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Preosteoblast Behavior on a Synthetic Calcium Phosphate Scaffold Coated with Cell Derived Matrix

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Abstract

Bone grafts are commonly used for the treatment of large bone defects. Biodegradable, synthetic bone grafts combined with cells and growth factors as a tissue engineered construct can provide benefits over the common autografts and allografts currently used. Calcium phosphate (CaP) materials offer high biocompatibility and chemical similarity to natural bone tissue. Biodegradable beta-tricalcium phosphate (β -TCP) scaffolds with an interconnected porous network have been created to promote tissue ingrowth and regeneration *in vitro* and *in vivo*. Extracellular matrix (ECM) coated on titanium and polymer scaffolds has been shown to improve cell response to those material surfaces. Here, we attempted to further improve the biocompatibility of the interconnected β -TCP ceramic scaffolds by generating a cell derived ECM on the ceramic surface *in vitro*. Cell derived ECM was generated on scaffolds for 1, 7, 14, 21, or 28 days of culture. Scaffolds were decellularized and sterilized to isolate the ECM coating on the β -TCP scaffolds. Osteogenic medium-soaked scaffolds treated for the same time periods as ECM coated scaffolds and autoclaved, untreated scaffolds were used as controls. The behavior of preosteoblasts seeded onto each scaffold was examined over a 28 day period. Cellular assays for cell number, alkaline phosphatase (ALP), and total protein (TP) were measured. Cell morphology changes on the scaffold were observed by scanning electron microscopy. The cell count on all scaffolds reached a plateau after 14 days. Specific ALP and specific TP slowly increased during the 28 days for all groups. New ECM was visible after 14 days of generation time on all groups. No significant difference was seen between the untreated scaffold control, the 1, 7, 14, 21, or 28 day ECM-coated scaffolds, or the 1, 7, 14, 21, or 28 day medium-soaked scaffolds (eleven groups). This study verifies the high biocompatibility of β -TCP scaffolds and suggests that a cell derived biological coating on the β -TCP scaffolds offers no significant changes to the attachment rates or differentiation rate of seeded cells. Future studies will seek to find other benefits of the ECM coating, like use as a bioactive agent delivery vehicle.

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**PREOSTEOBLAST BEHAVIOR ON A SYNTHETIC CALCIUM PHOSPHATE
SCAFFOLD COATED WITH CELL DERIVED MATRIX**

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Presented for
The Graduate Studies Council
The University of Tennessee
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In Partial Fulfillment
Of the Requirements for the Degree
Master of Science
In the Joint Graduate Program in Biomedical Engineering and Imaging
From The University of Tennessee
and
The University of Memphis

By
Daniel A. Young
May 2010

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DEDICATION

Dedicated to my family for their love and support. Mom, Dad, Michael, and Annabelle, thank you for making me feel at home, even when I'm hundreds of miles away.

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I would like to thank my advisor, Dr. Yunzhi Peter Yang, for supporting me throughout my graduate career. His academic support has been invaluable to my development as a researcher. My committee members, Dr. Joel Bumgardner and Dr. Satoru Ken Nishimoto, also have my thanks for their long term and long distance assistance in the details and overall objectives of this thesis. Special thanks to Dr. Denis Diangelo for taking up the position of my official research advisor when Dr. Yang became a member of the University of Texas Health Science Center.

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ABSTRACT

Bone grafts are commonly used for the treatment of large bone defects. Biodegradable, synthetic bone grafts combined with cells and growth factors as a tissue engineered construct can provide benefits over the common autografts and allografts currently used. Calcium phosphate (CaP) materials offer high biocompatibility and chemical similarity to natural bone tissue. Biodegradable beta-tricalcium phosphate (β -TCP) scaffolds with an interconnected porous network have been created to promote tissue ingrowth and regeneration *in vitro* and *in vivo*. Extracellular matrix (ECM) coated on titanium and polymer scaffolds has been shown to improve cell response to those material surfaces. Here, we attempted to further improve the biocompatibility of the interconnected β -TCP ceramic scaffolds by generating a cell derived ECM on the ceramic surface *in vitro*. Cell derived ECM was generated on scaffolds for 1, 7, 14, 21, or 28 days of culture. Scaffolds were decellularized and sterilized to isolate the ECM coating on the β -TCP scaffolds. Osteogenic medium-soaked scaffolds treated for the same time periods as ECM coated scaffolds and autoclaved, untreated scaffolds were used as controls. The behavior of preosteoblasts seeded onto each scaffold was examined over a 28 day period. Cellular assays for cell number, alkaline phosphatase (ALP), and total protein (TP) were measured. Cell morphology changes on the scaffold were observed by scanning electron microscopy. The cell count on all scaffolds reached a plateau after 14 days. Specific ALP and specific TP slowly increased during the 28 days for all groups. New ECM was visible after 14 days of generation time on all groups. No significant difference was seen between the untreated scaffold control, the 1, 7, 14, 21, or 28 day ECM-coated scaffolds, or the 1, 7, 14, 21, or 28 day medium-soaked scaffolds (eleven groups). This study verifies the high biocompatibility of β -TCP scaffolds and suggests that a cell derived biological coating on the β -TCP scaffolds offers no significant changes to the attachment rates or differentiation rate of seeded cells. Future studies will seek to find other benefits of the ECM coating, like use as a bioactive agent delivery vehicle.

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CHAPTER 1. INTRODUCTION

Bone graft surgeries are performed to enhance the healing of fractures and defects caused by trauma or disease. An estimated 7.9 million fractures occur in the United States annually, with approximately 1.5 million bone graft operations performed to treat those injuries.¹ Most large bone defects will not heal without the aid of bone grafts. Commonly an autograft treatment is performed to fill a bone void, using tissue from the patient's own body. Unfortunately the tissue harvest requires a second surgery, and only a limited amount of bone may be collected from the patient. If there is a need for large volumes of bone tissue, an allograft from a donor, usually a cadaver, may provide it. However, this donated tissue could potentially elicit an immune response or deliver diseased bone.² These complications show that despite the widespread use of these operations, neither is ideal. Tissue engineering seeks to develop a synthetic alternative at least as effective in bony healing. Such a material would not be limited by supply and be completely biocompatible, degrade safely after implantation and healing, and provide a stable scaffolding on which bone tissue could thrive.

The design of synthetic materials is inspired by the tissue it seeks to replace. Polymers, metals, and ceramics can each be manipulated to mimic some aspect of bone. The strength of bone, however, stems from its composite nature. The hard tissue can be approximated by a ceramic material, while the extracellular matrix (ECM) that permeates it acts like a reinforcing polymer. The two materials give bone its high mechanical strength and resistance to shear forces. At the porous center of long bones, the marrow produces stem cells for use by the entire body. Mimicking the composite and porous nature of bone has become a great challenge for tissue engineers, and one we hope to overcome with our unique ceramic scaffolds.

Our lab has focused on fabricating calcium phosphate (CaP) ceramics for treatment of bone void defects. Using beta-tricalcium phosphate (β -TCP) synthesized into a porous, biomimetic disc provides a biodegradable scaffold that can be integrated with developing bone tissue. In an attempt to mimic the composite nature of bone, a cell-deposited ECM will be laid on the scaffolds prior to use. Using the ECM as a way to enhance material interaction with developing cells has been performed *in vitro* by Datta et al. who saw increased cell number and calcium deposition when titanium mesh constructs were coated with ECM.³ The use of various polymers as a means of bioactive agent delivery has also been explored.⁴ However, using a natural polymer like ECM with a synthetic CaP scaffold to create a composite material has not been well characterized. There is a need to determine the amount of time required for a meaningful amount of ECM to be deposited on a ceramic surface and determine the effects such a coating has on cell behavior.

This project sought to determine what behavioral changes occurred for preosteoblast cells cultured on ECM-coated CaP scaffolds. An optimal cell culture time for ECM deposition on our unique porous CaP scaffolds was also investigated. Background information on the tissue engineering concepts necessary for understanding the project will be explained. Then the characteristics of the calcium phosphate scaffolds

will be examined. The characterization of the ECM deposition process will be explored, followed by characterization of cell behavior in response to ECM coated scaffolds. Finally, the significance of the results and future directions will be discussed.

CHAPTER 2. BACKGROUND

Bone Tissue

Bone is a complex and robust tissue that provides a source of primary cells and structural support for the entire body. It is a composite material with a rigid, mineralized portion integrated with an elastic phase of extracellular matrix throughout. The hard “bony” areas provide structure, anchors for articulation of muscles, and physical protection of internal organs. The elastic portion gives long bones torsional resistance and shock absorbing qualities. Within the bones, the marrow produces stem cells for development of blood and lymphocyte cells. Bones are highly vascularized, allowing the stem cells to easily distribute throughout the body via the circulatory system.⁵ The variety of functions bone tissue can perform ranges widely, but all the roles are important.

Characterization of the mechanical properties of bone is not straightforward due to their various and often irregular shapes. For most purposes discussed in this paper, long bones will be examined. The long bones in a human have very high compressive strength for their weight, though this is only along their longitudinal axis. If long bones are tested laterally, they do not exhibit such high compressive strength. This unevenness is due to the unique arrangement of osteocytes along the axis of load. Cellular feedback due to mechanical stress causes osteocytes to align themselves and strengthen in a particular direction. This phenomenon is readily demonstrated by astronauts who lose bone mass in low gravity environments, where very little mechanical stress is applied to the osteocytes.⁶

The compressive forces mentioned previously have been estimated to be between 137 MPa to 206 MPa in human long bones.⁷ The forces are approaching that of aluminum or mild steel, as pointed out by Barrère et al., though bone is much lighter than either of these metals. The Young's modulus of bone, a measure of the elasticity of a material, is estimated to be 41 MPa to 69 MPa, much less than the metals previously mentioned.⁷ Such high elasticity allows bone to function as a shock absorber when impacted.

Possessing high strength and shock absorbing qualities makes bone a much more interesting and multifunctional material than metals. The efficiency of bone can be attributed to its composite nature. The inorganic, ceramic-like material infused with an elastic polymer of non-mineralized extracellular matrix allows such a lightweight and robust tissue to exist.

Bone regeneration

In response to trauma or disease, a bone void will undergo a cascade of events to protect and then heal the tissue. First a protective layer of fibrous tissue forms around the injury site. Fibrous tissue grows more quickly than bone tissue can, and it fills the injury space quickly, within days.⁸ The temporary tissue that covers the wound protects the area from infection. The fibrous tissue also provides some stability while the surrounding

bone tissue gradually infiltrates and replaces the damaged tissue.

The surrounding bone tissue of a wound site continues to produce maturing osteoblasts and osteoclasts, two opposing cells that constantly remodel the bone. While osteoblasts produce mineralized bone and later convert to stable osteocytes, the osteoclasts dissolve mineralized tissue.⁹ Osteoclasts are derived from monocytic macrophages, and thus act similarly to phagocytic B-cells. The balance of both cells' function keeps bone strong and capable of adjusting to many incidents of trauma.

