ANTI-INFLAMMATORY PROTEIN TSG-6 PROMOTES EARLY GINGIVAL WOUND HEALING: AN IN VIVO STUDY

A Thesis

by

STACY RENAY BELTRAN

Submitted to the Office of Graduate and Professional Studies of Texas A&M University in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE

Chair of Committee, Kathy Svoboda
Committee Members, Lynne Opperman
Emet Schneiderman
Celeste Abraham
David Kerns
Head of Department, Jeffrey Rossmann

May 2014

Major Subject: Oral Biology

Copyright 2014 Stacy Renay Beltran
ABSTRACT

Human multipotent mesenchymal stromal cells (hMSCs) produce TNF-α-stimulated gene/protein 6 (TSG-6). TSG-6, a hyaluronan (HA)-binding protein, has been associated with the negative regulation of inflammatory tissue destruction. TSG-6 modulates proinflammatory cytokine cascades and enhances tissue repair. The aim of this study was to test the effects of recombinant human (rh) TSG-6 on the healing of an induced gingival wound within the first 2 days post-surgery.

Following gingival resection in one hundred twenty Sprague-Dawley rats (~400 g), 2 µg recombinant human TSG-6 (rhTSG-6) in 5 µL of Phosphate Buffered Saline (PBS) or the same volume of PBS solution was injected into gingival tissue approximating the surgical wound. Control animals did not receive injections. Examination of animals occurred at 1-2 hrs, 6-8 hrs, 24 hrs, and 48 hrs post-surgery (n=10 per group). Photographs were taken for a double blind clinical assessment at each time period. Tissue biopsy samples (4mm) and blood were collected at 1-2 hrs, 6-8 hrs, 24 and 48 hrs following surgery. Specimens were analyzed via histological analysis and enzyme-linked immunosorbent assays (ELISA) for quantification and comparison of inflammatory markers IL-1β, IL-6, TNF-α and myeloperoxidase (MPO) per treatment group. Weights were recorded for all animals pre- and post-surgery.

Animals injected with TSG-6 had significantly less severe inflammation based on clinical assessment scores at 6-8 (p=0.01228), 24 (p=0.01675), and 48 hours (p=0.0186). Sham and control animals had more weight loss at 24 and 48 hours. Based
on histological analysis, sham and control animals had more pronounced cellular infiltrate. Animals injected with TSG-6 had significantly less myeloperoxidase (MPO) (p=0.027) at 24 hours and IL-1β (p=0.027) at 24 & 48 hours. IL-6 showed marginal significant difference at 6-8 hours. There was no significant difference for TNF-α at any time point.

TSG-6 reduced post-operative gingival inflammation by modulating the inflammatory cascade; reducing levels of proinflammatory cytokines and cellular infiltrate. Gingival injection of TSG-6 may offer significant promise as an anti-inflammatory agent for patients requiring gingival surgery.
DEDICATION

I dedicate all of my accomplishments to my family. A special thank you to my parents, Al and Ellen, who have endlessly supported me. To my brother, Christopher, thank you for your encouragement and advice throughout my educational career.
ACKNOWLEDGEMENTS

Acknowledgements must truly be given to Dr. Kathy Svoboda, my mentor; this project could not have been accomplished without her guidance and dedication. Thanks to Dr. David Kerns, my graduate director, for his encouragement and support throughout this process. Thanks to all my additional committee members for their role in guiding my project. Thank you to Ryan Schmidgall, Jordan Payne, and Prashan Shanthakumar for helping me run histology samples and collect data. For help with statistical analysis, I’d like to thank Akash Seth. I also wish to extend my appreciation and gratitude to Connie Tillberg for her commitment and assistance throughout the surgical procedures and data analysis. And additionally, I wish to extend my sincere appreciation to Dr. Darwin Prockop for his generous support of my research.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>iv</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>v</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
<tr>
<td>CHAPTER I INTRODUCTION AND LITERATURE REVIEW</td>
<td>1</td>
</tr>
<tr>
<td>I.1 Inflammation</td>
<td>1</td>
</tr>
<tr>
<td>I.2 Gingivectomy</td>
<td>11</td>
</tr>
<tr>
<td>I.3 Cellular Components</td>
<td>16</td>
</tr>
<tr>
<td>I.4 Current Therapies</td>
<td>18</td>
</tr>
<tr>
<td>I.5 MSCs</td>
<td>20</td>
</tr>
<tr>
<td>I.6 TSG-6</td>
<td>21</td>
</tr>
<tr>
<td>I.7 Rodent Model</td>
<td>28</td>
</tr>
<tr>
<td>I.8 Aim</td>
<td>28</td>
</tr>
<tr>
<td>CHAPTER II ANTI-INFLAMMATORY PROTEIN TSG-6 PROMOTES EARLY GINGIVAL WOUND HEALING: AN IN VIVO STUDY</td>
<td>30</td>
</tr>
<tr>
<td>II.1 Synopsis</td>
<td>30</td>
</tr>
<tr>
<td>II.2 Introduction</td>
<td>31</td>
</tr>
<tr>
<td>II.3 Materials and Methods</td>
<td>33</td>
</tr>
<tr>
<td>II.4 Results</td>
<td>38</td>
</tr>
<tr>
<td>II.5 Discussion</td>
<td>41</td>
</tr>
<tr>
<td>CHAPTER III CONCLUSION</td>
<td>47</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>48</td>
</tr>
<tr>
<td>APPENDIX</td>
<td>58</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Model of the Complement Cascade</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>Gingivectomy Procedure</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>Surgical Procedure</td>
<td>58</td>
</tr>
<tr>
<td>4</td>
<td>Specimen Collection</td>
<td>60</td>
</tr>
<tr>
<td>5</td>
<td>Clinical Assessment</td>
<td>61</td>
</tr>
<tr>
<td>6</td>
<td>Box Plot Comparison</td>
<td>63</td>
</tr>
<tr>
<td>7</td>
<td>Histology</td>
<td>64</td>
</tr>
<tr>
<td>8</td>
<td>ELISAs</td>
<td>65</td>
</tr>
</tbody>
</table>
CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

I.1 Inflammation

Inflammation, a term coined by the ancient Egyptians and Greeks, is commonly used today in a variety of clinical settings. The word inflammation comes from the Latin word *inflammare* (to set on fire). In the 1st century AD., Aulus Cornelius Celsus, a Roman writer, described the four cardinal signs of inflammation as *calor, dolor, rubor,* and *tumor* (heat, pain, redness, and swelling)¹. This definition of inflammation recognizes what we know today as the “classical” acute inflammatory response. Two centuries after Celsus, the Greek physician, Galen promoted the idea that inflammation (especially pus) was not a superimposed pathology, but rather, part of the beneficial response to an injury. In 1871, Rudolf Virchow postulated that inflammation cannot be represented as a single process but rather constitutes various inflammatory processes. In addition, he introduced a fifth element to the four cardinal signs – *functio laesa,* or loss of function, denoting the restrictive function of inflamed tissue². As opposed to Galen, Virchow viewed inflammation as innately pathological.

The cellular processes and signals that underlie these cardinal signs occur at a subclinical level, and therefore solely defining clinical signs and symptoms presents with major limitations³. It was not until the 19th century, with advances in microscopy and cell biology that cell based definitions of inflammation came to the forefront. Varying cell populations stemming from both the blood and local tissue proliferation were being
discovered. We now know that the term inflammation encompasses a multiplicity of biological processes. And with advances in modern molecular biology, becoming knowledgeable in the complexities of inflammation at the cellular level has become critical to our understanding of how the body reacts to injury, manages insult and restores itself to health/function.

Inflammation is a defense reaction to the presence of any injurious stimulus. Such stimuli include infectious agents, foreign bodies, chemicals, thermal, mechanical and immunologic factors. Though there is a great deal of overlap, inflammation can be characterized as acute or chronic. If the cause of the initial injury is not completely abolished, acute inflammation will progressively give way to chronic inflammation, though there is no definitive time sequence.

Acute inflammation refers to a response that is rapid in onset and of short clinical duration. The acute inflammatory response has three major components: hemodynamic changes, alterations in the permeability of vessels, and changes in the location and concentration of white blood cells. In other words, injury to tissues triggers a specific acute response that involves an exudative reaction of fluid, serum proteins, and leukocytes that leave the bloodstream to enter the area of injury. The balance of intravascular hydrostatic pressure, which tends to force fluid out of vessels, and osmotic pressure, which opposes hydrostatic pressure by plasma proteins is referred to as Starling’s Law. Starling’s Law explains the movement and direction of fluid in and out of vessels. In brief, during the acute inflammatory response, swelling results from both an increase in the hydrostatic pressure and the permeability of vessels to plasma.
proteins. By leaving the venules and entering the extravascular space, proteins increase the osmotic pressure in tissues, drawing more fluid out of the vasculature and into the tissues. The fluid that accumulates in the tissues is termed edema fluid. Because fluid is lost from the vessels while blood cells are retained, the viscosity of the blood increases, which slows the movement of blood to the site of injury where supplies are needed. Active hyperemia, a process of increased “trafficking” of fluid and proteins results in dilation of arterioles at the site of injury due to congestion of blood and proteins within the microvasculature. When these vessels dilate, the cells of the endothelium contract to form gaps between the cells through which fluid and plasma proteins can move. Plasma proteins that leave through these gaps include albumin, fibrinogen, immunoglobulins, and other high molecular weight proteins.

