Biological Modification of Titanium Surfaces Using Bifunctional Peptides

BY

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ABSTRACT

HITOMI AKIMOTO: Biological Modification of Titanium Surfaces

Using Bifunctional Peptides

(Under the direction of Lyndon F Cooper, DDS, PhD)

The clinical success of endosseous implants is associated with the formation and maintenance of bone at implant surfaces. Based on *in vivo* observations, several generalizations have been derived regarding the nature of the interface. Most prominently, initial cell and molecular adhesion dictate the reactivity of the interface. The aim of this project is to use interfacial biomaterials (Ti-binding peptides) to graft cell adhesive peptides to titanium surfaces used for osseointegrated endosseous implants. We hypothesized that there is statistical significant difference in the differentiation of cells adherent with Ti-binding peptide treated and untreated on titanium surfaces. We used the peptide (AFF6008) can facilitate the adhesion of biologically active peptides to the titanium surface. Scanning electron microscopy was used to quantify cell adhesion on treated and untreated surfaces. Real time polymerase chain reaction was used to measure and compare osteoblast-specific gene expression, ALP and BSP, associated with osteoblastic differentiation in cells cultured on treated and untreated surfaces.

DEDICATION

To my grand parents,

Kiyoshi and Shima Sakamoto,

who always believed in me and gave me support throughout my whole life.

To my husband and Pochi;

who have been inspiring and encouraging with unreserved understanding throughout the course of my residency years.

ACKNOWLEDGMENTS

My sincere thanks to my mentor, Dr. Lyndon Cooper, for his valuable support and scientific guidance throughout the investigation.

I wish to extend my warmest thanks to all my graduate prosthodontic faculties, residents. Specially, I would like to thank Dr. Juanli Guo, Ms. Beth Stirling, Ms. Sara Valencia, Ms. Jin Yi Kwon, and Mr. Wallace Ambrose for the scientific guidance.

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TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF ABBREVIATIONS

ALP:	Alkaline phosphatase
BMP:	Bone morphogenetic protein
BSP:	Bone sialoprotein
cpTi:	Comercially pure titanium
DMEM:	Dulbecco's Modified Eagle's Medium
cDNA:	Complementary Deoxyribonucleic acid
EDTA:	Ethylenediamine tetra-acetic acid
GAPDH:	Glyceraldehydes 3-phosphate dehydrogenase
GRGDS:	Glycine-arginine-glycine-aspartate-serine
GRGES:	Glycine-arginine-glycine-glutamic acid-serine
hMCS:	Human mesenchymal cells
HCl:	Hydrogen chloride
IFBM:	Interfacial biomaterial
IgE:	Immunoglobulin E
LPS:	Lipopolysacharide
mRNA:	Messenger Ribonucleic acid
NaOH:	Sodium hydroxide
OC:	Osteocalcin
OPN:	Osteopontin
PBS:	Phosphate-buffered saline
PCR:	Polymerase chain reaction
PDGF-β:	Platelet derived growth factor beta
Real-time PCR	Real-time polymerase chain reaction
RNA:	Ribonucleic
SLA:	Sandblasted large-grit acid-etched
TGF-β:	Transforming growth factor β
Ti:	Titanium
TIMP:	Tissue inhibitors of metalloproteinases
UNC:	University of North Carolina

CHAPTER 1

INTRODUCTION

In this decade, endosseous dental implants have facilitated dramatically in dentoalveolar replacement. The use of endosseous implants to retain craniofacial prostheses (ears, noses, etc.) is also successful and may have even greater impact on the well being of the individual(Kosmidou, Toljanic et al. 1998; Abrahamsson, Zitzmann et al. 2001; Abrahamsson, Zitzmann et al. 2002; Abrahamsson, Berglundh et al. 2004; Albrektsson and Wennerberg 2004; Albrektsson and Wennerberg 2004) Implants are most recently implicated in craniofacial regeneration; distraction osteogenesis is a process that is being applied in the most challenging of regenerative situations.(McCarthy, Williams et al. 1998) Unfortunately, dental implant use requires sufficient quantity and quality of bone. A major impediment to endosseous implant use is the absence of bone that occurs most commonly in traumatic injuries to the craniofacial region and in aplasias and dysphasia's affecting the teeth, alveolar ridges and jaws of individuals affected with congenital or acquired defects. For these most challenging situations, further improvements in dental implant success is dependent on the clinical ability to create or replace bone at the implant surface.(Esposito, Hirsch et al. 1998)

Dental implant success is high where sufficient bone supports osseointegration. However, in locations of low bone density and volume, implant success is diminished.(Esposito, Hirsch et al. 1998) Implant placement in regenerated bone is required

several surgical procedures. Imparting osteoinductive or osteoconductive behavior to implants could improve bone formation at implants. Hydroxyapatite coating of titanium implants promotes osteoconduction.(Esposito, Hirsch et al. 1998) However, hydroxyapatite implant surfaces are also associated with peri – implant inflammation, bone loss(Iamoni, Rasperini et al. 1999) and increased failure rates after 5 – 7 years.(Salcetti, Moriarty et al. 1997) Titanium offers many biologic and mechanical advantages for creating tooth root analogs and serving a role in the surgical reconstruction of the craniofacial complex. Other methods of increasing bone formation at titanium surfaces are required.

Buser et al. demonstrated that increasing surface roughness beyond the machined titanium surface could increase (approaching that of hydroxyapatite) the amount of bone formed at the implant surface.(Buser, Nydegger et al. 1999) Gotfredsson also showed the benefit of rough titanium surfaces for bone formation early in healing of implants.(Block, Gardiner et al. 1996) In a series of reports, surface roughness parameters associated with reproducible increases in the extent of bone formed at the implant, will be identified.(Gotfredsen, Wennerberg et al. 1995) However, a biological theory or a biomechanical theory for this behavior remains undefined. A biomechanical theory of formed bone behavior at rough implant surfaces fails to identify the bone formation effects previously observed at rough implants. One possible explanation is greater cell adhesion on rough implant surfaces, however, cell culture studies do not fully support this. Other modifications to increase cell adhesion may be considered.

Many clues for tissue engineering success can be obtained by studying osseointegration, the process of bone formation and maintenance around an endosseous implant in function. Osseointegration as tissue engineering represents the planned cell activity within a given

matrix and cytokine environment. We strive to develop devices that are biocompatible, form tissues physiologically, and biomechanically equivalent to the missing structure. Further engineering of the cell, the matrix or the cytokines comprising a tissue forming device demands a complete understanding of the device's clinical or modeled clinical environments, including the phenotypic spectrum of the adherent cell population.(Hubbell 1995)

Based on investigations at the molecular level, implant substrate - osteoblast interactions may be characterized as specific, protein - mediated (indirect), dynamic and signal-generating events. Osteoblasts utilize integrin receptors to bind specific proteins absorbed on implant surfaces.(Kim and Mooney 1998) Some of these proteins present in serum are extracellular matrix proteins, or are expressed by osteoblasts. Examples include cell adhesion proteins such as collagen, fibronectin or osteopontin. This process is specific in two ways; first, adhesive proteins mediating attachment may be specific for bone or a particular implant surface and second, the osteoblast display of integrins is differentiation and matrix protein - specific.(Puleo and Bizios 1992) Compelling evidence for this is provided by the observation that cultured osteoblasts utilize different receptors for attachment to polystyrene, titanium and cobalt chrome alloy substrates.(Schneider and Burridge 1994)

Integrin receptors are heterodimeric transmembrane receptors that bind specifically to extracellular matrix proteins. Many of the integrins bind to a common peptide motif, Arg-Gly- Asp (RGD). Numerous investigators have attempted to use the RGD motif to activate different biomaterials using the RGD encoding peptides. While this has been attempted for metal surfaces, complex chemistries are often involved in covalently bonding the RGD peptide to the metal surface. More recent studies have demonstrated that placement of RGD

containing peptides onto titanium implants is possible but difficulties in complex chemistries remain.(Sinha and Tuan 1996)

General structureal and biological properties of noncollagenous proteins associated with the bons cell extracellular matrix.(Heinegard and Oldberg 1989)

The aim of this project is to use peptide affinity for titanium itself to simplify this strategy and permit the direct adhesion of peptides containing RGD to promote cell adhesion.

Scanning Electron Micrographic analysis of cell adhesion to AFF6008 (bifunctional peptide) treated titanium surfaces 2 and 8 hours following plating of human mesenchymal stem cells showed cell morphology. Early studies were performed using grit blast/etched surfaces. This image indicates the ability to identify spread and round cells adherent to the surface after early time points (2 hr) and the effectiveness of the surface treatment in directing cell adhesion as well as spreading (8 hr). Manual counting of cells (0 – 4 hours) enables definition of bifunctional peptide surface treatment effects on cell attachment and spreading. Quantification of human mesenchymal stem cell adhesion on titanium disks treated for 2 hours with 0 – 5 μM AFF 6005 was prepared. Triangle cells were associated with (non soluble RGD); Circler (soluble RGD). The results suggest that the peptide adheres to the titanium and directs RGD-dependent binding of osteoblastic cells.