Numerous proteins and signaling factors are also secreted locally to direct bone healing. In normal bone growth, proteins like alkaline phosphatase, osteocalcin, and osteopontin are secreted and used by maturing osteoblasts.¹⁰ Growth factors like bone morphogenetic protein 2 (BMP-2), which plays a role in osteoblast differentiation, can also be found. These same proteins are present in the wound healing environment, and there are numerous studies that exploit their growth functions.⁴ Being able to control the delivery of several key bioactive factors could greatly enhance bone regeneration. Due to the relatively short half-life of these local factors, methods of drug delivery become very important.

Bone tissue is constantly undergoing remodeling, and even in cases of fracture the same basic process continues. Knowledge of the local signaling and environmental factors that affect osteoblast development is key to enhancing the regeneration process.

Critical sized defects

There are some bone voids that cannot heal due to the large distance between areas of intact bone tissue. If the cells cannot bridge the gap to heal naturally over any period of time, it is referred to as a critical sized defect.

A common example of a critical sized defect in humans can be seen in children with cleft palates. The missing bone will not heal on its own without surgical intervention, so a bone graft surgery is necessary to restore the normal palate structure. In research, the rat cranial defects model is commonly used to examine bone healing efficacy of a material. Specifically for rat craniums, bone voids larger than 8 mm in diameter are considered critically sized. The top of the skull is the only non-load bearing area of bone, making it a good model for early in vivo studies of a new biomaterial.

Indicators of development and mineralization

Developing osteoblasts produce a number of detectable signaling proteins that can be used to determine their maturity. But before this can be measured, certain criteria must be met. For example, osteoprogenitor cells will only differentiate into mature, secretory osteoblasts if they have reached confluence, or growth arrest, and have an established extracellular matrix (ECM).¹¹ The day the proliferative phase has passed is an important milestone used as a reference point for further cell behavior.

A common early stage marker of osteoblast activity is alkaline phosphatase (ALP). After the proliferation period of preosteoblasts has finished (i.e. the cells have reached confluence), a measurable increase in ALP production occurs. The ALP levels rise for approximately 10 days following the post-proliferative phase, and the time it peaks is an indication of cell differentiation into the osteoblast phenotype.¹² Alkaline phosphatase is an intracellular protein, and while not unique to bone tissue, it is nonetheless convenient to test *in vitro* due to the ease of performing its calorimetric assay with cell lysate.

Later stages of maturity can be indicated by proteins like osteocalcin (OC) or osteopontin (OP), both of which are expressed post-proliferatively and likely linked to osteoclast resorption.¹²

The final stages of osteoblast maturation depend on reorganization of the extracellular matrix, which occurs between 12 and 18 days after proliferation has ceased. The remodeling of the ECM fibers also involves upregulation of genes necessary for matrix mineralization. When the ECM is rich with growth factors from the transforming growth factor beta (TGF- β) superfamily and other proteins, bone mineralization commences. At approximately 16 to 20 days post-proliferation, the ECM mineralizes and mature bone is formed.¹²

Tissue Engineering Concepts

Tissue engineering requires many biological concepts to be understood when designing a material. Several concepts that are important to the success of a composite ceramic and polymer biomaterial are described below.

Biocompatible

A material is considered biocompatible if a tissue's cells are capable of attaching, proliferating, and differentiating on its surface. There is an important distinction between this integration of tissue and material compared to inert materials. Even if a surface elicits no inflammation or wound response, it may not be considered biocompatible. If a material becomes encapsulated in fibrous tissue, this may indicate an inert reaction but still an undesirable foreign body response. A biocompatible material has cellular attachment directly to its surface without any fibrous tissue encapsulation.

Biodegradable

If a material is biodegradable, it can safely be left *in vivo* until it has completely dissolved. While this term does not suggest any particular time frame when a material must break down, it is generally assumed that a biodegradable material will show significant signs of degradation coincident with the time it takes the tissue to heal. In bone repair, this is particularly important in order to reduce the total number of surgeries. Ideally, one operation could be performed to implant the biomaterial. Then, the tissue

could heal through the degrading material, leaving only naturally developed tissue in its place.

Osteoinductive versus osteoconductive

An important part of the bone healing process is rebuilding bony tissue in areas where it no longer exists. In most cases this void is surrounded by intact bone. If a biocompatible material implanted in this void will support bone tissue growth through itself, it is called osteoconductive. If the same material is implanted at a site not in contact with bone, for example subcutaneously, it may not promote bone tissue formation. A material that does cause bone tissue growth in a location where none would have naturally occurred is called an osteoinductive biomaterial.

The tissue harvested for autografts and allografts is osteoinductive.¹³ Most synthetic materials are only osteoconductive. While the goal of synthetic biomaterials is intended to repair bone in areas already surrounded by existing tissue, osteoinductive materials do provide a stronger healing response after implantation. This response likely results from the presence of some growth factors, like bone morphogenetic protein 2 (BMP-2), that are absent on osteoconductive materials.¹⁴ Most synthetic calcium phosphate materials are considered osteoconductive.¹⁵⁻¹⁷

Calcium Phosphate Ceramics

The use of ceramics in bone tissue engineering dates back 40 years when Hulbert et al. suggested their use as permanent implants.¹⁸ The inorganic phase of bone mineral can be described as an impure calcium phosphate apatitic structure laced with carbonate groups and other trace elements.¹⁹ Synthetic substitutes of inorganic bone are derived from calcium orthophosphate salts, which constitute a wide variety of inorganic compositions and physicochemical properties.⁷ These calcium phosphate (CaP) based ceramics are used as coatings on metal prostheses, cements in non-load bearing applications, and in sintered forms as porous structures.²⁰⁻²² Currently, the most commonly used bone substitute materials are hydroxyapatite (HA) and beta-tricalcium phosphate (β -TCP), though these stoichiometrically pure forms lack the trace elements and non-apatitic functional groups that distinguish natural bone.^{19,23} Nevertheless, more than half the weight of bone is composed of an HA phase, and this similarity contributes to the strong biocompatibility these synthetic materials exhibit in developing tissue.²⁴

Though both HA and β -TCP are biodegradable, β -TCP breaks down to its constituent calcium and phosphate ions more readily in circulating body fluid than HA.²⁵ In choosing an appropriate ceramic composition for making our porous scaffolds, the solubility of β -TCP most closely matched our design goals. The dissolution of the calcium phosphate *in vitro* is dictated by the solution (pH, local calcium or phosphate concentration, temperature) and the material properties (crystal organization, surface area, density).⁷ When β -TCP is implanted *in vivo*, the ceramic degradation can be affected by other factors like serum proteins, enzymes, and osteoclast activity. The structure of β -TCP is similar enough to bone tissue that osteoclasts can also resorb the synthetic

material. Osteoclasts resorb bone tissue and β -TCP by secreting hydrochloric acid to lower the local pH, thus causing the calcium phosphate to dissolve.²⁶

Ideally, the synthetic material is solubilized and resorbed by osteoclasts at a rate equivalent to the mineralization of new bone. If this occurs, new tissue formation is not impeded by the presence of the scaffold, and the ionic components released from the CaP structure may be utilized by the developing tissue.²⁷ Successfully orchestrating the dissolution of a biomaterial and the growth of natural tissue in its place will be difficult, but the use of biocompatible and biodegradable components makes it a possibility.

Pore size

When a tissue graft is first exposed to cells, the integration between host tissue and foreign material surface largely determines the success of the implant. Despite the high biocompatibility of CaP materials, merely providing a biomimetic surface is not enough for full bone tissue integration. The architecture of the implant is critical to the long-term viability of a bone graft. In creating a synthetic analogue of bone, this means mimicking the porosity of cancellous bone.

The composite nature of bone can describe aspects besides its material composition. Spatially, the inorganic phase has two distinct areas, a dense cortical exterior layer for structural support and protection, and the interior porous cancellous matrix that houses the marrow and extensive vascularization. Many studies have explored the best architecture for bone ingrowth, but an interconnected porous network seems necessary for full tissue restoration.²⁸⁻³¹ Pores allow not only osteoblast cell migration but also endothelial cell penetration, so that vascularization can occur. Blood vessel supply is vital to any developing cells, so the pores must permit growth of vessels and the developing bone tissue.

Though an ideal pore size is difficult to identify, several groups have found approximate benchmark sizes that permit bone tissue growth. For interconnected networks, the limiting factor is the size of the interconnections and not the pores themselves. Bone graft materials with pore interconnection diameters smaller than 100 μm only support fibrous tissue ingrowth.¹⁸ Interconnections larger than 100 μm allow for osteoblast mineralization to occur, but the interconnections must be at least 300 μm for vascularization to complement the osteocyte development. So, for osteoid development in humans, an acceptable pore interconnection diameter for cell growth and vascularization is at least 300 μm .³² The CaP ceramics used in this study have a pore size of approximately 500 to 750 μm and pore interconnection size of at least 350 μm . Our scaffold fabrication method allows the pore size and pore interconnection size to be controlled, and our recent experiments have settled on this architecture to provide a balance of mechanical strength and cell viability.

Uses in tissue engineering

Ceramics are currently in use clinically for treating large bone defects. Bone cements and ceramic implants have been used successfully in dental and orthopedic applications for patients.^{33,2} The use of HA as a coating for other materials and implants speaks to the biocompatibility and resilience of these ceramics.³⁴ Most recently, porous and functionally graded ceramic structures are being examined as biomimetic replacements for auto and allografts.³⁵

Extracellular Matrix

The extracellular matrix (ECM) is a structural network secreted and maintained by fibroblast-like cells. It is a complex amalgamation of fibers and bioactive proteins, and its role changes drastically depending on the source of its development and its location.³⁶ In bone, the ECM is responsible for maintaining a stable network for osteoblast precursor cells to attach, proliferate, and differentiate on, then the ECM is modified to prepare for mineralization.¹¹ Calcium, phosphate, carbonate, and other minerals are deposited on the ECM by mature osteoblasts to create the apatite-like phase of bone. It is characterized by a polymer-like behavior due to the large weight percentage of collagen fibers that give it structure.³⁶

Proteins of bone extracellular matrix

In all instances of bone ECM, collagen type I is the most abundant protein. Collagen fibers provide a network for cells to attach and use for migration, and they give ECM its polymer-like properties. Collagen type I is a structural protein that polymerizes into a triple helix of collagen bands, held tightly to one another by hydrogen bonds.³⁷ Their defining glycine-X-Y amino acid sequence (where X and Y are other amino acids, usually proline or hydroxyproline) also makes the fibers easy to identify and forms a rigid, rod-like structure. The repeating sequence also allows the fibers to form into a triple helix that provides strength to the collagen type I structure. Beyond just a structural network, the ECM maintains the activity of other bioactive proteins and serves as a means of communication for attached cells.³⁸

Cells bind to collagen fibrils or other ECM proteins via transmembrane proteins, the integrins. The actin filaments inside of cells indirectly attach to the collagen network through these integrin anchors. It is the integrins which simultaneously provide a method of motility for the cells on the ECM and a direct line of communication between cells and the ECM.^{39,40}

Proteins like laminin and fibronectin, which function as adhesion molecules, can be found in the all types of ECM.^{41,42} Other proteins like bone sialoprotein, involved in ECM mineralization, and osteocalcin, involved in bone turnover, are preferentially expressed by developing bone-derived ECM.^{43,44} All of these proteins can be immobilized to the ECM structure, preserving their activity until released or until a cell contacts them. This allows the proteins to remain active for much longer than if they were

circulating in fluid, and also allows for local stimulation as the proteins are released.

