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Osteoinductivity of Demineralized Bone: A Quantitative In Vitro Assessment

Yihong Zheng
Old Dominion University

OSTEOINDUCTIVITY OF DEMINERALIZED BONE: A QUANTITATIVE IN VITRO ASSESSMENT

by

Yihong Zheng
Bachelor of Medicine, July 1988, Shanghai Medical University, China

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Approved by:

Lloyd Wolfinbarger, Jr., Ph.D (Director)

Keith A. Carson

Edgar Stillwell

ABSTRACT

OSTEOINDUCTIVITY OF DEMINERALIZED BONE: A QUANTITATIVE *IN VITRO* ASSESSMENT

Yihong Zheng
OLD DOMINION UNIVERSITY, 1991
Director: Dr. Lloyd Wolfinbarger, Jr.

Acid demineralized bone (DMB) implanted in extraosseous sites induces bone formation. *In vivo* studies have shown that DMB stimulates the differentiation of mesenchymal cells into osteogenic cells. In this study an *in vitro* system was developed to demonstrate the osteoinductivity of DMB quantitatively. This *in vitro* working system was used to assess the osteoinductivity of variously processed DMB products as a means to evaluate their processing protocols. A human dermal fibroblastic (HDF) cell line was initiated from tissue explants as a working model, along with a human periosteal (HPO) cell line as a control. Ground DMB processed by LifeNet Transplant Services was added to HDF cell cultures and changes in cell growth and functional characteristics were monitored over a 7-day incubation period. Cell proliferation rate decreased, as indicated by decreased total cell number and decreased rate of [^{3}H]-thymidine incorporation. Cell size decreased, using the amount of protein content per cell as a measure. Alkaline phosphatase (AP) activity also increased. These characteristics of stimulated HDF cells listed above are more typical of HPO cells rather than HDF cells used as a control, suggesting that DMB had stimulated HDF cells to display phenotypic characteristics

typical of osteogenic cells.

AP activity was chosen as a quantitative measure for osteoinductivity of various kinds of DMB products because it has been reported to be an exclusive indicator of osteoinducing cells and has been widely used as a marker for osteogenic cells. AP activities of HDF cells showed no significant difference when cultured with demineralized bone and ethanol-pretreated demineralized bone, indicating that ethanol pretreatment does not affect the osteoinductive potential of DMB.

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I. INTRODUCTION

Bone transplantation has become a routine surgical procedure in cases when a skeletal defect (caused by trauma, infection, degeneration, developmental malformation or neoplasia) occurs. There are three types of bone grafts: autografts, allografts and xenografts. Autografts are grafts from the same individual, allografts are grafts from different individuals of the same species, and xenografts are grafts from different species or nonbiological materials. Allografts are by far the most frequently used implants due to their better availability compared to autografts. They are superior to xenografts in that they resemble the original tissue physiologically. Usually a graft serves not only as a physical substitute for the defect but also as a triggering factor for bone regeneration.

Three major mechanisms are suggested to be responsible for the process of bone regeneration after a graft is transplanted: osteogenesis, osteoconduction and osteoinduction. Osteogenesis (Covey et al., 1989) occurs when viable osteoblasts and preosteoblasts are autogenically transplanted to form a new center of bone formation. Such cells can be provided by cancellous bone and marrow grafts. Osteoconduction (Covey et al., 1989; Glowacki and Mulliken, 1985) is the process of ingrowth of new bone from the recipient bed into the scaffold of an implant. Osteoinduction (Glowacki and Mulliken, 1985) is the transformation of connective tissue into osseous tissue in the

presence of inducers. Physiologically, osteoinduction occurs in response to native extracellular substances of calcifiable or calcified tissues such as epiphyseal cartilage or bone matrix. In other conditions, osteoinduction may occur when migratory mesenchymal cells are in contact with dentin, gall bladder epithelium, calcified connective tissue (Bigi and Ripaminti,1988; Ohgushi et al., 1988; Huggins and Urist, 1970), as well as synthetic materials.

Acid demineralized bone (DMB) is a commonly used allograft material. After being treated chemically, DMB is supposed to be free of calcium. It is widely used in periodontal medicine in powder form and in orthopaedics in forms of segments and chips. The special feature of DMB is that it has osteoinductive activity, in addition to the osteoconductive ability that is common in many other implants.

Urist, in 1965, demonstrated that DMB induced ectopic new bone formation in the soft tissue of experimental animals. He reported that after the implantation of DMB, connective tissue cells (mainly fibroblasts and macrophages) infiltration of the implant was observed, followed by the appearance of chondrocytes, which in turn differentiated to show osteoblast-like characteristics. This phenotypic change suggested that fully differentiated fibroblast cells retained transformability that could be elicited by inducers. It has been confirmed by many similar *in vivo* experiments (Howes et al., 1988; Lovell et al., 1989; Wientroub and Reddi, 1988; Wientroub et al., 1990; Reddi and Huggins, 1973; Huggins and Urist, 1970).

The hypothesis, as can be formulated from these *in vivo* studies, is that mature fibroblastic cells grown *in vitro* can differentiate into osteogenic cells upon the stimulation of DMB.

Because DMB is being used more than ever before, and tissue banks all over the world are processing bone, unfortunately with many different protocols, there is a need to establish a quantitative assay to analyze the osteoinductivity of DMB. This assay could then be used as a means to evaluate the validity and efficiency of the different processing protocols. The ultimate goal of banking of bone grafts is to provide safe materials while preserving the osteoinductivity of the materials to the maximum possible extent.

The objective of this study, therefore, was to establish a fibroblast cell culture model as a working system and to develop a quantitative assay as a measure of osteoinductivity of DMB *in vitro*, and to use this cell line model and the assay to evaluate the osteoinductivity of various kinds of DMB powder. Development of a quantifiable system for use in assessing osteoinduction should provide a means of evaluating different protocols used for preparation of DMB.

II. LITERATURE REVIEW

Bone Structure And Development

Bone is a special connective tissue that provides a physical framework and protection for the body. Its hardness is due to the deposition of minerals, mainly calcium, phosphate and carbonate, onto the relatively soft extracellular organic matrix.

Bones are classified according to their shapes into four types: long bones, short bones, flat bones and irregular bones. Their basic structures are the same: a dense outer layer and a spongy inner part with a medullary cavity in the center filled with bone marrow. The dense bone tissue is frequently referred to as compact or cortical bone, and the spongy bone as cancellous or trabecular bone. Outside the compact bone is the periosteum, a layer of dense connective tissue with nerves and blood vessels passing through it. A very thin layer of connective tissue, endosteum, lines the marrow spaces of bone.

The cells of bone proper are of three types: osteoblasts, osteocytes and osteoclasts. Osteoblasts are active bone-forming cells. They secrete osteoid or matrix vesicles, which form the organic constituent of the bone containing collagenous fibers

and ground substances. Matrix vesicles are membrane-bound and enriched in phospholipids, phosphate, alkaline phosphatase, pyrophosphatase, magnesium and ATP. When osteoblasts are eventually surrounded by their own secretions, which by then are calcified, they cease certain cytoplasmic activities and retreat to a relatively inactive state. At this point they are designated as osteocytes. Osteocytes are the principal cells of mature bone tissue. They can be reactivated to function as osteoblasts in fracture-healing processes when the surrounding calcified substances (or trabecula) are disrupted. Osteoclasts, unlike other osteogenic cells, are monocytes in origin (Vaughan, 1981). Their major function is to resorb the bone tissue and therefore they are engaged in bone remodeling and regulating the calcium level in the body. In periosteum, there are nerve cells, fibroblasts, osteoprogenitor cells and osteoblasts. Osteoprogenitor cells are derived from uncommitted mesenchymal cells and are destined to become osteoblasts. Fibroblasts are by far the largest population in connective tissue and are believed to retain the potential of differentiation into cells of other types even in adults (Reddi and Huggins, 1973, 1972; Huggins and Urist, 1970). The cells in endosteal linings are osteoblasts, but they are hardly distinguishable from the bone marrow cells, which are of two stem cell lineages: osteoprogenitor cells and hemopoietic cells, the latter predominating. Hemopoietic cells are the progenitors of leukocytes and erythrocytes.

The hardness of bone and its ability to withstand stress is attributed to the organization of organic and inorganic components in the extracellular matrix. The organic components are mostly proteins, of which 95% are type I collagen fibers and the

rest consist of proteoglycans, glycoproteins and various noncollagenous proteins that function as bone growth regulatory factors (Amenta, 1990). The collagens in bone are secreted as procollagens by osteoblasts and converted to collagens extracellularly by a procollagen aminoprotease, which removes the amino propeptides, and a procollagen carboxyprotease, which removes the carboxy propeptides. Collagen fibers are made of tropocollagen macromolecules. They align parallel to each other and give the ground substance an orderly appearance. A tropocollagen macromolecule is a right-hand triple helical polypeptide containing three left-hand helices. The stability of this conformation depends on the presence of proline and hydroxy proline. These molecules are rigid cyclic imino acids which limit the rotation of the polypeptide backbone and so contribute to the stability of the triple helix. Tropocollagen molecules are staggered longitudinally to form collagen fibers with spaces between the end of one triple helix and the beginning of the next. These so called "holes" may serve as nucleation sites for hydroxyapatite formation, as will be discussed later. The collagen plays a structural role in the bone.

Of the inorganic components, calcium, phosphate and carbonate are the major constituents, with lesser quantities of sodium, magnesium, potassium, chloride and fluoride. They are present as a mixture of hydroxyapatite crystals, amorphous calcium phosphate and other inorganic salts. The crystalline structure of hydroxyapatite provides the firmness and hardness of bone. It is believed that mineral deposits initially as amorphous calcium phosphate, which later becomes hydroxyapatite crystal (molecular formula: $Ca_6 (PO_4)_{10}(OH)_2$) alongside the collagen fibers. The surface ions of the

hydroxyapatite crystals are hydrated as a shell of water around the crystal+collagen combination, thus providing an efficient ion exchange environment (liquid) with the extracellular matrix. The abundance of calcium in the bone matrix permits it to serve as a calcium reservoir for regulation of the level of calcium ion in blood and tissue fluid.

Bone formation or osteogenesis takes place in two modes: intramembranous and endochondral. The essential process is the same: An organic matrix, the osteoid, is laid down by osteoblasts. This matrix becomes calcified with the deposition of amorphous and crystalline apatite. The bones of the calvarium of the skull are formed by intramembranous ossification whereas the basal bones of the skull and the majority of the bones of the skeleton are formed by endochondral ossification. Both processes may occur simultaneously at different locations within a single bone.

Intramembranous bone formation: In the fetus, a well-vascularized mesenchyme develops in presumptive regions where osteogenesis will occur. The stellate-shaped mesenchymal cells proliferate and organize to form a primordial mass of cells, which differentiate into osteoblasts. This process is characterized by the differentiation of intracellular structures necessary for matrix synthesis and a marked increase in alkaline phosphatase (AP) activity (Zernik et al., 1990). The matrix vesicles secreted by osteoblasts are later calcified when hydroxyapatite is deposited on it (Vaughan, 1981). As osteoblasts are gradually trapped within the calcified matrix they become osteocytes and the secretory function decreases. As a consequence the sizes of the cells are also

decreased. This leaves a space, the lacuna, around each osteocyte. Lacunae associate with one another via canaliculi, the spaces left by the retreat of the processes of the former osteoblasts. Since the osteoblasts are trapped simultaneously, the lacunae from the same generation of cells are lined up in a linear fashion, forming an osseous lamella. The formation of lamellae at the inner surface of a compact bone takes place along the primary marrow space, as the space is lined by a layer of osteoblasts and contains blood vessels and mesenchymal cells. The trapping of osteoblasts and the successive formation of lamellae occur in a concentric manner. The whole structure, the concentric lamellae and central canal with its blood vessels and associated connective tissue, is called a Haversian system or an osteon. As osteoblasts are trapped in the matrix, a new generation of osteoblasts, formed from division or differentiation of mesenchymal cells along the surface of the matrix, develops to maintain a constant layer of bone-forming cells. Osteoblasts and osteocytes actively participate through production of insoluble calcium phosphate, utilizing a mechanism involving mitochondria and extrusion of calcium salt from the cells, as will be discussed later.

Endochondral bone formation: this mode of bone formation involves the replacement of preformed cartilage with bone. As the cartilage model grows, the chondrocytes in its center hypertrophy, as a result of the breakdown of the proteoglycans of chondrocytes. This breakdown makes many free anionic sites available to bind calcium ions and thus facilitates the deposition of minerals within the matrix. The mineralization or calcification restricts the passage of nutrients to chondrocytes, causing

them to die. At the same time, some cells of the perichondrium (dense fibrous connective tissue surrounding cartilage) differentiate into osteoblasts, which secrete osteoid and eventually cause the perichondrium to calcify. This process gives rise to a thin plate of compact bone called periosteal bone collar, which is surrounded by the periosteum. A periosteal bud, consisting of osteoblasts and blood vessels, invades the disintegrating center of the cartilage model. Once in the center, the osteoblasts secrete osteoid, and an ossification center is established. This process continues until chondrocytes stop division and the whole cartilage model becomes calcified.

Bone is a dynamic tissue constantly remodeling itself to meet the functional changes of stress and strain resulting from growth, changing patterns of exercise and repair of wounds, as well as the structural changes occuring during embryonic development. The process of remodeling requires the removal of portions of existing bone followed by the addition and calcification of matrix in order to fit the evolving characteristics of a particular region in an ideal scheme. The removal or resorption of bone is spearheaded by the invasion of osteoclasts which are accompanied by blood vessels and perivascular connective tissue elements. The enzyme acid hydrolase secreted by osteoclasts dissolves hydroxyapatite and the collagenases secreted by fibroblasts digest collagen fibers. As resorption takes place, the advance of osteoclasts and their associated structures into the resorbing cavity creates a channel, called an erosion tunnel. Once an erosion tunnel has reached a certain size, the osteoclasts disappear and in their place osteoblasts differentiate from the perivascular connective tissue. Osteoblasts begin to lay

down new bone matrix along the inner wall of the erosion tunnel. The retreat of osteoclasts and the reappearance of osteoblasts may be a coupled process under the regulation of growth factors. As these resorption and formation processes go on, bone constantly adjusts itself to the needs of the body.

Regulatory Factors of Bone Remodeling

Bone remodeling is a complex process regulated by many local growth factors as well as systemic factors (hormones). These factors are small peptides that may act on cells of the same type (autocrine factors) or of different type (paracrine factors). They are mostly studied *in vitro*. Because bone is a heterogeneous organ, the growth factors present in the matrix may be secreted either by osteogenic cells or cells from adjoining tissue. Some of them may be systemic factors trapped in the matrix (Table 1). It is the local factors that play a critical role in regulating bone remodeling either by exerting their own effects or by mediating the effects of systemic factors (Canalis et al., 1988). Among these factors the most intensively studied are transforming growth factor ß (TGF-ß), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF) and bone morphogenetic protein (BMP). Because they show important biological effects on cultured bone cells, they are likely to have physiological significance in regulating bone growth *in vivo*. The following sections are devoted to the effects of individual local factors in cultured tissue. All factors except BMP can be secreted by both bone cells and extraosseous cells.

Table 1 Systemic Factors Affecting Bone Remodeling

Factors	Effects
parathyroid hormone (PTH)	couples the process of bone formation to bone resorption by enhancing the activity of local bone growth factors[1].
calcitonin	inhibits bone resorption by antagonizing PTH effect[2]; stimulates bone formation by increasing osteoblast activity, promotes collagen synthesis and mineralization; increases alkaline phosphatase activity[3].
Vit D	maintains calcium and phosphate homeostasis, participates in mineralization by increasing calcium transport through cell membranes[4]; small amount promotes bone formation while large amounts promote bone resorption[2].
Vit A	promotes chondrocyte functions; stimulates synthesis of lysosomal enzymes for osteoclast activity[5].
Vit C	essential for collagen synthesis[5].

1. Canalis etal., 1988
2. Guyton, 1986
3. Yamaguchi et al., 1989
4. Aralo et al., 1991
5. van de Graaff and Fox, 1988

TGF-ß is a polypeptide with a molecular weight of 25,000 daltons consisting of two subunits linked together by disulfide bonds. It is synthesized by many tissues, but bone and platelets are the major sources. TGF-ß is encoded by at least three genes: TGF-ß1, TGF-ß2 and TGF-ß3. TGF-ß1 and TGF-ß2 consist of two identical subunits. They exist in bone matrix and are likely to be present in bone cultures. TGF-ß1 is about 70% homologous with TGF-ß2 in amino acid sequence and they have very similar biological effects. TGF-ß3 is a heterodimer consisting of a TGF-ß1 and a TGF-ß2 chain. TGF-ß3 has not been reported in bone tissue. TGF-ß has been studied in many cell types (Kanda et al., 1990; Kanda et al., 1989; Kurokowa et al., 1986; Masui et al., 1985). In general it is a primary differentiation-inducing agent that promotes the proliferation and differentiation of progenitor cells and inhibits the growth of mature cells (Sporn et al., 1987; Masui et al., 1985). In bone tissue, TGF-ß stimulates DNA synthesis and cell replication. Cells of osteoblastic lineage are among the most sensitive to its mitogenic effect. TGF-ß also stimulates collagen synthesis, by increasing the number of osteoblasts and by regulating posttranscriptional mechanisms (Canalis et al., 1988; Sporn et al., 1987). TGF-ß has a major function in bone formation either by its own function or by association with other factors. Its release from bone matrix is increased by hormones that induce bone resorption such as parathyroid hormone (PTH). In addition, PTH enhances the binding of TGF-ß to osteoblastic receptors, and these two effects may be critical in the coupling of bone formation to bone resorption. TGF-ß is secreted in a latent form and can be activated in low pH conditions, such as may be associated with wound healing and bone resorptive environments (Graves and Cochran, 1990).

