	L-M120	A11	0	13.588	13.588	43.855	26.912	16.108		
	M-M115	11	2.251	7.994	10.245	45.597	34.709	11.314	13.711	3.390
70	A-M63	7	0	8.143	8.143	17.937	26.179	15.582		
	C-M64	7	0	8.314	8.314	16.792	16.8665	19.808		
	C-M35	A4	0	8.985	8.985	23.882	12.039	20.008	18.466	2.490
77	C-M69	A7	0	7.906	7.906	33.289	12.548	14.711		
	E-M66	7	0	30.317	30.317	40.422	23.933	32.023		
	G-M67	7	0	27.352	27.352	39	24.984	29.947	25.560	9.416
84	A-M68	A7		15.735	15.735	53.308	25.235	16.690		
	E-M70	A7	0	6.027	6.027	21.767	7.567	17.044		
	G-M71	A7	0	16.516	16.516	43.075	14.075	22.420	18.718	3.200
112	A-M14	A2	0	3.755	3.755	44.158	35.408	4.507		
	F-M88	8	0	13.769	13.769	43.631	31.468	15.494		
	E-M89	A9	0	5.449	5.449	37.57	12.249	9.859		
	H-M87	8	1.093	2.895	3.988	56.06	16.232	5.228	8.772	5.072
140	A-M15	A2	0	10.396	10.396	44.911	25.724	12.830		
	C-M7	A2	0	9.802	9.802	42.747	28.089	12.156		
	E-M16	4	4.061	11.219	15.28	44.364	20.724	19.013	14.666	3.765



Appendix E: % TYPE III COLLAGEN (MEDIUM LAYER) vs. DONOR AGE

APPENDIX F

Percentage of Type III Collagen to Total

Newly-synthesized Collagen with *In Vivo*

Aging and *In Vitro* Labelling

Raw Data (Yue, 1984)

TIME days	SAMPLE1 %	SAMPLE2 %	SAMPLE3 %	SAMPLE4 %	SAMPLE5 %	SAMPLE6 %	AVG %	STD %
0.00	5.60	2.59	3.94	8.19	8.81	10.10	6.54	2.95
1.00	3.76	6.44	4.76	7.55	9.35	7.56	6.57	2.04
2.00	5.08	5.47	6.35	7.14	6.67	5.63	6.06	0.79
3.00	4.96	8.94	5.17	6.70	9.01	13.97	8.13	3.36
4.00	11.29	5.65	6.13	9.88	14.54	12.18	9.95	3.49
5.00	5.22	6.86	5.49	10.02	13.07	8.69	8.23	3.01
6.00	8.68	5.18	5.33	10.86	9.37	10.44	8.31	2.49
7.00	5.43	5.03	5.31	7.53	7.73	8.15	6.53	1.42
8.00	5.16	5.63	4.00	6.50	6.70	7.00	5.83	1.35
9.00	3.56	5.72	3.48	5.10	5.20	7.20	5.04	1.22
10.00	3.96	3.95	2.59	5.10	10.99	8.04	5.77	3.14
14.00				5.34	4.40	3.55	4.43	0.90
21.00				4.64	3.61	4.93	4.39	0.69
28.00				2.48	2.32	2.65	2.48	1.07
35.00				2.40	4.41	1.17	2.66	1.64
42.00	1.78	2.05	2.13	3.25	3.96	3.45	2.77	0.90
49.00	0.65	1.26	2.40	4.30	3.52	2.70	2.47	1.36
56.00	1.07	1.02	2.30	2.44	3.20	3.36	2.23	1.01
63.00				3.06	3.89	4.20	3.72	0.59
70.00	0.00	0.00	1.30	5.72	2.11	3.98	2.19	2.28
77.00	2.26	0.00	0.00	0.00	2.06	0.00	0.72	1.12
84.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00
112.00				0.00	0.00	0.00	0.00	0.00
140.00				0.00	0.00	0.00	0.00	0.00