Uses in tissue engineering

A natural polymer, ECM has been used in several tissue engineering applications for its biocompatibility and ability to retain proteins. The ECM can be utilized to provide a reliable coating on which cells can readily adhere. For example, Wang et al. have used a synthetic human collagen to successfully bind proteins to a polymer surface, a surface that usually has little affinity for protein attachment.⁴⁵ Another instance of ECM improving upon cell-surface interaction is demonstrated by Datta et al. who used cell-deposited ECM to enhance osteoblastic differentiation on titanium fibers.³ Cells have also been observed to produce ECM and a number of osteogenic factors on polymer surfaces of polylactic-*co*-glycolic acid (PLGA) and polylactic acid (PLA).⁴⁶

A number of groups have studied the use of matrices like ECM as a delivery vehicle for bioactive agent delivery. The collagen fibrils of ECM naturally bind proteins like growth factors. In fact, bone morphogenetic protein-2 was discovered by being immobilized on collagen and consequently upregulating the differentiation of local osteoblasts.⁴⁷ One unique property of ECM that could assist this approach is its well-conserved structure across species, resulting in minimal rejection of even xenogeneic ECM implants.⁴⁸⁻⁵¹ This property makes it possible to generate ECM from any number of sources to function as a bioactive agent delivery vehicle.

Composite Materials: A Biologically Coated Synthetic Scaffold

Bone derives most of its impressive mechanical strength and resilience from two very different components. The hard tissue of bone is reinforced by the fibrous, biological polymer of ECM, allowing a light and porous material to have high compressive and shear strength. Combining two materials for a set of properties that is more meaningful than either one separately was the inspiration for developing an ECM coated ceramic scaffold.

A pre-laid ECM could assist cells in subsequent attachment and differentiation of osteoblast cells. Ideally, the ECM would be laid out, reorganized, and saturated with osteogenic growth factors for the second set of cells seeded on such a coated surface. The cell behavior enhancements that an ECM coating can provide has already been demonstrated on metal and polymer surfaces.^{3,46} Similar improvements could be seen for ceramic surfaces, but this combination has not been explored yet.

The time to develop such a biological matrix on a surface has also not been optimized. Previous studies that generated a cell derived matrix coating on a material used only one development time, usually about two weeks. Would a longer or shorter development time affect the ECM and subsequent cell behavior in any measurable way? This sort of characterization has yet to be examined.

Some preliminary experiments in our lab showed a significant increase in cell

number and early stage osteoblast activity biomarkers due to the presence of an ECM coating on ceramic scaffold surfaces. The matrix was allowed to develop for about two weeks, similar to other group's attempts. Based on previous research and the early results collected from our own experiments, we have decided to characterize a group of ECM coated ceramic scaffolds using biological assays and microscopic imaging.

Medium-soaked Scaffolds

To compare the effects that an ECM coated scaffold has on cell behavior, a positive control of scaffolds soaked in osteogenic medium will be used. Calcium phosphate materials are known to dissolve and then reprecipitate in simulated body fluid, causing fluctuations in the local calcium concentration.⁵² The surface morphology is also affected by this process, lowering surface roughness. These changes could potentially affect cell behavior independent of any biological matrix coating. For this reason, a positive control group of scaffolds soaked in the same osteogenic medium used to generate the ECM will be examined. Any cell behavior changes due to scaffold exposure to a medium containing osteogenic factors and fetal bovine serum may then be identified.

Osteoblast Precursor Cell Line

The cells used in this experiment are W-20-17 fibroblast cells, which are osteoblast precursor cells derived from mouse stromal cells. The cell line is widely used in the characterization of osteoblast response to bone morphogenetic protein 2 (BMP-2) and in modeling human osteoblast cell behavior. Our lab has primarily used this cell line in all previous experiments, so it is well characterized from first hand use.

These preosteoblasts have the potential to differentiate into osteoblasts, given certain biological signals. Confluence, or growth arrest, and the presence of a mature extracellular matrix are the minimum requirements for these cells to undergo differentiation into osteoblast cells.¹¹

Osteoblasts are derived from pluripotent mesenchymal stem cells, which can differentiate into a variety of potential lineages.⁷ The preosteoblasts used here are a particular lineage of these stem cells on the path towards the osteoblast phenotype. Four categories of osteoblast function and phenotype can be described. Active osteoblasts are cuboidal, mononuclear cells that actively produce proteins like ALP, osteocalcin, and osteopontin, as well as growth factors. These are secreted from the active osteoblast into the osteoid, or unmineralized matrix, to promote mineral deposition of calcium and phosphate on the mature ECM. When osteoblasts have become surrounded by mineralized tissue, their function changes from generation to maintenance, and they are called osteocytes. The third categorization is bone-lining cells, which are neither forming nor resorbing bone and can be found along the surface of bone. Lastly there are inactive osteoblasts which are morphologically the same as bone-lining cells.⁷

Summary

Tissue engineering faces a difficult task in bone repair. In creating a material that can compare with the complicated functions of bone, we looked first to identify important features of the natural tissue. Its composite nature seemed to be the most unique and influential aspect, so we used this concept to design a more robust material. A ceramic scaffold combined with a biologically derived polymer coating provides the benefits of a biomimetic synthetic ceramic and a complex natural polymer, in addition to being an unexplored combination of materials.

In designing the following experiments, several unexplored factors were identified. Full characterization of ECM development has not been done. The time it would take to develop a stable three dimensional ECM structure could be important, but only one generation period has been explored at a time in other studies. Calcium phosphate is already a biocompatible surface that is composed of ionic components useful for osteoblast mineralization. Introducing a positive control group that has only been exposed to osteogenic medium will provide insight on the interaction of the scaffold surface and physical protein adsorption.

Examining ECM and its effect on osteoblast behavior on a porous and interconnected calcium phosphate scaffold can shed light on some interesting challenges for tissue engineering. The concepts explored here were inspired by natural tissue, and similar approaches have seen success in improving the cell-surface interaction as measured by cell attachment and extent of differentiation. We hope that similar results will be found with this combination of a calcium phosphate scaffold with a unique architecture and cell derived coating of extracellular matrix.

CHAPTER 3. OBJECTIVES AND HYPOTHESES

Objectives

This project sought to determine the effects a cell deposited ECM coating on CaP scaffolds would have on preosteoblast proliferation and differentiation. Because this type of study has not been well characterized, the appearance and generation of the ECM will first be identified. To determine the extent of mineralization, cell behavior assays, and immunofluorescent staining, and electron microscopy was performed. The groups prepared for this study are summarized in **Table 3.1**. Sterile scaffolds were seeded with cells and fed osteogenic medium, gathering samples after 1, 7, 14, 21, and 28 days of culture (groups E1 through E28). Osteogenic medium was fed to the preosteoblasts generating ECM, so a positive control group of scaffolds exposed to only osteogenic medium was made, as well. Sterile scaffolds were given osteogenic medium and changed at the same time as their cell-seeded counterparts, gathering samples after 1, 7, 14, 21, and 28 days of medium-soaking (groups M1 through M28).

Next, the prepared scaffolds were all seeded with preosteoblast cells to determine what effects the scaffold treatment had on said cells. The groups used for this experiment are shown in **Table 3.2**. A control group of autoclaved scaffolds was used for comparison. Also, a group of cells seeded directly onto tissue culture plates in the absence of scaffolds was examined. The five groups of ECM coated scaffolds prepared as previously described were decellularized and then gas sterilized to isolate the ECM on their surface. The five groups of medium-soaked scaffolds were sterilized prior to cell seeding. Scanning electron microscopy and cell behavior assays were used to evaluate the cell-surface interactions. Samples were collected and measured at 1, 7, 14, 21, and 28 days.

The hypotheses of each section are stated below.

Table 3.1. Prepared scaffold groups. Scaffolds prepared in Stage I for further study.

Group abbreviation	Cells seeded	Scaffold	Days incubated
E1	+	+	1
E7	+	+	7
E14	+	+	14
E21	+	+	21
E28	+	+	28
M1	—	+	1
M7	—	+	7
M14	—	+	14
M21	—	+	21
M28	—	+	28

Table 3.2. Scaffold groups. Summary of scaffold groups and their treatments for use in Stage II.

Group abbreviation	Scaffold	Treatment	Purpose
A	+	Autoclave sterilized	Control
C	–	None (cells only)	Negative control
E1	+	Decellularized, gas sterilized	Experimental group
E7	+	Decellularized, gas sterilized	Experimental group
E14	+	Decellularized, gas sterilized	Experimental group
E21	+	Decellularized, gas sterilized	Experimental group
E28	+	Decellularized, gas sterilized	Experimental group
M1	+	Gas sterilized	Positive control
M7	+	Gas sterilized	Positive control
M14	+	Gas sterilized	Positive control
M21	+	Gas sterilized	Positive control
M28	+	Gas sterilized	Positive control

Notes: All groups were seeded with cells. All scaffold groups except ‘A’ were treated after Stage I preparation.

Hypotheses

Stage I (Chapter 5)

1. We hypothesized that the preosteoblast cells would develop more mass of extracellular matrix the longer they were cultured on CaP scaffolds.
2. We hypothesized that the scaffolds soaked in cell culture medium in the absence of cells would have their surface properties changed by dissolution and reprecipitation, as well as protein adsorption from the medium.

Both of these were tested qualitatively with imaging techniques.

Stage II (Chapter 6)

3. We hypothesized that the extracellular matrix generated from Stage I would enhance preosteoblast cell attachment and differentiation to the CaP scaffold surface compared to the sterile CaP control scaffolds.
4. We hypothesized that the medium-soaked CaP scaffolds would enhance preosteoblast cell attachment and differentiation to the CaP scaffold surface compared to the sterile CaP control group.

Cell behavior assays for cell number and cell activity were used to test these hypotheses. Imaging techniques were also employed to verify cell-surface interactions.