Fibroblast growth factor (FGF) is a polypeptide growth factor of two forms: acidic (aFGF) and basic (bFGF). aFGF has an isoelectric point of 5.6 to 6.0 and a molecular weight of 15,000 daltons, whereas bFGF has an isoelectric point of 9.6 and a molecular weight of 14,000 to 16,000 daltons. Both are single chain proteins which bind to the same receptors and their biological activities are similar (Mohan and Baylink, 1990; Canalis et al., 1988). However, they are the products of two different genes. Their best known functions are to induce endothelial cell proliferation and neovascularization. In bone tissue cultures, FGF stimulates DNA synthesis and cell proliferation. It also increases type I collagen synthesis, indicating that at least some of the cells of osteoblastic lineage are affected. There has been no report that FGF induces differentiation. The mitogenic effect of FGF can be enhanced by heparin, a substance that is abundant in callus of a bone fracture. These observations may lead to the conclusion that FGF plays an important role in wound healing, an idea that is further supported by the fact that FGF is only secreted after cell injury or death. No effects of FGF on bone resorption have been reported.

Platelet-derived growth factor (PDGF) is a highly cationic protein (isoelectric point: 9.8) with a molecular weight range from 28,000 to 35,000 daltons, depending on the site of proteolytic cleavage. These differences in molecular weight do not appear to affect its biological functions. PDGF is a chemotactic factor that attracts mesenchymal cells to wounds (Howes et al., 1988), and a mitogen to all cells of mesenchymal origin, including osteoblasts (Mohan and Baylink, 1990). PDGF stimulates cell growth by

stimulating resting cells in G_0 to enter the cell cycle at a point in G_1. PDGF also induces the target cells to secrete other growth factors that stimulate cells to progress from G_1 to S phase (Graves and Cochran, 1990). There is evidence that PDGF stimulates bone resorption via a mechanism that may involve prostaglandin synthesis (Mohan and Baylink, 1990; Canalis et al., 1988).

Insulin-like growth factor (IGF), also called somatomedin, is a growth hormone dependent peptide. Two structures have been identified: IGF-I and IGF-II. IGF-I, or somatomedin C (Sm C), is a basic peptide (isoelectric point: 8.4) with a molecular weight of 7649 daltons that can be synthesized by many tissues, including bone and cartilage. IGF-II has not been found in bone tissue. IGF-I promotes bone formation by two mechanisms: (1) enhancing the osteoblastic activity by increasing the secretion of bone matrix; (2) increasing the number of osteoblasts by stimulating the differentiation of osteoprogenitor cells (McCarthy et al., 1990; Canalis et al., 1988). It has no effect on bone resorption (Canalis et al., 1988).

Bone morphogenetic protein (BMP) was originally named for the acid-insoluble proteins extracted from acid-demineralized bone matrix that can induce bone formation (Urist et al., 1979). It is now understood to be a group of glycoproteins, BMP1, BMP2 and BMP3, the latter is also called osteogenin, with molecular weights of 14 KD, 16KD, 18KD, 22KD and 30 KD (Wang et al., 1988; Sampath et al., 1987; Urist et al., 1984; Urist et al., 1979). These glycoproteins have similar but independent osteoinductive

abilities. In normal physiological conditions, usually in neutral pH range, BMP is thought to be degraded by an endogenous BMPase that functions at neutral pH and temperatures between 37 to 40 C (Hirano and Urist, 1991; Vaughan, 1981). In acidic conditions, such as in the acid demineralization process and bone resorption process, BMPase is degenerated and BMP is activated. The major function of BMP is to stimulate osteoblastic maturation (Yamaguchi et al., 1991). It can also induce ectopic bone formation *in vivo* and *in vitro* (Wang et al., 1990; Katagiri et al., 1990). This shows the therapeutic potential of BMP in that it may promote *de novo* bone formation in humans (Wang et al., 1989) and therefore is of direct clinical interest.

Biochemistry of Mineralization

Mineralization occurs when calcium phosphate compounds deposit on extracellular matrix. This deposition is a necessary step in normal osteogenesis as well as in ectopic bone formations. Nucleation sites are required for mineral deposition because the calcium phosphate molecule itself is too small for aggregation. Zimmermann et al. (1991) summarized that these nucleation sites can be osteoid, phospholipids, necrotic cells and cell remnants, in addition to the "holes" in the collagen fibers, which for a long time were considered the only nucleation sites (c.f. Vaughan, 1981).

As described earlier, the components of matrix vesicles include small amounts of proteoglycans in addition to collagen fibers and glycoproteins. Proteoglycans are

negatively charged glucosaminoglycan chains covalently attached to a core protein, which plays a crucial role in the function of the compound (Klein-Nulent et al., 1987). Proteoglycans inhibit calcification by binding to free calcium ions, hence promoting calcium release from bone tissue (Klein-Nulent et al., 1990; Cochran ĕt al., 1990; Cochran, 1987; Tenorio et al., 1987). Fibroblasts and chondrocytes are the most productive among the many types of connective tissue cells that can produce proteoglycans. In endochondral osteogenesis and extraosseous soft tissue calcification (usually after injury), degradation of proteoglycans is observed (Klein-Nulent et al., 1990). This degradation may result from the proteolytic activity of the degenerated cells. As proteoglycans degrade, calcium ions are released. This event may facilitate the subsequent calcification process. The small amount of proteoglycans left in mineralized tissue are believed to be forms more resistant to hydrolysis.

The calcium ions needed in normal bone formation are provided by osteoblastic mitochondria, which trap and concentrate the intracellular calcium in the form of calcium phosphate crystals. Electron microscopic evidence shows that these calcium phosphate compounds are located in the intramitochondrial granules. Phosphates, on the other hand, are prepared from organic phosphate esters via the transphosphorylation action of alkaline phosphatase (AP) (Müller et al., 1991; Beertsen and van den Bos, 1991). It remains unclear where and how calcium and phosphate bind to each other. It is believed that magnesium and ATP levels may regulate the storage and use of calcium phosphate (Vaughan, 1981). In the process of matrix vesicle calcification, the membrane of the

vesicles are degraded after the vesicles bud off from the osteoblasts. This degradation causes the loss of magnesium, an inhibitor for apatite formation, and therefore initiates the deposition of amorphous calcium phosphate (reviewed by Vaughan, 1981).

AP is a polymorphic glycoprotein. Three gene loci encode two tissue-specific isoenzymes (intestinal and placental) and a third nonspecific isoenzyme found in liver, kidney, bone and circulatory system (Ragougy-Sengler et al., 1990; Fishman, 1990). Its natural substrate is unknown, although ß-glycerol phosphate is considered a good *in vitro* candidate and is widely used as a media supplement for bone cell cultures (Beertsen and van den Bos, 1991; Tenenbaum et al., 1989). Inorganic phosphates, as the products of the enzymatic reaction, are the natural inhibitors of AP. AP is a good marker for osteoinductive potential in cells. This potential can be verified in three aspects: (1) AP activity is clearly detectable in condensed mesenchymal cells prior to osteoid or mineral deposition during embryonic bone formation (Zernik et al., 1990); (2) High AP activity is a common biochemical feature of osteoinductive cells while in non-osteoinductive cells AP is invariably low (Wlodarski and Reddi, 1986); (3) In hypophosphatasia, an inherited disease resulting from defects in synthesis and formation of liver/kidney/bone isoenzyme of AP, patients show symptoms characteristic of defects in skeletal calcification. AP may also play a role in calcium deposition by directly affecting collagen fibers (Guyton, 1986).

Induced Bone Formation and DMB

Bone formation in extraosseous sites or ectopic ossifications can be induced in many circumstances. Atherosclerosis, arthritis and scar formation are the most common examples seen under natural pathological conditions. Ectopic ossification can also be induced artificially by osteoinductive cells (Ohgushi et al., 1988; Huggins, 1930), synthetic or natural calcium phosphate compounds (Grégoire et al., 1990; Santier et al., 1990), DMB (Reddi and Huggins, 1972, 1971; Urist, 1965), BMP (Wang et al., 1988; Sampath et at., 1987; Urist et al., 1979), and many other substances. These osteoinductive materials are used clinically for defect repairing and reconstruction in orthopaedic and periodontal medicine.

DMB implantation was reported more than 100 years ago, when Senn (1889) used acid-treated chips from ox tibia to repair cranial and long bone defects in his patients. The first DMB-induced extraosseous bone formation was reported by Urist in 1965. In that study DMB segments were implanted intramuscularly in the abdomens of rabbits, rats, mice and guinea pigs. Initially it was observed that circulating macrophages, fibroblasts and other cells of connective tissue origin infiltrated the implants. After partial resorption of matrix and vascularization of the area, chondroblasts were observed in the newly deposited matrix and they gradually differentiated, demonstrating osteoblast-like characteristics. The cells which transformed into osteogenic cells were believed to have been fibroblasts, as reported in an earlier study (Huggins, 1930). Upon stimulation,

the phenotypic expression of these responding fibroblast-like cells was obviously altered (Reddi and Huggins, 1972) with elevated AP activity (Reddi and Huggins, 1972b; Huggins and Urist, 1970).

The sequence of biochemical events after subcutaneous implantation of DMB includes the conversion of connective tissue into cartilage, which becomes calcified, invaded by host vessels and replaced by bone (Reddi and Huggins, 1972). This sequence is similar to the processes that occur in embryonic osteogenesis, in the elongation of long bones, and in the healing of fractures. The prominent event in induced osteogenesis occurs at the gene level in target cells. The mechanism of this phenotypic conversion is unknown.

The osteoinductivity of DMB is thought to be attributed to two factors: (1) a chemotactic-like factor that attracts blood vessels as well as mesenchymal cells and osteoprogenitor cells (if implanted in bony tissue) from the immediate vicinity; (2) a factor that induces mesenchymal and progenitor cells to transform into either chondrocytes or osteoblasts. BMP, as it is acid-insoluble, may be well preserved in DMB, and may serve in either or both of these events. There may also be other growth factors (e.g., TGF-ß) in the matrix and cell remnants that survived weak hydrochloric acid in the decalcification processes.

Fibroblast-like cells will transform into osteoblasts or chondroblasts in response

to the stimuli of DMB, depending on the oxygen tension and the nature of the compressive forces in the surrounding area, although chondroblasts will eventually degenerate and be replaced by osteoblasts (Bernick et al., 1989). Low oxygen tension favors chondrocyte formation while high oxygen tension favors osteoblast formation.

III. MATERIALS AND METHODS

Initiation and Maintenance of Cell Cultures

1. Human Dermal Fibroblastic Cell Line (HDF)

A human dermal fibroblastic cell line (HDF) was established as the working model. The tissue explants used to start the culture were obtained from the dermal tissue of the foreskin of 3-day-old infants during routine circumcision.

After the dermal tissue was dissected from the surgically removed foreskin, it was cut into 0.5 cm x 0.5 cm pieces and rinsed 3 times with Dulbecco's phosphate buffered saline solution (DPBS) supplemented with antibiotic mixture (final concentration: 1% penicillin/streptomycin and 0.1 mg/ml lincomycin), at room temperature, 10 minutes for each rinse. The pieces were transferred into T-25 tissue culture flasks containing 2 ml of Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotic mixture (final concentration: 1% penicillin/streptomycin and 0.1 mg/ml lincomycin). The flasks were inverted and placed into a 5% carbon dioxide humidified incubator. After the cells migrated out of the tissue and attached to the surface (approximately one week), the flasks were turned back to their regular position.

The medium was changed once each week. Upon reaching confluence the primary cultures were trypsinized and subcultured. The media were poured from the flasks and the cells were rinsed with 2 ml of warm trypsin (Gibco, 0.05% trypsin + 0.53 mM EDTA, 37 °C). They were then incubated with 2 ml of warm (37 °C) fresh trypsin for 2 minutes. Light microscopy (magnification: x100) was used to determine when most of the cells were dislodged from the surface of the T-25 flasks. After gently shaking the flasks, 2 ml of medium (with 10% FBS) was added to stop the trypsin reaction. The cell suspension (total volume: 4 ml in each T-25 flask) was transferred into a 15-ml centrifuge tube and centrifuged at 2000 rpm for 5 minutes. The supernatant was discarded and the cell pellets were resuspended in fresh maintenance medium (see below) and evenly distributed to 3 to 5 new flasks. Maintenance medium (5 ml) was added to each flask, followed by incubation. The cells were maintained in DMEM + 10% FBS, with medium changed twice a week, trypsinized and subcultured in 1:3 or 1:5 ratios upon achieving confluence. The antibiotic mixture was omitted in the medium after the first subculture. All the procedures were performed under sterile conditions. Cells used in experiments were from passage 6 to passage 10.

2. Human Periosteal Cell Line (HPO)

A human periosteal cell line (HPO) was initiated from freshly procured femoral periosteum of an adult cadaver donor, as a control osteogenic cell line model. The tissue was collected from the donor by the staff at LifeNet Transplant Services, a company that

processed donated tissues and organs, within 24 hours of death.

The procedure for starting this culture was identical to that of fibroblasts, except that α-modified MEM was used in place of DMEM.

Characterization of Cell Lines

1. Morphological Evaluation of Cell Lines

The morphological characteristics of HDF and HPO cell lines were determined and photographed with an Olympus FMT-001 microscope.

2. Characterization of Cell Lines in Culture

Cell growth data was derived for both HDF (passage 3 and 6) and HPO (passage 6) cell lines. Cells of passage 3 were seeded into T-25 flasks at a density of 1×10^5 cells per flask and maintained in the culture conditions as described above. At appropriate time intervals (12 hours for HDF cells and 24 hours for HPO cells) replicate flasks (3) of cells were harvested by trypsinization and the reaction was stopped by fresh medium. The number of cells in the cell suspensions were counted with a hemacytometer. The growth curves were derived by plotting the average number of cells against incubation time.

3. Alkaline Phosphatase Activities

AP activity in cell extracts was determined using a modification of the procedure described by Maranto and Schoen (1988). Cells cultured in T-75 flasks were dislodged by scraping in the presence of 1.5 ml of deionized (DI) water, after 1 rinse with 3 ml of DI water. The cell suspensions were sonicated 30 seconds at 30% intensity with a VirSonic Cell Disrupter (Model 16-850). n-Butanol (2 ml) was added to the sonicate and the mixture was vortexed. After centrifugation at 2500 rpm for 10 minutes, the aqueous layer (bottom) containing cell extracts was harvested for AP assays. Each AP assay was initiated by adding 0.8 ml 0.15 M 2-amino-2-methyl-1-propanol (pH 10.4) buffer solution to 1 ml of cell extract. The well-mixed solution was then equilibrated at 37 °C for 5 minutes, after which 0.2 ml of 100 mM of p-nitrophenylphosphate (PNPP) substrate (final concentration: 10 mM) was added. This solution was incubated at 37 °C for 30 minutes and the reaction then was stopped by adding 30 ul of 1 N NaOH. The absorbance was measured at 405 nm with a Beckman (Model 25) spectrophotometer. Authentic p-nitrophenol (PNP) was prepared in 0.15M 2-amino-2-methyl-1-propanol buffer diluted 1:2 with DI water, as standards (0.1 umole/ml, 0.2 umole/ml, 0.3 umole/ml, 0.4 umole/ml, 0.5 umole/ml) and the absorbance values were converted to the equivalent amount of PNP. Aliquots of cell extracts were used in BCA (Pierce) assays to determine the protein concentrations. The data were expressed as units of AP activity per gram of cell protein. One enzyme unit is defined as the activity that would convert one umole of PNPP (or, yield 1 umole of PNP) per minute at 37 °C.

To determine the kinetics of the AP from cell extracts, assays were carried out using different concentrations of substrate (final concentration: 0.3 mM, 0.9 mM, 1.5 mM, 2.1 mM and 3 mM, respectively, 3 replicate assays at each concentration). Each reaction mixture was placed in the Beckman spectrophotometer for 30 minutes and the absorbance was recorded continuously by an attached chart recorder. The changes of absorbances remained linear within 30 minutes at all the substrate concentrations. The reaction rates (the slopes of the changes of absorbances, abs/min, or converted to unit AP) were thus obtained and they were normalized to unit milligram of cell protein. The reaction rates were plotted against the substrate concentrations to generate a Michaelis-Menten kinetic curve. The double reciprocal plot of Michaelis-Menten curve was used to determine the specific Km and Vmax values. The concentration of substrate used in later experiments was chosen based on the calculated Km value such that substrate concentration was at or near saturation levels.