Chronic inflammation is characterized by an increasing production of small blood vessels, fibroblasts, in addition to an infiltration of chronic inflammatory cells such as lymphocytes, macrophages, and plasma cells. Collectively, these elements comprise chronic inflammatory tissue. T-helper cells and the macrophage are the key cells in initiating a chronic inflammatory response. Therefore, chronic inflammation is basically an immune response initiated in response to a persistent antigen; a response that persists for more than a few days or weeks. It is often associated with irreversible destruction of normal parenchyma, inducing a phasic process of destructive mechanisms (inflammatory-degenerative) and healing (scar tissue). Over time, this course may result in permanent loss of function in the affected parts of the body. Chronic inflammation may go unnoticed by patients because pain is often minimal or absent.
The cardinal signs (heat, pain, redness, and swelling) often prominent in acute inflammation are nominal in chronic inflammation. It differs from acute inflammation in that it is predicated almost entirely by cells of the immune system.

Pro-inflammatory immune mediators [cytokines, chemokines, C-reactive protein (CRP)] are generated from either cells or from plasma proteins. The liver proteins, plasma-derived mediators (e.g., kinins, complement proteins) present in the circulation as inactive precursors. These precursors must be activated by a series of proteolytic cleavages, in order to procure their biologic properties. The three interrelated enzyme systems which mediate vascular permeability and are involved in peptide formation, are the kinin, fibrinolytic, and complement systems. Hageman factor (XII), a key component of the blood clotting system, triggers both the kinin and fibrinolytic systems. Once activated, either during injury when it binds to damaged blood vessels or upon proteolytic cleavage by kallikrein and LPS, it converts prekallikrein to kallikrein, activating more Hageman factor. Kallikrein is a protease, cleaving kininogen to produce bradykinin. Bradykinin is capable of increasing the permeability of venules, inducing arteriolar dilation and thus cause pain. After forming a blood clot at the site of injury, the fibrinolytic system becomes activated to dissolve the clot. Fibrinogen is released from platelets after they adhere to sites of endothelial cell injury. Thrombin converts fibrinogen to fibrin. The generation of thrombin promotes platelet aggregation and secretion of inflammatory mediators by platelets. The fibrinolytic system also involves the activation of plasminogen to generate plasmin. Plasmin can cause vasodilation, digestion of fibrin, split activated Hageman factor into subunits that turn on
the kinin system, and can activate the complement system by cleaving C3 to produce C3 fragments. Once activated, complement becomes a powerful effector that mediates vascular responses to recruit phagocytic leukocytes, opsonizes targets of phagocytic cells and directly injures target cells or tissues. The complement system is made up of over 25 proteins and protein fragments found in the blood, normally circulating as inactive precursors and plays a key role in many acute inflammatory processes and host defenses. When stimulated, specific proteins are cleaved by proteases to release cytokines. These activated proteins can then initiate an amplifying cascade of further protein cleavages and cytokine release. The end result is the activation of the cell killing membrane attack complex (C5,6,7,8, and 9) which is directly responsible for the lysis of foreign organisms. The three biochemical pathways that stimulate the activation of the complement system include the classic, alternative, and lectin pathway. The classical pathway involves antigen:antibody complexes for activation, whereas the alternative and lectin pathways can be activated by C3 hydrolysis or antigens alone; without antibodies present. In all three pathways, C3 becomes activated, producing products C3a and C3b. C3a, called anaphylatoxin, causes mast cells and basophils to release histamine, resulting in vasodilation and increased vascular permeability. C3b binds to the surface of pathogens, facilitating opsonization for greater internalization by phagocytic cells.

This elaborate system is illustrated in the following diagram.
The complement system is by far the most important of the plasma protein systems of inflammation. Once it becomes activated, its constituents participate in virtually every inflammatory response.

Cell-derived mediators of inflammation are normally appropriated in intracellular granules. These mediators can be secreted promptly by granule exocytosis or can be synthesized de novo (e.g., prostaglandins, cytokines) in response to a stimulus. Platelets, neutrophils, monocytes/macrophages, and mast cells are the major cell types that produce mediators of acute inflammation. The local production of mediators, such as TNF-α, IL-1β, IL-6, and PGE₂ that occur within inflamed tissues may “spill” into circulation. Its circulatory involvement may prompt systemic sequelae, such as induction of endothelial dysfunction. Pro-inflammatory cytokines in circulation induce leukocytosis and production of acute phase proteins. Acute phase reactants include
fibrinogen, serum amyloid A protein, plasminogen activator inhibitor 1, complement proteins, lipopolysaccharide binding protein and soluble CD14 \(^\text{12}\).

Cytokines are low molecular weight proteins produced by inhabitant cells, such as epithelial cells, fibroblasts, phagocytes (neutrophils and macrophages), and immune cells (lymphocytes) and are associated with both the acute and early chronic phase of inflammation as well as in adaptive immunity\(^\text{13}\). Cytokines propagate inflammation, and regulate the extent and amplitude of response. IL-1\(\beta\) and IL-6 are signature innate cytokines that have well documented associations with inflammatory cell migration and osteoclastogenesis\(^\text{14}\). IL-1\(\beta\) exerts a range of inflammatory and immunomodulatory activities. It is produced primarily by monocytes and macrophages and also by T cells, natural killer (NK) cells, endothelial cells, fibroblasts, astrocytes, microglial cells, adrenal cortical cells, and pancreas \(\beta\)-cells\(^\text{15}\). It is a pleiotropic cytokine involved in host responses to microbial invasion, inflammation, immune regulation, metabolic reactions, hematopoietic processes and tumor progression\(^\text{16}\). It affects important cellular functions, such as decreasing protein synthesis and intracellular energy production, reducing DNA contents, and prompting \(\beta\)-cell apoptosis and necrosis\(^\text{17}\). A complex system of IL-1 family members and receptors tightly regulates IL-1\(\beta\) expression and function. It can activate a complex cascade resulting in signal amplification, transcription factor induction, and other cellular activities, including cell proliferation, differentiation, and apoptosis that play a pathophysiologic role in host protection and inflammation\(^\text{18}\). In addition, IL-1 is a key cytokine involved in extracellular matrix degradation. It had been up-regulated in patients with rheumatoid arthritis,
osteoarthritis, periodontitis and other inflammatory diseases and conditions\textsuperscript{19}. Interleukin-6 (IL-6) is a potent, pleiotropic Th2 (type 2 helper T cell) cytokine that regulates the immune defense response. It plays a role as both an anti-inflammatory and pro-inflammatory cytokine. It also plays a central role in the transition from the acute to the chronic phase of the inflammatory process. Elevated levels have been documented in an assortment of autoimmune diseases, such as inflammatory bowel disease, systemic lupus, rheumatoid arthritis, glomerular nephritis, etc.\textsuperscript{20}. Overproduction of IL-6 leads to the deposition of extracellular matrix proteins, development of inflammatory lesions and synthesis of acute phase proteins. TNF-α is a pleotropic cytokine that has many functions such as cell migration and tissue destruction\textsuperscript{21}. TNF-α also degrades extracellular matrix and causes bone resorption by acting to secrete matrix metalloproteinases (MMPs) and receptor activator of nuclear factor kappa-B ligand (RANKL) as well as up-regulating the production of IL-1β and IL-6\textsuperscript{22}.

Resident macrophages, mastocytes, dendritic cells, Kupffer cells, and histiocytes present certain receptors known as Pattern Recognition Receptors (PRRs) on their surface that are able to recognize molecules foreign to the body, collectively referred to as pathogen-associated molecular patterns (PAMPs). Upon injury, these cells undergo activation, whereby a pattern recognition receptor recognizes a pathogen-associated molecular pattern, causing a release of chemical mediators\textsuperscript{23}. Chemical mediators each have a specific function at some definite phase of the inflammatory reaction.

Upon stimulus, these chemical messengers intensify and propagate the
inflammatory response influencing, these previously described, vascular and hemodynamic changes. Following a brief period of vasoconstriction, arterioles dilate and the microvasculature at the site of injury becomes engorged with blood cells. With increased vasopermeability, blood flow to the wounded area can increase in as much as ten-fold. Plasma proteins, such as albumin, fibrinogen, and globulins leak into the extravascular compartment. As these proteins leave the vessels and enter the extravascular space, they increase the osmotic pressure in the tissue, drawing more fluid out of the vasculature and into the tissues; a process which facilitates vascular stasis. Vascular stasis describes an eventual complete stagnation of blood flow due to increased vascular permeability and hemoconcentration.

Selective migration of blood leukocytes to the site of inflammation occur by margination, diapedesis, and chemotaxis. During margination, leukocytes accumulate and adhere to the endothelial cells of blood vessels that occur at the site of injury. Adherence involves the interplay between specific adhesion molecules present on the surfaces of leukocytes and endothelial cells. Certain inflammatory mediators such as IL-1, TNF-α, chemotactic factors, and histamine stimulate the activation of these adhesion molecules. As white blood cells travel across the endothelium, a process known as diapedesis, they become part of the interstitial fluid. Interstitial fluid envelops blood vessels and tissue cells. The response of leukocytes to products formed during immunologic reactions, involves adherence and accumulation at the site of the reaction. Emigration and subsequent migration of white blood cells through tissue are due to chemotactic factors. Chemotaxis, therefore, is a cellular function that stimulates the
direction of migration of cells via chemical stimulus.