CHAPTER 4. BETA-TRICALCIUM PHOSPHATE SCAFFOLD CHARACTERIZATION

In bone tissue engineering, the use of calcium phosphate ceramics as a synthetic bone graft is as strong choice due to its similarity, and consequently biocompatibility, with bone. A beta-tricalcium phosphate (β -TCP) scaffold with a porous, interconnected network has been developed by our lab. It has solid struts, and the pore size can be adjusted. Scanning electron microscopy confirms the pore size to range between 500 and 750 μm . X-ray diffraction analysis confirms that the crystalline phase is primarily β -TCP with a minor hydroxyapatite (HA) phase. The template casting method used here provided scaffolds with a weight of 0.178 ± 0.015 g, diameter of 7.70 ± 0.23 mm, and height of 6.28 ± 0.36 mm.

Introduction

Biomaterials are being used to replace or enhance many different types of tissue. In bone tissue engineering, ceramics are being used to replace hard tissues, in coatings for metals, or as part of composite materials with polymers. Of the many types of ceramics available, the most commonly used for bone tissue repair are hydroxyapatite (HA) and beta-tricalcium phosphate (β -TCP). Natural bone has a crystal structure that resembles apatite, which is thought to contribute to the high biocompatibility of HA and β -TCP.²⁴ Though HA is mechanically strong and biocompatible, it has a slow degradation rate compared to β -TCP.²⁵ In designing a porous, biodegradable scaffold, our lab has chosen to focus on the development and use of β -TCP ceramics.

The architecture of porous scaffolds becomes very important when considering cell ingrowth and vascularization. Having a complete path through the bone graft allows bone tissue penetration and vessel recovery. Vascularization is necessary to provide sufficient nutrition to the regenerating tissue.⁵³ A completely interconnected porous network can provide this pathway for cell development on β -TCP scaffolds.

We are able to fabricate porous ceramic discs with solid struts, creating interconnected pores throughout the scaffold architecture. The pore interconnection size can be controlled by the use of a template casting method using sacrificial molds. The beta-tricalcium phosphate is converted to the desired crystal structure by high temperature sintering. This section will examine the crystal phase and pore structure of these fabricated scaffolds.

Materials and Methods

Scaffold fabrication

Porous beta-tricalcium phosphate (β -TCP) scaffolds were made as described previously.³⁵ Briefly, custom templates of paraffin wax were cast into plastic molds. A β -

TCP slurry was cast into the molds by exposure to low vacuum. Excess slurry was removed and the cast molds were dehydrated in ethanol at an elevated temperature of 35 to 55 °C for 48 hours. The temperature was increased to melt the wax and the scaffold green bodies demolded. The scaffolds were put through a graded dehydration process of increasing concentrations of ethanol over 8 hours and then allowed to dry for 2 hours. The green bodies were sintered at 1250 °C for 3 hours in a high temperature muffle furnace (Barnstead Thermolyne 46100). Scaffolds were cleaned in distilled (DI) water by ultrasonication and then autoclave sterilized prior to use.

Morphology by scanning electron microscopy

Scaffolds were sputter coated with gold at a thickness of 16.9 nm. Coated samples were imaged in a high vacuum environment at 30 kV beam energy in an FEI Quant 400 scanning electron microscope (SEM).

Crystal structure by X-ray diffraction

Sintered scaffolds were crushed into powder using a mortar and pestle. The powder was examined in a Siemens D5000 X-ray Diffractometer. Incident angles from 30 to 60 ° were recorded at a rate of 4.8 °/min.

Surface area analysis by gas adsorption

The sintered scaffold surface area was analyzed using a Micromeritics 2365 gas absorption machine. Nitrogen gas was used to infiltrate and fill the void spaces. The pressure difference between the scaffold tube and an empty control tube was used to calculate the surface area of scaffolds by BET analysis.

Results

Images gathered from the SEM machine are shown in **Fig. 4.1**. Pore interconnections ranging from 0.125 mm to 0.4 mm can be seen in the first picture, **Fig. 4.1(a)**. Arrows indicate pores through which another pore or pores can be seen, confirming the interconnected architecture of the scaffolds. A closer view of a pore surface in **Fig. 4.1(b)** shows the crystal structure of the sintered calcium phosphate ceramic. The continuous but tiled surface indicates crystallization of the beta-tricalcium phosphate material.

The X-ray diffraction (XRD) pattern in **Fig. 4.2** confirms the presence of β -TCP phase. Peaks at 31 ° and 34 ° in the β -TCP standard can be seen in the scaffold sample, as well as minor peaks at 47 ° and 52°. A standard for hydroxyapatite is also shown on the graph, and the scaffold sample exhibits minor HA peaks at 28° and 32.5 °.

The BET surface area analysis showed the scaffolds to have a $0.159 \pm 0.017 \text{ m}^2/\text{g}$

Figure 4.1. SEM images of CaP scaffolds. Electron microscopy images of a sterile scaffold at (a) 50x and (b) 2,500x magnification. Arrows in (a) indicate interconnected pores.

Figure 4.2. XRD pattern. Collected diffraction pattern from a crushed β -TCP scaffold with standards for β -TCP and HA. The scaffold sample shares peaks with both standards, indicating the presence of both phases.

surface area. A material density of 3.140 g/cm^3 , the density of pure β -TCP, was used for calculating the surface area.

Discussion

The interconnected network in the scaffolds provides structural support for the β -TCP scaffolds as well as a necessary pathway for cell proliferation. Other researchers have used pore interconnection diameters ranging from 100 to 500 μm , and the scaffolds examined here are of similar size. Studies show that a minimum pore interconnection size of 100 μm is needed for preosteoblast penetration, but pore interconnections must be larger than 300 μm to allow for full vascularization.³² The SEM images suggest that preosteoblasts seeded on the scaffold surface will be able to develop and attach to the porous CaP scaffold without being limited by pore interconnection size.

A stoichiometric β -TCP powder was used to create the scaffolds. After sintering, the material was expected to be crystallized into a β -TCP form. Comparison to standards of β -TCP and HA show that the scaffold material is primarily β -TCP with a minor phase of HA.³⁵

The gas adsorption method is very sensitive to surface roughness, since the nitrogen molecules can fill spaces much smaller than are usable by cells. The BET method of analysis is used to find the specific surface area, which accounts for very small variations in the surface. Thus, the surface area measured is actually much higher than is physically usable. The method used here measures a single layer of gas on the surface of the ceramic. Using this method of analysis to calculate the surface area of a typical CaP scaffold weighing 0.2 g gives a surface area of 318 cm^2 . For comparison, each well of a 24 well plate has a surface area of 2 cm^2 , and these 24 well plates are used for all cell culture work with the CaP scaffolds. Again, it is likely that the calculated surface area is much higher than what is actually usable by cells.

Conclusion

The porous scaffolds created here are appropriate for use in tissue engineering applications. Our own previous studies have shown the biocompatibility of the scaffolds using W-20-17 mouse preosteoblast cells. The performance of the porous β -TCP scaffolds are examined *in vitro* in the following chapters.

CHAPTER 5. GENERATING EXTRACELULAR MATRIX ON BETA-TRICALCIUM PHOSPHATE SCAFFOLDS

In bone tissue engineering, synthetic materials may be used in place of autograft or allograft tissue. This would provide a similar scaffold function without the need for a second surgery to harvest tissue as in autografts, or remove the risk of disease transmission that is present in allograft surgeries. One such synthetic material is calcium phosphate ceramics, which can be treated and shaped to fit large bone defects. Ceramic scaffolds used for treatment of large bone defects stimulate osteogenesis in critical sized defects. Though the materials are not osteoinductive, the proximity to healthy tissue allows the ceramic to function as a continuous scaffold for cells to attach and proliferate, resulting in bone tissue regeneration. A cell-deposited extracellular matrix (ECM) on the porous beta-tricalcium phosphate (β -TCP) scaffolds could enhance subsequent cell seeding. Scaffolds were seeded with W-20-17 cells and cultured in osteogenic medium for 28 days. Samples were removed at days 1, 7, 14, 21, & 28 days to measure ECM by looking for the presence of collagen type I, the most abundant protein found in all types of ECM. Scaffolds were decellularized to isolate the ECM coating on scaffolds. Immunofluorescence and electron microscopy were done to visualize the scaffold surfaces. Alkaline phosphatase, total protein, and double stranded DNA assays were performed to ensure osteoblast differentiation on the scaffolds. ECM deposition can be clearly seen after 14 days. The medium-soaked group exhibited smoother surface morphology over time. Varying amounts of ECM, increasing with time, were successfully generated and maintained on β -TCP scaffolds after decellularization.

Introduction

Large bone defects are treated with bone grafts at a rate of approximately 1.5 million operations per year in the United States.¹ The primary methods of treatment include autografts and allografts. Both of these treatments suffer from a lack of tissue supply, and they also require multiple surgeries to gather the necessary tissue and perform the implantation.

Synthetic biomaterials can provide the same beneficial effects of autograft bone tissues without the limit of supply. The goal of these synthetic materials is to create a substance that can generate a cell response similar to natural bone. The material must be biocompatible, biodegradable, and ideally osteoinductive. Ceramic and polymer materials tend to be osteoconductive rather than inductive, but cells readily attach and begin proliferation and differentiation on their surfaces.

Our lab focuses on beta-tricalcium phosphate (β -TCP) ceramic materials constructed by a casting and sintering method. The scaffolds have interconnected pores and possess the qualities mentioned before, being biocompatible, biodegradable, and osteoconductive. In order to improve the healing rate of the scaffolds, a cell-deposited layer of extracellular matrix (ECM) will be prelaidd prior to use. Datta et al. have shown that ECM synthesized on titanium constructs prior to seeding with marrow stromal cells

can enhance osteoblastic differentiation.³ We expect that a similar result will be seen on ceramic scaffolds with an ECM synthesized prior to cell seeding.

The process of depositing the ECM has not been fully characterized in our lab. We will examine the extent of ECM deposition over a 28 day period to determine an optimal time for culture. The ceramic scaffolds seeded with osteoblast precursor cells will be cultured with osteogenic medium. Samples and data will be collected every week during the study. Qualitative analysis of the ECM will be examined as well as the behavior of the cells depositing the ECM.

The goal is to confirm the ECM deposition on scaffolds, determine the effects of decellularization on the ECM, and finally define the starting parameters for the following experiments, which will examine effects on cell behavior due to a cell-synthesized ECM on β -TCP scaffolds.

Materials and Methods

All cell culture materials were purchased from Invitrogen unless otherwise noted. Control medium was made with 10% fetal bovine serum (FBS, #16000-044) in Dulbecco's modified Eagle medium (DMEM, #10313-039) with 1% antibiotic-antimycotic (PSA, #15240-062) and 1% L-glutamine (#25030-081).

Osteogenic medium was made by adding 50 mg/L of ascorbic acid (Sigma #A4544), 1.0×10^{-8} M dexamethasone (Fisher #AC23030-0050), and 0.01 M beta-glycerophosphate (Fisher #ICN15724150) to the control medium formula.

