Evaluation of the Effects of Recombinant Human Bone Morphogenetic Protein-6 On Periodontal Wound Healing in Induced Periodontal Defects in Rabbits (A Histological Study)

Thesis

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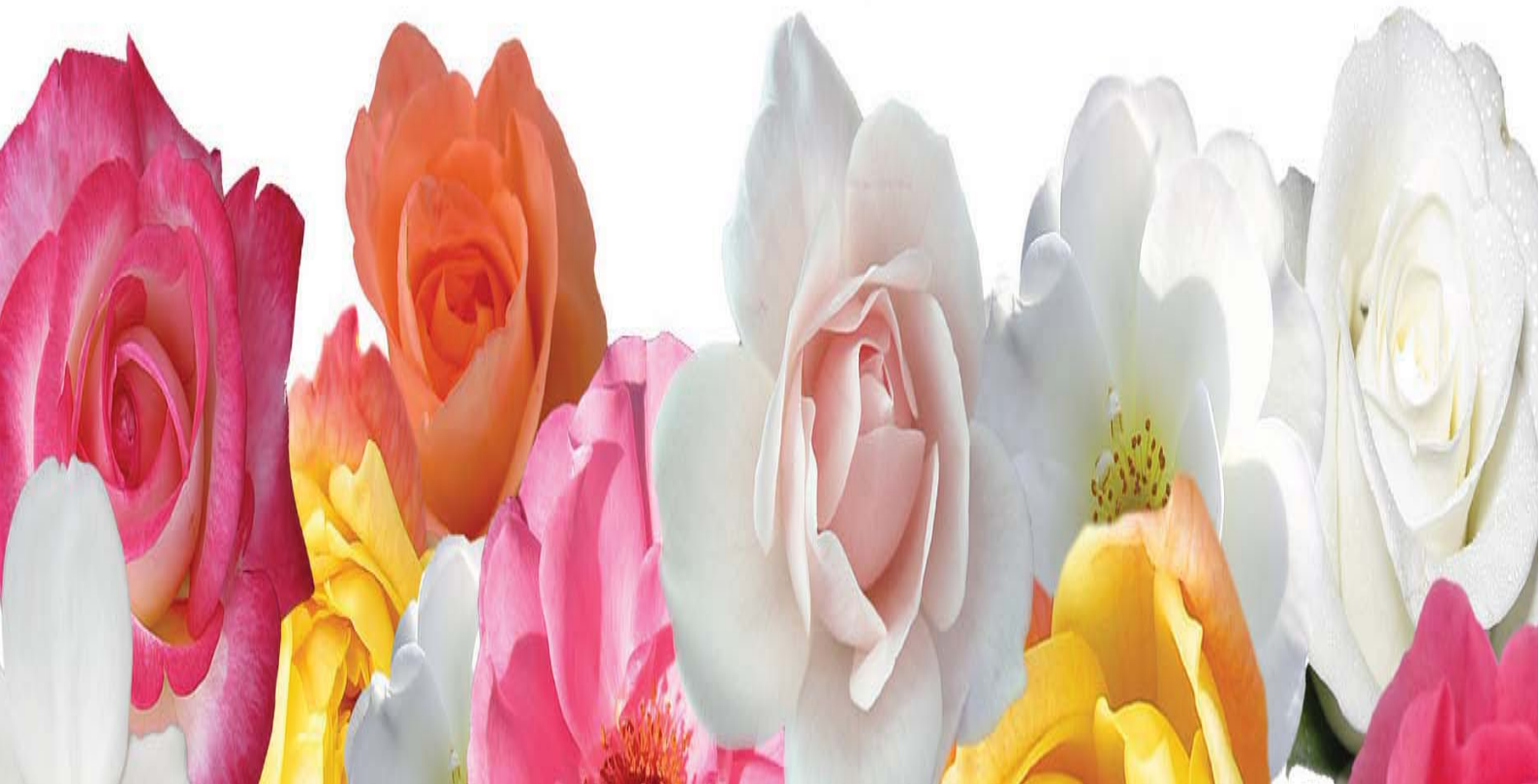
بسم الله الرحمن الرحيم

Dedication

This work is dedicated to

The Soul of My Grandmother



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Contents

Introduction and Review of Literature

Periodontal disease is characterized by microbially induced chronic inflammation of tissues that leads to destruction of tooth-supporting structures including alveolar bone, cementum and periodontal ligament. A challenge in the management of peridontitis is the predictable regeneration of tissues lost as a consequence of disease. ***(Bartold et al., 2000; Benatti et al., 2007)***.

