Response of Human Osteoblasts to Novel Silk-Based Biomaterials

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 RESPONSE OF HUMAN OSTEOBLASTS TO NOVEL SILK-BASED BIOMATERIALS

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B.D.S., University of Karachi, 1997

A Thesis
Submitted in Partial Fulfillment of the Requirements for the Degree of
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at the
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This thesis is dedicated to

My parents Chaudhry M. Aslam and Shamim Aslam

My wife Sharmeen

And

My son Faaris
ACKNOWLEDGEMENTS

Thanks be to Allah. I would like to begin by thanking my advisory committee consisting of Drs. Gloria Gronowicz, Alan Lurie, and Liisa Kuhn. Working with this committee has been a pleasure. Gloria has been a wonderful teacher and a mentor. A sincere thanks. In addition, I would like to acknowledge Ankur Jhaveri, Mary Beth McCarthy, Wenjian Zhang and Alex Pantschenko wonderful people I have had the honor of working with. All of them, especially Ankur, gave their time generously and without hesitation. I would like to show my appreciation for Dr. Lurie for his guidance and support. I would also like to express my gratitude for Dr. Liisa Kuhn who taught me valuable research skills. Finally, a special thanks to my family most importantly to my wife for her unending patience and unconditional support.
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INTRODUCTION

Silk offers a unique suite of structural and functional features to the field of craniofacial bone repair. No other biomaterial offers this combination of impressive and controllable mechanical and morphological properties such as biocompatibility, slow biodegradability, absence of bioburden concerns and options for chemical decoration due to the diversity of its surface-amino acid side chain chemistries (1). A comparison of mechanical properties of common silks (silkworm and spider dragline) to several types of biomaterial fibers and tissues commonly used today (Table 1) (1) suggests that silks provide a remarkable combination of strength and toughness. Distinguishing features of silks are high strength and excellent elasticity. Silks also display resistance to failure in compression that distinguishes them from other high performance fibers such as Kevlar. From a clinical perspective, silks are already widely used as suture materials and are being actively explored for other biomedical applications such as ligament repair. With an improved understanding of structure-function relationships, this window of applications for biomedical repairs should continue to expand. Aside from existing natural source of silk (B. mori silkworm; silk from sericulture), future options with the development of genetically engineered silk variants, would expand the set of silk protein structures available for use in vivo. Examples of potential applications include scaffolds for bone repair, artificial tendons or muscles, strong sutures and membranes for wound healing. Controllable structure and morphology, superior mechanical properties, ability to mineralize,
biocompatibility and slow biodegradability of silk will have an important impact in repairs related to bone defects in dentistry.

**Background:**

Silk and the tri-peptide arginine-glycine-aspartic acid (RGD)-decorated silk have been shown to be anabolic for bone formation (2). The RGD sequence is found in many of the extracellular matrix (ECM) proteins and serves as a binding site for integrins (transmembrane receptors) present on the plasma membrane of the osteoblasts. Integrin binding with RGD initiates a cascade of events that leads to the generation of intracellular signaling pathways responsible for cell adhesion, motility, shape, proliferation, prevention of apoptosis, and differentiation.

**Extracellular Matrix (ECM) Proteins and RGD**

The ECM is a part of the connective tissue and has a complex structure surrounding and supporting cells that are found within mammalian tissues. The ECM is composed of 3 major classes of biomolecules:

1. Structural proteins – Collagen and Elastin
2. Specialized proteins – Fibronectin, Laminin, Vitronectin etc.
3. Proteoglycans – These are composed of a protein core to which are attached long chains of repeating disaccharide units termed glycosaminoglycans (GAGs) forming extremely complex high molecular weight components of the ECM.

Osteoblasts bind to the ECM proteins in the bone matrix through integrins
that are present on the surface of osteoblasts. These ECM proteins include fibronectin, collagen, vitronectin, and osteopontin. Integrins are present in the plasma membrane of all mammalian cells. Integrin receptor binding and clustering culminate in the formation of focal adhesion sites where integrins connect the cytoskeleton and the ECM. Activation of these focal adhesion sites leads to a signaling cascade, which involves MAP kinase (ERK 1 and 2), its translocation to the nucleus and the activation of specific genes. This integrin-mediated signaling pathway affects proliferation and differentiation of osteoblasts.

The process of bone formation starts with derivation of osteoblasts from the newly organized mesenchyme. As osteoblasts differentiate, they synthesize and secrete the ECM proteins. During the process of bone formation, proteins are expressed in a temporal sequence. Major ECM proteins secreted by osteoblasts include fibronectin, type I collagen, bone sialoprotein, osteopontin and osteocalcin. A study has shown that fibronectin is the earliest bone matrix protein expressed in the process of bone formation (44, 45, 46). Fibronectin contains at least 6 tightly folded domains each with a high affinity for a different substrate such as heparan sulfate, collagen, fibrin and cell-surface receptors. Its cell surface receptor-binding domain contains the tri-peptide amino acid sequence RGD. RGD of fibronectin and other ECM proteins serve as a ligand for integrins. Data from fibronectin-null mutant mice embryos show defects in organization of mesoderm and somites suggesting its importance in migration, adhesion, differentiation, and proliferation of mesodermal tissue (50). Type I collagen is the most abundant protein in the bone matrix making up more than 90% of the organic matrix. It is the major component
of osteoid and bone, and is responsible for structural stabilization. Type I collagen mRNA levels have been shown to increase at the onset of mineralization (46). *Bone sialoprotein* is found at the mineralization front (52). It is believed to initiate mineralization (53, 54) as its levels are reported highest during early bone formation (46). It is deposited in the osteoid during mineralization and may be important in nucleating hydroxyapatite. Osteoclasts also bind to bone sialoprotein suing the αvβ3 integrin receptor. *Osteopontin* is also found at the mineralization front and is thought to control mineralization by inhibiting crystallization (55). It is expressed at a later stage in bone development and may be more important in remodeling bone. *Osteocalcin*, a marker of fully differentiated osteoblasts, is produced by mature osteoblasts during the process of mineralization. Its appearance is thought to be a signal for bone turnover (56) and differentiation of osteoclasts (57, 58).

