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EFFECTS OF MECHANICAL LOADING ON OSTEOBLAST FUNCTION USING A THREE DIMENSIONAL CELL/POLYMER MODEL

by

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ABSTRACT

Effects of Mechanical Loading on Osteoblast Function
Using a Three-Dimensional Cell/Polymer Model

by

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Mechanisms which trigger bone modeling/remodeling in response to changes in the mechanical environment are still unclear. In a three part study, effects of loading on osteoblast function were investigated using a three dimensional (3-D) cell/polymer model. The 3-D model has advantages of cell culture while maintaining the natural matrix architecture of bone. Such cell/polymer constructs have been shown to form bone *in vitro*.

Osteoblasts in 3-D cell/polymer constructs were cyclically loaded (5%). After five days, compressed constructs decreased in alkaline phosphatase activity, a marker of osteoblast maturation. After three weeks, loaded constructs showed lower alkaline phosphatase activity but higher RNA level of L-type calcium channels, involved in calcium signaling cascades. No difference was detected after twelve weeks. Results suggest osteoblasts sensed loading and altered functional activities in response. Use of the 3-D model to study other osteoblast functions under mechanical loading may increase understanding of regulated functional adaptation by bone.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF COMMON ABBREVIATIONS

1,25	1,25-dihydroxyvitamin D_3
2-D	Two Dimensional
3-D	Three Dimensional
ALPase	Alkaline Phosphatase
CS	Calf Serum
Dex	Dexamethasone
DMEM	Dulbecco's Modified Eagle Medium
ECM	Extracellular Matrix
FBS	Fetal Bovine Serum
GS	Gentamicin Sulfate
H and E	Hematoxylin and Eosin (stain for cells and tissue)
hr	Hour(s)
μE	Microstrain = 0.0001% deformation
min	Minute(s)
MTT	Thiazolyl Blue (stain for viability)
PBS	Phosphate Buffered Saline
PLGA	Poly(lactic-co-glycolicacid)
RT-PCR	Reverse Transcriptase - Polymerase Chain Reaction
s.d.	Standard Deviation
SE	Scanning Electron (microscopy / micrograph)
sec	Second(s)
VSCC	Voltage Sensitive Calcium Channel

CHAPTER 1
INTRODUCTION

> "Despite the fundamental role of the genome, sequential gene expression alone produces only a rough model of a bone; at critical points in development, movement and mechanical forces refine the shape of the bone." (Buckwalter et al., 1995b)

Bone is a dynamic system that responses to the stress and strain applied by external forces. Healthy bone is able to maintain structural integrity and functional support in response to the mechanical load it experiences. However, bone sometimes undesirably compensates for the change in the environment, such as microgravity or long term disuse, or is unable to respond sufficiently, such as when diseased or after trauma. By studying the effects of mechanical loading on bone formation, researchers can contribute to developing preventive therapy or cures to reduce bone loss and promote healing and maintenance of bone tissue.

With advances in space technology, the importance of countering the adverse effects of microgravity on the human body increases. Cessation of bone formation occurs with exposure to microgravity (Morey and Baylink, 1978). In addition, bone calcium density decreases (Smith et al., 1977) while risk of nephrolithiasis (renal stone formation) increases (Whitson et al., 1993). High levels of calcium and phosphate excretion and negative calcium balance all contribute to urinary supersaturation, heightening the potential for medical complications (Lane et al., 1993). Centrifugation experiments suggest that microgravity effects may be governed by the same mechanisms which regulate mechanical load effects (Kumei et al., 1996). By better understanding how bone behaves under load-compression, measures can be taken to prevent the onset of microgravity effects or improve

the slow and often incomplete recovery from bone loss (LeBlanc et al., 1990) in returning astronauts.

Similar decrease in bone formation and increase in bone resorption are observed with long term bed rest. Patients lose bone mass and density so are also at higher risk for renal stone formation (Hwang et al., 1988) and fractures. Like microgravity, long term bed rest decreases the demand on bone and encourages it to adapt to the reduced loading. Investigating how the growth and (re)modeling processes react to such changes in the environment will help define a method to maintain bone homeostasis during periods of extreme lightened load and to prevent excessive bone loss.

Knowledge about the mechanisms governing bone response to mechanical loading also has the potential to improve bone regeneration strategies. Presently, transplantation of bone cells expanded *ex vivo* is under investigation as a tissue engineering approach to regenerate bone (Thomson et al., 1995). The presence of the cells is believed to enhance the healing process through production of essential growth factors, extracellular matrix (ECM) components, and possibly by direct bone formation of surviving bone forming cells (Crane et al., 1995). Mechanical stimuli can be used to modulate *in vitro* bone formation to promote heightened expression of "desirable" genes in the cultured cells. These engineered grafts, active with signaling factors, can then be transplanted back into the patient as an alternative graft substitute to stimulate bone repair.

Studying the effects of mechanical loading on bone formation also promises innovative ways to treat bone loss from osteoporosis, osteopenia, fractures and bony surgery. Although the lack of mechanical load may not be the direct cause of such conditions, understanding the signaling pathway promoting or countering bone formation may prove useful. Mechanical stimulation has already been linked with enhanced fracture healing (Sandberg et al., 1993). Including mechanical loading as part of recovery therapy may improve patient progress and healing.

How mechanical loading alters the bone homeostasis is still not clear. Much has been learned through macroscopic observations. The next phase is to investigate the phenomenon at a microscopic level, to uncover the signaling pathways promoting the macroscopic response of bone to mechanical loading observed.

CHAPTER 2
BACKGROUND

A. Bone Biology

1) Cellular Population

Bone is a highly vascular organ which not only serves as the structural support of the body, but also plays a role in other, more delicate functions such as housing marrow and maintaining the balance of ions in the system. It is regulated and maintained by three main cellular populations. The cellular members of bone include osteoblasts, lining cells and osteocytes, and osteoclasts (Buckwalter et al., 1995a). Each of these cell types performs a different function in bone homeostasis.

Osteoblasts are derived from mesenchymal stem cells found in the bone canals, endosteum, periosteum and marrow. Their main function is to lay down the organic matrix (osteoid) which becomes the foundation of bone. Osteoblasts are round, polyhedral shaped cells usually found in and around sites of mineralization. These highly active cells have an abundance of endoplasmic reticulum, mitochondria, and large Golgi structures. Some osteoblasts also have cytoplasmic processes reaching deep into the bone system. Other possible roles assigned to osteoblasts include production of matrix vesicles regulating mineralization, initiation of modeling and remodeling processes, intercellular communication, and controlling electrolyte fluxes in body fluids. When osteoblasts accomplish their primary objectives, they become lining cells, embedded osteocytes, or migrate on to other sites (Buckwalter et al., 1995a).

Lining cells are less active and more elongated than their osteoblast predecessors. As the name suggests, these cells lie directly against the bone matrix. They respond to hormonal stimulation and are believed to be involved with recruiting osteoclasts for the

resorption process. Lining cells are sometimes referred to as surface osteocytes since they resemble the more differentiated cells but lie at the surface of bone (Marks and Cahill, 1986).

Mature osteocytes, the terminally differentiated osteoblasts which are encased in their self-secreted matrix, are also less active and have fewer organelles with less cytoplasm than osteoblasts. This population, which is in close contact with the deposited mineral, constitutes over 90 percent of all cells in bone. They maintain communication with each other as well as with osteoblasts and lining cells through an extensive network of processes and gap junctions. These traits suggest that osteocytes are involved with the signaling-transduction in bone as well as in modulating the ion balance in the body (Buckwalter et al., 1995a).

The large multinucleated cells responsible for resorption of bone are called osteoclasts. These cells are believed to be related to macrophages and derived from hematopoietic stem cells in the marrow. Active osteoclasts harbor an abundance of mitochondria for heightened energy. During bone resorption these cells first form a sealed attachment to the target surface, then release proton and acid proteases to solublize the mineral as well as digest the organic matrix. Phagocytotic behavior has also been noted in osteoclasts. These cells are stimulated by various hormones and growth factors coupled to the regulated behavior of the other bone cells (Buckwalter et al., 1995a).

The behavior of bone cells, particularly osteoblasts, has been examined by many *in vitro* studies. Cultures of primary cells and subcultured or transformed cell lines from donors of various species, ages, and physical sites have been shown to exhibit markers and perform functions expected of bone cells. For this work, primary rat osteoblasts from enzymatically digested neonatal calvaria (Ishaug et al., 1994) and from flushed adolescent femora bone marrow (Ishaug et al., 1997) were used. The calvaria source has been well characterized and, when supplemented with ascorbic acid and β-glycerol phosphate, exhibit

osteoblast-like markers including response to calcitropic hormone stimulation, high alkaline phosphatase activity, and mineralization (Bellows et al., 1986; Bellows et al., 1990; Puleo et al., 1991). Cultures from marrow extracts have also been previously examined (Maniatopoulos et al., 1988; Owen, 1988; Haynesworth et al., 1992). The harvested population contains osteoprogenitor cells which can be stimulated to express osteoblastic phenotype in culture, often by glucocorticoids such as dexamethasone (dex) (Kasugai et al., 1991). Culture environment is very important for determining the differentiated path of these pluripotent mesenchymal cells. Besides osteoblasts, cultured marrow cells have been shown to differentiate into chondrocytes, adipocytes, and myoblasts (Prockop, 1996).

The sequence of gene and protein expression during bone cell proliferation and function has been documented for bone formation *in vitro*. Figure 2-1 charts the timed expression for some osteoblast markers during bone formation by isolated rat calvaria cells in culture (Stein and Lin, 1993).Note that proliferation of the osteoblastic cells is inversely coupled with expression of markers for osteoblast function such as alkaline phosphatase (ALPase). Although the exact role of ALPase is unknown, it is generally associated with the onset of matrix secretion and maturation of preosteoblasts into osteoblasts. Activity levels in two dimensional (2-D) cultures decrease after further mineralization of matrix (Stein and Lin, 1993). Primary osteoblasts from other species such as fetal bovine cells (Ibaraki et al., 1992) and fetal chicken cells (Gerstenfeld et al., 1987) show similar patterns of ALPase activity, although the exact time course differs depending on the source of the cells and culture conditions. These trends also occur naturally *in vivo* (Aronow et al., 1990).

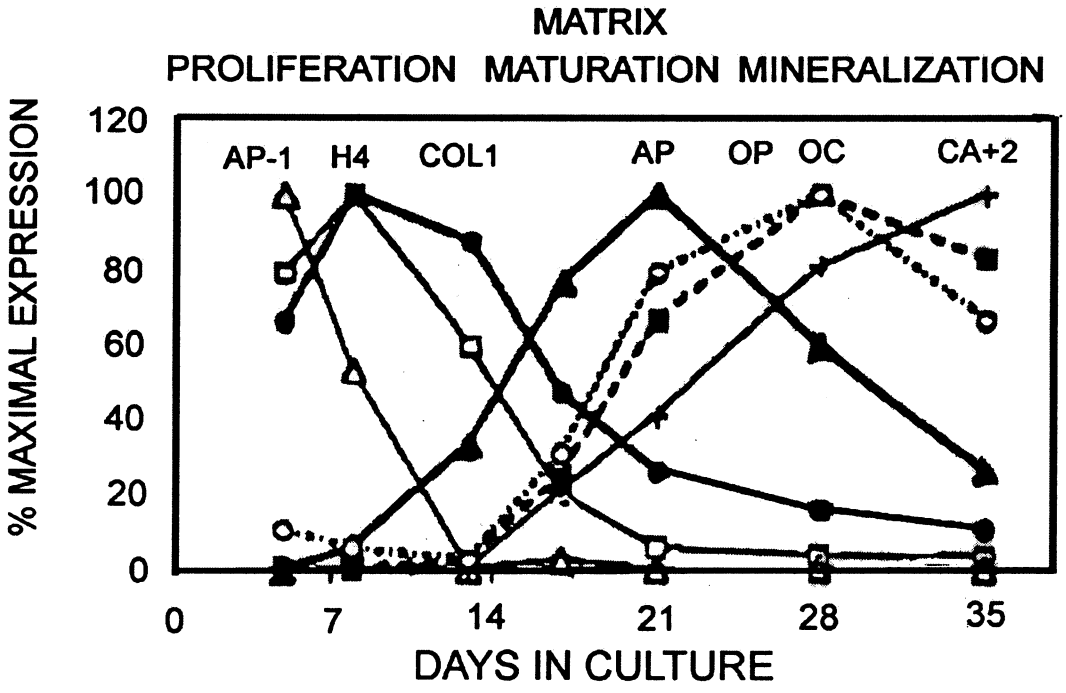


Figure 2-1. Regulated expression of various markers for rat calvaria-derived osteoblasts in culture. H4 (histone-DNA synthesis) and AP1 (c-*fos* and c-*jun*) are indicative of cellular proliferation. Note that AP (alkaline phosphatase) increases with the decrease of cell growth markers and peaks before the expression of mineralization markers such as OP (osteopontin), OC (osteocalcin), and Ca+2 (mineralization). Graph was adapted from Stein and Lian, (1993).

Recent work using reverse transcriptase - polymerase chain reaction (RT-PCR) showed that messenger RNA encoding L-type voltage sensitive calcium channels (VSCCs) exist in ROS 17/2.8 (rat osteosarcoma) (Caffrey and Farach-Carson, 1989) as well as primary calvaria-derived osteoblasts (Meszaros et al., 1996). Plasma membrane VSCCs in osteoblastic cells were down-regulated by 1,25-dihyroxyvitamin D (1,25) and most active during the growth stage of culture (Meszaros et al., 1996). They are believed to play an integral part in regulating cell cycle modulations that involve calcium signaling cascades. Because of their role in transducing external signals into intracellular responses in other cell types from skeletal muscle, cardiac muscle, and brain, it has been suggested that L-type VSCCs have a potential role relating mechanical loading to osteoblast response (Meszaros et al., 1996). Already, mechano-sensitive cation channels have been demonstrated in OHS-4 human osteosarcoma cell lines (Harter et al., 1995) and UMR-106 cell lines (Duncan and Hruska, 1994).

2) Extracellular Components

The matrix of bone is composed of both organic and inorganic elements. Most of the organic structure is collagen type I (90%) with some anchoring collagen type V and XII. Collagen fibrils contribute to cell attachment, migration and function, as well as guide mineralization of bone tissue. Bone matrix also includes various glycoproteins and proteoglycans which assist in cell or growth factor binding. In addition, some bone proteins contribute to the mineralization process. Osteonectin, osteopontin (bone sialoprotein), and thrombospondin bind calcium, while osteocalcin and phosphoproteins bind hydroxyapatite (Sandberg et al., 1993).

A reservoir of growth factors and other active molecules are found within bone. Some known include transforming growth factor-beta (TGF-β), bone morphogenic proteins (BMPs), insulin-like growth factors (IGF-1 and 2), platelet-derived growth factors

(PDGF), and interleukins (IL-1 and 6). All of these contribute to the regulatory behavior of bone (Buckwalter et al., 1995b).

The inorganic phase of bone is primarily in the form of hydroxyapatite crystals. Mineralization builds upon the organic base. Other forms of mineral deposits include whitlockite, octacalcium phosphate, brushite, and various amorphous forms of calcium phosphate (Buckwalter et al., 1995a). The inorganic component of bone houses almost 99% of the body's calcium as well as much of the phosphorous sodium and magnesium. As expected, the delicate balance of ions in the body is heavily dependent on bone homeostasis.

3) Mechanical Properties

Both phases of bone contribute to its mechanical properties; thus, bone is generally treated as a composite material. The tensile characteristics are attributed to the organic phase, especially collagen I, while compressive strength and anisotropy are based on the mineral phase (Hasegawa et al., 1994). Although most studies agree that the Young's moduli of bone in tension and compression are similar, a debate in literature exists over the strength, ultimate strain and work to failure of tension and compression (Rohl et al., 1991). Efforts to correlate measured results with tested specimen architecture have shown that the internal three dimensional (3-D) structure of bone defines its mechanical properties (Goldstein et al., 1993). The internal architecture of bone is, in turn, dependent on the intended function and location of the bone (Lanyon, 1992; Goldstein et al., 1983). Keaveny and Hayes (1993) provide a good summary of the mechanical properties of bone. For general orthopedic purposes, a compression strength of 5 MPa and modulus of 50-100 MPa are used as representative values for human trabecular bone (Van Audekercke and Martens, 1984; Thomson et al., 1995).

The three general categories of bone structures are short, flat, and long bones. Short bones include vertebral bodies, carpals, and tarsals. Cranial vault and vertebra lamina are both flat bones of different sizes. Long bones, sometimes called tubular bones, include the femur, humerus, and finger bones. Most bones protect the organ(s) they encase. Long bones are also designed for load bearing.

B. Bone as a Living Organ

1) Growth and Repair

The sequence of events during bone development and growth has been mapped through the efforts of many investigations. Bone is generally formed by three processes: endchondral, intramembraneous, and appositional development (Buckwalter et al., 1995b).

Embryonic development of long bones is by endchondral ossification. Initially, cells which differentiated into chondrocytes lay down a model of hyaline-like cartilage matrix. Mineralization and replacement by bone tissue occur after development of a periosteal covering coupled with enlargement of the chondrocytes and invasion of blood vessels bringing osteoblastic progenitor cells into the area. As the bone cells differentiate, they lay down more matrix over the calcified cartilage which is then remodeled into mature bone with the aid of osteoclasts.

Development of flat bones generally follows intramembraneous bone formation which does not build on a cartilaginous scaffold. Essentially, an accumulation of mesenchymal cells in multilayers begin paving the ECM path which becomes deposited with minerals. More bone matrix is laid down by differentiated osteoblasts. The matrix then mineralizes and is modeled into an organized tissue.

Appositional bone formation occurs during the growth and widening of most bones. As the name suggests, bone is expanded by the formation of more matrix and

osteoid layers by osteoblasts on the "old" bone, thus increasing its size. Modeling and remodeling also employ the apposition of new matrix at the sites resorbed by the osteoclasts. The layers of new osteoid mineralize to form new organized bone in the area resorbed.

In all cases, *in vivo* bone formation passes through three stages: proliferation of recruited cells, differentiation of functional osteoblasts which lay down matrix that mineralizes, and maintenance of the formed bone. This is also seen with *in vitro* bone formation. In addition, repair of bone often follows similar processes after initial wound healing responses. Briefly, inflammation initiates the recruit of inflammatory cells and undifferentiated mesenchymal cells. Granulated tissue then surrounds the wound while repair begins with a bony callus formation. The callus then serves as the scaffold for either endchondral or intramembraneous bone formation. Minor defects can also be repaired by apposition of new layers onto the old structure (Sandberg et al., 1993).

2) Modeling and Remodeling

Bone undergoes continuous modeling / remodeling activities. These processes normally maintain a balanced ionic concentration in the body as well as repair minor damages from everyday stress (microfractures). In healthy adults, bone is completely replaced approximately one time in ten years without compromising the mechanical properties of the skeletal support system (Buckwalter et al., 1995b). Figure 2-2 shows schematics of modeling process involving formation as well as resorption of bone (Eriebacher et al., 1995).

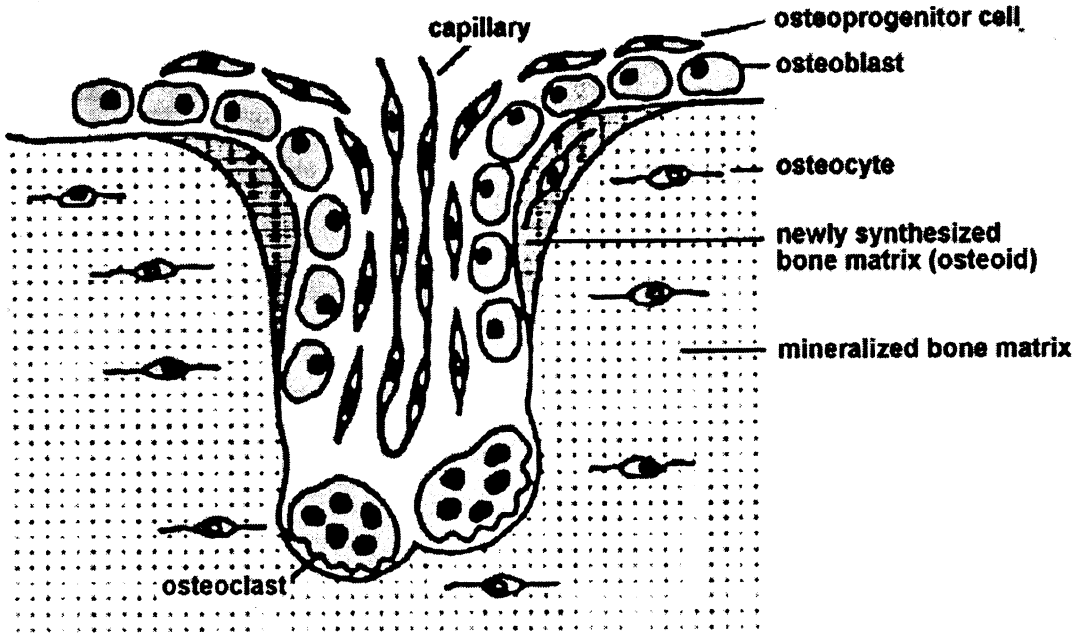


Figure 2-2. Bone modeling and remodeling. Bone is resorbed by osteoclasts, releasing osteocyte-osteoblasts and potential signaling molecules into the resorption lacuna. New capillary endothelial cells follow and advance into the area along with osteoprogenitor cells which lay down new matrix (osteoid). The refilled cavity then mineralizes into new bone tissue. Adapted from Eriebacher et al. (1995).

Bone has been observed to undergo functional adaptation, i.e. bone remodels itself in order to minimize experienced strain. The mechanical structure of skeleton is believed to follow the principal of maximum-minimum design (Fung, 1993). This theorem, first proposed in 1895 by Roux, states that nature created bone for maximum strength with minimum material for light weight and efficiency. In support, long bones have been shown to possess a "trajectory structure" as dictated by the expected strain of mechanical loading (Kummer, 1972; Fung, 1993). Mathematical models also have been created which correlate the stress lines to bone structure (Feldkamp et al., 1989). To maintain optimum design, bone responses to the change in environment. This behavior has been, and is, the subject of many investigations.

3) Effects of Mechanical Loading on Bone: Theories

In the late 1800's Julius Wolff presented his "law of bone transformation" describing the functional adaptation behavior of bone (Roesler, 1981; Bertram and Swartz, 1991). This "law" states that when the external demands of bone change, the organ alters its internal structure to adapt. Since then, much has been done to study the effects of mechanical forces on bone. Macroscopically, a wealth of understanding relating the morphology and physiology of bone to experienced load has been acquired. Nonetheless, specific mechanisms which trigger this phenomenon are not yet well understood.

It has been postulated that *in vivo* bone adaptation follows a feed-back theory with threshold levels determined by other systemic factors (Justus and Luft, 1970; Turner, 1991; Turner, 1992). Deformation of tissue, which triggers a response in bone, is often described in units of mechanical strain (E) as defined by change in length divided by original length, in one dimension. Small strains are sometimes reported as microstrains (μE), defined as 0.0001% deformation (Turner, 1991). Below 50-200 μE bone is expected to slow down formation activity and possibly up-regulate resorption. Microgravity and

long term disuse fall within this "window." For normal physiological range, approximately 200-1500 μE, bone maintains homeostasis. Above such range (>1500-2500 μE), bone generally heightens formation activity (Turner, 1991). A different reaction has been observed for extremely high strains which increased proliferation of osteoblasts but not formation of matrix. This has been correlated with orthodontic tooth movement where resorption is prominent (Burger and Veldhuijzen, 1993).

Many theories of possible signaling pathways linking the observed response in bone to mechanical loading are proposed. The effects of piezoelectric streaming potentials are being studied for their contributions (Cowin et al., 1991). Activation of cells by the fluid shear generated during mechanical movement remains a prominent theory (Reich et al., 1990). Direct signal transduction of shear through stretching of the substrate-cell attachment receptors (Ingber, 1994) is suggested as well as receptor activation by soluble factors that trigger the signaling cascades (Siddhanti and Quarles, 1994). Activation of calcium channels on the plasma membrane is also proposed as the trigger for mechano-transduction since similar vessels have been linked to stimulus response in muscle and brain tissue (Meszaros et al., 1996).

Most publications agree that the role of the gap junctions for intracellular signaling is important. However, controversy over the cellular population which plays the most critical role in signal-transduction in bone still exists. So far, osteoblasts and osteocytes have both been shown to respond to mechanical loading (Klein-Nulend et al., 1994).

Ongoing theoretical and experimental approaches are being taken to resolve these issues. Mathematical modeling, following the traditional approach of Wolff, has offers some valuable analysis of the structure-strain field correlation (Feldkamp et al., 1989) as well as support for broader theories (Weinans et al., 1992; Harrigan and Hamilton, 1994). *In vivo* studies continue to examine the overall behavior of bone within its natural environment (Biewener and Bertram, 1993). With the advances in technology, *in vitro*

investigations are now also possible. These studies aim to understand the phenomenon at a more microscopic level, to fill the gap connecting macroscopic observations and cellular and molecular activities.

