

EFFECTS OF TOBACCO ON HUMAN GINGIVAL FIBROBLASTS

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DEDICATION

To my parents, Shunquan Zhang and Jianhuang Liu, whose strength, wisdom and love help me survive and succeed.

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ABSTRACT

Weiping Zhang

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The negative health consequences of smoking are widely recognized, but there are still about 20% of the people in United States using tobacco products. Cigarette smoke condensate (CSC), the particulate matter of cigarette smoke, is comprised of thousands of chemicals (e.g., nicotine). Secondary only to bacterial plaque, cigarette smoking is a major risk factor for periodontal disease. Human gingival fibroblasts (HGFs) are the main cellular component of periodontal connective tissues. During the development of periodontal disease, collagen degradation occurs. Collagen is the major extracellular matrix component of the gingiva. The major extracellular matrix degrading enzymes produced by the HGFs are the matrix metalloproteinases (MMPs). The MMPs are mainly modulated by the tissue inhibitors of metalloproteinases (TIMPs). In this dissertation, three studies aimed at understanding the effects of tobacco on human gingival fibroblasts and their mechanisms have been conducted: the effects of CSC on HGF-mediated collagen degradation; comparison of the effects of CSC on HGFs with that of nicotine; and the combined effects of CSC and bacteria on HGFs.

The cell proliferation of HGFs decreased and cytotoxicity increased in HGFs treated with increasing concentrations of CSC. CSC increased the collagen degrading ability of the HGFs by altering the production and localization of MMPs and TIMPs.

Nicotine is one of the major components and the most pharmacologically active agent in tobacco. The percentage of nicotine in the CSC was 2.4%. CSC (100 µg/ml) increased the collagen degrading ability of the HGFs by affecting membrane associated MMP-2, MMP-14, and TIMP-2, but the level of nicotine in the CSC may only play a limited role in this process.

Porphyromonas gingivalis (*P. gingivalis*) is an opportunistic pathogen involved in periodontal disease. The combined effects of CSC and *P. gingivalis* supernatant increased HGF-mediated collagen degradation by destroying the balance between the MMPs and TIMPs at the protein and mRNA levels.

This project demonstrated that tobacco (with or without *P. gingivalis*) increased HGF mediated collagen degradation, as seen in the periodontal disease, through altering the MMPs and TIMPs.

L. Jack Windsor, Ph.D., Chair

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LIST OF ABBREVIATIONS

AVOVA	Analysis of Variance
CSC	Cigarette Smoke Condensate
DMEM	Dulbecco's Modified Eagle's Media
DMSO	Dimethyl Sulfoxide
ECM	Extracellular Matrix
FBS	Fetal Bovine Serum
GCF	Gingival Cervical Fluid
GRO- α	Growth Regulated Oncogene-Alpha
HGFs	Human Gingival Fibroblasts
IL	Interleukin
IRB	Institutional Review Board
LDH	Lactate Dehydrogenase
LPS	Lipopolysaccharide
MMPs	Matrix Metalloproteinases
MT-MMP	Membrane-Type Matrix Metalloproteinases
NIH	National Institute of Health
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
<i>P. gingivalis</i>	<i>Porphyromonas gingivalis</i>
RT-PCR	Reverse Transcription Polymerase Chain Reaction
PVDF	Polyvinylidene Fluoride

SD	Standard Deviation
SDD	Subantimicrobial-Dose Doxycycline
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SPSS	Statistical Package for Social Science
TIMPs	Tissue Inhibitors of Metalloproteinases
TPM	Total Particulate Matter
WST-1	Water-Soluble Tetrazolium-1

CHAPTER ONE

Introduction

Smoking is one of the main etiologies of human death every year. It is responsible for multiple cancers, cardiovascular diseases, respiratory diseases, and many other harmful health conditions. Furthermore, cigarette smoking is a risk factor for oral cancer, oral mucosal lesions, and periodontal disease.

Periodontal disease is a chronic infectious disease of the supportive tissues of teeth. It is the main cause of tooth loss among adults. Periodontal disease is very common and affects about 90% of the worldwide population (Pihlstrom et al., 2005). Just behind bacterial plaque, cigarette smoking is ranked second as a major risk factor for periodontal disease (Fang and Svoboda, 2005; Ryder, 2007) and even promotes its development (Barbour et al., 1997; Hilgers and Kinane, 2004; Pihlstrom et al., 2005; Rivera-Hidalgo, 2003). It is evident that smoking patients have greater bone loss, greater attachment loss, and deeper periodontal pockets than non-smoking patients (Bergstrom, 2004). Furthermore, the outcome of the treatment of periodontal disease is also less favorable in smoking patients (Bergstrom, 2004). In the USA, smoking contributes to about half of the risk of developing periodontal disease (Hujoel et al., 2003; Tomar and Asma, 2000).

During the development of periodontal disease, collagen degradation occurs. Collagen is the major extracellular matrix component of the gingiva. The major extracellular matrix degrading enzymes, produced by the human gingival fibroblasts (HGFs), are the matrix

metalloproteinases (MMPs). The MMPs play important roles in both physiological and pathological conditions. Several members of the MMP family have been shown to be involved in periodontal tissue destruction (Hannas et al., 2007; Sorsa et al., 2004). The MMPs, which are secreted by various cell types and found in most body fluids and tissues, are mainly modulated by the tissue inhibitors of metalloproteinases (TIMPs). The balance in the activities of the MMPs/TIMPs is considered to be important to periodontal health and tobacco may affect this balance (Verstappen and Von den Hoff, 2006).

Cigarette smoking condensate (CSC) is the particulate matter of cigarette smoke and contains thousands of chemicals (Gao et al., 2005b). Nicotine is one of the pharmacologically active agents in CSC. It has been used to study the effects of smoking on periodontal tissue. Nicotine can increase HGF-mediated collagen degradation at 250 µg/ml, partly through the activation of membrane-associated MMPs (Zhou et al., 2007). Nicotine (250 µg/ml), *Porphyromonas gingivalis* (*P. gingivalis*) lipopolysaccharide (LPS), or a combination of both agents can increase the production of multiple cytokines and growth factors in HGFs (Almasri et al., 2007). Other previous studies demonstrated that nicotine affects gingival blood flow (Johnson et al., 1991; Morozumi et al., 2004), cytokine production (Wendell and Stein, 2001), and connective tissue cells such as fibroblasts in aspects of cell morphology, attachment to substrates, and protein synthesis and secretion (Chamson et al., 1982; Peacock et al., 1993). However, nicotine is just one of the thousands of chemicals in CSC. Currently, there is little data on the effects of CSC

on HGFs. Thus, the aims of this study were to further examine the effects of CSC on HGFs.

Special Aims

The overall goal of this study was to examine the CSC stimulated collagen-degrading ability of HGFs. The study was accomplished by the following three specific aims.

Specific Aim 1: To assess the effects of CSC on the collagen-degrading ability of HGFs.

Periodontal disease has been recognized to be a host-mediated inflammatory disease. The host responses to periodontal infection plays a major role in the pathogenesis of periodontal disease (Pihlstrom et al., 2005). The health of periodontal tissues depends on the normal functions of periodontal cells. The attachment, migration, growth, and differentiation of periodontal cells are critical steps in the repair and regeneration of periodontal tissues. Human gingival fibroblasts (HGFs) are the main cellular component of periodontal connective tissues. Therefore, the normal functions of HGFs are very important to periodontal health.

During the development of periodontal disease, collagen degradation occurs. Collagen is the major extracellular matrix component of the gingiva. The major extracellular matrix degrading enzymes produced by the HGFs are the matrix metalloproteinases (MMPs). These enzymes are a group of zinc-dependent endopeptidases that include the collagenases, gelatinases, stromelysins, and membrane-associated MMPs. Several

members of the MMP family have been shown to be involved in periodontal tissue destruction and these include MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-13, and MMP-14 (Hannas et al., 2007; Sorsa et al., 2004). The MMPs are mainly modulated by the tissue inhibitors of metalloproteinases (TIMPs), which are secreted by various cell types and found in most body fluids and tissues.

Cigarette smoking is a risk factor for periodontal disease and cigarette smoking condensate (CSC) is the particulate matter of cigarette smoke. Currently, there is little data on the effects of CSC on HGFs. Thus, the goal of this specific aim was to examine the effects of CSC on the collagen-degrading ability of HGFs, as well as on the production of multiple MMPs and TIMPs.

Specific Aim 2: To compare the effects of CSC and nicotine on HGF mediated collagen-degradation and examine their mechanisms.

CSC is comprised of thousands of chemicals, which are powerful inducers of inflammatory responses (Gao et al., 2005b). CSC may destroy the balance of the MMPs/TIMPs and alter the localization of MMPs and TIMPs (especially membrane associated MMP-2, MMP-14, and TIMP-2) to promote the degradation of extracellular matrix (ECM), which occurs in periodontal disease (Zhang et al., 2009). Nicotine is one pharmacologically active agent in CSC. Many previous studies demonstrated that nicotine may play important roles in the development of periodontal disease (Almasri et al., 2007; Chamson et al., 1982; Johnson et al., 1991; Morozumi et al., 2004; Peacock et

al., 1993; Wendell and Stein, 2001; Zhou et al., 2007). However, there is no data that compares the effects of nicotine and CSC on HGFs. The goal of this specific aim was to compare the effects of CSC with nicotine on the collagen degrading ability of HGFs and the expression of selected MMPs and tissue inhibitors of metalloproteinases (TIMPs).

Specific Aim 3: To assess the combined effects of CSC and *Porphyromonas gingivalis* on the collagen degrading ability of HGFs.

Porphyromonas gingivalis (*P. gingivalis*) has been implicated as a major etiologic agent in the development and progress of periodontal disease (Andrian et al., 2006). *P. gingivalis* can disrupt MMP-9 production (Andrian et al., 2006; DeCarlo et al., 1998; DeCarlo et al., 1997) and elevate MMP-2 production in periodontal ligament cell cultures (Chang et al., 2002b). Purified gingipains, trypsin-like proteinases from *P. gingivalis*, can up-regulate MMP-8 and MMP-3 expression in rat mucosal epithelial cells (DeCarlo et al., 1998) and activate latent MMPs (DeCarlo et al., 1997; Grayson et al., 2003). Furthermore, *P. gingivalis* can up-regulate the collagen degrading ability of some human gingival fibroblast (HGF) cell lines, in part, by increasing MMP activation and by lowering the TIMP-1 protein level, as well as by affecting the mRNA expression of multiple MMPs and TIMPs (Zhou and Windsor, 2006).

Numerous studies have evaluated the effects of either *P. gingivalis* and/or nicotine on HGF function (Almasri et al., 2007; Bosco et al., 2007; Makino et al., 2008; Mineoka et al., 2008; Noiri et al., 2004). However, nicotine is just one of the active components in

CSC and no data about the combined effects of CSC and *P. gingivalis* on HGFs has been reported. Given the strong association between periodontal disease and *P. gingivalis*, as well as between periodontal disease and smoking, the goal of this specific aim was to investigate the combined effects of CSC and *P. gingivalis* on the collagen degrading ability of HGFs, as well as their effects on multiple MMPs and TIMPs at the protein and mRNA levels.

Background and Significance

Cigarette Smoking

The negative health consequences of smoking are widely recognized. However, still about 20% of the people in the United States use tobacco (Office-of-Applied-Studies, 2005). Smoking harms virtually every organ in the body. United States Surgeon General's Report linked cigarette smoking to lung cancer and cardiovascular disease more than 40 years ago (Guthrie, 1966). Since then, the list of smoking-related health effects has grown and cigarette smoking is recognized as the major preventable cause of death in the United States. Smoking individuals are generally less healthy with impaired immune systems and increased risk of infections. Studies have shown that smoking is associated with diabetes, skin diseases, and gastrointestinal diseases (Johnson and Guthmiller, 2007; Winn, 2001). Furthermore, passive smoking or involuntary exposure to tobacco smoke may result in severe health consequences (Johnson and Guthmiller, 2007; Winn, 2001).

CSC is a complex mixture that contains more than 6,000 chemicals (e.g., nicotine, cadmium, phenol, anthracyclic hydrocarbons, nitrosamines, heavy metals and chemical carcinogens) (Gao et al., 2005b). Based on the CSC manufacturer's information (Murty Pharmaceuticals, Lexington, KY), CSC is usually prepared by using a Phippps-Bird 20-channel smoking machine designed for Federal Trade Commission (FTC) testing. The smoke particulates are collected on Cambridge glass fiber filters from the University of Kentucky's standard cigarettes (1R3F: University of Kentucky, KY) and dissolved in dimethylsulphoxide (DMSO) at 40 mg/ml. Every cigarette produces 26.1 mg of CSC (Murty Pharmaceuticals, Lexington, KY) with 2.4% nicotine.

Periodontal Disease

Widely defined periodontal disease includes any inherited or acquired disorder of the periodontium. It may origin from development, inflammation, trauma, neoplasm, genetic disease, or metabolic disorders (Pihlstrom et al., 2005). However, the term periodontal disease usually refers to the common inflammatory disorders of gingivitis and periodontitis, which are caused by pathogenic microflora in the biofilm or dental plaque. These dental plaques form adjacent to the teeth on a daily basis (Pihlstrom et al., 2005). Gingivitis is the mildest form of periodontal disease. It is caused by bacterial biofilm, which accumulates on teeth adjacent to the gingiva (gums). Gingivitis does not affect the underlying teeth supporting structures and is reversible. Untreated gingivitis may develop into periodontitis. Periodontal disease results in loss of bone supporting tissues and connective tissues. Periodontitis is a major cause of tooth loss in adults. Periodontal disease is highly prevalent and can affect up to 90% of the worldwide population

(Pihlstrom et al., 2005). In addition to pathogenic bacteria in the dental plaque, genetic and environmental factors, especially tobacco use, contribute to periodontal disease.

Although bacteria are necessary for periodontal disease to occur, a susceptible host is also needed. The immune-inflammatory responses develop in the periodontal tissues in response to the chronic presence of bacteria plaque. These responses result in destruction of structural components of the periodontium, which leads, ultimately, to the clinical signs of periodontal disease (Pihlstrom et al., 2005).

Both the host and bacteria in periodontal biofilm can release proteolytic enzymes. These enzymes can damage tissue. Chemotactic factors, which recruit polymorphonuclear leucocytes (PMNs) into the tissues, can also be released; if sustained, various enzymes that break down tissues can be released by the PMNs (Pihlstrom et al., 2005). These enzymes include the MMPs, cathepsins and other proteinases (Ryder, 2007).

Tobacco use is one of the most significant risk factors for the development of periodontal disease. Smokers are much more likely to develop periodontitis than non-smokers (Bergstrom, 2004). Oral smokeless tobacco can also result in gingivitis and loss of tooth support tissue (Robertson et al., 1990). Furthermore, smoking has strong negative effects in response to periodontal treatment and other oral surgical interventions (Bergstrom, 2004). The proposed mechanisms for the negative periodontal effects of smoking may include but not be restricted to: decreasing immunoglobulin G2 production; shifting in neutrophil functions towards destructive activities (e.g., superoxide and hydrogen

peroxide generation); causing negative effects on cytokine and growth factor production (e.g., interleukin-1beta, interleukin-8, and tumor necrosis factor-alpha); and even inhibiting fibroblast growth, attachment and collagen production (Johnson and Guthmiller, 2007)

The health of periodontal tissues depends on the normal functions of periodontal cells. The attachment, migration, growth, and differentiation of periodontal ligament cells are critical steps in the repair and regeneration of periodontal tissues (Ryder, 2007). Human gingival fibroblasts are the main cellular component of periodontal connective tissues. Therefore, they are crucial in maintaining the connective tissues which support and anchor the teeth (Phipps et al., 1997).