The vascular changes and accumulated inflammatory exudate often result in clinical signs present on the skin. These changes, which consist of a flush, flare, and wheal, are referred to as the “Triple Response of Lewis,” and can be characterized by:

1) An immediate reddening, or flush, due to vasodilation; 2) A red flare that surrounds the injured area and spreads outward; and 3) A swelling, or wheal, as edema fluid collects due to increased vascular permeability. The substances that leave the vessels and enter the tissues are collectively called the exudate.

The amount of protein in an exudate is variable. Serous exudate is characterized as clear with low protein content and limited cells. This type of exudate is primarily associated with mild inflammation. Fibrinous exudate is rich in protein, especially fibrinogen. It typically forms a clot once it enters tissue, due to the formation of fibrin.

Suppurative (or purulent) exudate is rich in neutrophils (PMNs) and is typically recognized as pus.

The duration of vascular response is controlled by chemical mediators and can be identified as an immediate, delayed, or sustained response. Immediate transient vascular responses last about 10-15 minutes, and it is mediated primarily by histamine.

Immediate prolonged responses occur with more severe injury and may last for hours or even days. Chemical mediators include bradykinin, prostaglandins, leukotrienes, anaphylatoxins, oxygen-derived free radicals, etc. Delayed prolonged response to injury does not evoke and immediate response. This response is mediated by prostaglandins and leukotrienes. In the severest of injuries, a sustained response ensues, which involves
a marked vascular response comprising venules, capillaries, and arterioles. Vascular permeability immediately increases and remains high over a prolonged period of time. This type of response is mediated by all chemical mediators previously mentioned and involves an overlapping of the immediate (histamine-mediated) and delayed responses.

The events of the vascular response to injury, which include monocyte release of polypeptide cytokines and growth factors from peripheral blood, aim to concentrate and eradicate the injurious agents and eliminate damaged tissue constituents so that the body can begin to heal. The response, however, consists of alterations in blood flow, an upsurge in blood vessel permeability, and movement of fluid, proteins, and leukocytes from circulation to the site of tissue impairment. Collectively, these events explained by the behavior of the underlying cells and tissues, form the basis of which the Ancient Greeks and Romans derived their cardinal signs of inflammation centuries ago.

The immune system comprises a network of cells, tissues, and organs, highly specialized in defending our body against microbial invaders. Open bodily wounds serve as a conduit for invading pathogens, that to which our immune system must respond. In the field of medicine and dentistry, surgical insults, via blades, needles, lasers, etc. are made routinely upon rendering treatment. Subsequently, these initial acute wounds destroy cells, triggering the automatic response to injury and initiating the inflammatory cascade.

I.2 Gingivectomy

In the field of dentistry, specifically, periodontics, surgical procedures involve cutting or removing soft or hard tissues of the support structure to the teeth. According
to Genco and Rosenberg, the main objectives of periodontal surgery include: to gain surgical access to deep or tortuous pockets, to facilitate plaque control, to provide an environment for an adequate prosthesis, for periodontal regenerative therapy, and to correct cosmetic abnormalities. To provide access for thorough debridement of the tooth surfaces, surgical flaps, gingivectomy, and osseous contouring procedures are sometimes necessary. Hyperplastic or enlarged, fibrous gingival tissues often become an area of deep probing depths. These deep areas often serve as plaque retention areas and resective procedures help reduce bioburden. Periodontal surgical procedures such as crown lengthening, alveolar ridge augmentation, and correction of mucogingival defects help reduce inflammation and periodontal destruction. Resection of soft and hard tissues of the periodontium exposes subgingival margins of existing defective crowns or carious lesions. Gingivectomy or gingivoplasty often eliminates clefts, recontours bulbous gingiva and exposes the crowns of teeth that appear anatomically short, as occurs in altered passive eruption. As with most surgical procedures, each treatment mentioned would necessitate gingival incisions for access to root surfaces and bone.

Historically, periodontal procedures aim at reducing or eliminating pocket depths via resective procedures. The earliest form of resection was the gingivectomy. In 1792, Pierre Fauchard, now referred to as “the father of modern dentistry,” described the procedure and designed instrumentation specific to the removal of gingiva. Over a century later, in 1884, Robicsek described a similar technique of gingival excision and granulomatous tissue elimination. Pickerill in 1912 called this procedure ‘gingivectomy.’
A gingivectomy attempts to remove the soft tissue wall of the periodontal pocket by an external bevel incision that leaves a cut surface exposed to the oral cavity\textsuperscript{24}. As illustrated by Goldman in 1951, the primary incision is begun as far apically to the pocket depth as possible to achieve a long bevel\textsuperscript{28}. The gingiva is excised down to the base of the pocket. The epithelial attachment is removed during this process, leaving only a portion of the connective tissue of the gingival corium coronal to the alveolar crest\textsuperscript{29}. The knife is then extended deep into the interproximal areas so as to sever the existing papillae. The initial incision is made with a surgical knife or blade, extending interproximally as far as possible. After this is accomplished, the tissue is generally loosened and can easily be removed using a scaler or curette, as illustrated in the following pictures.

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{GingivectomyProcedure.png}
\caption{Gingivectomy Procedure.\textsuperscript{78}}
\end{figure}
Gingivectomy procedures are common with most periodontal procedures such as elimination of diseased tissue or other resective procedures such as crown lengthening.

In the case of open wounds, as in the gingivectomy procedure, many gingival vessels are severed and exposed. The gingival vasculature has been extensively studied in health and inflammation. Nuki and Hock evaluated vascular perfusion and vessel arrangement in cats and dogs. The gingival vasculature consisted of a microvascular bed, containing arterioles and precapillary venules. Numerous capillaries were found within the crestal gingiva, precapillary arterioles and postcapillary venules within the midgingival region, and small arterioles and venules predominated apical gingiva. The entire gingival vascular bed was affected with increasing severity of inflammation. Changes in vessel diameter and length as well as distortions of connecting capillaries and vessel loop formations were all observed. In 1963, Novaes and Kon studied the gingival and periodontal microcirculation during various phases of healing following the creation of a variety of surgical wounds in dogs. Gingivectomies were carried out on the labial side of the lower incisors. Intra-arterial injection with filtered Pelikan carbon black suspension was performed prior to animal sacrifice. Immediately after surgery, large vessels which had been cut were observed in direct contact with the surface of the wound. This was evident as the perfused material was released subjacent to and on the surface of the wound. By day -2, the specimen showed a thick clot covering the entire wound, bringing about an outer smoother surface. The connective tissue observed was dense and cellular, but showed little inflammation.

Epithelialization of the wound surface following gingivectomies in rats was
observed by Frederick Henning in 1968. By 24 to 48 hours, they found early epithelialization of the wound surface by migration of cells derived from the oral epithelium into the coagulum. “Reattachment”, however, was not achieved until 15 to 21 days after wounding and about 10 days after completion of epithelialization of the wounds. Upon histologic examination following gingival injury in a rat model, Stahl and Person found that the inflammatory response appeared prominent 24 to 48 hours after injury and the wound seemed epithelized 5 to 7 days post injury. Others found a blood clot and neutrophil leukocytes covering the wounded surface at 24 hours following gingivectomy in the rat. After 48 hours, the epithelium began to migrate from the remaining keratinized oral epithelium. Grevstad et al. reported similar observations. After performing interdental incisions to obtain a complete surgical transection of the gingival tissue in young albino rats, healing was evaluated via light and electron microscopy. At 24 hours, accumulations of inflammatory cells, were primarily found within the wound space. At the wound margins, striated fibrin strands appeared as remote, amorphous fragments along fiber bundles. In fact, for both incisional wounds of the gingiva and gingivectomies, within hours of injury, epithelial cells along the wound margins begin to migrate. Migration results from stimuli induced by locally released factors such as platelet-derived growth factor or epidermal growth factor and other cytokines. Upon injury, cells travel over the unprotected connective tissue surface releasing collagenase and plasminogen activator to boost collagen remodeling and promote dissolution of the fibrin clot formed. Within the initial phases of healing from a gingivectomy wound (days 1-2), the migrating epithelial
exterior is only 2-3 cells thick and forms a stratum basale. By day 5, complete coverage of the wound is observed and by day 7 a new stratum corneum is present as evidenced by matured epithelium 37.

Healing is commonly divided into three overlapping phases: 1) inflammation; 2) granulation tissue formation; and 3) matrix formation and remodeling. Neutrophils and macrophages dominate the early and late phases of inflammation, respectively 38. Within hours of injury, these inflammatory cells inhabit the clot to debride the wound of pathogens and necrotic debris through phagocytosis and formation of enzymes and toxic oxygen products. And though polymorphonuclear leukocytes can abolish toxic substances and provide protective mechanisms, these cells can also synthesize collagenases, acid hydrolases and neutral proteases that participate in destroying host tissue 39. These substances can break down collagen, proteoglycan ground substance and connective tissue.