In vitro studies can be divided into two subgroups: those using organ cultures and those using cell cultures. Organ culture studies try to maintain the natural environment of the bone while isolating the excised tissue for controlled strain application. Cell culture work, on the other hand, focuses on testing a specific cellular population to elucidate the role of the bone cells of interest. A review by Burger and Veldhuijzen (1993) presents some of the approaches and models used to apply strain on 2-D cell cultures, mostly of osteoblasts. A plethora of other experiments is also found in literature.

C. Basis for a New Approach

1) Three Dimensional (3-D) Cultures

Previous works with bone cell cultures suggest that, independent of cell source, the formation of multi-layer nodules always precedes mineralization. In addition, only in the 3-D nodules is bone-like tissue found. These observations suggest that bone cells need a 3-D configuration in order to mature and function as hard tissue (Bellows et al., 1986). It is suggested that 3-D culture systems would better allow the natural development of bone cells *in vitro* (Eriebacher et al., 1995). In order to produce 3-D structures similar to natural bone, groups are attempting to provide substitute scaffolds on which cells can attach, grow, and lay down matrix. Materials such as denatured collagen type I (Casser-Bette et al., 1990), polyphosphazenes (Laurencin et al., 1993) and various other potential bone graft substitutes (Begley et al., 1993) have successfully supported osteoblast attachment. 3-D osteoblast cultures based on biodegradable poly(α-hydroxy esters) scaffold systems are also possible (Thomson et al., 1995; Ishaug et al., 1997). The polymer scaffold serves as

an analogue for the natural ECM which directs proliferation, migration, and function. Seeded osteoblasts formed guided mineralization along the porous structure of the polymer into bone-like tissue (Ishaug et al., 1997).

2) Poly(α-hydroxy esters) Substrates

Poly(α-hydroxy esters) are linear aliphatic polymers including poly(glycolic acid) (PGA) and poly(DL-lactic acid) (PLA). PGA and PLA as well as their copolymer poly(DL-lactic-co-glycolic acid) (PLGA) are biodegradable. They break down mostly by bulk hydrolysis, although sometimes enzymatic attack can also interrupt the polymer chains (Holland et al., 1986). Degradation products (lactic acid and glycolic acid) can be excreted through urine or processed through metabolic pathways *in vivo* (Hollinger and Battistone, 1986).

Behavior of osteoblasts on poly(α-hydroxy esters) has been previously examined with thin films (Ishaug et al., 1994, 1996). These studies found that primary calvaria-derived osteoblasts readily attached to various formulations of these polymers. Attachment, proliferation, and migration of bone cells on 2-D polymer films were comparable to those on standard tissue culture treated polystyrene (TCPS). Cells on 75:25 PLGA substrates matched the increasing ALPase activity from week one to two as those on TCPS. Collagen synthesis was also similar and the cells formed mineralized nodules in the cultures. Initial seeding density was found to affect migration rate, but not proliferation rate. Also, the cells migrated as individuals in a monolayer and not a calcified tissue front.

Because poly(α-hydroxy esters) can be easily processed into various 3-D shapes as well as engineered to degrade at specified rates (Miller et al., 1977; Tamada and Langer, 1993), they hold potential for use in regeneration of tissue. First, the cells are harvested from a donor, preferably autologous. The cells are then expanded *in vitro*, seeded into the scaffold (and possibly treated to stimulate bone formation activity) then implanted back into

the patient at the defect site. The artificial scaffold is expected to degrade as new matrix is formed. With time, only natural bone tissue will remain in the body, thus avoiding complications typical of long term implants.

Already, work is under way to study implantation parameters of cultured calvaria and stromal cells on 3-D 75:25 foam scaffolds in rat models (Ishaug et al., 1997; Ishaug-Riley, 1997; Ishaug-Riley et al., 1997). Osteoblasts cultured on 3-D polymer foams proliferated and expressed differentiated function as demonstrated by the levels of high ALPase activity and matrix mineralization. *In vitro*, seeding density determined final cellular density in the constructs although it did not affect overall mineralization volume or penetration depth into the scaffold. Pore size was an important factor only in the corresponding *in vivo* study. After 49 days at an ectopic site in the rat mesentery, the mineralized volume and penetration depth of the cell/polymer constructs were greater for the scaffolds of pore diameter in the 150-300 μm range than in the 500-710 μm range. These results suggest that bone formation by osteoblast-transplantation via 75:25 PLGA scaffolds may be feasible for bone regeneration.

3) New Approach

In this thesis, the use of the 3-D osteoblast/PLGA 75:25 system to study effects of mechanical loading on bone formation is proposed. As osteoblast behavior on the polymer is known, mechanical load effects can be carefully deduced. Compared to standard 2-D cultures, the main advantage of the 3-D cell/polymer model lies in its ability to combine the appeal of cell culture with those of organ cultures and *in vivo* studies. As with other cell culture models, cell population and source as well as culture conditions are well-controlled parameters. However, the 3-D model preserves the matrix architecture similar to that of living bone. The 3-D culture allows the mechanical environment and cell-cell communication in bone to be mimicked without the complication of systemic response. The

results generated from cells cultured in the 3-D polymer constructs may provide the link between the results of previous *in vitro* studies using 2-D set ups and the sometimes contradictory results of organ cultures and *in vivo* investigations.

CHAPTER 3

OBJECTIVE

The project described in this thesis was designed to examine the effects of compressive mechanical loading on osteoblast function during the three different stages of bone formation. The research plan was based on the hypothesis that osteoblasts go through corresponding stages of differentiation *in vitro* on the three dimensional (3-D) cell/polymer model as during *in vivo* bone formation. It was also postulated that at all stages of maturation, osteoblastic cells respond to mechanical stimuli. We proposed the following specific aims to investigate the response to cyclic compressive loading on 3-D cell/polymer constructs with:

a) short term (5 day) cultures of highly proliferative cells (early stage of bone formation), with respect to cell proliferation and alkaline phosphatase activity;

b) mid term (3 week) cultures of differentiating-functional cells which lay down matrix (middle stage of bone formation), with respect to proliferation, alkaline phosphatase activity, and expression of voltage-sensitive calcium channels; and

c) long term (12 week) cultures of osteoblasts in a well mineralized matrix (late stage of bone formation), with respect to proliferation and mineralization.

CHAPTER 4

MATERIALS and METHODS

MATERIALS

A. Polymer Scaffolds

The polymer used for the 3-D scaffolds was 75:25 poly(DL-lactic-co-glycolic acid) (PLGA) from Birmingham Polymers (Birmingham, AL). Raw PLGA came in pellet form with weight average molecular weight of 63,400 and a polydispersity index (PI) of 1.8, as determined by gel permeation chromatography (GPC). PI is defined as the ratio of weight average to number average molecular weight. NaCl (Sigma Chemicals ,St.Louis, MO) sieved into diameters of 150-300 μm range was used. Solvents (dichloromethane and chloroform) were from Sigma Chemicals.

B. Cell Culture

Rats were from Sprague Dawley center in Houston, TX. Dulbecco's Modified Eagle Medium (DMEM) and Calf Serum (CS) were from Gibco-Life Technologies (Grand Island, NY). Fetal Bovine Serum (FBS) was from HyClone Laboratories (Logan, UT). All other chemicals and reagents used were of cell culture grade from Sigma Chemicals.

C. Analysis

Isotone II for 2-D cell counts was from Coulter (Miami, FL). RNA expression of calcium channels was performed with the RNA RT-PCR kit from Perkin-Elmer (Branchburg, NJ) plus other reagents of molecular biology grade from Sigma Chemicals 10% neutral buffer formalin and HEMO-De used for histology preparations were from Fisher-Scientific (Pittsburgh, PA). Hoechst 33258 dye for confocal microscopy was from Polysciences (Warrington, PA). All other reagents used were from Sigma Chemicals.

METHODS

A. Polymer Scaffolds

1) Polymer Processing

Polymer scaffold foams were processed using a solvent-casting and particulate leaching technique (Mikos et al., 1994) in combination with heat-compression molding (Thomson et al., 1995). The 75:25 PLGA foams were of approximately 90% porosity as determined by the weight percentage of salt during processing (Thomson et al., 1995). Pore sizes were directly correlated to the size of the salt particles used (Ishaug et al., 1997). Briefly, 0.5 g of polymer was dissolved in 4 mL dichloromethane. The solution was then casted into a Petri dish containing 4.5 g of NaCl (sieved, 150-300 μm in diameter) and dried on a leveler in the hood overnight. The dried composite was packed into a jacketed cylindrical Teflon mold 6 mm or 12 mm in diameter and heated (45 min at 90°C). A press (Model #3912, Carver, Wabash, IN) maintained 400 lbs compression on the heating polymer via an inserted bar. After cooling, the cylinders of polymer-salt composites were removed from the mold and cut into disks of 2 mm thickness with a diamond saw (Isomet 11-1180, Buehler, Evanston, IL). No lubricant was used to prevent introduction of other reagents or premature leaching of the salt by water. The disks were then leached in deionized-destilled water for 24 hr with three changes. Foams were first air then vacuum dried and kept desiccated under vacuum until use.

2) A GPC (Waters-Millipore, Milford, MA) equipped with a differential refractometer (Waters, Series 410) and UV detector (Waters, Model 486) was used. Samples were dissolved in analytical grade chloroform and syringe-filtered (0.45 μm PTFE

filter from Alltech, Deerfield, IL) before injected into the system of a Phenogel guard column (50 x 7.8 mm, 5 mm mixed bed, Phenomenex, Torrane, CA) and a Phenogel 0-10000 kDA column (7.8 x 300 mm, 5 mm mixed bed, Phenomenex) at 1 mL/min. External standards of polystyrene were used to calculate weight average molecular weight of the polymer.

B. Cell Culture

1) Marrow Stromal Cells

Marrow stromal cells were harvested from male Sprague Dawley rats, approximately six weeks old (150 - 170 g), using the method of Ishaug et al. (1997). Briefly, the rats were euthanized with ethyl ether in a glass container, according to an approved animal protocol meeting all federal guidelines for care and use of animals. The femurs were then excised aseptically and the soft tissue removed. The metaphyseal ends were excised and the marrow flushed from the midshaft using a syringe with 5 mL of primary media comprised of DMEM supplemented with 10% FBS and 25 μg/mL gentamicin sulfate (GS). The marrow suspension was then centrifuged at 500 g for 10 min to remove fatty cells in the supernatant. The cell pellet was resuspended in fresh media and plated in T-75 flasks. After 4 days, the cultures were rinsed with magnesium and calcium free phosphate buffered saline (PBS) to remove unattached cells. The cells were then allowed to expand for another 4-6 days in primary media before used in experiments.

2) Calvarial Osteoblasts

Calvarial osteoblasts were harvested from neonatal (less than one day old) Sprague Dawley rats according to procedures from Ishaug et al. (1994). In brief, after euthanization with ethyl ether in a glass container, the calvaria of 10 rats were excised aseptically and the

periosteum gently removed. The pieces were then minced and stirred in an enzymatic collagen/trypsin digestive cocktail for three 20 min periods at 37°C. The cocktail was comprised of 5 mL of the collagen mix : 0.33% collagenase type I in solution with PBS and 2.4% bovine serum albumin; and 1 mL of the trypsin solution (PET): 0.0625% trypsin with 0.0125% ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 0.625% polyvinylpyrrolidone, and 26 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) in 1.125% NaCl. The supernatant of the third digestion was collected and plated into T-75 flasks with primary media and allowed to expand. Cells passaged up to 5 times were used in experiments.

3) Three Dimensional (3-D) Culture

All cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2. Cells for experiments were seeded onto 3-D polymer substrates using a micropipet. Seeding densities were as specified in each experiment. The cells were supplied with complete media comprised of DMEM with 8 μg/mL GS, 10 mM Na β-glycerol phosphate, and 50 μg/mL L-ascorbic acid. Serum content of 10% calf serum (CS) or 10-20% FBS were added as specified. Also when noted, a supplement of 10 or 100 nM dexamethasone (dex) was included in the complete culture media.

C. Mechanical Loading

The Vitrodyne V-1000 (Chatelin, Greensboro, N.C) mechanical testing device was used to apply compressive loading to the 3-D *in vitro* cell/polymer constructs. The device was programmed for maximum displacement of 100 μm from contact with top surface of the 2 mm thick samples. A frequency of 0.3 Hz was used for cyclic loading experiments.

Corresponding load and duration of testing were monitored. The system was placed in a sterile hood and cleaned with 70% EtOH.

Two different types of compression probes were used: a single-probe and a tri-probe. Both consisted of stainless steel extensions connected to Teflon plates which contact the cell/polymer foams. The single probe was used with 6 mm diameter foams in 35 mm TCPS wells. The extension was directly connected to the load cell. The tri-probes were used to test 12 mm diameter specimens in flat bottom glass tissue culture tubes (Fisher Scientific, St. Louis, MO). Probes (96.75 ± 0.9 g, n=2) were set on the samples and an extension connected to the load-cell regulated compression. All components were autoclave-sterilized before use.

The 3-D cell/polymer constructs were kept in 1 mL media throughout the experiments to provide nutrients to the cells continuously. A dry heat bath (Fisher Scientific, Pittsburgh, PA) was used to maintain culture temperature at 37°C during testing.

D. Analysis

1) Cell Counting of 2-D Cultures

Total cell number of 2-D cultures was analyzed using a Coulter counter multisizer (model 0646, Coulter Electronics, Hialeah, FL). Briefly, the cells were released from the culture flasks by exposure to PET for 8-10 min in the incubator. Then, 200 μL of the resuspended cell solution (in media) was added to 10 mL of Isotone II (Coulter, Miami, FL) and 200 μL of 0.25% gluteraldehyde (Sigma). The diluted cell suspension was then counted.

2) Cell Counting of 3-D Cultures

3-D cell counts were determined with the DNA assay described in West et al. (1985) and Ishaug et al. (1997) using Hoechst 33258 flurochrome dye. Briefly, 3-D cultures were frozen (-20°C), thawed, then suspended in 1 mL cold 10 mM ethylenediaminetetraacetic acid (EDTA) (pH 12.3) and minced with surgical scissors. The suspension was sonicated (Sonic Dismembrator Model 300, Fisher) and then incubated in a 37°C bath for 20 min. While cooling on ice, the pH is balanced by 20 μL of 1 M KH_2PO_4 (pH 7.0). 1.5 mL of a 200 ng/mL Hoechst solution with 100 mM NaCl and 10 mM Tris was added to the cell suspension and emission was read at 456 nm on a fluorimeter (Amino-Bowman Series 2 Luminescence Spectrophotometer, Urbana, IL) (excitation at 350 nm). Readings from parallel polymer foams without cells were used to normalize results. DNA and cell standards were then used to convert readings to cell counts.

3) MTT Test for Viability

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (also known as thiazolyl blue) was used to stain for metabolically active cells in 3-D foams. Samples were rinsed in PBS before immersed in complete media without phenol red and 10 vol% of 5 mg/mL MTT stock solution. Samples were incubated for 4 hr then rinsed with PBS before visualized for the position of cells based on converted blue crystals trapped within metabolically active cells. For more quantitative analysis, the samples were immersed in equal amounts of 1:1 isopropanol/DMSO to solubilize the incorporated dye. Absorbance was then recorded at 570 nm with 630-690 nm as background. Parallel foams without cells were used as blanks for the readings.

4) Alkaline Phosphatase (ALPase) Activity

ALPase activity of the cultures was assessed as described in Ishaug et al. (1997). Briefly, 3-D cultures were rinsed in PBS and frozen (-20°C). After thawing, samples were minced with surgical scissors in 1 mL Tris (pH 8.0) and sonicated. 20 μL of the cell suspensions was then incubated at 30°C with 1 mL of the 16 mmole/L p-nitrophenyl phosphate solution from the Alkaline Phosphatase (kinetic) Diagnostic Kit 245 (Sigma). Absorbance was read at 405 nm at one min increments for up to 5 min using a UV/visible spectrophotometer (Hewlett Packard, Germany). Activity was calculated based on the slope of readings plotted against time and reported as units from reaction converting 1 μmole of p-nitrophenol phosphate per min per cell at 30°C (μmole/min/cell).

5) RNA Expression of L-type voltage sensitive Ca^{2+} channels (VSCC)

Total RNA was extracted using acid guanidinium thiocyanate (GTC) as described in Xie and Rothblum (1991). Briefly, the cells were washed in cold PBS before lysing in a diethylpyrocarbonate (DEPC)-treated RNA denaturing solution containing 1) Solution B: 4 M GTC (Fluka, Buchs, CH) and 25 mM Sodium Citrate at pH 7.0 (Fluka) mixed with 2) water saturated phenol, 2 M N-sodium laurylsarcosine (Fluka), and 720 μL β - mercaptoethanol (β-ME) per 100 mL of Solution B. Then, chloroform:isoamyl alcohol (24:1) was added and the emulsion was vortexed to promote phase separation. After 15-30 min on ice, it was centrifuged at 12,000 rpm for 20 min, 4°C. The aqueous portion was then saved and mixed with ice-cold isopropanol to precipitate RNA. The steps were repeated with final product vacuum dried after washing with 70% ethanol and centrifuged again at 12,000 rpm for 10 min, 4°C. The harvest was refrigerated as an ethanol precipitate until use.

RT-PCR was performed to amplify cDNA from harvested RNA. Procedures described were from Dr.Kamil Akanbi of UT-Houston Dental Branch. The RNA PCR Kit

(Perkin-Elmer, Branchburg, NJ) was used for the reverse-transcription and PCR. Thirty-five cycles of 30 sec at 94°C for denaturing, 30 sec at 60°C for primer annealing, and 90 sec at 72°C for polymerization were followed. Products were resolved by electrophoresis through a 1.5% agarose gel in Tris borate buffer. L19 primers (Meszaros and Karin, 1993) served as controls for the 246-base pair VSCC target sequence (Meszaros et al., 1996). Primers were from Dr. M.C. Farach-Carson of UT-Houston Dental Branch (Houston, TX). Densitometry comparison was performed to determine the ratio of control to load expression based on intensity of the bands in the autoradiograph.

6) Histology Preparation

Samples were rinsed in PBS and fixed in 10% neutral buffered formalin to prepare for histological evaluation as in Ishaug et al (1997). Before embedding in paraffin, samples were dehydrated through immersions of increasing ethanol dilutions (70%-100%), 50:50 EtOH/HEMO-De, HEMO-De, paraffin saturated HEMO-De, and three baths of molten paraffin. Tissue blocks were sectioned (5 μm) and stained. Hematoxylin and eosin (H and E) stains were used to visualize cells and tissue formation. Parallel sections were stained with the von Kossa method of 5% silver nitrate, for visualization of calcium phosphate deposits, countered by 0.5 % Safranin-O.

7) Histomorphology

Samples stained by the von Kossa method were analyzed for percent of the vertical cross section area that mineralized using digital imaging. Sample cross sections were scanned with a video camera (JVC, Model TK 107-OU) attached to an acquisition system (Quick Mapture, Data Translation, Marlboro, MA). With the NIH Imaging software (Version 1.54). Total sample cross section area was calculated by the software from pixels enclosed in the image manually outlined along the perimeter. Dark (mineralized) areas were

then quantified based on the gray level intensity of the silver nitrate stain. Dark pixels counted were then ratio-ed to the number from the total pixel number of the cross section previously defined. Ratios were reported as percent area mineralized per total cross section area.

8) Confocal Microscopy

3-D osteoblast cultures were incubated for 1 hr with 5 mg/mL of 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) in complete media. The samples were then rinsed with PBS and supplied with fresh media before viewing under a confocal microscope (Zeiss LSM Axiovert, Carl Zeiss, Germany). Depth projection of 16 sections was used to construct 3-D micrographs of the cultures.

CHAPTER 5

SHORT TERM STUDY

A. Experimental Design

To elucidate the effects of mechanical stimulus on the early stage of bone formation, an experiment to study the behavior of the early osteoblastic cells under load was designed. Mechanical loading has been shown to stimulate modeling and remodeling of bone which recruit osteoprogenitor cells from the proximal stroma to the formation site (Lanyon, 1992; Buckwalter et al., 1995b). The osteoblasts pass through a proliferative stage before differentiating into more mature functional cells (Lian and Stein, 1992). Previous studies with 3-D cell/polymer constructs suggest that cell proliferation occurs predominately during the first week after seeding, especially on foams seeded at a low initial density (6.83×10^5 cells / cm^2) (Ishaug et al., 1997). A five day experiment was, therefore, planned using the 3-D *in vitro* model with primary rat marrow stromal cells which has a rich osteoprogenitor population (Luria et al., 1987; Scutt et al., 1992).

Rat femora marrow cells which attached to the culture flask were expanded then seeded onto the 3-D scaffolds. Each PLGA foam (~90% porosity, 150-300μm pore diameter, 12 mm diameter, 2 mm thickness) was plated at ~5.0×10^5 cells / cm^2 top surface area to approximate the low seeding density used in Ishaug et al. (1997) in order to encourage high proliferation in culture. Cell/polymer constructs were incubated undisturbed in complete media for one day to allow maximum scaffold attachment. They were then transferred from 24-well plates to flat bottom tissue culture tubes for the experiment.

Cell/polymer constructs in the loaded sets were cyclically compressed at 0.3 Hz with the three-prong probes for 30 min/day during the four days after seeding. Control cultures were not subjected to load, but also were placed in the dry heat bath in the sterile

hood for the same duration. This precaution was to alleviate potential differences caused by the change in gas concentration during testing (air vs. 5% CO_2). The experiment was conducted twice, first in complete media with 10% FBS and second with 20% FBS. This was done because higher serum content has been shown to increase mitogenic activity and may increase detection of mechanical loading effects by increasing proliferation.

10 nM of dex was supplemented in the media of the Ishaug et al. study referenced (1997). Dex is a glucocorticoid often added in bone cell culture media to promote the expression of osteoblastic phenotype by the attached cells. Because the presence of dex may overshadow effects due to mechanical loading, its influence was also evaluated. One group (Group A) was supplemented with 10 nM dex in the complete media starting 6 hr after seeding and throughout the experiment. The other group (Group B) was cultured all five days without any exposure to the glucocorticoid. Both groups contained control and load sets for direct comparison of mechanical effects. Table 5-1 details the conditions experienced by the different sample groups. Assays were performed on day 5 to determine the effects of loading and dex treatments on proliferation and ALPase activity. Statistics was calculated by 2-factor ANOVA at the 95% and 99% confidence levels with a post-hoc Scheffe's F test for multiple comparison.

Table 5-1. Experimental Groups for Short Term Study

Sample Group	10 nM Dexamethasone Supplement	0.3 Hz Cyclic Mechanical Loading
Group A		
dex control	yes	no
dex load	yes	yes
Group B		
no dex control	no	no
no dex load	no	yes

B. Results

We tested for immediate cell viability after mechanical compression with a MTT staining test. Figure 5-1 shows a sample foam loaded one day after seeding. The foam was cyclically compressed for 30 min (0.3 Hz) with the tri-probes right before incubation with MTT reagent. Area shown with blue dye had attached enzymatically active cells with converted thiazolyl blue crystals trapped within the cytoplasm. This short experiment verified that immediately after mechanical loading, viable cells remained in the 3-D scaffolds.

Proliferation was different between the 10% and 20% FBS experiments. Figure 5-2 is of the DNA assay for total cell counts after five days of culture using 10% FBS media. No significant difference was noted in proliferation within Group A or Group B among the non-loaded controls and the mechanically loaded samples. Statistical analysis did distinguish an effect by dex between Group A and B ($p<0.01$). The glucocorticoid treatment increased the total cell numbers detected.

Figure 5-3 is the graph for total cell counts using the 20% FBS media for five days. Raw cell numbers were higher in this set which was seeded with the same initial density of cells as in the experiment using 10% FBS media. A more dramatic difference of the same trend in the 10% FBS experiment was seen. Again, mechanical loading caused no change in proliferation, but dex treatment significantly increased proliferation ($p<0.01$).

ALPase activity from the 20% experiment is shown in Figure 5-4. Statistical analysis detected a minor effect of loading ($p<0.05$) as well as a more pronounced effect of dex ($p<0.01$). ALPase activity in cell/polymer cultures were slightly lower from loading and much lower in the groups treated with dex, inversely coupled with results from cell counts. Again, a similar trend was seen in the experiment using 10% FBS, but those

changes were not statistically different (not shown). No significant interaction was detected between mechanical stimulation and dex treatment for any experiment or assay.