CHAPTER 2

MATERIAL AND METHODS

Titanium surface preparation

A preparation method described by Keller et al was used to provide a clean, passive Ti alloy surface for cell culture. Bulk Ti disks were prepared from cp Titanium. The 5mm diameter by 1.5mm thick sections were cut and polished by 600 grits with silicon carbide abrasive paper. These sections were washed, ultrasonically cleaned in ethyl methyl ketone. Then, they were rinsed five times in deionizer distilled water, and acid passivity in 40% nitric acid. Proper to use, these surfaces were sterilized by immersion in absolute ethanol.

Phage display emerged as a technology for the identification of peptides that bind to protein targets. It has evolved as a technology for the identification of peptides that bind to polymers, metals, and other materials.(Okamoto, Matsuura et al. 1998)

Preparation of peptides

The peptide AFF6008 was provided from Affinergy Inc. This peptide contains a proprietary titanium binding domain, a linker region and the RGD motif. Using the AFF6008 peptide a stock solution of 1mM was made in sterile phosphate buffered saline (PBS), and diluted in PBS to 10μM (100x), 1 μM (1000x), 0.1 μM (10,000x).

Assessments of IFBM-mediated cell adhesion

Scanning Electron Micrographic analysis of cell adhesion to AFF6008 treated titanium 2 and 8 hours following plating of human mesenchymal stem cells : Early studies were performed using grit blast/etched surfaces. This image indicates the ability to identify spread and round cells adherent to the surface after early time points (2 hr) and the effectiveness of the surface treatment in directing cell adhesion as well as spreading (8 hr). Manual counting of cells (0 – 4 hours) enables definition of bifunctional peptide surface treatment effects on cell attachment and spreading. Quantification of human mesenchymal stem cell adhesion on titanium disks treated for 2 hours with 0 – 5 μM AFF 6005 was prepared.

Human MSCs were plated onto 5 mm disks in 100 μl of DMEM containing 10% FBS at a concentration of 5 x 10^5 cells/ml and incubated in a humidified 5% CO_2 atmosphere at 37° C for 0~4 hours. Adherent cells and disks were rinsed three times with ice cold PBS and fixed for 60 minutes with 2.5 % glutaraldehyde, 2 % paraformaldehyde, and 0.1 M sodium-cacodylated buffer (pH 7.4) and post-fixed in 4% osmium tetroxide. The fixed cell layers were washed with PBS and dehydrated using graded ethanol solutions and critical point drying. Human MSC adhesion and morphology (spread versus round) were examined using SEM (Joel 6300 microscope; Joel USA, Inc., Peabody MA). Cell adhesion were determined by averaging the number of cells counted at low magnification (× 500) in three random areas per disk, and calculation of the mean counts from five disks for each of the different prepared surfaces. A single examiner using the presence of cell processes and the elongation of the central cytoplasm region as criteria for a spread cell shall manually score cell morphology. The numbers of spread and round cells were counted in three random areas per disk. Mean numbers were calculated for five disks for each of the different prepared surfaces.

Competition experiments using soluble GRGDS and GRGES peptides (Sigma, St. Louis MO) were used to demonstrate the specificity of cellular interactions.

Assessment of IFBM treatment on titanium adherent osteoblastic differentiation

Human MSCs were plated on titanium disks treated with IFBM (interfacial biomaterial) (AFF6008) or with PBS according to concentration (10μM) and time (4 hours). This results in optimal cell adhesion and spreading indicated in studies performed in assessments of IFBM-mediated cell adhesion. Twenty four hours following plating under such conditions, cells were examined for osteoblastic differentiation over a 14 day culture period.

Briefly, human MSCs (Clonetics, Inc) was treated with growth media containing osteogenic supplements (OS; 0.1μM dexamethasone, 0.05mM ascorbic acid and 10mM β-glycerophosphate). Cells were cultured for 1-14 days, replacing OS medium every other.(Matsuzaka, Walboomers et al. 1999) Osteoblastic differentiation was examined at 3, 7, and14 days as a function of one or another inflammatory agent. Results were quantified by scanning, and reported as mean ± SEM, n = 4 wells/group and compared by ANOVA, $P < 0.05$.

Real Time- PCR analysis were used to measure Alkaline phosphatase and BSP mRNA expression.(Harris and Cooper 2004) Reverse transcription reactions for all RNA samples were performed using 2 μg of total RNA to program standard Superscript™ reactions. Each 1st strand cDNA synthesis reaction generates sufficient cDNA to perform > 20 reactions. To account for potential variations among the 1st strand cDNA synthesis

reactions, synthesized cDNA were examined by GAPDH control reactions. Real time PCR reactions were performed using an ABI Thermocycler as described.(Diefenderfer, Osyczka et al. 2003) The relative abundance of bone specific mRNAs were used to demonstrate the effects of treatments on hMSC osteoblast differentiation. The fold induction of each mRNA at 3, 7 and 14 days is used to describe osteoblastic differentiation.

Statistical Analyses

All experiments were performed using 5 disks per time point and every experiment was reproduced using hMSC donor. 2 way ANOVA was used to statistically compare results between concentration and time.

CHAPTER 3

RESULT

Specification of cell adhesion to cpTitanium substrates

Human MSC adhesion and morphology (spread versus round) were examined using SEM. Cell adhesion was determined by averaging the number of cells counted at low magnification (× 500) in three random areas per disk, and mean counts from five disks for each of the different prepared surfaces. At 0.1μM the round cell and the spread cell increased at 5min, then the numbers decreased until 30min. At the 1.0 μM, the round cells and spread cells decreased in number at 5min. However, at 10μM, the round cells decreased and the spread cell increased at 5min. With time, the round cells transformed into spread cell patterns. In addition, the spread cells became more mature and the size expanded. (Figure 1)

Figure 2 a~d and Figure 3 a~e:

Demonstrate that titanium binding IFBM (interfacial biomaterial) mediates cell adhesion in substrate and legend specific manner

In the early stage of Ti binding RGD and polystyrene RGD, the size of the spread cell and round cell were small, similar to the morphology of dendric cells. They were spread out across the surface and grew as a monolayer. However, in the late stage, the dimension of the

spread cell increased and induced the cells to grow as multiple layers. In addition, the round cells grew as a continuous, thin monolayer across the surface of the existing monolayer.

The expression levels of BSP and Alkaline phosphatase mRNA determined by Real Time PCR is presented in Fig. 4 and 5. The data demonstrated that day 1, day 3, and day 7 of BSP expression, at which time the responding cells had not yet differentiated to osteoblasts as confirmed by the BSP. However, at day 14, the BSP expression had significantly increased. Moreover, when comparing cells with peptide and cells without peptide, the ones with peptide had significantly higher BSP expression. (Figure 4)

ALP is an enzyme expressed on the membrane of osteoblastic cells and has been used as a marker for osteoblastic activity. In this study, ALP expression was significantly higher with RGD peptide than without the peptide at day 7 and day 14, and the achievement of the maximum ALP activity was day 7. Both with the RGD peptide and without peptide showed very low ALP activity at day 1 and day 3. (Figure 5)

Specification of cell adhesion to cpTitanium substrates

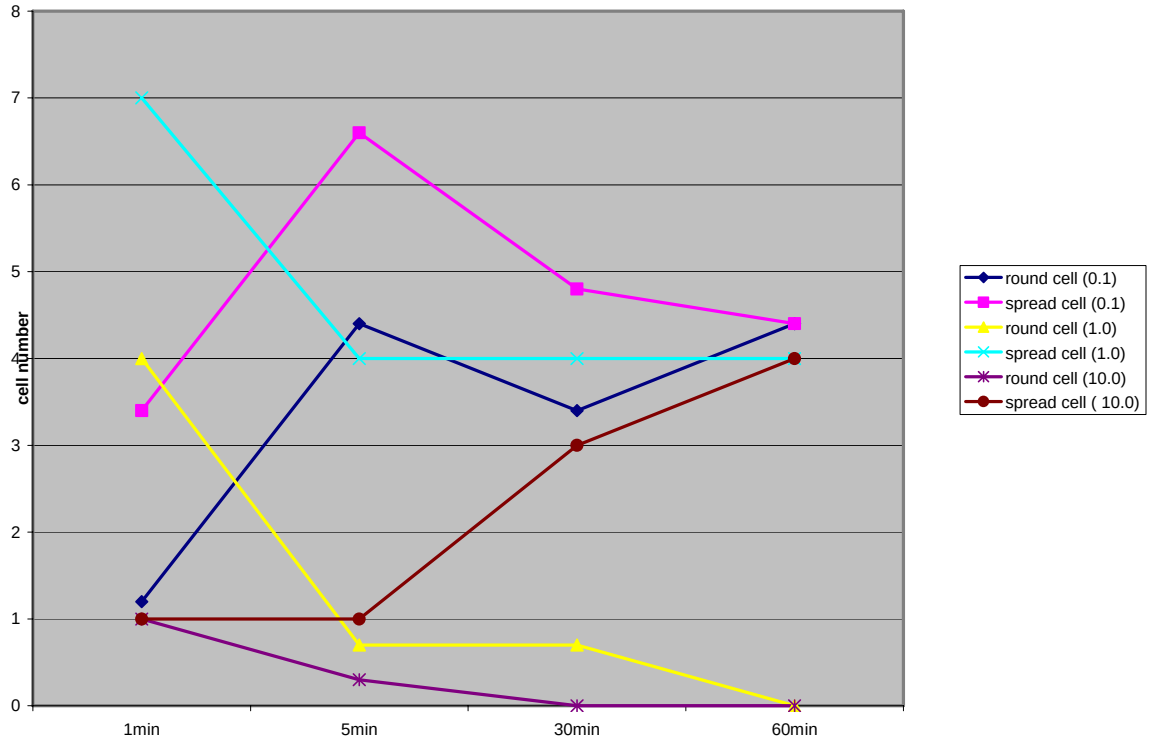

Figure 1
Assessment of IFBM treatment on titanium adherent osteoblastic differentiation