APPENDIX G

Cell Growth Rate Raw Data

AGE DAYS	CODE	Cell # at Confluency	Cell # less seeding concn.	Culture Time (days)	Growth Rate (#cells/day)	(Avg.)	(Std.Dev.)
0	A-C37	138000	98000	6	16333	20132	4609
	C-C84	217000	177000	7	25286		
	F-C82	209000	169000	9	18778		
1	A-C38	304000	264000	6	44000	51333	15616
	C-C50	456000	416000	6	69333		
	G-C52	284000	244000	6	40667		
2	A-C39	141000	101000	6	16833	25632	22340
	C-C54	194000	154000	9	17111		
	E-C59	217000	177000	18	9833		
	G-C40	275000	235000	4	58750		
3	A-C41	251000	211000	6	35167	35463	9356
	C-C42	310000	270000	6	45000		
	E-C56	276000	236000	9	26222		
4	C-C57	401000	361000	9	40111	49944	12786
	E-C44	378000	338000	6	56333		
	G-C45	271000	231000	6	38500		
	A-C43	429000	389000	6	64833		
5	E-C48	357000	317000	6	52833	51611	6233
	A-C46	309000	269000	6	44833		
	C-C47	383000	343000	6	57167		
6	E-C76	528000	488000	10	48800	56120	26797
	A-C72	337000	297000	5	59400		
	C-C74	404000	364000	5	72800		
	G-C77	464000	424000	5	84800		
	A-C23	114000	74000	5	14800		
7	E-C26	250000	210000	5	42000	41667	1521
	C-C29	255000	215000	5	43000		
	A-C24	240000	200000	5	40000		
10	A-C11	275000	235000	5	47000	45933	3317
	C-C17	283000	243000	5	48600		
	E-C18	251000	211000	5	42200		
14	A-C27	600000	560000	12	46667	30847	13688
	E-C31	215000	175000	8	21875		
	G-C36	328000	288000	12	24000		
21	C-C32	280000	240000	9	26667	25444	1829
	E-C33	277000	237000	9	26333		
	G-C34	250000	210000	9	23333		
28	A-C12	287000	247000	11	22455	32892	11664
	C-C1	316000	276000	9	30667		
	G-C2	450000	410000	9	45556		
35	A-C78	431000	391000	9	43444	43148	6756
	C-C28	488000	448000	9	49778		
	E-C19	366000	326000	9	36222		

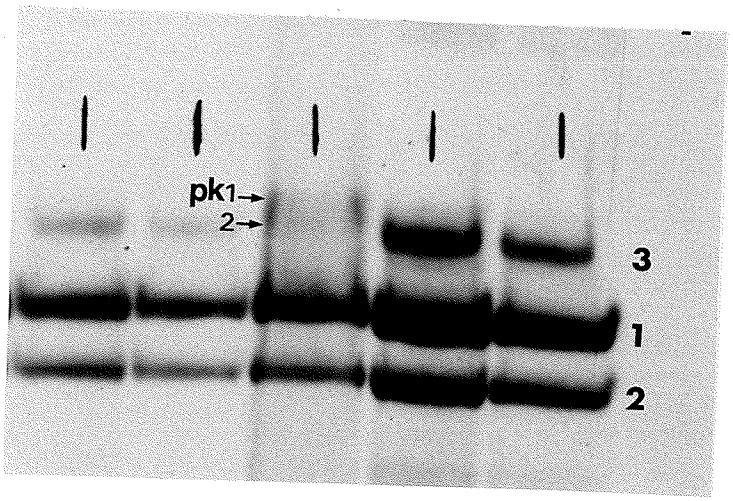
APPENDIX G con't

Cell Growth Rate Raw Data

AGE DAYS	CODE	Cell # at Confluency	Cell # less seeding concn.	Culture time (days)	Growth rate (#cells/day)	(Avg.)	(Std.Dev.)
42	A-C3	221000	181000	9	20111	35815	13693
	E-C4	451000	411000	9	45667		
	G-C5	415000	375000	9	41667		
49	G-C128	315000	275000	9	30556	29926	7601
	A-C126	238000	198000	9	22000		
	C-C127	375000	335000	9	37222		
56	C-C20	189000	149000	11	13545	16364	4325
	E-C25	275000	235000	11	21364		
	A-C6	196000	156000	11	14182		
63	A-C60	312000	272000	16	17000	12781	5967
	C-C61	177000	137000	16	8563		
70	A-C63	261000	221000	21	10524	10373	2951
	C-C64	332000	292000	21	13905		
	E-C65	258000	218000	21	10381		
	C-C35	167000	127000	19	6684		
77	C-C69	100000	60000	17	3529	6288	3000
	E-C66	110000	70000	12	5833		
	G-C67	154000	114000	12	9500		
84	A-C68	278000	238000	12	19833	11317	7399
	E-C70	145000	105000	17	6176		
	G-C71	175000	135000	17	7941		
112	A-C14	258000	218000	11	19818	23455	0
140	A-C15	319000	279000	8	34875	34417	588
	C-C7	310000	270000	8	33750		
	E-C16	317000	277000	8	34625		

APPENDIX H

PHOTOGRAPH OF FLUOROGRAM ILLUSTRATING COLLAGEN BANDS SEPARATED BY SDS-PAGE



1 = $alpha_1(I)$

2 = $alpha_2(I)$

3 = $alpha_1(III)$

→* Example of separated bands scanned as peaks 1 and 2

APPENDIX I

Comparison of the Proportion of Type III alpha-chains to Total Newly-synthesized Collagen in Cell Culture versus Organ Culture, as a Function of Age.