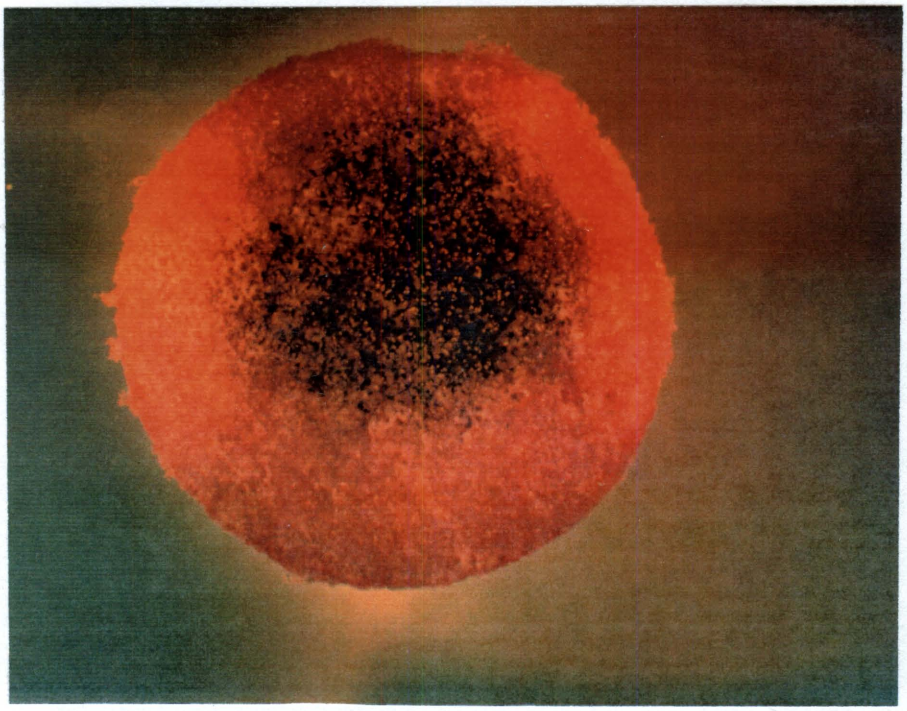


Figure 5-1. MTT staining of viable cells in cell/polymer constructs immediately following 30 min compression with the tri-probes. Metabolically active cells can be visualized from the blue stain of converted thiazolyl crystals within the cell's cytoplasm. (Construct diameter 12 mm)

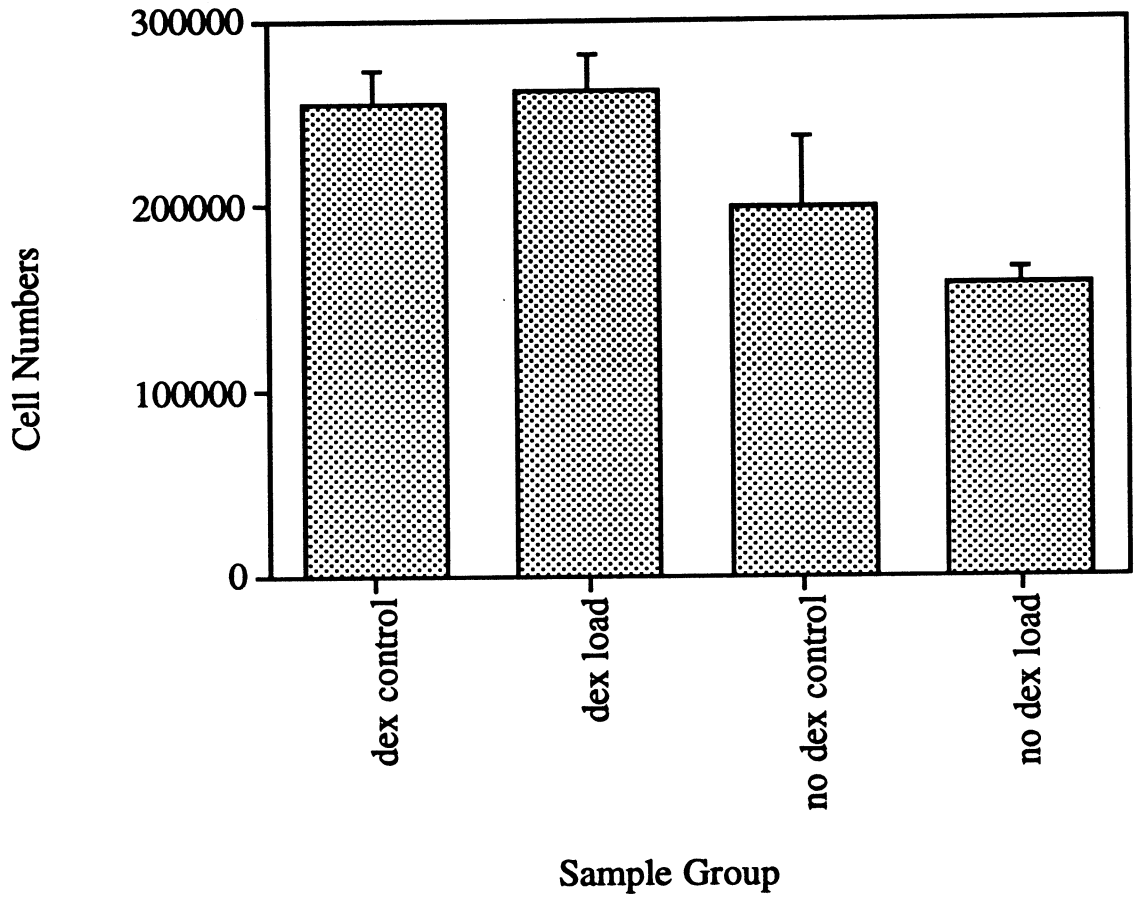


Figure 5-2. Cell numbers after 5 days of culture in complete media with 10% FBS, as determined with DNA assay. In these experiments, "control" groups were not mechanically loaded; "load" groups were exposed to four days of cyclic loading. No difference based on mechanical loading was detected. Group A was exposed to dexamethasone and are labeled "dex"; Group B was not exposed to the hormone and are labeled "no dex." Cell numbers from Group A (dex) were higher ($p<0.01$) than those of Group B (no dex). Data are means ± s.d. (n=3). Statistical significance was determined by a 2-factor ANOVA with a post-hoc Scheffe's F comparison.

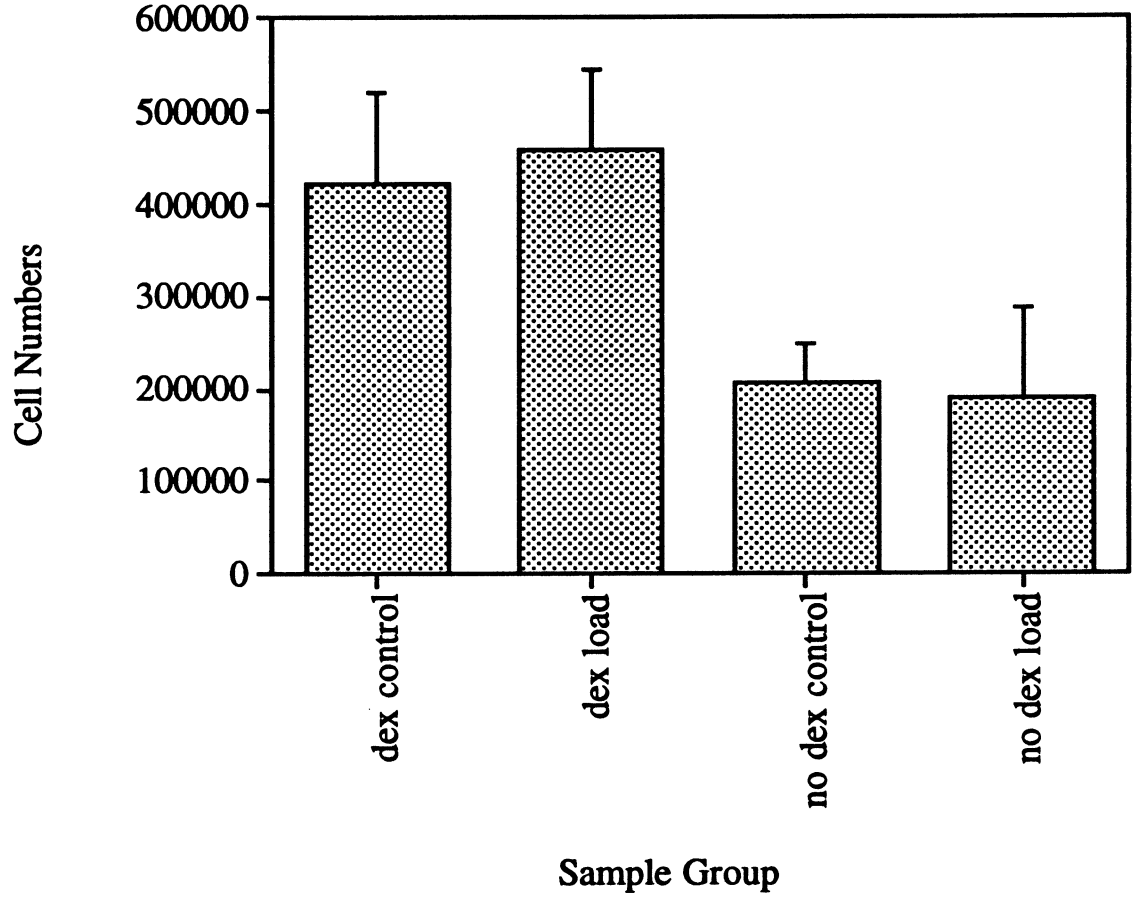


Figure 5-3. Cell numbers after 5 days of culture in complete media with 20% FBS, as determined with DNA assay. In these experiments, "control" groups were not mechanically loaded; "load" groups were exposed to four days of cyclic loading. No difference based on mechanical loading was detected. Group A was exposed to dexamethasone and are labeled "dex"; Group B was not exposed to the hormone and are labeled "no dex." Cell numbers from Group A (dex) were higher ($p<0.01$) than those of Group B (no dex). Data are means ± s.d. (n=4). Statistical significance was determined by a 2-factor ANOVA with a post-hoc Scheffe's F comparison.

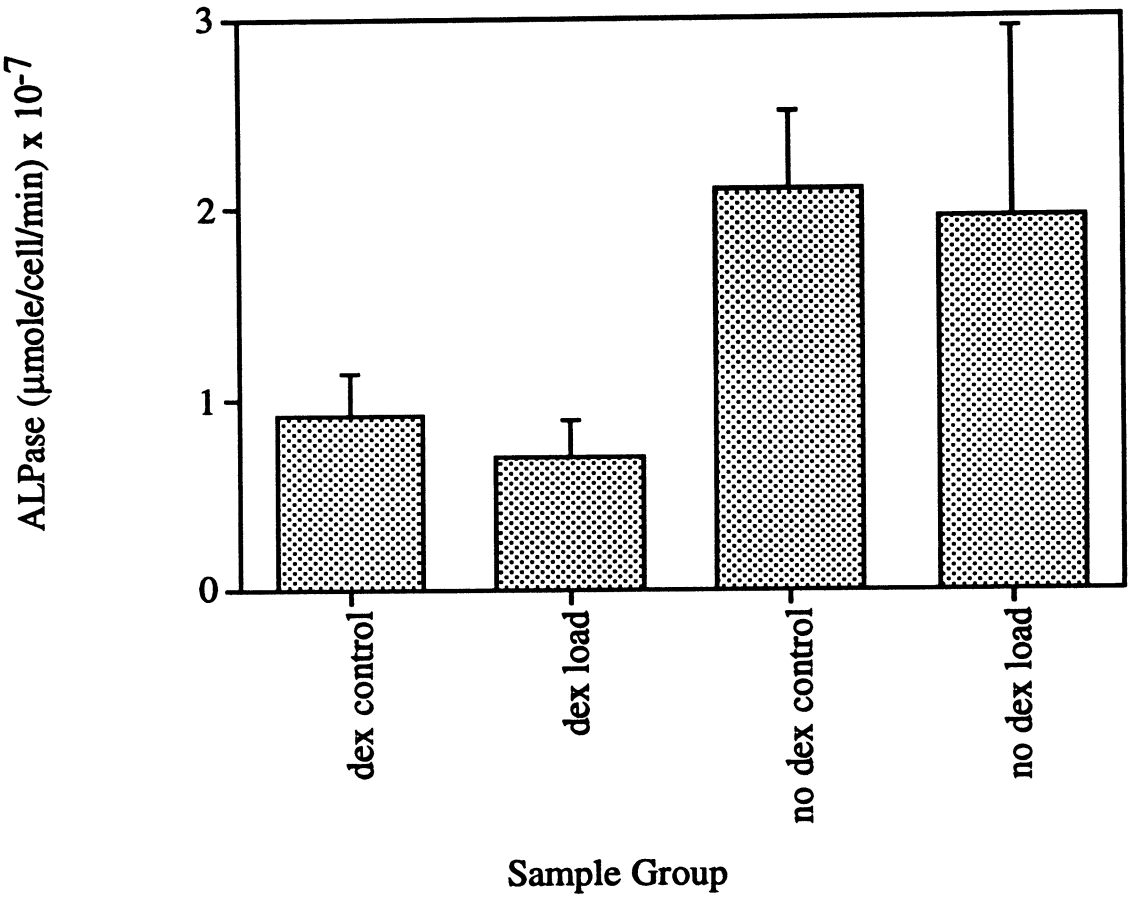


Figure 5-4. Alkaline phosphatase activity (ALPase) of 5 day cultures grown in 20% FBS. In these experiments, "control" groups were not mechanically loaded; "load" groups were exposed to four days of cyclic loading. A slight difference was observed due to mechanical loading ($p<0.05$). Group A was exposed to dexamethasone and are labeled "dex"; Group B was not exposed to the hormone and are labeled "no dex." A significant effect from dex ($p<0.01$) was detected. Statistical analysis was done with a 2-factor ANOVA and a post-hoc Scheffe's F test for multiple comparison. Data are reported as mean ± s.d. (n=4).

C. Discussion

Mechanical loading did not produce a significant change in proliferation of the marrow cells in early 3-D cultures. This suggested that the imposed strain was not geared to increase cell growth of osteoblast-stromal cells. In contrast, analysis of the ALPase activity for the short term study revealed an influence of mechanical loading, with the effect enhanced by increasing the FBS concentration. The change in ALPase activity indicated a response in the functional behavior of the cells after mechanical stimulation. It was postulated that the differentiation of the osteoblasts may have been altered by the external environment.

3-D culture systems showed increased cell growth due to higher FBS concentration and the addition of dex in the culture media. The increase from more FBS was expected as higher serum concentration has been known to enhance proliferative activity of cells [Life Technology communication]. The other observation was also consistent with results of some *in vitro* rat calvaria investigations which reported an initial increase in proliferation after treatment with dex (Bellows et al., 1986; Bellows et al., 1990). Since phenotypic expression is generally inversely related to proliferative activity (Owen et al., 1990), the coupling of higher cell numbers with lower ALPase activity in dex treated samples is not unusual. Controversy exists in literature over when (and how) dex is expected to modulate proliferation and when it affects only the differentiated expression of osteoblasts (Ibaraki et al., 1992). Results from the cell counts and ALPase activity showed that at day 5, exposure to dex enhanced proliferation but reduce ALPase activity of seeded stromal-osteoblasts in the 3-D constructs.

The high levels of ALPase activity for all samples after five days (~1-2 x 10^{-7} μmole/min/cell) suggested some expression of osteoblastic phenotype. Results were similar to those found in a previous study of rat marrow derived osteoblasts cultured on 75:25 PLGA scaffolds of ~150-300 μm pore size (as in this study). After 7 days in 10 nM dex,

cells from the Ishaug et al. (1997) study expressed ~1-3 x 10^{-7} μmole/cell/min, as read off a graph. When maintained for longer periods, those cultures formed mineralized bone-like tissue *in vitro*. Long term cultures of untreated (no dex) rat marrow cells on 3-D scaffolds have not been examined. However, it is suspected that such cells will eventually de-differentiate into fibroblast-like cells with much lower basal activity for ALPase. As seen with untreated human marrow cultures, these rat marrow cultures are not expected to form bone-like structures *in vitro* (Ashton et al., 1985).

The lower level of ALPase activity in marrow cells treated with dex matched the findings of some previous investigations (Shalhoub et al., 1992) but not others (Bellows et al., 1986). The effects of glucocorticoids on ALPase activity on osteoblastic cells, however, have been shown to depend on many experimental factors. For example, ALPase activity was affected by dex concentration (Canalis, 1983). It was also found that the effect was dependent on exposure duration. While the glucocorticoids increased ALPase activity for short exposure duration (≤ 24 hr), treatments of three days or more promoted the contrary (Bellows et al., 1990). The cells of Group A were constantly immersed in 10 nM dex supplemented media for 5 days; therefore, it is suspected that the discrepancy between the ALPase results from this study and those of some previous studies is related to the difference in exposure duration and concentration. Nonetheless, other factors may also be involved.

No interaction between the loading and dex treatments was detected, but the increase in FBS concentration from 10% to 20% enhanced detection of the effect on ALPase activity by mechanical stimulation. This observation suggested that mechanical loading may be involved with regulatory mechanisms of cell cycle modulations. More work, however, is needed to elucidate the exact nature of the correlation.

In vivo, the role of mechanical loading usually comes in later stages of bone formation (Buckwalter et al., 1995b). At the early phase of development, genetic

expression and hormonal regulation guide the activity of the cells. Mechanical loading then augments bone modeling and remodeling to determine the final mass, density and architecture of the organ (Lanyon, 1992). Although this phenomenon may be related to the delayed introduction of loading during natural circumstances, results from our model suggest that even with the introduction of mechanical loading at an early stage, hormonal (dex) effects dominate the regulatory activities of the osteoblasts. Some signs of loading-induced changes in ALPase activity of the cells were observed, but more significantly, the presence of dex affected proliferation and ALPase activity.

CHAPTER 6

MID TERM STUDY

A. Experimental Design

From previous studies, it is known that matrix secretion and maturation began in 3-D cultures during the second week after seeding, coupled with the slowing of proliferation rate (Ishaug et al., 1997; Ishaug-Riley, 1997; Appendix A). After two weeks of culture, evidence of ECM laid down by the cells as well as signs of mineralization (Figure A-6) can be seen. Based on these observations, experiments were designed to begin mechanical loading during the second week of culture to elucidate the effects of the stimulus on functional osteoblasts secreting ECM, simulating the second phase of bone formation.

Neonatal rat calvaria cells were chosen because they have been shown to develop into tissue-like organization similar to that of embryonic bone (Bellows et al., 1986; Bharagava et al., 1988) and have often been used to study proliferation/differentiation relations in bone cells (Stein and Lin, 1993). All cells were cultured in the presence of 10 nM dex in complete media, previously determined to stimulate maximal bone nodule formation in 2-D calvaria cultures (Bellows et al., 1990). Foams (~90% porosity, 150-300 μm pore diameter, 12 mm diameter, 2 mm thickness) were seeded at approximately 7.4 x 10^5 cells / cm^2 of top surface area. The seeding density was increased slightly from those used in the short term study to increase RNA levels for analysis.

Conditions for the first week were maintained for control and load sets to allow the osteoblasts to attach and proliferate. The cultures were transferred to the flat bottom tissue culture tubes one day prior to the start of loading. Testing began on day 7 when the onset of mineralization was expected. The load group was cyclically compressed at 0.3 Hz for 30 min/day during the next two weeks of the experiment. At the end of week 3, total cell

numbers and ALPase activity were determined. Statistical analysis was done with unpaired Student's-t tests. In addition, we also assessed the RNA expression of VSCCs which were previously observed to change with differentiation in primary rat calvaria and osteosarcoma cells (Meszaros and Karin, 1995). Comparison between load and controls were based on densitometry analysis.

B. Results

Mid term (3 week) experiments produced no pronounced difference in total cell numbers due to loading (Figure 6-1). ALPase activity, however, was lower ($p<0.05$) in the load compared to the control (based on an unpaired Student's t-test). Figure 6-2 shows the results graphically. RNA expression of VSCCs, on the other hand, was 5.7 times higher in the loaded samples compared to controls based on densitometry comparison (n=3). Figure 6-3 shows the agarose gel of the RT-PCR amplimers.

C. Discussion

No significant difference in proliferation was detected from the total cell numbers of the control compared to the load group. Because loading during the short term study also produced no increase in proliferation (Chapter 5), the lack of difference in cell numbers was not contributed to the delayed introduction of mechanical stimulus after the initial burst of proliferation expected during week one. We, therefore, conclude that mechanical loading of the 3-D cultures did not significantly heighten the proliferative activity of the cells during the second stage of *in vitro* bone formation.

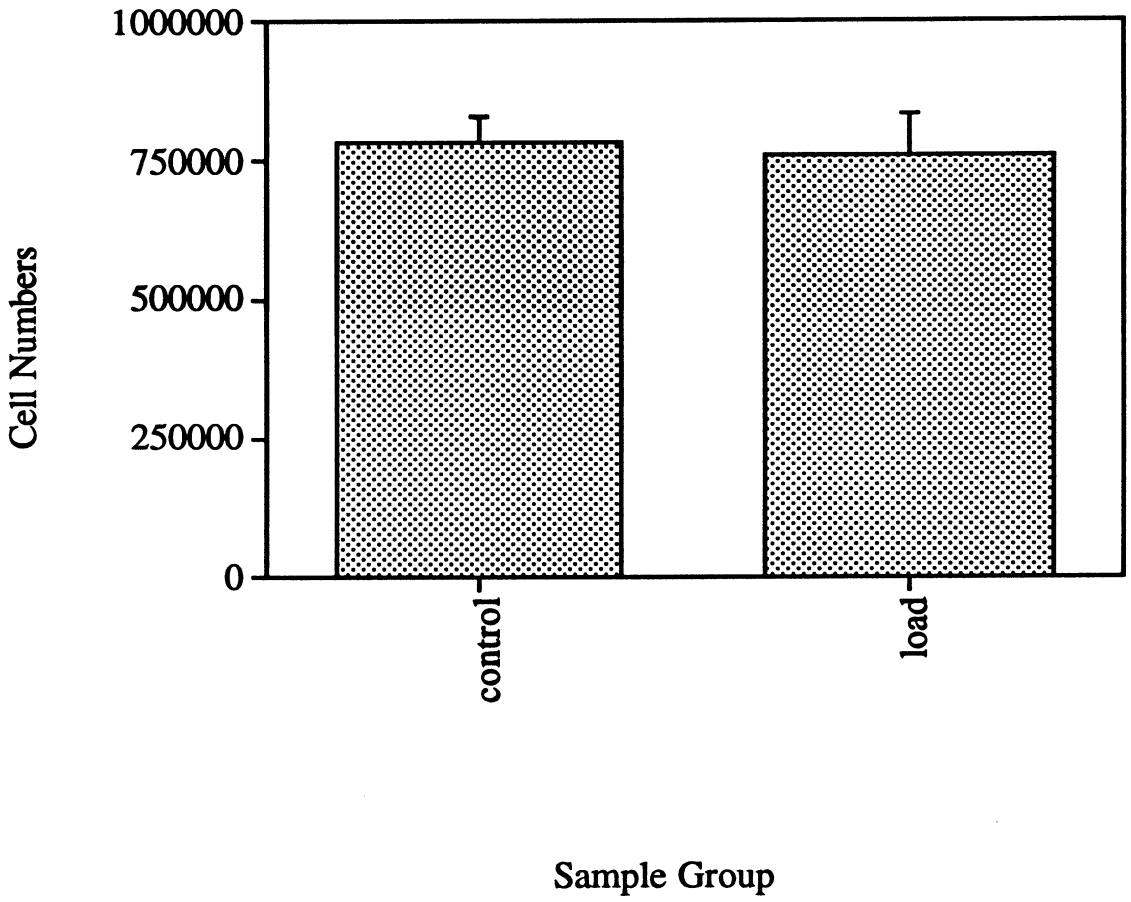


Figure 6-1. Cell numbers after 3 weeks. In these experiments, "control" constructs were not mechanically loaded; "load" constructs were cyclically compressed (0.3 Hz) for 30 min/day over 2 weeks (days 7-21). Data are reported as mean ± s.d. (n=3). No difference between the control and load groups was detected with an unpaired Student's T test.

