UCSF
UC San Francisco Electronic Theses and Dissertations

**Title**
Synthetic peptide analog of collagen enhances periodontal ligament fibroblasts attachment to hydroxyapatite

**Permalink**
https://escholarship.org/uc/item/9784095d

**Author**
Sadeghi, Mahyar,

**Publication Date**
1994

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

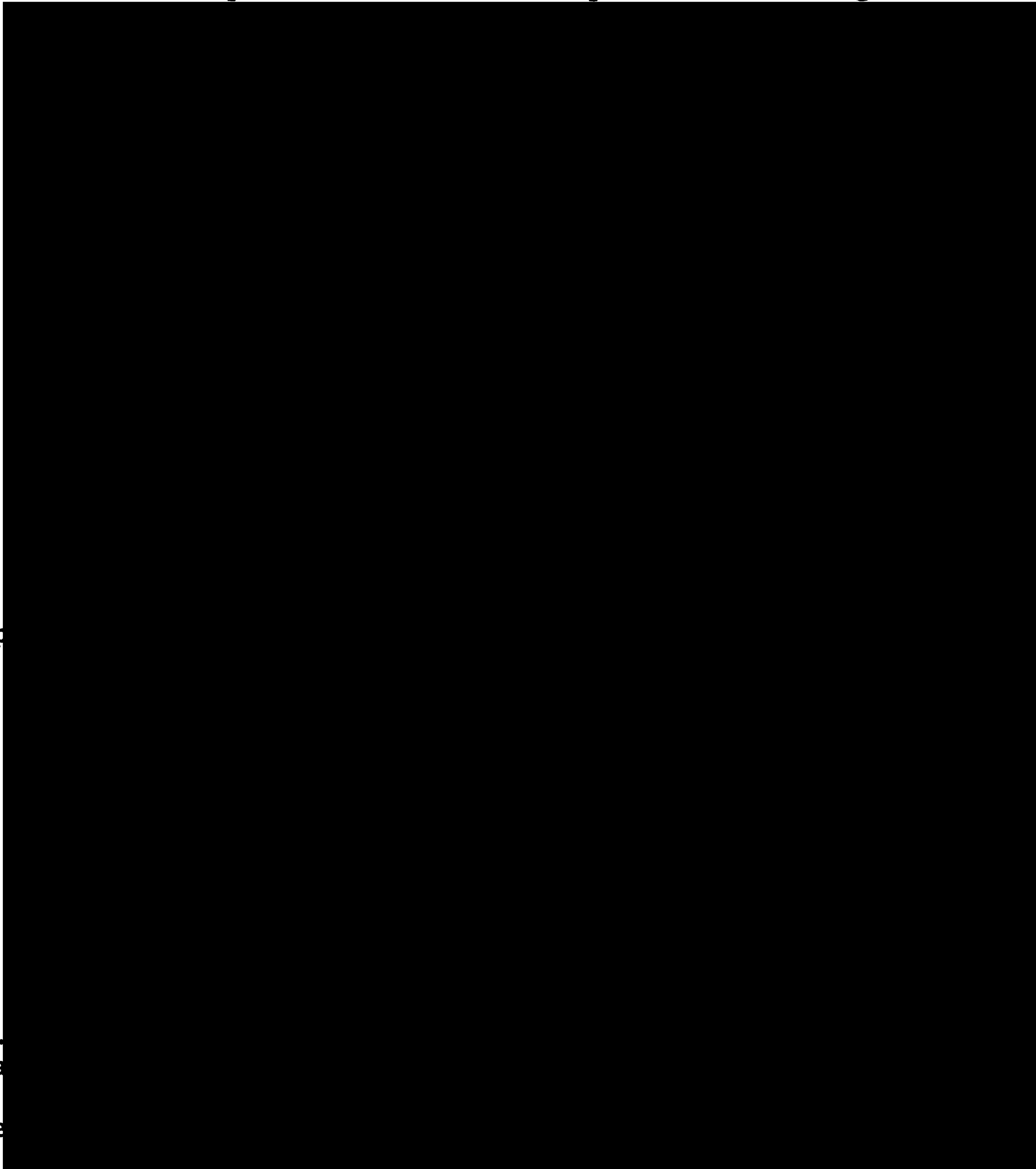
**Synthetic Peptide Analog of Collagen Enhances Periodontal Ligament Fibroblasts Attachment to Hydroxyapatite**

by

**MAHYAR SADEGHI, DDS**

**THESIS**

**Submitted in partial satisfaction of the requirements for the degree of**



**Ap**

**Da**

**De**

# DEDICATION

I would like to dedicate this thesis to my wife, Marjan, whose love and support throughout these years made my dreams come true; and to my newborn daughter for bringing so much joy to my life.
Many thanks to my family, Majid, Seemean, Maziar, and sormeh.
Your love has been inspirational.

# ACKNOWLEDGMENTS

I wish to thank Dr. Rajendra Bhatnagar for his guidance and support as my research mentor, committee chairman, and a friend.

I wish to thank Dr. Gary Armitage and David Richards for serving on my thesis committee and their invaluable assistance in the preparation of this text.

Thanks to Dr. Jing-Jing Qian and Dr. Anna Wedrychowska for helping me with research techniques and giving me valuable suggestions.

Thanks to Dr. Edward J. Taggart for his continual support and encouragement.

# ABSTRACT

In recent years, one focus of research in the field of periodontal regeneration has been on bioactive compounds that can be used to enhance osteogenesis and promote bone regeneration. In healing tissues, cells are anchored and migrate on collagen. A synthetic peptide (P-15), was developed that mimics the cell-binding activity of collagen (Bhatnagar and Qian, 1992 Trans. Orthoped. Res. Soc.17:106). P-15 is adsorbed in a concentration dependent manner on anorganic bone mineral (ABM) and is not eluted under physiological conditions. In the present experiments, we examined the attachment of periodontal ligament fibroblasts (PDLF) to anorganic bone mineral with and without P-15. Light and scanning electron microscopy indicated a greater number of PDLF were associated with ABM.P-15 than the control. Cells attached to ABM.P-15 seemed to be well anchored and assumed a normal spindle-like shape on, and between the particles. PDLF were prelabeled with $^3$H-thymidine and cultured on ABM.P-15 for several time points up to 24 hours. Prelabeled PDLF were also incubated with different concentrations of adsorbed ABM.P-15. Results showed that not only greater number of PDLF bind ABM.P-15 than the control, but also cell binding increased in a concentration dependent manner. Greater numbers of cells were observed in association with ABM.P-15 than with ABM alone. In another experiment adherent cells to ABM or ABM.P-15 were pulsed with $^3$H-thymidine and $^{14}$C-proline in order to monitor DNA and protein synthesis respectively. Cells on ABM.P-15 incorporated more radioactivity and showed greater increases in DNA synthesis with time compared to cells on ABM alone. Proline incorporation was also markedly greater in the P-15 group which showed a linear increase with time. These observations suggest

that P-15 provides the essential 3-D environment stimuli to promotes attachment of PDLF to ABM and significantly increase proliferation and protein synthesis. The ability of P-15 to enhance cell attachment and proliferation may prove to be a useful adjunct to periodontal regeneration procedures.

# TABLE OF CONTENTS


DEDICATION.......... iii

ACKNOWLEDGMENT.......... iv

ABSTRACT.......... v

TABLE OF CONTENTS.......... vii

LIST OF FIGURES AND TABLES.......... ix

I. INTRODUCTION.......... 1

II. REVIEW OF LITERATURE.......... 3

IIa. Collagen-Cell Interaction.......... 3

IIb. Native Collagen in the Dentinal Matrix.......... 5

IIc. Collagen/Hydroxyapatite Composite as a Graft Material.......... 6

IId. Immunogenicity of Collagen Implants.......... 6

IIe. Periodontal Ligament Cells.......... 7

IIf. "P-15".......... 9

III. MATERIALS AND METHODS.......... 12

IIIa. General.......... 12

IIIb. Attachment and Proliferation.......... 14

IIIc. Light and Scanning Electron Microscopy.......... 16

IV. RESULTS.......................................................... 18

IVa. P-15 Content of ABM.......................................... 18

IVb. Cellular Attachment to Uncoated and P-15 coated ABM, Light and Scanning Electron Microscopy.......................................... 19

IVc. Cellular Attachment to Uncoated and P-15 coated ABM, Quantitative Aspects.......................................... 20

IVd. Total Protein Assay.......................................... 21

IVe. Cell Proliferation and Cell Viability .......................................... 22

V. DISCUSSION.......................................................... 38

VI. BIBLIOGRAPHY.......................................................... 43

# LIST OF FIGURES AND TABLES


| | | |
|---|---|---|
| Figure 1. | P-15 content of ABM | 24 |
| Figure 2. | Cellular Attachment to Uncoated and P-15 coated ABM, Light Microscopy | 25 |
| Figure 3. | Cellular Attachment to Uncoated and P-15 coated ABM, Scanning Electron Microscopy | 27 |
| Figure 4. | Relative Cell Numbers Attached to Uncoated and P-15 coated ABM | 29 |
| Figure 5. | Attachment of PDL Cells to ABM containing different concentrations of adsorbed P-15 | 30 |
| Figure 6. | Total Protein Assay: Relative PDL Cell Attachment to P-15 Coated and Uncoated ABM | 31 |
| Figure 7A. | Relative Incorporation of $^{3}$H-Thymidine in PDL Fibroblasts Cultured on P-15 coated and Uncoated ABM. | 32 |
| Figure 7B. | Ratio of $^{3}$H-Thymidine Incorporation in PDL Fibroblasts on P-15 coated and Uncoated ABM | 33 |
| Figure 8A. | Relative Incorporation of $^{14}$C-Proline in PDL Fibroblasts Cultured on P-15 coated and Uncoated ABM | 34 |
| Figure 8B. | Ratio of $^{14}$C-Proline Incorporation in PDL Fibroblasts on P-15 coated and Uncoated ABM | 35 |
| Figure 9. | Ratio of Labeled Protein to Labeled DNA in culture on P-15 coated and Uncoated ABM | 36 |
| Table 1. | Comparison of $^{3}$H-Thymidine and $^{14}$C-Proline Uptake in PDLF Cultured on P-15 coated and Un-coated ABM | 37 |

# I. INTRODUCTION

Periodontitis is defined by the Glossary of Periodontal Terms (1992) as "inflammation of the supporting tissues of the teeth. Usually a progressively destructive change leading to loss of bone and periodontal ligament". Most of the treatment modalities for periodontal disease have traditionally focused on removal of the etiologic factors and surgical resection of the diseased tissue to create an environment that facilitates day-to-day removal of the disease-causing bacteria.

Long-term studies have demonstrated favorable results for both surgical and non-surgical approaches to treating and controlling periodontal disease (Pihlstrom, *et al.* 1983, Lindhe, *et al.* 1984, Isidor and Karring 1986). Despite several short-comings, it has been recognized that patients and therapists are able to maintain reduced and/or repaired periodontal tissues health and function (Hirschfeld 1978, McFall 1983). Nevertheless, the prospect of regeneration of lost periodontium is appealing and should be considered as one of the major goals of periodontal treatment. It is, therefore, of great clinical significance that materials and methods be developed so that the destroyed periodontium can be regenerated.

Regeneration is defined as reproduction or reconstitution of a lost or injured part to its original or prediseased state. According to the World Workshop in Periodontology (1989), successful periodontal regeneration must contain evidence of new bone, cementum, and functionally oriented periodontal ligament fibers. Early in the regenerative process, progenitor cells migrate into the injured tissue and through ill-defined biochemical and physiological changes presumably stimulate these cells to proliferate and

differentiate into the cells that can form bone, cementum, and fibrous attachments.

In recent years, principles of guided tissue regeneration with occlusive membranes have been applied to the process of periodontal tissue regeneration (Nyman 1982, Magnusson 1985). Using this technique, the cells from epithelium and gingival connective tissues can be physically excluded from the injured site, thereby maintaining a space for the migration of osteogenic cells. Although this technique has met with limited success, clinicians find it very demanding and not completely predictable. Numerous authors have suggested that bioactive compounds, such as growth factors (Lynch 1981 & 1991, Raines 1989, Libby 1985, Sporn 1978, Wirthlin 1989) and bone morphogenic proteins (BMP) (Lindholm 1988, Takagi 1982, Ferguson 1987, Johnson 1988) might be of value in periodontal regeneration procedures. Utilization of these bioactive compounds either with or without guided tissue regeneration may enhance osteogenesis and promote periodontal tissue regeneration in a more predictable manner.

Bhatnagar *et al.* (1992) have synthesized a 15 residue polypeptide (P-15) that mimics the cell-binding domain of the alpha chain of type I collagen molecules. Initial studies have shown that this compound binds avidly to cells and promotes cell migration into otherwise inhospitable environments (Qian and Bhatnagar 1994, submitted for publication). It is therefore conceivable that P-15 could be used as a bioactive compound to stimulate the migration and attachment of progenitor cells during periodontal regeneration.

# II. REVIEW OF LITERATURE

## IIa. Collagen-Cell Interactions

Some of the earliest events in tissue regeneration are the migration, attachment, and proliferation of the progenitor cells. Collagen-cell interactions appear to play important regulatory roles in a variety of cell activities. It has been known for many years that cells can attach to and migrate on collagen (Shore 1980). Erhmann and Gey (1956) reported that many strains of cell and tissue explants attached to the collagen substrate and this interaction improved their growth. Cells such as endothelial cells (Rixen 1989), hepatocytes (Sattler 1978, Rubin 1986), and chicken fibroblasts (Gey 1974) were found to have more extended life spans and better growth potential on collagen membranes. Saito (1993), reported that human mesangial cell proliferation may be regulated by the extracellular matrix components such as type I, III, and IV collagen.