Morphology of the titanium surfaces

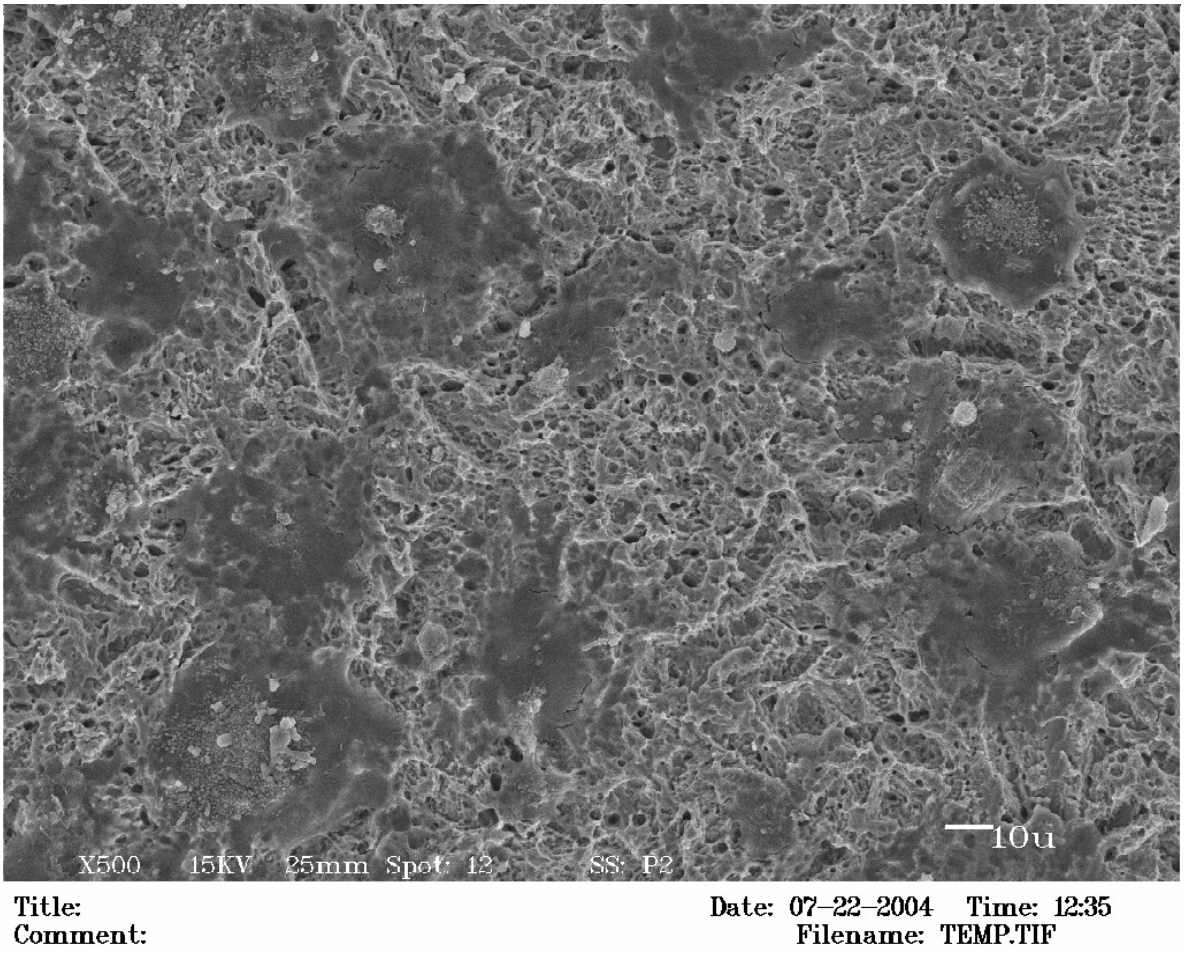

Figure 2-a
Scanning electron micrograph of the osteoblastic cell culture with Ti-binding RGD 30 min after seeding the culture. Bar = 10μm

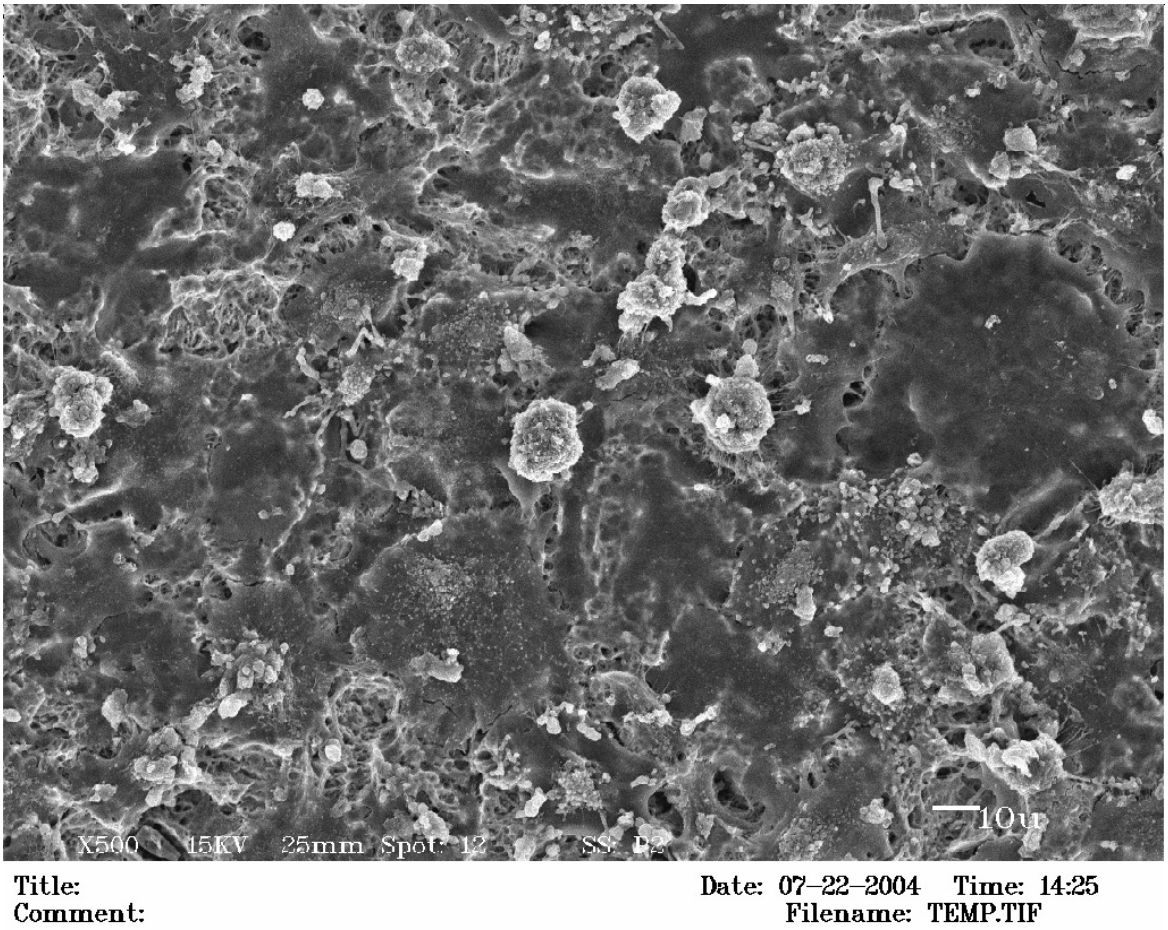

Figure 2-b
Scanning electron micrograph of the osteoblastic cell culture with Ti-binding RGD 60 min after seeding the culture. Bar = 10μm