4. [^{3}H]-thymidine incorporation

HDF cells were seeded at the density of 1.5 x 10^5/T-25 flask and cultured in 8.5 ml of the described maintenance medium for 2, 4 and 6 days before the incorporation experiments. On the days indicated the media were changed to 2 ml of F10 nutrient mixture containing [^{3}H]-thymidine (final concentration: 5 uCi/2.74 umole/ml) and the cultures were incubated for 4 hours at 37 °C in the CO_2 incubator. After 4 hours, the media were carefully removed and 2 ml of 10% trichloroacetic acid (TCA) was carefully

added to the flask to cover the attached cells. This procedure was followed by 3 rinses of DPBS, 5 ml for each time, with care being taken not to dislodge precipitated nucleic acids. The precipitate was finally dissolved in 1 ml of 1 N NaOH for 90 minutes at 60 °C. Aliquots of solution were used for scintillation counting and for assays to determine the protein concentration. Each aliquot used for scintillation counting was added to 7 ml of scintillation fluid (ScintiVerse, Fisher Scientific) and counted by a Beckman liquid scintillation counter. The counting errors were below 5%. Control [^{3}H]-thymidine (0.025 uCi/ 0.0137 umole in 5 ul) was counted and used to convert the counts per minute(cpm) values to equivalent amounts of thymidine. Data are expressed as the amount of thymidine incorporated by unit amount of cell protein (umole/mg).

Preparation of Ground Human Bone

The bone to be used in this study was provided by LifeNet Transplant Services (LN, 5809 Ward Court, Virginia Beach, VA 23455), and was equivalent to that bone material currently utilized in the production of the ground, demineralized bone product provided for clinical use. Bone, procured by LN staff from cadaver donors shortly (less that 24 hours) after death, was stored frozen at -84 °C until processed. For processing, the bone was thawed at room temperature, mechanically crushed to remove soft tissues such as bone marrow and periosteum, ground using a Telsa Analytical Bone Mill, sized (250-710 um in diameter), and refrozen. Ground bone was stored frozen until used in experiments. When used, all bone tissue was first thawed at room temperature. All the

procedures described were performed under aseptic conditions in order to insure sterility of bone products used in the cell culture assays.

1. LifeNet-Processed DMB

Specific details of the processing protocol are considered by LifeNet to be proprietary and are not available for disclosure in this document. DMB product provided by LifeNet was used in this study as an osteoinductive agent to the HDF cells and as a "standard" for the comparison to bone processed by known (described) procedures. In general terms, DMB produced by LifeNet involves the following processing steps. Ground frozen bone was thawed and washed in 100% ethanol (EtOH) overnight prior to demineralization. Demineralization was accomplished by stirring the bone in 0.5 N HCl solution for two to three days, with the last soak overnight. The acid extraction solutions were changed by centrifugation of the bone particles and resuspension in fresh acid. After demineralization, the bone was washed in distilled/deionized water 3 times, 30 minutes for each wash, followed by one 30-minute wash in potassium phosphate buffer solution. Then it was washed twice in water, after which the bone was freeze-dried under vacuum for 7 days. Aliquots were sealed under vacuum in stoppered vials.

2. Ethanol Pretreatment and Lipid Content Determination

Ground bone was stirred in 100% EtOH at room temperature in the ratio of 10

ml of EtOH to 1 gram of bone (Lee, 1990). This process consisted of 5 extractions, 60 minutes for each, followed by 3 washes in sterile deionized water, 30 minutes for each wash. Prior to changing solvents the mixtures were centrifuged at 2000 rpm for 5 minutes and the soaking solutions were saved and stored at 4 °C for determination of lipid content. After ethanolic extraction and washing the bone powder was immediately dried in a freeze drier (Labconco, model 4451F).

Cholesterol standards were prepared at 0.5, 1.0, 1.5 and 2.0 grams per 100 ml (expressed as weight per volume percentage) of 100% EtOH for use in calculation of lipid extracted from bone. Although absolute ethanol may be expected to extract a variety of compounds, of which lipids would constitute a relatively large percentage, cholesterol was chosen to provide a relative measure of the lipids extracted because it possesses a relatively good absorbance maximum at 290 nm. This absorbance maximum was determined using a scanning spectrophotometer (Beckman, model 26) scanning between 200 nm to 400 nm. As will be shown later, the ethanolic extracts of bone possessed similar absorbance spectra as cholesterol and thus it is suggested that although absorbance at 290 nm in bone extracts may measure compounds other than cholesterol, it permits a relative assessment of "lipids" extracted from bone. A cholesterol standard curve was derived by plotting absorbance values versus cholesterol concentrations. Concentrations of "lipids" in the samples were calculated using the curve. The total "lipid" extracted from one bone sample was the sum of the lipid contents in all 5 soaking solutions.

3. Mineral Extraction and Calcium Content Determination

Minerals were extracted from ground bone with 0.5 N HCl. The ratio of the amount of sample to solvent was 1 gram to 10 ml. The bone was constantly stirred in the HCl solution during the extraction and washing cycles. The process consisted of 5 successive extractions with fresh HCl solution, 60 minutes for each extraction, and 3 washes (60 minutes for each) in deionized water. Prior to changing solvents, the mixtures were centrifuged at 2000 rpm for 5 minutes and the extraction solutions were saved and stored at 4 °C for determination of calcium content. After extraction and washing the ground bone was immediately freeze-dried.

An Orion calcium-specific electrode, a reference electrode and a voltage meter (Corning, pH meter 125) were used to determine the calcium ion concentration in the extraction solutions (Lee, 1990). Calcium chloride standards at 0.01 M, 0.03 M, 0.05 M, 0.07 M, 0.1 M, 0.4 M, 0.6 M, 0.8 M and 1 M were prepared in 0.5 N HCl. The relative potential between the two electrodes in each standard solution was read and a standard curve was derived as millivolts versus concentrations of calcium chloride. The voltages of each bone extraction solution were read and converted to calcium concentrations. The total calcium extracted was the sum of calcium content in all five soaking solutions.

Total calcium content in fresh bone prior to demineralization was determined by

ashing the samples (about 1 to 1.5 gram each) in a muffle furnace (Sybron, Thermolyne 2000) at 700 °C for 5 hours (Bernick et al., 1989) after being oven-dried at 100 °C for 1 hour (American Scientific , Model DX-58). The residues were digested in 8 ml of 4 N HCl at 95 °C until completely dissolved. After cooling the solutions were diluted 1:8 with deionized water and their calcium content was determined as described using the calcium-specific electrode. The bone samples were weighed before and after ashing.

Statistical Analysis

Each data point consists of at least 3 replicate assays. The data were tested for normal distributions. Linear regression analyses were used to derive standard curves and analysis of variance (ANOVA) was used to determine the significance among treatment groups. Multicomparison tests (Don and Tukey) were used for comparing means of more than two groups. SAS statistical programs were used for all the analyses. Significance level was assigned at 0.05.

IV. RESULTS

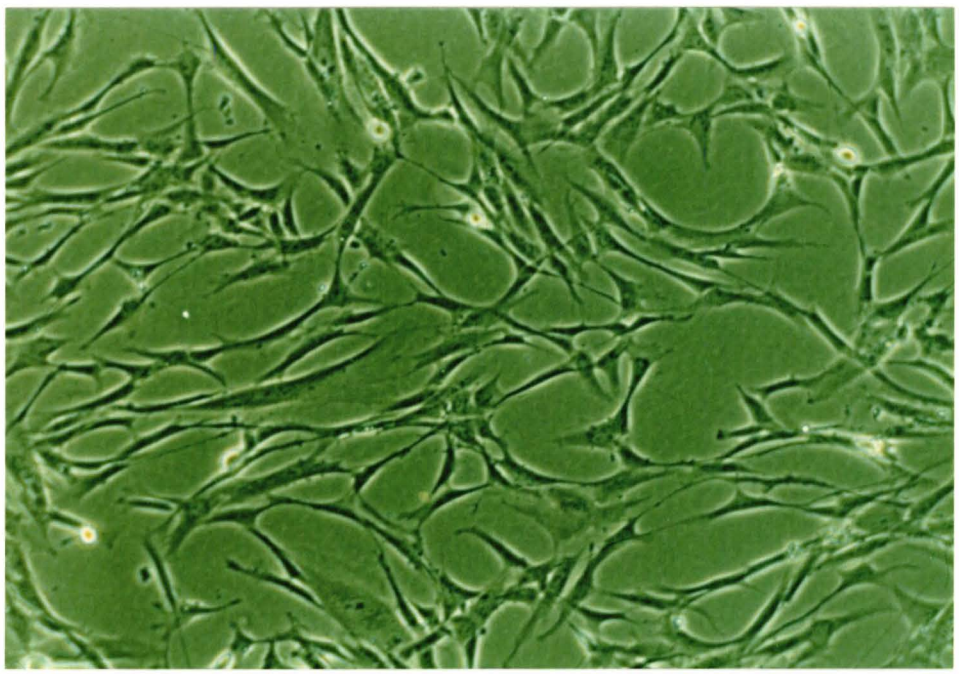
Characterization of Cell Culture Lines

1. Morphology

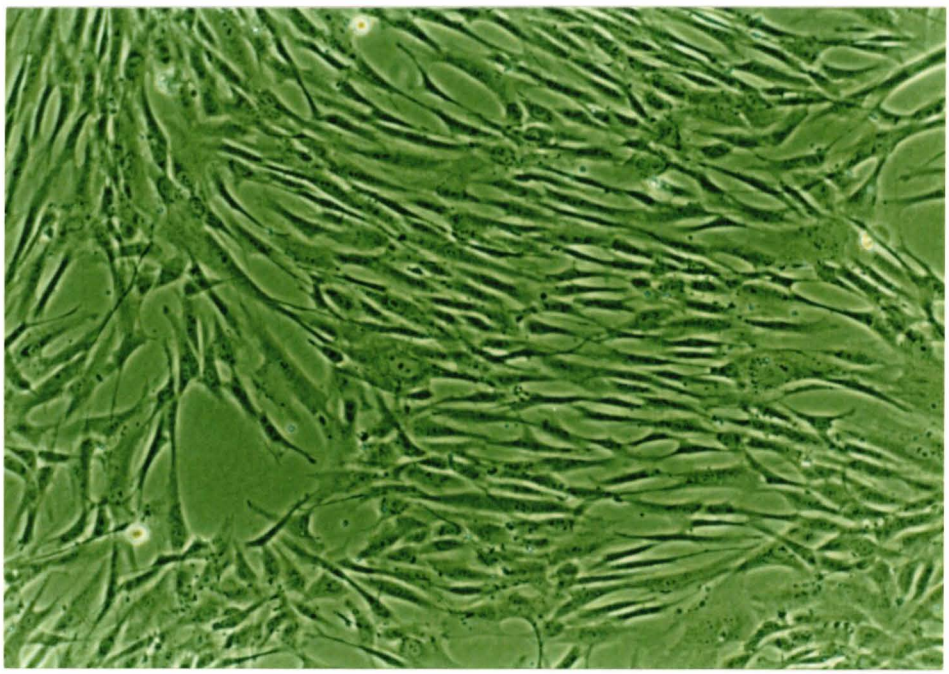
Using the light microscope, HDF cells could be seen as spindle- or stellate-shaped with fine homogeneous cytoplasm (Figure 1-a). The ovoid nuclei were pale compared to the cytoplasm and occasionally showed nucleoli. After confluence (Figure 1-b), cells became more elongated and tended to align as parallel arrays. Some cytoplasmic granules could be seen, and more nucleoli were observed. Confluence in this document is defined as the stage when cells are seen to physically contact each other and cover the available surface of the tissue culture flask.

HDF cells changed their shape when cultured in the presence of DMB (Figure 2-a), becoming larger, with more processes. Many cytoplasmic granules were seen even before confluence. Cells were less orderly aligned when they reached confluence (Figure 2-b), and nuclei were not easily visible either before or after confluence.

Fig.1 Light micrographs of human dermal fibroblast (HDF) cells cultured in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum, passage 4. Cells were spindle- or stellate-shaped with fine homogeneous cytoplasm. (a) On day 2. The ovoid nuclei are pale compared to the cytoplasm and nucleoli can be seen occasionally. (b) On day 3. Most cells reached confluence as shown in the center part of the photograph. Cells were more elongated and tended to align parallel to each other. Some cytoplasmic granules can be seen, and more nucleoli were visible. Magnification: x100.

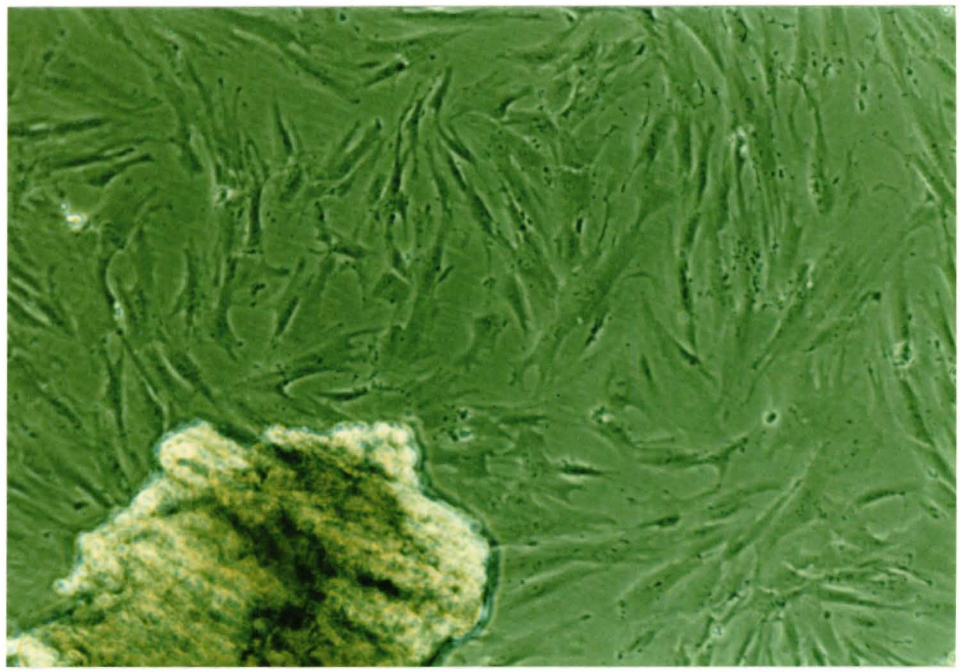


a



b

Fig. 2 Light micrographs of human dermal fibroblast (HDF) cells cultured in Dulbecco'smodified essential medium supplemented with 10% fetal bovine serum with the presence of demineralized bone particles, passage 7. Cells were larger and more dendritic, and nuclei were less easily visible than HDF cells cultured without demineralized bone particles. (a) On day 2. Many cytoplasmic granules appeared in the cytoplasm. One particle of demineralized bone can be seen in the lower left part of the photograph. (b) On day 5. Cells reached confluence. They were less orderly aligned compared to confluent HDF cells cultured without bone particles. Magnification: x100.



a

b

HPO cells appeared smaller than the HDF cells (Figure 3-a). They were also spindle or stellate in shape. The cytoplasmic area was pale and homogeneous without obvious granules either before or after confluence. The nuclear area of the HPO cells was darker than the cytoplasm, with no visible nucleolus (Figure 3-a,b). Confluent cells aligned in a less oriented manner (Figure 3-b).

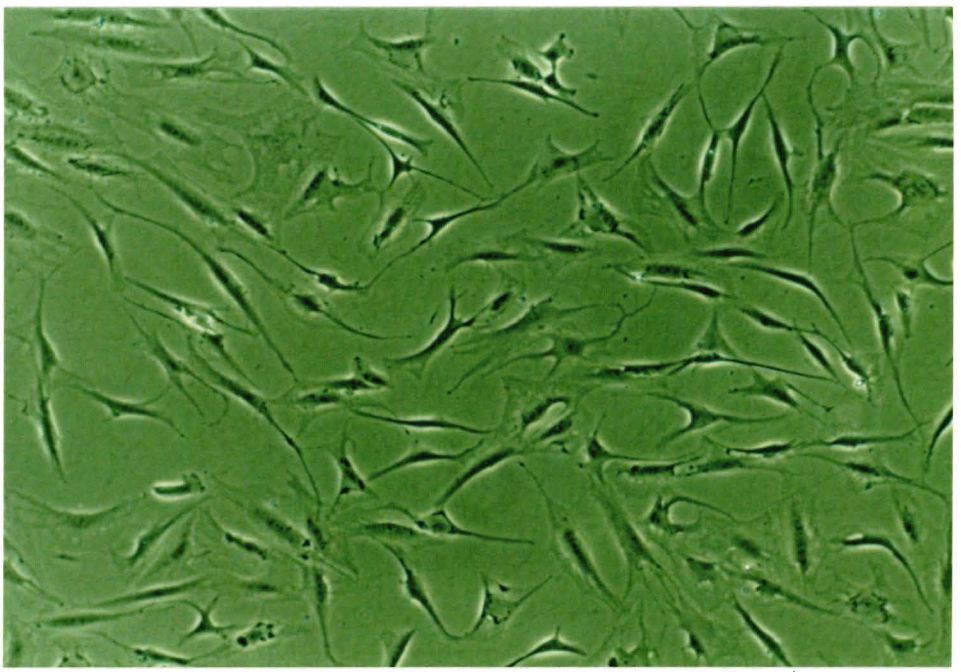
2. Growth Curve

Growth curves of HDF (Figure 4) revealed that at the seeding density of 0.4 x 10^4 cells/cm^2 in T-25 tissue culture flasks, the lag phase was 3 days followed by 2 days of logarithmic phase before reaching plateau (or saturation density). The saturation density was 8.7 x 10^4 cells/cm^2. The population doubling time was 17 hours during the logarithmic growth phase. Confluence of the cell culture was observed at day 4.