**Integrins**

Integrins are a large family of heterodimeric transmembrane glycoproteins, which are the principle receptors for ECM proteins, including collagen, fibronectin, and laminin. (4). Integrins consist of two transmembrane glycoprotein subunits, α- and β-chains, which are non-covalently bound. The length of the α-chain is 1008–1152 amino acids, with a cytoplasmic region of 22–32 amino acids, and a transmembranous part of 20–29 amino acids. The length of the β-chain is approximately 770 amino acids, with a cytoplasmic region of 20–50 amino acids,
and an intramembranous part of 26–29 amino acids. The extracellular domains of \( \alpha \)- and \( \beta \)-subunits interact with each other, creating a functional heterodimer. Both subunits contain disulfide bridges, protecting them from proteolysis. Integrins interact with ECM through the extracellular domain at the \( \alpha \)-subunits (5), with components of the cytoskeleton and signaling molecules through the intracellular domain of the \( \beta \)-subunit. Through these interactions, integrins can regulate many cellular functions, such as cell adhesion, motility, cell shape, proliferation, apoptosis, and differentiation (6).

Many combinations of \( \alpha \)- and \( \beta \)-subunits are possible, and each combination has the capacity to bind one or more ligands (see Table 2). There are 18 \( \alpha \)- and 8 \( \beta \)-subunits. The \( \alpha \)-subunit determines the specificity of binding to a particular ligand. There is also an overlap in specificity with many integrins capable of binding to more than one protein whereas, many proteins can act as ligands for more than one integrin. The same integrin molecule in different cell types can have different ligand binding activities as well, since additional cell type specific factors can interact with integrins to modulate their binding activity (5). Integrin recognition sites in many protein ligands consist of short linear sequences that contain an acidic residue. Additional discontinuous regions of the protein may provide secondary binding sites (4). One integrin subfamily has been shown to utilize RGD sequence (Arg-Gly-Asp) to recognize and bind ligands (5). The bone matrix contains many glycoproteins that have this integrin-binding sequence, such as type I collagen, fibronectin, vitronectin, thrombospondin and bone sialoprotein (7).
The integrin expression on osteoblasts has already been described in various studies (8, 9, 10). Human bone cells are capable of expressing a wide variety of integrins and have been shown to express integrin subunits α1, α2, α3, α4, α5, α6, αv, β1, β3, and β5 (6), but the pattern of integrin expression varied with the stage of bone development (9). Differences between studies on integrin expression can also be the result of cell source, or in vivo versus in vitro characterization. There are also technical reasons for differences, including the detection technique, method for fixation and permeabilization, antibody specificity, and immunostaining conditions.

The β1 integrins are the predominant adhesion receptor subfamily utilized by osteoblasts and osteoblast-like cells and are found in the bone cell receptors for collagen, fibronectin, laminin and vitronectin (7, 8, 9, 10, 11). Investigators have identified the integrin, fibronectin receptor, α5β1 as well as other integrins such as α3β1, α8β1 and αvβ3 in fetal rat calvarial tissue and cultured osteoblasts at all stages of differentiation (12). Blocking of the fibronectin receptor by addition of integrin specific RGD-peptide inhibited mineralization, disrupted osteoblast organization and mineralized matrix, decreased the osteoclast number in a mineralizing organ culture system and decreased calcification (13). Inhibition of the α5β1 function with monoclonal antibodies inhibited osteoblast differentiation (14). Another study showed that osteoblasts interact with the central cell-binding domain of endogenously produced fibronectin containing the RGD sequence during early stages of differentiation (12, 15). These interactions regulated bone nodule morphogenesis and the expression of mRNA for genes associated with osteoblast differentiation such as alkaline phosphatase and osteocalcin. These studies suggest
that fibronectin may play an important role in bone cell proliferation and early stages of differentiation, mediating the maturational pathway of bone cells.

Integrins play an important role in cell anchorage, migration, proliferation, differentiation and apoptosis (programmed cell death). Integrins can mediate a large number of different cellular responses to the ECM depending on cell type, the integrin receptors expressed by the cell, and the composition of the surrounding ECM. They are also mechanotransducers of force across the plasma membrane. The α- and β-subunit cytoplasmic domains do not have any intrinsic enzymatic activity, but couple with cytoplasmic proteins that nucleate the formation of large protein complexes containing both cytoskeletal and catalytic signaling proteins (16). The assembly of integrins, ECM proteins and intracellular protein complexes forms focal adhesion sites.

Integrins function as transmembrane linkers mediating the interactions between the cytoskeleton and the extracellular matrix. By influencing the cytoskeleton, integrins stabilize cell adhesion and regulate the shape, morphology, orientation and the movement of cells. The α-cytoplasmic domain contains highly divergent amino acid sequences while the β-cytoplasmic domain shows partial sequence conservation. The β-cytoplasmic (internal) domain plays a role in intracellular signaling whereas, the α- domain (external) regulates the specificity of the ligand-dependent interactions (16). The β-subunit connects to the cytoskeleton of actin filaments through proteins such as talin and α-actinin, and other intracellular attachment proteins like vinculin, paxillin and tensin in the focal adhesion sites. Talin and α-actinin bind to the integrin cytoplasmic domain in vitro
These assemblies of structural proteins may serve as a framework for the association of signaling proteins that regulate signal transduction pathways leading to integrin-induced changes in cell function and cell behavior.