Matrix Metalloproteinases (MMPs)

MMPs are a large family of zinc-dependent endopeptidases, which are capable of digesting the ECM. They include the collagenases, gelatinases, stromelysins, membrane-associated MMPs, and others (Table 1.1) (Birkedal-Hansen et al., 1993; Chaussain-Miller et al., 2006; Sorsa et al., 2004). At least 25 members of the MMP family have been discovered to date.

The MMPs are responsible for a wide range of proteolytic events. They play important roles in both physiological conditions (e.g., wound healing and tooth formation) and pathological conditions (e.g., inflammatory diseases and tumor invasion) (Sorsa et al., 2004). MMPs have also been shown to be involved in the functional regulation of host

non-ECM molecules, which include growth factors and their receptors, cytokines and chemokines, adhesion receptors and cell surface proteoglycans, and a variety of enzymes. As for all secreted proteinases, the catalytic activity of the MMPs is regulated at multiple points: (1) gene expression, (2) compartmentalization (i.e., pericellular accumulation of enzyme), (3) proenzyme (or zymogen) activation, and (4) enzyme inactivation, as well as (5) substrate availability and affinity (Ra and Parks, 2007). Most of the MMPs appear to be secreted as proenzymes. By cleaving the propeptides of the proMMPs, proMMPs can be activated to perform proteolysis. Some MMPs can activate other members of the MMP family. For example, MMP-14 can activate MMP-2 directly or indirectly by forming MMP-2-TIMP-2-MMP-14 complexes; MMP-2 and MMP-3 can activate MMP-9; and MMP-3 can activate MMP-1 (Sorsa et al., 2004).

TIMPs, which are secreted by multiple cell types and are found in most body fluids and tissues, are important endogenous MMP inhibitors. So far, four TIMPs (TIMP-1, -2, -3 and -4) have been identified (Sorsa et al., 2004). Usually, TIMPs bind to MMPs at a ratio of 1:1. The TIMPs fit into the active-site cleft of MMPs in a manner similar to that of the substrate. By forming complexes with MMPs, they may block activation of latent pro-MMPs or influence their ability to hydrolyze a particular substrate. The TIMP-2 molecule serves as a bridging molecule for the binding of latent pro-MMP-2 to active MMP-14 on the cell membrane. Another active MMP-14 in the cell membrane cleaves the propeptide domain of proMMP-2 in this trimolecular complex, which activates pro-MMP-2. Therefore, more TIMP-2 could be recruited to the cell membrane to enhance

MMP-2 activation (Strongin et al., 1995). The balance of the MMPs/TIMPs is important to health (Verstappen and Von den Hoff, 2006).

The MMPs play major roles in the development of periodontal disease. Several members of the MMP family (e.g., MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-13, and MMP-14) have been shown to be involved in periodontal tissue destruction (Sorsa et al., 2004). For instance, MMP-2 and MMP-9 participated in the tissue destruction seen in periodontitis (Hannas et al., 2007). The amount of the gelatinases (MMP-2 and MMP-9) increased during periodontal disease, while conventional periodontal treatment effectively reduced the levels of these enzymes (Makela et al., 1994). Furthermore, increased MMP-14 (membrane-type I MMP) occurred in the subepithelial fibroblasts in inflamed gingiva (Tervahartiala et al., 2000). MMP-14, by activating pro-MMP-2, -8, and -13, is responsible for collagenous and non-collagenous extracellular matrix destruction (Wahlgren et al., 2001). The subantimicrobial-dose doxycycline (SDD), a MMP inhibitor, along with the traditional mechanical therapies, has been widely used as an adjunctive systemic therapy in the management of periodontal disease (Giannobile, 2008).

CSC can also affect the production of MMPs. Exposure of endothelial cells to CSC resulted in the upregulation of MMP-1, MMP-8, and MMP-9 mRNA expression (Nordskog et al., 2005). Nicotine can bind to the root surfaces of teeth, and alter gingival and periodontal ligament fibroblast attachment and proliferation *in vivo* (Chang et al., 2002a; Gamal and Bayomy, 2002; Giannopoulou et al., 1999; James et al., 1999).

Nicotine itself may increase the destruction of the gingival ECM during smokeless tobacco use (Tipton and Dabbous, 1995). In an animal model, after cutaneous tissue exposure to cigarette smoke, an increase of basal MMP-2 (pro- and active form) occurs; however, TIMP-1 and 2 expression levels did not change, which could result in a net increase in matrix degradation (Fortino et al., 2007).

Porphyromonas gingivalis

P. gingivalis is a gram-negative black-pigmented strict anaerobic bacterium (Andrian et al., 2006). *P. gingivalis* produces a broad array of potential virulence factors, which are involved in tissue colonization and destruction, as well as in host defense perturbation. *P. gingivalis* has been implicated as a major etiologic agent in the development and progress of periodontitis. It is frequently isolated from periodontal pockets of periodontitis patients and it can produce multiple proteinases (Kinane et al., 2008).

P. gingivalis has been reported to affect the release and activation of certain MMPs from periodontal fibroblasts. *P. gingivalis* can disrupt MMP-9 production by gingival epithelial cells and interfere with ECM repair and re-organization (Andrian et al., 2006; DeCarlo et al., 1998; DeCarlo et al., 1997). Purified gingipains, which are trypsin-like proteinases and components of the *P. gingivalis* supernatant, can up-regulate MMP-8 and MMP-3 expression in rat mucosal epithelial cells (DeCarlo et al., 1998) and activate latent MMPs such as proMMP-1, -3, and -9 (DeCarlo et al., 1997). *P. gingivalis* Arg-gingipain in gingival cervical fluid (GCF) can also activate proMMP-2 (Grayson et al., 2003). Moreover, *P. gingivalis* has been shown to elevate MMP-2 production in

periodontal ligament cell cultures in a dose- and time-dependent manner (Chang et al., 2002b). Furthermore, partly by increasing MMP activation and by lowering the TIMP-1 protein levels, as well as by affecting the mRNA expression of multiple MMPs and TIMPs, *P. gingivalis* supernatant can up-regulate the collagen-degrading ability of some HGF cell lines (Zhou and Windsor, 2006).

Dissertation Outline

This dissertation is divided into five chapters. Chapter one (the current chapter) is a general introduction describing periodontal disease, MMPs/TIMPs, cigarette smoke, and their connection to each other. Chapter two describes the materials and methods of the three projects: project one investigated the non-toxic concentrations of CSC and their effects on HGF mediated-collagen degradation; project two compared the effects of CSC on the HGF-mediated-collagen degradation ability with those of nicotine; and project three investigated the combined effects of CSC and *P. gingivalis* supernatant on HGF-mediated-collagen degradation. Chapter three describes the results of three projects. Chapter four is the discussion. Chapter five concludes this dissertation and summarizes the collective significance of the data from these three studies. It also describes future directions for continuing research in this important area.

Table 1.1. Classification of the matrix metalloproteinase family

Collagenases	MMP-1, MMP-8, MMP-13, and MMP-18
Gelatinases	MMP-2 and MMP-9
Stromelysins	MMP-3, MMP-10, and MMP-11
Membrane-type MMPs (MT-MMPs)	MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, and MMP-25
Minimal-domain MMPs	MMP-7 and MMP-26
Others	MMP-12, MMP-19, MMP-20, MMP-21, MMP-22, MMP- 23, MMP-27, MMP-28

CHAPTER TWO

Material and Methods

Cell Culture

HGFs were cultured from explants of clinically healthy gingival connective tissue removed from a non-smoking patient undergoing crown-lengthening surgery as described previously (Zhang et al., 2009) at Indiana University School of Dentistry with Indiana University Purdue University Indianapolis (IUPUI) Institutional Review Board (IRB) approval (0609-60) and informed patient consent was obtained for the use of human gingival tissues. Briefly, the tissue was transported from the clinic to the laboratory in phosphate buffered saline (PBS) solution, washed with 70% ethanol, and rinsed in PBS to remove the ethanol. The washing and rinsing steps were repeated and then the tissues were minced into small fragments of approximately 1 mm³. The tissue pieces were then placed in cell culture dishes, air-dried, and incubated for 5-7 days at 37°C and 5% CO₂ in low glucose (1 g/L) Dulbecco's Modified Eagle's Media (DMEM) (Hyclone, Logan, Utah) supplemented with 15% fetal bovine serum (FBS) (Hyclone), 4 mM L-glutamine (Hyclone), 100 U/ml penicillin (Invitrogen, Carlsbad, CA), 50 µg/ml gentamicin(Invitrogen), and 2.5 µg/ml fungizone (Invitrogen). The cells that grew out of the explants were subcultured and maintained. Cells at passage 3 to 8 were utilized in these experiments.

Bacterial Growth and Culture Supernatant

The *P. gingivalis* ATCC 33277 supernatant was provided as a gift from Dr. J. Katz (University of Alabama at Birmingham School of Dentistry, Birmingham, AL). *P. gingivalis* was cultured on enriched trypticase soy agar plates with 3% sheep blood and then in brain heart infusion broth with 1 mg/ml of cysteine, 5 µg/ml of hemin, and 0.5 µg/ml of menadione (supplemented brain heart infusion broth) as described previously (Zhou and Windsor, 2006). *P. gingivalis* were incubated in an anaerobic chamber (Coy Laboratories, Ann Arbor, MI) with an atmosphere of 85% nitrogen, 10% hydrogen, and 5% carbon dioxide. When the bacterial growth yielded an OD₆₆₀ of 1.0-1.2, the culture supernatant was collected at 13,000 g for 20 min at 4°C. The collected supernatant was then filtered twice through 0.2 µm membranes and stored at -20°C until utilized.

Measurement of HGF Proliferation by Water-soluble Tetrazolium-1 (WST-1)

Assays in the Different Treatment Conditions

Mitochondrial dehydrogenase activities were measured using the WST-1 assay (Roche Applied Science, Penzberg, Germany) as described previously (Zhang et al., 2009). HGFs were detached by incubation for 5 min with 0.25% trypsin (Invitrogen), pelleted, resuspended in DMEM with 15% FBS, and then seeded (75,000 cells/well) in 6-well plates with 2 ml DMEM with 15% FBS. The plates were incubated for 24 hours to allow the cells to attach. The media were then removed and the HGFs exposed to 1 ml serum-free DMEM with different chemicals according to project conditions (Table 2.1.1, Table 2.2.1, and Table 2.3.1). The positive control was untreated HGFs and the negative control was serum-free DMEM without cells. After 72 hours, the media in the 6-well

plates was removed and the cells washed with 2 ml serum-free DMEM three times for 15 minutes. Cell proliferation reagent WST-1 (100 μ l WST-1 and 900 μ l serum-free DMEM) was added and the plate incubated for 2 hours at 37°C and 5% CO₂. A 100 μ l sample from each well of the 6-well plates were placed into a 96-well plate and the absorbance values of the samples against the negative control as the blank were measured using a microplate reader (Titertek, Multiskan MCC, Flow Laboratories, Mclean, VA) at 450 nm. The experiments were repeated three to four times and the mean value calculated. The absorbance values of each sample were compared with the untreated cell control by percentage in the following equation:

$$\text{Cell proliferation (\%)} = (\text{absorbance value of CSC treatment} / \text{absorbance value of no CSC treatment}) \times 100\%$$

Measurement of Cytotoxicity on HGFs by Lactate Dehydrogenase (LDH) Assays in the Different Treatment Conditions

Cellular membrane integrity was monitored using the permeability assay based on the determination of the release of LDH into the media. The Cytotoxicity Detection Kit^{PLUS} (Roche Applied Science, Mannheim, Germany), which measures the conversion of tetrazolium salt into a red formazan product, was used as described previously (Zhang et al., 2009). Cells were treated with 1 ml serum-free DMEM with different chemicals according to project conditions (Table 2.1.1, Table 2.2.1, and Table 2.3.1). The high control (total cell death) were generated by adding 1.9 ml of serum-free DMEM and 100 μ l lysis solution to the control cells as described by the manufacturer after 72 hours,

which gave the maximum release of LDH. The low control consisted of serum-free DMEM from the untreated control cells after 72 hours and gave the minimal release of LDH. Serum-free DMEM without HGFs was utilized as the background level of the assay. After 72 hours, media from each of the wells was transferred to a 96-well plate and 100 μ l of reconstituted mix, as per the manufacturer (Roche), was added to each well and the plates incubated for 15 minutes at room temperature. Absorbance was recorded at 490 nm in a microplate reader (Titertek, Multiskan MCC, Flow Laboratories, Mclean, VA). The experiments were repeated three to four times and the mean value calculated. The percentage release of LDH from the treated cells was calculated by comparing it to the maximum release of LDH. To determine the cytotoxicity, the absorbance values of the background were subtracted from that of the experimented samples and the cytotoxicity was calculated by the following equation:

$$\text{Cytotoxicity (\%)} = (\text{experiment value} - \text{low control}) / (\text{high control} - \text{low control}) \times 100\%$$

Collagen Degradation Assays

The collagen degrading ability of the HGFs was determined with a reconstituted collagen type I assay system as previously described by Birkedal-Hansen et al. (Birkedal-Hansen et al., 1984). Briefly, rat tail tendon type I collagen was dissolved in 13 mM HCl and then mixed rapidly on ice with one-fifth volume of a neutralizing phosphate buffer (prepared as follows: 40 ml of 0.2 M $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 7.4, 40 ml 0.1 M NaOH, 8.3 ml 5 M NaCl) to yield a final collagen concentration of 300 μ g/ml. Aliquots of 1.5 ml/well (450 μ g collagen/well) were dispensed in 6-well plates and incubated at 37°C for

2 hours. The collagen gels were dehydrated overnight in a laminar flow hood, washed 3 times for 30 min with sterile water, and then air-dried in the hood. Trypsin was used to assess the quality of collagen. Trypsin was not capable of cleaving the collagen, thus demonstrating that it was native and not denatured (data not shown). HGFs at 90% confluence were detached with 0.25% trypsin, pelleted, resuspended in DMEM with serum, and then seeded as single colonies (75,000 cells/well) in the center of a 6-well plate coated with collagen. After the cells attached, 2 ml serum-free DMEM with the different chemicals was added according the project requirements (Table 2.1.1, Table 2.2.1, and Table 2.3.1). Serum-free medium were utilized as experimental controls. After certain treatment periods according to different project conditions (Table 2.4), the cells were removed with 500 μ l of 0.1% Triton X-100 and 200 μ l of 0.25% trypsin (Invitrogen, Carlsbad, CA). The wells were then stained with Coomassie blue to visualize the collagen cleavage. The 6-well plates were scanned using the NIH Scion Image software (Scion Corporation, Frederick, MD) to measure the collagen degradation (the size of measurement area was defined by maximal collagen degraded area) and the average optical density (OD) value of collagen degradation area was determined. The average background values of each 6-well plate were subtracted from each OD value. The final OD values were compared to the control value (untreated group), and finally the relative value for each group was calculated by setting all the control to one. The experiments were repeated 3 times.