The W-20-17 mouse preosteoblast cells were obtained from ATCC. Primary antibody was obtained from Abcam (ab299999) and secondary antibody was purchased from Invitrogen (#A11008). Chemicals for the assays and bovine serum albumin (BSA) were purchased from Sigma unless otherwise noted. Scaffolds were fabricated in lab prior to use as described previously.³⁵

Scaffold fabrication

Porous beta-tricalcium phosphate (β -TCP) scaffolds were made as described previously.³⁵ Briefly, custom templates of paraffin wax were cast into plastic molds. A β -TCP slurry was cast into the molds by exposure to low vacuum. Excess slurry was removed and the cast molds were dehydrated in ethanol at an elevated temperature of 35 to 55 °C for 48 hours. The temperature was increased to melt the wax and the scaffold green bodies demolded. The scaffolds were put through a graded dehydration process of increasing concentrations of ethanol over 8 hours and then allowed to dry for 2 hours. The green bodies were sintered at 1250 °C for 3 hours in a high temperature muffle furnace (Barnstead Thermolyne 46100). Scaffolds were cleaned in DI water by ultrasonication and then autoclave sterilized prior to use.

Scaffolds used in this experiment weighed 0.178 ± 0.015 g with a diameter of

7.70 ± 0.23 mm and height of 6.28 ± 0.36 mm.

Cell culture

The W-20-17 cells were reconstituted from frozen stock. Cells were cultured in T-75 plates using control medium. Cells were incubated at 37 °C in a 5% CO₂ atmosphere and fed control medium every 3 to 4 days. Prior to seeding on scaffolds, cells were trypsinized for 2 minutes at 37 °C and diluted in control medium to the desired concentration. Cells were counted in a Coulter counter (Beckman Coulter Z2).

Development of extracellular matrix coating

Autoclaved scaffolds were placed in 24 well plates. An aliquot of approximately 50 µL control medium containing 1.0×10^5 W-20-17 cells was placed onto each scaffold. The cell solution and scaffold were incubated for 1 hour at 37 °C in a 5% CO₂ atmosphere. Then 2 mL of osteogenic medium was added to each scaffold well. The cells were cultured for 28 days. Osteogenic medium was changed every 3 days for the first 2 weeks and every 2 days after that. Samples were collected at days 1, 7, 14, 21, and 28 by removing medium and rinsing twice with phosphate buffered saline (PBS), then storing at -20 °C. Samples were seeded in duplicate 3 days apart and the sample readings were combined (n = 30).

Medium-soaked scaffolds

Another set of autoclaved scaffolds was prepared and soaked in 2 mL osteogenic medium for 28 days. Scaffolds were kept at 37 °C in a 5% CO₂ atmosphere. The scaffolds were kept in these conditions for 28 days, changing medium every 3 days for the first 2 weeks and every 2 days after that. Samples were collected at days 1, 7, 14, 21, and 28 by removing medium and rinsing twice with PBS, then storing at -20 °C. Samples were seeded in duplicate 3 days apart and the sample readings were combined (n = 30).

Immunofluorescence of extracellular matrix

Collagen type I was used as an indicator of ECM presence. After being collected from culture, scaffolds were brought to room temperature from -20 °C and fixed in 4% paraformaldehyde for 15 min. They were rinsed twice with PBS and blocked with 10% goat serum in 1% BSA-PBS for 30 min. The blocking agent was removed and scaffolds were submerged overnight at 4 °C in a 1:100 dilution of a mouse collagen type I antibody raised in rabbit. Scaffolds were rinsed twice with PBS and then immersed in a 1:250 dilution of Alexa Fluor 488, an anti-rabbit fluorescein conjugated secondary antibody raised in goat, for 1 hr at room temperature protected from light. Samples were rinsed twice with PBS and observed under a fluorescence microscope (Nikon TE2000-U).

This immunohistology was performed on thin versions of scaffolds approximately

half the weight and height of scaffolds used for quantification. The thin scaffolds weighed 0.085 ± 0.009 g and had a diameter of 8.11 ± 0.27 mm by 3.50 ± 0.25 mm height. Half the number of cells (5×10^4) was seeded onto these scaffolds, and half the volume of medium (1 mL) was used during feeding and culture. The decreased thickness allowed the scaffolds to be viewed under a microscope without the opaque β -TCP struts blocking light for viewing and recording images.

Scanning electron microscopy of scaffolds

Scaffolds were decellularized by rapid freeze-thaw cycling and rinsing with 0.2% triton. Scaffolds were fixed for 15 min using 4% paraformaldehyde and allowed to dry overnight. Using a sputter coater, samples were plasma sprayed with gold to be imaged with electron microscopy. An FEI Quanta 400 scanning electron microscope was used for all imaging, set to high vacuum mode and 30 kV beam energy.

Cellular behavior assays

At the end of cell culture and after rinsing, all scaffolds were moved to new plates and all samples were decellularized. The decellularization process had 700 μ L of 0.2% triton added to isolated scaffolds and their previous wells. The plates all went through rapid freeze-thaw cycling, which consisted of 3 cycles of -80 $^{\circ}$ C incubation for 20 minutes immediately followed by 37 $^{\circ}$ C incubation for 15 minutes. The scaffolds were ultrasonicated for 5 minutes to collect cellular debris ($n = 30$), while the well remains without scaffolds were scraped to dislodge and collect cellular debris ($n = 30$).

The cell lysate collected was tested for double stranded DNA, which can be measured to estimate cell number. A Quant-iT PicoGreen assay kit (Invitrogen # P11496) was used according to the manufacturer's instructions. Briefly, 50 μ L samples were added in duplicate to opaque 96 well microplates. An equal volume of a 1:200 dilution of picogreen was added to each well in the dark. After 5 min, plates were read in a BioTek FLx800 plate reader at 485 nm excitation and 530 nm emission. Standard curves were made using the provided dsDNA samples.

The cell lysate from decellularized scaffolds was also measured for the presence of alkaline phosphatase (ALP), an early stage indicator of osteoblast differentiation. Fifty μ L of each sample were added in duplicate to 96 well microplates. Each sample had 50 μ L of working solution, made of equal volumes 20 mM p-nitrophenyl phosphate (PNPP), 1.5 M 2-amino-2-methyl-1-propanol, and 1.0 mM MgCl_2 , added to it. Plates were incubated at 37 $^{\circ}$ C for 1 hr. The reaction was stopped by placing plates on ice and adding 100 μ L of 1 M NaOH. A Bio Rad model 680 microplate reader was used to measure the absorbance at 405 nm. For a standard, p-nitrophenol (PNP) was used.

A bicinchoninic acid (BCA) assay was run on cell lysate samples to measure total protein using a microBCA kit (Thermo Fisher #23235). Samples from days 7, 14, 21, and 28 were diluted 5x, while day 1 samples were measured undiluted. In duplicate, 130 μ L of sample were added to 96 well microplates. Working reagent was prepared fresh

according to manufacturer's instructions and 130 μ L was added to each well. Plates were gently shaken, covered, and incubated at 37 °C for 2 hrs. Plates were then brought to room temperature and the absorbance at 562 nm measured with a microplate reader. Standard curves were made using the provided bovine serum albumin (BSA).

Statistical analysis

Results are reported and shown on plots as average $\pm$ one standard deviation.

Results

Fig. 5.1 shows images of the scaffolds after immunofluorescent staining. A pair of images was taken using the brightfield for comparison, seen in **Fig. 5.1(a)** and **Fig. 5.1(b)**. Because no cells were present to generate ECM on the untreated scaffold, the fluorescent image in **Fig. 5.1(c)** appears dark. The green appearance in **Fig. 5.2(d)** results from the fluorescein conjugated secondary antibody bound to mouse collagen type I primary antibody reacting with a fluorescent lamp, filtered to provide excited light at 495 nm. The fluorescein conjugate emits light at 419 nm, giving a green appearance. The transparent sheet of cells indicated by a white arrow in **Fig. 5.1(b)** is clearly seen to fluoresce in **Fig. 5.1(d)**, also marked by a white arrow. This suggests the presence of collagen type I, a major component of extracellular matrix. The microscope camera took pictures in grayscale, so fluorescent images were pseudocolored for effect.

SEM images of decellularized scaffolds were taken at high magnification, as seen in **Fig. 5.2**. The untreated scaffold surface can be seen at 1,000x in **Fig. 5.2(a)**. Following it are images of scaffolds seeded with cells over the 28 day culture period. On day 14, **Fig. 5.2(d)**, cell depositions can be seen as thin fibers along the ceramic surface. They are marked by black arrows. By day 21, **Fig. 5.2(e)**, fibers have begun to cross pores and are thicker than in the day 14 scaffold. On day 28, **Fig. 5.2(f)**, the fibers can still be seen crossing between struts of ceramic material, and also sheets of cell matrix can be seen forming along the calcium phosphate surface, indicated by a black arrow.

Fig. 5.3 shows the cell behavior data collected from cell lysate over the 28 day preparation time. The first column, **Fig. 5.3(a)**, **Fig. 5.3(c)**, and **Fig. 5.3(e)** were collected and measured from cells on the scaffold surface. The second column, **Fig. 5.3(b)**, **Fig. 5.4(d)**, and **Fig. 5.3(f)**, were collected and measured from the cells remaining in the wells of cultured scaffolds. Cells were separated in this way to determine if any changes could be observed between cells attached to the scaffold surface and cells attached to the tissue culture plate surface.

The dsDNA plots represent the total number of cells from each group at a given time point. The scaffold group, **Fig. 5.3(a)**, shows a plateau after day 7, while the well remains group, **Fig. 5.3(b)**, plateaus at day 14. This suggests that cells on the scaffolds reached confluence more quickly than cells that had migrated or initially attached to the tissue culture plate surface.

Figure 5.1. Immunofluorescent staining. Brightfield images at 40x are shown of an untreated scaffold (a) and 28 day cell cultured scaffold (b). Respective fluorescent images at 40x are shown below that for the untreated (c) and 28 day cell cultured (d) scaffolds. A green fluorescent secondary antibody gives collagen a green appearance when viewed with a 419 nm filter. The arrows indicate cell deposited matrix.

Figure 5.2. SEM Images of ECM development. The progression of cells depositing extracellular matrix is shown at 1,000x magnification. Image (a) shows an untreated scaffold. Cell seeded samples were imaged after 1 (b), 7 (c), 14 (d), 21 (e), and 28 (f) days of culture. Arrows indicate cell matrix growth.

Figure 5.3. Cell behavior assays for cells on scaffold. Plots of data collected from scaffolds (a), (c), (e) and the well remains (b), (d), (f). Total dsDNA is shown in (a) and (b). Normalized ALP production per cell is shown in (c) and (d). Normalized total protein per cell is shown in (e) and (f). Data represent means $\pm$ standard deviation ($n = 30$) except (e) & (f) where ($n = 18$).