A large number of cell surface proteins have been shown to act as receptors for the extracellular matrix components such as laminin, fibronectin, and several types of collagen (Reichardth 1991, Hynes 1992). Prominent among these are proteins of the integrin family. Integrins are heterodimeric (contain $\alpha$ and $\beta$ subunits) glycoprotein complexes that are expressed by the variety of cells. Combination of different a and b subunits result in the production of several types of integrins. Members of integrin family that act as a collagen receptor usually contain a $\beta_1$ subunit which is also known as VLA-2. Cells such as fibroblasts, platelets, and melanomas are known to express this family of integrin ( Elices and Hemler 1989). Takada and Hemler (1989) Studied

VLA-2 and concluded that $\alpha 2\beta 1$ integrin specifically contains the collagen binding domain.

Others have suggested that distinct integrins such as $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_5\beta_3$ may also be involved in cell adhesion to different collagen isotypes (Vandenberg 1991, Lein 1991, Davis 1992).

In addition, collagen is known to participate in cell and tissue differentiation. In 1991, Griffith and Sanders investigated the effects of extracellular matrix components on the differentiation of chicken embryo tail bud mesenchyme. They concluded that type I collagen promotes myogenesis and chondrogenesis, while type IV collagen is involved in myogenesis alone. Fouser and co-workers (1991) reported that up-regulation of the $\alpha_1$(I)-collagen gene may be linked to phenotypic changes associated with the transition of quiescent endothelial monolayer cells to an angiogenic state. Karst, *et al.* (1988) in their studies stated that the physiological property of an isotype of collagen such as type I or type III is a significant factor in the development of organoid structures.

It has been postulated that 3-D interaction between cells and extracellular matrix components such as collagen play a major role in the cellular differentiation. Extracellular matrix, possibly trough integrins, stimulates signal transduction pathways into the cell (Hynes 1992) as well as cytoskeletal changes within the cell (Geiger 1992, Hay 1990). These changes will eventually lead to expression of specific genes and cellular differentiation. The literature contains numerous studies that substantiate the role of collagen in cell and organ differentiation, however review of all these reports is beyond the scope of this section. The reader is referred to a comprehensive review of the subject by Kleinman and Klebe (1981) and Hay (1990).

## IIb. Native collagen in the dentinal matrix

It has been suggested that partial demineralization of dentinal surfaces and exposure of collagen fibers, using citric acid, may promote the formation of a new periodontal attachment. Garrett, *et al.* (1979), used scanning electron microscopy and transmission electron microscopic techniques to study the topography of dentinal surfaces following acid etching. They concluded that acid treatment may cause exposure of collagen fibrils which are a part of the dentinal matrix, thus providing a suitable nidus for splicing with new fibers during the healing process. Others had a different hypothesis for the role of these exposed collagen fibrils. Polson (1984) postulated that the "mat-like" layer of exposed collagen fibrils that he observed following acid demineralization of dentinal surface may become a hospitable environment for cell attachment. Boyko (1979), reported an increased attachment of cells to citric acid-treated roots, confirming the importance of collagen fibril exposure in the enhancement of cellular attachment. In a histological study of wound healing in dogs, Register (1976), demonstrated that demineralization of the root surfaces using citric acid promoted regeneration with osteogenesis, cementogenesis, and periodontal ligament insertion from bone into new cementum layer.

The use of citric acid demineralization of root surfaces in humans has had mixed results. Albair, *et al.* (1982), reported significantly more teeth with fibrous attachment following treatment of the root surfaces with citric acid as compared to controls. Lopez (1993) implanted eighteen periodontally diseased roots which were treated with citric acid. The result showed that the diseased roots which had been treated with citric acid were more likely to form new

connective tissue attachment. In contrast, reports by Smith *et al.* (1986), Marks and Mehta (1986), Moor *et al.* (1987), and many others have failed to show any significant difference in the new connective tissue attachment between untreated and citric acid treated root surfaces.

## IIc. Collagen/Hydroxyapatite combination as a graft material

Collagen in combination with hydroxyapatite has been used as a graft material to enhance tissue repair and regeneration. In 1989, Mehlisch reported on a study in which deficient alveolar ridges were augmented with purified fibrillar collagen mixed with particulate hydroxyapatite. Results at 24 months showed a significant level of augmentation, both clinically and histologically. Similar results were also noted in a rabbit model for calvarial repair when hydroxyapatite impregnated with collagen was used (Thaller 1990).
Cornell and co-workers (1991) reported on a multicenter prospective trial of Collagraft® (a mixture of hydroxyapatite, tricalcium phosphate, and fibrillar collagen) combined with autogenous bone in the treatment of long bone defects. Results indicated that the Collagraft® mixture was as effective and safe as the autogenous bone graft alone. Conversely, Bell (1988) indicated that combination of hydroxyapatite, tricalcium phosphate, and collagen did not significantly induce mandibular healing in rats.

## IId. Immunogenecity of collagen implants

The usefulness of collagen as a versatile biomaterial in medical and dental applications (i.e., wound support, wound repair, tissue augmentation, cell attachment and growth etc.) has been well established. However, a major drawback in using collagen implants is its potential for eliciting a host immune reaction and delayed hypersensitivity. Collagen molecules contain

a number of antigenic determinants to which antibodies can be raised. Sensitivity and specificity of these antibodies have been tested in both human and bovine tissues (Nowack *et al.* 1976). Circulating anti-collagen antibodies have been isolated from patients who showed hypersensitivity reactions to reconstituted bovine collagen implants (Trautinger 1991, Ellingsworth 1986, Cooperman 1984). Even though cross-reactivity of antibovine collagen antibodies is rare, Trautinger (1991) reported some cross-reactivity to human type III collagen upon multiple challenges.

Systemic side effects to collagen implants such as a foreign body granulomatous reaction, erythema, and induration have been reported in approximately 0.4 to 5% of the patients who either received these implants or who were tested for hypersensitivity using injectable collagen preparations (Brooks 1982, Barr 1982).

## IIe. Periodontal Ligament Cells

The periodontal ligament is a narrow band of connective tissue that supports the teeth in the alveolar socket. As with other connective tissue structures, it is made up of a matrix and a cellular component. The most predominant cells in this compartment bear a close resemblance to the fibroblast cell lines, hence the name periodontal ligament fibroblast (PDLF). In spite of the morphologic similarities, comparison between these cells and other local connective tissue fibroblasts, namely gingival fibroblasts, have shown specific differences in function, growth characteristics, and protein and other macromolecule synthesis (Somerman 1988, Mariotti 1990). It has also been suggested that only PDLF cells are associated with regeneration of new periodontal ligament structures when reimplanted with demineralized roots in dogs (Boyoko 1981).

The regeneration of a functional periodontal ligament requires formation of bone, cementum, and collagen fibers. Thus, it is logical to assume the presence of a phenotypically heterogeneous population of PDLFs, that originates from a common progenitor cell line.
Melcher (1976), indicated that during the process of wound healing, subpopulations of the PDLF stem cells migrate into the injured area from the surrounding healthy periodontal ligament space and become involved in formation of a new periodontal attachment. This pattern of progenitor cell proliferation and migration into the periodontal ligament space following an injury was also noted by Gould (1980). Aukhil and Iglhaut (1988) examined periodontal regeneration in monkeys. They reported that "a limited zone of the PDL apical to the wound acts as a source of cells migrating into the wound".

Perera and co-workers (1980) studied the kinetics of PDL cells in the erupting mouse molar. They identified a stem cell population at the apical zone. This study also confirmed that the migration and differentiation pattern of PDLF was from the apical zone to the other zones in an occlusal direction. Others have also reported the presence of this population of progenitor cells predominantly around the paravascular sites ( McCulloch 1985, Gould 1983).

Even though the existence of a separate subpopulation of periodontal ligament fibroblasts has not been confirmed, it is evident that the pluripotent progenitor cells proliferate and subsequently differentiate into specific phenotypes. Recent investigations have focused on the characterization of this heterogeneous cell population. Piche *et al.* (1989) isolated two subpopulations of PDLF from explants of human periodontal tissues. The results of this experiment suggested the presence of "fibroblast-like" and " osteoblast-like" cell lines.

Special efforts have been directed toward identification of PDLF progenies that have terminally differentiated into osteoblasts and/or cementoblasts. Arceo (1991) reported that unlike gingival fibroblasts, PDLF cells showed significant levels of alkaline phosphatase activity and were able to produce "mineral-like nodules" *in vitro.* Nojima *et al.* (1990) examined bovine PDL cell cultures for the presence of osteoblast like cells. He indicated that PDL cells produced a higher levels of cAMP in response to parathyroid hormone. Nojima and his group also identified a protein in this cell culture that immunologically cross-reacted with bovine bone *gla* protein.

**IIf. "P-15"**

p-15 is a synthetic fifteen-residue peptide that is modeled after half the residues in a single turn of the $\alpha 1(I)$ chain of collagen (Bhatnagar 1992). The conformational studies that were done by Bhatnagar and co-workers on this synthetic 15-residue peptide indicated that this sequence ($^{766}$ GTPGPQGIAGQRGVV$^{780}$) has a high preference for $\beta$–strand conformation. The addition of triple helix promoting strings of glycine-proline-proline tripeptides to N- and C- terminals did not change the conformation of this peptide. Based on these studies, it was postulated that the presence of this unstable region in the collagen helix may cause a conformational perturbation, resulting in a uniquely accessible region which may act as a recognition and binding site for cells and other molecules. In the same study Bhatnagar observed that addition of P-15 to fibroblasts cultured on collagen-coated dishes significantly inhibited cell binding to the collagen substrate, indicating competition for the cell binding region of collagen. Thus it was suggested that the 15-residue synthetic peptide may mimic the cell binding domain of $\alpha 1(I)$ collagen molecule.

Qian and Bhatnagar (1994) Studied the ability of P-15 to promote cell attachment to hydroxyapatite. They reported an increased attachment of dermal fibroblast to the hydroxyapatite particles coated with P-15 as compared to hydroxyapatite alone . She also showed that the interaction of dermal fibroblasts with P-15 coated hydroxyapatite particles results in an induction of alkaline phosphatase in these cultures, suggesting an osteoblast-like phenotype. Other studies have indicated that P-15 facilitates infiltration of Fibroblasts into agaros gels (Bhatnagar *et al.* 1994).

Qian *et al.* (1994) investigated the effects of P-15 on monolayer cultures of dermal fibroblast. They show a concentration dependent increase in expression of collagenase and gelatinase. An *in vivo* evaluation was done by implanting P-15 coated and uncoated hydroxyapatite into the surgically created cranial defects in rabbits (Parsons, Bhatnagar, and Tofe 1994). This study suggested an increased ingrowth of bone in the cranial defects filled with P-15 coated hydroxyapatite as compared to uncoated hydroxyapatite.

## HYPOTHESIS

An ideal graft material for periodontal regeneration should provide a scaffolding for cells to migrate on and attach to (conduction). It should also be able to enhance proliferation and differentiation of progenitor cells to bone-, cementum-, and ligament- forming cells (induction).

Porous hydroxyapatite in various forms has been studied clinically as a graft material in periodontal defects (Kenney 1986 & 1988, Carranza 1987, Callan 1993). It has been postulated that porous hydroxyapatite provides a matrix for ingrowth of bone by allowing attachment and migration of osteogenic progenitor cells (Pillar 1983, Uchida, *et al.* 1985). However, clinically successful periodontal regeneration using hydroxyapatite alone as a graft material does not seem to be predictable.

In this study we are testing the potential of a combination of P-15 and porous hydroxyapatite as a graft material. Specifically, we hypothesize that adsorption of P-15 on a porous anorganic bone mineral (ABM, natural porous bovine hydroxyapatite) will promote the attachment and proliferation of periodontal ligament cells as compared to uncoated particles of ABM.

# III. MATERIALS AND METHODS

## IIIa. General

**P-15 Synthesis:**

P-15 was provided by Dr. Bhatnagar of the UCSF Laboratory of Connective Tissue Biochemistry. The 15 residue peptide, GTPGPQGIAGQRGVV (P-15), was synthesized by solid phase procedures, using symmetrical anhydrides for coupling of Boc-amino acids, except for glysine residues which were coupled with 1-hydroxybenzyl triazole. The peptide was purified by reverse phase HPLC using a C-18 column in a gradient of $H_2O$ and acetonitrile. The sequence was confirmed by sequence analysis (Bhatnagar *et al.*, Submitted for publication).

**Tissue culture:**

Primary cultures of human periodontal ligament fibroblasts were donated by Dr. Paul Johnson of the UCSF division of periodontology. These cultures were maintained in Eagles minimum essential medium (MEM) supplemented with 10% fetal bovine serum, and antibiotics (Fungizone 250 µg/ml, Streptomycin 10,000 units/ml, Penicillin 10,000 units/ml) (UCSF Cell Culture Facility). Cell cultures were kept in a humidified incubator at $37^{o}$ C with 5% $CO_2$. To avoid nonspecific binding of cells, glass petri dishes were siliconized (Sigmacote®, Sigma chemical Co. St. Louis, MO.), washed and allowed to air dry under UV to maintain sterility.