**Role of Integrins in Intracellular Signaling**

Integrins activate intracellular signaling cascades leading to changes in gene activity. Many of the signal transduction pathways identified previously for growth factors and cytokines are also activated by integrins. Signaling through both growth factor receptors and integrins seems to be necessary for optimal cellular responses such as cell adhesion, proliferation and migration (16). In addition, growth factor receptors, such as for platelet-derived growth factors and insulin-like growth factor I, interact with integrins and this interaction is required for growth factor signaling. The cytoplasmic tails of the integrins bind to signaling molecules, such as one or more kinases, which then initiate signaling cascades after integrin ligation and aggregation (17). Protein phosphorylation within focal adhesion sites is one of the earliest events found in response to integrin stimulation. Tyrosine phosphorylation has been shown to be a common response to integrin activation in many cell types (16).

**Focal Adhesion Kinase (FAK):** Focal adhesion kinase (FAK or pp125FAK), located in focal adhesion sites, is one of the major substrates for integrin-induced tyrosine phosphorylation (18) and induces intracellular signaling. FAK phosphorylation is important in regulating the formation of new focal adhesions. However, FAK activation may not be required for the maintenance of focal
adhesions (19). Cellular adhesion to fibronectin and other ECM proteins initiates integrin clustering resulting in FAK tyrosine phosphorylation and FAK activation. In fibroblasts, cell adhesion requires phosphorylation and activation of FAK. FAK activation is reduced in suspended cells (20). When suspended cells are replated on ECM substrates, FAK is rapidly phosphorylated and shows high levels of kinase activity.

In fibroblasts and endothelial cells, the auto-phosphorylation of FAK has been shown to be followed by further tyrosine phosphorylation and recruitment of other proteins including src to the focal adhesion sites. Intermediate steps involve the enzymatic activation of proteins by phosphorylation, which ultimately leads to the stimulation of MAP kinases.

**Mitogen Activated Protein Kinases (MAPK):** Integrins (16, 22, 23, 24, 25, 26) and growth factors (21, 22) activate the MAPK signal transduction pathway. The same pattern of MAPK signaling is used by different signaling pathways that involve distinct MAPK isoforms (22, 27). Extracellular Regulated Kinase 1 and 2 (ERK1/2) are two 44/42 kDa isoforms of MAPK. It is documented that the ERK 1/2 subfamily is activated by hormones, growth factors and integrins (28). ERK 1/2 have been shown to be phosphorylated in response to the adherence of human skin fibroblasts to fibronectin or by cross-linking of β1 integrins with antibody (29). This suggests that integrins are able to activate MAPK (ERK1/2). Phosphorylated MAP kinase translocates to the nucleus and is able to bind and regulate transcription factors (16, 26). The nuclear target proteins of MAP kinases include
fos and jun (members of the AP-1 transcription factor), which allows MAPK to control gene expression (22, 30).

The Activator Protein - 1 (AP-1) Transcription Factor: AP-1 transcription factor modulates the expression of genes involved in proliferation, differentiation and neoplastic transformation (30). It is characterized by its ability to alter gene expression in response to growth factors, cytokines, tumor promoters, carcinogenesis and increased expression of various oncogenes such as src and ras (31).

The AP-1 complex is a multiprotein complex composed of the products of the fos and jun gene families. Fos and jun are nuclear phosphoproteins which combine to form hetero- or homo-dimer complexes. While jun proteins can form homo-dimers and hetero-dimers with the fos proteins, fos proteins cannot form homo-dimers (31). Dimer formation is necessary to bind to the AP-1 recognition site on the DNA, which stimulates the transcription of genes that contain AP-1 consensus sequences in their promoter regions. Regulation of AP-1 activity in response to extracellular signals is determined by the levels of transcription of the fos and jun genes as well as by posttranslational modification (31). The AP-1 complex initiates early transcriptional events which lead to an increase in DNA synthesis and cell proliferation or it can affect other cellular processes such as differentiation. AP-1 sites are known to be present in the promoters of many bone specific genes, such as type I collagen (32), alkaline phosphatase (33), osteocalcin
(34, 35) and osteopontin (36). Fos has been found to have a critical role in regulating the development and activities of bone and cartilage cells (37). It can act both as a transforming oncogene or as cell type specific regulator of differentiation. Over expression of fos in transgenic mice causes transformation of cells in the chondrogenic and osteogenic lineages leading, in cooperation with jun, to the development of chondrosarcomas and osteosarcomas. Fos knockout mice lack osteoclasts and develop osteopetrosis (37). In vivo expression of c-fos has been shown to be associated with regions of fetal bone having the highest growth potential (37, 38). High levels of c-fos, c-jun and junB mRNA during the proliferative period of osteoblast development has been found (38). These findings suggest a fundamental role of AP-1 in cell proliferation.