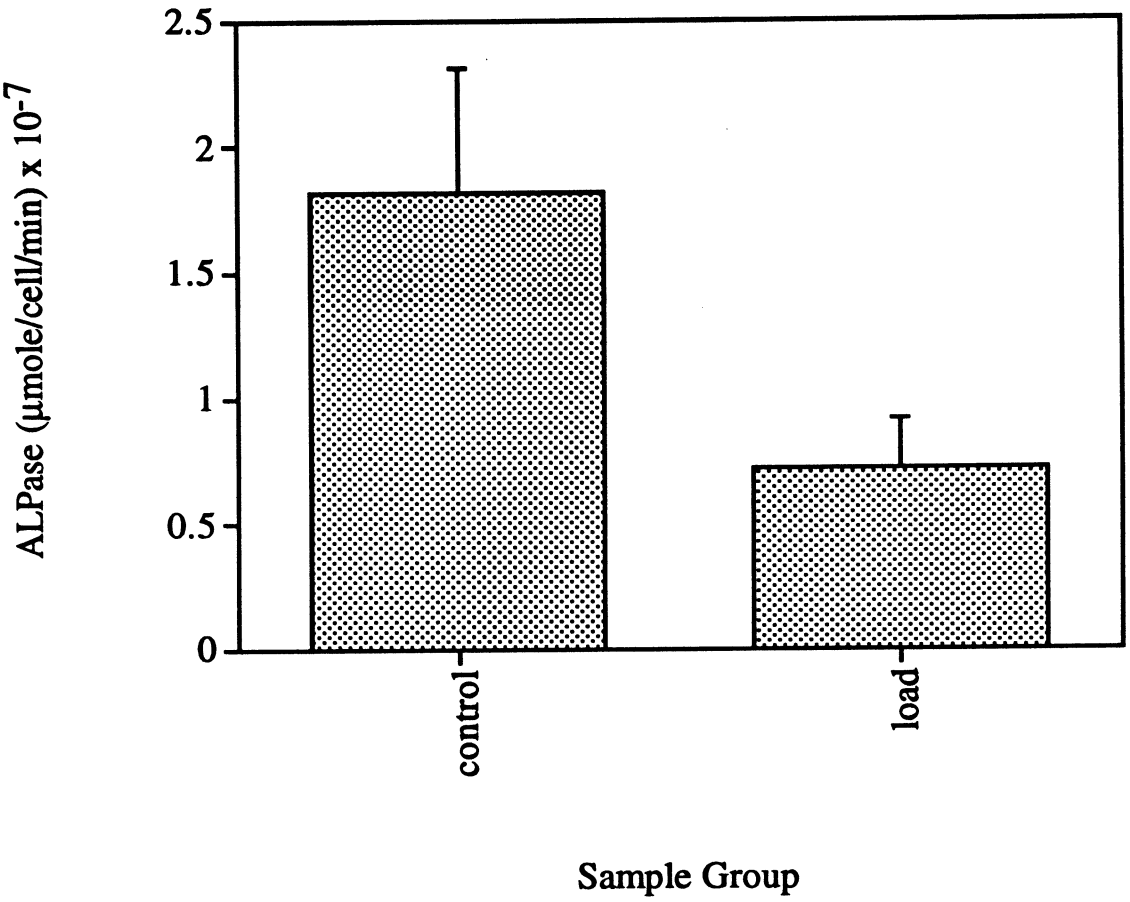


Figure 6-2. Alkaline phosphatase (ALPase) activity of osteoblasts in 3-D constructs after 3 weeks. In these experiments, "control" constructs were not mechanically loaded; "load" constructs were cyclically compressed (0.3 Hz) for 30 min/day over 2 weeks (days 7-21). Test samples exhibited significantly lower ALPase activity per cell ($p<0.05$) after 2 weeks of mechanical loading at 0.3 Hz. Statistical difference was determined with an unpaired Student's t-test. Data are reported as mean ± s.d. (n=3).

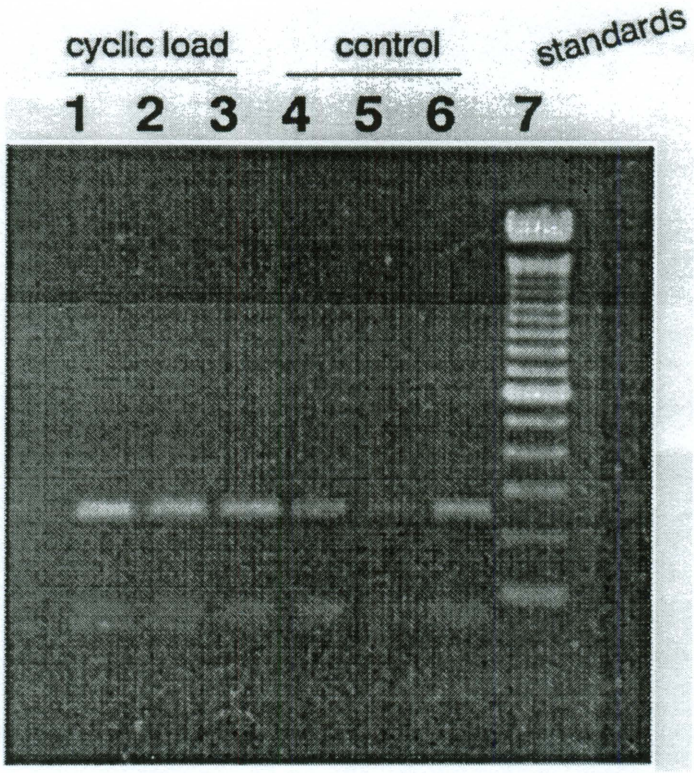


Figure 6-3. Autoradiograph of the agarose gel resolving the 246-base pair PCR amplimer for the a1 subunit of the L-type VSCC.) For quantitative analysis, relative intensity of the bands was evaluated densitometrically from autoradiographs as described in Meszaros et al, 1996. RT-PCR reactions were performed from total RNA preparations using each sample. RNA concentrations were determined spectrophotometrically (n=3).

Results from the ALPase activity suggested that loading produced changes in functional behavior of the cultured osteoblasts in the mineralizing culture. The lower level of ALPase activity of the load group was at first unexpected. ALPase is a known marker often used to detect bone growth (Leung et al., 1993) and mechanical movement, according to most theories, generally stimulates not reduces the formation of bone tissue. Recent theory of threshold limits for effects of mechanical loading differentiates, however, between response to extremely high and moderately high loading *in vivo* as well as *in vitro* (Burger and Veldhuijzen, 1993). Information from literature suggests that while moderate cyclic loading promotes bone formation marker expression such as ALPase, extreme mechanical strain (e.g., $\geq$ 22% uniaxial 2-D cyclic stretching) prolonged cell proliferation with corresponding lower ALPase levels (Imamura et al., 1990; Ozawa et al., 1990). It seems that cyclic compression of the 3-D cultures for 0.3 Hz with 100 μm maximum displacement produced a similar response.

ALPase activities of the 3 week control and load cultures both were within the order of magnitude expected for osteoblast-like cells (1×10^{-7} μmole/cell/min) (Ashton et al., 1985; Ishaug et al., 1994). The change in ALPase activity detected in loaded constructs may represent a change in culture maturation rate. Levels of ALPase activity in 2-D calvaria cultures increased with the change from proliferative phase to differentiated phase with the secretion of matrix proteins, then decreased as the culture further matures into the mineralization phase after week 3 (Stein and Lin, 1993). However, some previous studies using the 3-D PLGA system showed increasing ALPase activity even after 28 days (Ishaug et al., 1997). The study of Ishaug et al. (1997) with marrow cells in 10 nM dex expressed almost ten times higher ALPase activity after three weeks (~20×10^{-7} μmole/cell/min). In other previous calvaria studies by both Ishaug-Riley (1997) and as in Appendix A (100 nM dex complete media), ALPase levels were comparable to this study up to day 14 (~2×10^{-7}

μmole/cell/min). Thus, it is suspected that differences in cell source and other experimental methods contributed to the change in osteoblast response observed.

The expression of the L-type VSCCs involved in regulating calcium signaling activities and previously found in calvaria-derived primary osteoblasts of 2-D cultures as well as a osteosarcoma cell line was also evaluated (Meszaros et al., 1996). Osteoblasts in the 3-D cell/polymer cultures exposed to mechanical loading expressed 5.7 times higher L-type VSCC RNA than control cells as determined by densitometry comparison. The up-regulation of VSCC expression suggested that the cells were altering their responsiveness to external stimuli involved in calcium signaling, supporting the conclusion that the seeded calvaria osteoblasts in the loaded 3-D cultures were responding to mechanical stimulation.

The opposite responses in VSCC expression and ALPase activity to mechanical loading contrast that previously shown for 1,25-dihydroxyvitamin D_3 (1,25), a hormonal regulatory of bone. 1,25 down-regulated VSCC transcript levels (Meszaros et al., 1996) and up-regulated ALPase genes (Beresford et al., 1986; Kyeyune-Nyombi et al., 1989). A study relating 1,25 and mechanical loading may provide insight into the correlation of the two bone-regulatory factors.

A permanent deformation of the 3-D scaffolds subjected to loading was observed during this study. The 75:25 PLGA foams did not recover from repeated compression for two weeks at 30 min/day. Up to 40% permanent reduction in scaffold thickness (originally 2 mm) was seen, based on measurements of random 3 week samples (n=3). Foams examined were more stiff and compact than unloaded counterparts. Although no difference was detected with the DNA assay for total cell numbers, these potential problems prompted the reduction of loading in the long term study to 30 min/every 2 days with the single probes instead of the tri-probes.

CHAPTER 7

LONG TERM STUDY

A. Experimental Design

To examine the effects of mechanical loading on well mineralized cultures, experiments of twelve weeks with the 3-D cell/polymer model were designed. Up to eight weeks, mineralization was still increasing in 3-D cell/polymer constructs (Ishaug et al., 1997); therefore, a longer time point was selected to better study the response of a fully matured osteoblast culture. Scaffolds of 75:25 PLGA (~90% porosity, 150-300 μm pore diameter, 6 mm diameter, 2 mm thickness) were seeded with calvaria-derived osteoblasts. The experiment was conducted twice: Group A at 9.7×10^5 cells / cm^2 top surface area and Group B at 5.3×10^5 cells / cm^2 top surface area. The seeding densities were approximately those used in the mid-term and short-term experiments, respectively. Cells were supplied with complete media of 10% FBS and 10 nM dex as in the mid-term study and cultured under standard conditions for ten weeks before testing began. Previous studies using the 3-D constructs found no significant difference in mineralization volume / surface area based on seeding density under static conditions up to day 56; however, it was suggested that mechanical loading may alter the internal environment to reveal a late stage dependency on number of cells seeded (Ishaug-Riley, 1997).

During weeks 10-12, one set (load) was subjected to cyclic compression of 0.3 Hz for 30 min/ every 2 days using the single probe system. The loading regiment was reduced from that used in the three week study so as not to crush the foams. The controls were not exposed to mechanical loading, but were placed in the hood on the heating block for the same duration as tested samples to reduce difference introduced by the change in gas concentration.

Total cell numbers were determined for all the sample groups using the DNA assay. Because of the extremely long period of culture, some samples of Group A were lost to contamination and only duplicates were available for analysis. Group B was analyzed in triplicates. Imaging analysis of vertical histology sections stained with the von Kossa method was used to determine the percent of mineralized area to the total cross section area. Total cross section area was determined by manually outlining the image of the histology sample. Mineralized area was digitally selected based on the darker gray level of the silver nitrate stain compared to those of polymer, cells, and non-mineralized ECM. Values were reported as the ratio of mineralized area to total cross section area, in percentage. Four stained sections from two different samples for control and load (of each seeding density group) were taken to determine the mean value. All eight readings were included in statistical analysis of the effects of loading and initial seeding density.

B. Results

After 12 weeks of culture (2 weeks of loading), the total cell counts in the foams of the loaded samples were similar to the respective controls in both Group A and B (Figure 7-1). No statistical significance up to 95% confidence was found based on mechanical loading or seeding. Nonetheless, average values suggested that after mechanical loading Group A was higher than Group B. Figures 7-2a and b are sample histology images of loaded samples from Group A and B. Histomorphological analysis detected an interaction between seeding and loading on the percent area mineralized, $p<0.05$ (Figure 7-3). A 2-factor ANOVA and post-hoc Scheffe's F-test found the difference based on initial seeding density ($p<0.01$), but not mechanical loading. Mean $\pm$ range reported are based on four sections from each sample and two samples for each set (Group A control and load, Group B control and load).

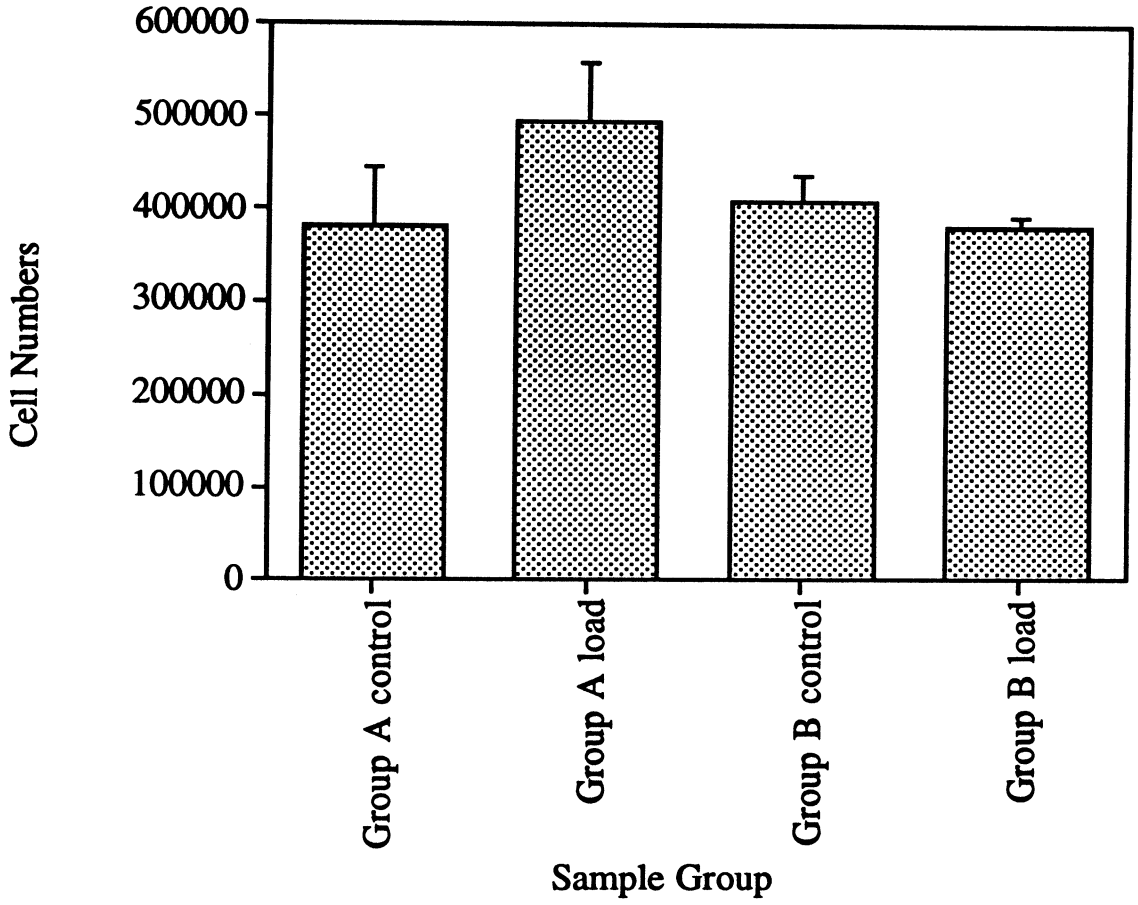
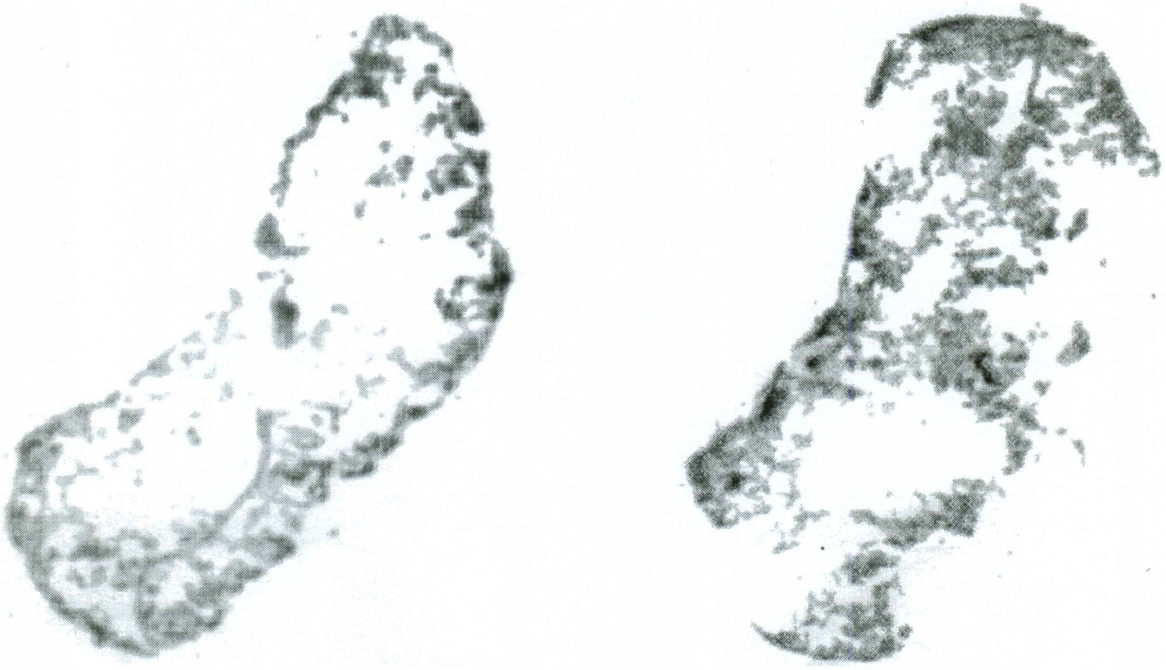


Figure 7-1. Total cell numbers of cell/polymer constructs after 12 weeks. In these experiments, "control" constructs were not mechanically loaded; "load" constructs were cyclically compressed (0.3 Hz) for 30 min/every 2 days during weeks 10-12. "Group A" was of higher initial seeding density. Data reported as mean ± range (n=2). "Group B" was of lower initial seeding density and reported as mean ± s.d. (n=3). No significant difference was detected among the groups based on ANOVA to 95% confidence level; however, raw numbers were higher for "Group A load" than for "Group B load".



(a) low seeding density

(b) high seeding density

Figure 7-2. Representative images of the vertical histology sections taken from cyclically loaded 12 week cultures stained with the von Kossa method to visualize mineralization. Dark areas have calcium phosphatase deposits: (a) low seeding density (Groups A) and (b) high seeding density (Group B). Note that the outer perimeters showed the darkest staining while interior of the scaffolds (center of image) had fewer mineral clusters.

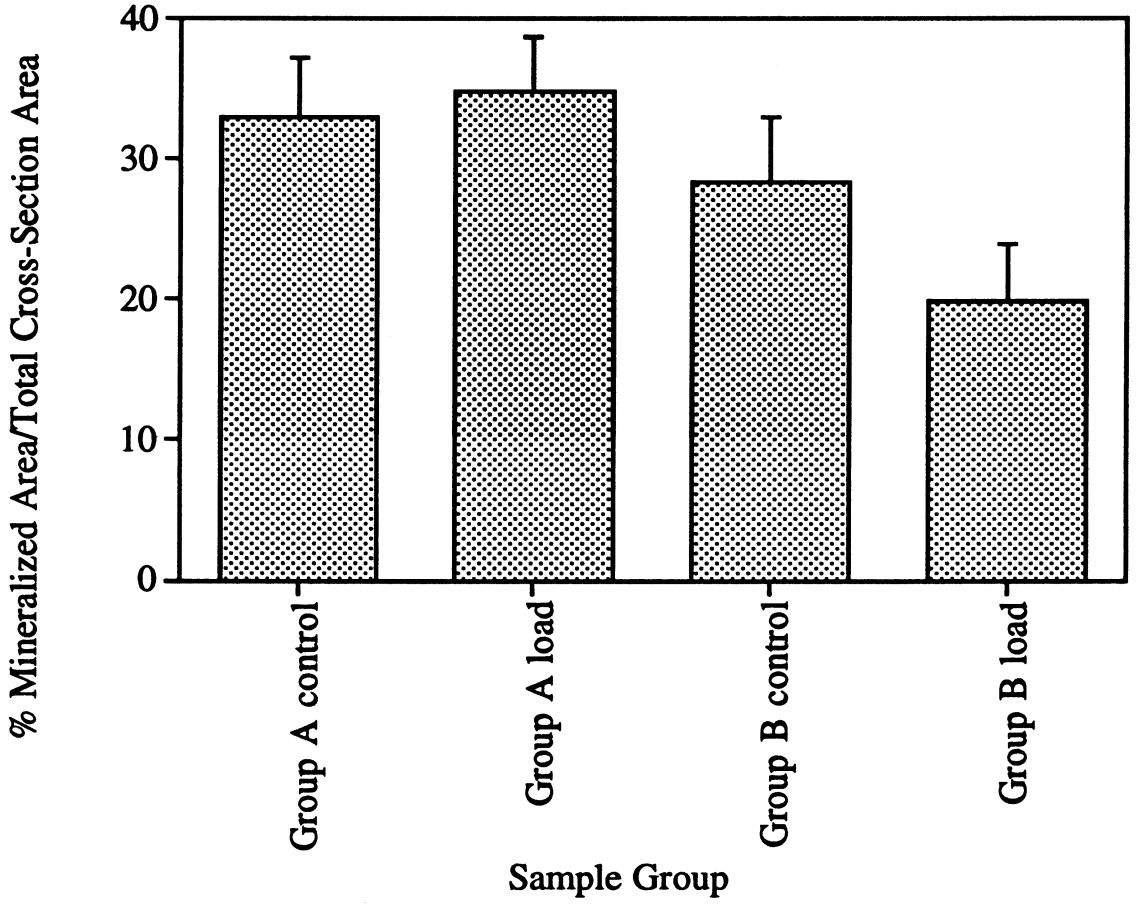


Figure 7-3. Percent mineralized area over total sample area of vertical histology cross sections stained by the von Kossa method for calcium phosphate deposits. The "control" constructs were not mechanically loaded; "load" constructs were cyclically compressed (0.3 Hz) for 30 min/every other day during weeks 10-12. Group A was of higher initial seeding density than Group B. Data are reported as mean ± range (n=2). Images of four sections from duplicate samples in each set were taken. An interaction between seeding density and loading was detected ($p<0.05$) and a significant difference based on seeding density ($p<0.01$) was found with 2-factor ANOVA and a Scheffe's F comparison.

C. Discussion

Results from the long term study did not show any significant effects of mechanical loading on 12 week-old mineralized cultures based on total cell counts and percent of mineralized cross section area. The low number of samples available made detection difficult; however, it is suspected that the limited source of mobile and active osteoblasts in a mature culture could not respond significantly to the change in mechanical strain without an accompanying osteoclast population to model or remodel. The cells present in the culture may have sensed the mechanical loading, but the terminally differentiated (and often matrix-entrapped) cells could not alter proliferation or mineralization.

Difference based on initial seeding density was coupled with mechanical loading. Although in static cultures, seeding density did not produce a significant difference in total cell counts or mineralization of long term (56 day) 3-D calvaria cultures (Ishaug-Riley, 1997), mechanical loading may have altered the internal environment to allow the "extra" cells to migrate and lay down more matrix. It was postulated that diffusion limitations prevented the penetration of mineralization into the center of the static cell/polymer constructs (Ishaug et al., 1997). If so, the mechanical movement may have enhanced the delivery of media with nutrients to the inner portion of the foams, thus allowing migration and survival of the osteoblasts. With a greater surface area to expand, the osteoblasts could lay down more matrix *in vitro*, as observed.

CHAPTER 8
CONCLUSIONS

The notion that composition and morphology of living bone are influenced by the mechanical environment surrounding the cells and tissue has been widely accepted for many years (Cowin, 1983). Still, the mechanisms which trigger the physiological behavior remain unclear. The use of 3-D cell cultures and the results of this work are intended to contribute to defining the effects of mechanical loading on bone at a microscopic level. The 3-D *in vitro* cell/polymer model was introduced to combine the simplicity of cell culture with the realism of a matrix architecture as in natural bone. The discovered osteoblast responses add to the understanding of functional adaptation at a microscopic level .

Proliferation and one marker expression were examined for the osteoblastic phenotype during *in vitro* models of the three stages of bone formation: proliferation of bone cells (5 days), differentiation of the cells coupled with matrix development (3 weeks), and maintenance of mineralized matrix (12 weeks). Cell counts found no difference in growth due to mechanical compression in any time period. The most significant differences from mechanical loading were observed during the early and mid term studies in ALPase activity, supplemented by the heightened RNA expression the calcium channels (VSCCs) for the 3 week cultures. These results suggest that functional expression of the cells was more affected than cell growth by mechanical loading. Loading during the last stage also seems to allow more mineral deposits by cultures with higher number of cells seeded. This phenomenon is believed to be caused by diffusion limitation in static cultures which prevented more penetration of osteoblasts into the internal area of the foams.