Next, the ALP per dsDNA plots are shown. Alkaline phosphatase is an early stage marker for osteoblast differentiation, and these plots are normalized to cell number to indicate the ALP production per cell. The scaffold group, **Fig. 5.3(c)**, shows a stable production of ALP per cell for the duration, while an increasing amount of ALP per cell can be seen in the well remains group, **Fig. 5.3(d)**. Following a peak in ALP production, cells are said to have begun osteoblast differentiation. However, no such peak was seen in either group.

Finally, the total protein per dsDNA plots represent the activity of measured cells. The assay measures all protein present in the cell lysate, and that value was also normalized to cell number with the dsDNA count. The cells collected from scaffolds, **Fig. 5.3(e)**, suggest a consistent level of activity over the 28 day culture period. However, the cells left in the well, **Fig. 5.3(f)**, show a high reading on the first day, followed by a sharp drop by day 7, and a steadily increasing protein production per cell over time. The high value on day 1 is likely an artifact of several factors, discussed later.

Discussion

The SEM images collected show that a cell derived matrix has been deposited after 14 days. The immunofluorescence images reinforce this with a depiction of cell matrix after 28 days of culture, and the cell behavior data also agrees with this by showing a plateau in cell number prior to this time.

The images of the scaffolds visibly show a cell derived coating of ECM on the scaffolds even after decellularization. That coating will be used to measure cell behavior when preosteoblasts are reseeded on the same scaffolds.

From the dsDNA data, a near zero cell number count for well remains on day 1 can be seen. The cell count then quickly increases to the same magnitude of the scaffolds within two weeks. The well remains represent cells that have migrated out from the scaffold, or were not well attached after the 1 hour incubation period but remained viable after adding the medium. Such a low value here suggests a high attachment rate of cells to the scaffold during the initial seeding.

The high reading on day one of total protein per dsDNA is likely an artifact of two confounding factors. The low dsDNA count on day one, coupled with the fact that the tissue culture plates are coated with fibronectin proteins, would give an artificially high reading not representative of actual cell behavior. The protein count at this time point likely includes the tissue culture plate coating, and dividing it by a low cell number results in a higher than expected value.

An increasing amount of ALP per dsDNA with time can be observed for the well remains groups. However, the cells on the scaffolds show a relatively low ALP production throughout the 28 days. Within two weeks of cell confluence, preosteoblasts that have begun differentiation towards mature osteoblasts show a peak in ALP production per cell. This was not observed, despite confluence being achieved at 7 or 14 days from cell seeding. The higher ALP production in the well remains group would

normally indicate more cells at a later stage of differentiation, but no ALP peak was observed in either group. We would expect higher amounts of ECM to be deposited by more mature osteoblasts, as the development of ECM is an important step towards generating mineralized tissue.¹¹

The low amount of ALP from lysed cells on the scaffolds could be a result of the protein being retained in the ECM, giving a lower measurement even at later time points. Regardless, the cells in either group are producing ALP, and the total protein per dsDNA plots show that the cells are active throughout the 28 day culture period. Qualitative imaging confirms a cell deposition on the scaffold surface, but the cell behavior data does not allow a statement to be made about the mineralization of the observed cell matrix.

Conclusion

The porous TCP scaffolds can be successfully coated in a cell-secreted ECM coating after 14 days. The coating is still visible after the decellularization process. The scaffolds prepared here are sufficient for further examination of the effects the cell derived matrix may have on subsequent cell seeding.

CHAPTER 6. PREOSTEOBLAST BEHAVIOR ON SCAFFOLDS COATED WITH EXTRACELLULAR MATRIX

Bone grafts are commonly used for the treatment of large bone defects. Biodegradable, synthetic bone grafts combined with cells and growth factors as a tissue engineered construct can provide benefits over the common autografts and allografts currently used. Calcium phosphate materials offer high biocompatibility and chemical similarity to natural bone tissue. Biodegradable beta-tricalcium phosphate (β -TCP) scaffolds with an interconnected porous network have been created to promote tissue ingrowth and regeneration *in vitro* and *in vivo*. Extracellular matrix (ECM) coated on titanium and polymer scaffolds improved cell response. Here, we attempted to further improve the biocompatibility of the interconnected β -TCP ceramic scaffolds by generating a cell derived ECM on the ceramic surface *in vitro*. Cell derived ECM was generated on scaffolds for 1, 7, 14, 21, or 28 days. Osteogenic medium-soaked scaffolds treated for the same time periods as ECM coated scaffolds and autoclaved, untreated scaffolds were used as controls. The behavior of preosteoblasts seeded onto each scaffold was examined over a 28 day period. Cellular assays for cell number, alkaline phosphatase (ALP), and total protein (TP) were measured. Cell morphology changes on the scaffold were observed by scanning electron microscopy. The cell count on all scaffolds reached a plateau after 14 days. Specific ALP and specific TP slowly increased during the 28 days for all groups. New ECM was visible after 14 days of generation time on all groups. No significant difference was seen between the eleven tested scaffold groups. This study verified the high biocompatibility of β -TCP scaffolds and suggested that a cell derived biological coating on the β -TCP scaffolds offers no significant changes to the differentiation or attachment rates of cells.

Introduction

Bone grafts are commonly used for the treatment of large bone defects. The primary methods of treatment, autografts and allografts, are limited by supply and the need for multiple surgeries, among other complications. Synthetic bone materials address these issues and can provide more specialized functions in bone tissue repair.

Tissue engineering designs aim to create implants that can improve the healing rate and tissue integration of a wound site. In long bone repair, the time to recovery can take weeks due to the relatively slow process of bone regeneration across a void. Particularly in large bone voids, cases exist where no regeneration would occur unless a graft is implanted. Though a porous, biodegradable beta-tricalcium phosphate (β -TCP) scaffold can successfully bridge a bone void and promote tissue regeneration even in critical sized defects, methods of accelerating the bone healing rate are always under investigation.

In natural bones, the extracellular matrix (ECM) that permeates the hard tissue provides torsional resistance and shock absorbing qualities to it. The naturally composite tissue possesses high compressive and shear strengths while maintaining a low weight

and a porous interior structure. In trying to mimic the tissue being replaced, composite materials can provide the combination of polymer and ceramic properties that makes bone unique.

In addition, the ECM naturally retains osteogenic proteins and is a primary structure in the mineralization of developing bone tissue. All of these roles of the two different components of bone inspired the approach of adding a cell derived ECM coating to synthetic, porous, β -TCP scaffolds. Due to the already biomimetic nature of calcium phosphate materials, a biological polymer coating of ECM has been proposed to enhance tissue regeneration.

Previous studies have found favorable results in cell proliferation and differentiation when an ECM coating was used on metal and polymer surfaces.^{3,46} Similar increases in cell attachment and differentiation are expected for an ECM coating on β -TCP scaffolds.

Little work has been done to characterize or optimize the ECM coating of other materials. Only one time period is chosen to allow cells to deposit their ECM on a surface. Examining the effects of ECM generated after 1, 7, 14, 21, or 28 days on the β -TCP scaffolds may provide an optimal growth time to improve subsequent cell seeding.

This experiment will examine the effects an ECM coating of β -TCP scaffolds has on preosteoblast behavior. Scanning electron microscopy and cellular assays for cell number and alkaline phosphatase (ALP), an early stage biomarker for osteoblast differentiation, will be used to characterize the cells. As a positive control, scaffolds soaked in medium for 1, 7, 14, 21, or 28 days without seeded cells will be examined. This control will be useful to compare the effect prolonged immersion has on the cell-surface interaction, independent of the matrix coating.

The goal is to determine what biological changes in cell behavior result from having an ECM coating on β -TCP scaffolds. Control groups will use medium-soaked scaffolds, untreated scaffolds, and cells cultured in osteogenic medium on tissue culture plates.

Materials and Methods

All cell culture materials were purchased from Invitrogen unless otherwise noted. Control medium was made with 10% fetal bovine serum (FBS, #16000-044) in Dulbecco's modified Eagle medium (DMEM, #10313-039) with 1% antibiotic-antimycotic (PSA, #15240-062) and 1% L-glutamine (#25030-081).

Osteogenic medium was made by adding 50 mg/L of ascorbic acid (Sigma #A4544), 1.0×10^{-8} M dexamethasone (Fisher #AC23030-0050), and 0.01 M beta-glycerophosphate (Fisher #ICN15724150) to the control medium formula.

The W-20-17 mouse preosteoblast cells were obtained from ATCC. Primary antibody was obtained from Abcam (ab299999) and secondary antibody was purchased

from Invitrogen (#A11008). Chemicals for the assays and bovine serum albumin (BSA) were purchased from Sigma unless otherwise noted. Scaffolds were fabricated in lab prior to use as described previously.³⁵

Scaffold fabrication

Porous beta-tricalcium phosphate (β -TCP) scaffolds were made as described previously.³⁵ Briefly, custom templates of paraffin wax were cast into plastic molds. A β -TCP slurry was cast into the molds by exposure to low vacuum. Excess slurry was removed and the cast molds were dehydrated in ethanol at an elevated temperature of 35 to 55 °C for 48 hours. The temperature was increased to melt the wax and the scaffold green bodies demolded. The scaffolds were put through a graded dehydration process of increasing concentrations of ethanol over 8 hours and then allowed to dry for 2 hours. The green bodies were sintered at 1250 °C for 3 hours in a high temperature muffle furnace (Barnstead Thermolyne 46100). Scaffolds were cleaned in DI water by ultrasonication and then autoclave sterilized prior to use.

Cell culture

The W-20-17 cells were reconstituted from frozen stock. Cells were cultured in T-75 plates using control medium. Cells were incubated at 37 °C in a 5% CO₂ atmosphere and fed control medium every 3 to 4 days. Prior to seeding on scaffolds, cells were trypsinized for 2 minutes at 37 °C and diluted in control medium to the desired concentration. Cells were counted in a Coulter counter (Beckman Coulter Z2).

Preparation of scaffolds

Two groups of scaffolds were prepared one month before the study began. The experimental group had an extracellular matrix produced by seeded cells for up to 28 days. A control group of scaffolds had no exposure to cells but was submerged in osteogenic medium for the same length of time as the experimental group. Scaffolds were prepared in 24 well plates for this process. Prior to processing, these scaffolds weighed 0.178 ± 0.015 g with a diameter of 7.70 ± 0.23 mm and height of 6.28 ± 0.36 mm.

To produce the ECM on scaffolds, autoclaved scaffolds were placed in 24 well plates. An aliquot of approximately 50 μ L control medium containing 1.0×10^5 W-20-17 cells was placed onto each scaffold. The cell solution and scaffold were incubated for 1 hour at 37 °C in a 5% CO₂ atmosphere. Then 2 mL of osteogenic medium was added to each scaffold well. The cells were cultured for 28 days. Osteogenic medium was changed every 3 days for the first 2 weeks and every 2 days after that. Samples were collected at days 1, 7, 14, 21, and 28 by removing medium and rinsing twice with phosphate buffered saline (PBS), then storing at -20 °C. After all samples had been collected, rapid freeze-thaw cycling was performed to decellularize the ECM coated scaffolds. First a detergent, 700 μ L of 0.2% triton in DI water, was added to each scaffold, and samples were incubated for 1 hr at 4 °C. Samples were cycled three times between -80 °C for 20 min

and 37 °C for 15 min. Scaffolds were ultrasonicated for 5 min to remove cellular debris, then the detergent was removed. Samples were rinsed twice with PBS, and then allowed to dry. All samples were sterilized with ethylene oxide gas prior to use.