**Anorganic bone mineral:**

ABM ( particles of 250-420 microns)was obtained from CeraMed Corp., Lakewood, CO. and coated with P-15. Coating was done according to Qian and Bhatnagar (1994, accepted for publication). Briefly, ABM was equilibrated with a solution containing different concentrations of P-15 in PBS ( ratio of 1.0 g ABM to 2.0 ml of solution) for 24 hours Coated ABM was washed several times with PBS and dried in a desiccator. The amount of adsorbed P-15 was determined by hydrolysis with 2N NaOH (for 2 hours at 100° C) and amino acid analysis.

**Assay for adsorbed P-15**

The peptide concentration was determined by measuring the $-NH_2$ content of peptide hydrolyzate using a fluorometric method (Jones 1981). In this method, a fluorophore adduct is formed by the reaction of o-phthaldialdehyde. The unreacted o-phthaldialdehyde does not Fluoresce. Adsorbed P-15 was hydrolyzed with 2.0 N NaOH at 100° C. 200 mg samples were placed in tubes containing 2 ml of 2.0 N NaOH and the tubes were sealed. Sealed tubes were placed in a boiling water bath for 2 hours. The hydrolyzates were neutralized with HCl and their pH were adjusted to approximately 9.0. The reaction mixture contained 1 ml of the hydrolyzate, 0.8 ml of 0.4 M sodium tetraborate buffer (pH 9.7), and 0.2 ml of o-phthaldialdehyde reagent solution (Sigma chemical Co. St. Louis, MO.). Resultant derivatized amino acids were measured with spectroflurometer (exitation at 340 nm and emission at 455 nm). Final amount of adsorbed P-15 was calculated using various concentrations of P-15 in PBS as a standard

curve (hydrolyzed and analyzed using the same method). Uncoated ABM particles serve as blank to compensate for the ABM background readings.

**Radioactive Material:**

Thymidine, [ methyl-$^{3}$H]- (specific activity of 2.0 μCi/mmol) and proline, L-[$^{14}$C(U)]- (specific activity of 250 μCi/mmole) were obtained from New England Nuclear.

## IIIb. Attachment and Proliferation:

**Radiolabeled thymidine and proline incorporation:**

In order to compare the number of cells attached and their viability on ABM and ABM.P-15, the incorporation of radiolabeled thymidine and proline was measured. Periodontal ligament cells were cultured on ABM and ABM.P-15 (in all experiments 0.3 g of ABM or ABM.P-15 was used).
The incorporation of radiolabeled Thymidine and proline was examined every 24 h, after 4 h pulse labeling with a mixture of 2 μCi $^{3}$H-thymidine and 0.5 μCi of $^{14}$C-proline in 1 ml of medium. Trichloracetic acid (10% w/v) insoluble pellets of the cultures were washed extensively with TCA to remove unincorporated radioactive precursors and incubated for 10 minutes with 0.1% SDS in 1 N NaOH, at 37$^{o}$ C. The amount of radioactivity was assayed in a Packard (model 1900 TR) scintillation counter. All of the experiments were done in triplicate.

**Cell binding assay:**

In order to maximize label incorporation into the cellular DNA, periodontal ligament cells were biosynthetically labeled with $^{3}$H-thymidine at half confluence level where they exhibit a high rate of DNA synthesis. After

reaching full confluence, the radioactive medium was removed and the cells were washed 3X with 50 μg/ml of non-radioactive thymidine. The amount of radioactivity contained in these cells was measured to be approximately 325 dpm per 1X $10^3$ cells.

**a) time course:** Harvested cells were resuspended in serum-free medium. Equal aliquots of cell suspensions were incubated with 0.2 g of ABM or ABM.P-15 for several time intervals ranging from 15 min. to 24 h. At each time point, the radioactivity in the attached and unattached cells was determined. A standard curve was constructed using known number of prelabeled cells plotted against dpm radioactivity.

**b) different concentrations of adsorbed P-15 on ABM:**
In order to determine whether P-15 specifically increases cell binding, the attachment of cells to P-15 coated ABM was measured as a function of adsorbed P-15. PDL cell preparation was done as was described above with the following modification:
Equal aliquot of $^3$H-thymidine pre-labeled periodontal ligament cells were incubated for 24 hours on 0.2 g of ABM with different concentration of adsorbed P-15. Control in this experiment consisted of the ABM without P-15.

**Protein Assay:**

4 X $10^5$ PDL cells were incubated with 0.2 g of either uncoated ABM or P-15 coated ABM in a total of 5 ml of MEM + 10% FCS for 6 and 14 days. Incubation was done in siliconized glass petri dishes to reduce the probability of nonspecific binding to the glass surfaces. Negative controls included 2 petri dishes with same amount of ABM or ABM.P-15, without cells. At each time point, The medium was removed and cells were washed once with 5 ml of cold PBS. Cells and ABM particles were collected in 1 ml of PBS and transferred into microcentrifuge tubes.

Following centrifugation for 3 minutes, PBS was removed and 500 μL of 0.1 N NaOH was added to each tube. The mixture was vortexed for 15 seconds and incubated for one hour in 65$^{o}$ C water bath.

100 μL aliquots of each sample was diluted 40 times with 0.2 M $NaHPO_4$ buffer (pH 7.4). 800 μL aliquots of the final sample was added to 200 μL of Bio-Rad protein assay dye and optical density was measured after 5 min. at 595 nm in a Shimadzu spectrophotometer (model UV-160).

Two standard curves were made using a) different concentrations of a standard protein solution (in this case Bovine Serum Albumin) and b) known cell numbers. The protein assay protocol was followed for each of these positive controls.

## IIIc. Light and scanning electron microscopy

### Light microscopy

All the light microscopic analysis were accomplished using an inverted Olympus microscope (IMT-2 model) at 20 or 40 times magnification. Light micrographs were obtained using an 35 mm Olympus camera attached to the microscope. Periodontal ligament cells were cultured for 14 days in the presence of ABM or ABM.P-15 as described earlier. Light micrographs obtained from these cultures are selected to represent the commonly observed relationship of PDL cells to the coated or uncoated ABM particles.

### Scanning electron microscopy

Periodontal ligament cells were cultured for 14 days in the presence of ABM or ABM.P-15 as described earlier. All specimens were washed with PBS and subsequently fixed with 2.5% (W/V) glutaraldehyde in 0.1 M cacodylate buffer. Samples were then dehydrated in a series of ethanol (25%, 50%, 75%,

90% twice, 100% twice) each for 10 minutes. Following this, the specimens were critical-point dried and mounted on metallic stubs. All the specimens were then coated with a gold-palladium alloy to a thickness of 30 nm and examined in a scanning electron microscope at 5 and 10 KV (model JSM-840A).

**Statistical analysis:**

A one-tailed Student's t-test was used to determine if there were statistically significant differences between the number of cells bound to ABM.P-15 and ABM alone. The same statistical method was also used to determine if there were differences between DNA and protein synthesis in cells bound to ABM.P-15 and ABM alone.

## IV. RESULTS

An *in vitro* model of cell culture on biomaterials such as hydroxyapatite is needed to examine the step-by- step events in this interactive process. Investigators have successfully cultured cells on hydroxyapatite substrate. Matsuda and Davis (1987) looked at the response of osteoblasts to bioactive glass. They concluded that this *in vitro* method can reproduce some aspects of known *in vivo* behavior of osteoblasts on these materials. Gregorie *et al.* (1990) described the influences of calcium phosphate biomaterials on human bone cells. They concluded that such *in vitro* model is useful in determining the biological response of cells.

Using the similar *in vitro* model, we examined the effect of P-15 on cell binding to the anorganic bone mineral (ABM) particles. If the attachment of cells to ABM is dependent on P-15, then cell binding should be proportional to the amount of P-15 adsorbed on the ABM particles. For this reason we prepared ABM particles that contained different amount of P-15.

### IVa. P-15 Content of ABM

Spectroflourometric amino acid analysis was used to measure the amount of adsorbed P-15 on the ABM particles. The adsorption of P-15 was calculated for each gram of ABM. The incubating solution contained different concentrations of P-15 dissolved in PBS. The results suggest that adsorption of P-15 is a dose-dependent and saturable process and is proportional to the concentration of P-15 in the incubation solution (Fig. 1). The surface adsorption reached an equilibrium around 250 µg of P-15 per ml of incubating solution.

At this concentration, approximately 217 ng of P-15 was adsorbed on to the surface of the ABM. Increasing concentration of P-15 in the solution did not yield increasing amount of adsorbed P-15.

The ability of the P-15 coated or uncoated anorganic bone mineral to support attachment of periodontal ligament cells was determined by microscopic examinations and binding assays.

**IVb. Cellular Attachment to Uncoated and P-15 coated ABM, Light and Scanning Electron Microscopy:**

In this experiment, the cultures prepared on dishes which were coated with 1% agarose. Under this condition the binding of cells to the dishes was abolished. Light microscopic observation of periodontal ligament cell cultures which were incubated for 14 days on an anorganic bone matrix (ABM), uncoated or coated with P-15 peptide, is shown in Fig. 2. As seen in Fig. 2, 1% agarose effectively blocked the attachment of cells to the dishes and cells are only observed in contact with the ABM particles. The relationship of periodontal ligament cell to uncoated ABM is presented in Fig. 2A, C, and E. Periodontal ligament cells were clearly seen at the periphery of the anorganic bone matrix particles, suggesting potentially a hospitable environment for cellular attachment. However, frequently cells in contact with uncoated ABM rounded up and lost their typical stretched spindle-shape configuration.

In contrast to the uncoated particles, P-15 coated ABM showed the ability to host a greater number of cells around the periphery. Many cells formed cellular bridges between particles( Fig. 2B, D, and F). These interparticle cellular bridges were seen rarely in the uncoated ABM cultures (Fig. 2C). Fig. 2D demonstrates the multilayered arrangement of the cells in the network of interparticle cellular bridges associated with P-15 coated ABM.

Fig 2D represents only one focal plain, however, looking through the other focal plains also confirmed the 3-D cellular arrangement. In the cultures associated with P-15 coated ABM, the intercellular bridging of the periodontal ligament cells often results in the contraction and clustering of the particles of ABM (Fig. 2F)

These cultures were examined at higher magnifications using scanning electron microscopy. Figure 3A and 3C represent two different magnifications of cultures of uncoated ABM particles. Very few cells are attached to the surface of the ABM particle. At this magnification, the porous surface of the ABM particle is readily seen in Fig. 3A. It also appeared that cells were colonizing inside the pores. Scanning electron micrographs of PDL cell cultures on P-15 coated ABM show a significantly greater number of cells stretched across the particle (Fig. 3D). In Fig. 3B a densely populated particle is visible. Aside from more cells, a large amount of materials, possibly extracellular matrix, is seen covering the P-15 coated particle as compared to the uncoated ABM particles (Fig 3A and 3B). The accumulation of cells and other materials on The ABM particle (Fig 3B) made it difficult to easily discern the topography of the ABM surface as seen in Fig 3A.

**IVc. Cellular Attachment to Uncoated and P-15 coated ABM, Quantitative Aspects:**

Kinetics of cell binding and binding as a function of P-15 contents of ABM were determined using $^{3}$H-thymidine pre-labeled periodontal ligament fibroblasts.

**Kinetics of cell binding**: in this experiment, maximally coated ABM particles were used to determine if cell binding followed a specific kinetic process. The results for both P-15 coated and uncoated cultures are presented

in the Fig. 4. The results demonstrate a rapid binding phase which slowed down to a plateau followed by a slight detachment of cells at the later time points. The binding profiles were similar for both cultures. These results also suggest no significant difference in binding activity between the cultures for the first two hours. After two hours of incubation, ABM.P-15 supported an average of 23500 ± 1263 cells. The same amount of uncoated ABM bound 16488 ± 1668 cells. The difference in cell number showed, after 2 hours, the binding efficiency of P-15 coated ABM ( ABM.P-15) were significantly greater than uncoated ABM ($p < 0.005$) (Fig 4).

**Binding as a function of P-15 contents of ABM:** binding assays were performed with different concentration of adsorbed P-15 on ABM. The results of this experiment can potentially tell us whether P-15 is responsible for enhanced cell attachment. As shown in the Fig. 5, The ability of P-15 coated ABM particles to support attachment of periodontal ligament cells increased with the increasing concentration of the adsorbed P-15. Fig. 5 also shows that the concentration of 133 ng adsorbed P-15 seems to be the saturation point for the cell attachment sites. The data suggest that at this point an equilibrium has been reached and increasing the concentration of adsorbed P-15 above this point will no longer significantly change the binding ability of the P-15 coated ABM particles.

### IVd. Total Protein Assay

In this experiment, total protein content of periodontal ligament cells was used as a measure of the number of the cells attached to the ABM particles at 6 and 14 days incubation with either P-15 coated or uncoated ABM. Initially, a standard curve was constructed which indicated the amount of

total protein in different concentration of periodontal ligament cell suspension. Subsequently this standard measurement was used to calculate the number of cells associated with the test cultures. The results of this experiment showed that attached cells continue to proliferate on the beads regardless of their coating status. At both time points, however, a greater amount of periodontal ligament cells were associated with the P-15 coated ABM cultures than uncoated control particles (Fig. 6). The difference between these two cultures were statistically significant for both 6 days ( $p < o.ooo3$) and 14 days ($p < 0.00004$).

**IVe. Cell Proliferation and Cell Viability**

The results from microscopic and quantitative cell binding assays suggested that more cells are associated with P-15 coated ABM as compared to uncoated ABM particles. The next logical step was to determine if the cells are viable and actively proliferating. Viability and the proliferative state of the cell that were attached to the anorganic bone mineral particles were confirmed measuring DNA and protein synthesis. PDL cell cultures on P-15 coated and uncoated ABM were pulse labeled with either $^{3}$H-thymedine or $^{14}$C-proline.