The application of periodontal regenerative biomaterials such as autografts, allografts, xenografts, guided tissue regeneration (GTR) procedures, and enamel matrix proteins has been pursued with varying degrees of success to regenerate lost tooth support ***(American Academy of Periodontology, 2005).***

These therapeutic measures demonstrate some success as regenerative biomaterials in clinical practice. However, the ability to completely and predictably regenerate damaged periodontal supporting structures has not been achieved in humans. Several key complicating factors that represent challenges to predicable periodontal regeneration are the contamination of periodontal wounds with tooth associated biofilms of anaerobic bacteria, the nature of transmucosal and soft tissue environment which allows entry of pathogens into wounds, the existence of multiple junctional complexes, and stromal cellular interactions and the effects of occlusal forces that deliver intermittent loads in axial and transverse dimensions ***(McCulloch, 1993; Giannobile, 2008).***

The lack of predictable outcome when using passive therapies as osteoconductive matrices and GTR led to the development of treatment designed to stimulate cells responsible for regeneration. This is an approach considered to be an application of tissue engineering. Tissue engineering aims at developing techniques for fabrication of new tissues to replace damaged tissues based on principles of cell biology, developmental biology and biomaterials ***(Narem and Sambanis, 1995; Reddi, 1998; Bartold et al., 2000).***

The main requirements for producing an engineered tissue are the appropriate levels and sequencing of regulatory signals, the presence and numbers of responsive progenitor cells and an appropriate extracellular matrix or carrier construct. Tissue engineering involves the production of cells, a tissue composite or an organ that is grown in tissue culture and then transplanted into a patient. Tissue engineering also involves directing or accelerating natural tissue healing, a process often known as in vivo tissue engineering ***(Lynch et al., 2008)***.

In vivo tissue engineering involves induction of progenitor cells of the host tissue to migrate into extracellular matrices or scaffolds. The scaffolds provide means to support cell migration and retention in place long enough to affect the desired repair. Scaffolds also prevent soft tissue collapse and facilitate blood clot stabilization. Growth factors and differentiation factors serve to stimulate the migration of native cells into the site to be repaired and increase proliferation of these cells to populate the scaffold through specific mitogenic and chemotactic effects. By increasing the number of cells in the treatment site, growth and differentiation factors enhance matrix formation and increase the

potential to drive the healing process toward regeneration ***(Lynch, 2005; Lynch et al., 2008).***

Growth and differentiation factors are natural biologic mediators that regulate crucial cellular events involved in tissue repair such as DNA synthesis, chemotaxis, differentiation and matrix synthesis. Growth and differentiation factors exert their effects by binding to specific cell receptors that transduce signals to the cell nucleus via signal transduction pathways. Examples of growth and differentiation factors present locally in bone, cementum and healing tissues include platelet derived growth factor (PDGF), transforming growth factor-beta (TGFβ), acidic fibroblast growth factor (aFGF), basic fibroblast growth factor(bFGF), insulin-like growth factors (IGFs), cementum derived growth factor and bone morphogenetic proteins ***(Giannobile, 1996).***

The expression of growth and differentiation factors, cytokines and chemokines following tissue injury and possibly during peridontitis may regulate the healing process. The objective for administration of these factors in treatment of periodontal disease is to enhance the normal wound healing response which may be of insufficient magnitude to promote regeneration of periodontal ligament, cementum and alveolar bone. ***(Giannobile, 1996)***.

Growth factors and bone morphogenetic proteins have been the focus for considerable basic and clinical research to evaluate the possible role of these factors in periodontal wound healing ***(Bartold et al., 2000; Lynch et al., 2008).*** With advances in recombinant technology, proteins may be now be synthesized and subsequently concentrated and purified in large quantities allowing for development and commercialization of

recombinant growth factor-matrix combination products which can be applied for periodontal and peri implant regeneration ***(Lynch, 2005; Lynch et al., 2008)***.