Medium-soaked scaffolds were similarly prepared. Another set of autoclaved scaffolds was soaked in 2 mL osteogenic medium. Scaffolds were kept at 37 °C in a 5% CO₂ atmosphere. The scaffolds were kept in these conditions for 28 days, changing osteogenic medium every 3 days for the first 2 weeks and every 2 days after that. Samples were collected at days 1, 7, 14, 21, and 28 by removing medium and rinsing twice with phosphate buffered saline (PBS), then storing at -20 °C. Samples were thawed, dried, and sterilized with ethylene oxide gas.

Cell seeding on scaffolds

Scaffolds were separated into 24 well plates according to collection time. Scaffolds previously prepared over a 28 day period consisted of two experimental treatments, scaffolds seeded and cultured with cells and scaffolds soaked in osteogenic medium. Each treatment had scaffolds collected at days 1, 7, 14, 21, and 28. This totaled 10 groups, five time points for both ECM coated scaffolds and medium-soaked scaffolds. An eleventh group of scaffolds was prepared and not treated except by autoclave sterilization to function as a scaffold surface control.

On day 0, all scaffolds were seeded with 1.0×10^5 W-20-17 cells at passage 9 in a 50 μ L aliquot. The cell solution and scaffold were incubated for 1 hour at 37 °C in a 5% CO₂ atmosphere. Then 2 mL of osteogenic medium was added to each scaffold well. The cells were cultured for 28 days. Osteogenic medium was changed every 3 days for the first 2 weeks and every 2 days after that. Samples were collected at days 1, 7, 14, 21, and 28 by removing medium and rinsing twice with PBS, then storing at -20 °C.

A twelfth group consisting of cells seeded directly on tissue culture plates was also started to serve as a cell behavior control. Each well was seeded with 1.0×10^5 W-20-17 cells at passage 9 in a 50 μ L aliquot. Then 1 mL of osteogenic medium was added to each well. The cells were cultured at 37 °C in a 5% CO₂ atmosphere for 28 days. Osteogenic medium was changed every 3 days for the first 2 weeks and every 2 days after that. Samples were collected at days 1, 7, 14, 21, and 28 by removing medium and rinsing twice with PBS, then storing at -20 °C.

The cell culture was performed in duplicate, seeded three days apart. Each of the twelve groups had a total of 6 scaffolds (or wells in the case of the cells on tissue culture plates) to be measured at each time point (n = 6).

Cellular behavior assays

At the end of cell culture and after rinsing, all scaffolds were moved to new plates. The isolated scaffolds and their previous wells had 700 μ L of 0.2% triton added to them. The plates all went through rapid freeze-thaw cycling. The scaffolds were

ultrasonicated for 5 minutes to collect cellular debris ($n = 6$), while the well remains without scaffolds were scraped to dislodge and collect cellular debris ($n = 6$).

The cell lysate collected was tested for double stranded DNA, which can be measured to estimate cell number. A Quant-iT PicoGreen assay kit (Invitrogen # P11496) was used according to the manufacturer's instructions. Briefly, 50 μL samples were added in duplicate to opaque 96 well microplates. An equal volume of a 1:200 dilution of picogreen was added to each well in the dark. After 5 min, plates were read in a BioTek FLx800 plate reader at 485 nm excitation and 530 nm emission. Standard curves were made using the provided dsDNA samples.

The cell lysate from decellularized scaffolds was measured for the presence of alkaline phosphatase (ALP), an early stage indicator of osteoblast differentiation. Fifty μL of each sample were added in duplicate to 96 well microplates. Each sample had 50 μL of working solution, made of equal volumes 20 mM p-nitrophenyl phosphate (PNPP), 1.5 M 2-amino-2-methyl-1-propanol, and 1.0 mM MgCl_2 , added to it. Plates were incubated at 37 °C for 1 hr. The reaction was stopped by placing plates on ice and adding 100 μL of 1 M NaOH. A Bio Rad model 680 microplate reader was used to measure the absorbance at 405 nm. For a standard, p-nitrophenol (PNP) was used.

A bicinchoninic acid (BCA) assay was run on cell lysate samples to measure total protein using a microBCA assay kit (Thermo Fisher #23235). Samples from days 7, 14, 21, and 28 were diluted 5x, while day 1 samples were measured undiluted. In duplicate, 130 μL of sample were added to 96 well microplates. Working reagent was prepared fresh according to manufacturer's instructions and 130 μL was added to each well. Plates were gently shaken, covered, and incubated at 37 °C for 2 hrs. Plates were then brought to room temperature and the absorbance at 562 nm measured with a microplate reader. Standard curves were made using the provided bovine serum albumin (BSA).

Scanning electron microscopy of scaffolds

Some scaffolds were set aside for imaging without decellularization. These scaffolds were brought to room temperature from -20 °C, then fixed for 15 min using 4% paraformaldehyde. Samples were rinsed twice in PBS and allowed to dry overnight. Using a sputter coater, samples were plasma sprayed with gold to be imaged with electron microscopy. An FEI Quanta 400 scanning electron microscope was used for all imaging, set to high vacuum mode and 30 kV beam energy.

Statistical analysis

Results are reported as average $\pm$ standard deviation. As described before, all measurements were performed twice. Results are considered significantly different if the p -value < 0.05 . Two-way ANOVA was performed on data groups and time. Significance was tested pairwise using the Holm-Sidak method.

Results

The results of the cell behavior assays are shown below in **Fig. 6.1** through **6.6**, with odd numbered figures corresponding to measurements taken from cells on the scaffold surface and even numbered figures for the measurements from cells remaining in the well after isolating the scaffold. In each plot, the legend lists the control group(s) first, followed by the experimental groups. For each time point, the vertical bars represent those same groups, in the order they are listed, from left to right.

For plots titled “from scaffolds,” all group abbreviations end in ‘S’ to indicate the measurements were taken from cells on the scaffold surface. The first letter indicates from which group of scaffolds the cells were collected. The ‘AS’ group represents the control group of autoclaved, untreated scaffolds. Groups labeled ‘ExxS’ are from the ECM coated group, with the number in the middle (where ‘xx’ is 1, 7, 14, 21, or 28) telling the number of days the scaffold was allowed to culture before being frozen, decellularized, and sterilized prior to seeding again. The ‘MxxS’ groups represent the medium-soaked scaffolds, also with the number stating how many days the scaffold was prepared before being frozen, sterilized, and then seeded in this experiment.

The plots titled “from well remains” have an extra group, ‘C,’ that was collected from cells seeded directly onto tissue culture plates in the absence of any scaffold. The other groups had a scaffold in the well until decellularization, and end in ‘W’ to represent this. Here, the ‘AW’ group was result of cells remaining in the well of the untreated, autoclaved control scaffold; the ‘ExxW’ group are the well remains of the scaffolds cultured for the indicated number of days (where ‘xx’ is 1, 7, 14, 21, or 28) before beginning this experiment; and the ‘MxxW’ group is the well remains of scaffolds soaked in medium for the indicated number of days before beginning this experiment.

Though the measurements for cells on the scaffolds and cells remaining in the wells were done separately, the cells that were found in the wells were a direct result of cell migration from the seeded scaffolds.

In all plots, the asterisk (*) indicates a significant difference (p -value < 0.05) between the indicated group and the AS or AW control group. Significance between time points is pointed out in the figure descriptions.

The dsDNA assays, which represent cell number from each source, are shown first. In **Fig. 6.1**, the cells from all scaffold groups can be seen to plateau after 14 days. There was no significant difference amongst any of the scaffold groups. On day 7, group E14S was about the same magnitude as day 1. A problem with cell seeding likely caused this low reading. A plateau had been reached at day 14, as no significant difference was seen between time points 14, 21, and 28. In **Fig. 6.2**, the cell culture control group, C, had a very high count on day 1 compared to the other 11 groups. Because the cells were seeded directly onto the plate surface, the dsDNA count is very high at this time, whereas the well remains of scaffolds must migrate out of the scaffold and into the well. The low number of cells in the wells this early suggests a high retention of seeded cells across all scaffold groups. As with the cells from scaffolds, none of the groups were significantly

Figure 6.1. dsDNA from scaffolds. Lysate from scaffolds was collected and measured for dsDNA to give an estimation of cell number. Groups in the legend marked with (*) are significantly different from the AS control, the cells from the untreated scaffold group. There was no significant difference between time points 14, 21, or 28. Data represent means $\pm$ standard deviation ($n = 6$).

Figure 6.2. dsDNA from well remains. Lysate from the wells of scaffolds was collected and measured for dsDNA to give an estimation of cell number. Groups in the legend marked with (*) are significantly different from the AW control, the cells from the untreated scaffold group's well remains. There was no significant difference between time points 14, 21, or 28. Data represent means $\pm$ standard deviation (n = 6).

Figure 6.3. ALP per dsDNA from scaffolds. Lysate from scaffolds was collected and measured for ALP to indicate cell differentiation. This number was normalized by dividing by cell number to give ALP produced per cell. Groups in the legend marked with (*) are significantly different from the AS control, the cells from the untreated scaffold group. Only time point pairings 1 versus 7 and 1 versus 14 were significantly different. Data represent means $\pm$ standard deviation (n = 6).

Figure 6.4. ALP per dsDNA from well remains. Lysate from the wells of scaffolds was collected and measured for ALP to indicate cell differentiation. This number was normalized by dividing by cell number to give ALP produced per cell. Groups in the legend marked with (*) are significantly different from the AW control, the cells from the untreated scaffold group's well remains. All time point pairings were significantly different except day 1 versus 14. Data represent means $\pm$ standard deviation (n = 6).

Figure 6.5. Total protein per dsDNA from scaffolds. Lysate from scaffolds was collected and measured for total protein to indicate cell activity. This number was normalized by dividing by cell number to give total protein produced per cell. Groups in the legend marked with (*) are significantly different from the AS control, the cells from the untreated scaffold group. All time point pairings were significantly different. Data represent means $\pm$ standard deviation ($n = 6$).

Figure 6.6. Total protein per dsDNA from well remains. Lysate from the wells of scaffolds was collected and measured for total protein to indicate cell activity. This number was normalized by dividing by cell number to give total protein produced per cell. Groups in the legend marked with (*) are significantly different from the AW control, the cells from the untreated scaffold group's well remains. All time point pairings were significantly different. Data represent means $\pm$ standard deviation ($n = 6$).

different from one another. After day 14, the cell count of the well remains also reached a plateau, as indicated by the lack of a significant difference between time points 14, 21, and 28.