1.3 Cellular Components

Neutrophils, the most abundant of the leukocytes and the first to infiltrate tissues in the early stages of an acute inflammatory response, are myeloid-derived antimicrobial professional phagocytes than can also execute pathogens extracellularly, link the innate and adaptive arms of the immune response, and help promote inflammatory resolution and tissue healing 40. The human neutrophil originates from a precursor pool of committed progenitor cells and is followed by two secretory stages: the promyelocyte and the myelocyte. During each of these stages a distinct type of secretory granule is produced – azurophils (solid black) during the promyelocyte stage, and specific granules
(light forms) during the myelocyte stage. During the course of differentiation in the bone marrow, all of the blood granulocytes synthesize and package into secretory granules a number of enzymes (some with digestive and antibacterial abilities) and other substances. The mature form of the neutrophil is characterized by a multilobulated nucleus and cytoplasm containing primarily glycogen and granules. After the myelocyte stage, cells are released into the blood where they circulate and migrate to tissues, killing and digesting pathogens. They are the most common cells found at the sites of inflammation.

Neutrophilic differentiation and maturation are stimulated by a cytokine, granulocyte colony-stimulating factor, elaborated by activated macrophages. Macrophages, a key participant in the mechanisms of host defense, are ultimately derived from bone marrow precursors. They spend a brief time in the blood stream as circulating monocytes before taking up their definitive location as mature macrophages. The earliest recognizable forms are the replicating monoblasts and promonocytes. Like granulocytes, monocytes form granules during differentiation in the bone marrow. Monocyte granules, however, are smaller than those of the neutrophils and comparatively inconspicuous. Monocytes differentiate very rapidly in marrow (1-3 days), usually undergoing from 1 to 3 divisions before entering the blood, where they circulate for about 1 day. One of the most critical steps in their life history is their delivery to tissue sites both within and outside of the vasculature, where they further differentiate, within a day or so, into long-lived (60-120 days) and versatile macrophages.
On the basis of function, the blood leukocytes are usually divided into two main groups: the phagocytes, such as granulocytes and monocytes; and the nonphagocytes, the lymphocytes. The granulocytes include the polymorphonuclear neutrophil (PMN) [50-70% in blood], the eosinophils [1-5% in blood], and the basophil [0-1% in blood]. The lymphocyte [20-40% in blood] and monocyte [1-6% in blood] comprise the mononuclear cells. Of the phagocytes, the neutrophils and monocytes (macrophages) play a major role in host defense. These cells are discriminatory and efficient in their ingestion, and effectively destroy invaders intracellularly. The term phagocytosis describes the process of engulfment and ingestion of large particles by the cell or phagocyte to form a phagosome (or food vacuole), which in turn fuse with lysosome and become phagolysosome. The engulfed material is eventually digested or degraded and either released intracellularly to undergo further processing or extracellularly via exocytosis. With their phagocytic properties, neutrophils along with the other pro-inflammatory cells, propagate the swelling and edema often experienced after a surgical procedure.

I.4 Current Therapies

Efforts to control inflammation to date have been focused on the use of pharmacological agents that inhibit pro-inflammatory mediator pathways. Non-steroidal anti-inflammatory drugs (NSAIDs), for example, target COX-1 and COX-2-dependent pathways inhibiting generation of prostanoids. Newer classes of inhibitors target lipoxygenase pathways and leukotriene (LT) production or TNF-α. Side effects of these agents, however, prohibit their extended use. Glucocorticoids are the most effective
anti-inflammatory drugs currently available for treatment of chronic inflammation. The mechanisms by which they effect repression of inflammatory gene expression remain incompletely understood. Glucocorticoids reduce the expression of chemokines, cytokines, inflammatory enzymes, adhesion molecules, and other inflammatory proteins, lowering the inflammatory cell burden. They exert their anti-inflammatory actions via a complex interplay between glucocorticoid receptor (GR)-mediated transcriptional regulation and signal transduction within target tissues. Glucocorticoids target specific cell populations to combat hyperactivation of the immune system, at both the transcriptional and cellular level. Corticosteroid complications, however, include metabolic derangements (particularly hyperglycemia), adrenal insufficiency, and critical illness myopathy. Dexamethasone, a steroid medication often given post-surgery via IM, IV, or oral administration to reduce inflammation also suppresses the body’s usual immune response. Side effects include stomach irritation, vomiting, headache, dizziness, insomnia, muscle weakness, depression, anxiety, vision problems, absent or irregular menstrual periods, etc.

With the progression of research in the field medicine, however, scientists are well on their way to finding more innocuous ways to reduce or eliminate this inflammatory response, so often causing pain and discomfort to patients alike. In procedures such as gingivectomies, gingivoplasties, or crown lengthening where a laceration induces an acute inflammatory response, tissue repair is of paramount importance to post-operative wound healing and patient well-being.
I.5 MSCs

Human adult stem/progenitor cells from bone marrow, referred to as mesenchymal stem cells or multipotent mesenchymal stromal cells (hMSCs), have been at the forefront of modern research. They are of interest because they can readily be isolated from human donors or patients, expand rapidly for 30 or more population doublings in culture, and differentiate into several cellular phenotypes in vitro and in vivo. These and related properties prompted testing the therapeutic potential of the cells in animal models and in clinical trials for a large number of diseases. Throughout their course of study, evolving data has led to the development of various hypotheses regarding their role in wound repair. Prockop (2011), identified MSCs as ‘guardians of inflammation,’ and summarized the shifts in paradigms. Initially, the cells were thought to provide a niche for culture of hematopoietic cells. Cells were then reconnoitered as reparative cells that might engraft and differentiate to replace injured cells. Engraftment and differentiation was observed in rapidly growing embryos, after local administration of large concentrations of the cells or after extreme injury. However, repair of tissues and functional improvements were more frequently observed without long-term engraftment of MSCs. More recently, data suggest that the cells only transiently appear in injured tissues under most conditions, but while present, respond by paracrine secretions or cell-to-cell contacts/ cross-talk with injured cells to limit tissue destruction or enhance repair by a variety of mechanisms. These mechanisms include upregulating genes that can modulate excessive inflammatory and immune reactions and enhance proliferation and differentiation of other stem cells. Among these therapeutic
factors, MSCs express the multipotent anti-inflammatory protein tumor necrosis factor (TNF)-α-stimulated gene/protein 6 in response to tissue injury.

**I.6 TSG-6**

TNF-α-stimulated gene/ protein 6, or simply TSG-6, is a hyluronan (HA)-binding protein that has since been implicated in the negative regulation of inflammatory tissue destruction. TSG-6 DNA was originally isolated from a cDNA library prepared from TNF-α - treated human fibroblasts. It is secreted by different cells, such as fibroblasts, upon stimulation by the proinflammatory cytokines tumor necrosis factor or interleukin-1.

**I.6.1 Structure**

The TSG-6 protein has a molecular mass of ~35 kDa. The solution structure of the link module was identified by Kohda et al. via NMP spectroscopy, revealing a compact fold containing two α-helices and two anti-parallel β-sheets, each comprising three β-strands. This link module of TSG-6 shows varying degrees of homology to members of the hyaladherin family of proteins. The N-terminal half of the TSG-6 protein (the link module) binds various glycosaminoglycans (GAGs), including HA, chondroitin sulfate, heparin, and heparin sulfate. Its amino acid sequence shows 36-40% identity with members of the hyaladherin family, including the CD44 receptor, cartilage link protein, and proteoglycan core proteins.

CD44 is identical to the hyaluronate receptor, mediating the adhesion of a variety of different cells to HA in the extracellular matrix and on cell surfaces. CD44 is expressed on a large variety of mammalian tissues mediating many different cell
adhesion events involving cell-cell interactions. Aggrecan, the proteoglycan core protein and link protein are important structural components of the extracellular matrix. Versican, the closely-related proteoglycan core protein of fibroblasts, and aggrecan contain at their amino terminus HA binding motifs. In cartilage, these core proteins form a ternary complex with both HA and link proteins, which determine the structural integrity of the extracellular matrix of cartilage.

The C-terminal half of TSG-6 forms a CUB domain, a structure that has been defined exclusively on the basis of homologies and certain structural elements. The term CUB domain is derived from the initials of three representatives of the family that reflects the diversity of this group: the complement components C1r and C1s are serine proteases, Uegf – a gene involved in the development of sea urchin embryos, and bone morphogenetic protein 1 (BMP-1) – a metalloproteinase. The structure of the CUB domain of TSG-6 has not been solved, however, it is likely that the CUB domain of TSG-6 shares the general structural features of those CUB domains whose 3D structures have been identified. Among these, β-sheets have been found around 15 hydrophobic and 4 aromatic amino acid residues that form the core of the CUB domain. It is presumed that the CUB domain structure found within TSG-6 is similar in structure. In addition, two disulfide bridges, which have been identified among most other CUB domains, have been found within the putative CUB domain of TSG-6. So far, no functions other than involvement in protein-carbohydrate and protein-protein interactions have been attributed to the CUB domain. The limited sequence homology between the CUB domains of various proteins precludes conclusions about any
particular binding specificity. To date, only fibronectin has been shown to bind to the CUB domain of TSG-6. TSG-6 is unique in that it possesses both a link module and a CUB domain.