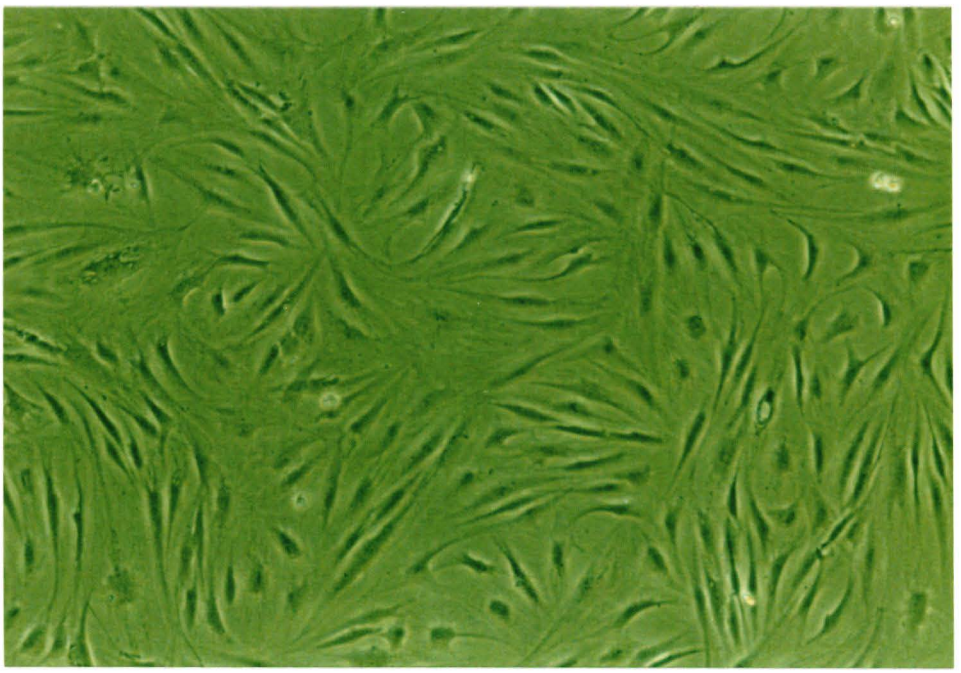
When subcultured at the density of 0.67 x 10^4 cells/cm^2 in T-75 flasks in the presence of 10 mg of LN-DMB, the HDF cells changed their growth characteristics (Figure 5). The lag phase was 2 days, followed by atypical logarithmic and plateau phases. The saturation density was 2.0 x 10^4 cells/cm^2. The population doubling time calculated during the atypical logarithmic phase was 33 hours. Cell confluence was reached at day 5.

The growth patterns of HPO cells were very different when compared to the HDF

Fig. 3 Light micrographs of human periosteal (HPO) cells cultured in α-modified essential medium supplemented with 10% fetal bovine serum, passage 5. Cells are spindle or stellate in shape. The cytoplasmic area appeared pale and homogenous without obvious granules. The nuclear area of HPO cells was darker than the cytoplasm, with no visible nucleolus. (a) On day 3. (b) On day 10. Confluent cells aligned in a less oriented manner. Magnification: x100.



a



b

Fig. 4 Growth curve of HDF cells (passage 3) cultured in DMEM supplemented with 10% FBS. Cells were seeded at 1 x 10^5/ T-25 flask. At every time interval 3 replicate flasks of cells were harvested by trypsinization and the numbers of cells were counted with a hemacytometer. Values represent the mean$\pm$SE. T_D designates population doubling time.

GROWTH CURVE OF HDF

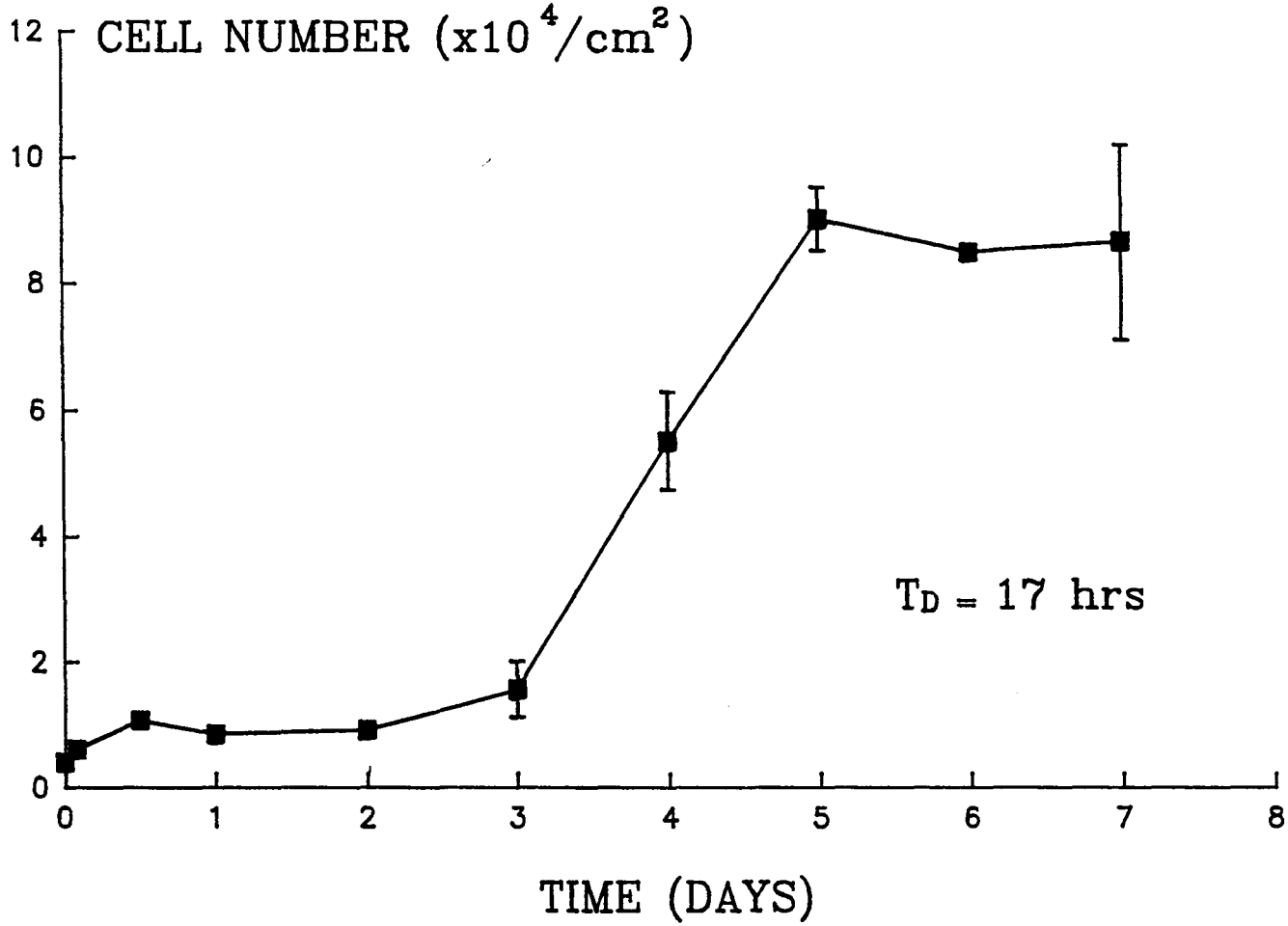
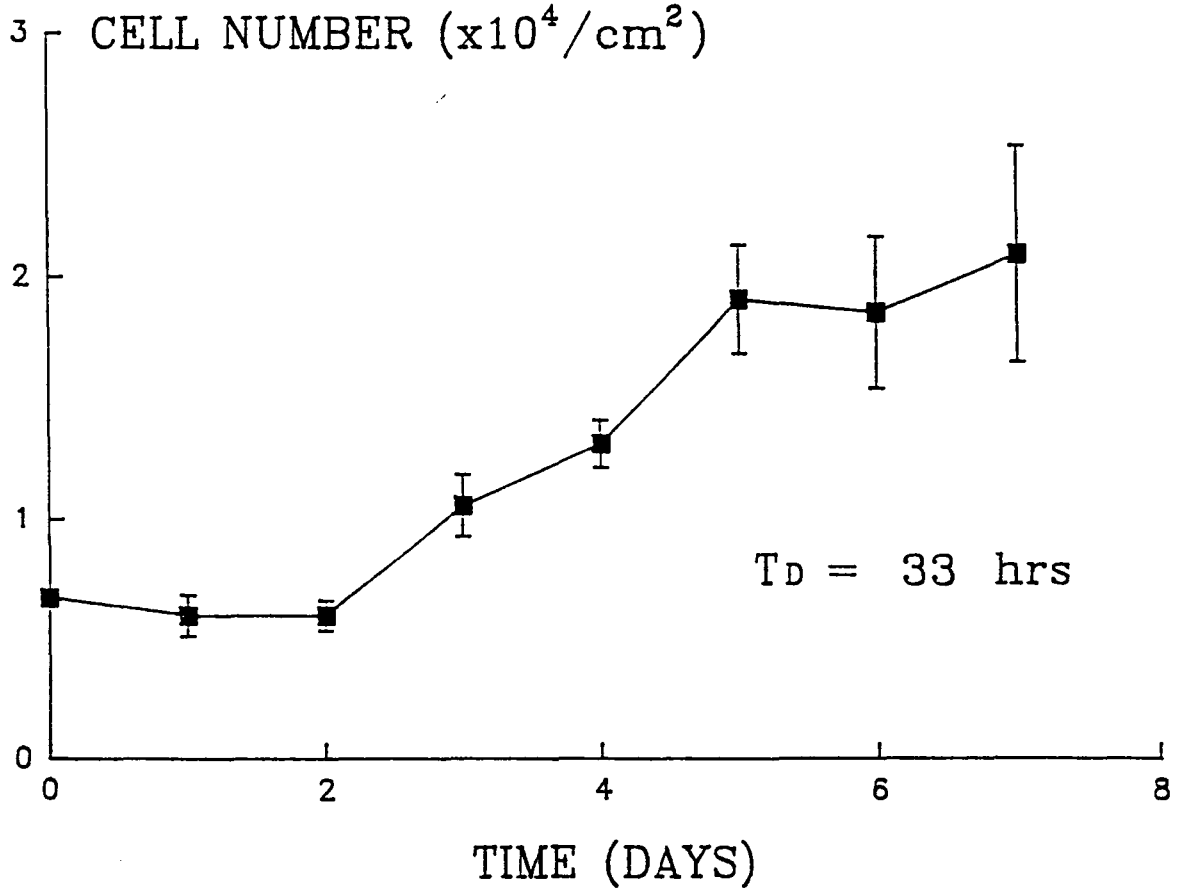


Fig. 5 Growth curve of HDF cells (passage 6) with the presence of 10 mg LN-DMB cultured in DMEM supplemented with 10% FBS. Cells were seeded at 5 x 10^5/T-75 flask. At every time interval 3 replicate flasks of cells were harvested by trypsinization and the numbers of cells were counted with a hemacytometer. Values represent the mean$\pm$SE. T_D designates population doubling time.

GROWTH CURVE OF HDF WITH LN-DMB



cells (Figure 6). As a mixed population, HPO cells displayed no distinct lag, logarithmic, or plateau phase. Seeded at a density of 0.67×10^4 cells/cm^2 in T-25 flasks, the cell number increased slowly but persistently, in a heterogeneous way. The cell density at the end of the observation period was 2.5×10^4 cells/cm^2. The population doubling time was calculated to be 215 hours and it was determined according to the trend over the entire observed period of 12 days. Cell confluence was observed at day 9 or day 10.

3. Cell Size Change and Cell Growth

While the cell numbers were counted each day during the period of cell growth, the protein content was also determined daily using the BCA protein assay. The protein content in each cell (designated as average cell size) was thus obtained by dividing the milligram of protein by total number of cells. This cell size value changed during growth for all the cell cultures (Figure 7). In general, all cells started from a large size and ended with a smaller one. In HDF cells, the size changed dramatically when the number changed dramatically (in logarithmic phase), and then stabilized when the cell numbers became stable. For HPO cells and HDF cells cultured with DMB, the average cell sizes decreased gradually as the cell numbers increased slowly, with no obvious quick-change phase or stable phase. The size of HDF was 0.94 ± 0.01 ng protein/cell at day 1 of growth and 0.12 ± 0.02 ng protein/cell at day 7. With the presence of 10 mg LN-DMB, the cell size at day 2 was 0.54 ± 0.03 ng protein/cell and then decrease to

Fig. 6 Growth curve of HPO cells (passage 6) cultured in α-MEM supplemented with 10% FBS. Cells were seeded at 5 x 10^5/ T-75 flask. At every time interval 3 replicate flasks of cells were harvested by trypsinization and the numbers of cells were counted with a hemacytometer. Values represent the mean$\pm$SE. T_D designates population doubling time.

GROWTH CURVE OF HPO

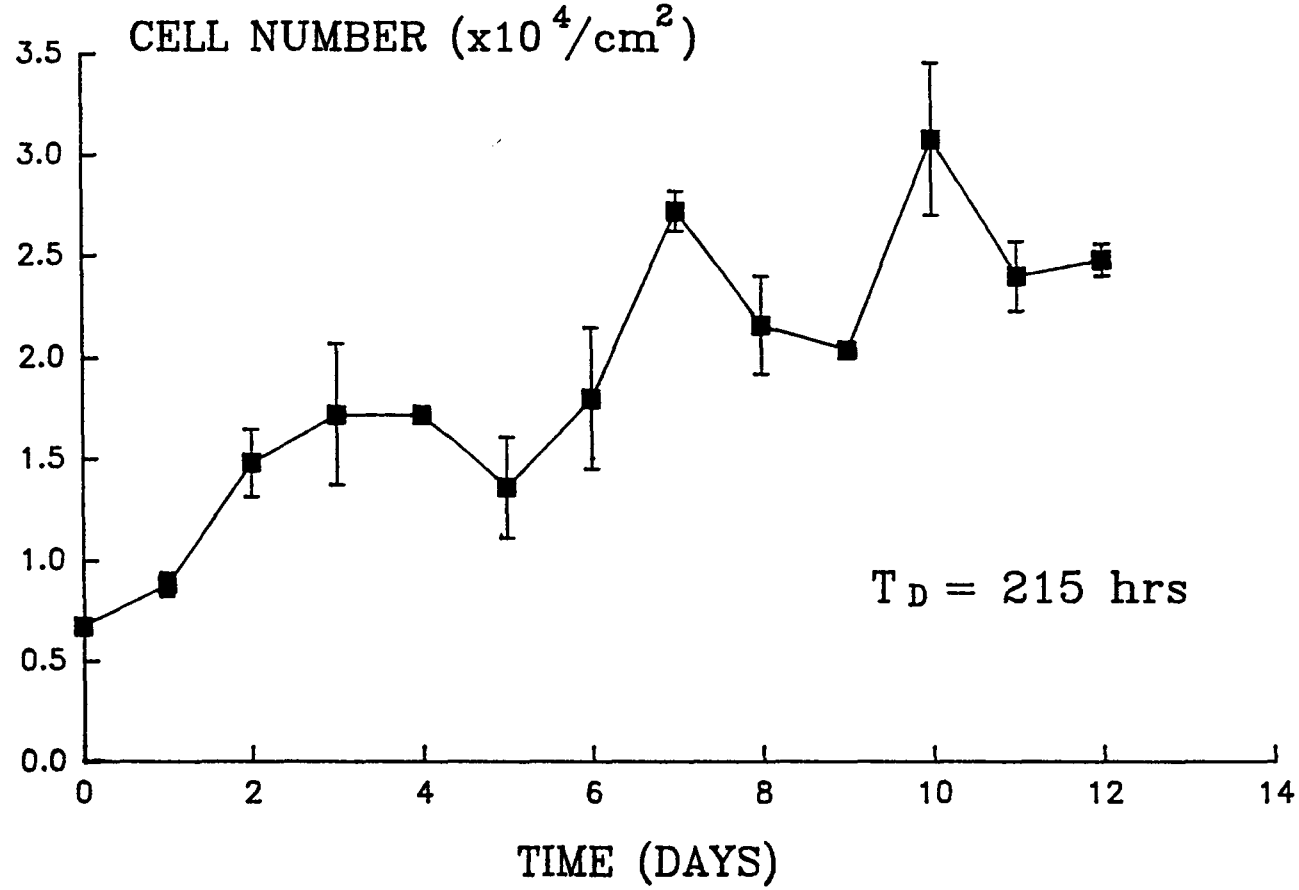
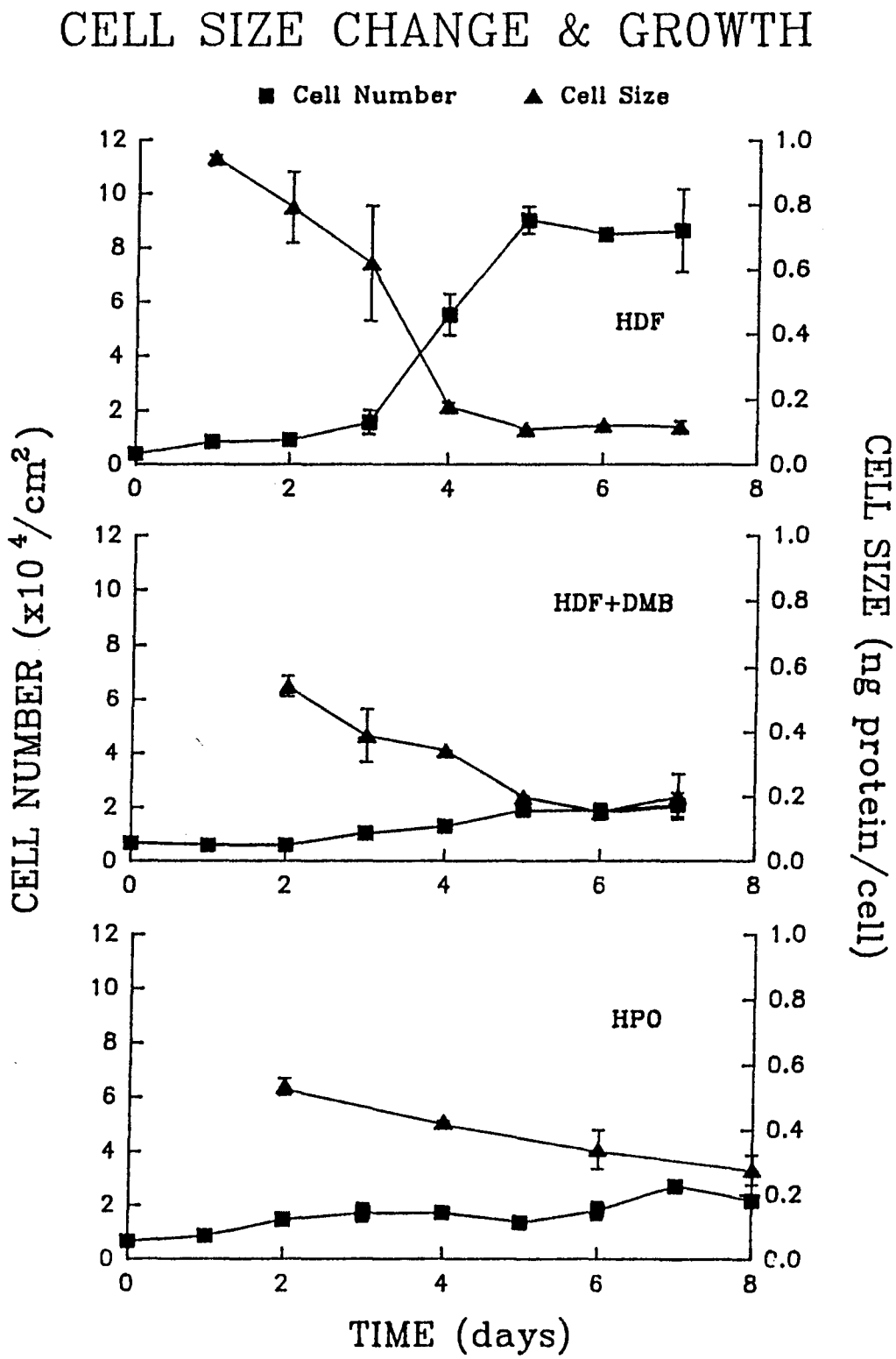