Gelatin Zymography

HGFs were cultured in serum-free DMEM (Hyclone, Logan, Utah) with the different chemicals according the project conditions (Table 2.1.1, Table 2.2.1, and Table 2.3.1). After three or five days (Table 2.5), the conditioned media from the HGF cells were collected. The cell membrane extracts were then prepared utilizing the Mem-PER Eukaryotic Membrane Protein Extraction Regent Kit (Pierce, Rockford, IL) and the PAGEprep™ Protein Clean-Up and Enrichment Kit (Pierce, Rockford, IL). The concentrations of total protein in the concentrated media and membrane extracts were determined according to the Bio-Rad protein assay protocol (Bio-Rad Laboratories, Hercules, CA). The same amount of concentrated HGF media and membrane extracts by protein levels were mixed with non-reducing loading buffer (without β -mercaptoethanol) and resolved at 200 V in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels copolymerized with 1 mg/ml gelatin. The gels were then washed with solution 1 (50 mM Tris, pH 7.5, 3 mM NaN_3 , 2.5% Triton X-100), solution 2 (50 mM Tris, pH 7.5, 3 mM NaN_3 , 5 mM CaCl_2 , 1 μM ZnCl_2 , 2.5% Triton X-100), and solution 3 (50 mM Tris, pH 7.5, 3 mM NaN_3 , 5 mM CaCl_2 , 1 μM ZnCl_2) for 20 minutes each. The gels were then incubated in fresh solution 3 at 37°C overnight and then stained with Coomassie blue to visualize the proteolytic bands. The gelatin gels were scanned using the NIH Scion Image software to measure the gelatin degradation (the size of measurement area was defined by maximal gelatin degraded area) and the average OD value of gelatin degradation area was determined. The average background values of each gels were subtracted from each OD value. The final OD values were compared to

the control value (untreated group) and finally the relative value for each group was calculated by setting all of the controls to one. The experiments were repeated 3 times.

Western Blots Analyses

HGFs were cultured for three days or five days in serum-free DMEM with the different chemicals according the project conditions (Table 2.1.1, Table 2.2.1, and Table 2.3.1). The conditioned media were then collected and concentrated, and the membrane extracts (Table 2.6) prepared as described above. The concentrations of the total protein in the concentrated media were determined according to the Bio-Rad protein assay protocol (Bio-Bad Laboratories, Hercules, CA). The same amount of total protein of untreated and CSC treated concentrated media was resolved in 10% SDS-PAGE gels at 200 V. The proteins on the gels were transferred to polyvinylidene fluoride (PVDF) membranes at 0.08 amps overnight using blotting buffer (2 mM Tris-HCl, pH 8.3, 192 mM glycine, and 10% methanol). The membranes were then incubated in 5% milk in PBS solution with 0.1% Tween-20 (PBS-T, pH 7.4) (Invitrogen, Carlsbad, CA) for 1 h to block nonspecific binding. The membranes were then incubated with primary antibodies at 4°C for overnight. The primary antibodies utilized in the study were described in Table 2.7 and Table 2.8. The membranes were then washed three times with PBS-T and incubated with anti-mouse or anti-rabbit secondary antibodies (Amersham, Piscataway, NJ) for 1h at room temperature. The membranes were then developed with the ECLTM (kit Amersham, Piscataway, NJ) according to the manufacturer's protocol. The blots exposed X-ray films were scanned using the NIH Scion Image software to measure the bands (the size of measurement area was defined by maximal detected band) and the

average OD value of the detected antibody band area was determined. The average background values of each X-ray film were subtracted from each OD value. The final OD values were compared to the control value (untreated group) and finally the relative value for each group was calculated by setting all of the controls to one. The Western blots were repeated 3 times.

Reverse Transcription PCR (RT-PCR)

To determine the mRNA levels of MMPs and TIMPs (Table 2.10) expressed by the HGFs treated with the different agents for 48 hours (Table 2.1.1, Table 2.2.1, and Table 2.3.1), the total RNA was extracted using the Qiagen mini kit (Qiagen, Valencia, CA). The mRNA and primers (Zhou and Windsor, 2006) were mixed with the Qiagen OneStep RT-PCR mix (Qiagen, Valencia, CA) according to the manufacturer's protocol (Table 2.9). RT-PCR was performed using a Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, CA). The reverse transcription was accomplished at 50°C for 30 min and terminated at 95°C for 15 min. PCR was then performed for 30 cycles with denaturation at 95°C, annealing at 50°C, and extension at 72°C for 1 min each. The PCR products were then resolved in 0.8% w/v agarose gels at 120 V and stained with ethidium bromide. The gels were photographed under ultraviolet light and then analyzed with the NIH scion Image software (Version beta 4.03). The cyclophilin bands were utilized for standardization of the RT-PCR results. The relative mRNA expression levels of the treated and untreated cells were then determined. The experiments were repeated 3 times.

Statistical Analyses

The data were presented as mean and standard deviation (SD). Statistical analyses were performed by One-way Analysis of Variance (ANOVA) and the Tukey's test in the Statistical Package for Social Science (SPSS) 11.5 (SPSS Inc., Chicago, IL) (Zhou et al., 2007). The level of significant was set at $p < 0.05$.

Table 2.1. Treatment conditions for project one

Treatments
800 µg/ml CSC
400 µg/ml CSC
200 µg/ml CSC
100 µg/ml CSC
50 µg/ml CSC
25 µg/ml CSC
12.5 µg/ml CSC
6.25 µg/ml CSC
3.125 µg/ml CSC
0 µg/ml CSC

CSC: cigarette smoke condensate (Murty Pharmaceuticals, Lexington, KY) diluted in serum-free DMEM.

Table 2.2. Treatment conditions for project two

Treatments
100 $\mu\text{g/ml}$ CSC
2.4 $\mu\text{g/ml}$ Nicotine
0 $\mu\text{g/ml}$

CSC: cigarette smoke condensate (Murty Pharmaceuticals, Lexington, KY) diluted in serum-free DMEM. Nicotine (Sigma Chemical Co., St. Louis, MO) diluted in serum-free DMEM. The percentage of nicotine in the CSC was determined to be 2.4% by the Clinical Pharmacology Laboratory (San Francisco General Hospital, San Francisco, CA).

Table 2.3. Treatment conditions for project three

Treatments
100 $\mu\text{g/ml}$ CSC plus 10% (v/v) <i>P. gingivalis</i> supernatant
50 $\mu\text{g/ml}$ CSC
10% (v/v) <i>P. gingivalis</i> supernatant
50 $\mu\text{g/ml}$ of CSC plus 10% (v/v) <i>P. gingivalis</i> supernatant
0 $\mu\text{g/ml}$ CSC

CSC: cigarette smoke condensate (Murty Pharmaceuticals, Lexington, KY) diluted in serum-free DMEM. For gelatin zymography, collagen degradation assay, Western blots, and RT-PCR, 50 $\mu\text{g/ml}$ CSC plus 10% (v/v) *P. gingivalis* supernatant has not be used as treatment because its cytotoxicity to HGFs.

Table 2.4. Treatment periods of collagen degradation assays for the different projects

Project	Treatment Periods
Project one	3 days
Project two	3 days
Project three	1, 3, 5, and 7 days

In project three, a synthetic MMP inhibitor (GM6001, 100 nM, Chemicon, Temecula, CA) were utilized in the inhibition experiments.

Table 2.5 Samples for gelatin zymography in the different projects

Project	Treatment Periods
Project one	Conditioned media and cell membrane extracts at three days
Project two	Conditioned media and cell membrane extracts at three days
Project three	Conditioned media at five days

In project two, a MMP inhibitor, 1, 10-phenothroline (10 mM) (Sigma-Aldrich, St. Louis, MO), was utilized in the inhibition experiments.

Table 2.6. Samples for Western blots in the different projects

Project	Treatment Periods
Project one	Conditioned media and cell membrane extracts at three days
Project two	Conditioned media and cell membrane extracts at three days
Project three	Conditioned media and cell membrane extracts at five days

Table 2.7. MMPs and TIMPs antibodies used in the Western blots

Anitibody	Clone	Company	Concentrations
monoclonal MMP-1 (Ab-5)	Clone III 12b	Neomarkers, Fremont, CA	5 µg/ml
monoclonal MMP-2 (Ab-2)	Clone V B3	Neomarkers, Fremont, CA	5 µg/ml
monoclonal MMP-3 (Ab-2)	Clone II D4	Neomarkers, Fremont, CA	5 µg/ml
polyclonal MMP-14	AB8221	Chemicon, Temecula, CA	0.3 µg/ml
monoclonal TIMP-1 (Ab-1)	Clone 102B1	Neomarkers, Fremont, CA	5 µg/ml
monoclonal TIMP-2 (Ab-1)	Clone T2-101	Neomarkers, Fremont, CA	5 µg/ml

Table 2.8. MMPs and TIMPs detected in the Western blots in the different projects

Project	Treatment Periods
Project one	MMP-1, -2, -3, and -14; TIMP-1 and -2.
Project two	MMP-2 and -14; TIMP-2.
Project three	MMP-1, -2, -3, and -14; TIMP-1 and -2.

Table 2.9. RT-PCR primers for cyclophilin, MMPs and TIMPs

mRNA	Primer
Cyclophilin (247 bp):	(f)CCGTGTTCTTCGACATT (r)GCCAGGACCCGTATGCT
MMP-1/collagenase-1 (721 bp):	(f)GAAGTTGATGAAGCAGCCCAGATGT (r)CAGTTGTGGCCAGAAAACAGAAGTGAAA
MMP-2/gelatinase-A (364 bp):	(f)ATCCGTGGTGAGATCTTCTTCTT (r)AGCCAGGATCCATTTTCTTCTT
MMP-3/stromelysin-1 (659 bp):	(f)GACACCAGCATGAACCTTGTT (r)GGAACCGAGTCAGGTCTGTG
MMP-14/MT1-MMP (670 bp):	(f)GATAAACCCCAAAAACCCACCTA (r)CCCTCCTCGTCCACCTCAATG
TIMP-1 (534 bp):	(f)CCTTCTGCAATTCCGACCTCGTC (r)CGGGCAGGATTCAGGCTATCTGG
TIMP-2 (433 bp):	(f)TGGAACGACATTTATGGCAACC (r)ACAGGAGCCGTCACCTTCTTGTGAT

f: forward; r: reverse

Table 2.10. MMPs and TIMPs detected by RT-PCR in the different projects

Project	Treatment Periods
Project one	None
Project two	MMP-2, and -14; TIMP-2.
Project three	MMP-1, -2, -3, and -14; TIMP-1 and -2.

CHAPTER THREE

Results

Project One: Cigarette Smoke Condensate Affects the Collagen-Degrading Ability of Human Gingival Fibroblasts

Morphology of the HGFs

The HGFs attached and spread rapidly to take on a typical long spindle-shaped parallel alignment appearance in the 6-well plate without CSC (Figure 3.1.1). At low CSC concentrations, the HGFs remained normal in appearance (Figure 3.1.2). In contrast, CSC-treated HGFs showed a rounded appearance with a haphazard arrangement and detached from the 6-well plate at the higher concentrations (above 200 $\mu\text{g/ml}$; Figure 3.1.3). When the concentrations reached 800 $\mu\text{g/ml}$, almost no HGFs were still attached (data not shown).

Measurement of the Cell Proliferation by WST-1

After exposure to CSC at 800 $\mu\text{g/ml}$, 400 $\mu\text{g/ml}$, 200 $\mu\text{g/ml}$, 100 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$, 25 $\mu\text{g/ml}$, 12.5 $\mu\text{g/ml}$, 6.25 $\mu\text{g/ml}$, and 3.125 $\mu\text{g/ml}$, the amount of cell proliferation was 6.86 \pm 5.36%, 7.32 \pm 5.49%, 46.79 \pm 26.39%, 90.29 \pm 42.43%, 81.56 \pm 24.71%, 80.43 \pm 29.36%, 64.85 \pm 30.10%, 60.09 \pm 12.92%, and 81.85 \pm 38.24%, respectively, when compared to the untreated control HGFs (100%; Figure 3.1.4). Overall, the CSC had a negative effect on the growth of the HGFs compared to the control. However, various concentrations of the CSC had different effects on the growth of the HGFs. There were

no concentration dependent relationship between the CSC concentrations and negative effects at CSC concentrations less than 200 µg/ml. When the concentration of CSC reached 200 µg/ml, cell proliferation was reduced to 46.79% and decreased significantly when compared to the untreated HGFs ($p=0.023$).

Measurement of Cytotoxicity of HGFs by LDH

Generally, the CSC had a toxic effect on the growth of HGFs despite some variations (Figure 3.1.4). After exposure to CSC at 800 µg/ml, 400 µg/ml, 200 µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, and 3.125 µg/ml, the amount of cytotoxicity was $70.43\pm 11.13\%$, $32.42\pm 24.62\%$, $10.59\pm 16.44\%$, $10.93\pm 19.66\%$, $8.97\pm 17.22\%$, $-0.80\pm 4.52\%$, $4.43\pm 10.356\%$, $1.23\pm 6.49\%$, and $4.22\pm 4.25\%$, respectively (Figure 3.1.5). The CSC at concentrations of 400 µg/ml and 800 µg/ml demonstrated significant toxic effects on the HGFs ($p=0.001$ and $p=0.000$, respectively)

Collagen Degradation

Untreated HGFs (control) cleaved the collagen underneath the cell colonies and formed a small transparent area (Figure 3.1.6). At low concentrations of CSC (≤ 25 µg/ml), the area of collagen degradation increased a little but was not statistically significant (Figure 3.1.7) ($p>0.05$). This increase in collagen degradation was especially evidence when the concentration of CSC reached 100 µg/ml ($p=0.002$). When the concentration of CSC reached 200 µg/ml or higher, the collagen degrading ability decreased probably as a result of the CSC's negative effects on the cells in regard to proliferation and toxicity.

Gelatin Zymography

In the HGF conditioned media with or without CSC, gelatinase-A (MMP-2, 72/62kDa) bands were detected (Figure 3.1.8). The proMMP-2 (72kDa) production at low concentrations of CSC (≤ 100 $\mu\text{g/ml}$) showed little differences among the various concentrations of CSC (Table 3.1.1). At high concentrations of CSC (≥ 200 $\mu\text{g/ml}$), the levels of both the pro and active forms of MMP-2 decreased compared to the untreated HGFs. However, at low concentrations of CSC (≤ 100 $\mu\text{g/ml}$), the active form of MMP-2 (62kDa) increased with increasing CSC concentrations. This increase was especially evident when the concentration of CSC reached 25 $\mu\text{g/ml}$. Furthermore, MMP-9 (gelatinase B) could not be detected by zymography (data not shown).

In the HGF membrane extracts with or without CSC, gelatinase-A (MMP-2, 72/62kDa) bands were detected (Figure 2.9). The proMMP-2 (72kDa) production was not much different among the various concentrations of CSC (Table 3.1.2). However, the active form of MMP-2 (62kDa) increased more than 16-fold compared to the control at concentrations above 50 $\mu\text{g/ml}$. The production of MMP-9 (gelatinase B) could not be detected by zymography of the membrane extracts (data not shown).

Western Blots Analyses

Both pro-MMP-1 (52 kDa) and active MMP-1 (42 kDa) were detected in the conditioned media (Figure 3.1.10). Compared to the control group, the highest levels of both pro and active MMP-1 were produced when the concentration of CSC was 100 $\mu\text{g/ml}$ (Table 3.1.3).

In the HGF conditioned media with or without CSC, gelatinase-A (MMP-2, 72/62kDa) bands were detected (Figure 3.1.10). Similar to the results of the zymography, the proMMP-2 (72kDa) production increased at 25 and 50 $\mu\text{g/ml}$ CSC, and decreased at 800 $\mu\text{g/ml}$. At other concentrations of CSC, proMMP-2 showed little differences (Table 3.1.3). At 25, 50, and 100 $\mu\text{g/ml}$ CSC, the active form of MMP-2 (62kDa) increased with increasing CSC concentrations. At higher concentrations of CSC (400 and 800 $\mu\text{g/ml}$), the active form of MMP-2 (62kDa) decreased. In the HGF membrane extracts with or without CSC, gelatinase-A (MMP-2, 72/62kDa) bands were detected (Figure 3.1.11). The proMMP-2 (72kDa) production increased at higher concentrations of CSC (≥ 25 $\mu\text{g/ml}$) (Table 3.1.4). However, the active form of MMP-2 (62kDa) increased more than 2.8 folds at CSC treated concentrations ≥ 3.125 $\mu\text{g/ml}$ compared to the control.