As shown in Fig. 7A and 7B, during the normal growth period of the culture, cells in association with P-15 coated ABM incorporated an average of 2 to 3 fold more radioactive thymidine into their DNA than cells on uncoated ABM. This difference in the radioactive incorporation was highly significant for all the time points (Table 1). After six days of incubation cells in association with both P-15 coated and uncoated ABM incorporated less radioactivity. Nevertheless, this drop was greater in cell cultures associated with uncoated ABM.

Pulse labeling the PDL fibroblasts cultures with $^{14}C$-proline showed enhanced label uptake by the cells incubated with ABM.P-15 as compared to ABM alone (Table 1) (Fig. 8A). The ratio of the $^{14}C$-proline incorporation into the cells associated with P-15 coated ABM and uncoated ABM continuously increased throughout the experimental period as shown in Fig. 8B. These results are suggestive of possibly more viable cells in association with P-15 coated ABM. Fig. 8A shows that at first two days, a negligible amount of proteins are being synthesized by the cells associated with uncoated ABM. However, at the same time points, cultures associated with P-15 coated ABM show an active protein synthesis which may suggest a more hospitable environment for cellular growth.

Fig. 9 shows the ratio of $^{14}C$-proline / $^{3}H$-thymidine incorporation (corresponds to the ratio of protein / DNA synthesis) is greater in the P-15 coated ABM cultures. Protein synthesis by the cells in contact with P-15 coated ABM started from the first day of incubation and continues to rise as a function of time up to day five and remained at that level for the remainder of the experimental period. PDL cell cultures over uncoated ABM did not start to make significant amounts of protein until day 3; and after the third day protein to DNA ratio either stayed the same or decreased.

# P-15 Content of ABM

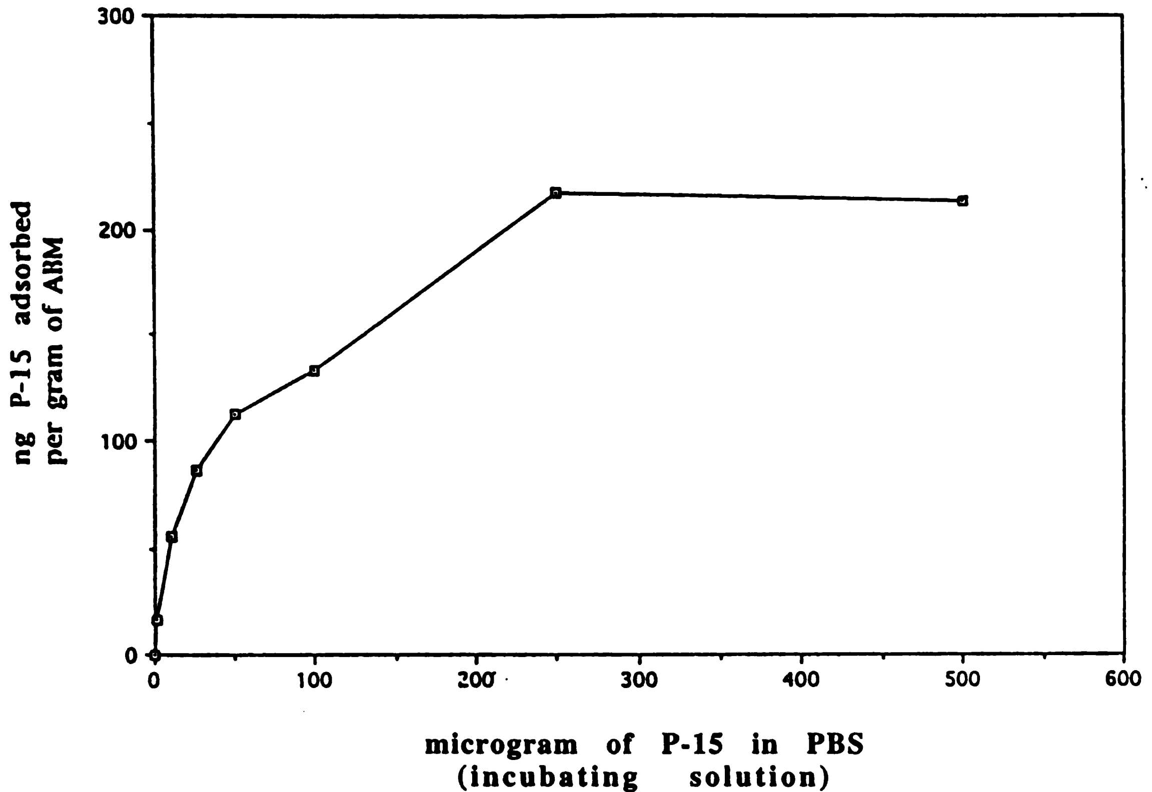



**Figure 1:** This graph shows the amount of adsorbed P-15 onto the ABM particles. Adsorption of P-15 seems to be a dose dependent phenomenon and is proportional to the concentration of P-15 in the incubation solution. At around 250 μg of P-15 per ml of incubating solution, the surface adsorption reached an equilibrium. At this concentration, approximately 217 ng of P-15 was adsorbed on to the surface of the ABM.

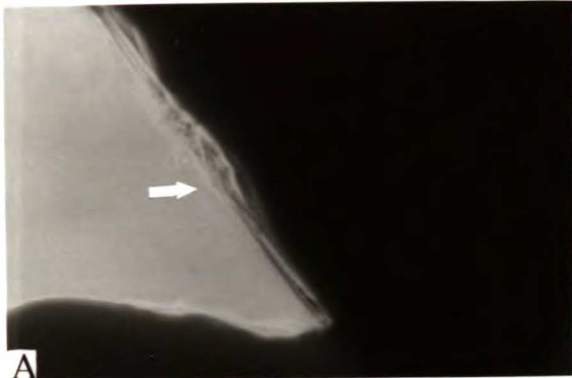
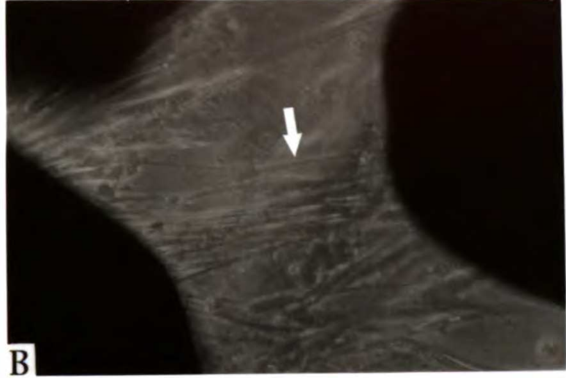
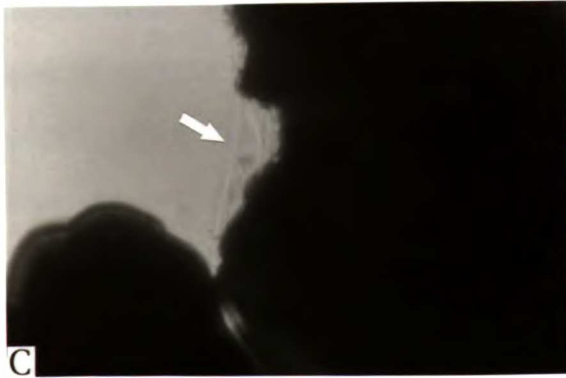
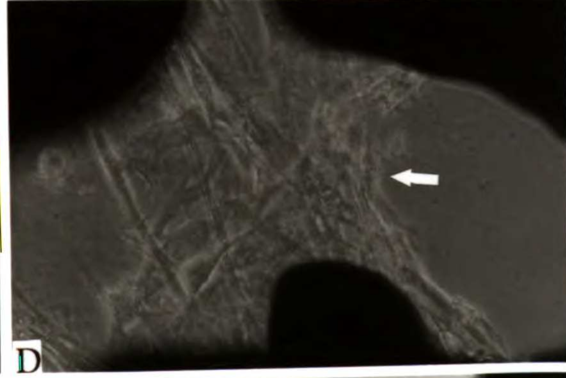
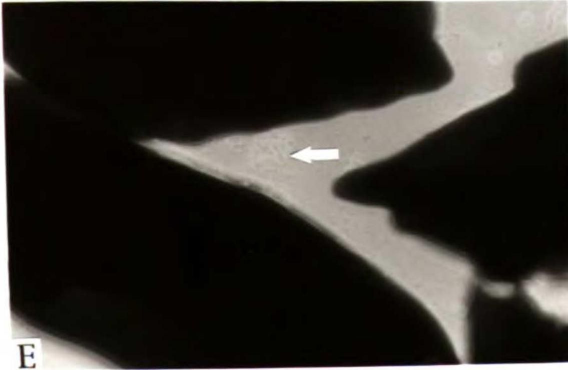
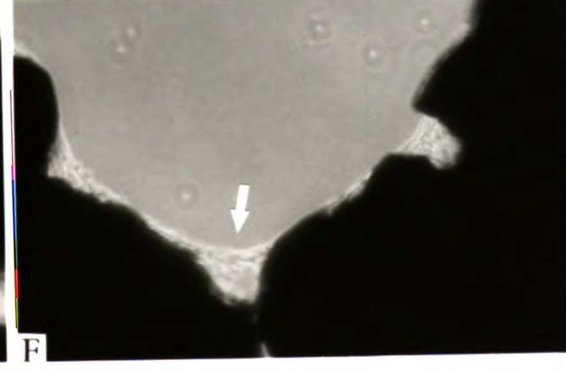
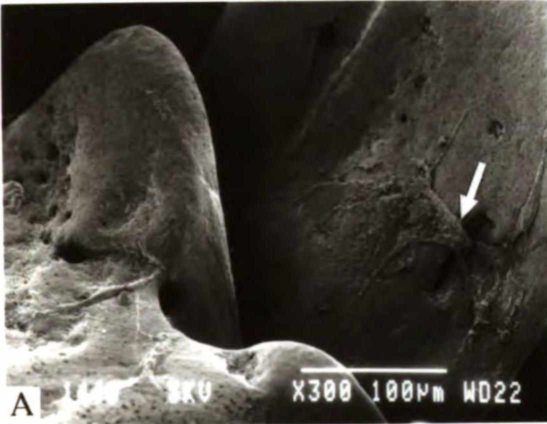
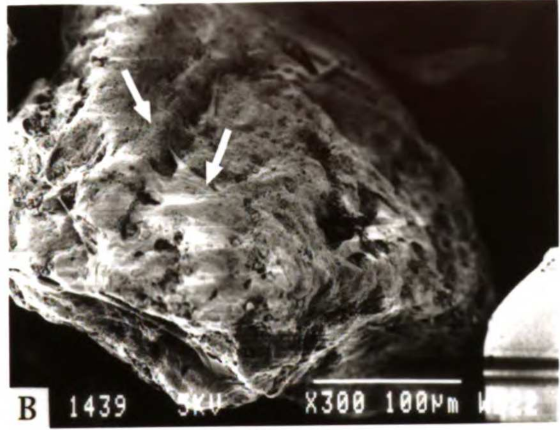
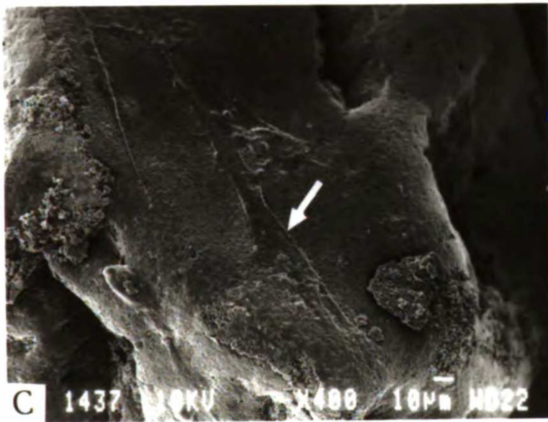







**Figure 2:** Light micrographs of 14 days old PDL cell cultures on uncoated or P-15 coated ABM. Relatively fewer cells are associated with uncoated ABM **(A)** than P-15 coated ABM **(B)**. Frequently PDL cells were observed stretching between particles forming cellular bridges **(B)**. Occasionally these interparticle cellular bridges were seen with uncoated ABM **(C)**. **(D)** Demonstrates the multilayered arrangement of the cells in the network of interparticle cellular bridges associated with P-15 coated ABM. The arrow points out to one focal plain of cells. **(E)** Frequently, PDL cells in association with uncoated ABM round up and lose their normal configuration. **(F)** The intercellular bridging of the PDL cells often results in the contraction and clustering of the particles of P-15 coated ABM. Light micrographs were taken with inverted phase Olympus microscope at 20X magnification.


A
X300 100µm WD22


B
1439
X300 100µm


C
1437
10µm WD22

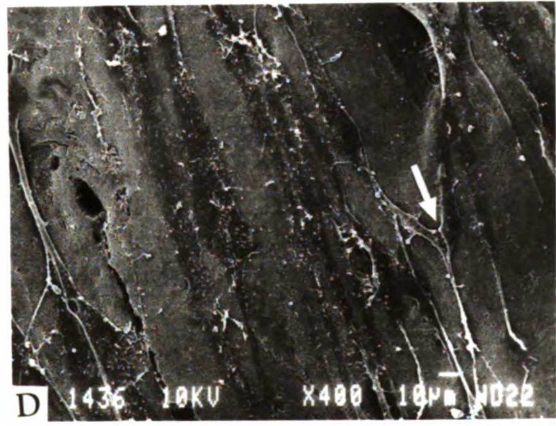

D
1436 10KV
X400 10µm WD22

**Figure 3:** Scanning electron micrographs of 14 days PDL cell cultures on ABM.P-15 or ABM alone. **(A)** and **(C)** Show PDL cells on an anorganic bone mineral particle without P-15. **(B)** and **(D)** show similar culture of PDL cells but incubated with anorganic bone mineral which is coated with P-15. **(A)** The arrow points out the porous surface of the ABM particle and PDL cells colonizing inside the pores. **(B)** The arrows in this figure show the densely populated P-15 coated ABM particle. It seems that the normal surface of this particle is covered by cells as well as possibly extracellular matrix. **(C)** the arrow points to the few number of cells are attached to the surface of the uncoated ABM particle. **(D)** The arrow points to one of the many cells that are stretched across the particle of P-15 coated ABM. It appears in these photomicrographs that significantly more cells are attached to the ABM particles coated with P-15 than the control particles. Photomicrographs **(A)** and **(B)** are taken at X 300 and photomicrographs **(C)** and **(D)** are taken at X400 magnification.