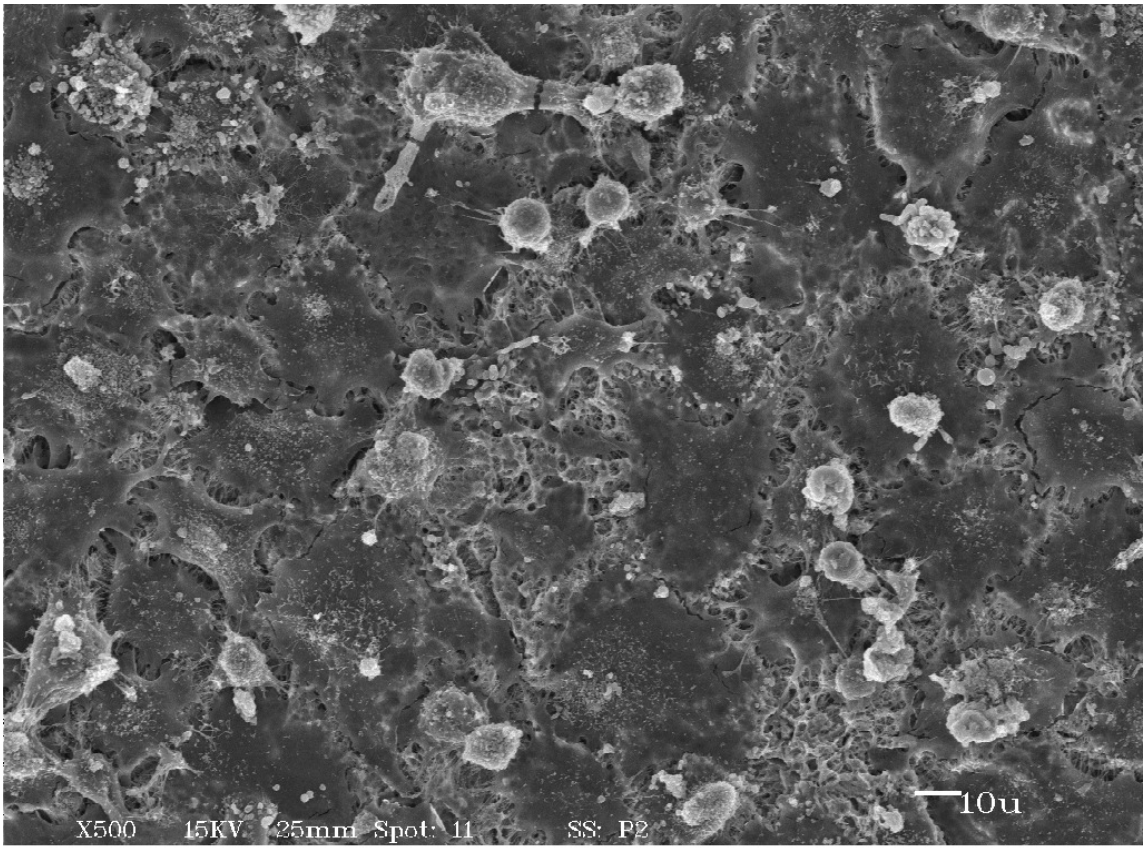

Figure 2-c
Scanning electron micrograph of the osteoblastic cell culture with Ti-binding RGD 120 min after seeding the culture. Bar = 10μm

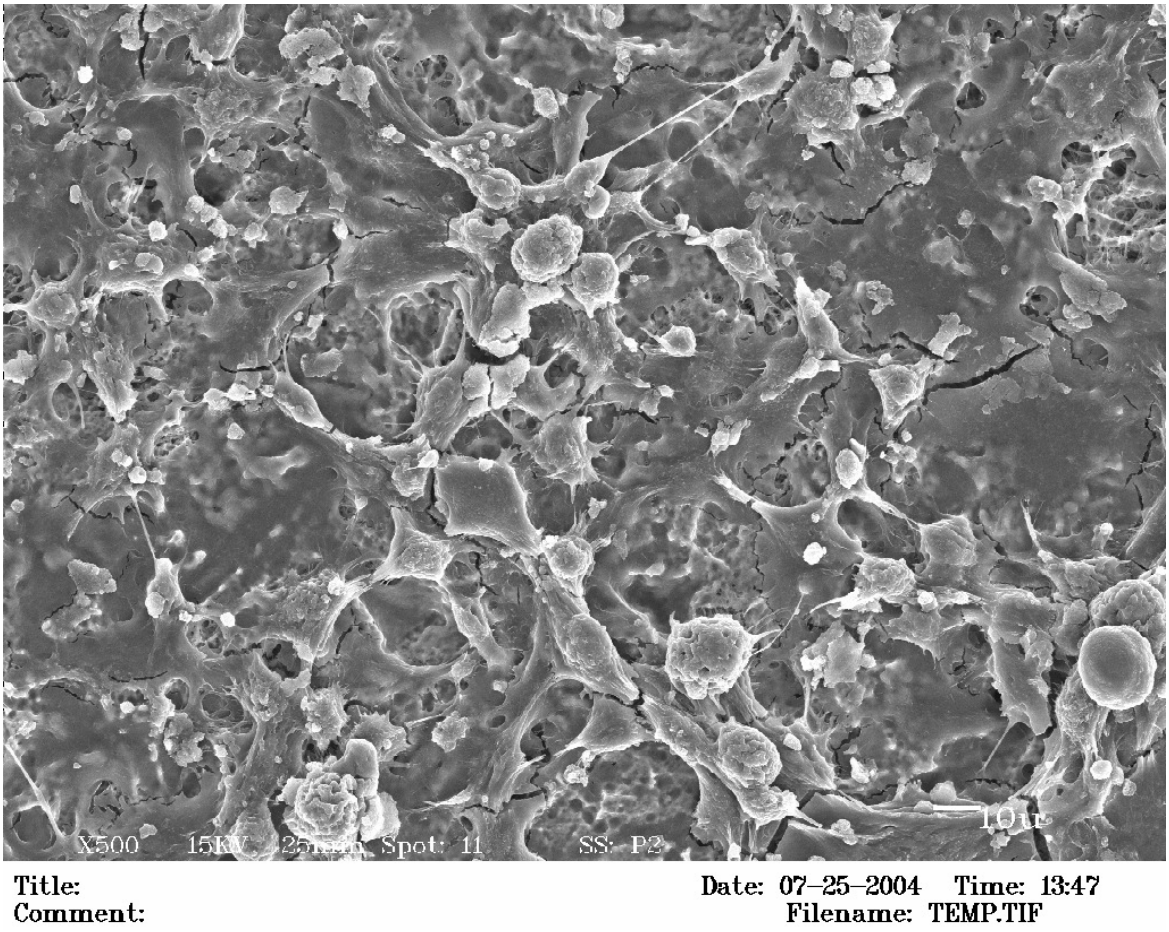

Figure 2-d
Scanning electron micrograph of the osteoblastic cell culture with Ti-binding RGD 240 min after seeding the culture. Bar = 10μm

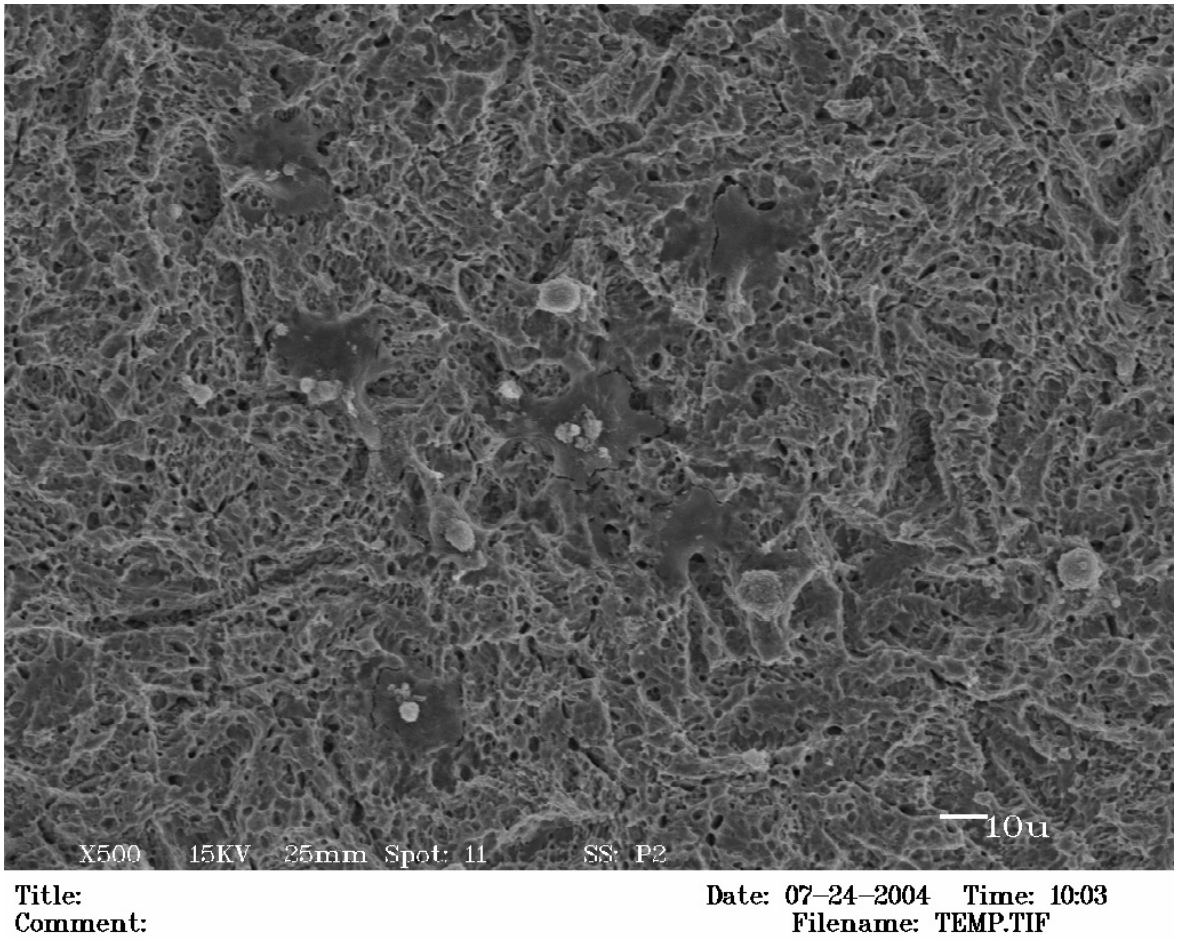

Figure 3-a
Scanning electron micrograph of the osteoblastic cell culture with Polystryene binding RGD 30 min after seeding the culture. Bar = 10μm