I.6.2 TSG-6 Expression

TSG-6 is not expressed constitutively in unstimulated cells or tissues; its expression can be induced in response to a variety of factors. The rapid upregulation of TSG-6 in the presence of the pro-inflammatory cytokines TNF and IL-1 is consistent with its involvement in inflammatory processes. This association was confirmed by the detection of TSG-6 protein in synovial fluids of patients with a variety of conditions including rheumatoid arthritis, osteoarthritis, Sjögren’s syndrome, polyarthritic gout, and osteomyelitis. TSG-6 has not been detected in synovial fluids obtained at autopsy from accident victims with no known joint disease. Both chondrocytes and articular synovial cells, isolated from patients with rheumatoid arthritis, were shown to produce TSG-6 in culture. When stimulated with IL-1 or TNF in vitro, both cell types produced increased amounts of TSG-6. Additional studies have confirmed the expression of TSG-6 in human chondrocytes, demonstrating TSG-6 in IL-1-stimulated chondrocytes obtained from patients with osteoarthritis.

The first indication of an anti-inflammatory activity of TSG-6 came from a study using recombinant human TSG-6 protein in the murine air pouch model of acute inflammation. Wisniewski and colleagues examined the effects of TSG-6 on experimentally induced inflammation. Recombinant TSG-6 protein, injected together with a pro-inflammatory agent, e.g. carrageenan or IL-1, into the air pouch lumen
revealed an inhibitory effect of the locally administered TSG-6 on the IL-1-induced cellular infiltration. When compared to that of systemic dexamethasone treatment, TSG-6 proved to be as effective as dexamethasone in inhibiting IL-1-induced neutrophil infiltration. Histological analysis of the air pouch lining and the underlying tissue showed that TSG-6 treatment resulted in a significant resolution of the edema and of the tissue destruction induced by the injection of carrageenan. Minrescu et al. examined the effect of recombinant TSG-6 on inflammatory joint disease induced in DBA/1K mice. Mice receiving the CIA (collagen induced arthritis) immunization were given twelve intraperitoneal doses of TSG-6, three days before the expected onset of the disease symptoms. They found that there was a decrease in the disease incidence, arthritis index, and footpad swelling in TSG-6 treated animals, producing a potent ameliorative effect.

In 2009, Lee and colleagues, studied the anti-inflammatory effects of human mesenchymal stem cells in a model of myocardial infarction. hMSCs were infused i.v. in a mouse model with ligation of the anterior descending coronary artery. They found that the i.v. injection significantly reduced the early inflammatory response and size of the myocardial infarcts. Three weeks later, significant improvements were also observed in left ventricular function as observed by echocardiography. Quantitative assays, however, demonstrated that only a small fraction of the infused MSCs were recovered in the heart after injection and within 24 hrs. There was an absence of stem cells by 48 hours. RNA was extracted from the mice 10 hours after infusion and was assayed. It was found that the hMSCs were activated to upregulate the expression of over 50 human
genes. Among these was TSG-6. In an effort to study the role TSG-6 played, they infused hMSCs with an siRNA knockout of the TSG-6 gene into the mouse. In this model, they found that the hMSCs had little or no effect in the MI model. Results of the experiment demonstrate that hMSCs were activated to secrete TSG-6, and the TSG-6 decreased the early and excessive inflammatory response in the heart that caused the destruction of cardiac tissue.

In a subsequent article published in 2011, Gavin Roddy and colleagues, demonstrated that systemically administered hMSCs reduced inflammatory damage to the cornea without engraftment and primarily by secretion of TSG-6 in response to injury signals from the cornea. Similar therapeutic effects were reproduced by i.v. or topical administration of TSG-6. Corneas of six-week-old male Lewis rats were injured by brief exposure to alcohol followed by mechanical debridement of the corneal and limbal epithelium with a surgical blade. Immediately following injury, rats either received hMSCs via i.v. or i.p. Other rats were treated with siRNA for TSG-6. Others received topical application of 8µg of rhTSG-6 in 20µL PBS or PBS alone. Results revealed i.v. infused hMCs markedly decreased neutrophil infiltration, production of proinflammatory cytokines, and development of opacity in the cornea. Infusion, via i.p. of the hMSCs was also effective in subduing inflammation and preventing the opacity in the cornea. <10 hMSCs were present in the corneas of rats at day 1 and 3 after i.v. or i.p. administration. Those rats that received the siRNA knockdown of the TSG-6 gene showed no effective healing of the cornea. To test that signals from injured corneal cells would activate hMSCs to express TSG-6, hMSCs were
co-cultured with human corenal epithelial cells injured with ethanol. They found that TSG-6 expression was increased approximately 13-fold in hMSCs. TSG-6 secretion by hMSCs was therefore both necessary and sufficient to reduce corneal inflammation. These results are consistent with those previous findings that i.v. injection of hMSCs secreted TSG-6 to improve cardiac function by decreasing inflammation in a mouse model of myocardial infarction ⁶⁵.

A related series of experiments validated that direct injection of rhTSG-6 into the anterior chamber of the rat eye also decreased excessive inflammation in the injured cornea. In a recent study by J. Oh and colleagues, 2 µg recombinant human TSG-6 or PBS solution was injected into the anterior chamber of rats’ eyes that were exposed to chemical and mechanical injury ⁶⁶. The eyes injected with the TSG-6, had markedly reduced inflammatory damage, attributed to the ability of TSG-6 to inhibit proteases and to suppress neutrophil migration into the site of inflammation. The levels of proinflammatory cytokines, chemokines, and matrix metalloproteinases were also decreased. To study a dose response to the therapy, varying doses of TSG-6 (0.0002-2µg) were injected into the anterior chamber of the eyes immediately following injury. A dose-dependent response was observed, as significant improvements in the anti-inflammatory effects were associated with higher concentrations of TSG-6. To investigate the window of opportunity for TSG-6 to be administered following an acute injury, 2µg TSG-6 or PBS solution at different time points (0-24 hours) after injury was administered. Injection within 4 hours after injury significantly decreased inflammation in the cornea. The same results were seen when injection was delivered 2 hours after
injury. TSG-6 was not effective, however, if administered 8 – 24 hours after injury, at which time neutrophils had intensely infiltrated the cornea. The mechanism by which TSG-6 inhibits neutrophil invasion has not been well-defined. Several reports suggest an inhibition of the migration neutrophil extravasation of neutrophils by down-regulating the protease network; specifically mediated by its potentiation of inter-α-inhibitor anti-plasmin activity. Plasmin has an important role in the proteinase cascade activated during inflammation which results in extracellular matrix degradation and cellular infiltration. The anti-inflammatory activity of TSG-6 was attributed to its ability to inhibit components in the inflammatory network of proteases and to suppress neutrophil migration into the site of inflammation.

TSG-6, secreted by activated MSCs following injury, has thus been found to modulate the cascade of proinflammatory cytokines and enhance tissue repair. The mechanism of action observed by the anti-inflammatory effects of TSG-6 is not well understood. However, in recent studies, Inter-α-inhibitor, an abundant plasma protein with anti-proteolytic activity, consisting of three polypeptides: two heavy chains and one light chain, has been shown to bind to hyaluronon molecules via covalent bonds, a reaction thought to be mediated by TSG-6. TSG-6 has been shown to be tightly bound to the hyaluronon-linked heavy chains. This complex has been implicated in stabilizing extracellular matrix by cross-linking hyaluronan molecules and protecting hyaluronan against fragmentation by reactive oxygen species, enhancing tissue repair. Though the anti-inflammatory properties are likely to be attributable to more than one mechanism, as witnessed by its diverse biologic properties, including the down-regulation of plasmin...
activity, inhibition of neutrophil migration, as well as antiresorptive properties, much research is needed to clarify its physiologic role in wound healing among humans.\textsuperscript{71-73} And though the role of endogenous TSG-6 in inflammation requires further investigation, its potent anti-inflammatory effect suggests a role for TSG-6 in a negative feedback control of the inflammatory response.

1.7 Rodent Model

Efforts to contribute new knowledge in biological sciences, including periodontology, have come not only from human sources, but from animal models and cell cultures. Rodents, in particular, provide unique characteristics to evaluate microbial and host responses to compliment primate and human periodontal studies.\textsuperscript{74} Rats and mice are the most commonly used animals in research and testing. In fact, rats and mice account for approximately 90\% of all mammals used in scientific endeavors. The commercial availability, plus their small size, high reproductive rate, and minimal costs for purchase and maintenance have made them the most commonly used laboratory animal species. The contribution of animal studies to our understanding of medicine has proven vital. They have enabled researchers to unveil new insights in clinical experiments, which could otherwise not be performed on humans for ethical or logistical reasons.

1.8 Aim

This study is designed to test the hypothesis that TSG-6 protein will decrease the acute inflammatory response following gingival wounding in a rat model. This
experiment could provide valuable data on the feasibility of using TSG-6 for human clinical studies.
CHAPTER II

ANTI-INFLAMMATORY PROTEIN TSG-6 PROMOTES EARLY GINGIVAL WOUND HEALING: AN IN VIVO STUDY

II.1 Synopsis

II.1.1 Background

Human multipotent mesenchymal stromal cells (hMSCs) produce TNF-α-stimulated protein 6 (TSG-6). TSG-6 modulates proinflammatory cytokine cascades and enhances tissue repair. This study tested the effects of recombinant human (rh) TSG-6 on gingival wound healing within the first 2 days post-surgery.