ProMMP-3 (59/54 kDa) were detected in the conditioned media at all the different concentrations of the CSC and the active forms of MMP-3 (49/44 kDa) were also detected (Figure 3.1.10 and Table 3.1.3). However, the production of the active form of MMP-3 decreased sharply compared to control above 100 $\mu\text{g/ml}$ and this may be due to cell death.

MMP-14 (64 kDa) was also detected in the conditioned media. Compared with the control group, CSC increased the production of MMP-14 at concentrations of 12.5 $\mu\text{g/ml}$ and 200 $\mu\text{g/ml}$ (Figure 3.1.10; Table 3.1.3). However, the production of MMP-14 increased more than 2-fold in the membrane extracts from HGFs at the concentration of 50 $\mu\text{g/ml}$ (Figure 3.1.11; Table 3.1.4).

TIMP-1 (28.5 kDa) levels in the conditioned media increased at concentrations of CSC at and above 6.25 $\mu\text{g/ml}$. Generally, increasing CSC concentrations led to increased production of TIMP-1 compared to the control until cytotoxicity occurred (Figure 3.1.10; Table 3.1.3).

Generally, TIMP-2 (21 kDa) production in the conditioned media decreased with increasing concentrations of CSC (Figure 3.1.10; Table 3.1.3). However, TIMP-2 presence in the HGF membrane extracts increased at the concentrations of 50 and 100 $\mu\text{g/ml}$ (Figure 3.1.11; Table 3.1.4). TIMP-2 levels in the membrane extracts decreased at and above 200 $\mu\text{g/ml}$.

Table 3.1.1. Expression of MMP-2 in HGF conditioned media at the different CSC concentrations compared to the control by gelatin zymography

	3.125 µg/ml	6.25 µg/ml	12.5 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml	400 µg/ml	800 µg/ml
	vs. control								
proMMP-2	1.30±0.34	1.12±0.20	0.93±0.22	1.77±1.13*	1.76±1.21*	1.45±0.81*	0.64±0.50	0.44±0.25*	0.26±0.15*
MMP-2	1.09±0.08	1.23±0.09	0.80±0.22	1.62±0.53*	1.44±0.10*	1.43±0.28*	0.83±0.40	0.43±0.30*	0.30±0.10*

Table 3.1.2. Expression of MMP-2 in HGF membrane extracts at the different CSC concentrations compared to the control by gelatin zymography

	3.125 µg/ml vs. control	6.25 µg/ml vs. control	12.5 µg/ml vs. control	25 µg/ml vs. control	50 µg/ml vs. control	100 µg/ml vs. control	200 µg/ml vs. control	400 µg/ml vs. control
proMMP-2	1.25±0.02	1.26±0.21	1.27±0.03	1.07±0.02	1.40±0.02	1.68±0.05*	0.93±0.03	1.25±0.05
MMP-2	5.68±0.29*	5.36±0.59*	8.87±0.88*	5.19±1.52*	16.64±2.20*	31.88±2.62*	23.73±2.32*	27.16±2.86*

40 * denotes significant differences from control value at p<0.05

Table 3.1.3. Expression of MMPs and TIMPs in HGF conditioned media at the different CSC concentrations compared to the control by Western blots

	3.125 µg/ml	6.25 µg/ml	12.5 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml	400 µg/ml	800 µg/ml
proMMP-1	0.80±0.04	0.98±0.03	1.22±0.02	0.86±0.06	0.72±0.04	1.43±0.07*	1.04±0.07	1.56±0.06*	0.93±0.01
MMP-1	0.77±0.03	1.28±0.05	1.17±0.03	0.95±0.03	0.77±0.06	1.35±0.07	0.65±0.02	0.94±0.02	0.89±0.05
proMMP-2	1.26±0.43	1.10±0.29	0.88±0.32	1.47±1.03*	1.44±1.01*	1.39±0.34	0.76±0.40	0.59±0.35	0.27±0.25*
MMP-2	1.18±0.18	1.09±0.19	1.12±0.42	1.96±0.43*	1.81±0.31*	1.44±0.18*	0.96±0.41	0.27±0.32*	0.20±0.15*
proMMP-3	1.05±0.02	1.04±0.02	1.14±0.02	1.14±0.05	0.93±0.04	1.21±0.06	1.17±0.08	0.96±0.03	0.89±0.06
MMP-3	0.96±0.01	0.80±0.02	0.90±0.02	0.94±0.02	0.80±0.03	0.90±0.11	0.22±0.02*	0.11±0.01*	0.15±0.01*
MMP-14	1.09±0.01	1.08±0.06	1.97±0.09	1.17±0.06	1.20±0.02	1.57±0.04*	1.29±0.02	1.18±0.04	1.19±0.11
TIMP-1	1.34±0.01	3.49±0.04*	4.56±0.06*	4.35±0.05*	5.91±0.05*	6.45±0.05*	6.20±0.16*	3.83±0.09*	5.59±0.09*
TIMP-2	1.28±0.01	1.02±0.02	0.97±0.06	0.92±0.04	0.41±0.02*	0.31±0.04*	0.40±0.02*	0.44±0.02*	0.34±0.01*

* denotes significant differences from control value at p<0.05

Table 3.1.4. Expression of MMP-2, MT1-MMP, and TIMP-2 in HGF membrane extracts at the different CSC concentrations compared to the control by Western blots

	3.125 µg/ml	6.25 µg/ml	12.5 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml	400 µg/ml
	vs. control							
proMMP-2	1.18±0.12	1.57±0.71	1.41±0.23	1.66±0.32*	1.82±0.22*	2.11±0.15*	2.19±0.63*	1.65±0.25*
MMP-2	2.86±0.29*	3.01±0.59*	4.48±0.88*	4.36±1.02*	4.08±1.29*	5.52±1.92*	4.82±1.32*	3.12±1.56*
MMP-14	0.97±0.02	1.04±0.03	0.79±0.06	1.13±0.06	2.13±0.14*	1.23±0.03	1.67±0.06*	0.78±0.05
TIMP-2	1.46±0.05	1.50±0.11*	1.74±0.12*	1.02±0.03	2.98±0.13*	2.47±0.11*	0.88±0.04	0.09±0.01*

* denotes significant differences from control value at p<0.05

Figure 3.1.1. CSC-free HGFs (negative control). HGFs attached to the plate displayed a typical long spindle-shaped parallel alignment appearance (magnification $\times 100$).

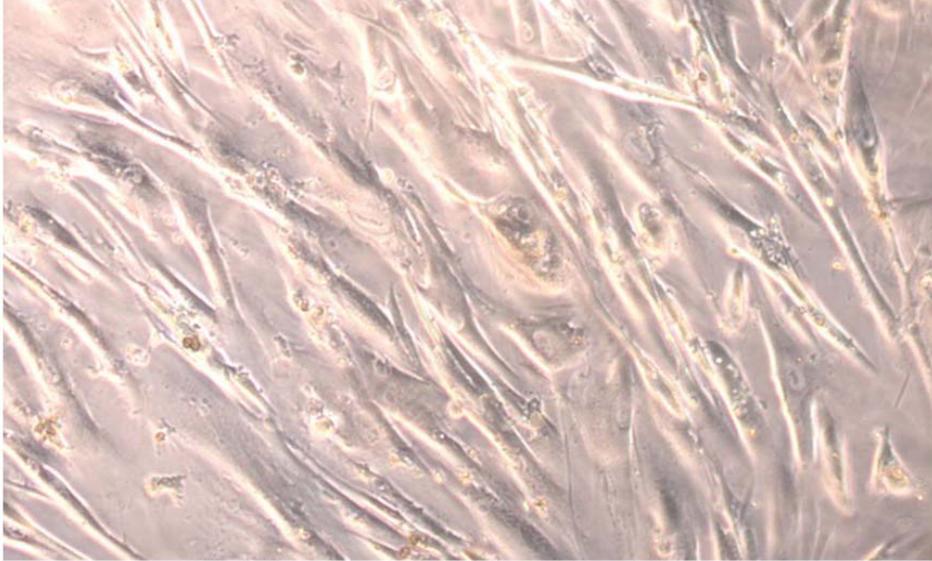


Figure 3.1.2. 3.125 $\mu\text{g/ml}$ CSC-treated HGFs. HGFs displayed a normally typical long spindle-shaped parallel alignment appearance (magnification $\times 100$).

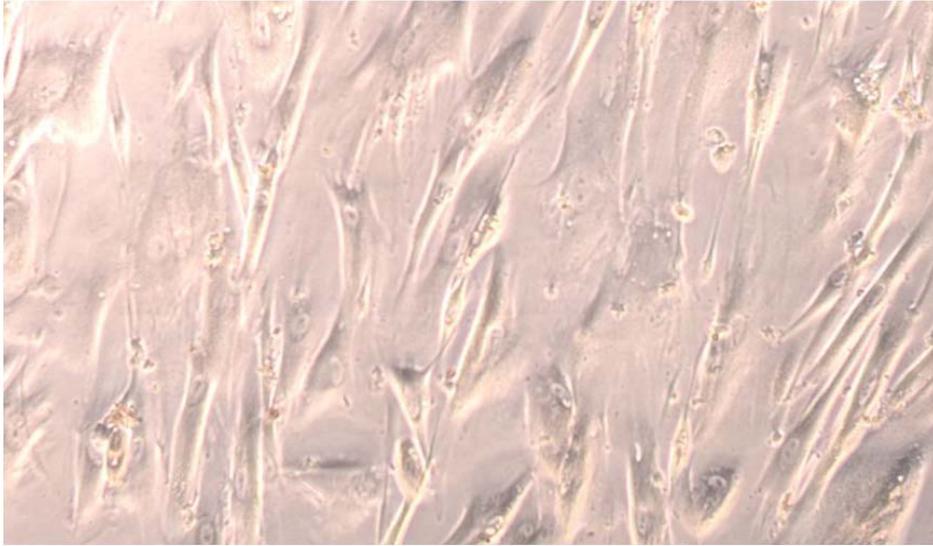


Figure 3.1.3. 200 $\mu\text{g/ml}$ CSC-treated HGFs. HGFs displayed a round appearance with a haphazard arrangement and most of the HGFs detached from the plate (magnification $\times 100$).

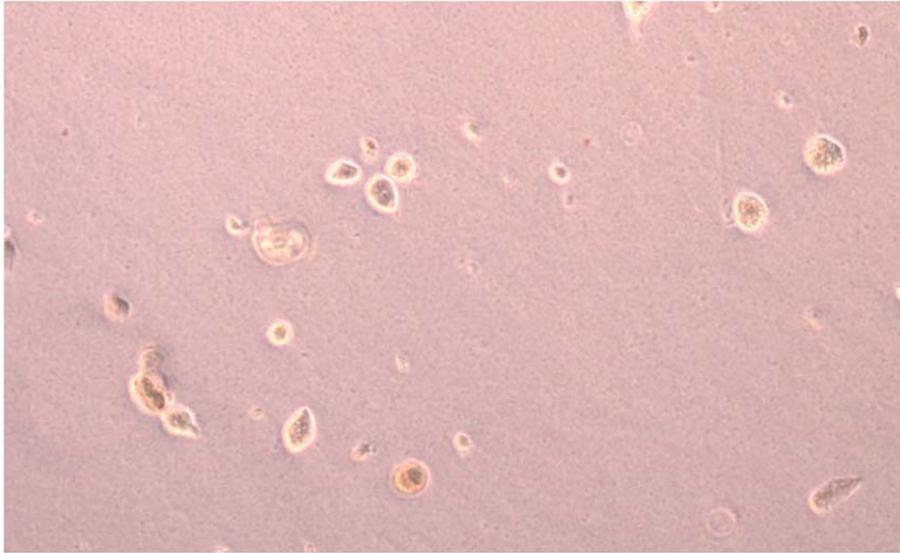


Figure 3.1.4. Cell proliferation (%) of CSC-treated HGFs. The cell proliferation of CSC treated HGFs was lower than that of the untreated HGFs. Data were analyzed by one-way ANOVA and Tukey's test multiple comparison tests: * denotes significant differences from control value at $p < 0.05$.

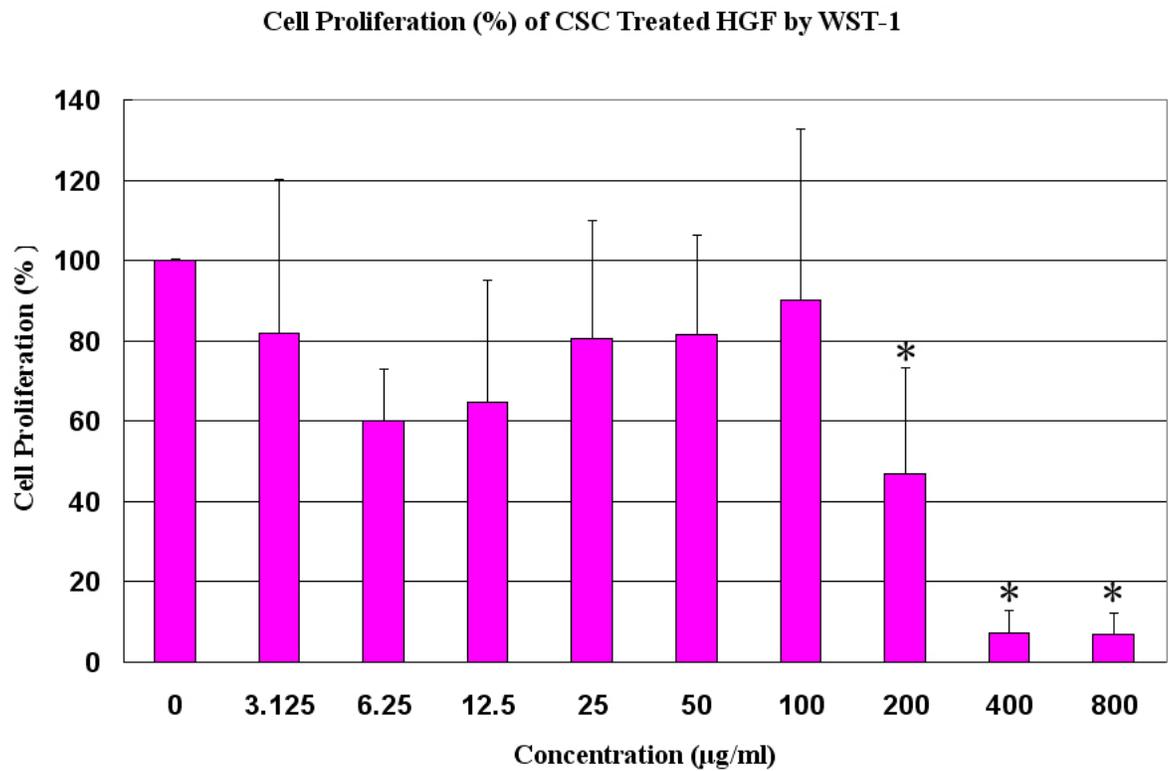


Figure 3.1.5. Cytotoxicity (%) of CSC-treated HGFs. The cytotoxicity of CSC treated HGFs was higher than that of CSC-free HGFs. Data were analyzed by one-way ANOVA and Tukey's test multiple comparison tests. * denote significant differences from control value at $p < 0.05$.