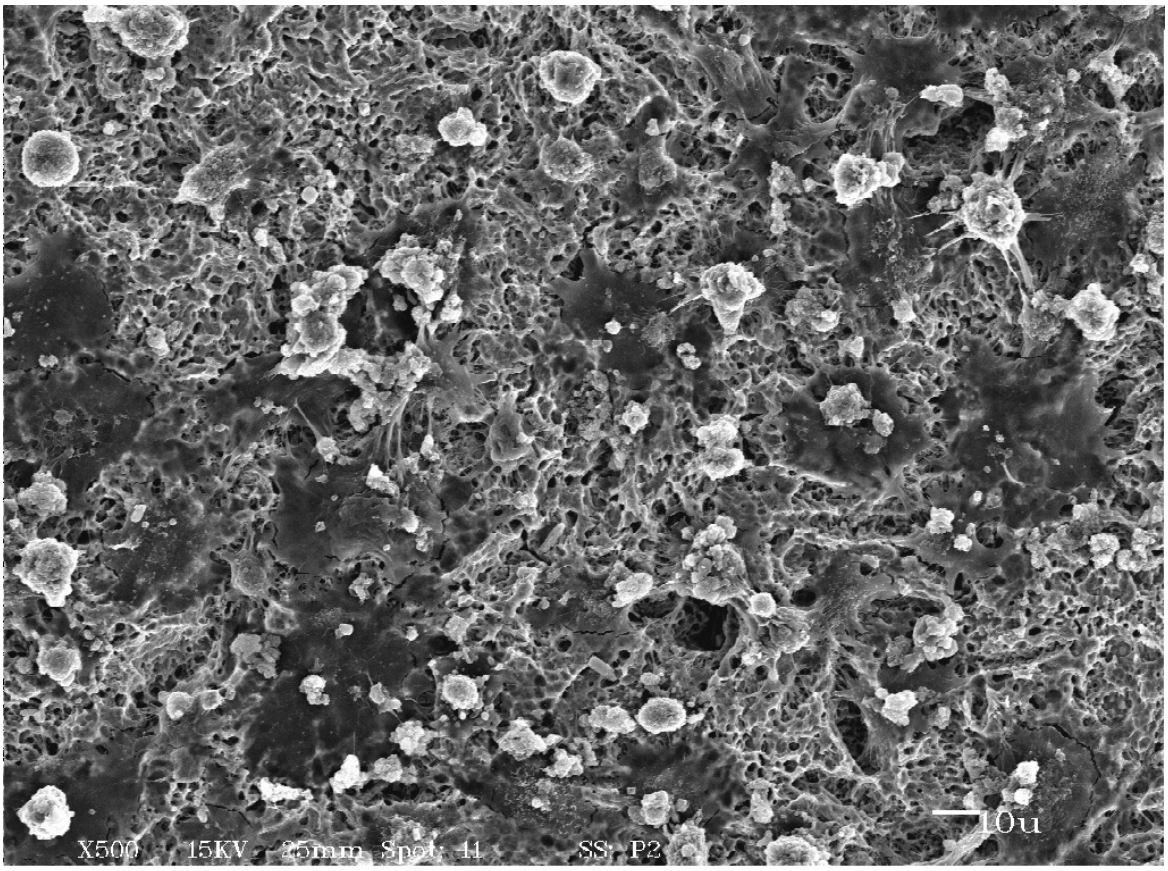

Figure 3-b
Scanning electron micrograph of the osteoblastic cell culture with Polystryene binding RGD 60 min after seeding the culture. Bar = 10μm

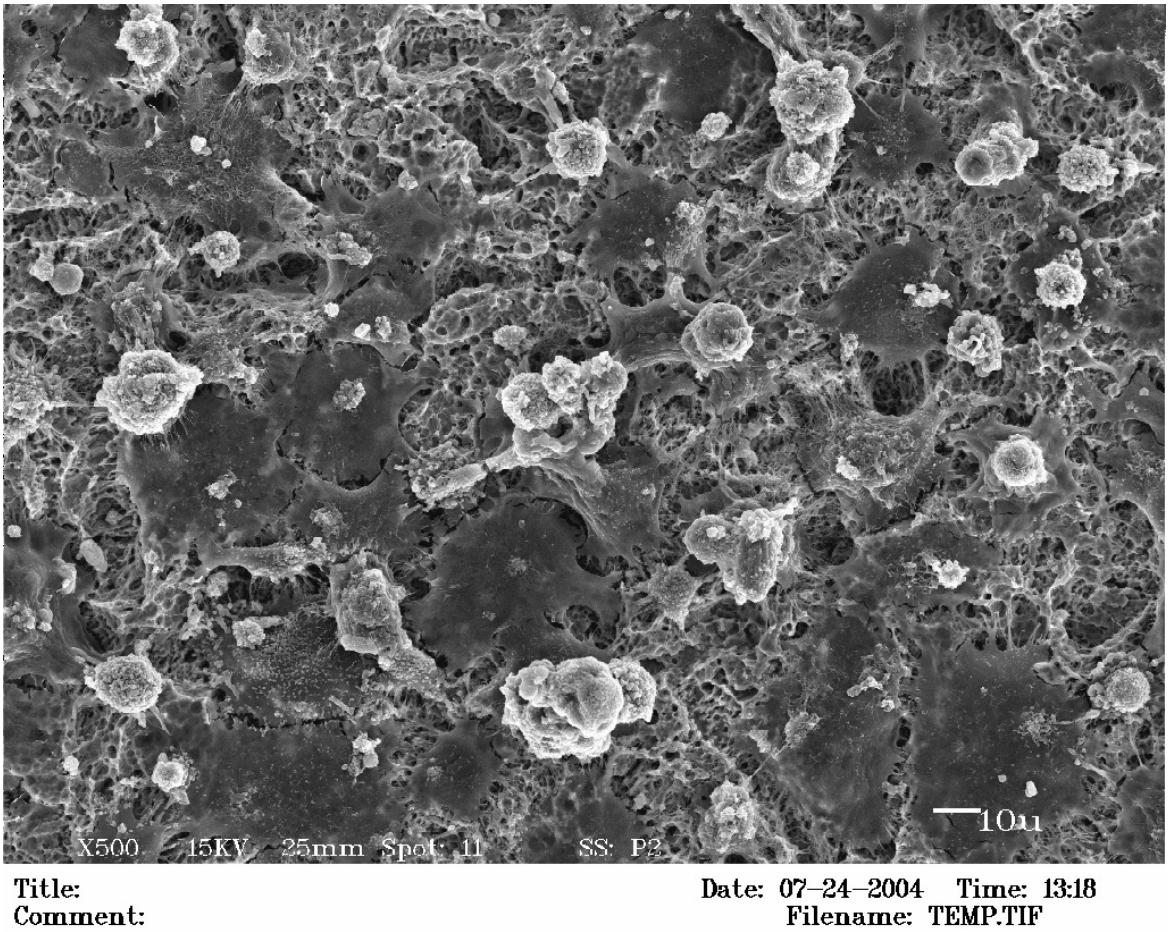

Figure 3-c
Scanning electron micrograph of the osteoblastic cell culture with Polystryene binding RGD 120 min after seeding the culture. Bar = 10µm