From this series of experiments, it can be concluded that osteoblastic cells cultured on the 3-D scaffolds sensed the mechanical compression applied to the entire construct and responded with changes in functional behavior, most obviously during the period when

they were expected to differentiate and lay down matrix. Since this is but the first attempt to study bone behavior with such a system, however, it would be unwise to generalize that cells were not otherwise affected by mechanical loading. Only a few of many markers known for osteoblastic cells were examined. More experiments are needed to further elucidate the full effect of mechanical loading on bone formation at the cellular and molecular level.

The present work opened the door to a new generation of investigations using 3-D cell cultures to study the formation of bone under mechanical loading. For example, changes in expression of genes associated with cell division and growth would provide more insight into the responses not detectable by cell counting. Testing for matrix proteins known to up-regulate production during the second or third phases of bone formation, such as osteopontin and osteocalcin, would also provide more details about changes in the later phases and further clarify the ALPase and VSCCs response observed in this work. It is also important to examine potential signaling pathways to determine which are directly triggered by mechanical stimulus and which are secondary responses solicited by the initial reaction. Use of the 3-D scaffolds is important to better mimic the structure of natural bone without losing the simplicity of cell culture.

The limitations of this particular model must be acknowledged. 75:25 PLGA was chosen as the primary scaffold material for the 3-D culture based on previous work (Ishaug et al., 1994; Ishaug et al., 1996; Ishaug et al., 1997). The advantage of using this biodegradable system is that proliferation, ALPase, and mineralization behavior of osteoblastic cells on these foams are known. Also, knowledge gained with this system can be directly transferred to bone regeneration strategies. However, the degradable characteristics so appealing for tissue engineering applications may not be ideal for studying mechanical loading effects. Changes in degradation behavior of the scaffold due to the mechanical (and fluid) movement may complicate the cellular and molecular

responses detected. Although the media was changed every other day to prevent side-effects due to degradation products, release of glycolic and lactic acid can increase the acidity of the environment and also alter cellular development.

The most serious drawback of this model is the permanent deformation after repeated loading at 30 min/day for displacement of 100 μm. Not only can cellular responses be thus complicated, but mathematical modeling of the strain experienced by the cells is difficult because of the instability of the internal matrix system. Other materials may be more suitable as the scaffold of 3-D *in vitro* models for bone formation under load. A material that is more elastic (such as silicon rubber) or stronger (such as ceramics) may provide a better mechanical environment to study bone formation. The porous 75:25 PLGA foams served as a first generation scaffold for the 3-D *in vitro* model. Such a system, if successful, will stimulate more research leading to the clarification and unification of the numerous results presented in literature and solve the century old mystery of adaptive bone transformation.

APPENDIX A

PRELIMINARY ONE AND TWO WEEK STUDIES

A. Experimental Design

Previous studies showed that under no load (static) conditions osteoblasts grown in 75:25 poly(lactic-co-glycolic acid) (PLGA) foams expressed high levels of alkaline phosphatase (ALPase) and formed mineralized bone-like structures (Ishaug et al., 1997). Experiments of one and two weeks were designed to verify cell survival and high ALPase activity in the 3-D *in vitro* model after a brief loading regiment. Results from these studies were used to modify the load application system and improve the 3-D model for other studies on bone formation.

75:25 PLGA foams (~90 % porosity, 150-300 μm pore diameter, 6 mm diameter, 2 mm thickness) were used. The foams were seeded with primary rat marrow stromal cells at seeding densities of approximately 5.0×10^5 cells / cm^2 top surface area for Set 1. Because concurrent experiments indicated that lower seeding density on the 3-D constructs does not significantly affect cell behavior in terms of ALPase activity (Ishaug et al., 1997), seeding was reduced for Set 2 to approximately 3.5×10^5 cells / cm^2 top surface area to reduce "donor" demand. Both are considered low seeding densities compared to those use in the referenced study. Complete media (10% calf serum) supplemented with 100 nM dex (Bellows et al., 1986; Shalhoub et al., 1992) provided nutrients to the cultures. Media was changed every other day before mechanical treatment.

Cell/polymer constructs were compressed 70 min with either cyclic (0.3 Hz) or static (holding at maximum displacement) loading. The control sets were also placed in the hood on a heated dry bath for the same duration to alleviate potential side-effects from exposure to air. Table A-1 details experimental parameters of the two-part study.

Table A-1. Experimental Parameters of Studies

Set	Seeding Density (x 10^5 cells)	Period of Loading	Loading Regiment	Total Culture Period
1	5.0	days 1-7	10 min/day	7 days
2	3.5	days 1-14	10 min/every 2 days	14 days

Total cell numbers and ALPase activity were assessed for cyclic (0.3 Hz load), static (holding load), and control (no load) sample groups. These assays were used to monitor changes in proliferation and signs of differentiated behavior due to mechanical loading. Histology sections of the samples were stained with H and E for cells and tissue formation as well as with the von Kossa method to visualize calcium phosphate deposits. In addition, improvements and modifications of the model for other studies were made based on gained experience.

B. Results

1) Set 1

Total cell numbers as well as ALPase activity were not different (ANOVA, 95% confidence level) among control samples and those subjected to cyclic and static loading for one week (Figures A-1 and A-2). Confocal micrographs confirmed the presence of cells in a 3-D configuration (Figure A-5a). Histology sections showed scattered cells throughout all foams, but no mineralization after 7 days. Figures A-6a and b show sample histology sections stained with H and E and with the von Kossa method. Based on these assays no detectable difference can be made between loaded samples and controls in proliferation or functional behavior.

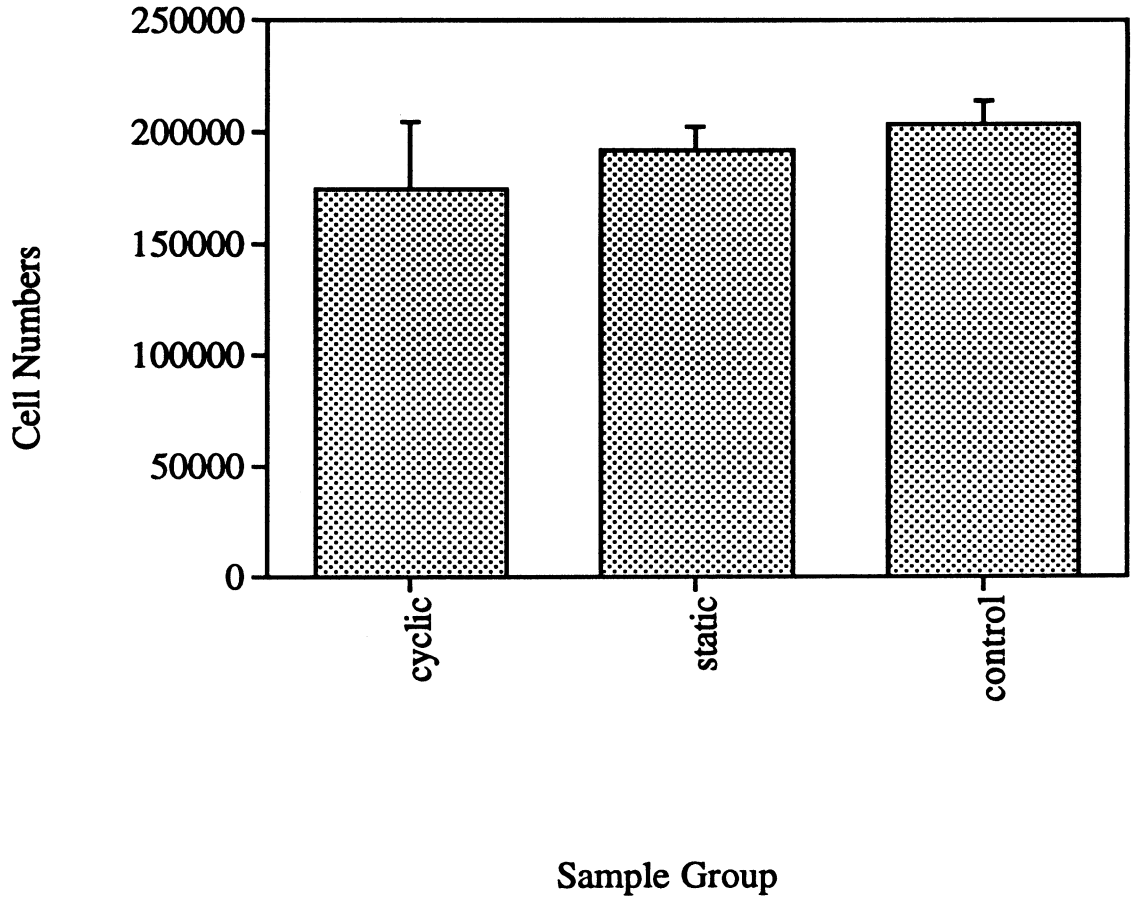


Figure A-1. Cell counts from cell/polymer constructs as determined with a DNA assay after one week. The 3-D cultures were cyclically compressed 10 min/day at 0.3 Hz (cyclic), held at 100 μm compression (static), or cultured without mechanical stimulation (control). No significant difference was noted among the sample groups based on ANOVA, 95% confidence level. Data are reported as mean ± s.d. (n=3).

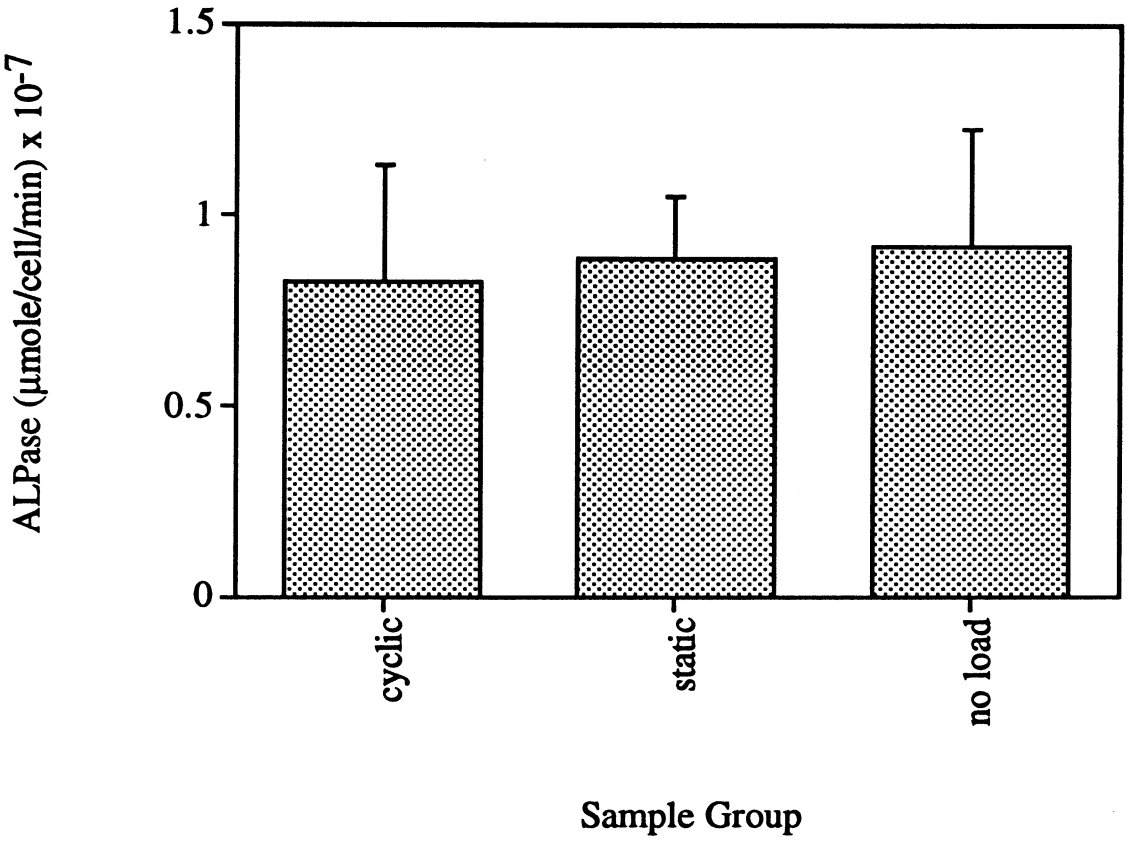


Figure A-2. Alkaline phosphatase activity (ALPase) after 1 week. The 3-D cultures were cyclically compressed for 10 min/day at 0.3 Hz (cyclic), compressed and held at 100 µm compression (static), or not exposed to mechanical compression (control). Results of the three groups were similar after 1 week of culture. Data are reported as mean ± s.d. (n=3).

2) Set 2

Again, proliferation was not significantly affected by mechanical loading based on the total cell numbers (Figure A-3). In addition, no statistical difference (ANOVA at 95% confidence level) could be discerned among the three sample groups based on ALPase analysis, although the trend shows decreasing means from cyclic to static to control. Figure A-4 depicts the results in graphic form. Confocal micrographs (Figure A-5b) as well as histology sections (Figure A-6c) confirmed cell attachment in 3-D configuration after two weeks of culture. Cells lined the pores in the culture with most of the population along the outer perimeter of the scaffold. Signs of mineralization were also visible with the von Kossa stain (Figure A-6d).

C. Discussion

Although no difference was detected due to mechanical loading either after one or two weeks of compression at 70 min, cells in the 3-D constructs at both seeding densities attached and expressed levels of ALPase activity indicative of osteoblast-like phenotype (Ashton et al., 1985). The levels of ALPase activity for Set 1 at ~1 x 10^{-7} μmole/cell/min were comparable to those found for previous studies using 3-D cell/polymer constructs after one week (Ishaug et al., 1997). Results from Set 2 (between 1.5 to 2 x 10^{-7} μmole/cell/min), however, were much lower than those in that same study after two weeks at ~10 x 10^{-7} μmole/cell/min. The increase of ALPase activity from week 1 to week 2 was not as dramatic as in the Ishaug et al. (1997) study, even for the control samples. This may be attributed to the difference of 10% CS instead of 10% FBS. FBS has been shown to be more mitogenic than CS. In addition, the level of dex added in the media for this study was 100 nM instead of 10 nM. In a study by Ishaug-Riley (1997) using calvaria osteoblasts on 3-D PLGA foams with 100 nM dex, ALPase activity was of the same level found in this

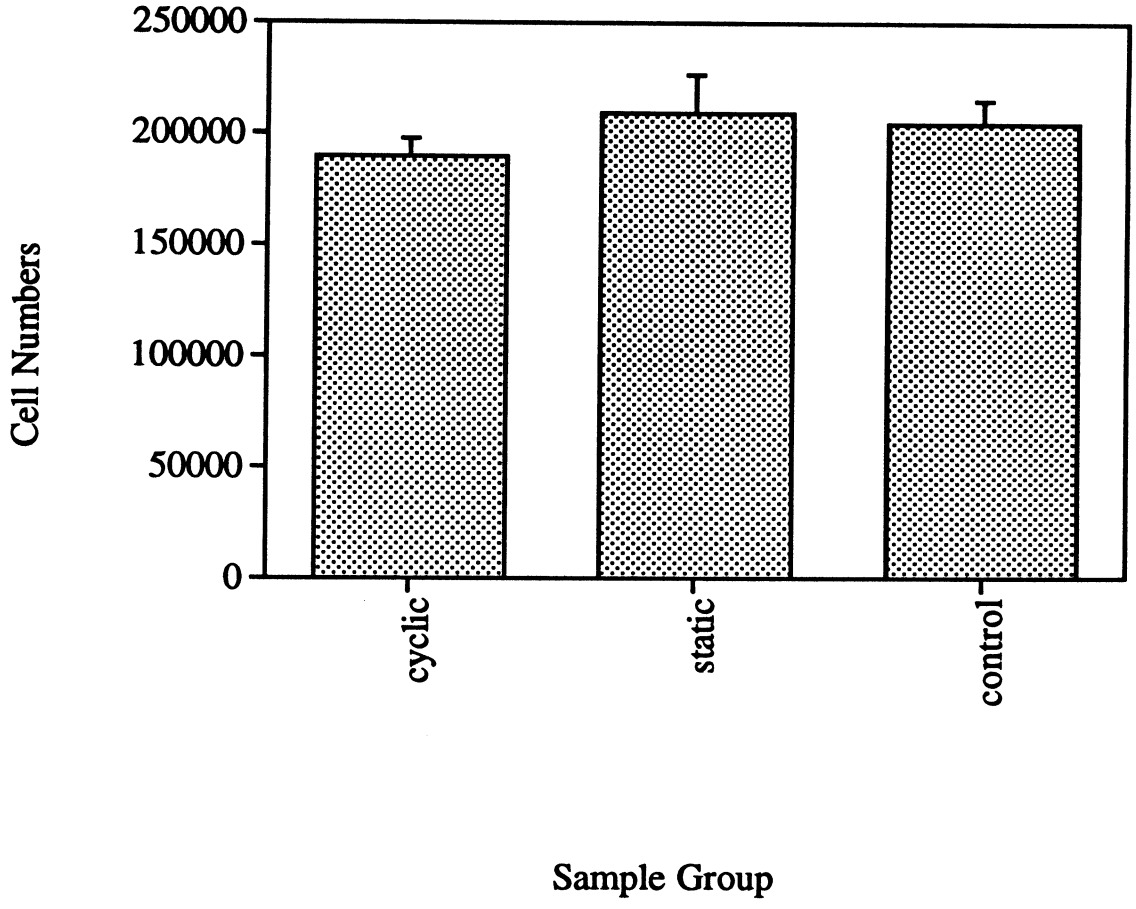


Figure A-3. Cell counts of the 3-D cell/polymer constructs after two weeks based on a DNA assay. Samples were cyclically compressed for 10 min/ every 2 days at 0.3 Hz (cyclic), compressed and held at 100 μm displacement for 10 min/every 2 days (static), or not subjected to mechanical loading (control). Total cell numbers were similar for all groups based on ANOVA, 95% confidence level. Data are reported as mean ± s.d. (n=3).

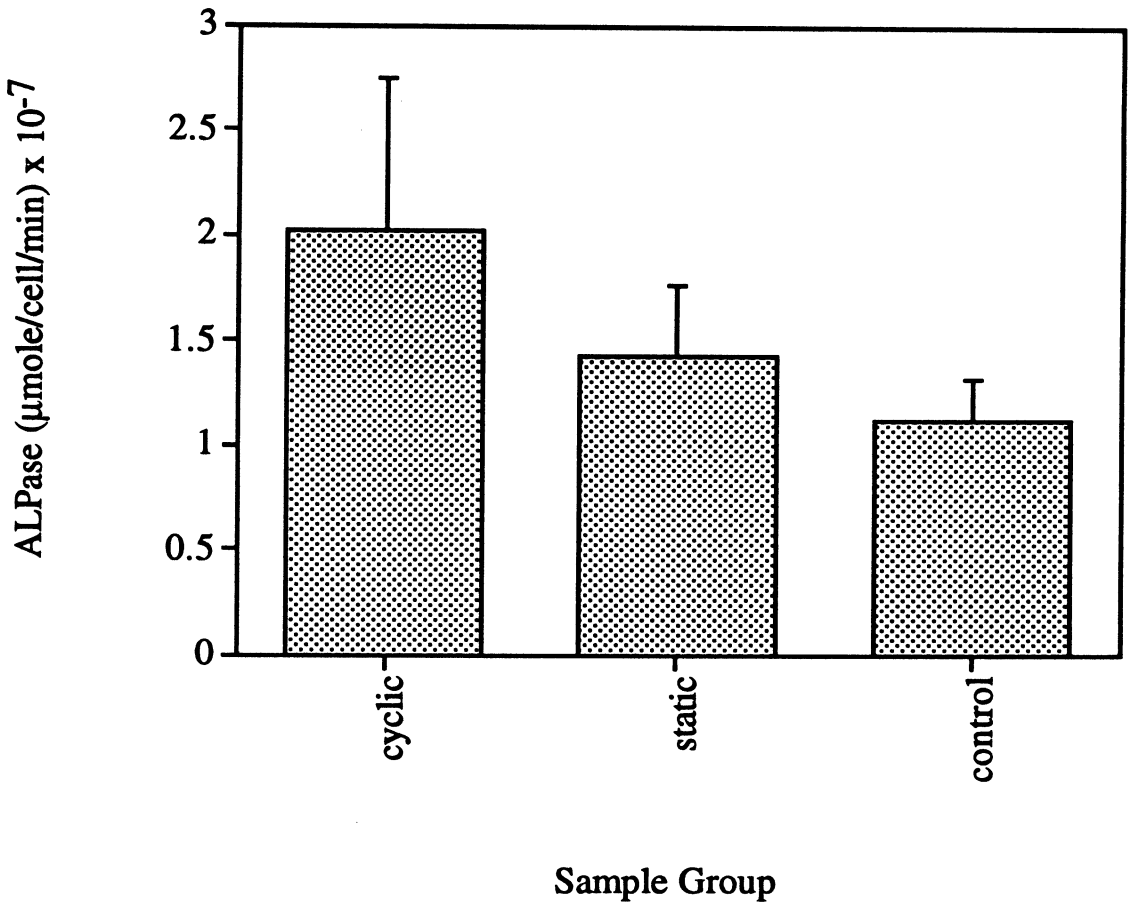
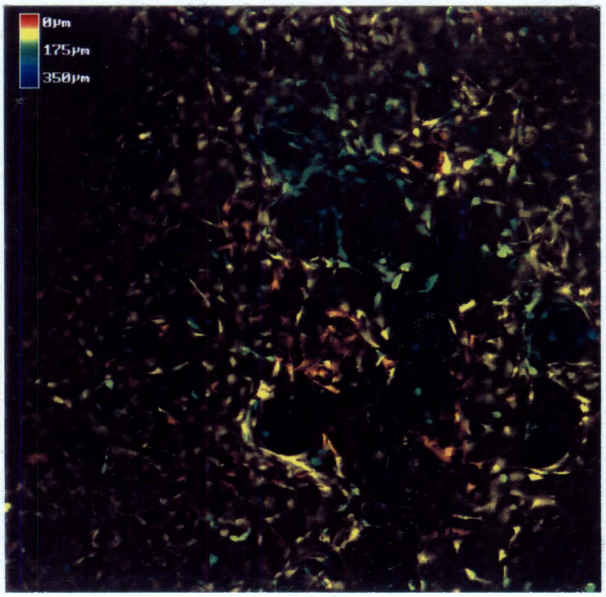


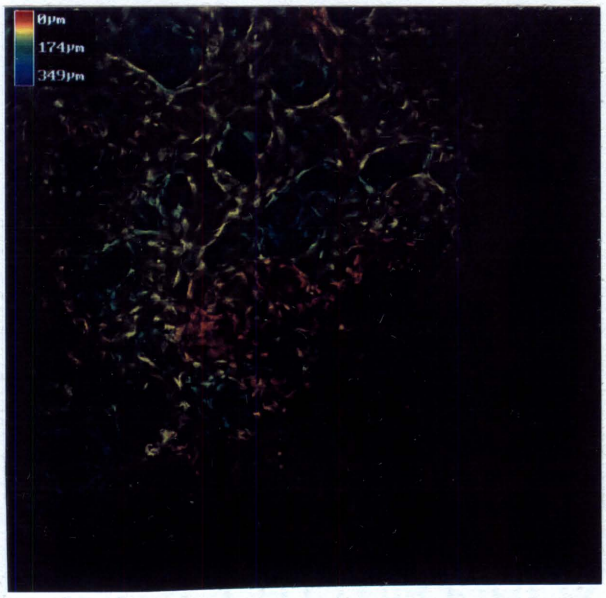
Figure A-4. ALPase activity on cells in 3-D constructs after two weeks. Samples were subjected to cyclic loading at 0.3 Hz (cyclic), compression and holding at 100 µm maximum displacement (static), or no mechanical loading (control). A trend of decreasing activity is seen from cyclic to static to control, but results were not statistically different (ANOVA, 95% confidence level). Data are reported as mean ± s.d. (n=3).

Figures A-5 a and b. Confocal micrographs of cyclically loaded samples: (a) Set 1 after 1 week and (b) Set 2 after 2 weeks. Viable cells were stained with BCECF-AM and viewed at 100x. Images were digitally color coded for depth (16 sections). Red is closest to the surface of the foams. Control and statically loaded samples also looked similar (not shown).