# Relative Cell Numbers Attached to ABM and ABM.P-15

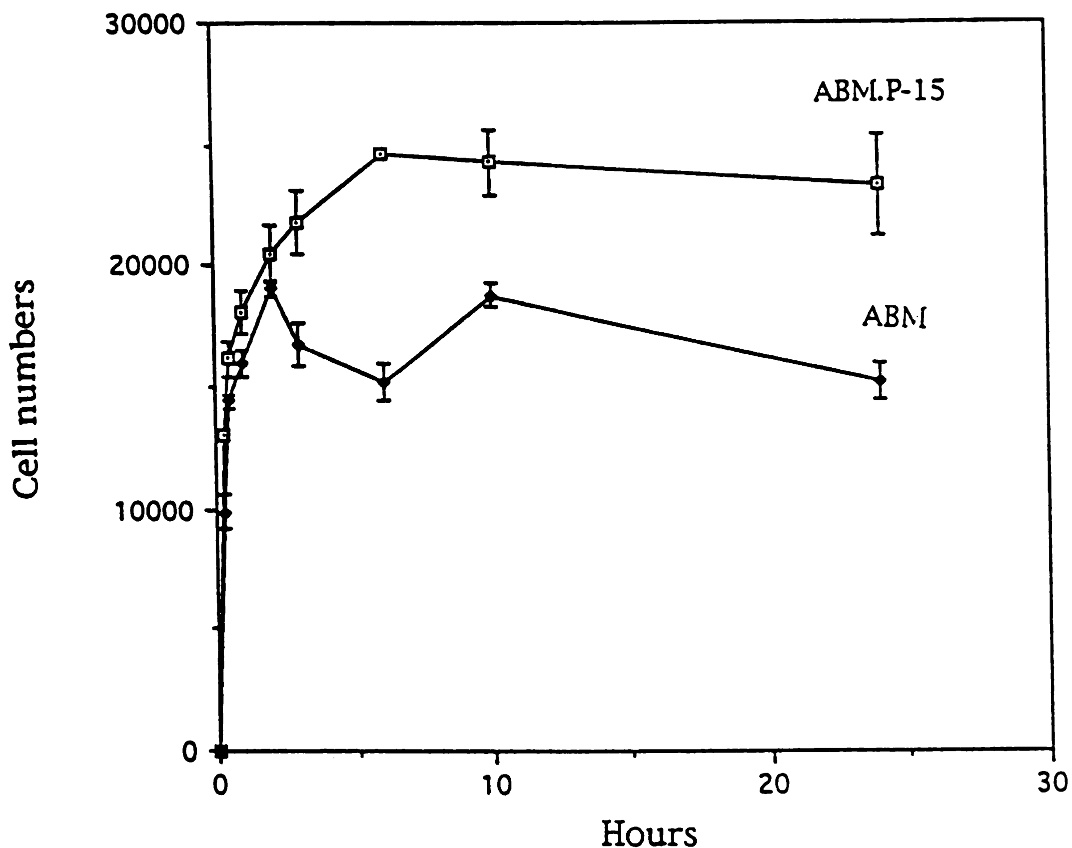



**Figure 4:** The results from time course cell binding assays using $^{3}$H-thymidine pre-labeled PDL cells are shown here. After two hours, efficiency of binding of cells on P-15 coated ABM is on average 50% greater than cells on un-coated ABM.

# Attachment of PDL Cells to ABM Containing Different Concentrations of Adsorbed P-15

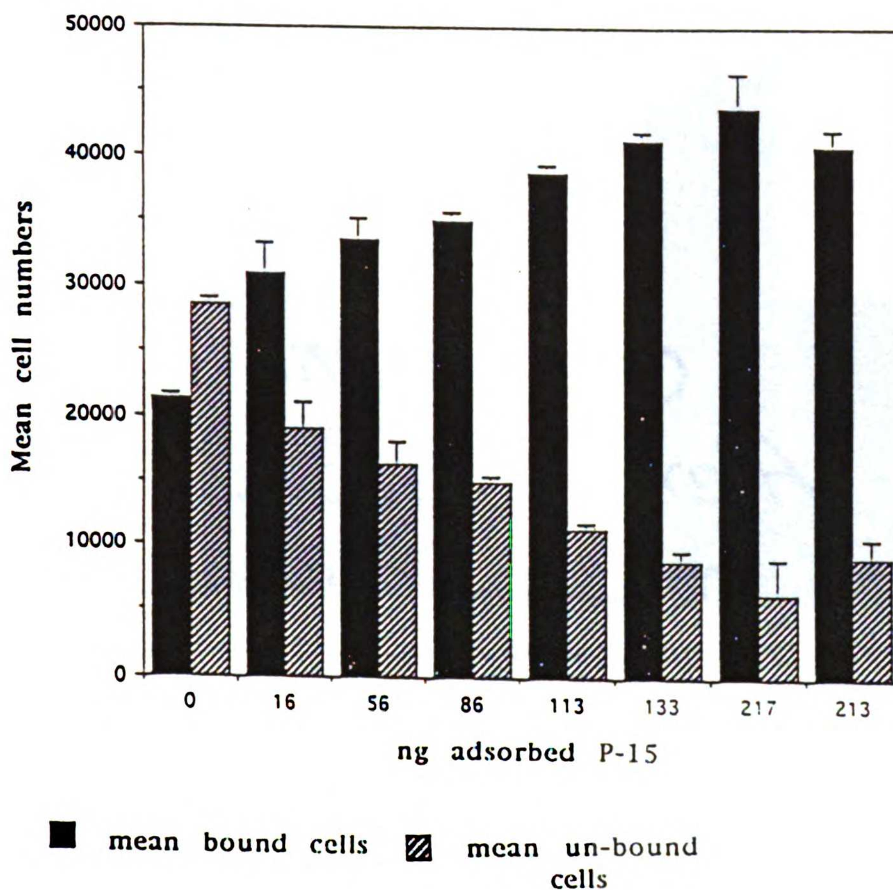



**Figure 5:** The graph shows that the attachment of PDL cells to P-15 coated ABM particles increases with the increasing concentration of the adsorbed P-15. Also the concentration of 133 ng adsorbed P-15 seems to be the saturating point for the cell attachment.

## Relative PDL Cell Attachment to P-15 Coated and Un-coated ABM

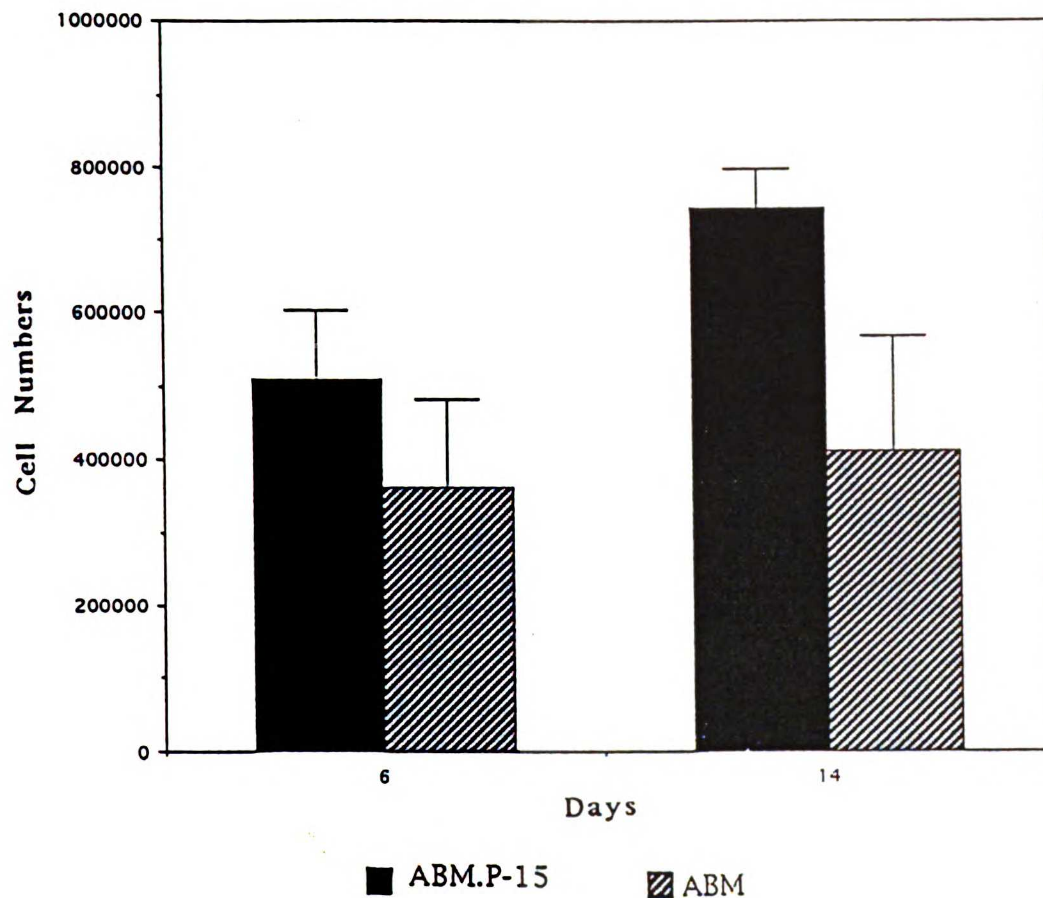



**Figure 6:** Total protein assay was used to measure the number of cells which are attached to the ABM particles. At both time points, a greater number of cells were attached to the p-15 coated ABM than the un-coated particles.

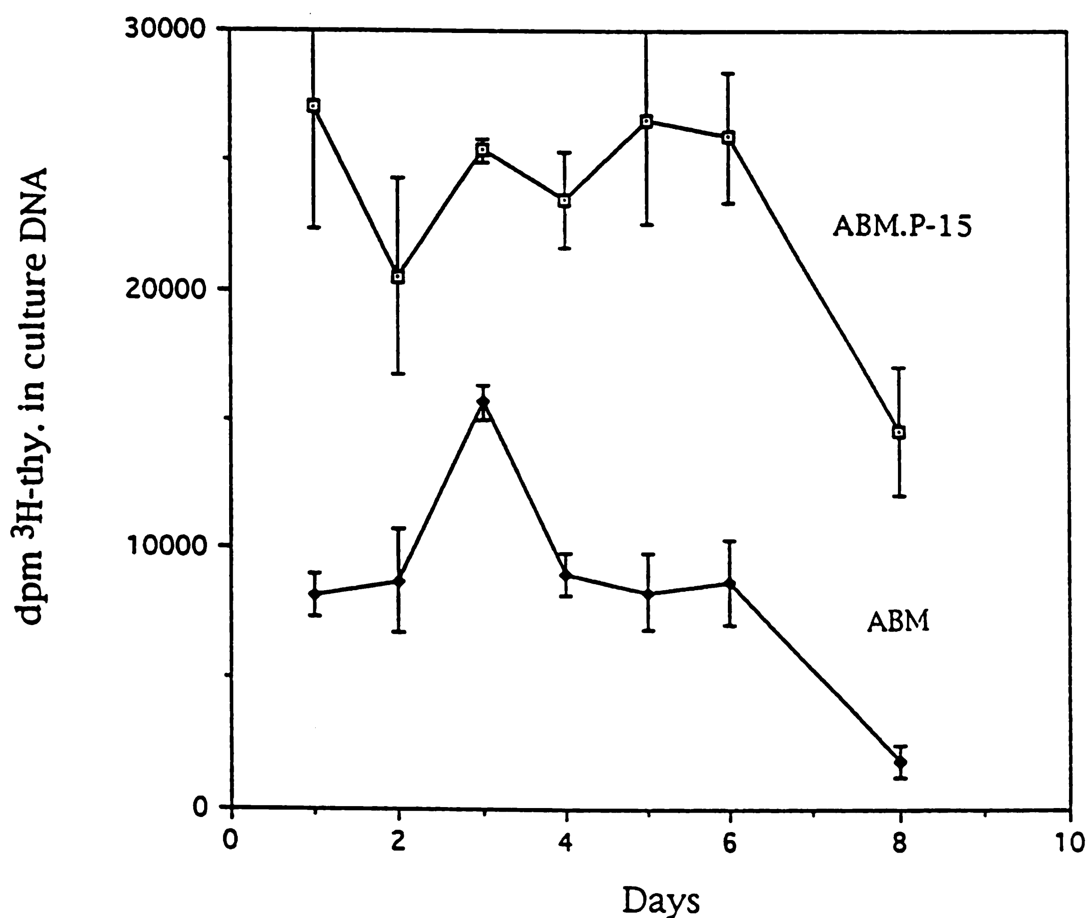



**Figure 7A:** Periodontal ligament cells in association with P-15 coated ABM (ABM.P-15) incorporated a greater amount of $^{3}$H-thymidine as compared with cells on un-coated particles (ABM).