Fig. 7 Cell size change during growth. HDF cells were cultured in DMEM supplemented with 10% FBS (top). The same medium was used when LN-DMB was present (middle). HPO cells were cultured in α-MEM supplemented with 10% FBS (bottom). Cell size was designated by the amount of protein present per unit number of cells. Values represent mean$\pm$SE of 3 replicate assays.


CELL SIZE CHANGE & GROWTH
Cell Number
Cell Size
HDF
HDF+DMB
HPO
CELL NUMBER (x10⁴/cm²)
CELL SIZE (ng protein/cell)
TIME (days)
0
2
4
6
8
10
12
0.0
0.2
0.4
0.6
0.8
1.0

0.20±0.07 ng/cell at day 7. In HPO cells the size at day 2 was 0.53±0.03 ng protein/cell and 0.28±0.05 ng protein/cell at day 8 of growth.

[^{3}H]-Thymidine Incorporation

[^{3}H]-thymidine incorporation experiments were performed using HDF cells (seeded at 1.5 x 10^5/ T-25 flask) with and without the presence of 3 mg of LN-DMB after 2, 4 and 6 days of incubation (Figure 8). The amount of thymidine taken up by unit amount of cell protein was decreased significantly from day 2 to day 4 and from day 4 to day 6 in both control (HDF) and treatment (HDF+DMB) groups ($P<0.05$). At each day, the thymidine uptake rate was significantly lower in treatment (DMB) groups than in control groups ($P<0.05$). It was 63% of the control at day 2, 50% at day 4 and 65% at day 6 ($P>0.05$).

Alkaline Phosphatase Activity

1. AP Kinetics

Authentic p-nitrophenol (PNP) was used as standard for the AP assay (Figure 9). The kinetics of AP from HDF cell extract was determined by assaying for enzyme activity at different substrate concentrations. The reaction rates, (abs/min/g cell protein or unit/g cell protein) were plotted against their respective substrate concentrations and

Fig. 8 [^{3}H]-thymidine incorporation into HDF cells. [^{3}H]-thymidine was incorporated into HDF cells (seeding density: 1.5 x 10^5/ T-25 flask) cultured for 2, 4 and 6 days with and without the presence of 3 mg LN-DMB. The amount of thymidine taken up per unit amount of cell protein was decreased significantly from day 2 to day 4 and from day 4 to day 6 in both control (HDF) and treatment (HDF+DMB) groups ($P<0.05$). At each day, the thymidine uptake rate was significantly lower in treatment groups than that in control groups ($P<0.05$). Values represent mean$\pm$SD of 6 replicate assays.

[^{3}H]–THYMIDINE INCORPORATION

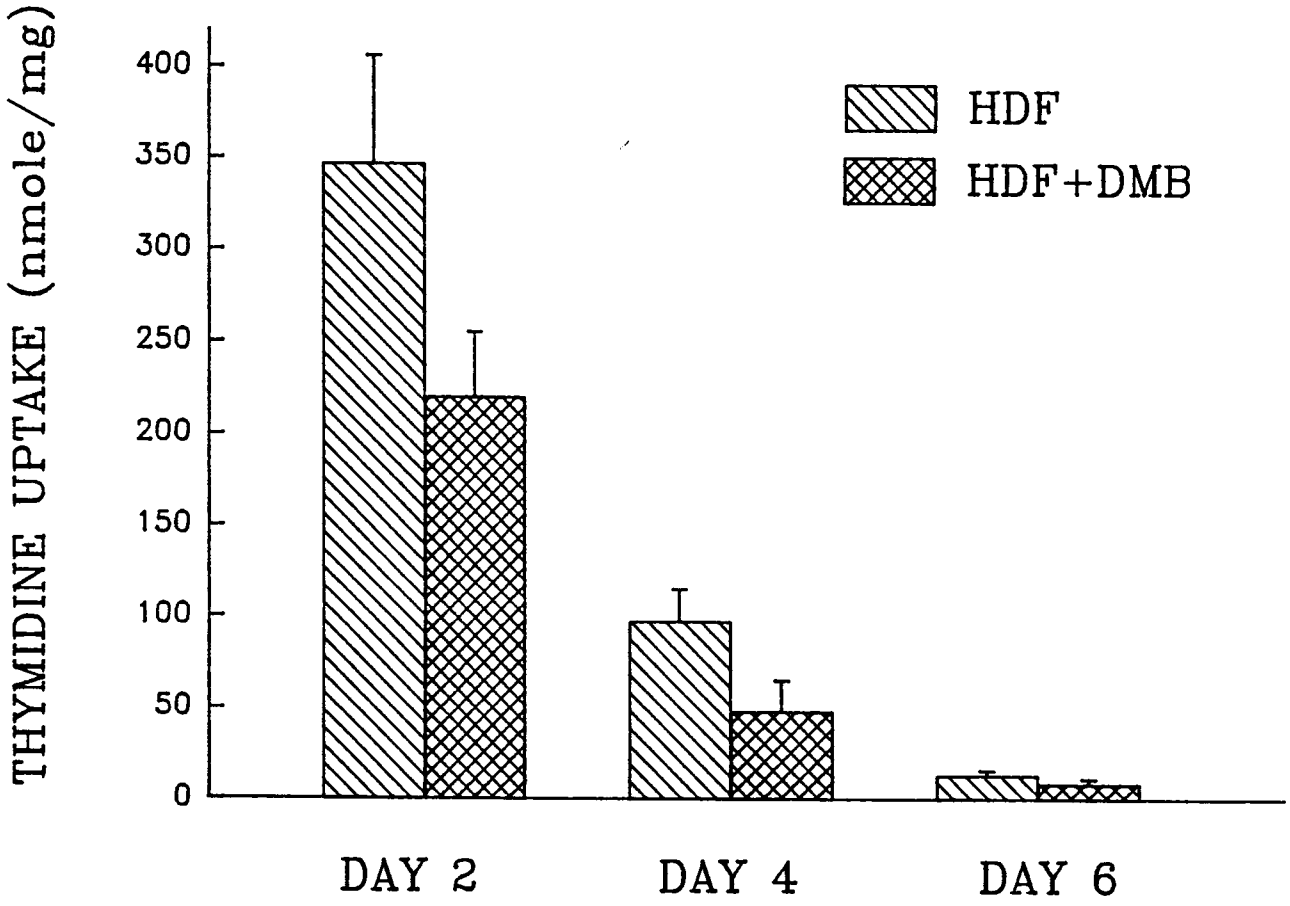
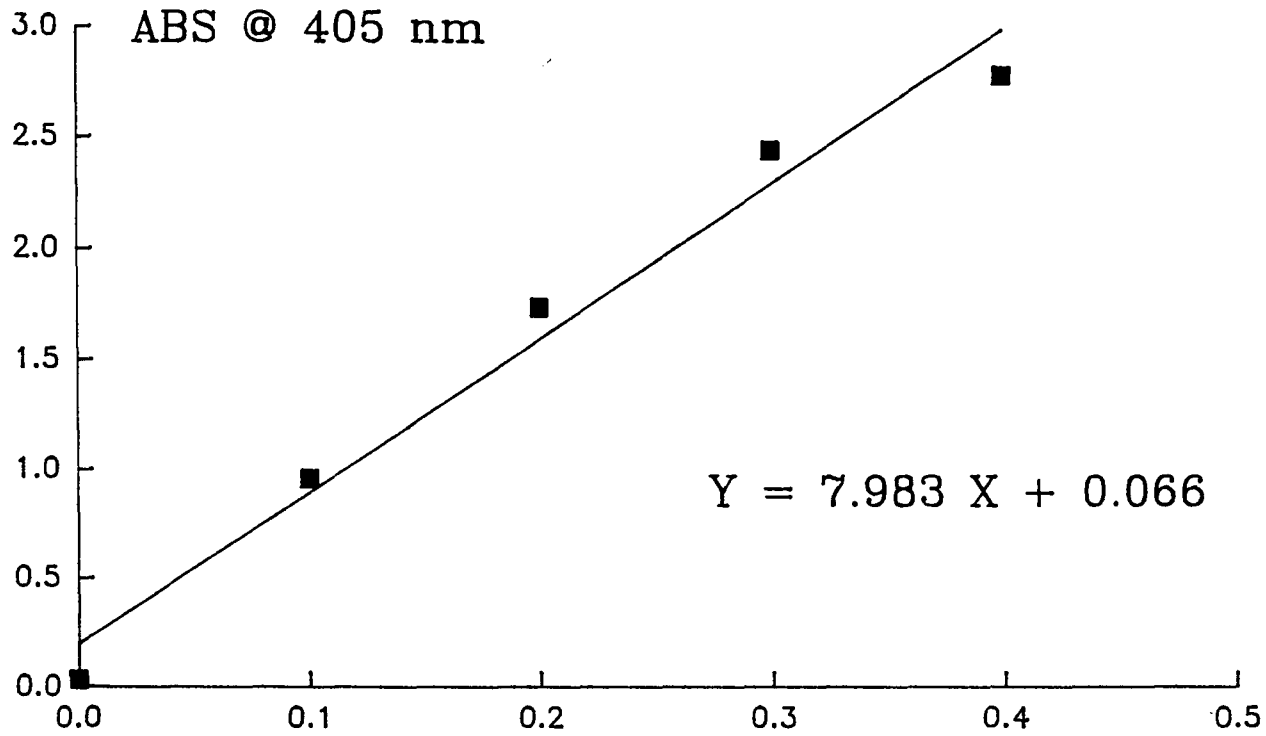


Fig. 9 PNP standard curve for alkaline phosphatase assay. Authentic PNP (p-nitrophenol) was prepared in 0.15 M 2-amino-2-methyl-1-propanol buffer diluted 1:2 with deionized water as standards and their absorbances at 405 nm were correlated to equivalent amount of PNP.

PNP STANDARD



the acquired curve (Michaelis-Menten plot) showed a classical pattern with a rapid linear increasing phase, a slower intermediate phase and a saturation phase (Figure 10). The specific Km and Vmax values were derived from the double reciprocal plot (Figure 11). Vmax was 14.8 unit/g and Km was 14.24 mM.

2. AP Activity Change during Growth of HDF

In HDF cells (Figure 12), AP activities did not change significantly over the 7-day observation period ($P > 0.05$). Confluence was observed microscopically at day 4. In the presence of DMB, however, AP activities significantly increased at days 6 and 7 ($P < 0.05$). There were no differences from day 1 to day 5 and between day 6 and day 7 ($P > 0.05$). Cell confluence was achieved at day 5, one day before the AP activity started to increase. Therefore, in the following experiments cells were assayed for alkaline phosphatase one day after they reached confluence (Figure 1-b, 2-b, 3-b).

3. AP Activity and the Amount of DMB

HDF cells cultured in the presence of 10 mg, 20 mg, 30 mg, 40 mg and 50 mg of LN-DMB per T-75 flask (seeding density 5 x 10^5/T-75 flask) were assayed for AP activity one day after they reached confluence (Figure 13). There was no significant difference among the various treatment groups ($P > 0.05$). AP activities in all groups were significantly higher ($P < 0.05$) than that of the control group (i.e., HDF without

Fig. 10 Michaelis-Menten kinetics of alkaline phosphatase from HDF extracts. Cells were assayed one day after they reached confluence. Values represent mean$\pm$SD of 3 replicate assays.

ENZYME KINETICS OF ALKALINE PHOSPHATASE FROM HDF

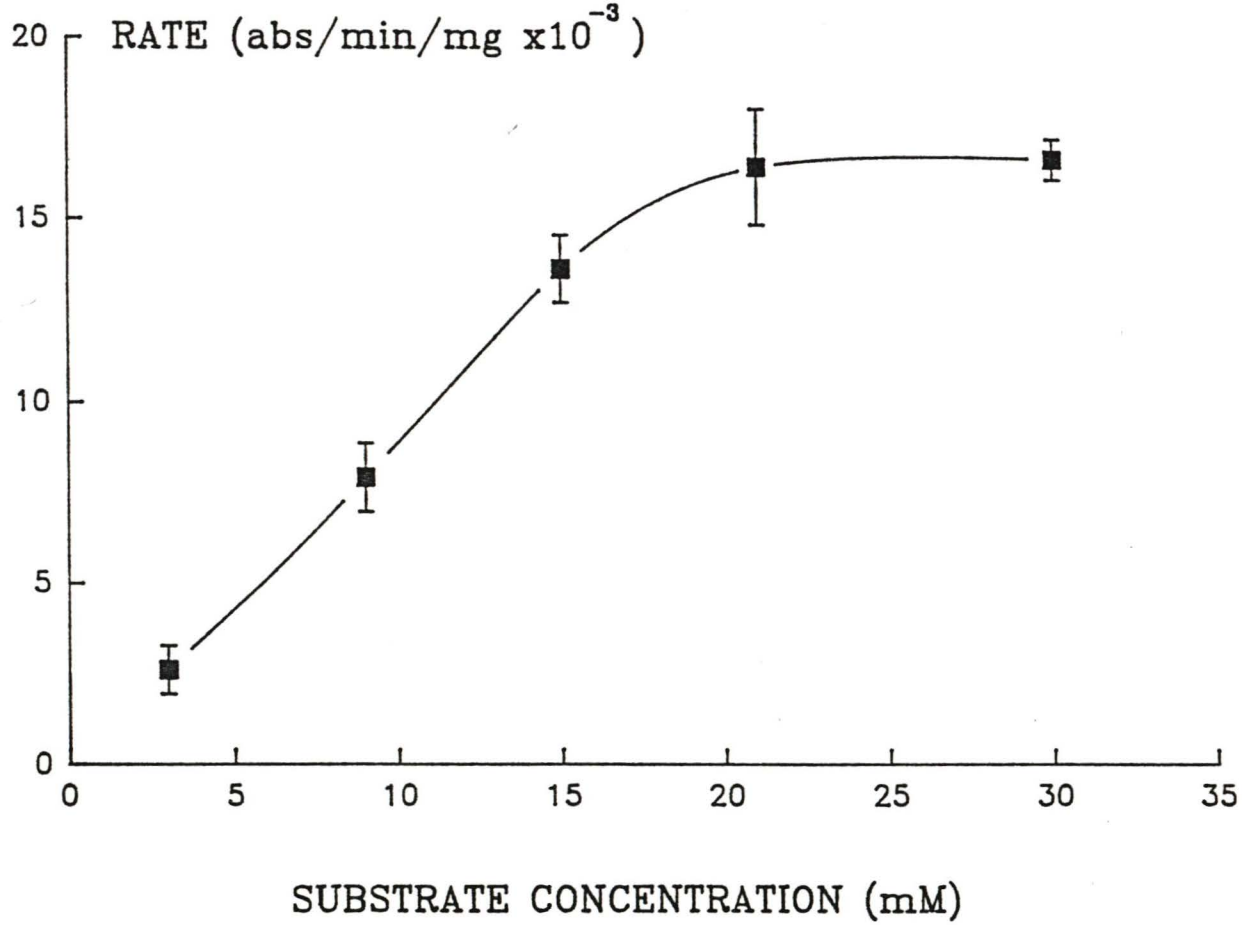


Fig. 11 Lineweaver-Burk (double reciprocal) plot of Michaelis-Menten curve. When $1/[V] = 0$, $1/[S] = -1/Km$; when $1/[S] = 0$, $1/[V] = 1/Vmax$.