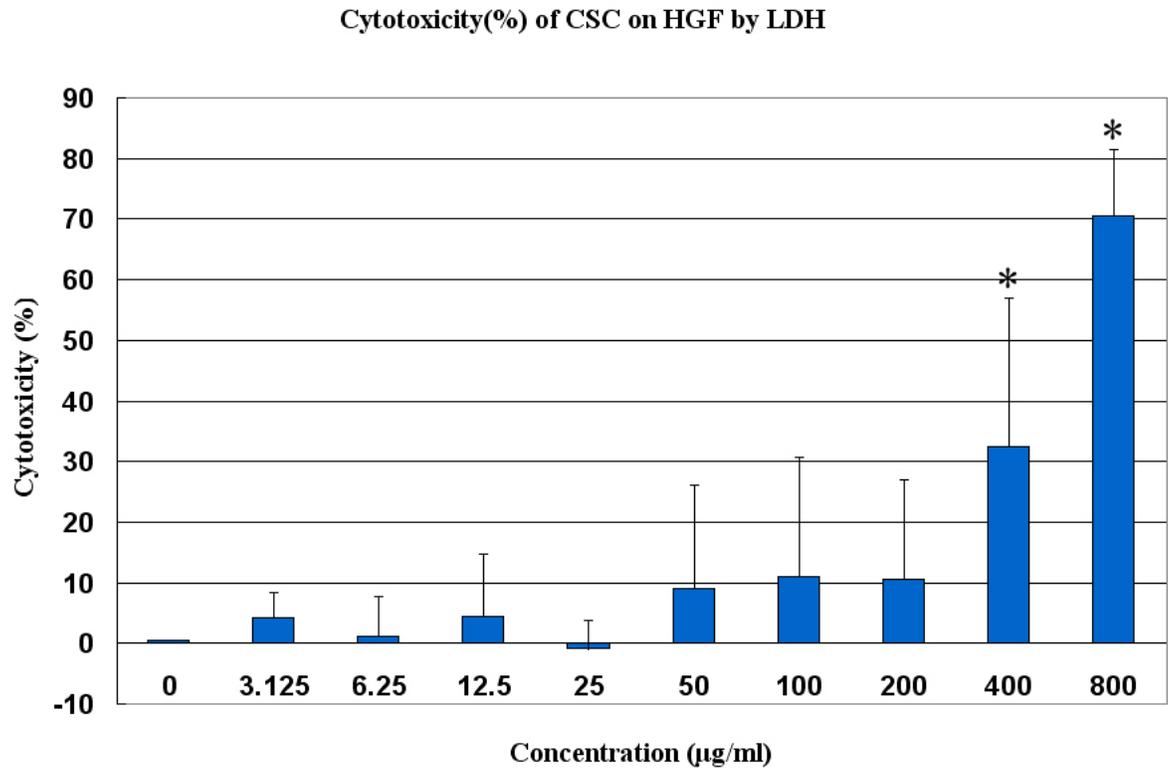


Figure 3.1.6. Effects of the different concentrations of CSC (0-800 $\mu\text{g/ml}$) on the collagen-degrading ability of HGFs.

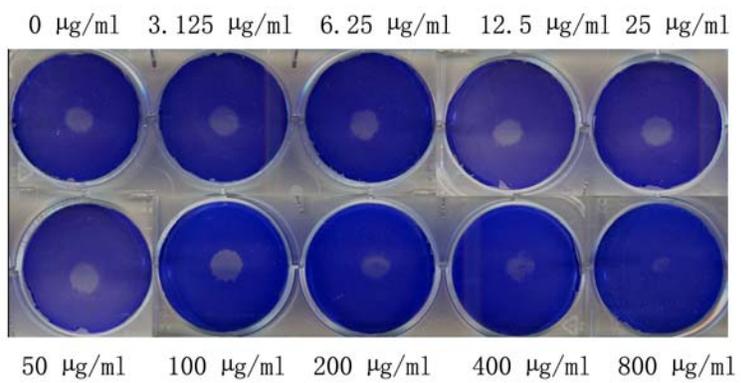


Figure 3.1.7. The statistical analyses of HGFs at the different concentrations of CSC (0-800 $\mu\text{g/ml}$) treatment compared to the control group (without CSC). Data were analyzed by one-way ANOVA and Tukey's test multiple comparison tests. * denotes significant differences from control value at $p < 0.05$.

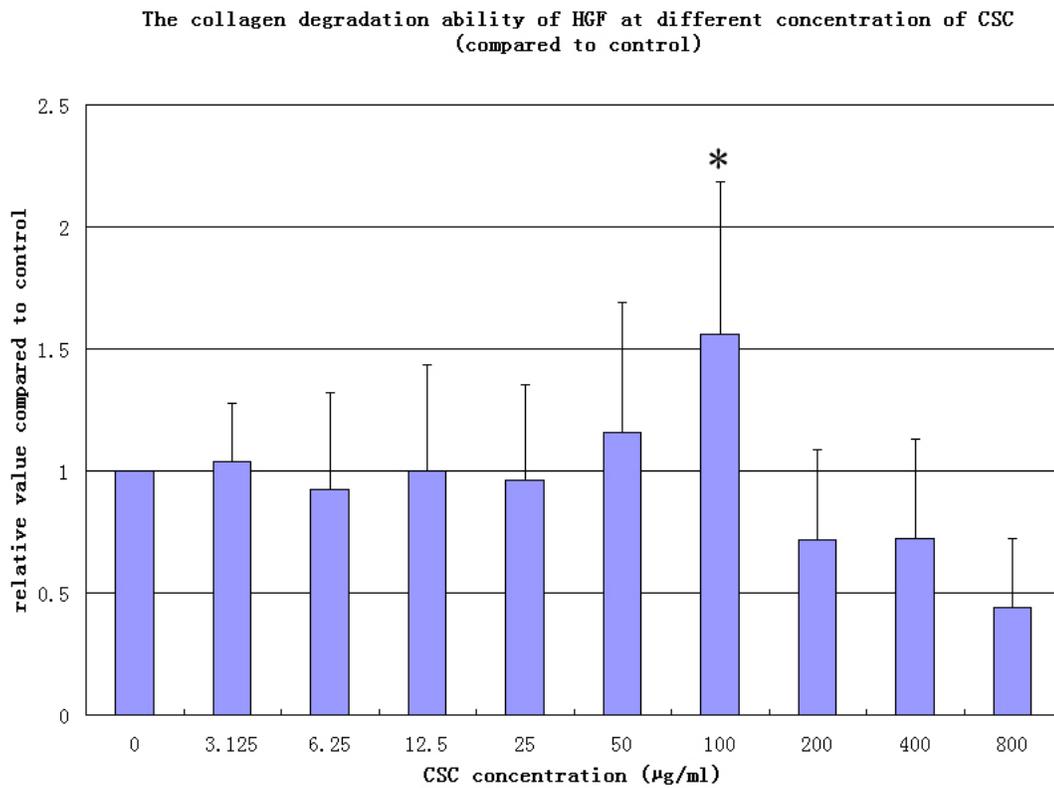


Figure 3.1.8. Gelatin zymography of the HGF-conditioned media treated with the different concentrations of CSC (0-800 $\mu\text{g/ml}$) for 72 hours.

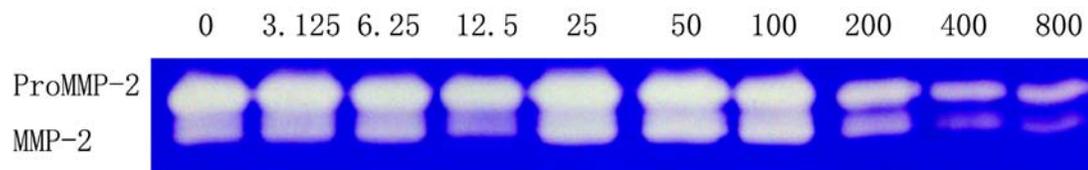


Figure 3.1.9. Gelatin zymography of membrane extracts from the HGFs treated with the different concentrations of CSC (0-400 $\mu\text{g/ml}$) for 72 hours.



Figure 3.1.10. Western blots analyses for selected MMPs and TIMPs in HGF-conditioned media treated with the different concentrations of CSC (0-800 $\mu\text{g/ml}$) for 72 hours.

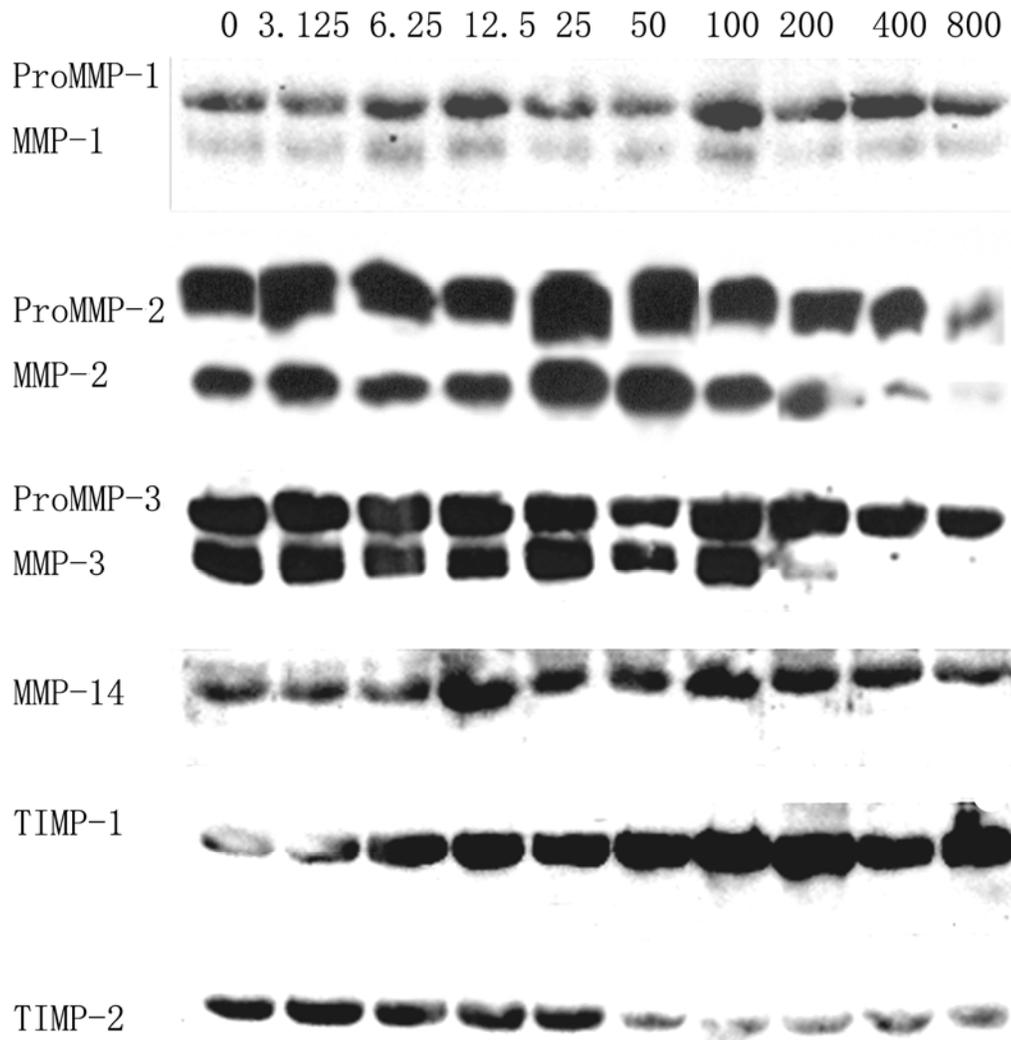
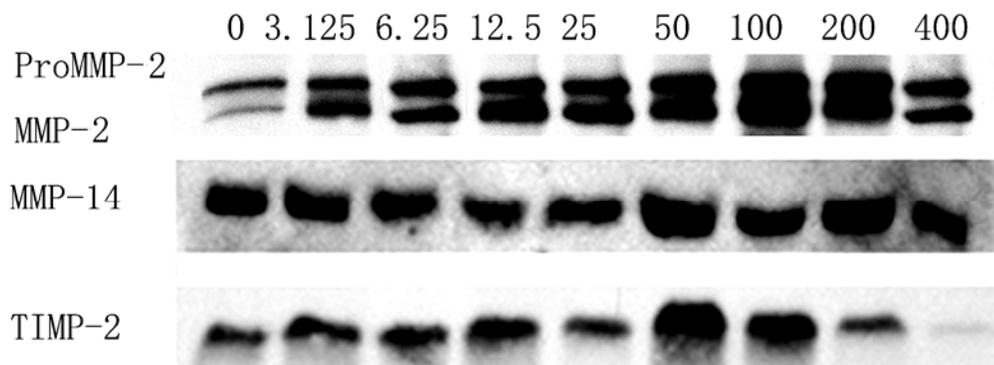


Figure 3.1.11. Western blots analyses for MMP-2, MMP-14 and TIMP-2 in the membrane extracts from HGFs treated with the different concentrations of CSC (0-400 $\mu\text{g/ml}$) for 72 hours.



Project Two: Effects of Cigarette Smoke Condensate and Nicotine on Human Gingival Fibroblast-mediated Collagen Degradation

Collagen Degradation

Cell morphology was examined every 24 hours. The HGFs displayed normal long spindle-shaped parallel alignment (data not shown) during the three days of treatment with 100 $\mu\text{g/ml}$ CSC or 2.4 $\mu\text{g/ml}$ nicotine. CSC at the concentration of 100 $\mu\text{g/ml}$ significantly increased the collagen degradation of HGFs at 72 hours (1.57 ± 0.48 fold, $p=0.011$) when compared to the control. However, nicotine at a concentration of 2.4 $\mu\text{g/ml}$ did not alter the collagen cleaving ability of the HGFs (1.01 ± 0.29 fold, $p=0.99$) (Figure 3.2.1 and 3.2.2).

Gelatin Zymography

Pro and active MMP-2 (72/62 kDa, respectively) bands were detected in the HGF conditioned media (Figure 3.2.3 and 3.2.4). No significant difference in the pro-MMP-2 (72 kDa) protein levels were detected with the different treatments ($p>0.05$). The active MMP-2 (62 kDa) levels increased in the media of the CSC-treated group (1.93 ± 0.55 fold, $p=0.04$) when compared to the control. However, active MMP-2 did not significantly increase in the nicotine-treated group (1.22 ± 0.18 fold, $p=0.72$).

Pro and active MMP-2 (72/62 kDa) were detected in the HGF membrane extracts (Figure 3.2.5 and 3.2.6). The proMMP-2 (72 kDa) levels were not altered in the CSC or nicotine treatment groups (1.21 ± 0.19 fold, $p=0.49$ and 1.42 ± 0.31 fold, $p=0.11$, respectively).

Active MMP-2 (62 kDa) increased significantly in the CSC-treated group (5.24 ± 1.42 fold, $p=0.002$) when compared to the control. There was no significant difference in the level of active MMP-2 in the HGF membrane extract of the nicotine treated group (1.01 ± 0.29 fold, $p=0.99$) when compared to the control.

Western Blots Analyses

Pro-MMP-2 (72 kDa), active MMP-2 (62 kDa), MMP-14 (64 kDa) and TIMP-2 (21 kDa) were all detected in the conditioned media (Figure 3.2.7 and 3.2.8). CSC increased the level of active MMP-2 (1.36 ± 0.18 , $p=0.043$) when compared to the control group. However, CSC did not alter the levels of pro-MMP-2, MMP-14 and TIMP-2 (1.16 ± 0.27 fold, $p=0.42$; 1.11 ± 0.04 fold, $p=0.068$; and 0.71 ± 0.67 fold, $p=0.69$, respectively) in the conditioned media. Nicotine did not alter the levels of pro-MMP-2, active MMP-2, MMP-14 and TIMP-2 (0.95 ± 0.23 fold, $p=0.93$; 0.95 ± 0.31 fold, $p=0.91$; 0.98 ± 0.09 fold, $p=0.89$; and 1.00 ± 0.5 fold, $p=1.00$, respectively) in the conditioned media.