**Objectives**

**Rationale:**

Previous work in our laboratory demonstrated that silk-based biomaterials and RGD-decorated silk were osteogenic by increasing differentiation of osteoblasts, and stimulating mineralization (2). Additionally, an in vivo study has shown bone formation in intramuscular implants of silk films seeded with rat mesenchymal stem cells (MSCs) (3). However, it was not known whether silk and silk-RGD stimulated bone formation through the activation of integrins and the MAP kinase signaling pathway.
Goals:

Our goal was to study integrin expression, proliferation and mineralization in primary human osteoblasts grown on tissue culture plastic, purified silk films, purified silk films covalently decorated with the tripeptide RGD and control tripeptide RAD.

Hypothesis:

Our hypothesis was that decoration of silk films with RGD would enhance proliferation and mineralization by up-regulating specific integrin subunits in human osteoblasts.
MATERIAL AND METHODS

Cell culture

Human bone fragments, which are normally discarded after surgery, were obtained from healthy patients undergoing foot surgery and immediately minced. The use of this tissue was approved by the Institutional Review Board (IRB) at The University of Connecticut Health Center. The human osteoblasts (HOBs), used in our experiments, were obtained from these fragments by culturing them at 37°C and 5% CO₂ in DMEM/F12 Ham (Sigma, St. Louis, MO) with 15% fetal bovine serum (FBS) and 100 units/ml penicillin G and 100 μg/ml of streptomycin sulfate (Beresford, 1984 #243).

When the tissue culture dishes were almost confluent, the HOBs were collected by trypsinization and plated at 10,000 cells/cm² in six-well Costar dishes (Corning Costar Corp., Cambridge, MA) in DMEM/F12 Ham with 15% fetal bovine serum (FBS) and 100 units/ml penicillin G and 100 μg/ml of streptomycin sulfate (Beresford, 1984 #243). The plating sequence (six wells/plate) is shown in Table 3.

Preparation of Silk Substrates

Silk films were prepared according to Sophia et al., (1). Briefly, cocoons from B. mori silkworms were boiled for 1 hour in an aqueous solution of 0.02M Na2CO3 and 0.3% (w/v) Ivory soap solution, and then rinsed thoroughly with water to extract the glue-like sericin proteins. The extracted silk was then dissolved in 9M LiBr solution overnight at 37°C giving a 10% (w/v) solution. This solution was
dialyzed in water using a Slide-a-Lyzer dialysis cassette (Pierce, MWCO 2000),
lyophilized, and the regenerated silk fibroin dissolved in hexafluoro-2-propanol
(HFIP, Aldrich) to give a 2–3% (w/v) silk fibroin solution. Films were formed by
covering the bottom of the 6-well plates (Costar) with 140 µl of the aqueous (after
dialysis) solution under sterile conditions. The films were air dried for 4–5 hours.
Once the films were dried, they were contacted with a 90/10 (v/v) methanol/water
solution for 10 minutes to induce a conformational change in the silk to prevent
resolubilization in the cell culture media. The films were then dried overnight
before use. In general, films cast from water solutions dried with non-uniform
thickness and tended to crack after the methanol treatment, whereas the HFIP-cast
films had uniform thickness and surfaces and remained so after the methanol
treatment. Therefore, only the HFIP-cast films were used for cell culture studies.

**Covalent coupling of peptides to silk fibroin films:** Silk films were modified
by covalent coupling of RGD and RAD peptides. Briefly, after hydration of silk
films in PBS (pH 6.5) for 1 hour, exposed carboxyl groups of aspartic acid and
 glutamic acid amino acids were activated by reaction with an appropriate volume of
0.5 mg/mL 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC)
and 0.7 mg/mL N-hydroxsuccinimide (NHS) solution (Pierce) in phosphate-
buffered saline (PBS) for 15 minutes at room temperature. After activation, silk
films were rinsed extensively to remove excess EDC/NHS and were reacted with 1
mL of either 0.01, 0.05, or 0.1 mg/mL glycine-arginine-glycine-aspartic acid-serine
(GRGDS) peptide (Sigma, St. Louis, MO) or the negative control peptide glycine-
arginine-alanine-aspartic acid-serine (GRADS; Sigma) in PBS for 2 hours at room
temperature. To complete the reaction, matrices or films were rinsed extensively in distilled water and air dried.

**Proliferation**
During 72 hours of culture, $[^3]$H thymidine (5 μCi/ml) was added to the cells for the last 4 hours. Cell layers were then extracted twice for 5 minutes with 10% trichloroacetic acid (TCA) and lysed in 0.5 N NaOH for 10 minutes. Liquid scintillation counting was performed to measure radioactivity in the lysates (Packard Instrument Co., Downers Grove, IL).

**Mineralization**
At 7 days of plating, culture medium was changed to αMEM + 15% FBS + 1% Penicillin/Streptomycin (mineralization medium). In addition to the change in medium, osteoblasts were fed with 50 μg/ml ascorbic acid every other day to promote mineralization. Day 1 for this group was day 8 of plating. On day 35 of culture, cells were extracted twice for 30 minutes with 5% TCA. Calcium content in the cell extract was measured colorimetrically using a calcium kit (Sigma, St. Louis, MO).

**Integrin Expression**
Western blots were utilized to probe for integrin expression. Protein extraction was accomplished by using RIPA cell lysis buffer (50 mM tris-HCl pH 7.4 + 150 mM NaCl + 1 mM EDTA + protease inhibitor cocktail + 1% sodium deoxycholate + 0.1% SDS + 0.004% sodium azide). Cell lysate from each time point and for each substrate was transferred to an eppendorf tube. Each sample was
spun at 10,000 G for 30 min. BCA protein assay (Pierce, 23225) was performed to
determine the concentration of samples. Samples were stored at -20° C. A sample
of 50 μg of protein was concentrated to 15ul using a lyophylizer. An equal volume
(15 μl) of sample buffer was added to each sample. The final samples were heated
at 95°C for 5 min.