II.1.2 Methods

Following gingival resection in 120 Sprague-Dawley rats, 2 µg rhTSG-6 in 5 µL of phosphate buffered saline (PBS) or the same volume of PBS solution was injected into gingival tissue approximating the surgical wound. Control animals did not receive injections. Tissue biopsies and blood were collected at 1-2, 6-8, 24 and 48 hrs post-surgery (n=10 per group). Specimens were analyzed via histological analysis and enzyme-linked immunosorbent assays (ELISA) for quantification and comparison of inflammatory markers IL-1β, IL-6, TNF-α and myeloperoxidase (MPO). Photographs were taken for a double blind clinical assessment at each time period. Weights were recorded for all animals pre- and post-surgery.

II.1.3 Results

Animals injected with rhTSG-6 had significantly less severe clinical inflammation at 6-8 (p=0.01228), 24 (p=0.01675), and 48 hours (p=0.0186). Sham and
control animals had more weight loss at 24 and 48 hours. Sham and control animals had more pronounced cellular infiltrate. rhTSG-6 treated animals had significantly less myeloperoxidase (MPO) (p=0.027) at 24 hours and IL-1β (p=0.027) at 24 & 48 hours. IL-6 showed marginal significant difference at 6-8 hours, but there was no significant difference for TNF-α.

II.1.4 Conclusion

rhTSG-6 reduced post-operative gingival inflammation by reducing levels of proinflammatory cytokines and cellular infiltrate and may offer significant promise as an anti-inflammatory agent for gingival surgery.

II. 2 Introduction

Inflammation is the body’s normal protective response to an injury, irritation, or surgery. Acute inflammation refers to a response that is abrupt in onset and of short duration. If the cause of the initial injury is not completely eliminated, acute inflammation will gradually give way to chronic inflammation, though no definitive time sequence.

The acute inflammatory response has three major components: hemodynamic changes, alterations in the permeability of vessels, and changes in the location and concentration of white blood cells⁴. Injury to tissues triggers a specific acute response that involves an exudative reaction of fluid, serum proteins, and leukocytes that leave the blood stream to enter the area of injury, often causing symptoms of pain, heat, redness and swelling. The events of the vascular response to injury, which also include release of polypeptide cytokines and growth factors from peripheral blood monocytes, aim to
localize and eliminate the injurious agents and remove damaged tissue components so the body can begin to heal.

Human adult stem/progenitor cells from bone marrow, referred to as mesenchymal stem cells or multipotent mesenchymal stromal cells (hMSCs), have been at the forefront of modern research. Throughout their course of study, evolving data has led to the development of various hypotheses regarding their role in wound repair. Among their therapeutic factors, MSCs express the multipotent anti-inflammatory protein tumor necrosis factor (TNF)-α-stimulated gene/protein 6 in response to tissue injury.47

TNF-α-stimulated gene/protein 6, or simply TSG-6, is a hyaluronan (HA)-binding protein that has since been implicated in the negative regulation of inflammatory tissue destruction. TSG-6 DNA was originally isolated from a cDNA library prepared from TNF-α-treated human fibroblasts.49 It is secreted by various cells, such as fibroblasts, upon stimulation by the proinflammatory cytokines tumor necrosis factor or interleukin 1. The rapid upregulation of TSG-6 in the presence of the pro-inflammatory cytokines TNF and IL-1 is consistent with its involvement in inflammatory processes.58 This association was confirmed by the detection of TSG-6 protein in synovial fluids of patients with a variety of conditions including rheumatoid arthritis, osteoarthritis, Sjögren’s syndrome, polyarthritic gout, and osteomyelitis.59 There are no studies to date that evaluate the properties of TSG-6 on gingival inflammation. Therefore, this study aims to test the effects of recombinant human (rh) TSG-6 on early gingival wound healing in an animal model.
II.3 Materials and Methods

The experimental model used in this study involved the creation of an acute inflammatory lesion; localized to the in the maxillary and mandibular gingival tissue in a rat model. The hyluronan (HA)-binding protein, rhTSG-6 (purchased from R & D Systems), was used to test its anti-inflammatory effects following injury. This protocol was submitted for review and approved by the Institutional Animal Care and Use Committee at Baylor College of Dentistry – a member of Texas A&M University System.

II.3.1 Study Animals

One hundred twenty, adult male (DOB:12/14/12), Sprague Dawley rats, (Harlan Laboratories), weighing approximately 400 grams were used in this study in an attempt to minimize interspecies variance. All animals were housed in individual polycarbonate cages and provided with standard rat chow pellets and water ad libitum. Rats were separated into three groups (experimental, sham, control) and were numbered for identification purposes via tail markings. All animals were given a 7-day acclimation period with a 12-hour light/12-hour dark cycle. Rats were monitored daily throughout the experimental period. General anesthesia was induced by administering Ketamine (0.08mL/100g) and Xylazine (0.04mL/100g) intramuscularly. Following onset of anesthesia, the animals were weighed and placed on a surgical table. An aseptic surgical technique was utilized. All protocols for anesthesia, post-operative care, and necropsy of the animal subject were approved by the Institute of Animal Care and Use Committee prior to commencement of study.
II.3.2 Surgical Procedures

Ketamine (0.08mL/100g) and Xylazine (0.04mL/100g) were combined for use as a general anesthetic and given as an intraperitoneal injection using a 1 ml syringe 23-25 gauge 5/8 inch needle. All rats were weighed prior to surgical procedures. Animals were numbered and divided among three groups:

Group 1: Experimental (40 animals)—gingivectomy + TSG-6
Group 2: Sham (40 animals)—gingivectomy + PBS
Group 3: Control (40 animals)—gingivectomy

Using a Miltex stainless steel disposable #12 scalpel, under 3.5x magnification via dental loupes, a standardized gingival wound was produced in one hundred and twenty animals. The wound consisted of a gingivectomy performed on the mandibular and maxillary anterior marginal and attached gingiva, removing a triangular piece of tissue between the central mandibular and maxillary incisors. Incisions were performed at an angle of 45° and at 2mm from each tooth surface. All measurements were made with a Hu-Freidy PCPUNC15 perio probe (Figure 1, A). The scalpel was inserted in the keratinized tissue so as to obtain a complete surgical transection of the gingiva (Figure 1, B). A Gracey 11-12 curette was utilized to remove excised gingival tissue. Immediately after tissue removal, 2 µg recombinant human TSG-6 (rhTSG-6) in 5 µL of PBS solution was injected into the gingival tissue approximating the surgical wound in forty experimental rats (Group 1). The depth of needle insertion, for delivery of the solution, was standardized using a rubber stopper 1 mm from the needle point (Figure 1, C). Injections were made with a 3/10cc insulin syringe 29 guage ½” needle. The same
volume of PBS solution was injected into the gingival tissue in forty additional rats (Group 2). The forty control animals (Group 3) were not injected following gingivectomy. All animals (Groups 1, 2 & 3) were assessed at 4 different time points:

1-2 hrs: 10 animals
6-8 hrs: 10 animals
24 hrs: 10 animals
48 hrs: 10 animals

Intra oral photographs were taken of all animals at time of sacrifice.

II.3.3 Specimen Recovery Procedures

At the time points allocated (1-2, 6-8, 24, and 48 hrs) post-surgery, all animals per specified group were euthanized. Euthanasia was accomplished via CO2 chamber, for ease of handling, prior to decapitation. Weights were recorded for all animals before decapitation.

At the time of sacrifice, blood was collected into test tubes from each animal. Samples were placed on ice into 15ml polypropylene tubes with 0.5M EDTA. Blood was spun down at 3000 rpm for 20 minutes. Plasma was collected and frozen at -20°C until further analysis of inflammatory markers could be assessed via ELISA assays.

Random allocation performed prior to surgery determined the distribution of maxillary and mandibular gingival tissue per method of analysis – histology or myeloperoxidase assay (MPO).

Tissue specimens were obtained from the incisional border at the site of injury with a 4mm Tru-Punch sterile disposable biopsy punch (Sklarcorp) (Figure 2).
Maxillary and mandibular specimens were separated, according to randomization chart, and placed into bottles labeled for histopathology, containing 4% paraformaldehyde, or MPO assay. All specimens were frozen until termination of all surgical procedures.

II.3.4 Gingival Surface Evaluation and Clinical Outcome Analysis

At the time of sacrifice, gingival wounds in the mandibular arch were photographed utilizing a Digital SLR camera with a DG 105 mm f/2.8 Macro EX Sigma Lens at a distance of approximately 12 inches from the specimen. Four photographs from each group, from each time point, were selected for clinical evaluation. Each photograph was then assigned a number corresponding to the degree of inflammation observed by 18 blinded volunteers comprised of periodontists and periodontal residents. All volunteers were given an initial description and photograph corresponding to the severity of inflammation to be observed. Subjects were asked to determine the severity of gingival inflammation using criteria presented to each subject before the survey. Severity was determined on a 3-point – (1) mild, (2) moderate, and (3) severe.