# Ratio of $^{3}$H-Thymidine Incorporation in PDL Fibroblasts on ABM.P-15 and ABM

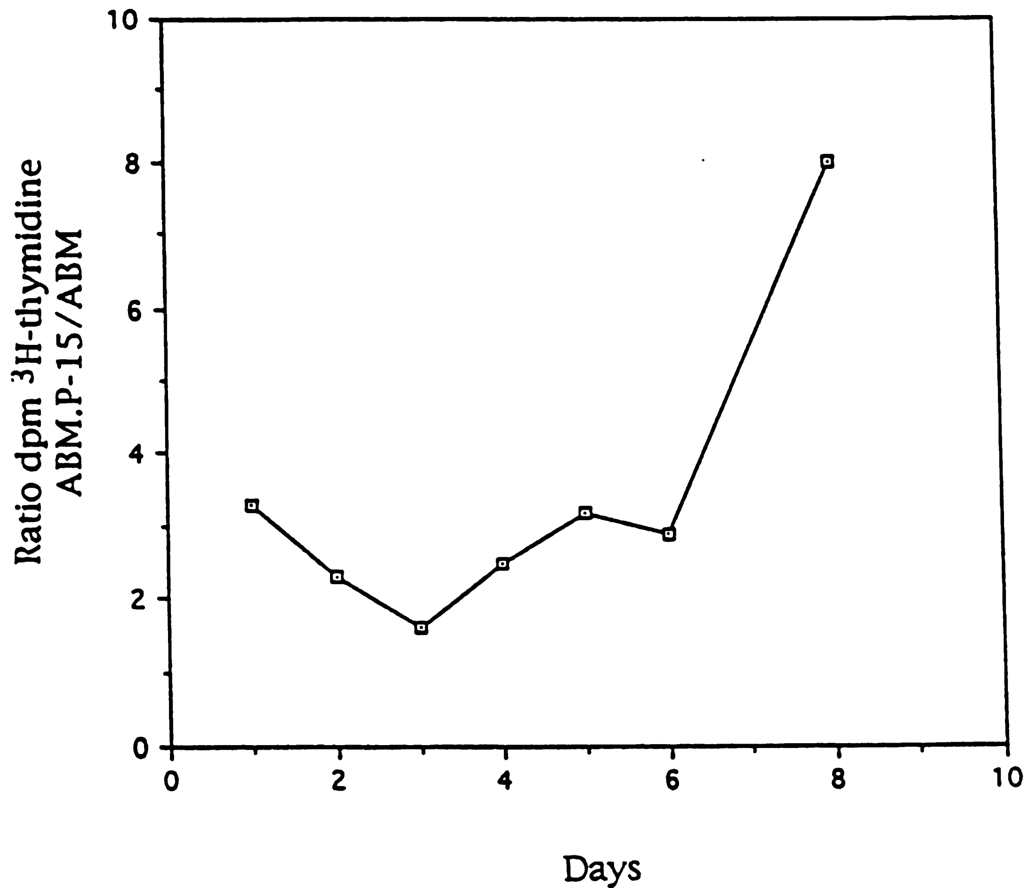



**Figure 7B:** During the normal growth period (1 to 6 days) PDL cells associated with P-15 coated ABM (ABM.P-15) incorporated 2 to 3 times greater amounts of radioactivity in to their DNA.

# Relative Incorporation of $^{14}C$-Proline in PDL Fibroblasts on ABM.P-15 and ABM

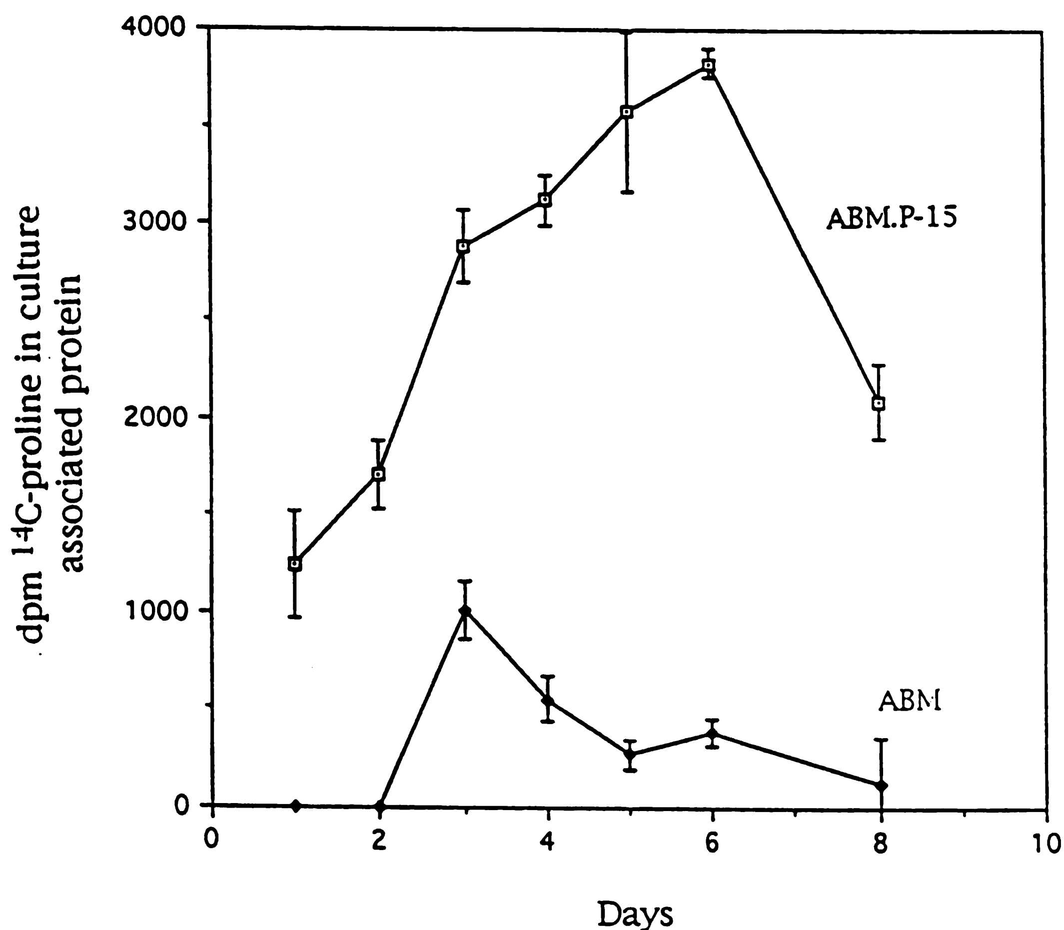



**Figure 8A**: Periodontal ligament cells cultured on P-15 coated ABM (ABM.P-15) incorporated a greater amount of $^{14}C$-proline into their proteins than cells cultured on un-coated particles (ABM).

# Ratio of $^{14}$C-Proline Incorporation in PDL Fibroblasts on ABMP-15 and ABM

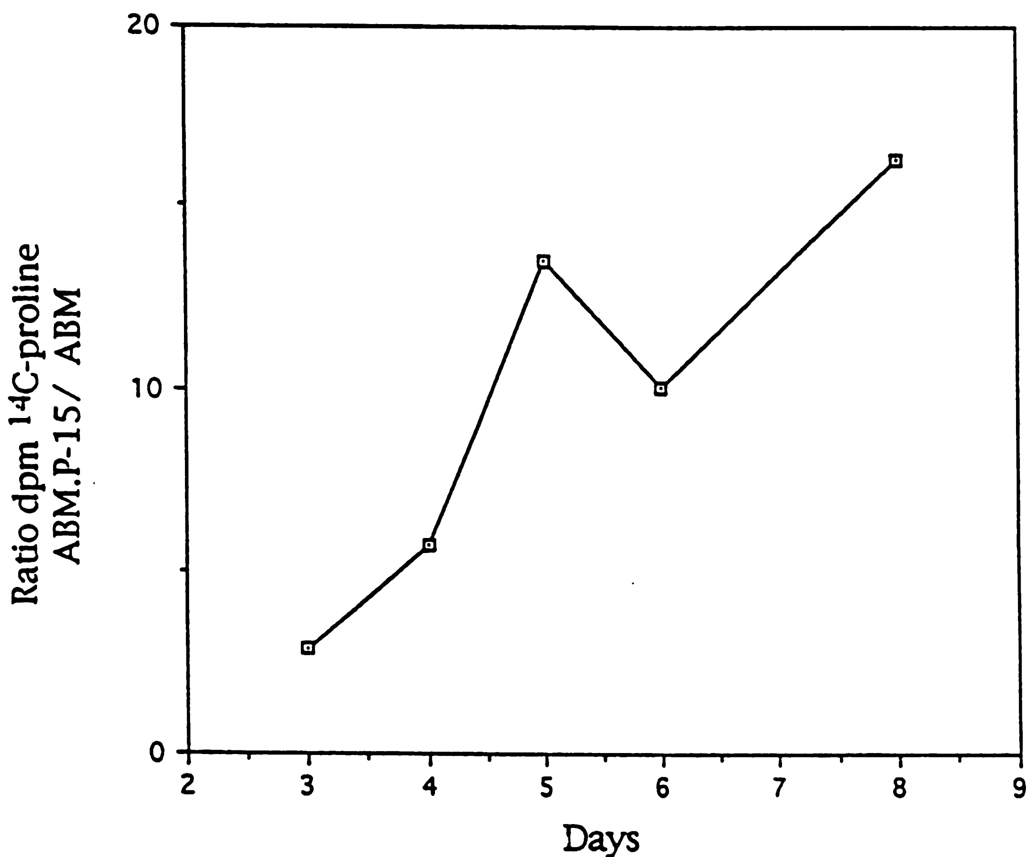



**Figure 8B**: Periodontal ligament cells associated with P-15 coated ABM (ABM.P-15) continuously incorporated more $^{14}$C-proline into their proteins than PDL cells associated with un-coated ABM (ABM).

# Ratio of labeled protein to labeled DNA in culture on ABM.P-15 and ABM

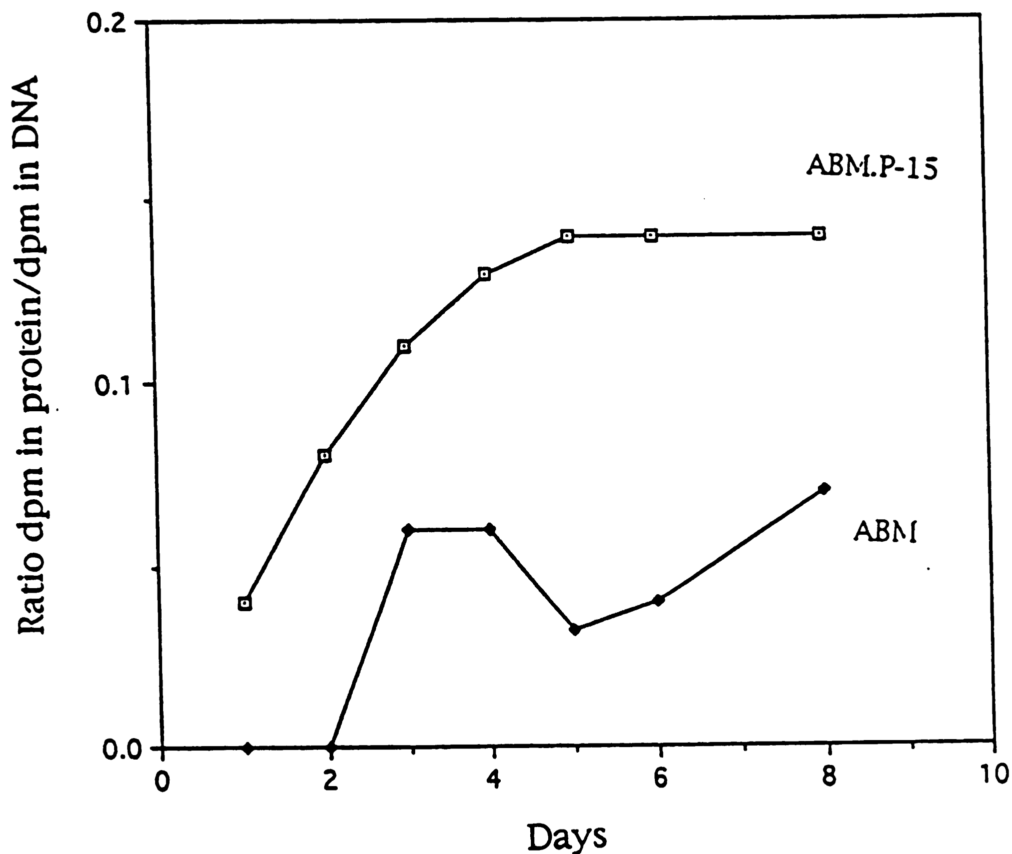



Figure 9: Periodontal ligament cells cultured on P-15 coated ABM (ABM.P-15) show a greater ratio of 14C-proline/ 3H-thymidine incorporation than cells cultured on un-coated ABM (ABM). It seems that P-15 coated ABM provides a much more hospitable environment for PDL cells than un-coated particles.