DOUBLE RECIPROCAL PLOT

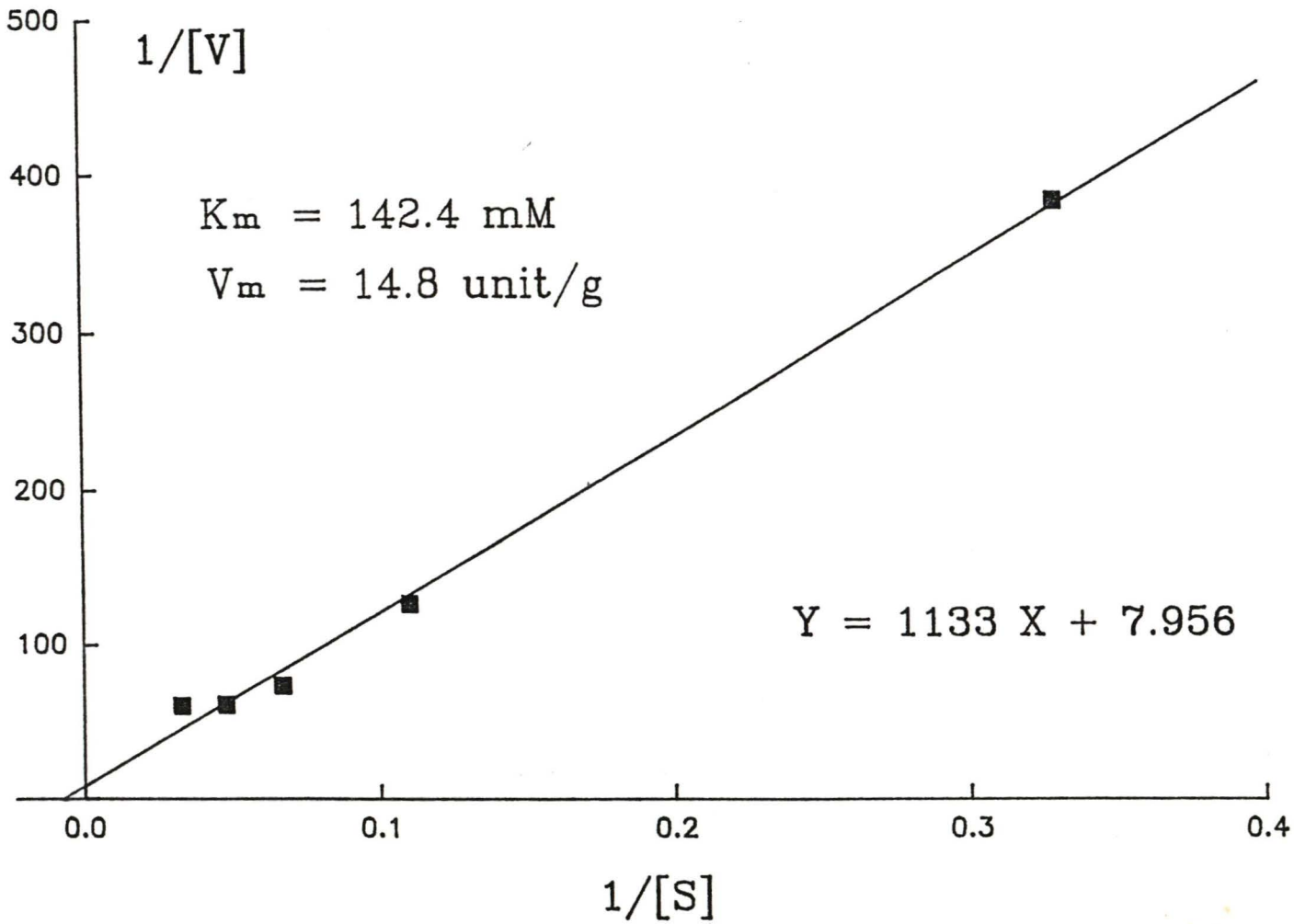


Fig. 12 Alkaline phosphatase activities during 7-day incubation time of HDF cells with and without exposure to DMB. Control HDF cells (without DMB) reached confluence at Day 4. When LN-DMB (10 mg per T-75 flask) was added, confluence was reached at Day 5. There was no significant change ($P > 0.05$) of AP activities over the 7-day incubation period for control HDF cells. In HDF+DMB (10 mg LN-DMB per T-75 flask) groups, however, AP activities were much higher ($P < 0.05$) in confluent cells (Day 6 and Day 7). The AP activities of HDF+DMB were significantly higher than those of control HDF cells at Day 1, 2, 6 and 7 ($P < 0.05$). Values represent mean$\pm$SD of 7 replicate assays.

ALKALINE PHOSPHATASE IN HDF

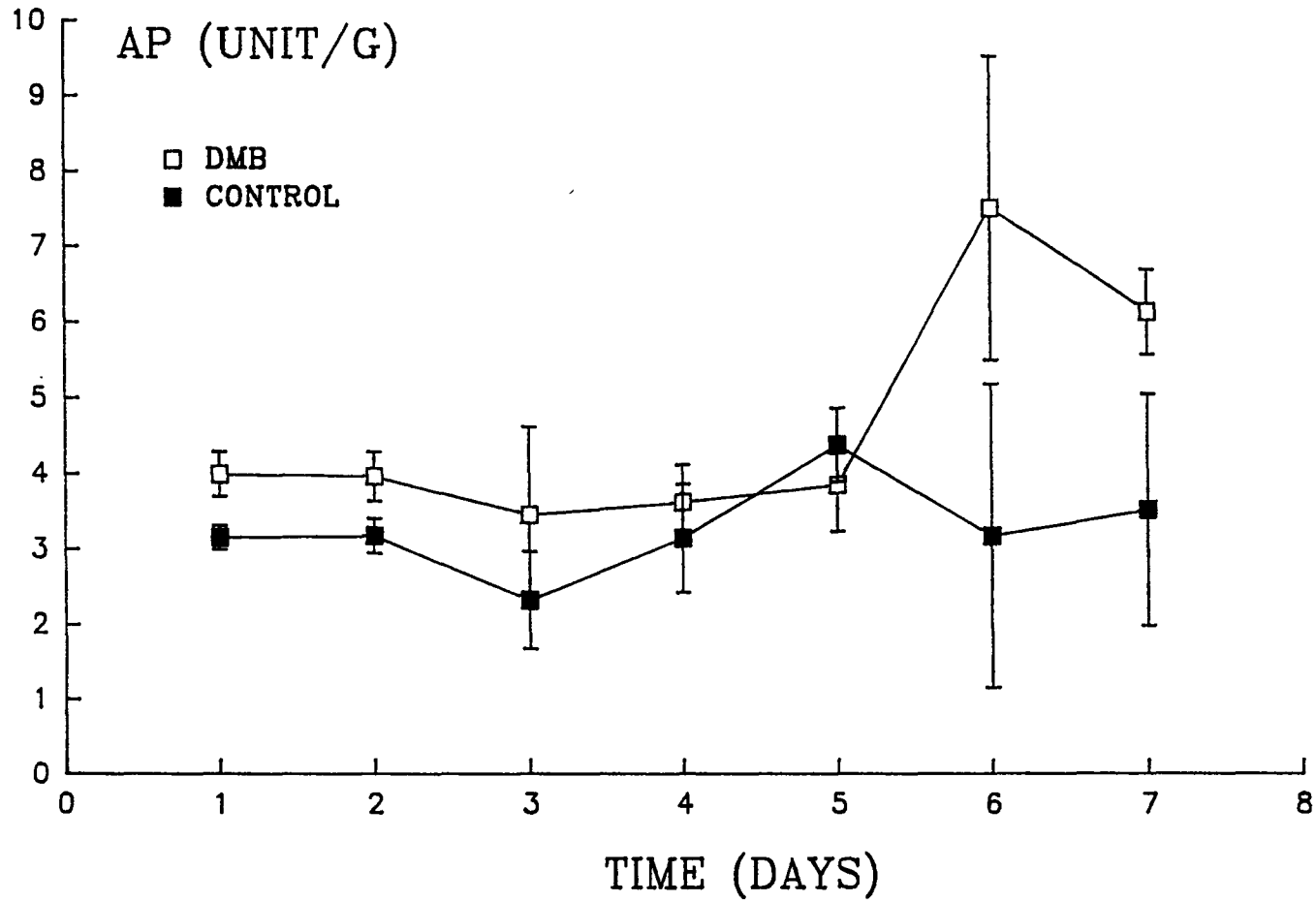
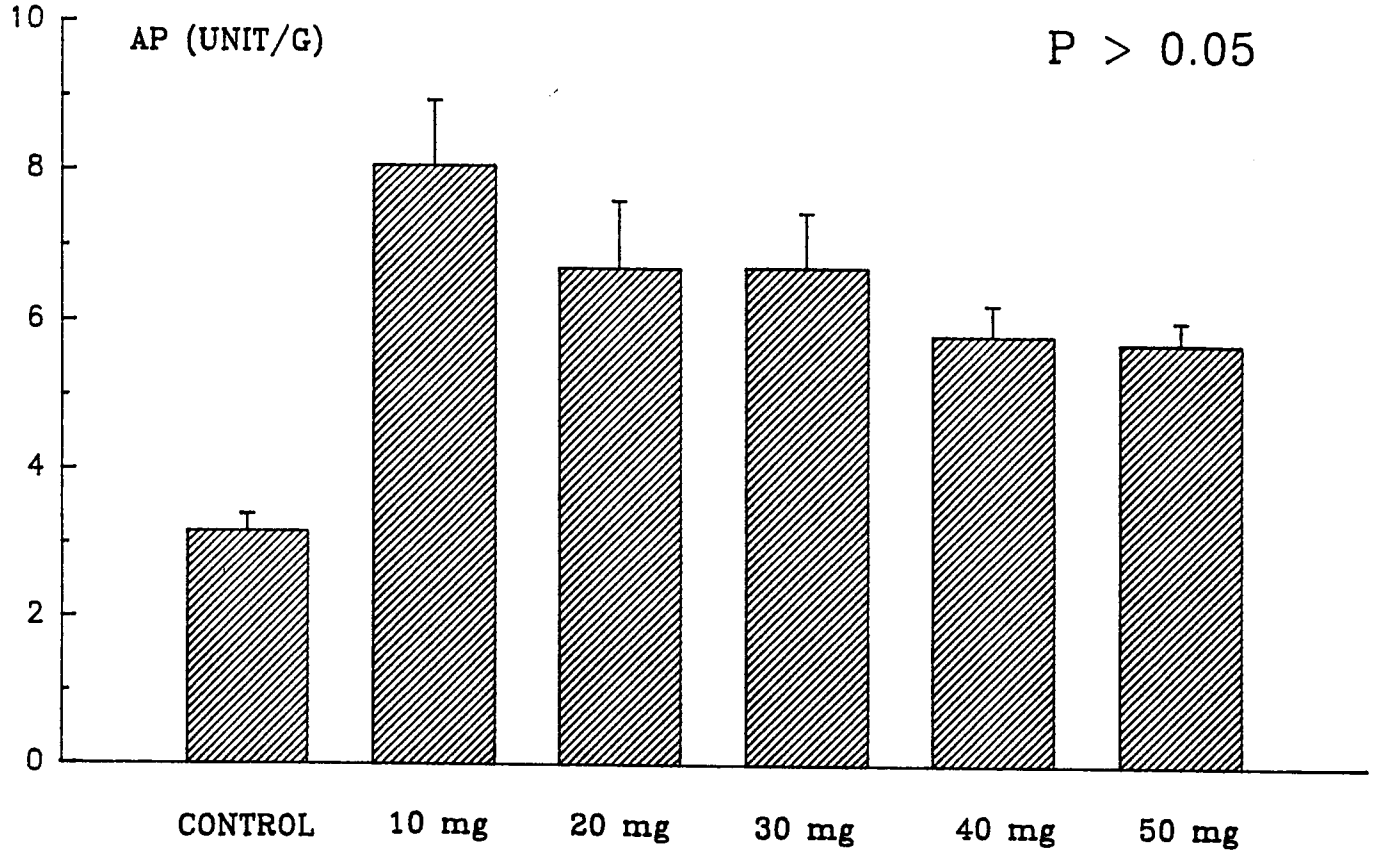


Fig. 13 Alkaline phosphatase activities in confluent HDF cells exposed to different quantities of DMB. HDF cells cultured in the presence of 10 mg, 20 mg, 30 mg, 40 mg and 50 mg of LN-DMB per T-75 flask (seeding density 5 x 10^5/ flask) were assayed one day after they reached confluence. There was no significant difference among various groups with DMB ($P > 0.05$). AP activities in all the groups with DMB were significantly higher than that of the control group ($P < 0.05$). All the cells cultured with DMB reached confluence at day 5, while control HDF cells reached confluence at day 3 or 4. Values represent mean $\pm$ SD of 5 replicate assays.

AP IN CONFLUENT HDF WITH DMB



DMB). It was decided that 10 mg of ground bone/ 5 x 10^5 cells/ T-75 flask would be the standard dose used in later experiments.

Preparation of Ground Human Bone

The sole purpose of this part of the study was to provide different kinds of processed bone to add into HDF cell cultures. By assaying AP activity of the cell extracts, the osteoinductivity of each kind of ground bone could be estimated.

1. Scanning Electron Microscopy of Fresh and Demineralized Ground Bone

Scanning electron microscopy was used to visualize the LN-processed ground DMB used as a standard in this study. Figure 14-a is a representative electron micrograph of fresh ground dried bone fragments, revealing a dense network and fine granular structure. As may be seen in Figure 14-b, the fine granular structure is less dense in demineralized bone than in fresh nondemineralized bone fragments.

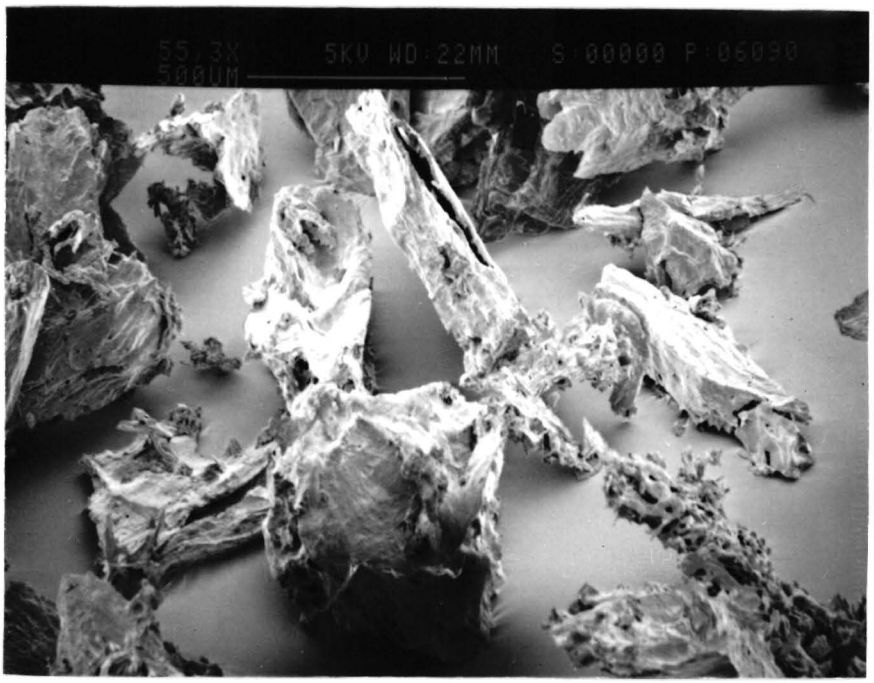
2. Ethanol Pretreatment

Lipids were extracted from ground human bone using absolute ethanol (100% EtOH). Although this solvent was expected to differentially extract different lipids (e.g., cholesterol and phospholipids) and other ethanol-soluble components from the bone,

Fig. 14 Electron micrographs of ground human bone, with particle size ranging from 250 um to 710 um. (a) Fresh human bone, revealing a dense network and fine granular structure. Magnification: x54,000. (b) Demineralized human bone, showing a less dense granular structure than that in fresh nondemineralized bone. Magnification: x55,000.