Pro-MMP-2 (72 kDa), active MMP-2 (62 kDa), MMP-14 (64 kDa) and TIMP-2 (21kDa) were detected (Figure 3.2.9 and 3.2.10) in the membrane extracts from the HGFs. CSC increased the levels of active MMP-2, MMP-14, and TIMP-2 (1.56 ± 0.17 fold, $p=0.003$; 1.57 ± 0.19 fold, $p<0.001$; and 1.82 ± 0.73 fold, $p=0.019$, respectively) in the HGF membrane extracts when compared to the control group. However, nicotine did not significantly affect the levels of proMMP-2, active MMP-2, MMP-14, and TIMP-2 (1.10 ± 0.19 fold; $p=0.62$; 1.09 ± 0.031 fold, $p=0.79$; 1.04 ± 0.12 fold, $p=0.92$; and 1.30 ± 0.48 fold, $p=0.53$, respectively) when compared to the control.

RT-PCR

MMP-2, MMP-14 and TIMP-2 mRNA levels in the 100 µg/ml CSC treated HGFs increased 2.11 ± 0.13 ($p=0.02$), 1.85 ± 0.17 ($p<0.001$) and 2.01 ± 0.04 ($p<0.001$) folds, respectively, when compared to the control (Figure 3.2.11 and 3.2.12). However, nicotine (2.4 µg/ml) did not significantly alter the mRNA levels of MMP-2, MMP-14 and TIMP-2 when compared to the control group (1.62 ± 0.07 fold, $p=0.10$; 0.90 ± 0.01 fold, $p=0.257$; and 1.65 ± 0.02 fold, $p=0.09$, respectively).

Figure 3.2.1. Effects of CSC (100 $\mu\text{g/ml}$) and nicotine (2.4 $\mu\text{g/ml}$) on the collagen-degrading ability of the HGFs.

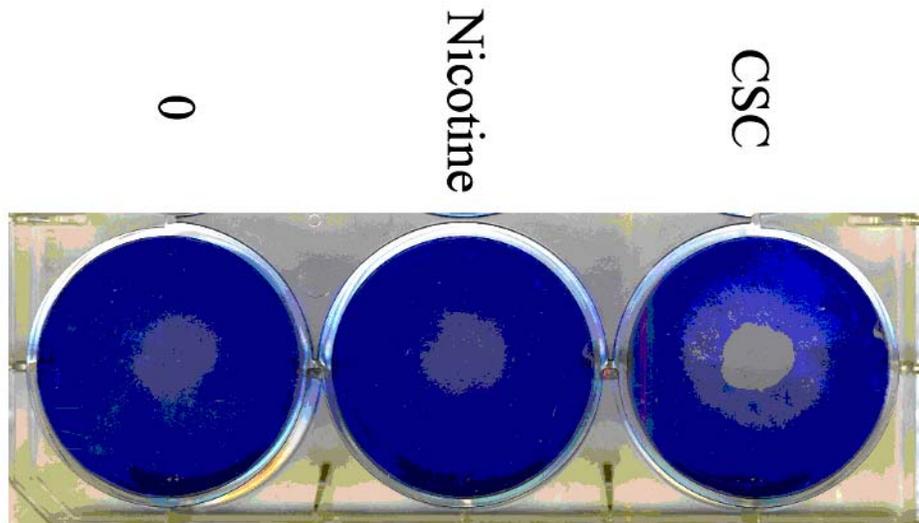


Figure 3.2.2. The graphic representation of the treated HGFs compared to the control HGFs on their collagen-degrading ability. * denotes significant difference, $p < 0.05$.

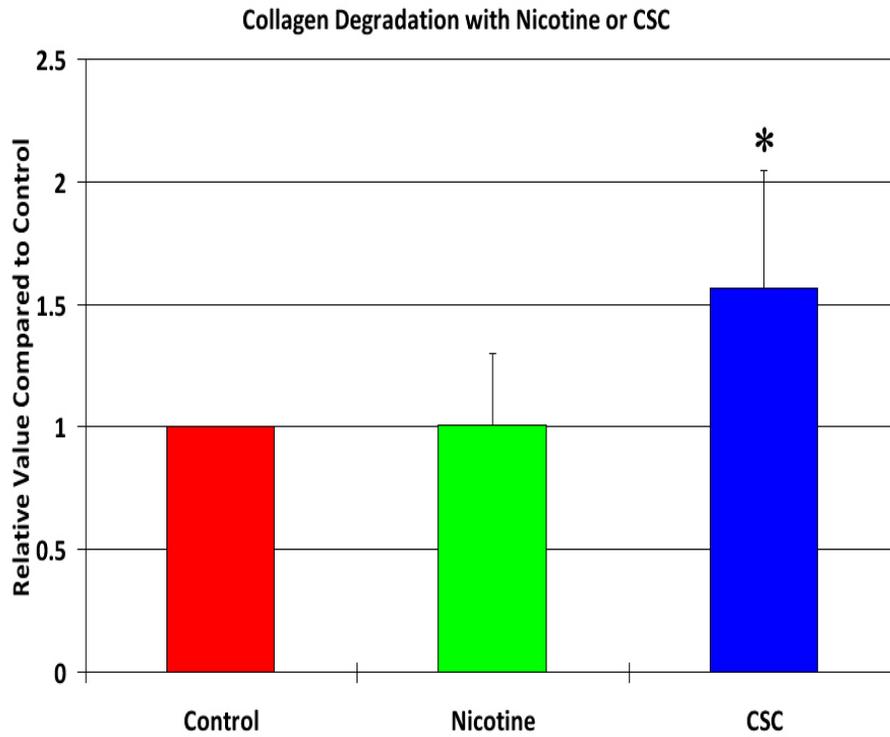


Figure 3.2.3. Gelatin zymography of conditioned media from HGFs treated with CSC (100 $\mu\text{g/ml}$) or nicotine (2.4 $\mu\text{g/ml}$) for 72 hours.

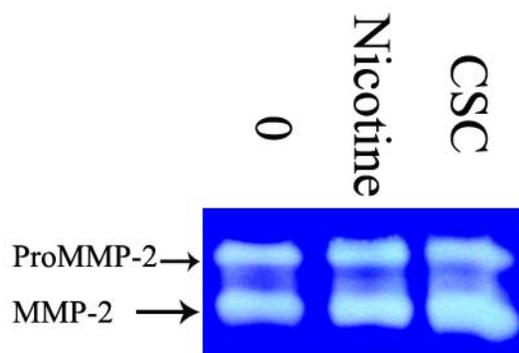


Figure 3.2.4. The graphic representation of gelatin zymography of conditioned media from treated HGFs compared to the control HGFs. * denotes significant difference, $p < 0.05$.

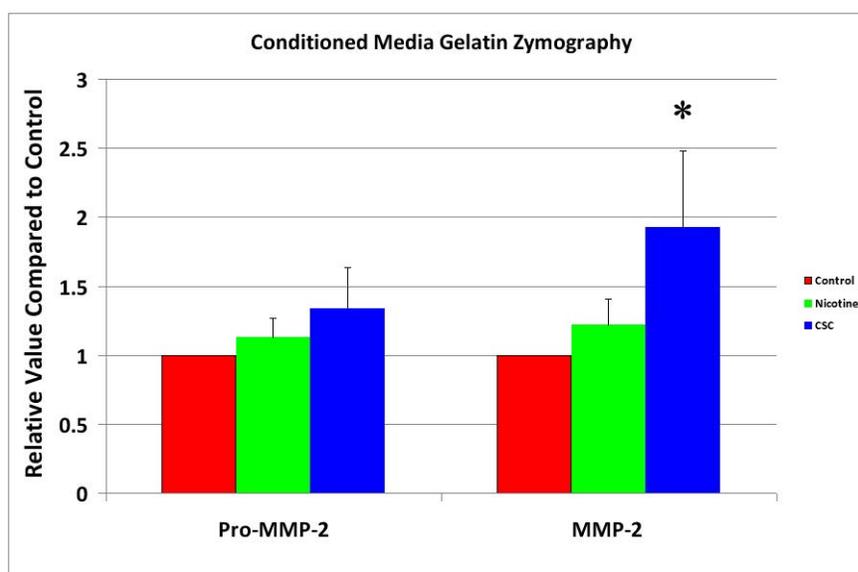


Figure 3.2.5. Gelatin zymography of the membrane extracts from HGFs treated with CSC (100 $\mu\text{g/ml}$) and nicotine (2.4 $\mu\text{g/ml}$) for 72 hours.

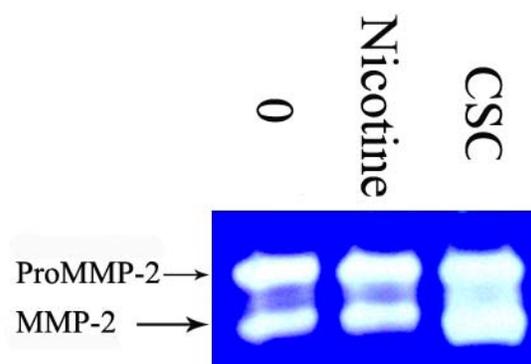


Figure 3.2.6. The graphic representation of gelatin zymography of the membrane extracts from treated HGFs compared to the control HGFs. * denotes significant difference, $p < 0.05$.

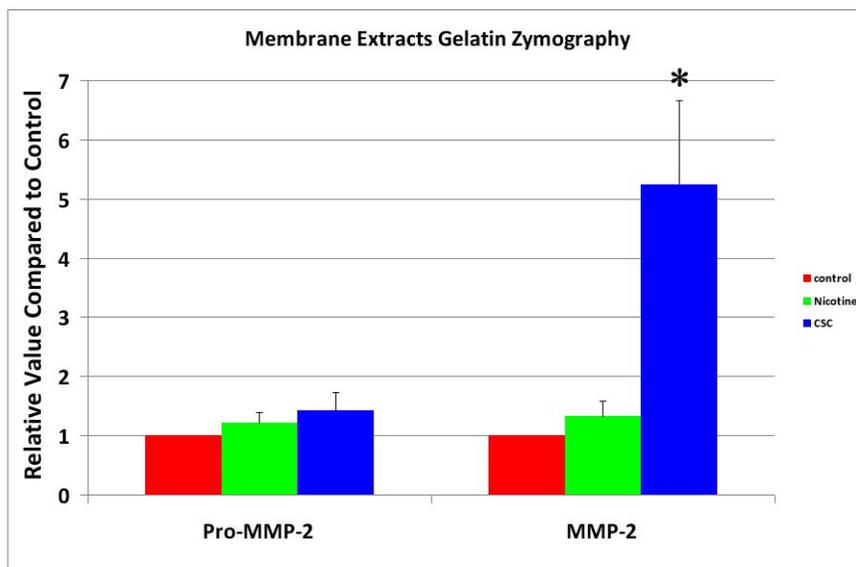


Figure 3.2.7. Western blots analyses for MMP-2, MMP-14, and TIMP-2 in conditioned media from HGFs treated with CSC (100 μ g/ml) or nicotine (2.4 μ g/ml) for 72 hours.

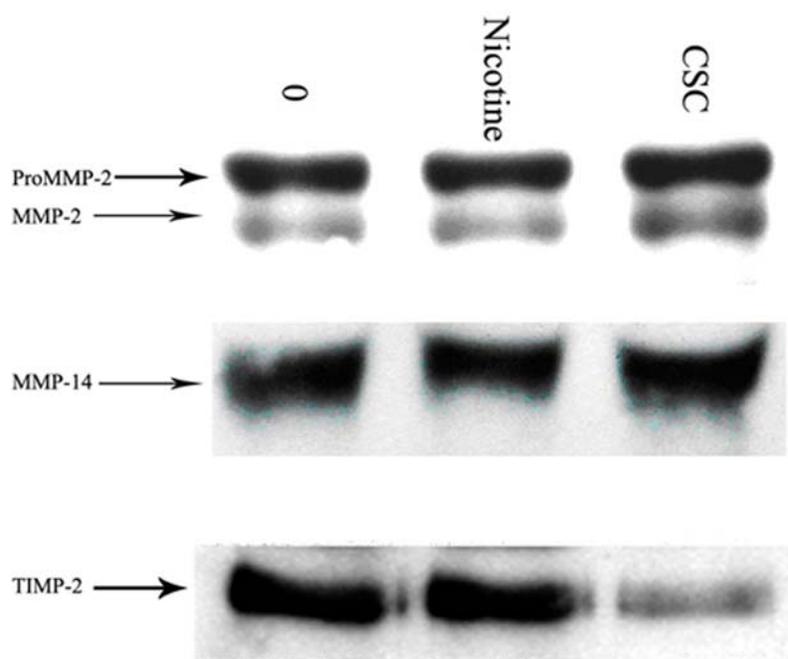


Figure 3.2.8. The graphic representation of Western blots analyses for MMP-2, MMP-14, and TIMP-2 in conditioned media from treated HGFs compared to the control HGFs. * denotes significant difference, $p < 0.05$.

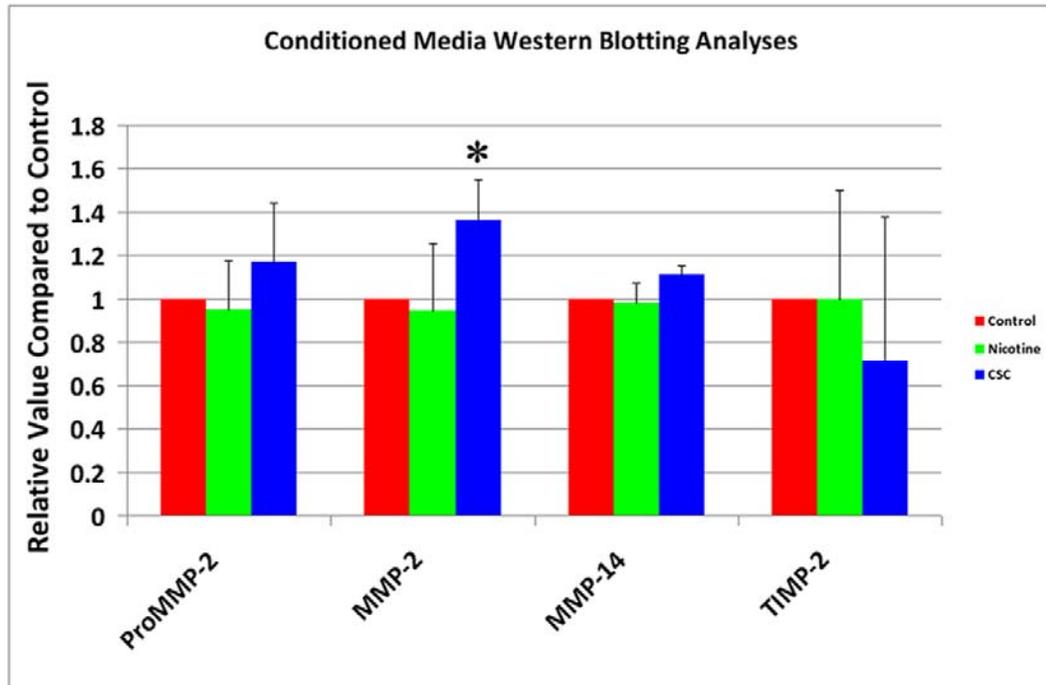


Figure 3.2.9. Western blots analyses for MMP-2, MMP-14, and TIMP-2 in the membrane extracts from the HGFs treated with CSC (100 $\mu\text{g/ml}$) or nicotine (2.4 $\mu\text{g/ml}$) for 72 hours.