Bone Morphogenetic Proteins:

Bone morphogenetic proteins (BMPs) belong to a group of proteins called transforming growth factor-β (TGF-β) super family, and this gene family currently includes at least 43 members. The proteins of TGF-β superfamily regulate many biological processes including cell growth, differentiation and embryonic pattern formation. This group of proteins includes transforming growth factors β (TGF-β1 though TGF-β5), activins and inhibins, growth and differentiation factors and bone morphogenetic proteins. ***(Ripamonti and Reddi, 1994).***

Discovery of BMPs:

The history of BMPs began with observation that demineralized bone matrix is able to induce ectopic bone formation in subcutaneous and intramuscular pouches in rodents. ***In 1965, Urist*** made this discovery and he described this phenomenon as bone induction or osteoinduction. Because of the histologic transformation of soft tissue into bone, he named this activity bone morphogenetic protein; although it was unclear whether a single or unique protein was responsible for the observed bone formation ***(Urist, 1965; Vande Pulte and Urist, 1965).***

The bone induction process has been studied extensively. Histological analysis showed that cartilage appears 5-10 days after implantation of active demineralized bone matrix. This cartilage

mineralizes by day 7-14 and is subsequently replaced by new bone. After 21 days hematopoietic bone marrow formation can be observed ***(Urist et al., 1973; Sampath and Reddi, 1983; Reddi et al., 1987).***

Then, advances in biotechnical techniques and the advent of biotechnology allowed the purification and subsequent molecular cloning of the factors responsible for the osteoinductive activity in bone. The purification process included removal of the mineral component of bone by acid, extraction of the active component from the remaining organic matrix of bone using chaotropic agents as guanidine Hcl or Urea and multiple column chromatography steps ***(Wozney et al., 1988)***. Beginning with kilogram quantities of bone, microgram quantities of purified osteoinductive material eventually were obtained ***(Wozney et al., 1988)***.

The dissociative extraction of the demineralized bone matrix with chaotropic agents yielded an insoluble component mainly type I collagen and a solubilized protein extract containing the putative osteogenic proteins ***(Sampath and Reddi, 1981)***. The single implantation of insoluble collagenous bone matrix or the lyophilized protein extract in the subcutaneous space of the rodents did not induce endochondral bone differentiation. But, When the soluble signals of the extracted matrix were reconstituted with the insoluble signal of the insoluble collagenous matrix, the osteogenic activity was restored ***(Sampath and Reddi , 1981)***.

The purification of proteins in the osteoinductive extract provided amino acid sequence data for isolation and molecular cloning of complementary DNA clones. Analysis of these clones indicated that the bone-inductive extract consisted of a family of related proteins which

were named the "bone morphogenetic proteins" after the original activity ***(Wang et al., 1988; Celeste et al., 1990)***.

Classification and chemical structure of BMPs:

The BMPs 2-16 are the presently known members of the BMP superfamily ***(Ripamonti, 2006)*** and they can be divided into different subgroups according to how closely they are related to each other structurally. One subgroup contains BMP-2 and BMP-4 that are 92% identical and differ mainly in the amino acid terminal region with BMP -2 containing a heparin binding domain ***(Wozney, 1988)***. In another group, BMP-5, BMP-6, BMP-7 (known as osteogenic protein-1 [OP-1]) , BMP-8A (OP-2) and BMP-8B (OP-3) are present ***(Celeste et a.l, 1990; Ozkaynak et al., 1990; Ozkaynak et al., 1992 ; Lynch et al., 2008).***

These are slightly larger molecules than BMP-2 and BMP-4 and there is an approximate 72% amino acid identity between the 2 groups ***(Ripamonti and Reddi, 1994)***.