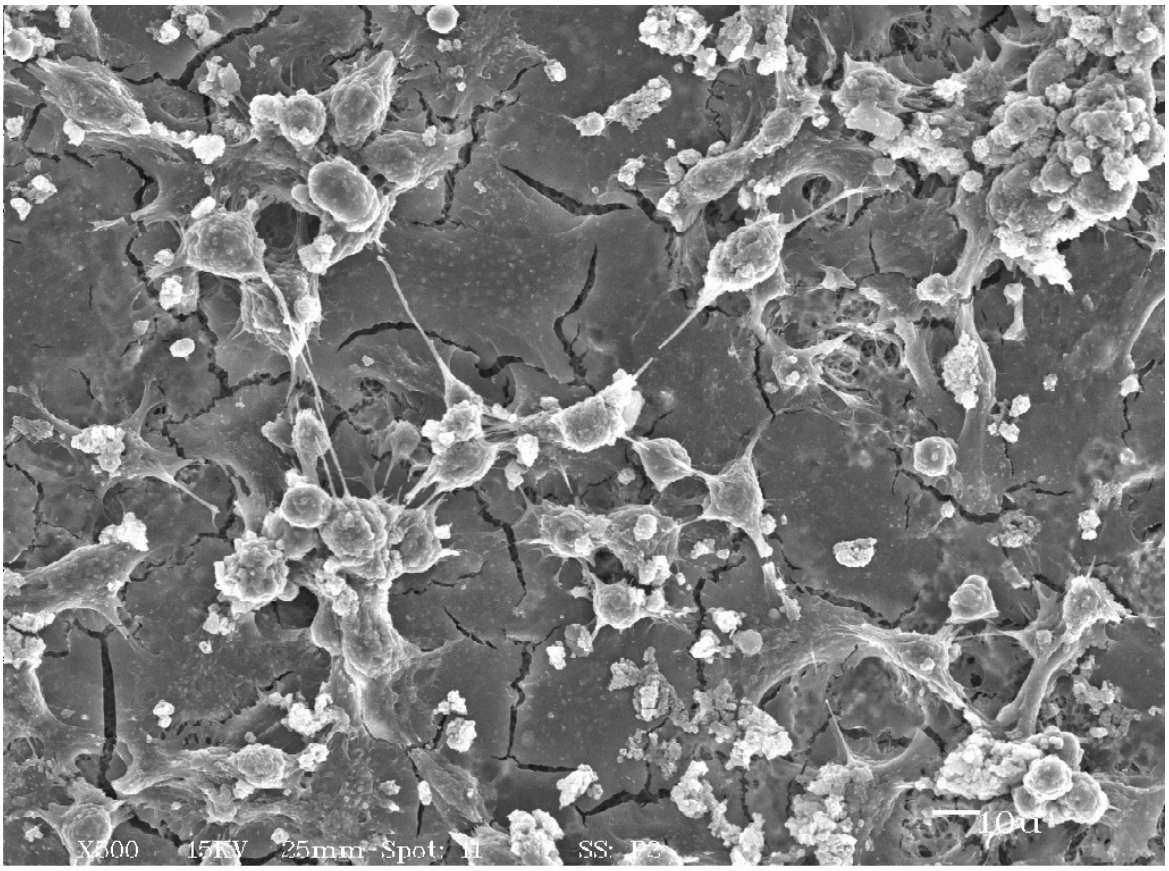

Figure 3-d
Scanning electron micrograph of the osteoblastic cell culture with Polystryene binding RGD 240 min after seeding the culture. Bar = 10μm

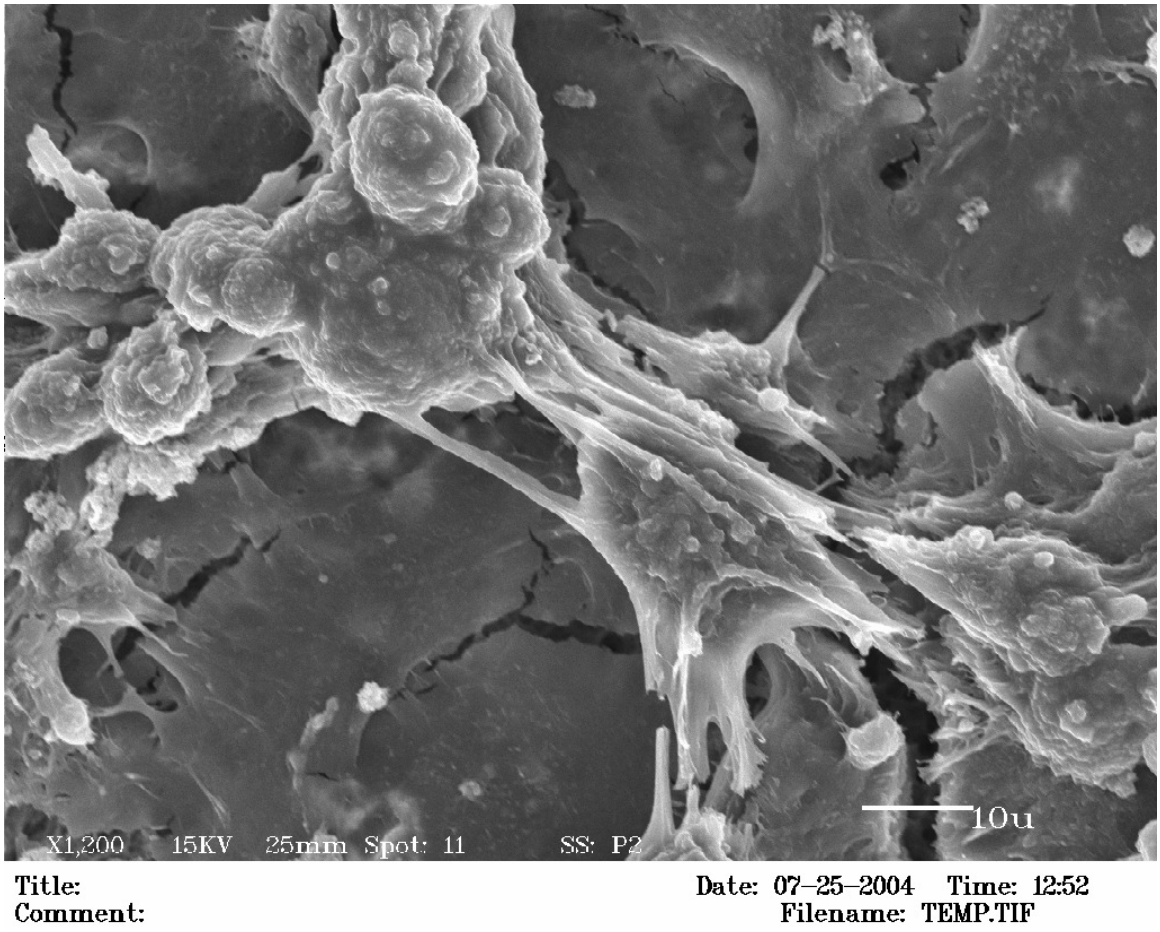

Figure 3-e

Scanning electron micrograph of the osteoblastic cell culture with Ti-binding RGD 240 min after seeding the culture. Bar = 10μm