**Table 1**
**Comparison of $^{3}H$-thymidine and $^{14}C$-proline uptake in PDLF cultured on ABM.P-15 and ABM**

| Days of incubation | mean dpm $^{3}H$-tymidine | | mean dpm $^{14}C$-proline | |
|---|---|---|---|---|
| | ABM.P15 | ABM | ABM.P-15 | ABM |
| 1 | ** 27051 ± 4688 | 8201 ± 804 | **1238 ± 273 | 0 |
| 2 | *** 20477.5 ± 3788 | 8754 ± 2032 | *1704 ± 181 | 0 |
| 3 | *** 25346.5 ± 506 | 15623 ± 657 | ***2883 ± 195 | 1004 ± 150 |
| 4 | *** 23433.5 ± 1876 | 9053 ± 824 | ***3121.5 ± 126 | 548 ± 117 |
| 5 | *** 26497 ± 3994 | 8317.5 ± 1481 | ***3579 ± 413 | 266.5 ± 82 |
| 6 | *** 25863 ± 2489 | 8695 ± 1629 | **3829 ± 69 | 379 ± 69 |
| 8 | *** 14493 ± 2456 | 1812 ± 617 | ***2088.5 ± 190 | 128 ± 222 |

($p < 0.0005$) ***
($p < 0.005$) **
($p < 0.01$) *

## IX. DISCUSSION

It is well known that cellular functions during development and/or regeneration are modulated through an intricate interaction between the cells and their extracellular matrix environment. Molecular components of the extracellular matrix are involved in the regulating processes such as cellular migration, adhesion, proliferation, and differentiation (Reviewed by Hay, E.D. 1990, Terranova 1986, Reichardth 1991, Hynes 1992).
Studies have shown that prior to attachment to the substrata, cells need to produce and deposit extracellular matrix proteins such as collagen, fibronectin, and laminin. (Kleinman 1981, Grinnell 1979). It has also been postulated that matrix protein can stimulate and inhibit cellular growth and proliferation (Terranova 1986). Other studies have indicated that cells such as endothelial cells attach to collagen, fibronectin, and laminin. This interaction will lead not only to proliferation, but also influence phenotype changes of these cells (Madri 1982, Terranova 1980).

Cells from mesenchymal origin are thought to play a major role in bone development and regeneration (Hall,1987). Periodontal ligament cells are a multi-potential cell population that originate from the mesenchymal cells in the dental follicle. Studies have shown that periodontal ligament cells are involved in development, repair, and regeneration of the periodontium (Melcher 1976, Gould in 1980. Aukhil and Iglhaut in 1988). It is, therefore, likely that the interactions between periodontal ligament cells and their extracellular matrix are responsible for the variety of functions attributed to this cell population. In order to enhance periodontal regeneration, a suitable physiologic environment is necessary for the precursor periodontal ligament cells to migrate, attach, and proliferate. Several investigators have looked for bioactive materials and modifiers that can enhance cellular migration,

attachment, proliferation, and differentiation. Such materials can provide the regenerative environment needed for these progenitor cells to reestablish the lost periodontal tissues.

To achieve this goal, investigators have attempted to stimulate cell proliferation using different growth factors and biologic response modifiers such as platelet-derived growth factors, endothelial cell growth factor, interleukin-1. Rutherford (1992), looked at the effects of PDGF, IGF and dexamethasone on fibroblast proliferation in culture. He noted that these growth factors alone or in combination (except IGF alone) significantly enhance cellular proliferation. Takeshita (1992) reported that interleukin-1b alone or in combination with TNF-$\alpha$ and TGF-$\beta$ stimulate DNA synthesis in periodontal ligament cells. The mitogenic effects of platelet-derived growth factors on human periodontal ligament cells was investigated by Oates and co-workers in 1993. They reported that both PDGF-AA and PDGF-BB have a major mitogenic effect on this cell population.

Terranova, *et al.*. (1986,1989) have tried to apply growth factors to the root dentin and access the migration of periodontal ligament cells and endothelial cells over these surfaces. their data indicated that coating of the dentinal surfaces with basic fibroblast growth factor stimulates migration of human periodontal ligament cells and endothelial cells.

Others have used different methods and/or bioactive compounds to modify and expose the organic matrix of mineralized oral tissues such as dentin. These biomodifiers should allow selective repopulation of the wound by periodontal ligament cells rather than gingival epithelial cells or fibroblasts. Terranova (1986, 1987), treated root surface dentin with tetracycline-HCl and reported a greater fibroblast attachment to these treated surfaces. In the same study, he also noted that the migration and proliferation of periodontal

ligament cells was enhanced over the dentinal surfaces which were preconditioned with tetracycline-HCl and treated with either fibronectin or endothelial growth factor. Other studies also show a greater attachment of periodontal ligament cell or other fibroblasts to fibronectin, tetracycline modified surfaces (Fernylhogh 1983). Several studies have looked at the advantages of citric acid conditioning of the root dentin. these studies demonstrated that citric acid treatment and partially demineralizing the root surface results in exposure of the collagen matrix and increased attachment of periodontal ligament cells (Boyko 1980, Pitaru 1984, Lowenberg 1985).

P-15 is a cell-binding domain of type I collagen and can be expected to modulate attachment, migration, proliferation, and differentiation. In the current study, we attempted to promote the attachment and proliferation of PDL cells by exploiting normal physiologic cell function, using this synthetic peptides (P-15). We examined the potential stimulation of attachment and proliferation of periodontal ligament fibroblasts utilizing anorganic bone mineral (ABM) coated with this synthetic peptide (P-15).

In a light and electron microscopic study, Qian and Bhatnagar (1994 accepted for publication) have reported that, when compared to uncoated ABM controls, P-15 coated anorganic bone mineral (ABM.P-15) promote attachment of dermal fibroblasts. In agreement with the results of Qian and Bhatnagar (1994), our light and scanning electron microscopic data suggest an increased attachment of periodontal ligament cells to P-15 coated ABM compared to uncoated control particles. Results from our cell binding experiments indicate that initial binding of human PDL cells to P-15 coated ABM is greater than that of uncoated ABM. The adhesion of PDL cells to the P-15 coated ABM seems to be concentration dependent.

The binding equilibrium at higher concentrations of adsorbed P-15 suggests that cells have adhered to all the available P-15 ligands.

The observed differences in the amount of radioactive precursor incorporation into DNA and proteins not only indicate greater cell attachment, but may suggest that P-15 provides a premissive environment for cells proliferation and for cellular activity. The biochemical measurements in this study confirms the findings of Qian and Bhatnagar (1994). They also reported enhanced DNA and protein synthesis by the fibroblasts in association with P-15 coated ABM particles. Therefore, our data support the concept that ABM particles coated with P-15 may provide a more hospitable environment, promoting the attachment and proliferation of periodontal ligament cells as compared to uncoated particles of ABM.

It has been suggested that cell-to-cell and cell-to-extracellular matrix adhesion may play a major role in initiating morphogenetic changes during the developmental stages of an organ. Geiger and Ayalon (1992) refer to a number of interdependent processes which leads to specific gene expression. These genotypic changes eventually result in the modulation of cell differentiation. These processes include a) initial receptor mediated binding b) alterations in cellular cytoskeleton c) effect of these alteration on cellular behavior. These processes seem to be highly dependent on the spatial and temporal mechanisms. Opas (1989) reported that the biochemical composition of the extracellular matrix is responsible for the regulation of the phenotype differentiation in epithelial cells. The mechanism which controls these regulatory events is thought to be tied to the cytoskeletal alteration following cell spreading on the substratum. Fernandez (1989), demonstrated that during the differentiation of fibroblast to adipocytes, expression of the transmembrane linkage protein between extracellular matrix and

microfilaments is closely regulated. These regulatory events alter the adhesion property of the fibroblast and causes their differentiation.

Normal cell growth on different surfaces requires cell spreading. Our light and electron microscopic observations suggest that periodontal ligament cells in association with P-15 coated ABM are more likely to assume a spindle shape by spreading their pseudopodia. In contrast, cells on the control ABM frequently rounded up and stopped growing ( Fig. 2E). The result of radioactive proline and thymidine uptake into the cellular protein and DNA confirms the initial delay in cell growth in uncoted ABM cultures. Light microscopic examination of the P-15 coated ABM cultures revealed a number of interparticle cellular bridges (Fig. 2B, 2D). At times, these cellular bridges contracted and formed clusters of particles (Fig. 2F). It is therefore, conceivable that the observed cell to matrix interactions in P-15 coated ABM cultures result in a series of events leading to cellular differentiation.

In this study, we asked a major question regarding the bioactivity of P-15 coated anorganic bone mineral (ABM) particles. Will P-15 coated ABM particles promote attachment and proliferation of periodontal ligament cells as compared to uncoated particles of ABM? The results of our study clearly support this hypothesis. Indeed, P-15 coated ABM particles promoted attachment and proliferation of periodontal ligament cells. This finding may prove to be clinically significant since P-15 coated anorganic bone mineral can be used as an adjunct to the periodontal regeneration procedures.

Considering the significance of periodontal ligament cell differentiation in overall scheme of periodontal tissue regeneration, future efforts should be directed toward investigating the stage of differentiation of this cell population in our current *in vitro* model.

## VI. BIBLIOGRAPHY


1. Albair, W., Cobb, C., Killoy, W.: Connective tissue attachment to periodontally diseased root after citric acid demineralization. J. Perio. 53:515, 1982.

2. Arceo, N., Moehring, J., Foster, R., Somerman, M.: Human periodontal cells initiate mineral-like nodual invitro. J. Perio. 62:499, 1991.

3. Aukhil, I., Petersson, E., and Suggs, C.: Periodontal wound healing in the absence of periodontal ligament cells. J. Periodont. 58: 71, 1987.

4. Aukhil, I. and Petersson, E.: Effect of citric acid conditioning on fibroblast cell density in periodontal wounds. J. Clin. Periodont. 14: 80-84, 1987.

5. Aukhil, I., and Iglhaut, J.: Periodontal ligament cell kinetics following experimental regenerative procedures. J. Clin. Periodont. 15: 374-382, 1988.

6. Barr, R., King, D.: Necrobiotic granulomas associated with bovine collagentest site injection. J. Amer. Acad. of Dermat. 6:867, 1987.

7. Bell, R., Beirne, O.: Effectof Hydroxyapatite, tricalcium phosphate, and collagen on the healing of defects in the rat mandible. OralMaxiofacial Surg. 89:594, 1988.

8. Bhatnagar, R. and Qian, J.: A synthetic peptide related to collagen supports cell attachment and migration. Trans. Orthop. Res. Soc. 17:106, 1992.

9. Bhatnagar, R. and Qian, J.: Molecular mechanisms in the interaction of collagen: identification of cell binding domain. Submitted for publication.

10. Bhatnagar, R. and Qian, J.: Design of collagen-mimetic biomaterials. Trans. Soc. Biomaterials 17:309, 1994

11. Bhatnagar, R.: UCSF US patent Application.

12. Brooks, N.: A foreign body granuloma produced by an injectable collagen implant at a test site. J. Derm. Surg. Oncol. 8:2, 1982.

13. Boyko G. et. al.: Cell attachment to demineralized root surfaces in vitro. J. Perio Res., 15: 297, 1980.

14. Callen, D., et al.: Use of bovine-derived hydroxyapatite in the treatment of edentulous ridge defects: A human clinical and histological case report. J. Perio. 64:575, 1993.

15. Carranza, F., Kenney, E.: Histologic study of healinf of human periodontal defects after placement of porous hydroxyapatite. J. Perio. 58:682, 1987.

16. Cornell, C., et al.: Multicenter trial of Collagraft as bone graft substitute. J. of Orthopedic Trauma 5:1, 1991.

17. Cooperman, L., Michaeli, D.: The immunogenicity of injectable collagen. I. A 1-year prospective study. J. of Amer. Acad. of Derm. 10:638, 1984.

18. Davis, G.: Affinity of integrins for damaged extracellular matrix: $a_v b_3$ binds to denatured collagen type I through RGD sites. Bioch. and Biophy. Res. Communications 182:1025, 1992.

19. Egelberg, J.: Regeneration and repair of periodontal tissue. J. Perio. Res. 22:233,1987.

20. Ellingsworth, L., et al.: The human immune response to reconstituted bovine collagen. The J. of Immunology 136:877, 1986.

21. Elices, M., Hemler, M.: The human integrin VLA-2 is a collagen receptor on some cells and a collagen/laminin receptor on others. Proc. Nat. Acad. Sci. USA 86:9906, 1989.

22. Erhmann, R. and Gey, G.: The growth of cells on a transparent gel of reconstituted rat tail collagen. J. National Cancer Inst., 16:1375, 1956

23. Ferguson, D., Davis, W.: Bovine bone morphogenic protein fraction-induced repaired of craniotomy defects in the rhesus monkey. Clin. Orthop., 219:259,1987.

24. Fernandez, J., Ben-ze'ev, A,: Regulation of fibronectin, integrin and cytoskeleton expression in differentiating adipocytes: inhibition by extracellular matrix and polylysine. Differentiation 42:65, 1989.

25. Fernylhogh, W., Page, R.: Attachment growth and synthesis by human gingival fibroblasts on demineralized or fibronectin treated normal and diseased tooth roots. J. Perio. 54:133, 1983.

26. Frouser, L., et al.: Transcriptional activity of the a1(I)-collagen promotor is correlated with the formation of capillary-like structures by endothelial cells in vitro. The J. of Biological Chem. 266:18345, 1991.

27. Geiger, B., Ayalon, O.: Cadherins. Annu. Rev. Cell Biol. 8:307, 1992.

28. Gregoire, M. et al.: The influence of calcium phosphate biomaterials on human bone cell activities. An in vitro approach. J. of biomedical Material Res. 24:165, 1990.