Next, the ALP per dsDNA plots reveal the alkaline phosphatase produced from cells, normalized to cell number. This early stage biomarker of osteoblast activity gives an indication of the state of cell differentiation. One to two weeks after preosteoblast cells reach confluence, a peak in ALP production occurs that confirms their differentiation towards mature osteoblasts. **Fig. 6.3** shows a stable amount of ALP produced per cell on all scaffold surfaces, with no significant difference between time points except for day 1 versus 7 and day 1 versus 14. **Fig. 6.4** shows the behavior of cells on the tissue culture plates with a higher magnitude over time than the cells from scaffolds. There is a significant increase in ALP production per cell over time, as only the day 1 versus 14 pairing showed no significant difference. All the ECM-coated groups well remains showed a significant difference in ALP per dsDNA compared to the AW control. On day 1 for both groups the ALP production appears very high. This is likely a result of the normalization calculation of dividing by a low cell count and not a result of high osteoblast activity.

The total protein amount from the micro BCA assay was also normalized to cell number. This value gives a measure of cell activity in general. As with the ALP per dsDNA plots, the first time point shows artificially high values that are more likely a result of low dsDNA values than exceptionally high protein amounts. For the cells from scaffolds, **Fig. 6.5** showed no significant difference between any groups, though all the time points were significantly different from one another. However, no upwards or downwards trend in protein production was seen over time. Similarly, **Fig. 6.6** displays very similar protein per cell counts for all twelve groups for all time points. Here, only a significant difference was found between the cell control group C and AW. However, this difference is likely another artifact of the high dsDNA count for the C group on day 1. No other significant differences between groups was measured. As with the scaffold groups, all time points were significantly different from one another though no consistent trend could be determined.

SEM images from the different groups are shown to compare cell deposition on the different treated scaffold surfaces over time. A low magnification (50x) and high magnification (2,500x) image are shown side-by-side to show the distribution of cell deposited matrix across the scaffolds. Each scaffold sample was fixed at the designated time point. Samples were gold coated to allow electron microscopy imaging of the non-conductive samples. In all SEM figures, arrows indicate cell deposits of matrix. **Fig. 6.7** shows the untreated, autoclaved scaffold control group, 'A.' On day 1, **Fig. 6.7 (a)** and **Fig. 6.7 (b)** show no evidence of cell deposition on the control scaffold. On day 14 for the control scaffold, **Fig. 6.7 (c)** shows evidence of matrix deposition across the surface, while **Fig. 6.7 (d)** shows a thin sheet of cell matrix developing across a ceramic pore with thin fibers crossing the ceramic surface. On day 28, matrix deposition can still be seen in the low magnification image of **Fig. 6.7 (e)**, while a sheet of matrix can be seen across the ceramic surface in **Fig. 6.7 (f)**.

Figure 6.7. SEM images of control scaffold. Images (a), (c), & (e) were taken at 50x magnification. Images (b), (d), & (f) taken at 2,500x. The samples were collected at days 1 (a), (b); 14 (c), (d); or 28 (e), (f). Arrows indicate cell deposited ECM.

Fig. 6.8 shows a similar layout of images from the 14 day medium-soaked scaffold group, 'M14.' Both **Fig. 6.8 (a)** and **Fig. 6.8 (b)** show no signs of cell deposition, as would be expected with only one day of exposure to cells. Here, the ceramic surface appears smooth in comparison to the untreated scaffold of **Fig. 6.7 (a)** and **Fig. 6.7 (b)**. The exposure to osteogenic medium for 2 weeks allowed the inorganic materials to dissolve and precipitate on the surface, creating a smoother scaffold surface compared to the autoclaved-only scaffold. Fibers of cell deposited matrix can be seen in **Fig. 6.8 (c)**, and a close up of the matrix-surface interaction can be seen in **Fig. 6.8 (d)**. By day 28, similar structures of matrix are visible in **Fig. 6.8 (e)** and **Fig. 6.8 (f)**.

Lastly, in **Fig. 6.9** images from the 14-day cell cultured scaffold group, 'E14,' are shown at low and high magnifications. Some ECM deposits from the preparation stage can be seen on day 1, indicated by arrows in **Fig. 6.9 (a)**. A cell sheet can be seen in the upper left and top of the E14 scaffold in **Fig. 6.9 (b)**, indicated by a white arrow. This cell matrix was present from the scaffold's previous 14 day cell culture prior to this experiment, as no such observations were made in the control group, A, or medium-soaked, M14, group. **Fig. 6.9 (c)** and **Fig. 6.9 (d)** show the E14 scaffold surface with newly developed thin fibers of cell matrix growth across its surface, pointed out by white arrows. These thin fibers were not seen on day 1, suggesting that the cells recently produced it and that they are not remains from the previous culture period. On day 28, the scaffold surface of **Fig. 6.9 (e)** and **Fig. 6.9 (f)** also show deposits of ECM, though these images can not indicate which of the deposits are from the preparation stage and which were newly developed over the 28 day culture period.

Discussion

For all groups, SEM images showed a visible amount of cell deposited matrix present after 14 days of culture. The medium-soaked groups, regardless of soaking time, did not significantly affect the matrix development. The ECM coated scaffolds saw new matrix developed on their surface after day 14, but none of the five coated groups exhibited any significant effect on the ECM development.

It was hypothesized that the pre-laid ECM coating would enhance cell behavior by increasing attachment and speeding the process of differentiation. From the images obtained, it appears the cells continued to generate new ECM on the pre-existing ECM, much like their behavior on medium-soaked or untreated, autoclaved scaffolds.

Cell behavior on the treated scaffold groups showed little change compared to the cells cultured on untreated scaffolds. From the assays tested, no group stood out as having drastically affected preosteoblast behavior. The consistent behavior of the cells for all groups suggests that the untreated calcium phosphate material is already very biocompatible. Other groups that explored an ECM coating as a means for enhancing cell behavior on a material examined metal or polymer surfaces, materials that have no biological analogue.^{3,46} The scaffolds composed of calcium phosphate closely resemble hard bone tissue in chemical composition and structure, which contributes to high cell attachment and differentiation on the scaffold surface.²⁴ Treatment of the CaP surface

Figure 6.8. SEM images of 14-day medium-soaked scaffold. Images (a), (c), & (e) were taken at 50x magnification. Images (b), (d), & (f) taken at 2,500x. The samples were collected at days 1 (a), (b); 14 (c), (d); or 28 (e), (f). Arrows indicate cell deposited ECM.

Figure 6.9. SEM images of 14-day ECM-coated scaffold. Images (a), (c), & (e) were taken at 50x magnification. Images (b), (d), & (f) taken at 2,500x. Samples were collected at days 1 (a), (b); 14 (c), (d); or 28 (e), (f). Arrows indicate cell deposited ECM.

with a cell derived matrix coating or by soaking in medium may not be necessary, or even capable of improving scaffold biocompatibility.

As with the previous chapter, the lack of an ALP peak after cell confluence does not allow any statements to be made about the state of cell differentiation in the presence of scaffolds. While extracellular matrix can clearly be seen after two weeks, it is possible that mineralization of the ECM has not begun by day 28. The plots of ALP per dsDNA and total protein per dsDNA do suggest that the measured osteoblasts are active and confluent, so further investigation into the state of differentiation of cells on scaffolds is necessary.

Conclusion

Differences in the surface morphology can be seen between ECM coated, medium-soaked, and untreated scaffolds before 14 days of culture. By day 28, no distinguishing differences can be seen in the cell matrix coating of any of the eleven scaffold groups. There were no measurable changes in preosteoblast cell behavior due to the surface treatments as indicated by dsDNA, ALP, and total protein assays. The porous β -TCP scaffolds exhibited a high level of biocompatibility with or without any surface treatment. A cell derived matrix coating is a viable modification of CaP scaffolds that does not negatively impact the performance of the scaffold.

CHAPTER 7. DISCUSSION AND CONCLUSIONS

Overall Findings

The porous and interconnected beta-tricalcium phosphate scaffolds were characterized and found to be similar in composition to the bone tissue they seek to replace. Preosteoblast cells seeded on their surface produced a visible amount of extracellular matrix after 14 days of culture, and that biological coating can still be seen after fairly harsh treatments of decellularization, drying, and sterilization. The cell derived matrix persists through a subsequent cell seeding, and can still be seen even after another 28 days of culture. From qualitative analysis, more mass of ECM is deposited over time on the scaffolds cultured with cells. Also, the surface morphology of the medium-soaked scaffolds appeared smoother than the control, likely due to dissolution and reprecipitation of the calcium phosphate.

The biological coating on porous calcium phosphate scaffolds had no significant effects on preosteoblasts, according to assays for cell number and an early stage marker for osteoblast differentiation, alkaline phosphatase. New ECM was developed even on scaffolds with existing ECM. The morphology of the newly developed ECM was similar to that generated on both medium-soaked and sterile, untreated scaffolds. The cells behaved comparably on ECM coated, medium-soaked, and untreated scaffold surfaces.

The biocompatibility of the untreated calcium phosphate scaffolds may already be sufficient for maximum cell attachment. The high seeding density could also have masked any potential benefits of the ECM coating on the scaffolds.

Conclusions

It is possible to generate a cell-derived biological polymer coating on the calcium phosphate ceramic scaffolds. However there appear to be no beneficial or negative effects on cell behavior compared to cells on an untreated scaffold surface.

CHAPTER 8. FUTURE DIRECTIONS

Though benefits to cell attachment and differentiation were not observed with this cell derived matrix coating on beta-tricalcium phosphate scaffolds, a biological polymer coating can serve other purposes. Bioactive agent delivery is one direction that would be very interesting to pursue for this application. Another approach to aid ceramic biomaterial research is improving mechanical strength, which may be possible with this approach.

Extracellular matrix naturally retains the activity of bound proteins for local cellular use. In fact, the discovery of BMP-2 occurred when the protein was bound to its natural carrier, collagen.⁴⁷ Being able to deliver specific bioactive agents, like the growth factor BMP-2, would greatly increase the range of uses this synthetic bone graft material has in tissue engineering. For instance, the use of CaP scaffolds to enhance regeneration of vessels alongside bone growth has been examined by some groups.⁵³ Delivering bioactive agents known to promote neovascularization could improve the overall quality of tissue developed on the ceramic scaffold.

Though not examined in this experiment, mechanical stability provided by a pervasive polymer coating is a potential area of interest. Bone tissue itself derives many of its unique properties through this composition of materials. A thorough study on mechanical strength in compressive and shear forces may reveal an additional benefit to this cell derived matrix coating.

Most importantly, the use of the matrix for osteogenic factor retention should be examined. Either by immunostaining, ELISA, or more cell behavior assays, the ability of the decellularized and sterilized ECM to provide a structural support for bioactive agents should be examined. Without this ability, the ECM coating offers little benefits to the already biocompatible surface of these porous calcium phosphate scaffolds.

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VITA

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