Statistical Test: Two-way Analysis of Variance

TC = tissue culture values
OC = organ culture values

Phase (days)	Culture	No. of samples	Type III (mean)	Standard Deviation	Standard Error	F'	p	Diff.
1: 0-21	TC	37	16.536%	6.653	1.094	0.0001	0.0001	9.488%
	OC	60	7.048%	2.794	0.361			
2: 22-56	TC	15	15.743%	3.234	0.835	0.0001	0.0001	13.232%
	OC	24	2.511%	1.037	0.212			
3: 57-84	TC	13	8.678%	3.126	0.867	0.0341	0.0001	7.317%
	OC	21	1.361%	1.834	0.400			

APPENDIX J

COLLAGEN BIOSYNTHESIS

The tropocollagen molecule is the simplest building block unit of collagen in the extracellular space. It is approximately 15A in diameter and 2800 to 3000 A in length with a molecular weight of about 300,000 (Barbanell et al., 1978; Gay and Miller, 1978). A tropocollagen molecule consists of three alpha-chains, each of which are individually coiled in a left-handed minor helix. As a unit, these three polypeptide chains are intertwined into a right-handed, rod-like triple superhelix (Gay and Miller, 1978).

The primary structure in each alpha-chain is composed of a repeating triplet (Glycine-X-Y)n of about 1052 amino acid residues (Barbanell et al., 1978; Prockop et al, 1979a). One-third of the amino acid residues in each alpha-chain are glycine. One-quarter of the remaining amino acids are proline and hydroxyproline since they usually occupy the X and Y positions, respectively (Cheah, 1985). Each alpha-chain is coiled in a left-handed helix due to the frequent presence of these latter two residues.

Hydroxyproline is found almost exclusively in collagen; subsequently, any changes in its content may be indicative of changes in collagen metabolism (Prockop, 1979a; Pinnell

et al., 1987). Hydroxyproline serves to stabilize the collagen triple helix (Jimenez et al., 1973; Pinnell et al., 1987); therefore, its absence results in structurally unstable collagen (Berg and Prockop, 1973) which is not secreted from cells at a normal rate (Jimenez et al., 1973).

Other constituent amino acids most commonly occupy the X position of the tropocollagen molecule. Alanine, a neutral amino acid, makes up about 9%, while lysine and hydroxylysine, important in the formation of intermolecular linkages (Davison, 1978; Bentley, 1979; Eyre, 1980), constitute 1.3% of the total amino acids in the molecule.

Collagen secretion is the net result of synthesis and intracellular degradation. Collagen formation follows the normal path of protein synthesis with unique post-translational modifications to the primary molecule (Kivirikko and Risteli, 1976; Barbanell et al., 1978; Minor, 1980). These modifications include the removal of the signal peptide, independent hydroxylation (Pinnell et al., 1987) of specific proline and lysine residues (which depends on the presence of ascorbic acid (Levene et al., 1972; Cardinale and Udenfriend, 1974; Ten Cate et al., 1976; Murad et al., 1981)), glycosylation of specific hydroxylated residues, pro-alpha chain association, intra- and inter-chain disulphide bond formation, and the formation of a triple helical structure. Depending on the alterations in the activity of the enzyme systems which mediate these modifications, characteristic

changes in the rate of collagen synthesis may occur (Hall, 1976).

The enzyme protocollagen proline hydroxylase, which mediates the hydroxylation of proline residues, has been shown to fall to one-tenth the value apparent in rat fetal tissue during the first 100 days of life (Heikkinen, 1969). Based on decreasing levels of radioactive proline incorporation, there is a subsequent overall reduction in the rate of synthesis of proto- and tropo-collagen with increasing age. Similar age changes are also evident in human skin (Uitto, 1971) where the activity of this enzyme has been shown to progressively decrease from 1 to 50 years of age; this decrease in enzyme activity is reflected by an 85% decrease in soluble collagen formation.

Procollagen, also referred to as soluble or transport collagen, is secreted and converted to the insoluble tropocollagen molecule in the extracellular space. In this process, the non-helical propeptides at the amino and carboxyl terminals are enzymatically cleaved by procollagen peptidase. It has been suggested (Hall, 1976) that this removal of the procollagen peptide may occur in a series of stages, and it may be that the retention of part of the procollagen peptide in the extracellular environment temporarily prevents the quarter-staggered alignment of tropocollagen molecules which is typical of mature collagen. Although the development of mature collagen is

time-dependent, it is not a true age-related phenomenon since the conversion occurs soon after synthesis and as a required modification for normal function. Nonetheless, this observation has allowed comparative assessments of collagen stability, since the mature fibre retains its characteristic 67nm periodicity (Hay, 1981) unchanged until the death of the organism (Hall, 1976).

The helical portions of the procollagen molecules are bound to the cell membrane (Goldberg and Burgeson, 1982) and aggregate by way of a cell-mediated multistep process (Trelstad, 1982) to form collagen fibrils. Fibril formation is a critical event since most, if not all, of the physiologic roles of fibrillar collagen are achieved by the protein in the fibrous or solid state (Gay and Miller, 1978). For type I collagen, for example, this process proceeds most efficiently following procollagen conversion into tropocollagen. In contrast, the fibrillar networks derived from type III collagen retain some portions of their procollagen sequences (Gay and Miller, 1978) which may render this latter collagen phenotype more susceptible to extracellular degradation (Miller et al., 1976a).