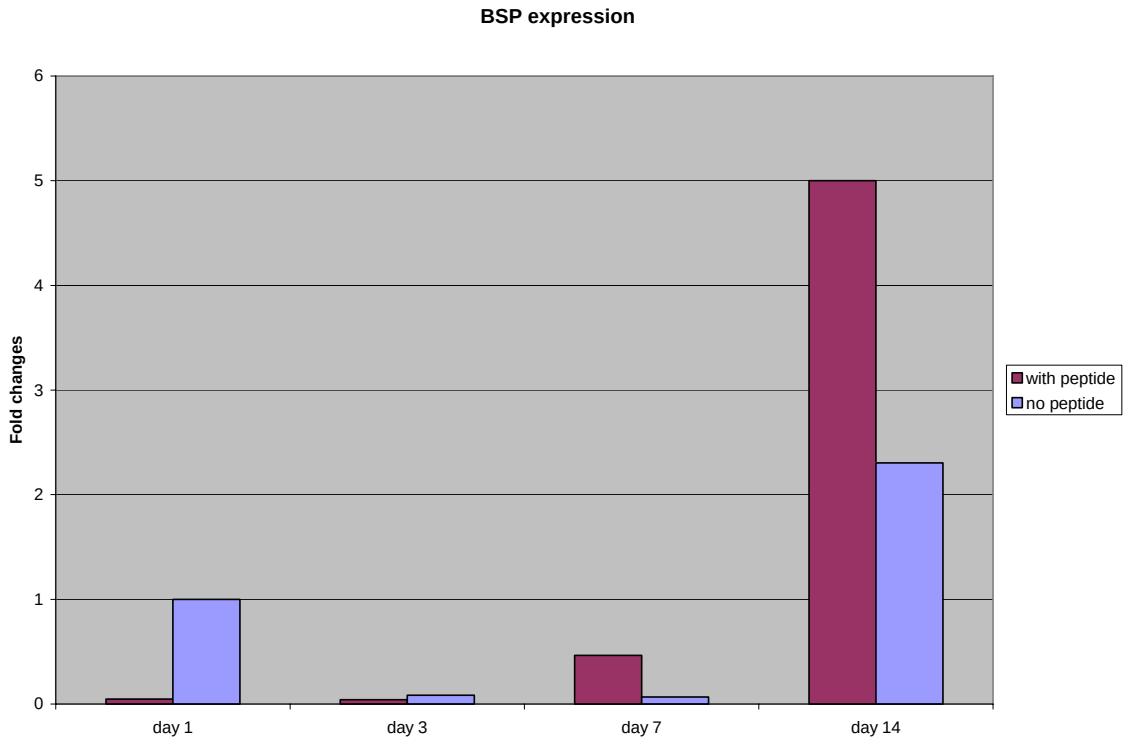

Figure 4
The expression levels of BSP mRNA determined by Real Time PCR

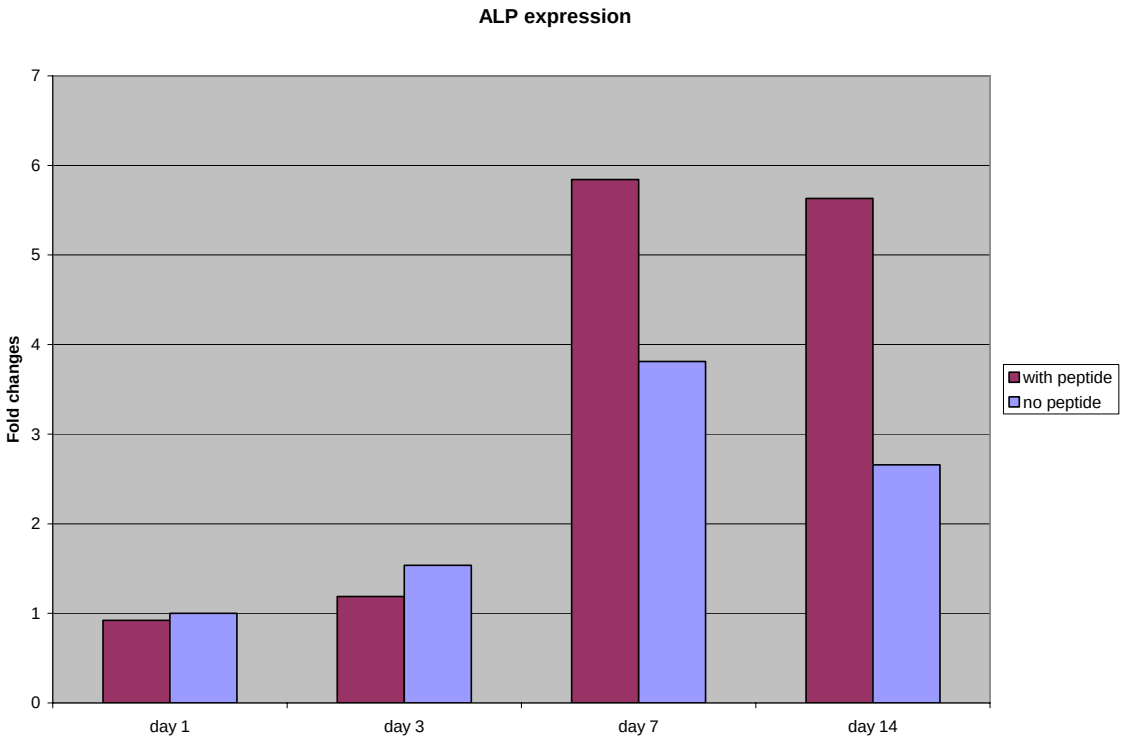

Figure 5
The expression levels of Alkaline phosphatase mRNA determined by Real Time PCR

CHAPTER 4

DISCUSSION

The success of osseointegration is determined by the integration into tissue of surrounding biomaterial substances. Osseointegration of dental implants is dependent on the attachment and spreading of osteoblast-like cells on the implant surface. One way to measure whether the implant surface might be favorable for this phenomenon is to incubate osteoblast-like cells on prepared titanium surfaces in vitro and determine initial attachment to these surfaces. There may be optimum surface microroughness that can affect initial cellular events such as attachment and spreading.

Osteoblast differentiation and responses during osseointegration are influenced by the implant surface microtopography, associated extracellular matrix proteins and their respective interin receptors. (Schneider and Burridge 1994; Lincks, Boyan et al. 1998; Okamoto, Matsuura et al. 1998; Cooper, Masuda et al. 1999; Shin, Zygourakis et al. 2004)

Many studies have been directed at attempts to comprehend the interaction of osteoblasts with various dental implants, exam cell adhesion and morphology, DNA synthesis, integrin and extracellular matrix expression and enzyme activity, and describe a variety of cellular responses to various implant surface conditions.(Bowers, Keller et al. 1992; Schneider and Burridge 1994; Masuda, Salvi et al. 1997; Biesalski, Knaebel et al. 2006)

Bone growth is related to differentiation while it is formatting throughout the life of the organism through tissue remodeling. For example, osteoclastic bone resorption must correspond to osteoblastic activity resulting in new bone formation. The differentiation stages of osteoblasts are apparent during formation of bone tissue. Adherence of osteoblasts produce high levels of type I collagen, alkaline phosphates, and extracellular matrix mineralization. Moreover, mineralization influences cell shape and metabolic changes in the osteoblasts, which develop into the osteocytes surrounded with mineralized bone matrix. In general, the source of stem cells of osteoblasts is a pluripotent mesencymal fibroblast.

Specific genes encode proteins that support proliferation by functioning as transactivation factors and as proteins that play a primary role in packaging newly replicated DNA into chromatin, as in histones during this proliferation period, and fundamental to the development of bone cell phenotype, several genes associated with formation of the extracellular matrix are actively expressed. Theses specific genes are gradually down-regulated during subsequent stages of osteoblast differentiation. With the decline in DNA synthesis, the expression of alkaline phosphates (enzyme activity and mRNA), a protein associated with the bone cell phenotype, increases more than 10-fold immediately after the down-regulation of proliferation. During this period extracellular matrix undergoes a series of modifications in composition and organization that renders it competent for mineralization stages, and cellular levels of alkaline phosphates mRNA decline.(Stein, Lian et al. 1990)

Morphology of the titanium surfaces

One of major factors in the success of implants is their surface property. The layer of macromolecules and water influences the behavior of cells when the cell starts to adhere on the implant surface. In many *in vivo* studies, rough surfaces were found to produce better bone fixation than smooth surfaces.(Thomas and Cook 1985; Carlsson, Rostlund et al. 1988; Buser, Schenk et al. 1991) Several *in vitro* studies have also demonstrated that surface roughness affects cell response. The osteoblast cells exhibit greater initial attachment to rough Ti surfaces. Moreover, most recent studies of surface roughness have focused on cell attachment and shown better cell attachment on rough surface. The result of surface texture on later time frames of cell differentiation, matrix production, and calcification is as important as cell bond and will have a major role in implant prognosis and success.

Bowers et al determined the morphology between a sandblasted surface and an acid edge surface of titanium. Sand blasted surfaces were highly irregular in morphology with many small (< 10 μm) depressions and indentations among flatter-appearing areas of various sizes. Acid etching also produced irregular surfaces whose morphology differed from that produced by sandblasting. The acid solution appeared to remove bulk metal from the surfaces with irregular shaped defects varying in size of up to approximately 20μm. In general, synthesis on rougher surfaces was greater than seen on smoother surfaces.(Bowers, Keller et al. 1992)

Scanning electron microscopy was used to determine the morphologic characteristics of cells as a function of the various surfaces and time intervals used in this project. Early stages of our study show that hMSC demonstrated similar morphologic appearance on both prepared surfaces. The shape of the cells is spherical with a round cell in the center. They spread in all directions and adhere to titanium surfaces. In the late stage, cell morphology

was similar, yet there were more flatter and spread out cells on the Ti-binding RGD surface than on the other surface. Moreover, the round cells multiplied more on the flat spread out cells than they did in early stages.

When comparing the morphology of the shape of the osteoblast on sandblasted surfaces and acid edge surfaces, our result shows that sandblasted surfaces have more spread type of cells than the acid edge surfaces. Furthermore, the speed of the spread osteoblast is faster on the sandblasted surface than the acid edge surface at the same time. Our results show that in the early stages of the sample, the sizes of the spread cell and the round cell were small. In addition, the round cell did not show cell division. On the other hand, in the late stages of the sample, the cells exhibited more division and demonstrated more spread on the spread cell surfaces. When comparing Ti-binding RGD and Polystyrene binding RGD, the Ti -binding RGD show greater growth speed than the other.

Many studies proved that the peptides attempt to develop more osteogenic inducing surface in order to improve oseeous differentiation the metallic surfaces by themselves.(Ferris, Moodie et al. 1999; Rezania and Healy 1999)

Zreiqat et al examined the effects of chemically modifying Ti-6AI-4V surfaces with common RGD sequence, on the modulation of bone remodeling. Their results showed that human bone derived cells (HBDC) grown for 7 days on RGD surfaces displayed significantly increased levels of osteocalcin, and pro-collagen IαImRNAs, compared with the production by HBDC grown on the native Ti-6AI-4V.(Zreiqat, Akin et al. 2003)

Rammelt et al. examined between the collagen RGD peptide and chondroitin sulfate. They found that an increased expression of osteogenic factors (osteocalcin, procollagen

mRNA) after 7days and an increased concentration of osteoblast differentiating factors. (IL-6, osteoprotegerin) (Rammelt, Illert et al. 2006)

Ferris et al evaluated the quality and quantity of the new bone formed in response to titanium rods surface-coated with the peptide sequence Arg-Gly-Asp-Cys (RGDC) using gold-thiol chemistry and implanted in rat femurs. Their study suggested that an RGDC peptide coating enhance titanium rod oseeointegration in the rat femur.(Ferris, Moodie et al. 1999)

Germanier et al presented to examine early bone apposition to a modified sandblasted and acid-etched (SLA) surface coated withan Arg-Gly-Asp (RGD)- peptide modified polymer (PLL-GPEG/PEG-RGD) in the maxillae of miniature pigs. Then they concluded that the (PLL-G-PEG/PEG-RGD) coating may promote enhaced bone apposition during the early stages of regeneration.(Germanier, Tosatti et al. 2006)

Furthermore, Elmengaard et al shows encouraging results as cyclic RGD coating on unloaded press-fit titanium implants significantly increased bone formation on and around the implant.(Elmengaard, Bechtold et al. 2005)

Current study provides evidence that the highest adherence of cell attachment is obtained on the rough, irregular surfaces created by sand blasting with Ti-binding RGD. At 240 minutes, the cells had flattened on the surface and had intimated or integrated on the cells on the surface. If implant substrates can alter the cellular morphology following initial attachment, then it is imaginable that implant topography may affect osseointegration.(Stanford and Keller 1991) In addition, the results from SEM show that surface roughness of the implant with Ti-binding RGD peptide affects the biological responses of osseointegration.