Criterion 7.5% acrylamide gels (Bio-rad, 345-0043) were used to separate
the proteins. Pre-stained (Bio-rad, 161-0372) protein standards were added to the
first well, and 30ul of final samples were added to the remaining wells.
Electrophoresis was performed at 200V for 45 min. The gel was equilibrated in
tank buffer for 5 min. Proteins from the gel were transferred to an Immobilon paper
using a blotter tank (Bio-rad, 170-4071) at 100V for 1hr.

Protein detection was accomplished by chemiluminescence. The blot
(Immobilon) paper was blocked for 1hr. in blocking buffer (5% non-fat milk in
Tween-20 Tris buffered saline (TTBS)) at room temperature. It was incubated at
4°C overnight with primary antibodies, one at a time (monoclonal mouse anti-
human antibody for α1, α2, α5, αV, β1 or β3 (Santa Cruz BioTechnologies, Santa
Cruz, CA), 1: 100 concentration in 5% non-fat milk dissolved in Tris-buffered
saline (TBS)). The blot was rinsed with wash buffer [1x TBS (20mM tris, 150mM
NaCl, pH 7.5), 0.05% Tween-20 (add 0.5 ml Tween-20) (Sigma, P-9416) into 1L
TBS solution]. It was then incubated for 1 hour at room temperature in secondary
antibody [goat anti-rabbit IgG HRP (Pierce, 31430) 1: 10,000 in 5% milk in
TTBS].
The detection of bands involved the use of a mixture of luminol/enhancer and peroxide buffer (Bio-rad, 170-5040). Blot was incubated in the substrate mix for 5 min. X-ray films were exposed to the blot using various exposure times and developed.

**Densitometric and Statistical Analyses**

The densitometry of bands in the autoradiographs and chemiluminescence films was scanned and quantitated by Kodak 1D Image Analysis Software (Eastman Kodak Company, Rochester, NY). The data are representative of two or more individual experiments. Statistical analysis was performed by a two-way analysis of variance using Microsoft® Excel® (Student–Neuman–Keuls; p< 0.05)
RESULTS

**Proliferation**

Proliferation of osteoblasts on tissue culture Plastic, Silk, Silk-RGD and silk-RAD was measured at 72 hours. A graph of the data for proliferation is shown in *Figure 1*.

No significant difference was found in proliferation between Plastic and Silk-RAD groups (p-value= 0.355). There was a 2.8 fold increase in [³H]-thymidine incorporation for Silk compared with the Plastic group but this was not significant (p-value=0.331). However Silk-RGD group had a 5.8 fold increase in [³H]-thymidine incorporation that was significant at a p-value of 0.006. Osteoblast proliferation was also 22 fold higher on Silk-RGD compared to Silk-RAD (p-value=0.002).

**Mineralization**

Mineralization, determined by measuring calcium content of the matrix on all four substrates, was measured after 5 weeks of culture. A graph of mineralization data is shown in *Figure 2*.

A significant increase in calcium deposition was seen on Silk compared to Plastic substrates (p-value=0.04). Significant stimulation of mineralization was observed for Silk-RGD vs. Plastic and Silk-RAD vs. Plastic substrates with p-values of <0.05. There was no significant difference in calcium deposition on Silk-RGD compared to Silk-RAD group (p-value=0.62).
Integrin Expression

Integrins are important cell surface receptors responsible for attachment of osteoblasts to ECM proteins. In this study, \(\alpha_1, \alpha_2, \alpha_5, \alpha V, \beta_1\) and \(\beta_3\) integrins were studied for their role in osteoblast function when grown on four different substrates Plastic, Silk, Silk-RGD and Silk-RAD.

Expression of each of these six integrins was studied at 0 time, 24-hour and 7-day time points by Western blot (Figure 3). Twenty-four hour integrin expression was compared to 0 time (Figure 4) and 7-day integrin expression was compared to 24-hour by densitometric scans of the Western blots (Figure 5).

Between 0 and 24 hours, osteoblasts on Plastic decreased the \(\alpha_1\) and \(\alpha_2\) expression 0.6 and 0.4 fold (Figure 4). Whereas, \(\beta_1\) and \(\beta_3\) expression increased 3.2 and 2.2 fold. No significant change was observed for \(\alpha_5\) and \(\alpha V\). On Silk, \(\alpha_1\) expression decreased 0.7 fold. However, \(\alpha_5\) expression increased 2.2 fold. No significant changes were found for \(\alpha_2, \alpha V, \beta_1\) and \(\beta_3\) on silk. On Silk-RGD group, \(\alpha_2\) expression decreased 0.5 fold while \(\alpha_5\) and \(\beta_1\) expression increased 2.2 and 1.9 fold. No change was seen for \(\alpha_1, \alpha V\) or \(\beta_3\) expression on Silk-RGD. Between 0 and 24 hour in the Silk-RAD group, there was a decreased expression of \(\alpha_1, \alpha_2\) and \(\alpha V\) by 0.5, 0.5 and 0.7 fold. Whereas, an increased expression of 2.8 and 1.5 fold was observed for \(\alpha_5\) and \(\beta_1\) respectively with no significant change in \(\beta_3\).