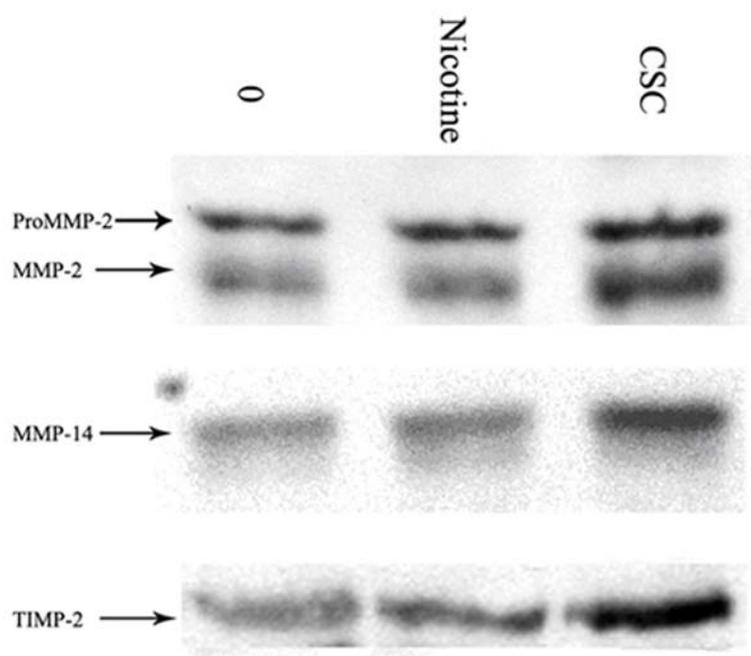


Figure 3.2.10. The graphic representation of Western blots analyses for MMP-2, MMP-14, and TIMP-2 in the membrane extracts from treated HGFs compared to the control HGFs. * denotes significant difference, $p < 0.05$.

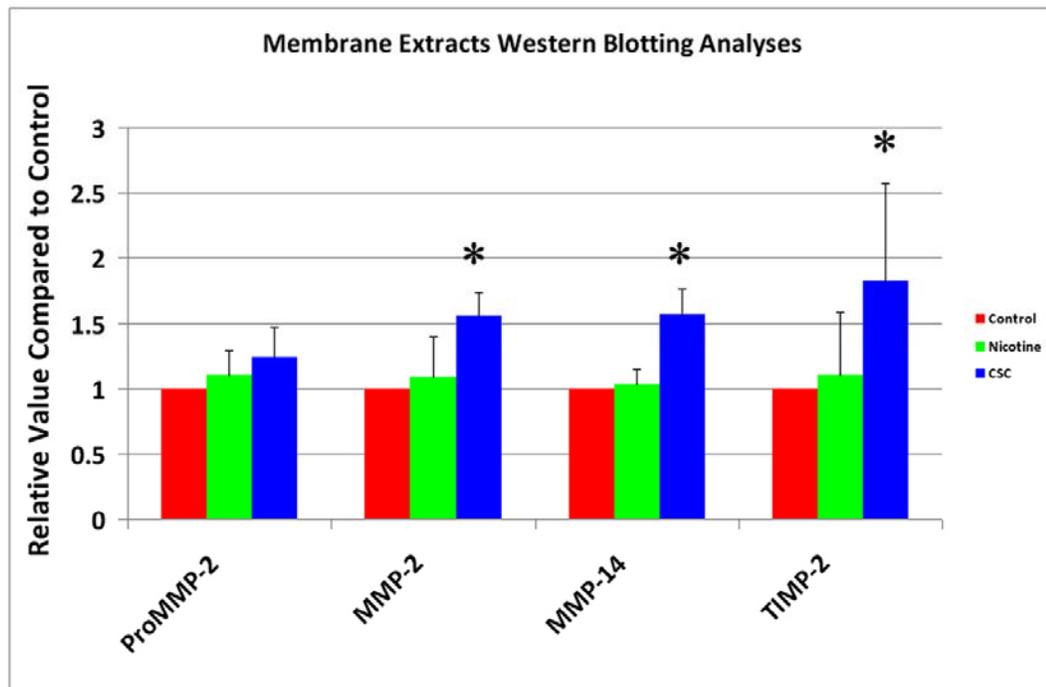


Figure 3.2.11. Effects of CSC (100 $\mu\text{g/ml}$) or nicotine (2.4 $\mu\text{g/ml}$) on MMP-2, MMP-14, and TIMP-2 mRNA expression. Cyclophilin (CYP) served as the control.

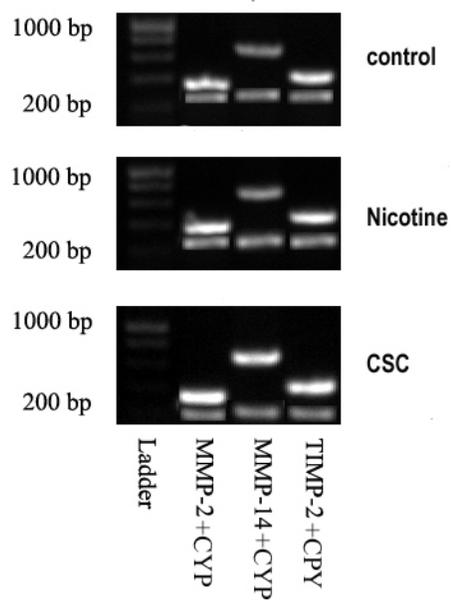
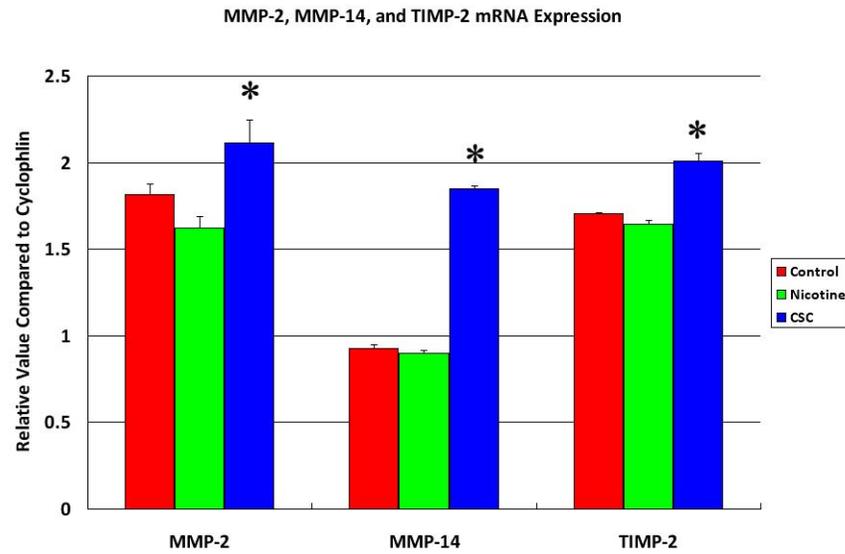


Figure 3.2.12. The graphic representation of MMP-2, MMP-14, and TIMP-2 mRNA expression of treated HGFs compared to the control HGFs. * denotes significant difference, $p < 0.05$.



Project Three: Combined Effects of Cigarette Smoke Condensate and *Porphyromonas gingivalis* on the Collagen Degrading Ability of Human Gingivalis Fibroblasts

Morphology of the HGFs

The HGFs attached and spread rapidly to take on a typical long spindle-shaped parallel alignment appearance in the 6-well plate without CSC at 3 days (Figure 3.3.1). When HGFs was treated with 50 µg/ml of CSC plus 10% (v/v) *P. gingivalis* supernatant for 3 days, the HGFs remained normal in appearance (Figure 3.3.2). However, the number of HGFs attached on the wells decreased when treated with 100 µg/ml of CSC plus 10% (v/v) *P. gingivalis* supernatant for 3 days (Figure 3.3.3).

Measurement of the Cell Proliferation by WST-1

After exposure to 50 µg/ml of CSC, 10% (v/v) *P. gingivalis* supernatant, 50 µg/ml of CSC plus 10% (v/v) *P. gingivalis* supernatant, or 100 µg/ml of CSC plus 10% (v/v) *P. gingivalis* supernatant for 3 days, the amount of cell proliferation was 85.8%, 62.9%, 82.3%, and 25.8%, respectively, when compared to the untreated control HGFs (100%; Figure 3.3.4.). Overall, 50 µg/ml of CSC, 10% (v/v) *P. gingivalis* supernatant, and 50 µg/ml of CSC plus 10% (v/v) *P. gingivalis* supernatant treatments had no effects on the growth of the HGFs when compared to the control (p=0.93, p=0.45, and p=0.88, respectively). However, the treatment of 100 µg/ml of CSC plus 10% (v/v) *P. gingivalis* supernatant had a negative effect on cell proliferation (p=0.003).

Measurement of Cytotoxicity of HGFs by LDH

Little effect was observed on the growth of HGFs after exposure to 50 µg/ml of CSC, 10% (v/v) *P. gingivalis* supernatant, or 50 µg/ml of CSC plus 10% (v/v) *P. gingivalis* supernatant for 3 days with cytotoxicity of 0.3% (p=0.86), 0.5% (p=0.56), and 5.0% (p=0.91), respectively, when compared to the control. However, after exposure to the treatment of 100 µg/ml of CSC plus 10% (v/v) *P. gingivalis* supernatant, the amount of cytotoxicity was 21.8% (p=0.009) (Figure 3.3.5).

Collagen Degradation

At day 1, almost no collagen degradation was noted among any of four groups (Figure 3.3.6). At day 3, untreated HGFs (control) cleaved the collagen underneath the cell colonies and formed a small transparent area. 50 µg/ml of CSC or 10% (v/v) *P. gingivalis* supernatant treated HGFs increased the collagen degradation by 1.2 fold and 1.4-fold, respectively, when compared to the control group. CSC at 50 µg/ml plus 10% (v/v) *P. gingivalis* supernatant treated HGFs increased the degradation of the collagen 1.7 fold. At day 5, 50 µg/ml of CSC plus 10% (v/v) *P. gingivalis* supernatant treated HGF degraded all of the collagen in the wells. At day 7, both 10% (v/v) *P. gingivalis* supernatant treated HGFs and 50 µg/ml of CSC plus 10% (v/v) *P. gingivalis* supernatant treated HGFs degraded all of the collagen in the wells. The MMP inhibitor (GM6001) inhibited collagen degradation by the HGFs treated with CSC at 50 µg/ml plus 10% (v/v) *P. gingivalis* supernatant (Figure 3.3.6).

Gelatin Zymography

Gelatinase-A (MMP-2, 72/62 kDa) bands were detected in the HGF conditioned media (Figure 3.3.7). Both proMMP-2 (72 kDa) and the active form of MMP-2 (62 kDa) were detected in all of the treatment groups. The active form of MMP-2 increased in the combined treatment group compared to the other groups. Furthermore, the production of MMP-9 (gelatinase B) could not be detected by zymography (data not shown) and the MMP inhibitor (1, 10-phenothroline) inhibited the gelatin degradation in the zymograms (Figure 3.3.7).

Western Blots Analyses

Pro-MMP-1 (57/52 kDa) was detected in the conditioned media from the control and the CSC treated HGFs. Both pro-MMP-1 (57/52 kDa) and active MMP-1 (42 kDa) were detected in the conditioned media from the *P. gingivalis* and CSC plus *P. gingivalis* treated HGFs (Figure 3.3.8). The 47 kDa active form of MMP-1 was detected in the conditioned media from CSC plus *P. gingivalis* (Figure 3.3.8). Similar to the results of the zymography, the gelatinase-A (MMP-2, 72/62 kDa) bands were detected and the active form of MMP-2 (62kDa) protein increased in the treated groups when compared to the control group (Figure 3.3.8). ProMMP-3 (60/54 kDa) were detected in the conditioned media of all the samples. The active forms of MMP-3 (50/44 kDa) were detected in the conditioned media from *P. gingivalis* and CSC plus *P. gingivalis* treated HGFs (Figure 3.3.8). MMP-14 (64 kDa) was detected in the conditioned media of all the samples (Figure 3.3.8). Both TIMP-1 (28.5 kDa) and TIMP-2 (21 kDa) were detected in the conditioned media from all the samples (Figure 3.3.8). However, TIMP-2 decreased

in the treated groups when compared to control group (Figure 3.3.8). In the cell membrane extracts, proMMP-2 (72kDa), MMP-2 (62kDa), MMP-14 (64 kDa) and TIMP-2 (21 kDa) were detected in all the groups. However, there was a lower molecular weight fragment of MMP-14 (44 kDa) in the 10% *P. gingivalis* treated group and CSC plus *P. gingivalis* treated group (Figure 3.3.9).

RT-PCR

The mRNAs for MMP-1, MMP-2, MMP-3, MMP-14, TIMP-1, and TIMP-2 were detected in both the untreated and treated HGFs (Figure 3.3.10). MMP-1, MMP-2, and MMP-14 mRNA levels in the 50 µg/ml of CSC plus 10% (v/v) *P. gingivalis* supernatant treated HGFs all increased about 1.7 fold when compared to the control. MMP-3 mRNA level in 50 µg/ml of CSC plus 10% (v/v) *P. gingivalis* supernatant treated HGFs increased more than 1.4 fold when compared to the control. Interestingly, the MMP-3 mRNA level in 50 µg/ml of CSC treated HGFs increased 2.7 fold when compared to the control. The TIMP-1 and TIMP-2 mRNA levels in 50 µg/ml of CSC plus 10% (v/v) *P. gingivalis* supernatant treated HGF increased about 1.3 and 1.4 fold, respectively. The other MMP/TIMP mRNA levels in the treated HGFs were basically unchanged when compared to the control group (Figure 3.3.11).

Figure 3.3.1. Morphology of untreated HGFs (negative control) at 3 days (Bar=50 μm , magnification $\times 100$).

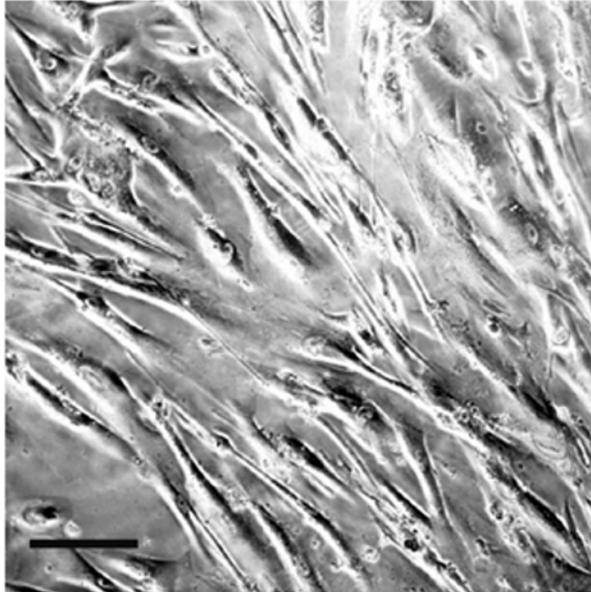


Figure 3.3.2. Morphology of 50 $\mu\text{g/ml}$ of CSC plus 10% (v/v) *P. gingivalis* supernatant treated HGFs at 3 days (Bar=50 μm , magnification $\times 100$).

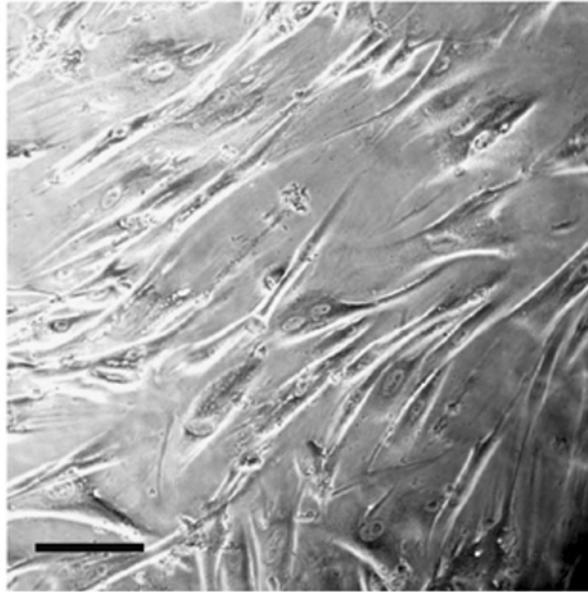


Figure 3.3.3. Morphology of 100 $\mu\text{g/ml}$ of CSC plus 10% (v/v) *P. gingivalis* supernatant treated HGFs at 3 days (Bar=50 μm , magnification $\times 100$).