29. Gey, G., Svotelis, M., Foard, M., Bang, F.: Long term growth of chichen fibroblasts on a collagen substrate. Exp. Cell Res., 84:63, 1974.

30. Glossary of Periodontal Terms: 3rd edition, 1992

31. Gould, T. et al.: Migration and division of progenitor cell population in periodontal ligament after wounding, J. Perio. Res. 15:20, 1980.

32. Griffith, C., Sanders, E.: Effects of extracellular matrix components on the differentiation of chick embryo tail bud mesenchyme in culture. Differentiation 47:61, 1991.

33. Grinnell, F., Feld, M.: Initial adhession of human fibroblasts in serum-free medium. Possible role of secreted fibronectin. Cell 17:117, 1979.

34. Hall, B.: Earliest evidence of cartilage and bone development in embryonic life. Clin. Orthop. 225:255, 1987.

35. Hay, E. D.: Cell Biology of Extracellular Matrix. Plenum, New York, 1990.

36. Hirschfeld, L., Wasserman, B.: A long term survey of tooth loss in 600 treated periodontal patients. J. Perio. 49:225, 1978.

37. Hynes, R.: Integrins: versatility, modulation, and signaling in cell adhesion. Cell 69:11, 1992.

38. Iglhaut, J., Aukhil, I., et al.: Progenitor cell kinetics during guided tissue regeneration in experimental periodontal wounds. J. Perio. Res. 23:107-117,1988.

39. Isador, F., Karring, T.,: Long-term effect of surgical and non-surgical periodontal treatment. A 5 year clinical study. J. Perio. Res. 21:462, 1986.

40. Johnson, E., Urist, M.: Bone morphogenic augmentation grafting of resistant femral nonunions. Clin. Orthop., 230:253, 1988.

41. Jones, B., Paabo, S., Stern, S.: Amino acid analysis and enzymatic sequence determination of peptides by an improved O-phthalaldehyde precolumn labeling procedure. J.Liq. Chromatog., 48:549, 1981.

42. Karst, W., Merker, H.: The differentiation behavior of MDCK cells grown on matrix components and in collagen gels. Cell differentiation 22:211, 1988.

43. Kenney, E., et al.: Bone formation within porous hydroxylapatite implants in human periodontal defects. J. Perio. 57:76, 1986.

44. Kenney, E., et al.: The use of a porous hydroxylapatite implant in periodontal defects. II. Treatment of class II furcation lesions in lower molars. J. Perio. 59:67, 1988.

45. Kleinman, H, Klebe, R.: Role of collagenous matrices in adhesion and growth of cells. J. Cell Biology 88:473, 1981.

46. Lein, P., et al.: The NC1 domain of type IV collagen promotes axonal growth in sympathetic neurons through interaction with the $a_1b_1$ integrin. The J. of Cell Biology 113:417, 1991

47. Lindhe, J., Westfelt, E., Nyman, S.: Long-term effect of surgical/nonsurgical treatment of periodontal disease. J. Clin. Perio. 1984.

48. Lindholm, T. et al.: Bovine bone morphogenic proteininduced repair of skull trephine defects in sheep. Clin. Orthop. 227:265, 1988.

49. Lopez, N.: Connective tissue regeneration to periodontally diseased roots, planed and conditioned with citric acid and implanted into the oral mucosa. J. Perio. 55:381, 1984.

50. Lynch, S. et.al.: Growth factors in wound healing . Single and synergistic effect on partial tickness porcine skin wounds. J. Clin. Inves. 84:640, 1989.

51. Lynch, S. et al.: A combination of platelet derived and insulin-like growth factors enhances periodontal regeneration. J. Clin. Perio. 16:545, 1989.

52. Lynch, S. et al.: The effects of short term application of a combination of platelet derived and insulin-like growth factors on periodontal wound healing. J. Perio. 62:458, 1991.

53. Libby, P. Wyler, D.: Differential effects of human interlukin-1 on growth of human fibroblasts and vascular smooth muscle cells. Arteriosclerosis 5:186, 1985.

54. Lowenberg, B., Aubin, J., et al.: Attachment, migration, and orientation of human gingival fibroblasts to collagen coated, surface demineralized, and untreated root slices. J. Dental Research 64:1106, 1985.

55. Madri, J., Williams, S.: Endothelial cell matrix cell interactions. Prog. Hemost19:137, 1982.

56. Magnusson, I., Nyman, S., Karring, T., Egelberg, J.: Connective tissue attachment formation followingexclusion of gingival connective tissue and epithelium during healing. J. Perio. Research 20:201, 1985.

57. Mariotti, A., Cochran, D.: Characterization of fibroblasts derived from human periodontal ligament and gingiva. J. Perio. 61:103, 1990.

58. Marks, S., Mehta, N.: Lack of effect of citric acid treatment of root surfaces on the formation of new connective tissue attachment. J. Clin. Perio. 13:109, 1986.

59. Matsuda, N. Lin, W.: Mitogenic, chemotactic, and synthetic responses of rat periodontal ligament fibroblastic cells to polypeptide growth factors invitro. J. Perio. 63:515, 1992.

60. Matsuda, T., Davis, J.: The in vitro responces of osteoblast to bioactive glass. Biomaterials 8:275, 1987.

61. McCulloch, C.: Progenitor cell population in the periodontal ligament of mice. The Anatomical Record 211:258, 1985.

62. McCulloch, C., Bordin, S.: Role of fibroblast subpopulation in periodontal physiology and pathology. J. Perio. Res. 26:144, 1991.

63. McFall, W.: Tooth loss in 100 treated patients with periodontal diseases-A long-term study. J. Perio. 53:539, 1983.

64. Mehlisch, D.: Collagen/Hydroxyapatite implants for augmenting deficient alveolar ridges:A 24 month clinical and histologic summary. Oral Surg. Oral Med. Oral Path. 68:505, 1989.

65. Melcher, A. H.: On the repair potential of periodontal tissues. J Perio 47: 256-60,1976.

66. Moore, J., Ashley, F., Waterman, C.: The effect of healing of the application of citric acid during replaced flap surgery. J. Clin. Perio. 14:130, 1987.

67. Nyman, et al.: New attachment folowing surgical treatment of human periodontal disease. J. Clinical Perio. 9:290, 1982.

68. Nojima, N., et al.: Fibroblastic cells derived from bovine periodontal ligaments have the phenotype of osteoblasts. J. Perio. Res. 25:179, 1990.

69. Nowack, H., et al.: Preparation and use in immunology of antibodies specific for type I and type III collagen and procollagen. J. of Immuno. Methods. 12:117, 1976.

70. Oats, : Effects of growth factors on human PDL fibroblasts. J. Perio. 64:142,1993.

71. Opas, M.: Expression of the differentiated phynotype by epithelial cells in vitro is regulated by both biochemistry and mechanics of the substratum. Developmental Biology 131:281, 1989.

72. Parson, R., Bhatnagar, R., Tofe, A.: In vivo evaluation of anorganic bovine HA with adsorbed bioactive synthetic peptides. Trans. Soc. Biomaterials 17:125, 1994

73. Perera, K, Tonge, C.: Fibroblast cell population kinetics in the mouse molar periodontal ligament and tooth eruption. J. Anat. 133:281, 1981.

74. Piche, J., et al.: Initial characterization of cells derived from human periodontia. J. Dent. Res. 68:761, 1989.

75. Pillar, P.: Powder metal-made orthopedic implants with porous surfacefor fixation by tissue growth. Clin. Orthop. rel. res., 176:42, 1983.

76. Pihlstrom, B., et al.: Comparison of surgical and nonsurgical treatment of periodontal disease. A review of current studies and additional results after 6 1/2 years. J. Clin. Perio. 10:524, 1983.

77. Pitaru, S., et al.: The influence of morphological and chemical nature of dental surfaceson the migration, attachment, and orientation of human gingival fibroblasts in vitro. J. Perio. Research 19:408, 1984.

78. Polson, A., et al.: The production of a root surface smear layer by instrumentation and its removal by citric acid. J. Perio. 55:443, 1984.

79. Qian, J., and Bhatnagar: Design of bone matrices: Enhanced cell attachment to anorganic bone mineral in the presence of a synthetic peptide related to collagen. J. of Biomedical and Material Research. Submitted for publication, 1994.

80. Qian, J. et al.: Increased cell attachment, migration, and differentiation on hydroxyapatite in the prtesence of a collagen-analogue. Trans. Soc. Biomaterials 17:126, 1994.

81. Qian, J. Wedrychowska, A., Bhatnagar, R.: accepted for publication, 1994.

82. Raines, E. et al.: Purification of human platelet-derived growth factor. Methods Enzymol. 109:749, 1985.

83. Raines, E. et al.: Interleukin-1 mitogenic activity for fibroblasts and smooth muscle cell is due to PDGF-AA. Science 243:393, 1989.

84. Register, A., Burdick, F.: Accelerated reattachment with cementogenesis to dentin, demineralized in situ. II. Defect repair. J. Perio. 47:497, 1976.

85. Reichardth, L.: Extracellular matrix molecules and their receptors: Functions in neural development. Ann. Rev. Neurosci. 14:531, 1991.

86. Rixen, H., Et al.: Interaction between endothelial cells and basement membrane components. Experimental Cell Biology 57:315, 1989.

87. Rubin, K., et al.: Hepatocyte adhesion to collagen. Isolation of membrane glycoproteins involved in adhesion to collagen. Experimental cell research 164:127, 1986.

88. Sattler, R., Mayne, R., Holtzer, H.: Ultrastructure of adult rat hepatocytes cultured on floating collagen membranes. Cancer Res., 38:1539. 1978.

89. Shor, S.: Cell proliferation and migration on collagenous strata. J. Cell Sci. 41:159, 1980.

90. Smith, B., et al.: The effectiveness of citric acid as an adjunct to surgical treatment procedures in human. J. Clin. Perio. 13:701, 1986.

91. Sporn, M. Roberts, A.: Polypeptide transforming growth factors isolated from bovine source and used for wound healing in vivi. Science, 219:1329, 1983.

92. Somerman, M., Foster, R., sauk, J.: Biochemical analysis of periodontal cell activities. The biolo. mechan. of Tooth Movement and Craniofacial Adaptation 291-300, 1992.

93. Takada, Y., Hemler, M.: The primary structure of VLA-2 collagen receptor a2 subunit (Platelet GPIa): Homology to other integrins and the presence of possible collagen binding domain. J. Cell Bio. 109:3978, 1989.

94. Takagi, K., Urist, M.: The reaction of dura to bone morphogenic proteinin repair of skull defects. Ann. Surg. 196:100, 1982.

95. Takeshita, A., Zhon, G.: Effect of interlukin-1 beta on gene expression and function of fibroblastic cell derived from human periodontal ligament. J. Perio. Res. 27:250, 1992.

96. Terranova, V. et al.: Laminin receptor on human breast carcinoma cells. Proc. National Acad. Sci USA 80:444, 1983.

97. Terranova, V. et al.: A biochemical approach to periodontal regeneration: tetracycline reatment of dentin promotes fibroblast adhesion and growth. J. Perio. Res. 21:330, 1986.

98. Terranova, V. et al.: Regulation of cell attachment and number by laminin and fibronectin. J. Cell Physio. 127:473, 1986.

99. Terranova, V. et al.: Chemotaxis of human gingival epithelial cells tp laminin: A mechanism for epithelial cell apical migration. J. Perio. 57:311, 1986.

100. Terranova, V. et al.: Extracellular matrices and polypeptide growth factors as mediators of functions of cells of the periodontium. J. Perio. 58:371, 1986.

101. Terranova, V. et al.: A biochemical approach to periodontal regeneration- AFSCM: assay for specific cell migration. J. Perio. 58:247, 1987.

102. Terranova, V. et al.: Repopulation of dentin surface s by periodontal ligament cells and endothelial cells- Effects of basic fibroblastgrowth factor. J. Perio. 60:293, 1989.

103. Thaller, S., et al.: The split calvarial bone graft donor site: The effects of surgicel and hydroxyapatite impregnated with collagen. Ann. of Plastic Surg. 25:435, 1990.

104. Trautinger, E., Kokoschka, M., Menzel, E.: Antibody formation against human collagen and C1q response to a bovine collagen implant. Derm. Res. 283:395, 1991.

105. Rutherford, R., Trailsmith, M.: Synergistic effects of dexamethasone on PDGF mitogenesis in vitro. Arch. Oral Bio. 37:139, 1992.

106. Uchida, A., Nade, s., McCartney, E., Ching, W.: Bone ingrowth into three different porous ceramic implanted into the tibia of rats and rabbits. J. Orthop. Res., 3:65, 1985.

107. Vandenberg, P., et al.: Characterization of a type IV collagen major cell binding site with affinity to the a1b1 and the a2b1 integrins. The J. of Cell Biology 113:1475, 1991.

108. Wirthlin, R.: Growth substances: Potential use in periodontics. J Western soc. Perio. 37:101, 1989.

109. World Workshop in Clinical Periodontics: American Academy of Periodontalogy, 1989.

LIBRARY

**For reference**

Not to be taken from the room.

632961

3 1378 00632 9612

RETURN TO the circulation desk of any
University of California Library

or to the

NORTHERN REGIONAL LIBRARY FACILITY
Bldg. 400, Richmond Field Station
University of California
Richmond, CA 94804-4698

ALL BOOKS MAY BE RECALLED AFTER 7 DAYS

- 2-month loans may be renewed by calling (510) 642-6753
- 1-year loans may be recharged by bringing books to NRLF
- Renewals and recharges may be made 4 days prior to due date

DUE AS STAMPED BELOW

MAY 02 2004

DD20 15M 4-02