Between 24 hours and 7 days, osteoblasts on Plastic showed decreased expression of \(\alpha_1\) and \(\alpha V\) by 0.4 and 0.5 fold (Figure 5). Whereas, increased expression of 2.6, 6.0, 2.2, and 1.8 fold was seen for \(\alpha_2, \alpha_5, \beta_1\) and \(\beta_3\) respectively. On Silk, \(\alpha_1\) and \(\alpha V\) expression decreased 0.7 and 0.5 fold while \(\alpha_2, \alpha_5, \beta_1\) and \(\beta_3\)
expression increased 14.7, 2.3, 4.2 and 2.2 fold respectively. Silk-RGD group showed decreased α1 expression by 0.4 fold. Whereas, α2, α5, αV, β1 and β3 showed increased expression of 2.1, 2.7, 1.3, 1.9, and 1.7 fold respectively. Between 24 hours and 7-day Silk-RAD groups, α1 expression decreased 0.6 fold while α2, α5, αV, β1 and β3 showed increased expression of 2.8, 1.6, 3.0, 2.8 and 2.5 fold respectively.
DISCUSSION

*In vivo,* human osteoblasts use integrins to attach to the extracellular matrix (ECM) proteins. Osteoblasts are able to attach to the ECM proteins at the bone/implant interface and perhaps to the implant material itself via integrins (39, 40). Specifically, integrins recognize and bind to extracellular matrix proteins such as collagen, fibronectin, laminin, vitronectin etc. In this study, integrins α1, α2, α5, αV, β1 and β3 that are well documented to be expressed by human osteoblasts (6, 10), were studied for their interactions with novel silk-based biomaterials and control materials. Since integrins initiate intracellular signaling pathways that affect cell proliferation and differentiation we evaluated osteoblast proliferation, mineralization (calcium deposition) and integrin expression.

Osteoblast adhesion to and proliferation on an implant material are the first vital steps in determining biocompatibility of the substrate. When an implant material is placed in a tissue defect (*in vivo*) or in culture medium (*in vitro*), proteins will adsorb to its surface. This protein layer will subsequently mediate the interaction of the implant material with the cells arriving from the surrounding tissue. Integrins are the adhesion molecules involved in the process of cellular adhesion. They can also pass information from the ECM to the cell and from the cell to the ECM (6).

Cell adhesion can be divided into two phases: The attachment phase and the adhesion phase. Cellular attachment takes place rapidly with short-term events, such as physicochemical linkages between cell and material involving ionic forces, van der Waals forces, etc. (41). The adhesion phase occurs in the longer term and
involves various biological molecules such as extracellular matrix proteins, cell membrane proteins, and cytoskeleton proteins. These molecules interact together to induce signal transduction regulating transcription factors, other proteins and gene expression (41). Integrins are the primary family of cell membrane proteins that mediate adhesion of cells to substrates.

The expression of integrins in osteoblasts has been demonstrated in vivo and in vitro. In human bone sections, osteoblasts expressed α1 and α5 sub-units although a subpopulation of osteoblastic cells expressed α2 and α7 (9). When primary human osteoblasts adhered to fibronectin, type I collagen and laminin coated polystyrene dishes in the presence of serum, β3 and α7 integrins were found in focal contacts. The α4 and β1 subunits were also expressed by primary human osteoblasts on these substrates. The α2, α6 and α7 integrins were expressed on control dishes, α2, α3 and α7 on collagen coated dishes and α6 and α7 on laminin-coated dishes. The β3 subunit was expressed on all surfaces except on laminin-coated dishes (42). Adhesion to fibronectin-, collagen- and laminin-coated dishes is inhibited by antibodies to the β1 integrin subunit, however, the antibody to α5 affects adhesion only to fibronectin (43). An understanding of the integrin subunits involved in osteoblast adhesion provides essential information for biomaterial improvement and notably for identification of the proteins, which may be useful to adsorb on materials before implantation.

Bone is formed with the sequential deposition of ECM proteins. Fibronectin has been shown to be produced first, by pre-osteoblasts undergoing proliferation in prenatal mice calvariae that have not begun to mineralize (46). As pre-osteoblasts
develop, type I collagen is expressed. From this time onwards, collagen expression increases gradually, while fibronectin drops precipitously. Type I collagen is the major ECM protein, comprising 90% of the matrix, and is critical for mineralization. Increased type I collagen expression has been correlated with an increase in calcium deposition/mineralization (46). In our study, decreased expression at 24 hours of both \( \alpha_1 \) and \( \alpha_2 \) (collagen integrins) substantiates this finding as little mineralization is occurring at this time. By 7 days, there is a 2 – 15 fold increase in \( \alpha_2 \) expression and a 2 – 4 fold increase in \( \beta_1 \) expression on all four substrates. This data suggest that collagen expression is gradually increasing with the increase in expression of collagen integrins \( \alpha_1 \) and \( \alpha_2 \). Our 5-week mineralization (calcium deposition) data shows that most mineralization occurs on Silk-RGD substrate. It can be postulated that osteoblasts are attaching, proliferating and mineralizing on Silk-RGD substrates more than on Plastic and Silk substrates. In case of Silk-RGD this could be explained as increased recognition of RGD as a ligand by the fibronectin (\( \alpha_5 \)) and collagen (\( \alpha_1 \) and \( \alpha_2 \)) integrins of the osteoblasts.