The assessment criteria provided, is as follows:

1 = mild inflammation or with slight changes in color and texture but not in all portions of marginal or papillary gingiva. Non-bleeding.

2 = moderate, bright surface inflammation, erythema, edema and/or hypertrophy of marginal or papillary gingiva.

3 = severe inflammation; erythema, edema and/or gingival hypertrophy of the unit or spontaneous bleeding, papillary, congestion or ulceration.
II.3.5 Histological Processing/ELISAs

Individual specimens were prepared for histological analysis using standard procedures for biopsy specimens. Tissue samples were fixed in 4% paraformaldehyde at 4°C. After dehydration, specimens were immersed in paraffin and processed for sectioning. Paraffin embedded specimens were sectioned at 5 microns thickness. Slides were stained with hematoxylin and eosin and studied under light microscope.

Tissue for ELISA testing was homogenized in 500ul TPER with HALT + EDTA as a protease inhibitor. Specimens were spun down for 20 minutes at 4°C 3000rpm. Supernatant was then pulled off and frozen at -20C until used for ELISA analysis.

II.3.6 Statistical Analysis

Before the initiation of this study, the sample size was determined to be 10 animals per treatment group per time point based on a power test with $\alpha = 0.05$. A Difference Z-test was performed on pre-surgical weight (PreSW) versus post-surgical weight (PostSW). Weights were tested separately and plotted using a boxplot. Simultaneous paired t-tests were performed to determine between which groups exhibited a significant difference. To analyze the presence of significant differences for each protein and enzyme at each point of time between each subgroup a Kruskal-Wallis Rank Sum Test for Two-Sided One-Way ANOVA was performed. For all tests level of marginal significance is $0.05 \leq \alpha \leq 0.1$, and the level of significance $\alpha < 0.05$. The survey data was analyzed using Kruskal-Wallis Rank Sum Test. A plot with associated p-
values at each time category was produced. Because the data is nonparametric, and n<4, the Mann-Whitney two sample comparison test was forgone.

II.4 Results

II.4.1 Clinical Assessment

From the time of gingivectomy until the time of sacrifice (hrs to days), no signs of infection or adverse healing were noted. Clinical evaluation at the time of sacrifice for the 1-2 hour group revealed erythematous and edematous gingival tissue. Tissues continued to appear inflamed and swollen at 6-8 hours post-surgery. By 24 hours, gingiva appeared moderately erythematous. An early wound covered with a blood clot was generally present. At 48 hours, gingival tissue appeared generally pink and non-bleeding. Results of the survey data are presented on Figure 3. The means of each group at each time point were plotted. Overall, the data conclusively indicates that at each time point, Group 1 (TSG-6) showed lower inflammation as a result of visual analysis by the subjects at 6-8 hrs (p=0.01228), 24 hrs (p=0.01675), and 48 hrs (p=0.01806). There was a significant difference between Group 1(TSG-6) and Group 2 (PBS) (p<0.001) and a significant difference between Group 1 and Group 3 (Control) (p=0.016). Additionally, Group 2 showed the highest levels of inflammation at all points. Unexpectedly, Group 3 showed an unusual score increase between 6-8 hrs and 24 hrs.

II.4.2 Weights

All animals were weighed at baseline and at time of sacrifice. The mean weights for the 24-hr and 48-hr group were tabulated. The difference from the mean pre-surgical to post-surgical weights for the 24-hr Group 1 (TSG-6), Group 2 (PBS) and Group 3
(Control) were 1.8, 8.9, and 10.8 grams, respectively. The difference from the mean pre-surgical to post-surgical weights for the 48-hr Group 1 (TSG-6), Group 2 (PBS) and Group 3 (control) were 5.7, 12.1, and 11.8 grams, respectively. The mean with a 95% confidence interval was tabulated for all animals and presented in Figure 4. It is evident that overall, post-surgical weights were lower than pre-surgical weights. Differences in mean weights were -3.8, -11.1, and -16.3 for Group 1, 2 and 3, respectively. A box plot comparison of the differences in pre-surgical and post-surgical weights versus group illustrates that the Group 1 (TSG-6) showed a lower loss in weight (calculated as $|PostSW - PreSW|$) than the other two groups (Figure 4). Additionally, simultaneous paired t-tests were performed to determine between which groups exhibited a significant difference post-surgically. There was a significant difference between Group 1 (TSG-6) and Group 2 (PBS) ($p < .001$) and a significant difference between Group 1 and Group 3 (Control) ($p = .016$). There was no significant difference between Groups 2 & 3.

II.4.3 Histology

Three tissue specimens per treatment group and time point were analyzed. Specimens chosen for histologic analysis were obtained from the same animals utilized in the visual assessment examination. H & E stain showed similar amounts of cellular infiltrate at 1-2 hours post-surgery. Cellular infiltrate increased over the subsequent hours analyzed. Generally, Groups 2 & 3 (PBS & Control) showed markedly more infiltrate compared to Group 1 (TSG-6) at 6-8, 24, and 48 hours (Figure 5).
II.4.4 ELISAs

Myeloperoxidase (MPO) assay revealed an increase in MPO levels between 1-2 hours and 24 hours, but a slight decrease between 24 and 48 hours. All MPO levels showed similar values at the 1-2 hour time point, and thus showed no significant difference. There were marginal significant differences noted at 6-8 and 48 hours (p=0.66) and a significant difference between the three groups at 24 hours (p=0.027). Group 1 showed a 43.8% reduction in MPO levels at 24 hours when compared to Group 3. The group treated with TSG-6 had the lowest levels between 6-8 and 24 hours. The control group showed the highest level of MPO for 24 to 48 hours. For each data point n=3 (Figure 6).

Blood samples for Interleukin 1β (IL-1β) were analyzed via ELISA; however, levels of IL-1β were not detectable, indicating no systemic involvement. Tissue samples were therefore utilized to assess cytokine levels. Data revealed an increase in IL-1β between 1-2 and 24 hours, but decrease between 24 and 48 hours except Group 1. The test showed no significant difference at the initial point, 1-2 hours (p=0.670), but there were marginal significant differences at 6-8 hours (p=0.051), and a significant difference between the three groups at 24 and 48 hours (p=0.027). Group 1 showed a 68.0% reduction in levels of IL-1β at 24 hours when compared to Group 3. Overall, the group treated with the TSG-6 has the lowest levels between 6-8 hours, 24 hours, and 48 hours. For each data point n = 3 (Figure 6).

Interleukin 6 (IL-6) was elevated between 1-2 hours and 6-8 hours, but there was a decrease in IL-6 levels between 6-8 hours and 24 hours and between 24 hours and 48
hours. Overall, the group treated with TSG-6 had the lowest levels between 6-8 hours and 24 hours. The control group showed the highest level of IL-6 for 6-8, 24, and 48 hours. There was a marginal significant difference at 6-8 hours (p=0.099) (Figure 6). Group 1 showed a 50.9% reduction in levels of IL-6 at 6-8 hours when compared to Group 3.

II.5 Discussion

Healing of periodontal tissue after surgical treatment has long been a subject of interest. In the current study, the anti-inflammatory effects of tumor necrosis factor-α-stimulated gene protein 6 (TSG-6) was evaluated following injection into an acute gingival wound in Sprague-Dawley rats. Within the limits of this study, injection of 2 µg TSG-6 was found to accelerate wound healing by reducing inflammatory cell infiltrate and reducing cytokines IL-1β and IL-6. Significant differences were seen at 6-8, 24, and 48 hours, as observed clinically and microscopically.

Similar findings have been reported when 2µg TSG-6 were injected into the anterior chamber of rats’ eyes following mechanical injury. In addition, TSG-6 reduced corneal inflammation and opacity in a dose-dependent manner. In transgenic mice, its anti-inflammatory effects were observed when an over expression of the gene decreased inflammation and joint destruction following induced arthritis. Likewise, TSG-6 was associated with a lower density of macrophages and infiltrating leukocytes, reduced levels of pro-inflammatory cytokines, and increased levels of anti-inflammatory cytokines following infusion of MSCs into the CNS of post traumatic brain injuries in rats. The anti-inflammatory activity of TSG-6 has been attributed to its ability to
suppress neutrophil migration into the site of inflammation and inhibit components in
the inflammatory network of proteases.  

The present study showed that intra-gingival injection of rhTSG-6 decreased the
infiltration of neutrophils, pro-inflammatory cytokines, and reduced clinical signs of
inflammation following gingival transection within the first 2 days following surgery.

All rats were weighed pre-surgically and post-surgically immediately prior to
decapitation. Weight differences were analyzed and compared between 24 and 48 hour
groups. Simultaneous t-tests were performed to determine between which groups
existed a significant difference. All animals injected with TSG-6 showed less weight
loss compared to animals not injected with TSG-6. Animals not injected with the
protein evidently ate less or stopped eating after surgery, indicating that in addition to
decreasing inflammation, TSG-6 may have some influence on pain perception. Pain is a
subjective sensation. From the site of injury, impulses travel to the central nervous
system and to higher centers of the brain responsible for determining the magnitude of
pain. Vasodilation and increases in vascular permeability at the site of injury result in
heat, redness, and swelling; while exuded fluid produces pressure on sensory nerve
endings. Pain in an animal is often inferred from the absence of normal behaviors such
as alertness, mobility, groomed coat, or a good appetite. One can speculate that the
animals that received TSG-6 were experiencing less pain/discomfort, primarily
associated with a reduced state of inflammation, and subsequently were able to maintain
their eating habits compared to control animals.