A third subgroup and more distantly related to the other factors include BMP-3 (also called osteogenin) and growth differentiation factor 10 (GDF-10) ***(Sampath et al., 1987; Cunningham et al., 1995)***. The molecules BMP-12, BMP-13 and BMP-14 appear to be the human homologues of mouse growth and differentiation factors GDF-7, GDF-6 and GDF-5 respectively ***(Dube and Celeste, 1995; Celeste et al., 1995)*** which are implicated in limb cartilage and bone development in mice ***(Storm et al., 1994)***. BMP-1 is not related to the BMP family. It does not show osteoconductivity and has recently been identified as procollegn C-

proteinase ***(Reddi, 1994)*** that cleaves procollagen fibrils as well as chordin which is a peptide that binds and antagonizes the actions of BMP-2 and -4. (Tab. 1)

Tab. 1 Classification of BMPs*

BMP Subfamily	BMP Molecule	Synonym
BMP-2/4	BMP-2	BMP-2A
	BMP-4	BMP-2B
BMP-3	BMP-3	Osteogenin
	BMP-3B	GDF-10
BMP-7	BMP-5	
	BMP-6	Vgr-1
	BMP-7	OP-1
	BMP-8A	OP-2
	BMP-8B	OP-3
CDMP/GDF	BMP-12	CDMP-3 or GDF-7
	BMP-13	CDMP-2 or GDF-6
	BMP-14	CDMP-1 or GDF-5
Miscellaneous	BMP-9	GDF-2
	BMP-10	
	BMP-11	GDF-11
	BMP-15	
	BMP-16	

* BMP-1 is not included because it is not a member of the TGF-β superfamily. GDF=growth differentiation factor, Vgr=vegetal related, OP=osteogenic protein, and CDMP=cartilage-derived morphogenetic protein.

(Lynch et al., 2008).

Structural Characteristics of BMPs:

The proteins BMP-2 through BMP-9 have the following similar structural characteristics: hydrophobic secretory major sequence, a large region propeptide and mature region domain. This mature domain contains the active substance of the molecule. Within the mature domain there are seven cysteine remains which are characteristics of TGF- β super family. BMP-8 which contains the eighth cysteine in this region is an exception. ***(Wozney, 1989; Wozney et al., 1990).***

The hydrophobic secretory leader (the amino acid terminal) is believed to target the precursor to the secretory pathway, whereas the propeptide (the prodomain) may assist in folding and dimerization. The mature domain (the carboxy terminal fragment) contains 110 to 140 amino acids with six cysteine residues forming three disulfide bonds within each monomeric unit. The seven cysteine residue is involved in the formation of dimers ***(Wozney, 1989; Wozney et al., 1990).***

For the BMPs, the mature domain is cleaved, monomeric unit dimerize by a cysteine-disulfide bridge and glycosylated intracellularly. The dimer is expressed in active form. TGF-β (unlike BMPs) is not glycosylated, but secreted in a latent state, and is activated extracellularly. While assembly can produce homodimers, heterodimers are also produced. And along with glycosylation variability, become factors influencing activities. In heterodimers, one might consist of a BMP-2 subunit and BMP-7 subunit with any possible combination of subunits ***(Wozney, 2002)***.

Signaling mechanism of BMPs:

BMPs initiate signaling from the cell surface by interacting with two distinct serine/thereonine kinase receptors. The receptors are type I and type II. Both receptors have a cysteine-rich extracellular domain and intracellular serine-thereonine rich domain. There are three distinctive type I receptors and three distinctive type II receptors. Different BMPs bind with different affinity to the BMP receptor complexes ***(Yamashita et al., 1996)***.

Upon ligand binding, type II receptor forms a heterodimer with type I receptor and the constitutive kinase of type II activates type I receptor and initiates the signal transduction cascade by phosphorylating downstream nuclear factors which then translocate to the nucleus to activate or inhibit transcription (***Nohe et al., 2002)***.

After receptor activation, BMPs, TGF-β and activin signal via Smads (a fusion of Sma gene in nematode *C.elegans* and Mad gene in *Drosophila*). At least eight Smads have been isolated in mammals. These include receptor regulated Smads that can be activated by BMPs such as Smad1, 5 and 8, or TGF-β and activin activated such as Smad 2 and 3 with a common TGF-β, BMP mediator Smad 4. In addition, there are inhibitory Smads, Smad 6 and 7 that can bind with activated Smad complexes and deactivate signal transduction. The inhibitory Smads constitute negative BMP signaling feedback loop ***(Kretzschmar and Massague, 1998; Kawabata et al., 1998).***