Successful adhesion of osteoblast to the implant material leads to proliferation. It has been demonstrated that cellular proliferation in osteoblasts is triggered by the activation of integrins with fibronectin and collagen. Both type I collagen and fibronectin support early proliferation in the absence of exogenous growth factors. Substratum-supported cell growth decreased with protein kinase inhibitors, indicating that osteoblast integrins signal through kinase-mediated pathways (44). It has been shown that fibronectin may be critical in the early stage of bone formation owing to its ability to stimulate osteoblast progenitor
proliferation and by the induction of c-fos and c-jun. In contrast, type I collagen affects these parameters less than fibronectin. In our study, human osteoblasts showed significantly high proliferation on Silk-RGD substrate. Silk substrate showed lesser proliferation and was followed by the Plastic group. Silk-RAD substrate showed minimal proliferation. Silk decorated with RGD acts as a suitable substrate for osteoblast integrins to attach. This result corresponds with a 2.5 fold increase in α5 (fibronectin integrin) and 2 fold increase in β1 expression on Silk-RGD substrate at 24 hours. This data is supported by another study that shows a gradual increase in fibronectin expression from day 1 to day 3 in neonatal mice calvariae (46). Fibronectin expression has been shown to coincide with the condensation of pre-osteoblasts prior to calcification and decreases once bone mineralization commences (46).

Mineralization (calcium deposition) data in this study showed significant difference between Silk and Plastic substrates with a p-value of 0.04. Comparison of Silk-RGD and Silk-RAD with Plastic group showed significant differences with p-values of <0.05. No statistically significant difference was seen for Silk-RGD compared to Silk-RAD group with a p-value of 0.62. This is in contrast to another study that showed a significant difference between Silk-RGD and Silk-RAD groups at 2 and 4 weeks of culture (2). The main differences that might explain this phenomenon are the cell population and the length of culture time. Our study used primary human osteoblasts cultured for 5 weeks compared to the other study that used Saos-2 osteoblasts-like cells cultured for 2 and 4 weeks. Another possible explanation is an assumption that at 5 weeks primary human osteoblasts cultured
on Silk-RAD are catching up with Silk-RGD group in mineral deposition. In any case, further studies using primary human osteoblasts and Saos-2 osteoblast-like cells cultured for the same length of time under similar conditions are warranted to correctly explain this difference.

The ultimate therapeutic goal for tissue and bone loss due to periodontal disease, trauma or metabolic bone disease is regeneration of tissues to a normal or pre-disease state. Such regeneration requires differentiation of reparative cells and production of appropriate amounts of extracellular matrix components in a precise temporal and spatial manner to form specific types of soft and hard connective tissues. This goal can be achieved with the guide of a biocompatible matrix scaffold. A biomaterial scaffold with appropriate cell signaling molecules attached can initiate this cellular differentiation and ultimately the repair.

Millions of Americans are afflicted with periodontal disease. It has been estimated that at least 35% of the dentate U.S. adults aged 30 to 90 have periodontitis, with 21.8% having a mild form and 12.6% having a moderate or severe form (47). Periodontal disease causes destruction of supporting structures of teeth namely alveolar bone, periodontal ligament and cementum. Periodontitis can lead to loss of attachment, resorption of alveolar bone, destruction of connective tissue matrix and cells and may lead to loss of teeth. Regeneration of damaged periodontium especially alveolar bone can be achieved by the activity of local progenitor cells found in paravascular locations of the periodontal ligament (PDL) (48). However, a biomaterial scaffold is needed to provide a structure for the progenitor cells to regenerate the damaged tissue, alveolar bone in this case.
Bone regeneration techniques are also needed, particularly in the field of oral and maxillofacial surgery. Trauma, including surgical removal of odontogenic and non-odontogenic tumors in the oral and maxillofacial region causes bone damage and injury that interferes with normal function and is often disfiguring. Patients with congenital disorders may carry severe inherited bone and soft tissue defects. It is estimated that over 300,000 procedures are performed in the US each year to correct deformities specifically in the craniofacial region.

Silk has a long history of use as suture material and is FDA approved. It is biocompatible, less immunogenic and inflammatory than collagens or polyesters such as PLGA (1). Silk degrades and resorbs slowly and has minimal negative impact on surrounding tissues. Silk exhibit strength, flexibility and resistance to compression, properties that exceed all other known natural fibers and even rival some synthetic high performance fibers. Silk fibroin in the form of 3D matrices also exhibit mechanical properties that exceed commonly used polymeric biomaterials such as calcium phosphate, even prior to efforts at optimization (1). An ideal biomaterial should degrade slowly enough to maintain porosity for cell ingrowth and new tissue formation. Silk achieve these outcomes without chemical cross-linking, unlike collagens. Silk can be reprocessed into many different biomaterial formats, including nano-diameter fibers via electro-spinning, hydrogels and porous 3D matrices. An ideal biomaterial should be functionalizable with cell growth factors. Silk can be decorated with different biological factors to control cell attachment and signaling because of the nature of their surface chemistry.

Specifically, silk can be decorated with RGD to improve cell attachment,
proliferation and mineralization. Our data shows that decoration of silk with RGD enhances proliferation of and mineralization by primary human osteoblasts. If 3D silk scaffolds decorated with RGD are developed, these could have many dental and maxillofacial applications such as treatment of bone loss due to periodontal disease, jaw pathology, trauma and congenital defects. Before this goal could be achieved, work needs to be done to study the signaling pathways and gene expression mechanisms initiated by osteoblast integrin activation in response to peptide decorated silk substrates.
TABLES
Table 1

Comparison of mechanical properties of common silks (silkworm and spider dragline) to several types of biomaterial fibers and tissues commonly used today