54.2X 5KV WD:21MM S:00000 P:06093
500UM

a


55.3X 5KV WD:22MM S:00000 P:06090
500UM

b

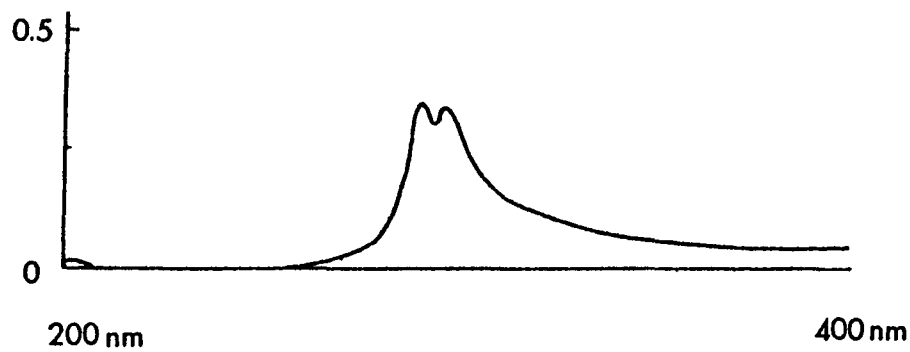
cholesterol was chosen to be monitored as a relative measure of the extraction process. Cholesterol is a common component of mammalian lipids and is soluble in absolute ethanol. Using 100% ethanol as a reference, the absorbance spectrum of cholesterol between 200 nm to 400 nm was scanned (Figure 15). The peak was at 290 nm which is similar to that of the eluent solution obtained from ethanol extraction of bone (Figure 16). This wavelength was used for the cholesterol standards (Figure 17) and eluent solutions of ethanol-extracted bone.

Although cholesterol standards and ethanolic extracts of bone had similar absorbance spectra, the absorbance of the latter cannot be directly correlated with the cholesterol concentration at that absorbance point because in the eluent solution, the absorbance value at 290 nm was influenced by all of the components present in the solution, i.e., the total lipids extracted would be different from the value calculated for cholesterol concentration from the standard curve. This special assay was used only to monitor and standardize the ethanolic extraction process. As seen in Table 2, the "lipids" extracted per gram of bone by the indicated process had an average concentration equivalent to 0.146% of cholesterol.

3. Mineral Extraction

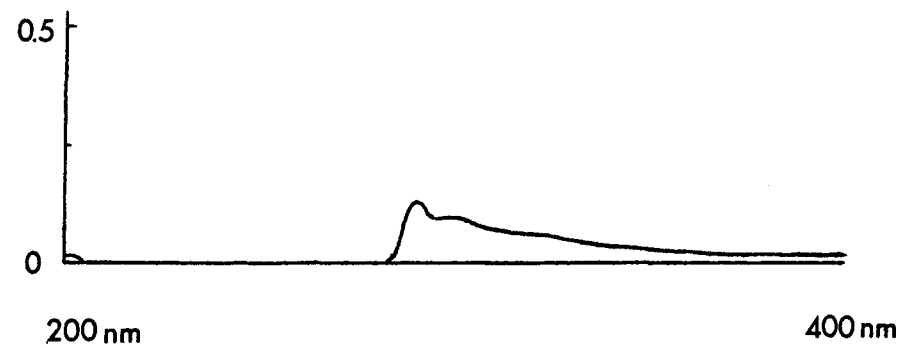
Because calcium is the main cationic mineral component of bone, its concentration was chosen to be monitored during the demineralization process. Standard calcium

Fig. 15 Absorbance spectrum of cholesterol in absolute ethanol between 200 nm and 400 nm. The peak of absorbance was at 290 nm.



PEAK : 290 nm

Fig. 16 Absorbance spectrum of eluent solution, from ethanol pretreatment of bone, between 200 nm and 400 nm. The peak absorbance was exhibited at 290 nm.



PEAK : 290 nm

Fig. 17 The cholesterol standard curve. Cholesterol standards were prepared in absolute ethanol and the absorbances were read at 290 nm. Concentration was expressed as grams of cholesterol per 100 ml of 100% ethanol.

CHOLESTEROL STANDARD

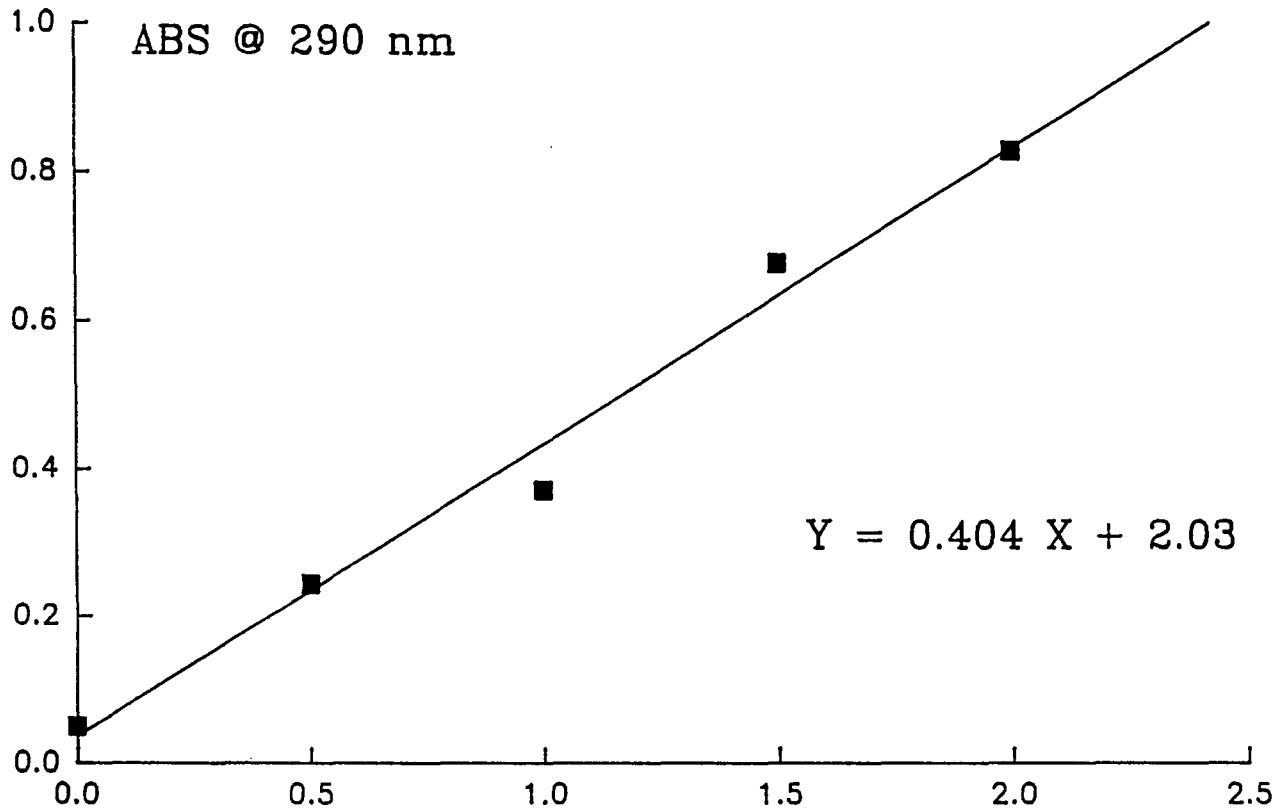


Table 2 Ethanol Treatment of Ground Fresh Human Bone

No.	a (gram)	b (%)	c (%/gram)	d (%/gram)
1	5.00	0.686	0.137	
2	2.05	0.297	0.146	0.146±0.009
3	2.93	0.462	0.154	

a. dry weight of fresh bone before the ethanol treatment process.

b. total concentration (equivalent to gram of cholesterol per 100 ml of ethanol or %) of "lipids" in the eluent solution from 5 rounds of ethanol extractions (weight of bone to volume of ethanol: 1 gram to 10 ml).

c. total concentration of "lipids" in extraction solution (b) divided by the original weight of the bone sample (a), i.e., the concentration of "lipids" extracted from each gram of fresh bone.

d. average of (c), expressed as mean±SD.

concentrations were read as relative potentials (mV) using the electrode specific for calcium ions. The standard curve is shown in Figure 18. Before demineralization processes, the total calcium content in bone samples was determined.

The total calcium content and its percentage in human bone varies from donor to donor. Since all of the ground bone samples processed in this study (except LN-DMB) were from donor No. 90-500, an 18-year-old male, the results (Table 3) may not be representatives of all bone. The total calcium ion consists of 34.6% of the total dry weight of fresh bone.

The demineralization process was monitored using the same calcium ion-specific electrode. Both fresh and ethanol-treated bone were demineralized. All extraction solutions were measured for the relative potential (mV). Calcium concentrations were then calculated from the standard curve. The results are shown in Table 4.

AP Activity in HDF cells with Different Bone Products

AP activity in HDF cells cultured with different kinds of processed bone and in HPO cells were measured one day after the cells reached confluence (Figure 19). The data showed no significant difference in AP activities among HDF cells with fresh bone powder, ethanol-treated bone powder and the HDF cell control ($P > 0.05$). They were significantly lower than all the other groups ($P < 0.05$). While the demineralized and

Fig. 18 The calcium electrode calibration standard curve. Calcium chloride standards were prepared in 0.5 N HCl. Relative potentials of each standard were read and plotted against the concentration (the latter in logarithmic scale). The curve is reliable when calcium concentration is within the range between 0.01 M and 1 M.

CALCIUM STANDARD

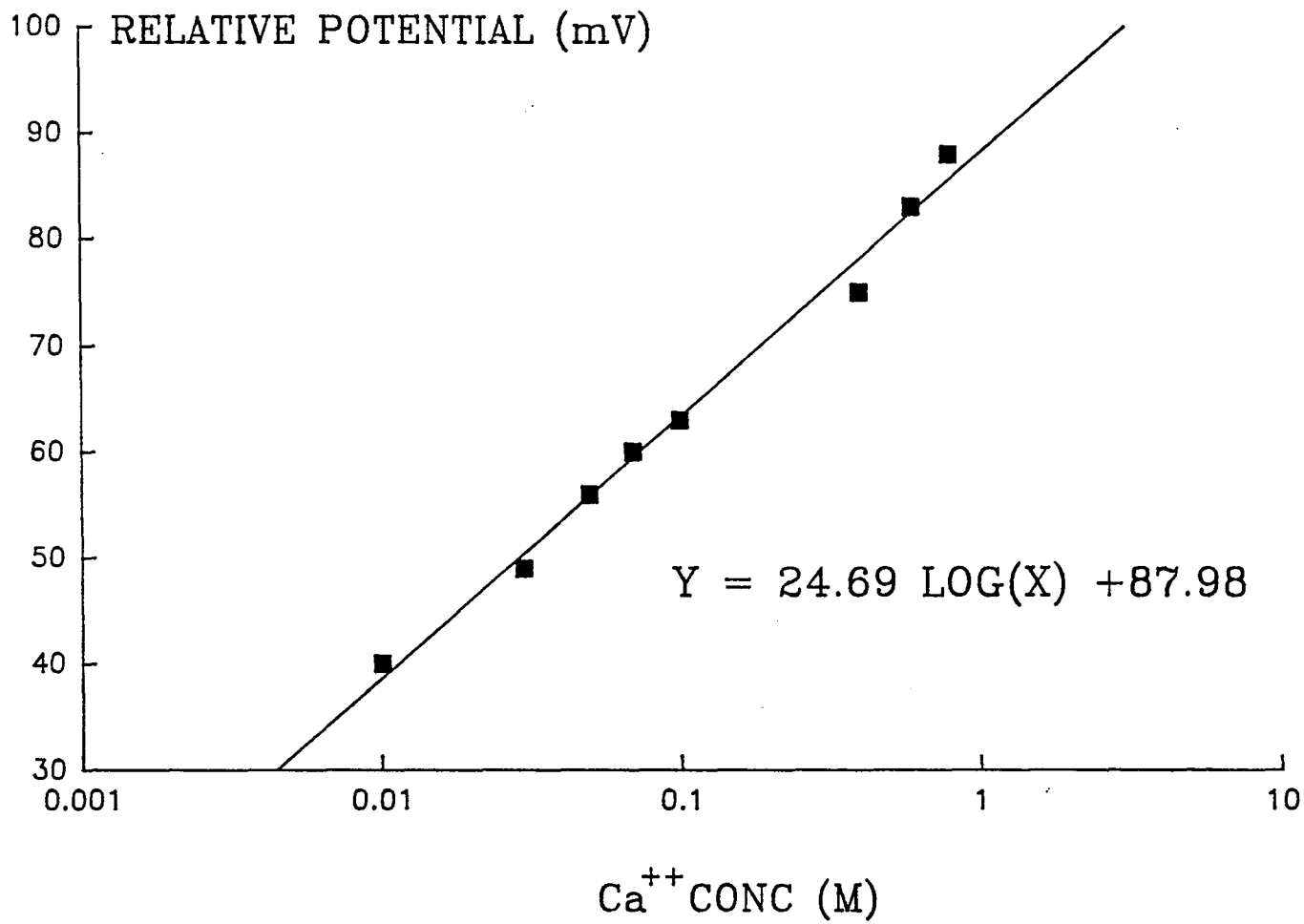


Table 3 Total Calcium Content in Fresh Human Bone from Donor No.90-500

No.	a (g)	b (g)	c (%)	d (g)	e (%)	f (%)
1	1.2534	0.8295	33.8	0.4352	34.7	
2	1.1063	0.7325	33.8	0.3968	35.9	34.6±1.3
3	1.4364	0.9513	33.8	0.4787	33.3	

a. dry weight of fresh bone before ashing.

b. weight of residue substance (presumably inorganic matter) after ashing.

c. weight loss (presumably organic matter) during ashing process in percentage.

d. the amount of calcium element (Ca^{++}) present in the ashing residue.

e. the percentage of calcium element (dry weight basis) in fresh bone.

f. the average value of (e), expressed as mean±SD.

Table 4 Mineral Extraction from Ground Human Bone with 0.5 N HCl

sample	n	a (g)	b (g)	c (%)	d (%)
fresh	2	4.93 3.06	1.48 0.98	30.0 32.0	31.0±1.7
ethanol treated	2	3.14 4.58	1.21 1.48	38.5 32.3	35.4±4.1

n. number of replicates

a. weight of bone before mineral extraction process.

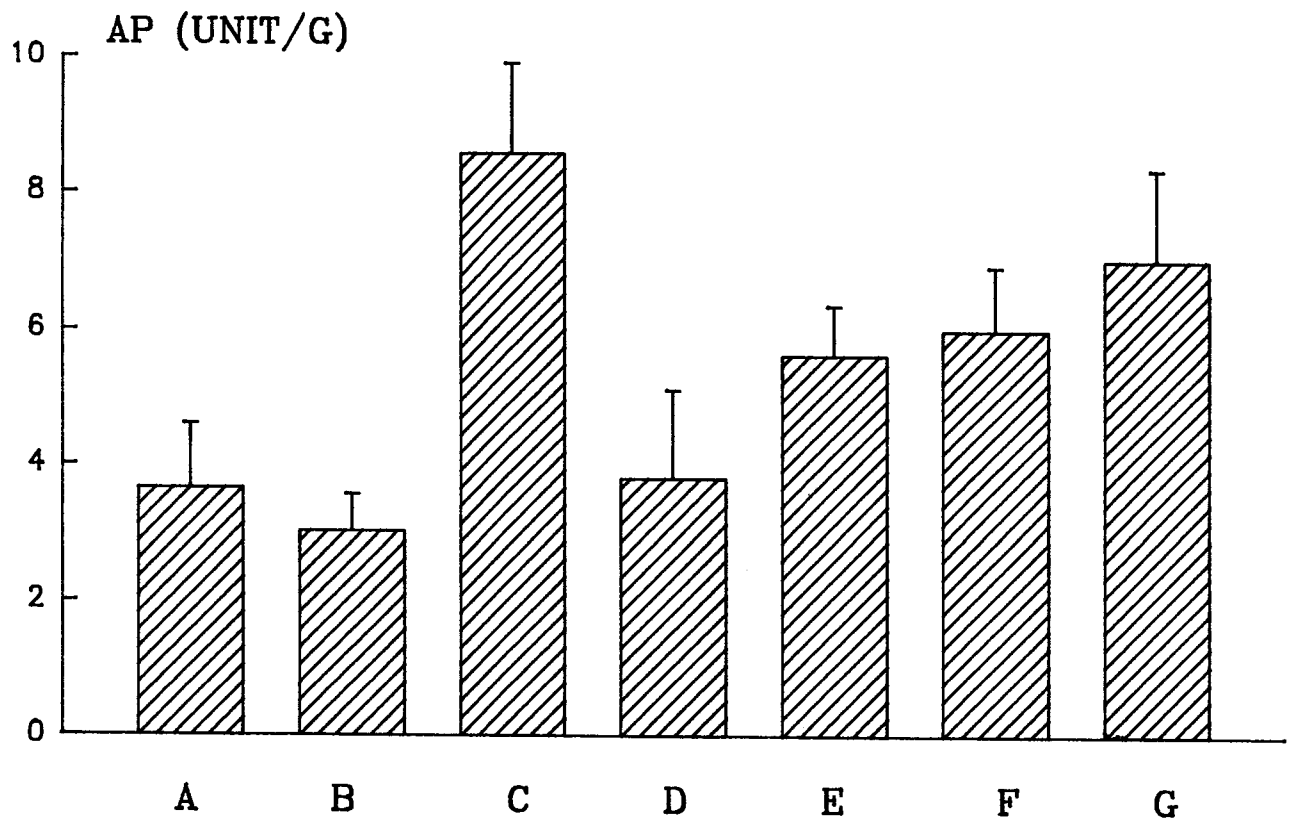
b. amount of calcium element (Ca^{++}) measured from extraction solution.

c. calcium element extracted from each bone sample in weight percentage.

d. average of (c) expressed as mean±SD.