(a)

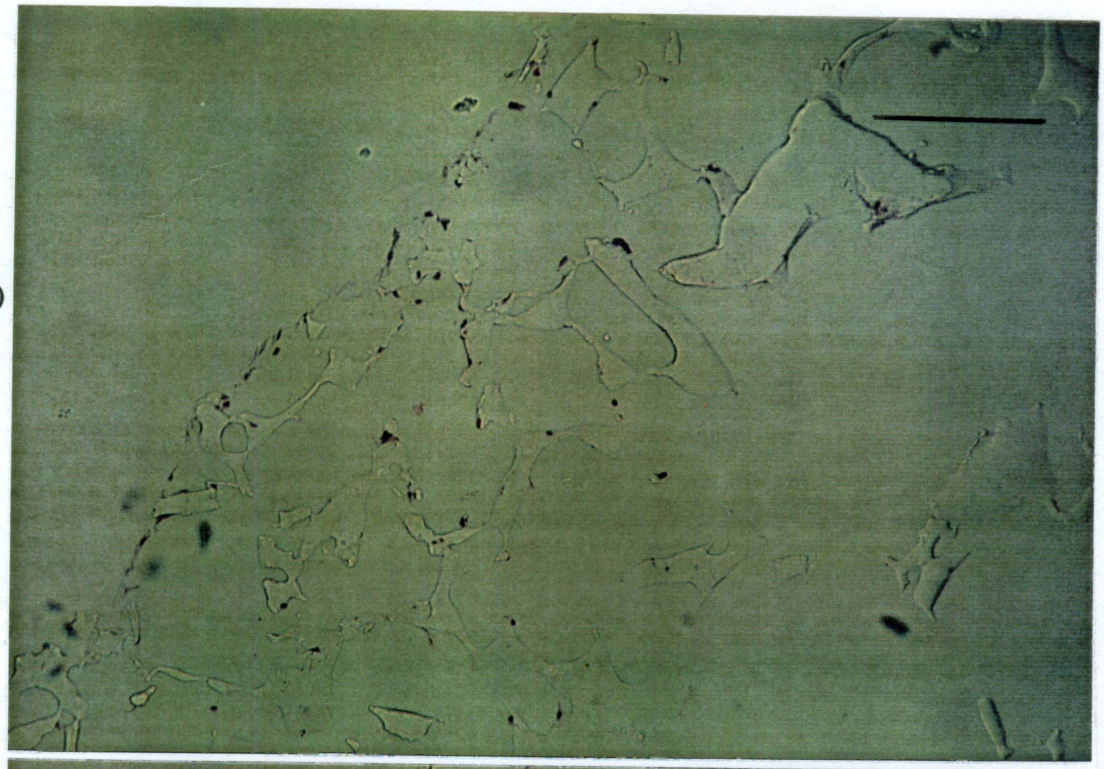


(b)

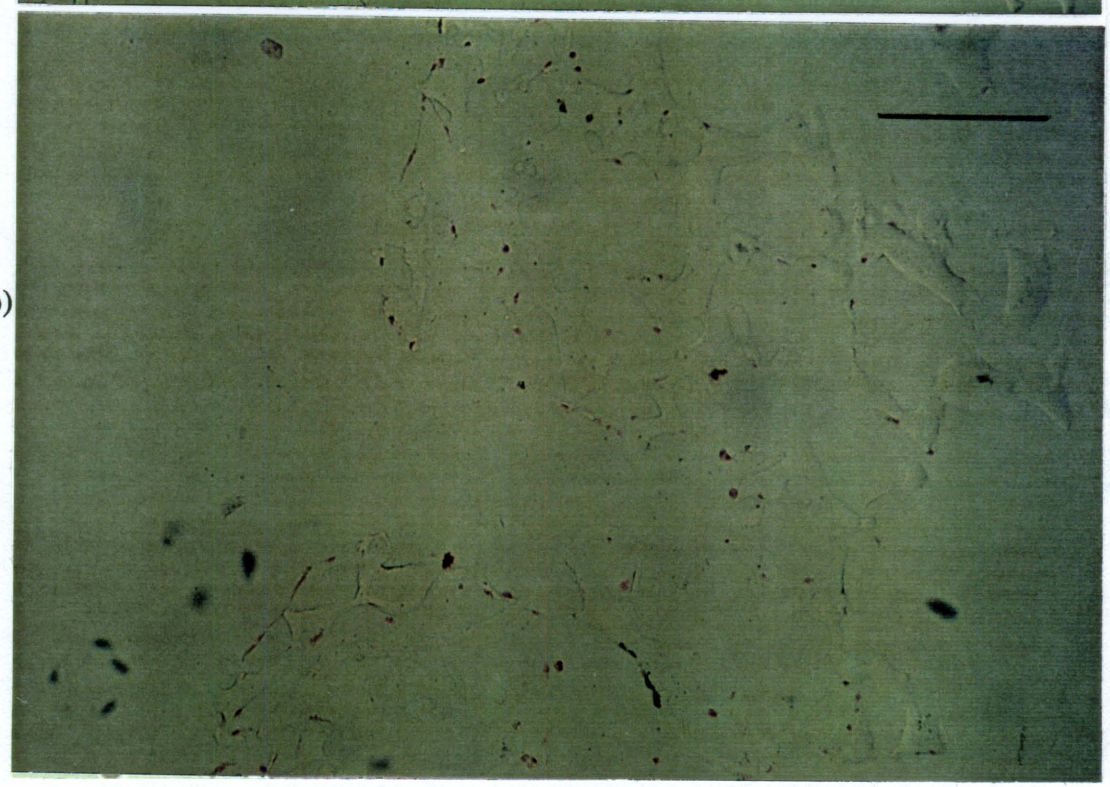


Figures A-6 a and b. Histology sections from cyclically loaded samples of Set 1 (1 week). (a) is stained with H&E for cells and tissue formation. (b) is stained with the von Kossa method to show mineral deposits. (bar = 80 μm)

(a)

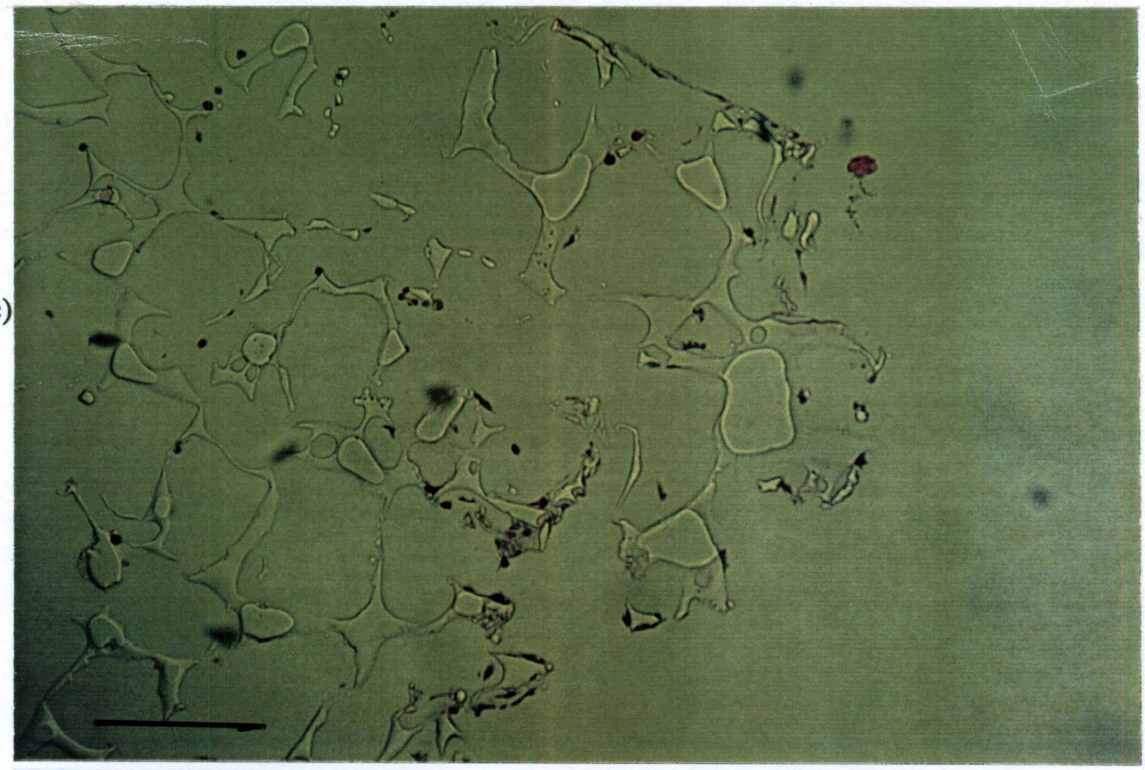


(b)

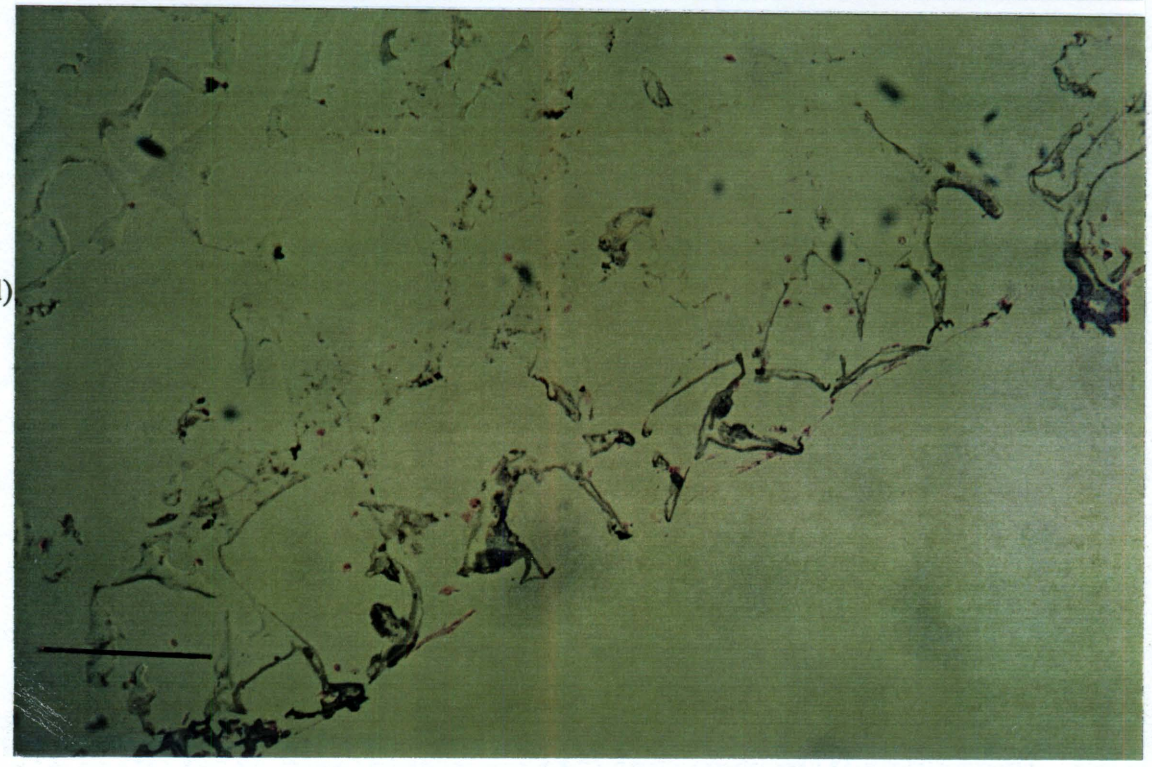


Figures A-6 c and d. Histology sections from cyclically loaded samples of Set 2 (2 weeks). (a) is stained with H&E for cells and tissue formation. (b) is stained with the von Kossa method to show mineral deposits. (bar = 80 μm)

(c)



(d)



study after two weeks (1.5 to 3 x 10^{-7} μmole/cell/min). Evidence in literature agrees that osteoblastic cells have a dose dependent response to dex (Canalis, 1983). Also, seeding densities and final cell numbers were different between the studies, although Ishaug et al. (1997) found no significant effect of these parameters on ALPase activity.

Cell presence and survival were visually confirmed with confocal microscopy. Histology sections stained with H and E and by the von Kossa method also showed that the cells within the foams laid down ECM along the polymer scaffold which, given time, mineralized. The concentration of cells and mineral deposits around the outer perimeter of the foams was also seen in the previous study referenced (Ishaug et al., 1997). It was suggested that seeded cells and the ECM formed reduced diffusion into the middle of the foams; thus physical blockage limited the nutrients at the core of the foams and discouraged migration of the cells. The short regiment of mechanical loading did not seem to cause a visible difference in the construct dimensions, cell concentration, or mineralization pattern in this study.

Results from the experiments showed that exposure to 100 μm compression of the entire construct, either cyclic or static for 70 min, did not produce significant differences in proliferation or ALPase activity compared to respective non-loaded controls within each set. The trend seen in the 2 week study (Set 2) as well as references in literature (review, Burger and Veldhuijzen, 1993) suggest that cyclic loading stimulates more bone forming activity than static loading. In addition, loading for longer duration may produce more detectable changes in osteoblast behavior for proliferation and ALPase activity. Longer culture periods may also produce more pronounced changes in these particular markers. Based on these studies, the experimental design was modified for the work described in the main body of the thesis to concentrate on cyclic loading. Loading time was also extended to 30 min/day to better model reambulatory schedules (Thompson et al., 1996). In addition, culture media was changed to include FBS and 10 nM dex.

APPENDIX B

DACRON-POLY(GLYCOLIC ACID) FABRICS

A. Introduction

The use of weft-knitted Dacron-poly(glycolic acid) (PGA) fabrics provided by Dr. C. C. Chu of Cornell University (Ithaca, NY) was investigated for osteoblast culture. Unwoven PGA meshes had been previously used to seed chondrocytes and osteoblasts for implantation into animal models (Vacanti et al., 1993). However, their potential as a substrate for *in vitro* bone formation was not known. In this study, the behavior of rat stromal cells cultured on Dacron-PGA fabrics was examined up to 32 days. Assay for cell proliferation, ALPase activity, and mineralization were performed.

The knitted Dacron-PGA (K-6) fabrics examined were previously described in Yu and Chu (1993). Briefly, Dacron yarns (200 deniers) and PGA yarns (90 deniers) were knitted with a Lamb circular knitting machine into tubular fabrics. The fabrics contained 31% PGA by weight fraction with a void volume of 84%. Dacron, the other component, is biocompatible and has been used successfully as a non-absorbable non-thrombogenic vascular material. This was incorporated with the PGA to maintain a minimal mechanical integrity as the degradable component disappears. *In vitro* degradation showed the most noticeable changes during the 30-60 day immersion period in PBS solution of pH 7.4 at 37°C. Previously, these knitted PGA fabrics were tested for use as synthetic vascular grafts. In this study, the use of the Dacron-PGA fabrics as a scaffold for culturing marrow stromal osteoblasts for bone regeneration was examined.

B. Experimental Design

The behavior of the stromal-osteoblasts on three variations of the K-6 fabrics: control, those irradiated at 4 Mrad, and those irradiated at 10 Mrad were studied. Radiation treatment was to increase the degradation rate of the fabrics. Control fabrics were previously determined to remain intact up to 30 days. 4 Mrad and 10 Mrad fabrics were expected to degrade faster with increasing treatment dosage. Fabrics were pre-wetted (sterilized) in 100 % ethanol for 45 min followed by three 15 min rinses of PBS and two 1.5 hr rinses in complete media before the introduction of cells.

Primary rat marrow stromal cells were seeded onto the fabric disks (7 mm diameter, 0.5 mm thickness) at 1 x 10^6 cells/cm^2 top surface area as in a previous study using 3-D PGA constructs for culturing chondrocytes (Freed et al., 1993). The cell/polymer disks were incubated at standard culture conditions with complete media supplemented by 15% FBS and 10 nM dex as in Maniatopoulos et al. (1988). Culture media was changed every other day to reduce potential effects of degradation products. Confocal micrographs of cultures from days 4 and 7 were taken to verify cell attachment on the knitted fabric yarns. Cell counts were determined for days 1, 4, 7, 14, and 32 to detect proliferation activity. ALPase activity were assessed for cultures of 4, 7, 14, and 32 days. In addition, histology sections of days 32 were stained either with H and E or by the von Kossa method to visualize tissue formation or mineral deposits, respectively. Quantitative results were analyzed for statistical significance at 95% confidence level. Paired ANOVA with post-hoc Scheffe's test were used to detect difference within the same sample group at different time points. Unpaired ANOVA with post-hoc Scheffe's test was used to compare different sample groups at the same time point.

C. Results

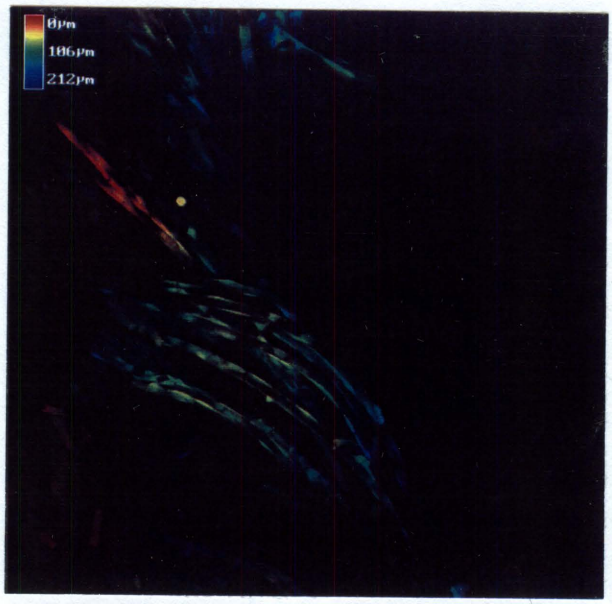
Cells were seen on the Dacron-PGA fabrics as shown by the confocal micrographs for both day 4 and day 7 (Figures B-1a and b). Cell counts showed proliferation on all fabric groups over the 32 days examined. Figure B-2 presents the results in graphic form. The plateauing of the cell numbers between days 14 and 32 suggested a decrease in proliferation with culture time. Cell counts on control fabrics were consecutively lower; however, the difference was not statistically significant based on triplicate sampling.

ALPase activity increased from day 4 to day 7 for all samples. On day 7, control samples were higher than the 10 Mrad set ($p<0.05$), however the difference was not detected at other time points. Cells on the 4 Mrad samples continued to increase ALPase activity up to day 32. However, ALPase levels for samples from the 10 Mrad group increased from day 7 to day 14, but tested for a similar level at day 32. ALPase activity between the 4 Mrad samples were slightly higher on day 32 than on the 10 Mrad ones, but the difference were not statistically significant. Figure B-3 compares the ALPase activity over the time examined.

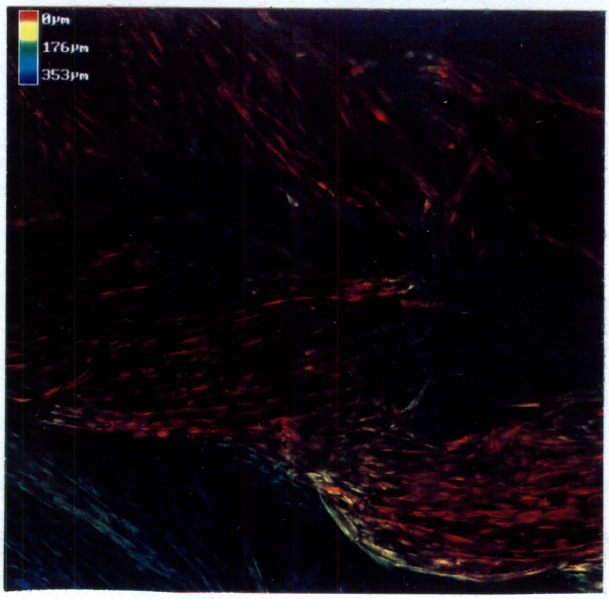
Histology sections of day 32 samples showed cell presence and matrix formation as well as signs of mineralization on control (Figures B-4a and b), 4 Mrad (Figures B-5a and b), and 10 Mrad (Figures B-6a and b) fabrics. No quantitative measurements were determined because seeded fabrics curled up in culture and histology sections were not of the same proportions. However, control samples consistently showed more areas of dense cellular population and mineralization.

Figures B-1 a and b. Confocal micrographs of Dacron-PGA samples stained with BCECF-AM at 100x. Cells attached on all three variations of fabrics. A 4 day sample is shown in (a) and a day 7 sample is shown in (b).

(a)



(b)



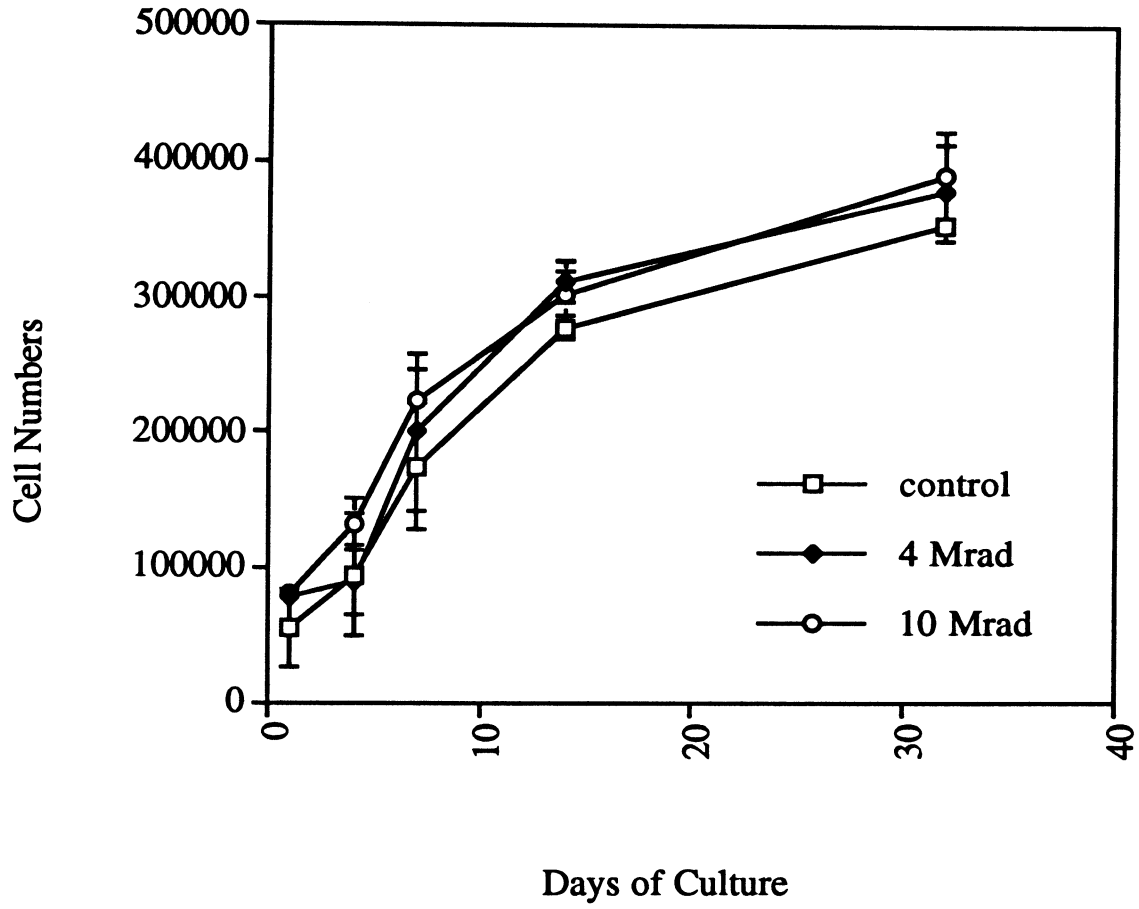


Figure B-2. Proliferation up to 32 days of rat marrow stromal cells on Dacron-PGA fabrics based on DNA assay. In these experiments, "control" samples were unirradiated K-6 PGA fabrics. "4 Mrad" samples were irradiated at 4 Mrad to increase degradation rate. "10 Mrad" samples were irradiated at 10 Mrad and expected to degrade the fastest. By day 32, a slowing of growth was detected. All samples showed a similar cell numbers for all the time points assayed based on unpaired ANOVA. Data are reported as mean ± s.d. (n=3).