Real-time PCR results demonstrated that upregulation is the key to osteoblast specific gene expression, such as ALP and BSP. Both enzymes initiate phosphate precipitation as an early indicator of osteoblastic differentiation. The ALP enzyme is often used as a marker for increased osteoblastic metabolic activity and an early indicator of osteoblastic differentiation. (Aubin 1996)

The Arg-Gly-Asp (RGD) peptide

The Arg-Gly-Asp (RGD) represents a strategy to control biological interactions at the cell-material interface. The tri-peptide sequence arginine-glycine-aspartic acid (RGD) is found in the proteins of the extracellular matrix (ECM), with various transmembrane receptors.(Humphries 1990)These peptides are known to improve the tissue-material contact owing to highly specific binding to cellular membrane receptors known as integrants, thereby promoting the adhesion and migration to borosilicate glass and titanium surface using silanisation chemistry. A tryptophan residue was incorporated into the amino acid sequences of selected peptides to facilitate the detection of the covalently bound peptides. (Senyah, 2005)

The RGD sequence is found in many extracellular matrix proteins, such as fibronectin, osteopontin, bone sialoprotein and collagen type I. The immobilization of synthetic RGD- peptides is an attempt to control the cell response at the material tissue interface. The extent to which the peptides attract cells and allow settlement differs largely depending on their amino acid composition and length of the overall peptide sequence. Our

study shows that the Ti-binding RGD stimulate more cell adhesion than Polystyrene banding RGD.

Stein et al mentioned that a reciprocal and functionally coupled relationship between the decline in proliferate activity and the subsequent induction of genes associated with matrix maturation and mineralization is supported by a temporal sequence of events in which an enhanced expression of alkaline phosphates occurs immediately after the proliferate period, and later an increased expression of osteocalcin and osteopontin at the onset of mineralization. In addition, mineralization induces cell shape and metabolic changes in the osteoblast, which develops into the osteocyte surrounded by mineralized bone matrix or flattened bone lining cell apposed to the mineralized surface.(Stein, Lian et al. 1990)

Bone sialoprotein (BSP)

Bone sialoprotein (BSP) is an acidic phosphoprotein isolated from bone matrix containing very high concentration of O-glycosidically linked sialic acid residues. It appears to be present only within bone tissue upon Northern blot analysis. BSP has a peptide backbone of 33,600 Da and total weight of approximately 59,000Da. As with osteopontin, this molecule contains a significant concentration of acid amino acids (32% are Glutamate), including a ten amino acidic repeated sequence of glutamic acid that is thought to convey mineral binding properties to the molecule. Upon sequencing, a RGD sequence was detected that appears to convey specificity for the vitronectin integrant receptor. Therefore, much like osteopontin (BSP-I), bone sialoprotein has cell attachment properties, although apparently

with less affinity. (Stanford 1991). BSP is transiently expressed very early and then unregulated again in differentiated osteoblasts forming bone. (Davis 2000) Moreover, the addition of dexamethasone or other factors as BMPs increases the number of bone nodules or bone colonies in calvarias-derived and bone-marrow stromal-cell cultures, suggesting the presence of "inducible" osteoprogenitor cell populations as well. Real Time PDR showed significant increases in BSP expression in cells grown on roughened with RGD peptide, as compared without RGD peptide. The response is to increase production of a factor that promotes early stages of bone formation.

Alkaline phosphates (ALP)

The expression of alkaline phosphates mRNA and enzyme activity before the initiation of osteoblast mineralization suggests that alkaline phosphates may be involved preparing the extracellular matrix for the ordered deposition of mineral.(Stein, Lian et al. 1990) The induction of these mineralization-associated genes may reflect an acquisition of osteoblast properties necessary for signaling bone turnover *in vivo*. The patterns of expression of the ALP and the synthesis of the endcoded proteins, and determined biochemically and by histochemical staining, demonstrate that a temporal sequence of gene expression exists during the culture period associated with development of the extracellular matrix and reflects maturation of the osteoblast phenotype in vitro.(Lian and Gundberg 1988)

By combined thymidine labeling and in situ autoradiography with alkaline phosphates histochemistry, it has been possible to establish a direct relationship between proliferation

and initiation of tissue-specific gene expression at the single-cell level during the osteoblast developmental sequence. The down-regulation of proliferation induces the expression of some genes that are normally expressed later in the osteoblast developmental sequence is derived from experiments that establish that inhibition of DNA synthesis in activity proliferation of cell growth genes.(Owen, Aronow et al. 1990)

This is equivalent with a fourfold increase in alkaline phosphates mRNA levels, and it indicates that the premature down regulation of proliferation induces the expression of an early marker for the extracellular matrix maturation period of the osteoblast development sequence.(Majeska, Nair et al. 1985)

The recent studies showed that the actual role of the ALP stimulates the mineralization of the bone. ALP is an early marker of osteogenic differentiation. While this enzyme activity is present in all cell membranes, it is found in higher levels in cells which mineralize their matrix such as osteoblasts. As osteoblasts mature, they produce extracellular matrix vesicles which are enriched in alkaline phosphates specific activity; because of this specific enrichment, an alkaline phosphate is the marker enzyme for this extracellular organelle. Matrix vesicles are associated with the onset of calcification and they contain enzymes necessary for matrix modification necessary for crystal deposition and growth. The results of this study show clearly the effects of RGD modified peptide.

Wang et al revealed that a higher level of ALP activity at the cranial site may promote the initiation of mineral deposition in the presence of BSP. Our result shows that the ALP expression had increased at day 7. However, the BSP expression started increasing at day 14. That is consistent with that ALP in matrix mineralization starts earlier than BSP

expression.(Wang, Zhou et al. 2006) In general, the induction of mineralization-associated genes may reflect an acquisition of osteoblast properties.

Stein et al used rat osteoblast development sequence to explain that gene expression had three distinct periods; proliferation, extracellular matrix maturation, and mineralization; and those patterns suggest two important transition points on osteoblast development sequence which are important regulatory signs. (They might be very important for the progressive expression of the bone cell phenotype to proceed. In the beginning, proliferation decreases and gene expression connects the extra cellular matrix and maturation is initiated. Then, mineralization starts increasing. In addition, the study confirms the day 20 restriction point for mineralization by showing that mineralization occurs prior to osteoblast growth. The specific genes encode proteins that support proliferation by functioning as transactivation factors and as proteins that play the primary role in packaging newly replicated DNA into chromatin, as in histones during this proliferation period, and fundamental to the development of bone cell phenotype, several genes associated with formation of the extracellular matrix are actively expressed. These specific genes are gradually down-regulated during subsequent stages of osteoblast differentiation. With the decline in DNA synthesis, the expression of alkaline phosphates (enzyme activity and mRNA), a protein associated with the bone cell phenotype, increases more than 10-fold immediately after the down-regulation of proliferation. During this period the extracellular matrix undergoes a series of modifications in composition and organization that renders it competent for mineralization stages, and cellular levels of alkaline phosphates mRNA decline.(Stein, Lian et al. 1990; Boyan, Lossdorfer et al. 2003) In this study, ALP expression was significantly higher with RGD peptide than without peptide at days 7 and 14, and the achievement of the

maximum ALP activity was day 7. Both with RGD peptide and without peptide showed very low ALP activity at day 1 and day 3.However, at days 7 and 14 the ALP activity increased dramatically. This result support that ALP is usually up regulated at the earlier stages of osteoblast differentiation.(Bancroft, Sikavitsas et al. 2002)

The current study shows that different implant surfaces and RGD modified surface microtopographies can influence the consequences in expression of the key to osteogenic genes as ALP and BSP. This suggests that the interaction of the osteoblasts with the extracellular matrix components on the different modified implant surface can effect gene expression. Moreover, the AFF 6008 peptide were capable of mediating specific interaction with hMSC derived osteoblasts.

Consequently to the significant effect of the peptide on the differentiation and mineralization of hMSC, the level of expression of the ALP and BSP was significantly affected by the AFF 6008 peptide.

CHAPTER 5

CONCLUSION

This investigation is to use peptide affinity for different surfaces implants and permit the direct adhesion of peptides containing RGD to promote cell adhesion. Consequently to the significant effect of the peptide on the differentiation and mineralization of hMSC, the level of expression of the ALP and BSP was significantly affected by the AFF 6008 peptide in the early stage of osseointegration.

The results, furthermore, suggest that additional RGD coating may be effective in early formation of bone implant contacts.

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