Fig. 19 Alkaline phosphatase activities in confluent HDF cells exposed to different bone products. A: HDF cells without bone (HDF control), n=9; B: HDF cells with 10 mg of unprocessed bone, n=7; C: HDF cells with 10 mg of LN-DMB, n=7; D: HDF cells with 10 mg of ethanol treated bone, n=7; E: HDF cells with 10 mg of demineralized bone, n=6; F: HDF cells with 10 mg of ethanol treated demineralized bone, n=6; G: HPO cells without bone (HPO control), n=7. All the cells were seeded at the density of 5 x 10^5/ T-75 flask. There was no significant difference among group A, B and D ($P>0.05$), but all three of them were significantly lower than other groups ($P<0.05$). There was no significant difference among groups E, F and G ($P>0.05$). However, group E and F were significantly lower than C ($P<0.05$) while G and C were not different ($P>0.05$). Values represent mean$\pm$SD of 6 to 9 replicate (designated as n) assays.

AP ACTIVITY IN HDF WITH VARIOUS GROUND BONE



ethanol-treated demineralized groups were not significantly different in AP activities from the control HPO cells ($P > 0.05$), they were lower than that of LN-DMB group ($P < 0.05$), which was not significantly different from HPO control. Figure 19 also shows that the AP activity of HPO cells was 2 times higher than that of HDF cells; AP activities of HDF cells treated with LN-DMB, ethanol-treated demineralized bone and demineralized bone was 2.4, 1.7 and 1.6 times, respectively, higher than the AP activity of HDF cell control.

V. DISCUSSION

The osteoinductivity of demineralized bone (DMB) has long been the subject of much research. Most classical experiments used *in vivo* models involving mice, rats, rabbits and guinea pigs. DMB segments or powder were implanted into various extraosseous sites (subcutaneously and intramuscularly) of the animals and the formation of new bone was later observed (Reddi and Huggins, 1973; Reddi and Huggins, 1972a; Reddi and Huggins, 1972b; Huggins and Urist, 1970; Urist, 1965). It was therefore postulated that DMB has osteoinductivity, and this osteoinductivity would stimulate mesenchymal cells to differentiate into osteogenic cells.

Similar cell types in different animals may respond differently to the same osteoinductive agents. According to Hirano and Urist (1991), rats and rabbits are predisposed to respond to DMB matrix. Mice respond consistently to collagen-free implants of bone morphogenetic protein (BMP). Rats respond to BMP incorporated in bone matrix. Guinea pigs are resistant to demineralized bone matrix, BMP incorporated in bone matrix, or collagen-free purified BMP, but are susceptible to uroepithelium-induced bone morphogenesis. Dogs, monkeys and humans are resistant to implants of BMP in all forms and may require an activator or unmasking factor (Urist, 1972). Baboons respond to implants of demineralized allogenic bone. These genetic species-

linked predispositions are key considerations in laboratory studies and clinical applications.

Beginning in the early 1980s, researchers began to study *in vitro* cell culture models as a means to assess the osteoinductivity of DMB and other substances. The most frequently used cells were periosteal cells and osteoblasts (Nakahara et al., 1990; Letton et al., 1990; Vukicevic et al., 1989; Orly et al., 1989). In these studies it was observed that DMB enhanced the osteogenic expressions of the cultured cells. Osteoblasts and periosteal cells are committed bone-forming cells and it has been suggested that their use in the demonstration of osteoinductivity is not appropriate, because osteoinductivity implies a process involving the differentiation of cells of mesenchymal origin into osteogenic cells. There are few reports of *in vitro* cell culture systems using human fibroblast models, although a mouse embryonic fibroblast cell line has been successfully induced to differentiate into osteoblastic cells by recombinant human BMP (Katagiri et al., 1990).

In this study a human fibroblast cell line was used to demonstrate the osteoinductivity of DMB. According to the hypothesis, these fibroblast cells would differentiate into osteogenic cells upon stimulation by DMB. A periosteal cell line was also used in the study, as a control of differentiated osteogenic cells. Cell growth characteristics, proliferation rates and alkaline phosphatase activities of confluent cells were determined to demonstrate differentiation of the fibroblast cells following exposure

to an osteoinductive bone matrix.

Human periosteal cells, consisting of osteoblast cells, osteoprogenitor cells and a variety of other cell types, grow slowly *in vitro*. Koshihara et al. (1989) reported that a human periosteal cell line cultured in α-MEM supplemented with 10% FBS had a population doubling time of 40 to 60 hours. In another study (Gotoh et al., 1990), an osteoblast cell line cultured in α-MEM supplement with 10% FBS, 5mM of ß-glycerophosphate and 50 ug/ml L-ascorbic acid was reported to have a population doubling time of 40 hours, and cell confluence was reached at day 7 when seeded at 1.0 - 1.7 x 10^4 cells/cm^2. Growth characteristics of a chick periosteal cell line (Nakahara et al., 1990) cultured in Ham's F-12 medium supplemented with 10% FBS showed that cell confluence was reached at day 10 - 12, when seeded at 1.3 - 2.5 x10^4/cm^2. In the present study, HPO cells reached confluence at day 9 or 10 when seeded at 0.67 x 10^4/cm^2, similar to those previous reports, with a population doubling time of 215 hours (Figure 6). The growth curve showed that cell numbers increased slowly with time, with no obvious lag phase, logarithmic phase or plateau phase. This observation can be partly explained by the report that osteoblast-like cells do not exhibit contact inhibition (Nijweide et al., 1982). The density of cells at the end of the observation period was 2.5 x 10^4 cells/cm^2.

In contrast, fibroblast cells grow very fast *in vitro* and are probably the easiest of all cell types to grow in serum-containing media (Ham, 1984). It was reported

(Hayflick and Moorhead, 1961) that a human lung fibroblast cell line cultured in buffered Eagle's Medium supplemented with 10% calf serum had a population doubling time during logarithmic phase of approximately 24 hours, and cell confluence was reached at the 4th or the 5th day. In the present study, the HDF cells displayed typical growth kinetics with a lag phase, a logarithmic phase and a plateau phase (Figure 4). The population doubling time was 17 hours during logarithmic phase and confluence was reached at day 4. The saturation density was 8.7×10^4 cells/cm^2. The growth characteristics were similar to those reported by Hayflick and Moorhead (1961).

The growth characteristics of HDF cells changed when DMB was added to the cell culture (Figure 5). The growth curve displayed an atypical logarithmic phase and plateau phase. The number of cells increased slowly with a rate very similar to that of HPO cells. The population doubling time in the atypical logarithmic phase was 33 hours, and cell confluence was delayed to day 5. Saturation density was 2.0×10^4 cells/cm^2. These changes indicate that the overall growth pattern of HDF cells exposed to DMB closely approximated that of HPO cells and was consistent with the suggestion that a factor, or factors, present in the DMB stimulated the fibroblastic cell population to differentiate into osteogenic cells.

The [^{3}H]-thymidine incorporation study (Figure 8) showed that the rate of thymidine incorporated into DNA decreased in HDF cells over the observed period. This observation indicated a decreasing rate of DNA replication and hence suggested a

decreasing rate of cell division. The results were consistent with the growth curve study in which cell numbers stopped to increase after confluence was reached. Because there is always a latency time between DNA replication (S phase) and cell proliferation (M phase), it was perfectly reasonable that in the HDF cell culture DNA proliferation rate decreased from day 4 while the cell proliferation rate (cell number) started to decrease from day 5. The same pattern of decreased rate of thymidine incorporation and its relation to decreased rate of cell proliferation also occurred when HDF cells were cultured with DMB. The presence of DMB in HDF cell cultures reduced the amount of thymidine incorporation at each sampling time, presumably as a result of a reduction in number of cells replicating their DNA. This observation was consistent with the decreased number of HDF cells with number of days in culture in the presence of DMB, as seen in the growth curve data (Figure 7). However, this result was contradictory to the report by Katagiri et al. (1990) that the rate of [^{3}H]-thymidine incorporation into DNA increased in a mouse embryonic fibroblast cell line when these cells were cultured with recombinant human bone morphogenetic protein (rhBMP). In that study mouse embryonic fibroblast cells were induced to differentiate into osteoblastic cells by rhBMP in cell culture conditions. It can be inferred that an increase in DNA synthesis in the presence of rhBMP indicated an increase in rate of cell proliferation, but "induced" differentiation of fibroblasts (rapid proliferation cell population) into osteoblasts (slow proliferation cell population) would more likely demonstrate a decreased DNA replication rate, which would be consistent with the results of the present study.

"Cell size" (expressed as amount of protein per cell) was used as another measure to describe the growth characteristics of cells in culture. Ceccarini and Eagle (1971) used similar parameters to define the growth characteristics of their cell lines. In their study, cells cultured in various serum-free media were assayed for their total cell numbers and total protein contents, as a measure of their growth characteristics and hence as an assessment of the influences of different media on cell physiology. These parameters can serve as a proxy for "cell size" (amount of protein per cell). In the present study (Figure 7) the values of cell size were derived from total cell number and total cell protein; therefore, this "cell size" parameter should serve as a valid measure to describe the pattern of cell changes as a function of population increase.

The patterns of "cell size" changes over the observed period were very different between HDF cells and HPO cells. For HDF cells, the "sizes" decreased rapidly as cell numbers increased rapidly, and stayed at a low level when the cell numbers stayed at a high level in plateau phase. For HPO cells, the "cell sizes" decreased gradually as the numbers increased gradually. When HDF cells were cultured with DMB, however, the changes of both the "sizes" and numbers resembled those of HPO cells, indicating that DMB had affected the HDF cells such that they displayed growth characteristics very similar to those of HPO cells.

Alkaline phosphatase (AP) activity is a very important early marker of osteogenic cells. High alkaline phosphatase activity was detected in condensed mesenchymal cells

prior to the increase of type I collagen synthesis and osteoid or mineral deposition (Zernik et al., 1990). According to Wlodarski and Reddi (1986), high alkaline phosphatase activity is a property shared by all osteoinducing cells but not by nonosteoinductive cells. Many classical works on osteogenesis have used AP activity as a characteristic of osteogenic expression and it has been reported that the AP activity of cultured human osteoblastic cells (passage 6) is 3.3 times higher than the AP activity of cultured human fibroblastic cells (Koshihara et al., 1989). In the present study (Figure 19), the AP activities of the periosteal cell line (passage 6) were approximately 2 times higher than that of the fibroblastic cell line (passage 7). Because the percentages of each cell type present in periosteal cells varies among individuals, it is to be expected that values of AP_{HPO}/AP_{HDF} may differ from study to study. When HDF cells were cultured with DMB, their AP activity increased to 2.4 times that of the HDF cells cultured in the absence of DMB. This value was at the level of AP activity of HPO cells. Since high alkaline phosphatase activity is a marker for all osteoinducing cells (Wlodarski and Reddi, 1986), the results suggested that some factors in demineralized bone matrix affected the HDF cells, causing them to be phenotypically similar to osteogenic cells. These results with alkaline phosphatase activity measurements support the hypothesis that fibroblastic cells can differentiate into osteogenic cells following appropriate stimulation(s), i.e., the presence of DMB. In a recent report (Katagiri et al., 1990), increased AP activity was also used as a marker for the presence of osteoblastic cells when a mouse embryonic fibroblastic cell line was incubated in the presence of BMP, a substance presumably present in DMB, causing them to differentiate into osteoblastic

cells *in vitro*.

In the present study, cells were assayed one day after they reached confluence because HDF only exhibited elevated AP activity at that time of culture (Figure 12). Other research groups have also observed a 24-hour delay in the increase in AP activity in osteoblastic cells after confluence (Masquelier et al., 1990; Sautier et al., 1990). These phenomena can be explained by the suggestion that a three-dimensional structure is necessary for cell differentiation to occur (Sautier et al., 1990; Tenenbaum and Heersche, 1986). Cells adapted to *in vitro* culture conditions usually lose some of their native characteristics of growth and function. This loss is presumably due largely to the dissociation of cells from a three-dimensional geometry and their propagation on a two-dimensional substratum. As the cells spread out, they become mobile and start to proliferate. When cells reach confluence, cell-cell contacts and interactions are more or less restored and these factors might lead to the expression of specific cellular characteristics (e.g., high alkaline phosphatase activity). However, the exact mechanisms for these interactions are unknown.

HDF cells are clearly a suitable *in vitro* model for use in quantitatively assessing the osteoinductive potentials of variously processed bone matrices. Although the direct and indirect evidences showed that HDF cells, when cultured with DMB, differentiated into osteoinducing cells, it is not clear whether they became osteoblasts, osteoprogenitor cells, or were at some other stage of the differentiation process. Analyses for specific

cellular markers (e.g., osteocalcin and type I collagen) in future studies will help to clarify this issue.

The second part of the work presented in this thesis included the extraction of minerals from ethanol-treated bone and from unprocessed bone. The products, designated as ethanol-treated demineralized bone (ETDMB) and demineralized bone (DMB), were tested for their osteoinductivity using AP assays. In addition, ethanol-treated nondemineralized bone and unprocessed bone were also used in AP assays for osteoinductivity (Figure 19).

The procedure of ethanol treatment was originally incorporated into bone-processing procedures for sterilization and dehydration purpose. Ethyl and isopropyl alcohols have been proven to be effective and safe disinfectants (Mulliken and Glowacki, 1980). Another property of alcohols is that they extract lipids. Previous research had suggested that lipid removal prior to demineralization with HCl improved the subsequent demineralization of bone (Lee, 1990). The mechanism is not clear, although the hydrophobic property of lipids may restrict the access of acid to the mineral components of bone. It can be seen from the electron microscopic photographs presented (Figure 14) that lipid-extracted DMB appears to have a less dense texture. Despite all the influences that lipids have on demineralized bone, it has been reported that exposure to alcohol does not affect the osteoinductive properties of bone matrix (Mulliken and Glowacki, 1980). The results of AP assays in this present study are consistent with these previous

observations (Figure 19). There was no difference in AP activities between HDF cells exposed to demineralized bone with and without ethanol treatment, and between HDF cells treated with unprocessed bone and ethanol-treated bone. There was, however, a clear increase in AP activities when demineralized bone was used in the cell cultures. The present study was not designed to evaluate whether lipid extraction facilitates demineralization. However, AP activities of HDF cells exposed to DMB and ETDMB showed no significant difference. The osteoinductivity (induced AP response) of LifeNet-processed DMB is apparently better than the other demineralized bone products used in this study. This observation indicates that the processing protocol currently used by LifeNet Transplant Services provides a ground demineralized bone product capable of stimulating increased AP activities in our *in vitro* bioassay system.

Osteoinductivity is largely affected by calcium phosphate content, because calcium causes bone resorption (Vaes, 1988) and phosphate inhibits AP activity (Tietz, 1976). When nondemineralized bone graft is implanted into a host, bone resorption occurs prior to new bone formation. This process, the resorption of graft bone followed by the laying down of new bone, is called "creeping substitution" (Covey and Albright, 1989; Glowacki and Mulliden, 1985). When demineralized bone is used, the resorption process is bypassed. It was also reported that osteoblast cells participate in phagocytosis of synthetic calcium phosphate implants (Grégoire et al., 1990) and that the alkaline phosphatase activity of osteoblasts decreases after the phagocytosis of calcium phosphate. When bone materials undergo demineralization with HCl, the mineral compounds (mainly

calcium and phosphate in the form of hydroxyapatite crystals) become soluble substances ($CaCl_2$, HPO_4^{2-}, $H_2PO_4^-$, etc.) and eventually are washed out of the bone. Data presented in the present study (Figure 19) revealed that all the demineralized bone, whether it was ethanol-treated or not, induced high AP activity in HDF cells and the enzyme levels reached those of HPO cells. There was no significant increase in AP among those cells treated with nondemineralized bone.

It is concluded from the results of the experiment that DMB has osteoinductive ability and this osteoinductivity is not affected by ethanol treatment. By comparison nondemineralized bone does not have good osteoinductive ability.

The results of this study supported the hypothesis that fibroblastic cells can differentiate into osteogenic cells upon stimulation with demineralized bone. Human dermal fibroblastic cell line used in this study responded to DMB and was shown to be a very good *in vitro* model for assessing osteoinductivity, and AP activity assay was a successful quantitative measurement. The main purpose of evaluating osteoinductivity of demineralized bone is to assure the clinical effectiveness of these bone implants. Therefore, a human cell line model is considered of much advantage over other animal cell line models with regards to the interests of clinical research.

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