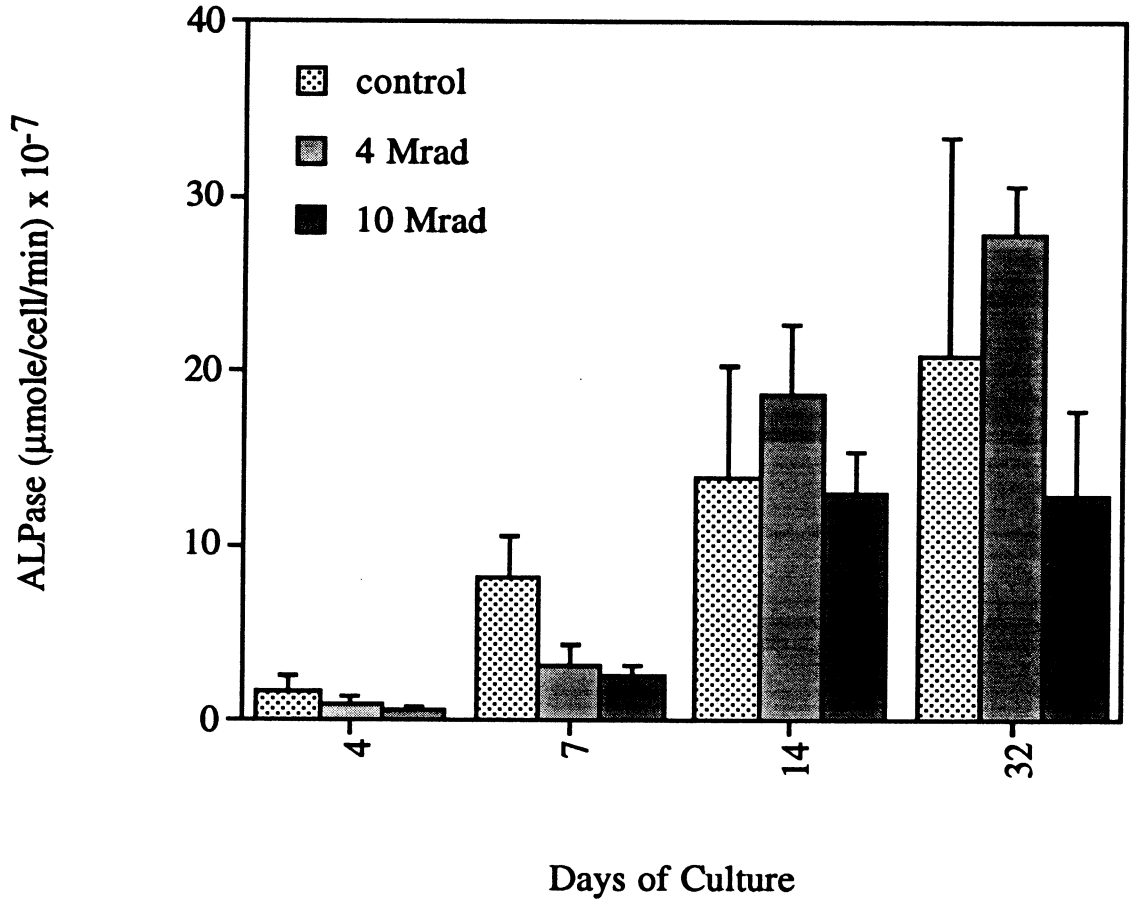


Figure B-3 ALPase activity for rat marrow stromal cells cultured on Dacron-PGA fabrics. The "control" samples were unirradiated K-6 PGA fabrics. "4 Mrad" samples were irradiated at 4 Mrad to increase degradation rate. "10 Mrad" samples were irradiated at 10 Mrad and expected to degrade the fastest. Control samples showed higher activity on day 7 compared to 10 Mrad samples based on unpaired ANOVA with post-hoc Scheffe's test ($p<0.05$). Control ALPase levels, however, did not increase as the other sets. 4 Mrad samples continued to increase up to day 32, but 10 Mrad samples showed similar levels of activity on days 14 and 32. Data are reported as mean ± s.d. (n=3).

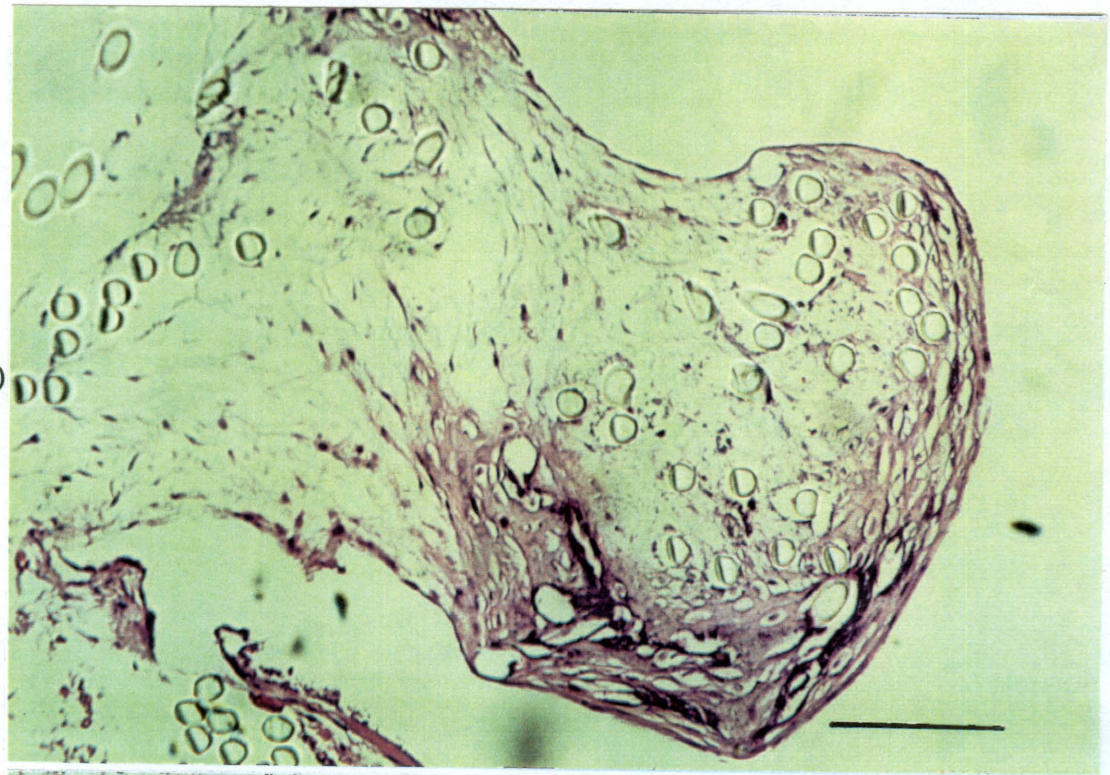
D. Discussion

Rat marrow stromal cells proliferated and expressed high ALPase activity on the Dacron-PGA fabrics both with and without irradiation treatment. No change due to the expected difference in degradation rate of the fabrics was detected for proliferation. ALPase results showed that modulation in functional behavior in the cells on day 7 of culture was dependent on fabric characteristics ($p<0.05$). Levels of ALPase activity on all fabric scaffolds were similar to those for marrow stromal cells cultured on 3-D 75:25 PLGA foams at similar time points despite 5% higher FBS here (Ishaug et al., 1997). At day 7, osteoblastic cells on Dacron-PGA constructs as well 75:25 PLGA expressed ALPase activity between 1 to 5 x 10^{-7} μmole/cell/min. K-6 controls were slight higher at ~8 x 10^{-7} μmole/cell/ min. Levels in both studies were between 10-15 x 10^{-7} μmole/cell/ min for day 14. Day 28 ALPase activities reported in the Ishaug et al. (1997) study were in the same range as those of control and 4 Mrad constructs (20-25 x 10^{-7} μmole/cell/ min). 10 Mrad Dacron-PGA levels, however, were like those from day 14. Overall results suggested that the cells on the Dacron-PGA were expressing osteoblastic phenotype. In support, histology sections also showed signs of mineralizing matrix after 32 days of culture.

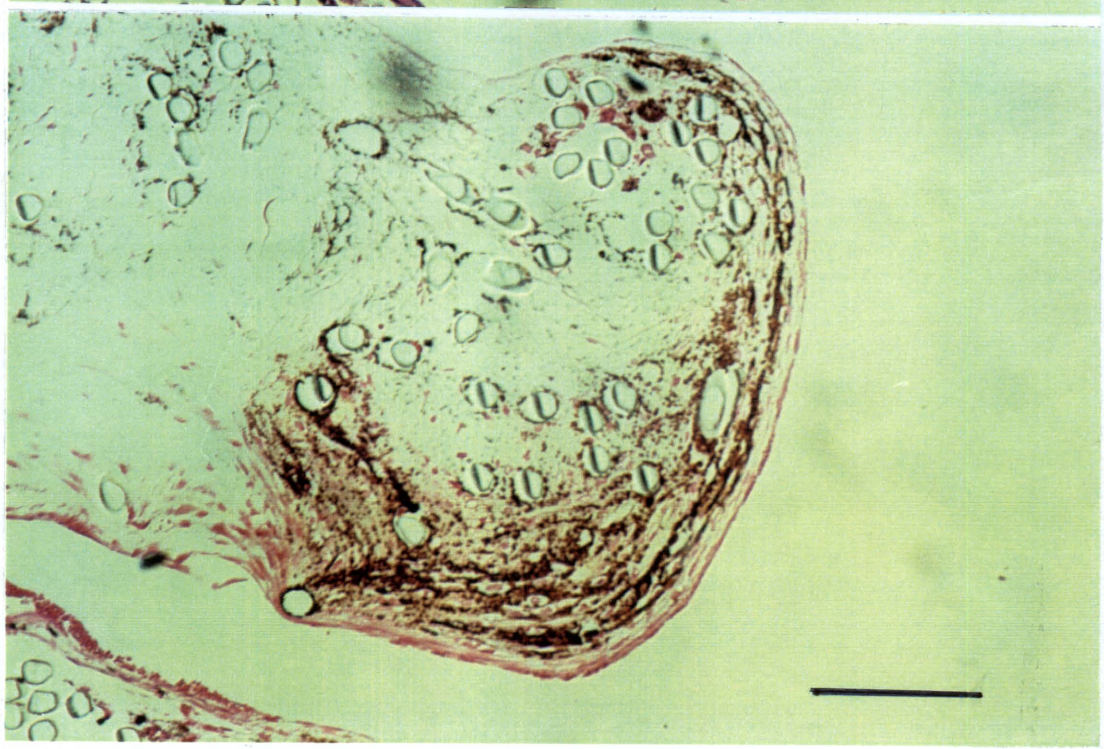
Difference in rate of fabric degradation affected ALPase activity on day 7, but the effect was not seen at other time points. Histology sections showed more matrix and mineral presence on control fabrics compared to treated fabrics. After 32 days in culture media, signs of fabric degradation were limited to debris found on the bottom of the culture dish, increased, and detection of glycolic acid in the media by the method of Niederwieser et al., 1978 (not shown). The non-degradable Dacron is believed to retain the macroscopic form of the constructs. A more detailed study of the degradation behavior is needed to define the link between the response of the cells to the condition of the scaffold before definitive conclusions can be made.

Figures B-4a and b. Histology sections of day 32 control Dacron-PGA fabric cultured with rat marrow stromal cells. Samples were stained with H and E for cells and matrix formation (a) or with the von Kossa method for calcium phosphate deposits (b). (bar = 80 μm)

(a)

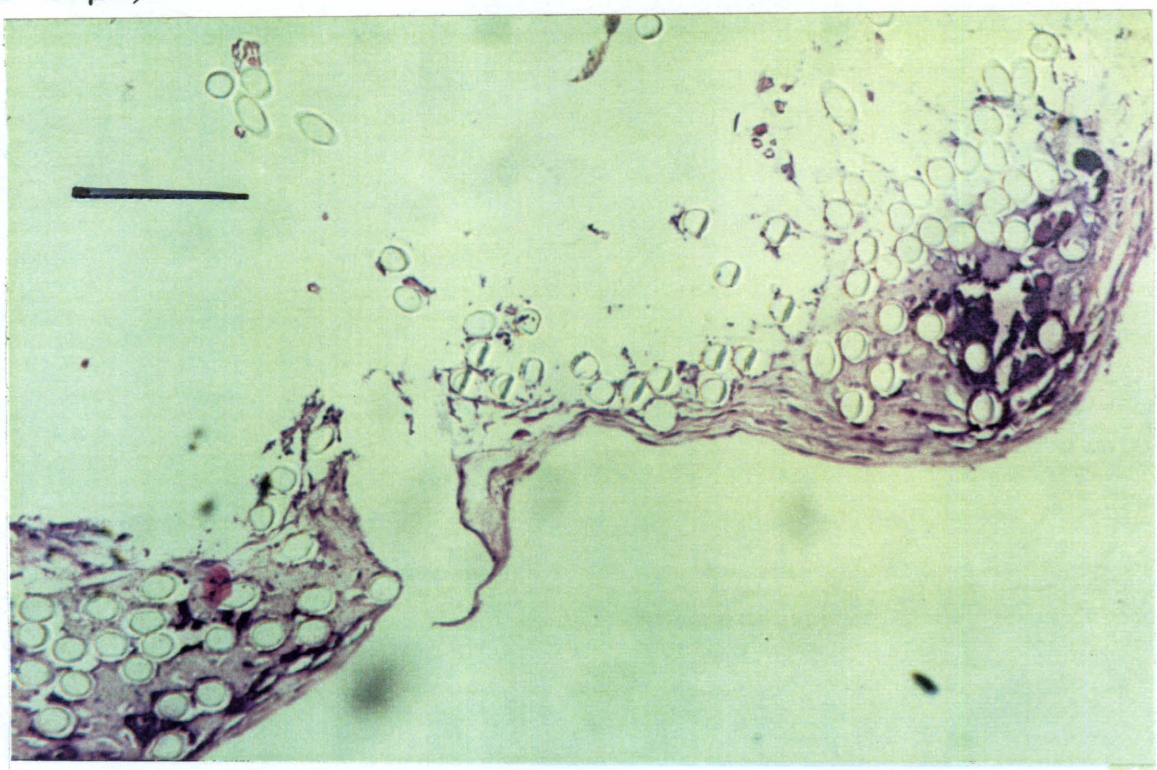


(b)

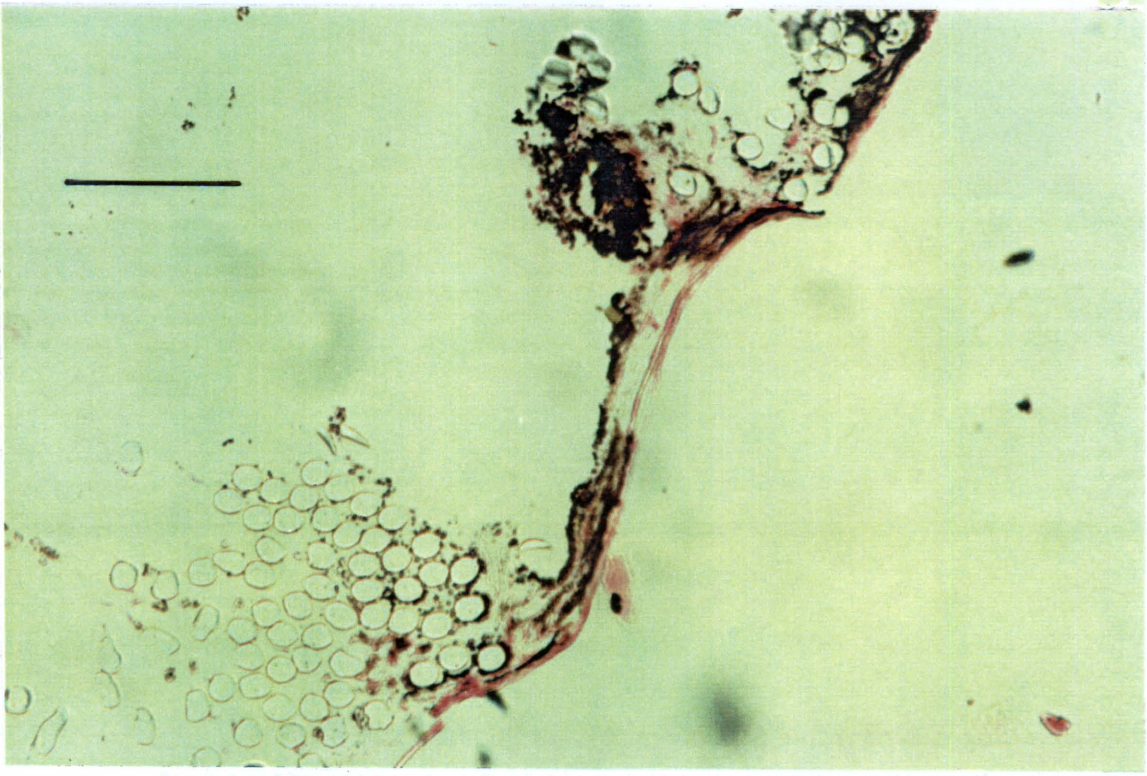


Figures B-5a and b. Histology sections of day 32 Dacron-PGA fabrics irradiated at 4 Mrad and cultured with rat marrow stromal cells. Samples were stained with H and E for cells and matrix formation (a) or with the von Kossa method for calcium phosphate deposits (b). (bar = 80 μm)

(a)

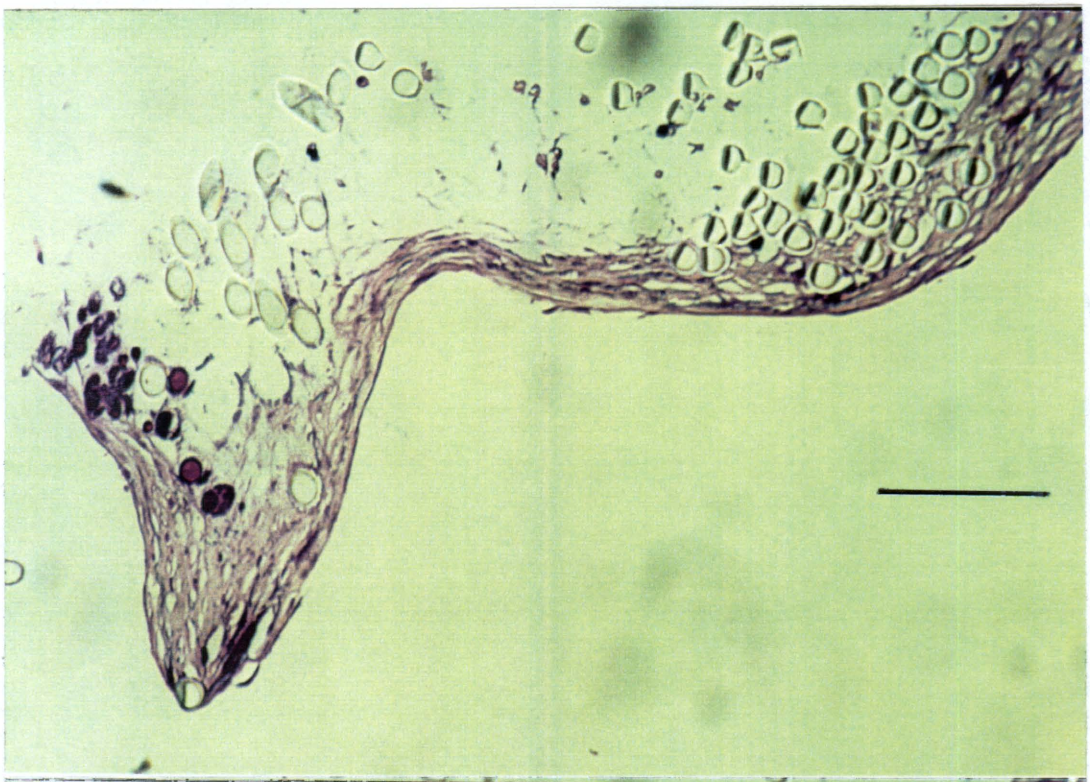


(b)

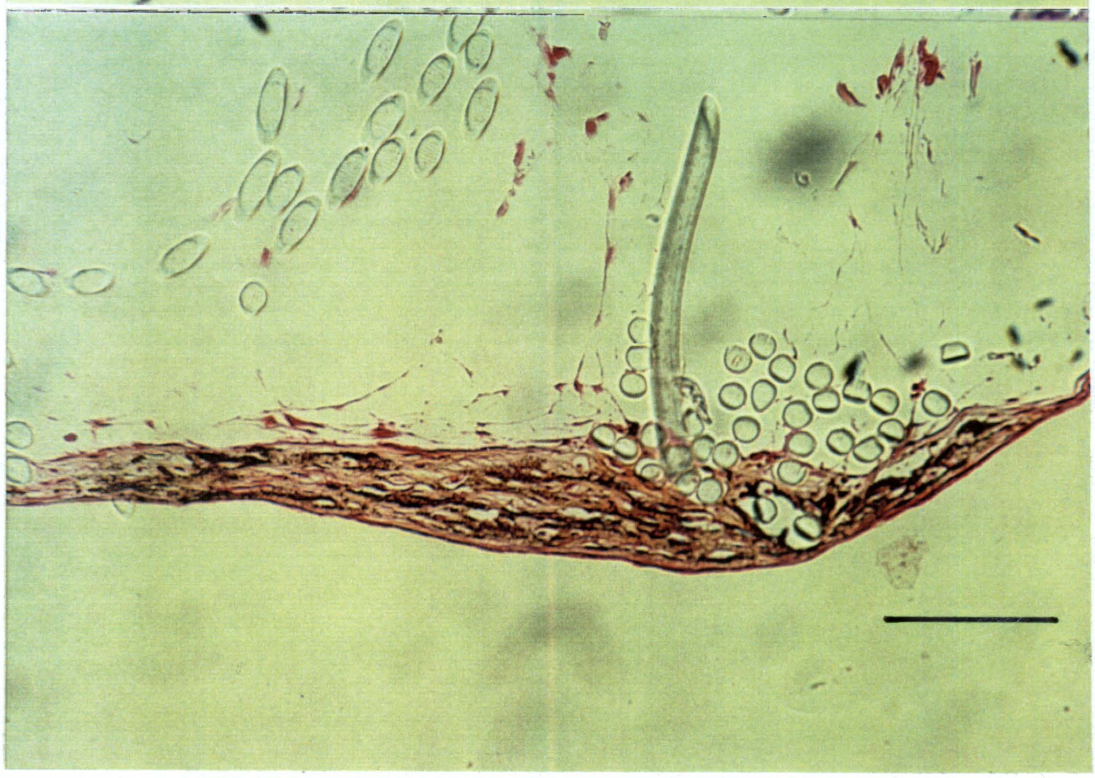


Figures B-6a and b. Histology sections of 32 day 10 Mrad treated Dacron-PGA fabrics cultured with rat marrow stromal cells. Samples were stained with H and E to visualize cells and matrix formation (a) or with the von Kossa method for calcium phosphate deposits (b). (bar = 80 μm)

(a)



(b)



APPENDIX C

FIBER-REINFORCED 75:25 PLGA COMPOSITES

A. Introduction

Fiber-reinforcement technology was employed to produce stronger PLGA-coated composites that more closely resemble the mechanical properties of bone. The material properties of the composites as well as its potential as substrate for culturing osteoblasts for use as a model of bone formation *in vitro* were examined.

Weft-knitted fibers are used as reinforcing agents because of their special mechanical properties which form an anisotropic composite material, similar to natural bone. The composites can be made stronger in the direction of loading. Figure C-1 illustrates the characteristic shape of the weft-knit fibers. Materials formed with weft-knitted hybrid yarns are easily adaptable to free form shapes, with better drapability and mechanical stability in all three spatial dimensions (Mayer et al., 1994). Weft-knitted carbon, steel, and glass fiber reinforced thermoplastics are presently being investigated for developing advanced medical devices, automotive parts, and other free form applications with strong, tough, and lightweight material requirements. Steel and carbon knits is often used as the single-filament and multi-filament models, respectively, for developing processing methods. These fibers are not biodegradable.

B. Experimental Design

1) Composite Processing

The knitted fibers were first desized/cleaned in a sonicated bath of chloroform before processing to enhance matrix bonding. While still wet, the fiber knits were gently

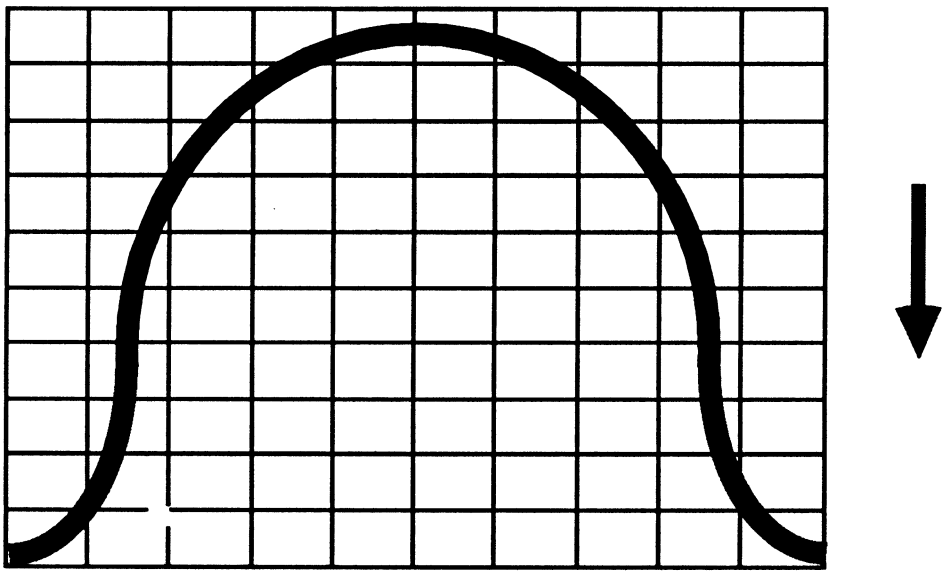


Figure C-1. Weft shape of the knitted fibers used as reinforcement agents to form composites with anisotropy. Arrow depicts the direction of maximal strength because of the alignment of the fibers.

dipped into a polymer solution and allowed to dry into a polymer covered sheet (prepreg). Meanwhile, thin films of polymer also were casted and allowed to dry. The prepreg/polymer laminates were then combined in a high-vacuum heat press to form a composite. The method was developed using carbon knits as the multi-fiber model and steel knits as the single-fiber model. Knitted fibers were provided by Professor Wintermantel and Dr. Mayer of ETH (Zurich, Switzerland). The polymer used was 75:25 PLGA (Birminghman Polymers) dissolved in chloroform (F.E.R.O.S.A., Barcelona, Spain).