Within hours of injury, inflammatory cells (predominantly neutrophils and
monocytes) populate the clot. These cells cleanse the wound of microorganisms and necrotic tissue via phagocytosis and release of enzymes and toxic oxygen products. Histologically, cellular infiltrate was less pronounced in specimens obtained from animals receiving rhTSG-6 injections, most notably at 6-8, 24 and 48 hours.

In this study, quantification of neutrophils was assessed both morphologically and biochemically. Myeloperoxidase (MPO), released from the granules of neutrophils and monocytes in response to the activation of leukocytes plays an important role in several inflammatory conditions. MPO is a lysosomal protein that produces hypochlorous acid (HOCl) from hydrogen peroxide and chloride anion. The neutrophil uses the hypochlorous acid, which is cytotoxic, to kill bacteria and other pathogens. Hypochlorous acid is an extremely powerful oxidant that rapidly attacks a wide range of target molecules. For a quantitative measure of neutrophil infiltration, gingival specimens were assayed for MPO concentration. Because TSG-6 has been shown to directly suppress neutrophil infiltration, assessment of MPO concentration was chosen for evaluation, as opposed to C-reactive protein (CRP). CRP is an acute phase protein synthesized by the liver in response to factors released predominately by macrophages and fat cells. As anticipated, TSG-6 had significantly decreased levels of MPO in the gingiva. This finding correlates with our histological observations; reduced cellular infiltrate in specimens injected with TSG-6.

A similar pattern was observed in data obtained by assays of the tissue for expression of proinflammatory cytokines. The expression of proinflammatory cytokines, IL-1β and IL-6, were markedly reduced by TSG-6 treatment. Similar findings
in expression of cytokine levels were seen when TSG-6 was injected into the eyes of rats following injury. Neutrophils, monocytes, and other cells produce innate immune cytokines such as IL-1, IL-6, and TNF-α after being summoned to the site of injury by chemokines. In this study, levels of TNF-α (data not shown) were undetectable. These findings parallel those of Oh et al and colleagues. In their study, TNF-α was not detected in treated or untreated corneas by ELISA or real-time PCR analysis. TNF-α, while known to stimulate the acute phase reaction, is produced chiefly by activated macrophages. It is a potent chemoattractant for neutrophils and promotes the expression of adhesion molecules on endothelial cells. Inconsistencies with other data sets in this study remain unclear.

In addition, it is of interest that intragingival injection of TSG-6 had no effect on plasma cytokine levels as determined by ELISA, indicating intra-gingival injection of 2µg rhTSG-6 had no systemic involvement. TSG-6 acted locally on tissues to down regulate inflammatory mediators. This is in contrast to earlier findings by Getting et al and colleagues in which as little at 1 µg TSG-6 was administered at a site remote from the inflammatory environment. Reductions in neutrophil influx and inhibitions of IL-1β were observed. Their findings suggest that TSG-6 does not need to be given local to the site of inflammation but, rather, acts via the circulation. Further studies are needed to corroborate these findings.

The mechanism of action observed by the anti-inflammatory effects of TSG-6 is not well understood. However, in recent studies, Inter-α-inhibitor, an abundant plasma protein with anti-proteolytic activity, consisting of three polypeptides: two heavy chains
and one light chain, has been shown to bind to hyaluronon molecules via covalent bonds; a reaction thought to be mediated by TSG-6. TSG-6 has been shown to be tightly bound to the hyaluronon-linked heavy chains. This complex has been implicated in stabilizing extracellular matrix by cross-linking hyaluronan molecules and protect hyaluronan against fragmentation by reactive oxygen species, enhancing tissue repair. Though the anti-inflammatory properties are likely to be attributable to more than one mechanism, as witnessed by its diverse biologic properties, including the down-regulation of plasmin activity, inhibition of neutrophil migration, as well as antiresorptive properties, much research is needed to clarify its physiologic role in wound healing among humans.

Inflammation, though protective in nature, can cause cellular damage if not controlled. For instance, neutrophil granules contain more than 20 proteolytic enzymes, of these, elastase, collagenase, and gelatinase have the greatest potential to act as mediators of tissue destruction. These enzymes are able to attack extracellular matrix. In addition, hypochlorous acid, induced by myeloperoxidase, can inactivate key proteinase inhibitors while activating latent neutrophil proteinases. Lysosomal enzymes also become susceptible to host tissue. Leakage of lysosomal enzymes during phagocytosis or cell death may result in tissue damage. Reverse endocytosis and regurgitation during feeding (phagocytosis) may also permit release of these lytic enzymes into surrounding tissues. Damage to host tissue is almost inevitable. Efforts to control or limit the inflammatory burden are paramount in restoring the body to health.

Resective surgeries in non-inflamed tissue are common practices in the field of
periodontics for treatment of altered passive eruption, over grown gingiva, crown lengthening, gingival recontouring, gingival flap retraction for access to impacted teeth/roots, or for implant placement. Extension of the incision as well as tissue manipulation and length of surgery could affect the extent of swelling. Post-operative discomfort resulting from swelling and pain following different surgical strategies is an area of great interest. Attention has been focused on non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroid therapy administered by diverse routes (orally, IM, IV, topically) before or after surgery. Side effects of corticosteroids include, but are not limited to hyperglycemia, adrenal insufficiency, and reductions in immune response. Efforts to find new anti-inflammatory agents that reduce post-operative discomfort are well underway.

Future studies are needed to investigate and substantiate our prediction that TSG-6 decreases pain as well as inflammation. Placing animals into feeding chambers and/or analyzing pain pathways and alternations thereof could provide significant insight into the molecular mechanisms of TSG-6. Replications of these experiments with larger treatment groups would be of great value. This study, though primitive in nature and small in sample size, demonstrates that a single intragingival injection of TSG-6 reduced local post-operative inflammation, limiting destruction of the tissue by the normal but excessive inflammatory response to a sterile injury.
CHAPTER III
CONCLUSION

To the best of our knowledge, this is the first invivo study to compare the clinical and histological effects of TSG-6 on gingival inflammation in an animal model. Within the limits of the study, injection of 2µg rhTSG-6 significantly reduced post-operative inflammation by decreasing levels of proinflammatory cytokines IL1β (68%) and IL-6 (50.9%), neutrophil infiltration (43.8%), erythema, and bleeding within the first few hours and days after injury. TSG-6 offers substantial promise for patients undergoing gingival resective surgery.
REFERENCES


APPENDIX

Figure 3. Surgical Procedure

A. Pre-Surgical Measurements
(PCPUNC15 perio probe)

B. Surgical Transection of the Gingiva
(Miltex stainless steel disposable #12 scalpel)
Figure 3. Continued

C. Injecting 2µg TSG-6/ PBS Solution
(3/10cc insulin syringe U-100 29g ½” needle w/ rubber stopper 1 mm from tip end)
Figure 4. Specimen Collection
(4mm Tru-Punch sterile disposable biopsy punch)
Figure 5. Clinical Assessment

A. 24 HRS Post-Surgery.
Group 1 (rh TSG-6), 2 (PBS), 3 (CONTROL); respectively.

B. 48 HRS Post-Surgery.
Group 1 (rh TSG-6), 2 (PBS), 3 (CONTROL); respectively.
Figure 5. Continued

C. Mean Score With Respect To Time By Group

![Graph showing mean score with respect to time by group](image)
Figure 6. Box Plot Comparison

<table>
<thead>
<tr>
<th></th>
<th>Pre Surgery (PreSW)</th>
<th>Post Surgery (PostSW)</th>
<th>Difference (D)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 3 Control</strong></td>
<td>412.6 (365.4, 459.8)</td>
<td>396.3 (371.9, 420.7)</td>
<td>-16.3 (-66.9, 34.3)</td>
</tr>
<tr>
<td><strong>Group 2 PBS</strong></td>
<td>401.9 (381.0, 422.7)</td>
<td>390.8 (369.4, 412.1)</td>
<td>-11.1 (-23.2, 1.0)</td>
</tr>
<tr>
<td><strong>Group 1 TSG-6</strong></td>
<td>398.2 (370.1, 426.2)</td>
<td>394.4 (366.4, 422.4)</td>
<td>-3.8 (-10.7, 3.2)</td>
</tr>
</tbody>
</table>
Figure 7. Histology
H & E stain of specimens per treatment group per time point

<table>
<thead>
<tr>
<th>Time Point</th>
<th>TSG-6</th>
<th>PBS</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2 hrs</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>6-8 hrs</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>24 hrs</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>48 hrs</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>
Figure 8. ELISAs

A. MPO Assay
Units of MPO are $\frac{ng}{ug}$ of protein

B. IL-1β
Units of IL-1β are $\frac{pg}{ug}$ of protein
Figure 8. Continued

C. IL-6
Units of IL-6 are \( \frac{pg}{\mu g} \) of protein