Altman, G. et al., 2003 (1)
<table>
<thead>
<tr>
<th>Material</th>
<th>UTS (MPa)</th>
<th>Modulus (GPa)</th>
<th>% Strain at break</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. mori silk (w/ sericin)</td>
<td>500</td>
<td>5 - 12</td>
<td>19</td>
</tr>
<tr>
<td>B. mori silk (w/o sericin)</td>
<td>610 - 690</td>
<td>15 - 17</td>
<td>4 - 16</td>
</tr>
<tr>
<td>B. mori silk</td>
<td>740</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Spider silk</td>
<td>875 - 972</td>
<td>11 - 13</td>
<td>17 - 18</td>
</tr>
<tr>
<td>Collagen</td>
<td>0.9 - 7.4</td>
<td>0.0018 - 0.046</td>
<td>24 - 68</td>
</tr>
<tr>
<td>Collagen X-linked</td>
<td>47 - 72</td>
<td>0.4 - 0.8</td>
<td>12 - 16</td>
</tr>
<tr>
<td>PLA</td>
<td>28 - 50</td>
<td>1.2 - 3.0</td>
<td>2 - 6</td>
</tr>
<tr>
<td>Tendon (comprised of mainly collagen)</td>
<td>150</td>
<td>1.5</td>
<td>12</td>
</tr>
<tr>
<td>Bone</td>
<td>160</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>Kevlar (49 fiber)</td>
<td>3600</td>
<td>130</td>
<td>2.7</td>
</tr>
<tr>
<td>Synthetic Rubber</td>
<td>50</td>
<td>0.001</td>
<td>850</td>
</tr>
</tbody>
</table>

a. Bombyx mori silkworm silk - determined from haeve (multithread fibers naturally produced from the silk worm coated in sericin).
b. Bombyx mori silkworm silk - determined from single brins (individual fibroin filaments following extraction of sericin).
d. Nephila clavipes silk produced naturally and through controlled silking.
e. Rat-tail collagen Type I extruded fibers tested after stretching from 0% to 50%.
f. Rat-tail collagen dehydrothermally cross-linked and tested after stretching from 0% to 50%.
g. Polylactic acid with molecular weights ranging from 50,000 to 300,000.
Table 2
Ligand specificity of integrin receptors of human bone cells

Saito et al., 1949; (11)
<table>
<thead>
<tr>
<th>Integrin receptor</th>
<th>ECM protein</th>
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<tbody>
<tr>
<td>$\alpha_1\beta_1$</td>
<td>Laminin, Collagen</td>
</tr>
<tr>
<td>$\alpha_2\beta_1$</td>
<td>Laminin, Collagen</td>
</tr>
<tr>
<td>$\alpha_3\beta_1$</td>
<td>Fibronectin (RGD site)</td>
</tr>
<tr>
<td>$\alpha_\nu\beta_1$</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>$\alpha_\nu\beta_3$</td>
<td>Fibronectin, Vironectin, Fibrinogen, Thrombospondin, von Willebrand factor, Osteopontin, Bone Sialoprotein</td>
</tr>
</tbody>
</table>
Table 3
The sequence of plating human osteoblasts is shown for assaying proliferation at 72 hours of culture, for determining integrin expression at 24 hours and 7 days, and measuring mineralization at 5 weeks of culture. Each plate consisted of six wells and was plated at a density of 10,000 cells per cm². Surface area of each well was 9.6 cm². This experimental sequence was repeated three times.
<table>
<thead>
<tr>
<th></th>
<th>0 time</th>
<th>24 hours</th>
<th>72 hours</th>
<th>07 days</th>
<th>35 days</th>
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</thead>
<tbody>
<tr>
<td>Plastic</td>
<td></td>
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<td>Proliferation</td>
<td>1 plate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Integrins</td>
<td>2 plates</td>
<td>2 plates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mineralization</td>
<td>1 plate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Silk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proliferation</td>
<td>1 plate</td>
<td></td>
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<td></td>
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<tr>
<td>Integrins</td>
<td>2 plates</td>
<td>2 plates</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Mineralization</td>
<td>1 plate</td>
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<td></td>
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<td></td>
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<tr>
<td>Silk-RGD</td>
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</tr>
<tr>
<td>Proliferation</td>
<td>1 plate</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Integrins</td>
<td>2 plates</td>
<td>2 plates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mineralization</td>
<td>1 plate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Silk-RAD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proliferation</td>
<td>1 plate</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Integrins</td>
<td>2 plates</td>
<td>2 plates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mineralization</td>
<td>1 plate</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
FIGURES
Figure 1

Proliferation of human osteoblast. Plastic, Silk, Silk-RGD and Silk-RAD substrates at 72 hours of culture, determined by [3H]-thymidine incorporation (DPM = Disintegration Per Minute). No significant difference was found between Plastic and Silk-RAD groups (p-value=0.355) and Plastic and Silk groups (p-value=0.331). Significant differences were demonstrated between Plastic and Silk-RGD groups at a p-value of 0.006 and between Silk-RGD and Silk-RAD groups at p-value=0.002.
Figure 2

Mineralization (calcium deposition) by human osteoblasts at 5-weeks. A significant difference was found between Silk and Plastic substrates with a p-value of 0.04, between Silk-RGD and Plastic and between Silk-RAD and Plastic with p-values of <0.05. No statistically significant difference was seen for Silk-RGD compared to Silk-RAD with a p-value of 0.62. Error bars are standard errors of the means.
Figure 3

Western blots of integrin expression by human osteoblasts. At 0 time, 24 hours and 7 days.
Figure 4

Relative differences in integrin expression by human osteoblasts. Determined by densitometric scans of the Western blots in Figure 3 at 24 hours compared to 0 time.
Figure 5

Relative differences in integrin expression in human osteoblast cultures. At 7 days compared to 24 hours of culture determined by densitometric scans of the Western blot in Figure 3
BIBLIOGRAPHY