2) Material Characteristics

Scanning electron (SE) microscopy was used to visualize the fiber-matrix interface characteristics such as wetting and intra-bundle penetration in the multi-fiber composite. Porosity values were estimated based on total weight and volume of the composites and the density of each components: r (carbon fibers) of 1.8 g/cm^3, r (steel fibers) of 7.8 g/3, and r (PLGA)of 1.3 g/cm^3.

The elastic (E) moduli of the composites were determined to estimate mechanical properties. Guidelines from ASTM D790-92 (Standard Test Methods for Flexural Properties of Unreinforced and Reinforced Plastics and Electrical Insulating Materials) were followed. Accordingly, a 4-point bending test was used on dry samples to generate load deflection data. Four samples of carbon-fiber reinforced and four samples of the steel fiber reinforced composites were tested. The E modulus was calculated using the following equation: $E_B = 0.21\ (L^3\ m\ /\ b\ d^3)$, where E_B is the modulus of elasticity in bending (MPa), L is the support span (mm), b is the width of beam tested (mm), d is the depth of beam tested (mm), and m is the slope of the tangent to the initial straight-line portion of the load-deflection curve generated from the 4-point bending test (N/mm).

3) Cell Culture

Cell culture test was used to determine initial feasibility of the composites for supporting bone cells *in vitro*. Marrow stromal cells were seeded (8.4 x 10^6 cells/scaffold) onto the ethylene oxide sterilized composite scaffolds and nourished with complete media (10% CS and 10 nM dex). Samples were incubated at 37°C under a 5% CO_2 humidified environment for up to 18 days. The MTT test was used to assess cell viability after 6 and 18 days of culture on the composite scaffolds. Enzymatically active and viable cells convert MTT into crystals trapped within the cytoplasm. The crystals were then released and solubilized in 1:1 DMSO/isopropanol. Spectrometric readings were normalized with control composites without any cells. Two samples of each kind of composite were available for each time point.

C. Results

1) Material Properties

The SE micrographs show wetting of a carbon fiber by the polymer solution (Figure C-2) and intra-bundle penetration into the multi-fiber carbon strands (Figure C-3). Figure C-4 shows that the fibers covered in a sheath of polymer. The PLGA matrix held the fibers together and formed a polymer surface for the composite. Steel fibers were also encased within the polymer matrix, exposing a PLGA exterior.

The carbon and steel fiber composites were processed in the same manner. Nonetheless, the approximate porosity and the E moduli were different. The multi-filament carbon fiber-PLGA composites were estimated to be approximately 75% porous and the single strand steel fiber-PLGA composites were 90% porous. The determined elastic (E) modulus of the composites from four samples were 238.11 ± 126.83 MPa for carbon fiber composites and 46.65 ± 6.18 MPa for steel fiber composites (Figure C-5).

Figure C-2. SE micrograph showing wetting of PLGA polymer solution on carbon fiber used as reinforcement agent. (bar = 23.1 μm)

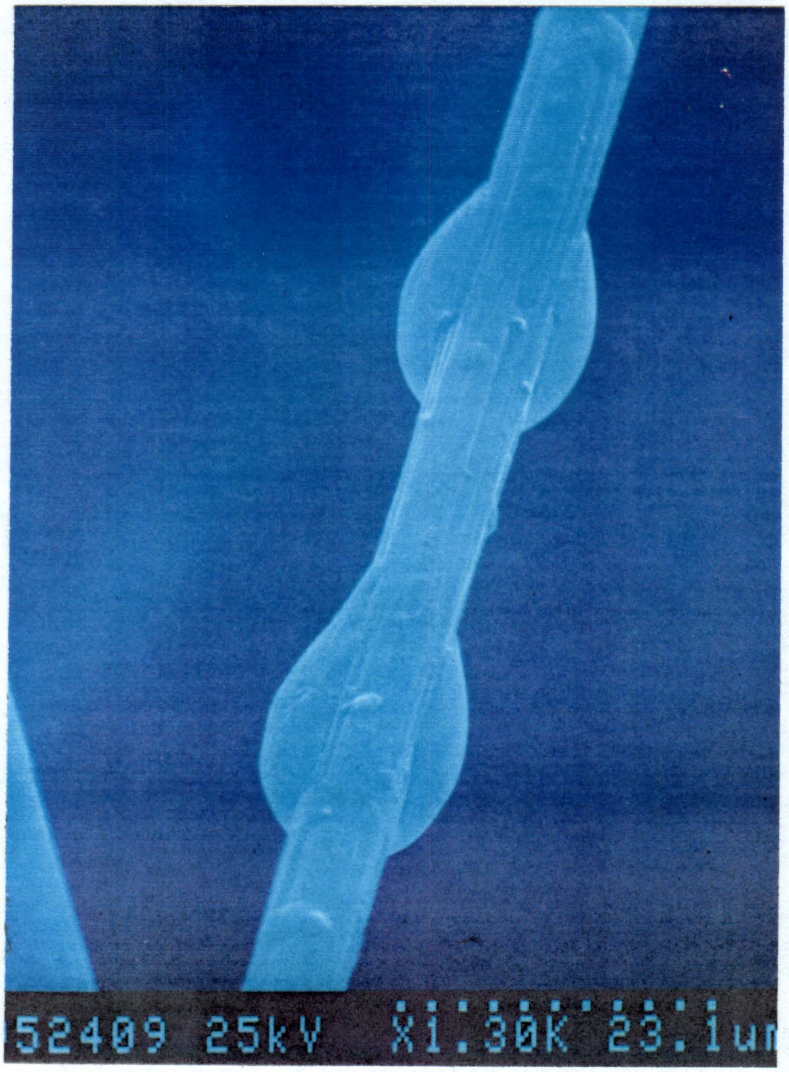


Figure C-3. SE micrograph of inter-fiber penetration by PLGA/ chloroform solution into the multi-filament bundle of carbon fibers. (bar = 75 μm)

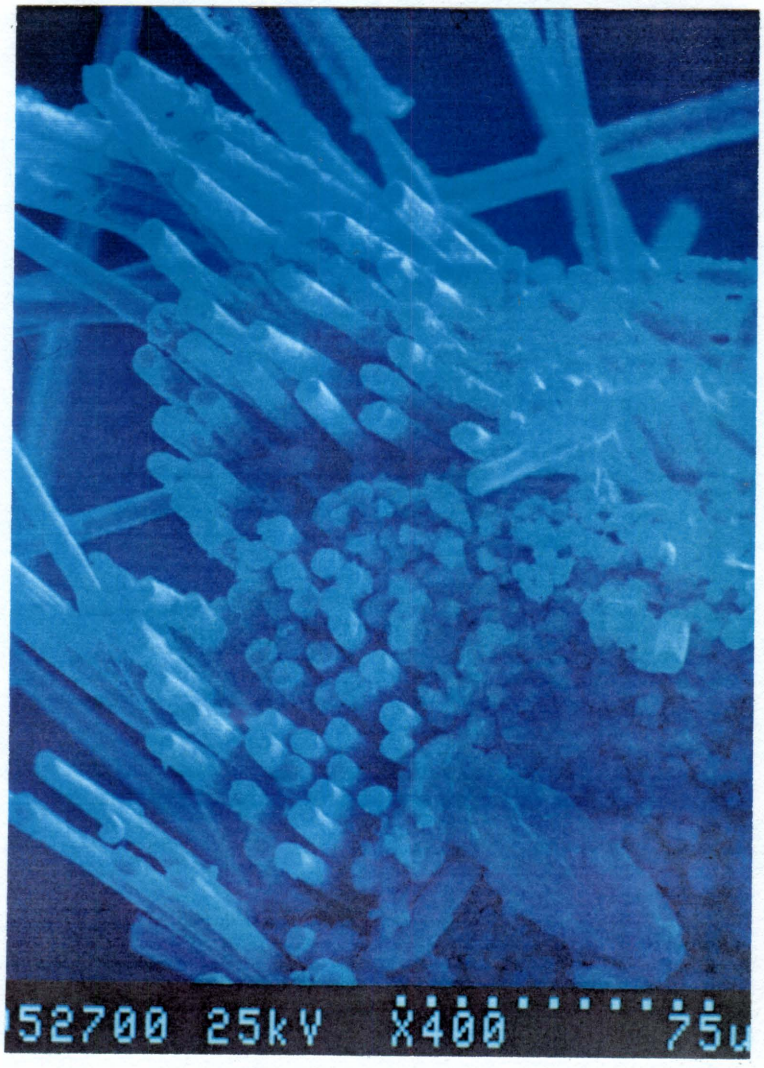
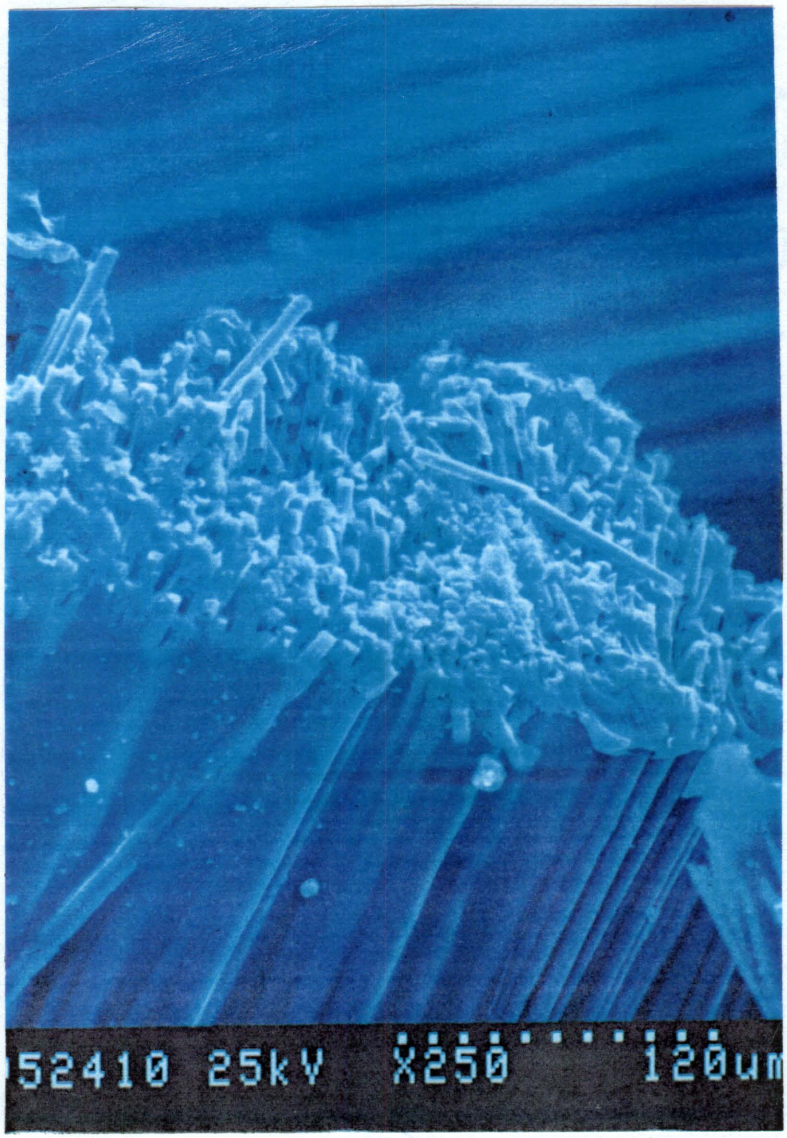


Figure C-4. SE micrograph of the PLGA sheath around the carbon fiber bundles forming a polymer-only surface on the composite. (bar = 120 μm)



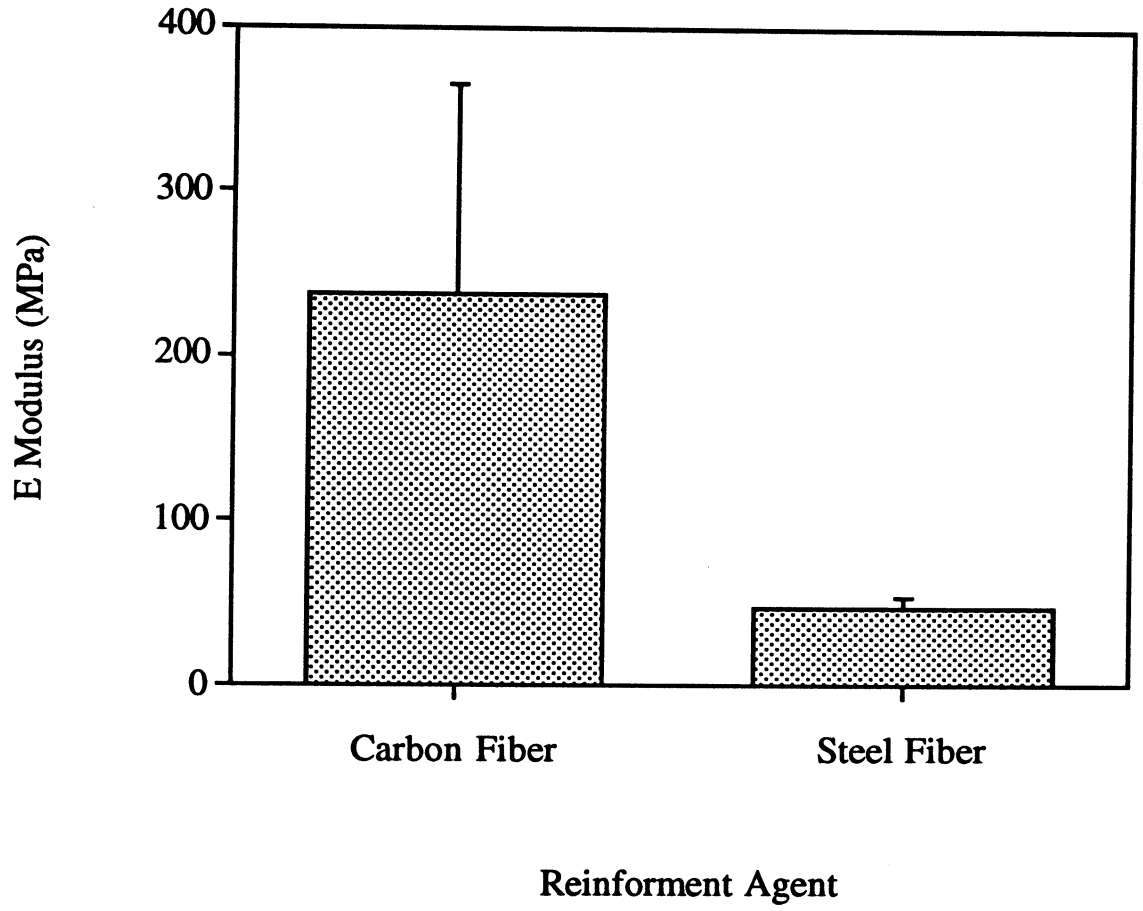


Figure C-5. Elastic modulus of bending (E Modulus) for carbon fiber- and steel fiber-reinforced composites with PLGA matrix. Values were calculated from a 4-point bending test (ASTM D790-92) and reported as mean ± s.d. (n=4). Carbon fibers were used as the multi-filament model and the steel-fibers were used as the single-filament model for composite processing.

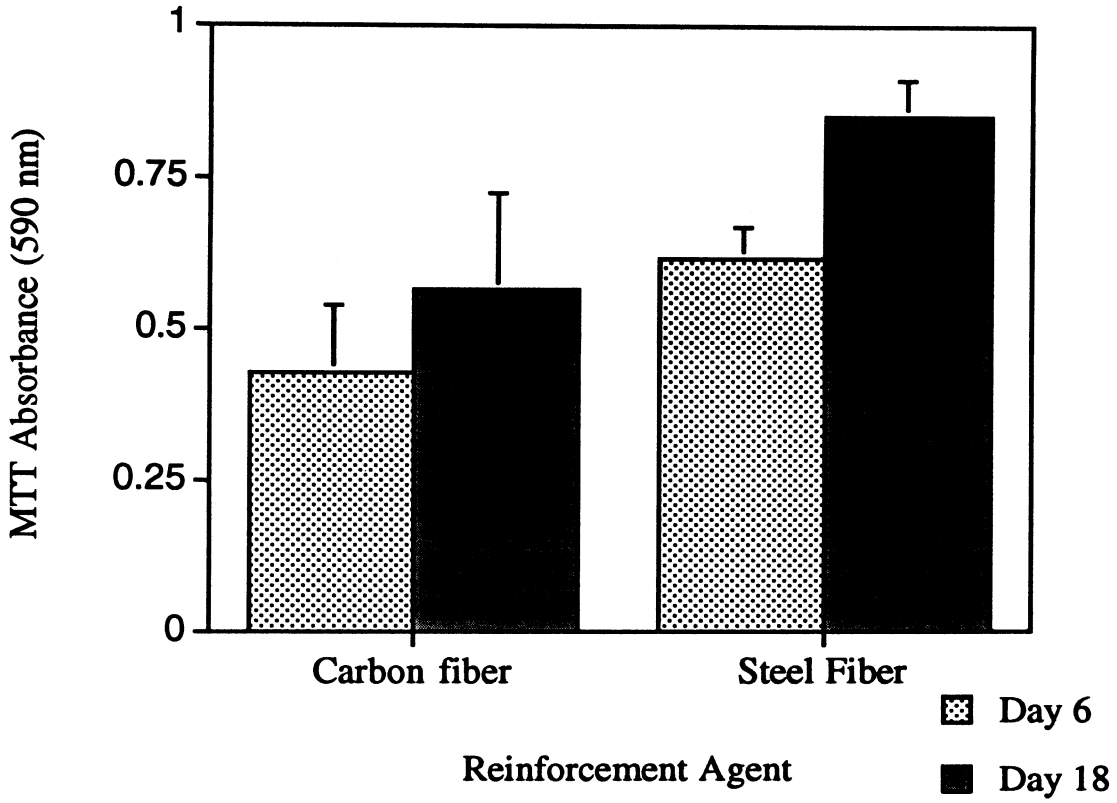


Figure C-6. Positive absorbance readings of MTT uptake by viable marrow osteoblast-like cells cultured on fiber-reinforced composites at days 6 and 18. Background absorbance from composites without cells was subtracted as blanks. Results show cell survival on both carbon fiber and steel fiber reinforced PLGA. Mean ± range are presented (n=2).

2) Cell Culture

Cell viability was shown with the MTT test for days 6 and 18 of culture, normalized to controls of composite without cells (Figure C-6). Samples were rinsed and removed to new wells before the dye was released and solubilized with isopropanol/ DMSO. Presence of converted MTT dye verified enzymatic activity in and viability of the cultured cells.

D. Discussion

Processing of the multi-filament composite was less reproducible than that for the single-fiber model; the total weight and E modulus found were less uniform for these composites. This was due to the difficulty in handling the brittle carbon fibers since binding and penetration were satisfactory based on SEM studies. A large batch processing set up for making the polymer-fiber prepegs would reduce fraying of the fibers. Ease of handling would increase the reproducibility for improved production of more uniformed multi-filament fiber-polymer composites.

The processing methods used to make the single-strand or multi-filament fiber reinforced composites can easily be adapted for manufacturing other soluble biocompatible thermoplastics with a variety of fiber reinforcing agents, such as titanium or ceramics. Osteoblast proliferation and function on the composites need to be studied further to determine if the behavior of the cells are dependent only on the matrix or if the reinforcement agents affect cellular behavior. This is especially important when using a degradable matrix. Based on the cell culture test, these composites can be used for short term *in vitro* cultures and offer a potential alternative as scaffold for growing bone cells.

APPENDIX D

TOTAL RAT MARROW EXTRACT AS CELL SOURCE

A. Experimental Design

75:25 PLGA foams (~90% porosity, 150-300 μm pore diameter, 6 mm diameter, 2 mm thickness) were seeded directly with marrow cells flushed from adolescent rats (50.0 x 10^5 cells / cm^2 top surface area, all cells between 6-32 μm in marrow extract). Complete media with 10% calf serum and 100 nM dex was provided to allow cell growth and provide necessary ascorbic acid and β-glycerol phosphate to induce marrow cells with osteoblastic phenotype. This study was done to test if total bone marrow extract can be directly plated onto the scaffolds to form bone-like structures *in vitro* without *ex vivo* expansion and removal of hematopoietic cells.

B. Results and Discussion

Confocal microscopy showed a 3-D arrangement of the cells in all 6 weeks old cultures (Figure D-1). Histology sections stained with H and E confirmed that osteoblastic cells in the cultures survived to attach, proliferate and deposit matrix along the scaffold pores (Figures D-2). The majority of the cells and tissue were around the surface of the scaffold as in 3-D cultures with other osteoblastic cell sources. Mineralization visualized from the von Kossa staining method was also concentrated on the outer perimeter of the scaffolds (Figures D-3).

Polymer scaffolds seeded with the entire marrow extract formed mineralized bone-like tissue *in vitro*. This finding suggests that entire marrow extracts may also have potential for growing bone-like tissue *in vitro* and in bone regeneration strategies.

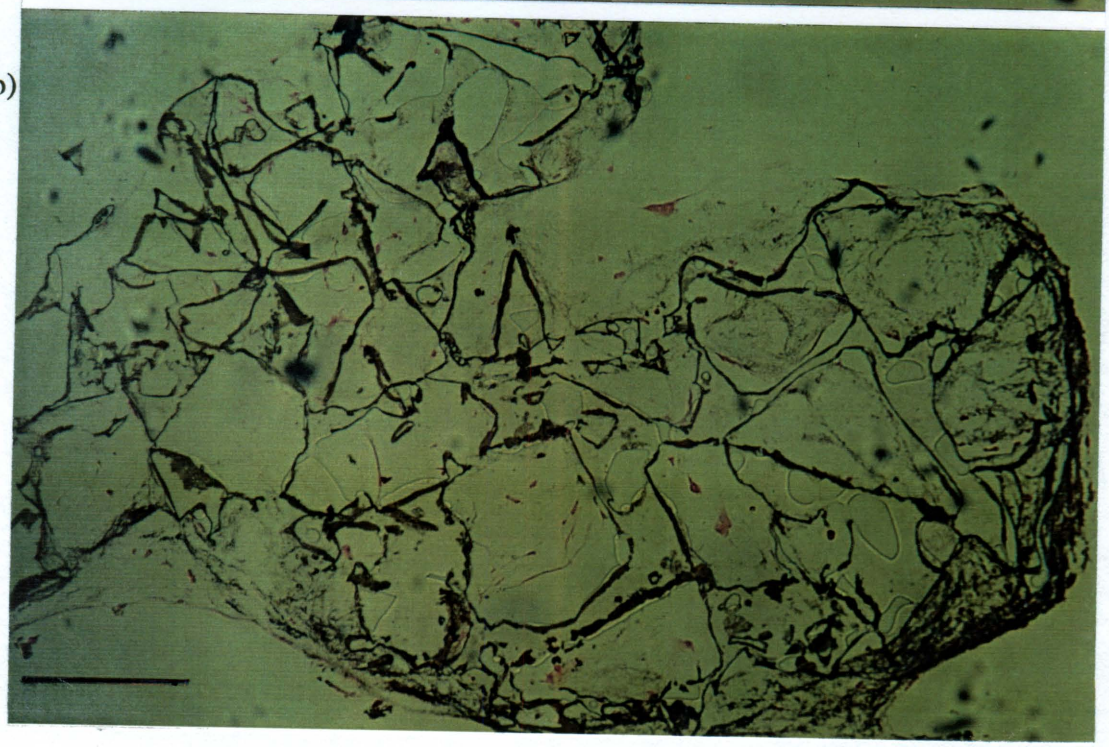
However, for the other studies presented in this work, well characterized osteoblastic cell sources were used to examine the effects of mechanical loading on osteoblasts cultured on the 3-D polymer scaffolds.

Figure D-1. Confocal micrograph of three dimensional culture seeded with rat marrow extract at 100x. Cells were stained with BCECF-AM for visualization.

Figure D-2. Histology preparations of cell/polymer construct seeded with total rat marrow extract. Sections were stained with (a) H and E to show cell and matrix deposit in the 3-D cultures after 6 weeks or (b) von Kossa to show mineral deposit. (bar = 80 μm)

(a)

(b)



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