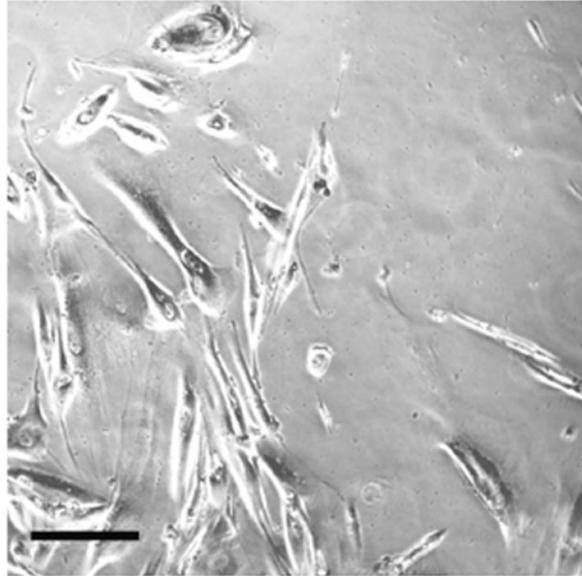


Figure 3.3.4. Cell proliferation (%) of HGFs by WST-1 at 72 hours. Results were presented as mean and standard deviation (SD). Error bars represent SD and N=4. The level of significance was set at $p < 0.05$. * denote a significant difference compared to the control.

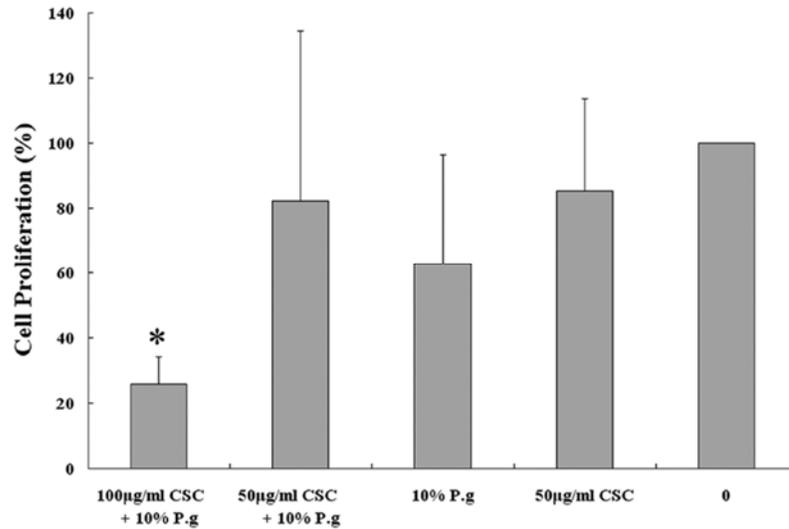


Figure 3.3.5. Cytotoxicity (%) of HGFs by LDH at 72 hours. Results were presented as mean and standard deviation (SD). Error bars represent SD and N=4. The level of significance was set at $p < 0.05$. * denote a significant difference compared to the control.

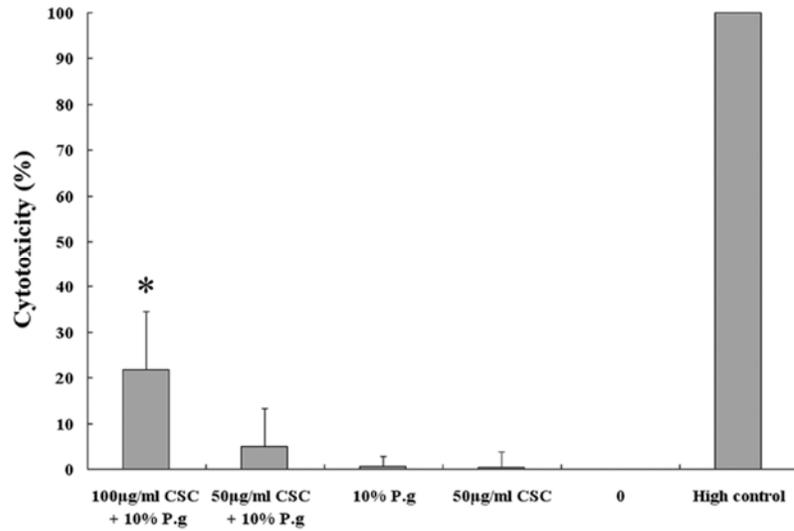


Figure 3.3.6. Effects of 50 $\mu\text{g/ml}$ of CSC, 10% (v/v) *P. gingivalis* supernatant, 50 $\mu\text{g/ml}$ of CSC plus 10% (v/v) *P. gingivalis* supernatant, or 50 $\mu\text{g/ml}$ of CSC plus 10% (v/v) *P. gingivalis* supernatant, as well as 100 nM GM6001, on the collagen-degrading ability of HGFs at 1, 3, 5, and 7 days.

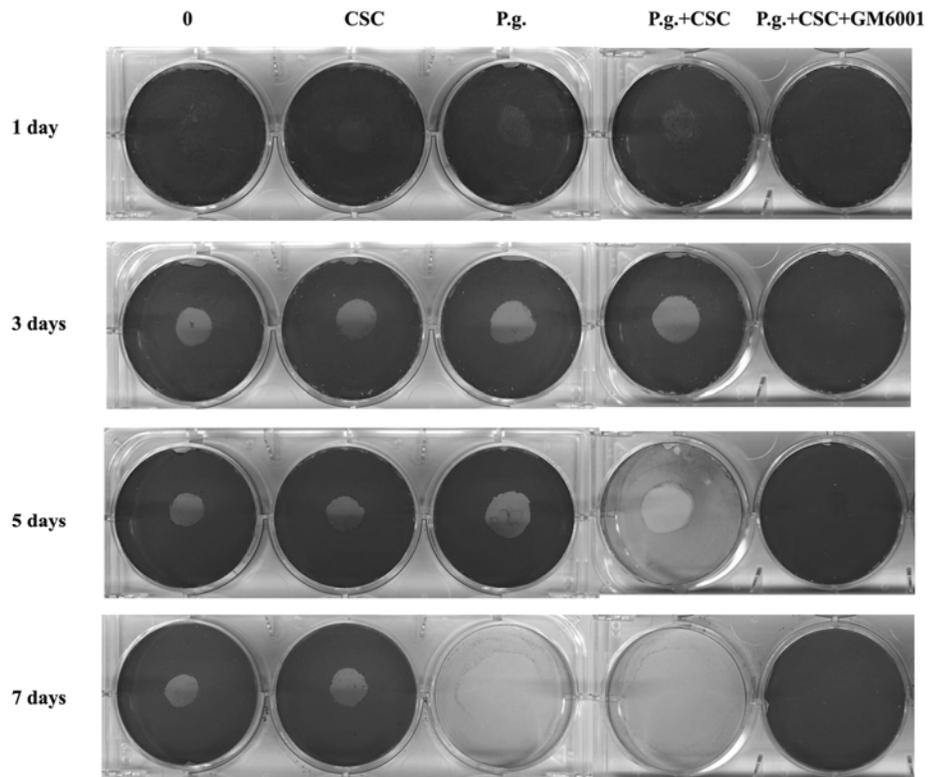


Figure 3.3.7. Gelatin zymography of the HGF-conditioned media with or without 10 mM 1, 10-phenothroline.

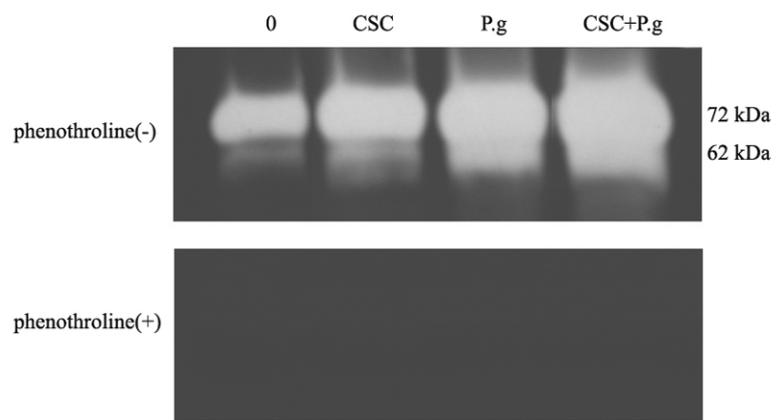


Figure 3.3.8. Western blots analyses for selected MMPs and TIMPs in the HGF-conditioned media.

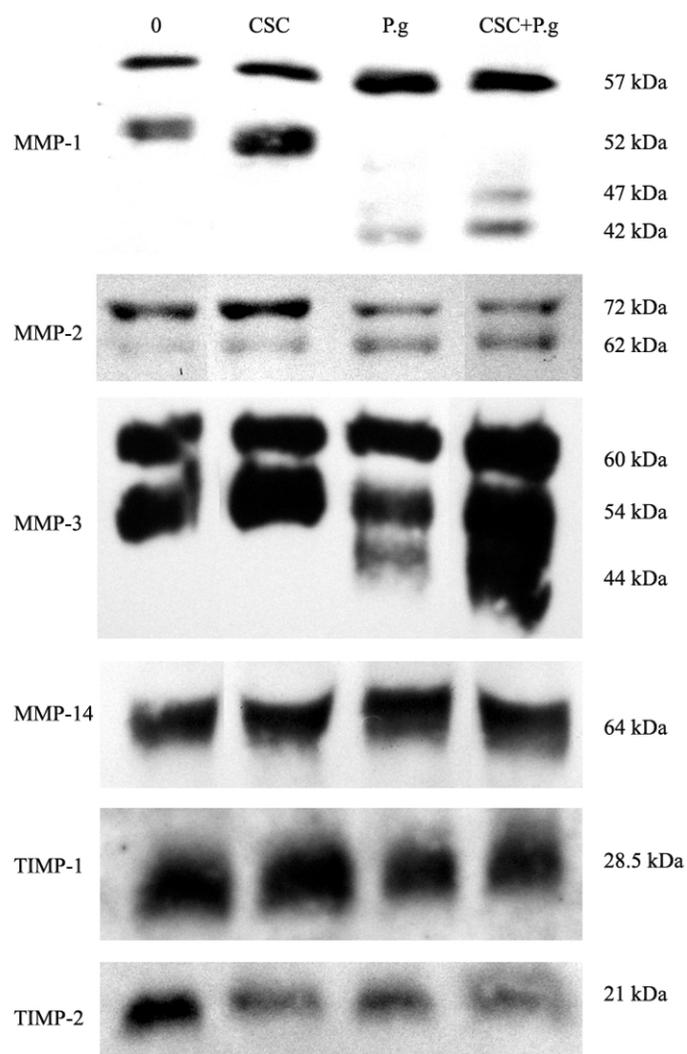


Figure 3.3.9. Western blots analyses for MMP-2, MMP-14, and TIMP-2 in the cell membrane extracts of HGFs

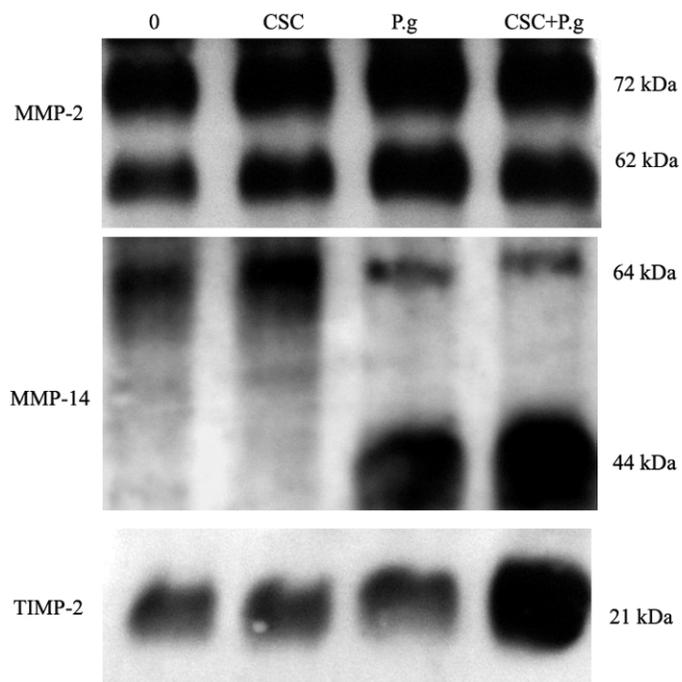


Figure 3.3.10. Effects of 50 $\mu\text{g/ml}$ of CSC, 10% (v/v) *P. gingivalis* supernatant, or 50 $\mu\text{g/ml}$ of CSC plus 10% (v/v) *P. gingivalis* supernatant on the mRNA expression of MMPs and TIMPs. RT-PCR results of the RNA extracted from untreated HGFs, 50 $\mu\text{g/ml}$ of CSC, 10% (v/v) *P. gingivalis* supernatant, or 50 $\mu\text{g/ml}$ of CSC plus 10% (v/v) *P. gingivalis* supernatant treated HGFs for 48 hours. The cyclophilin bands were utilized for standardization of the RT-PCR results.

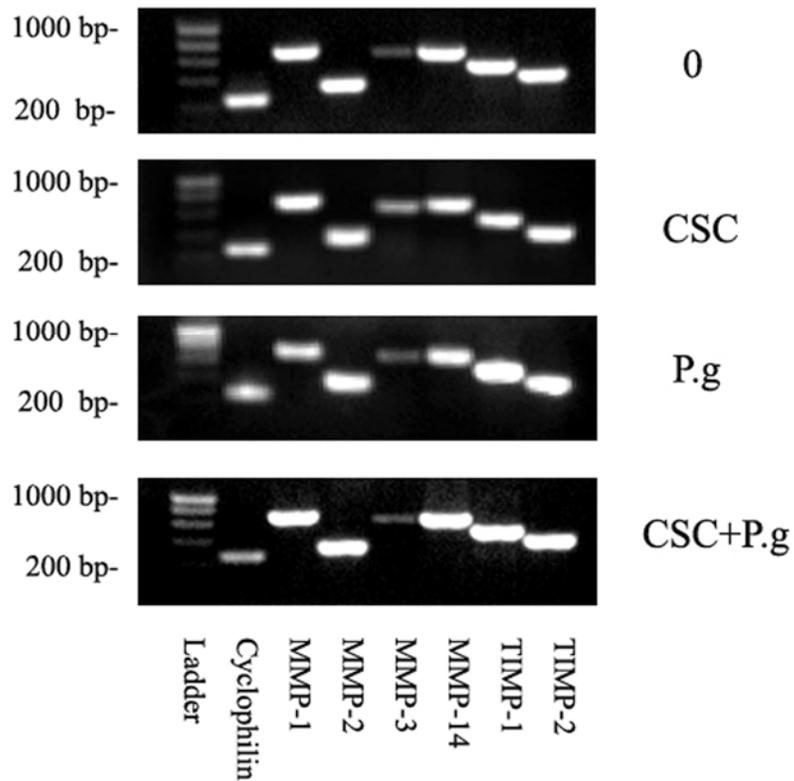
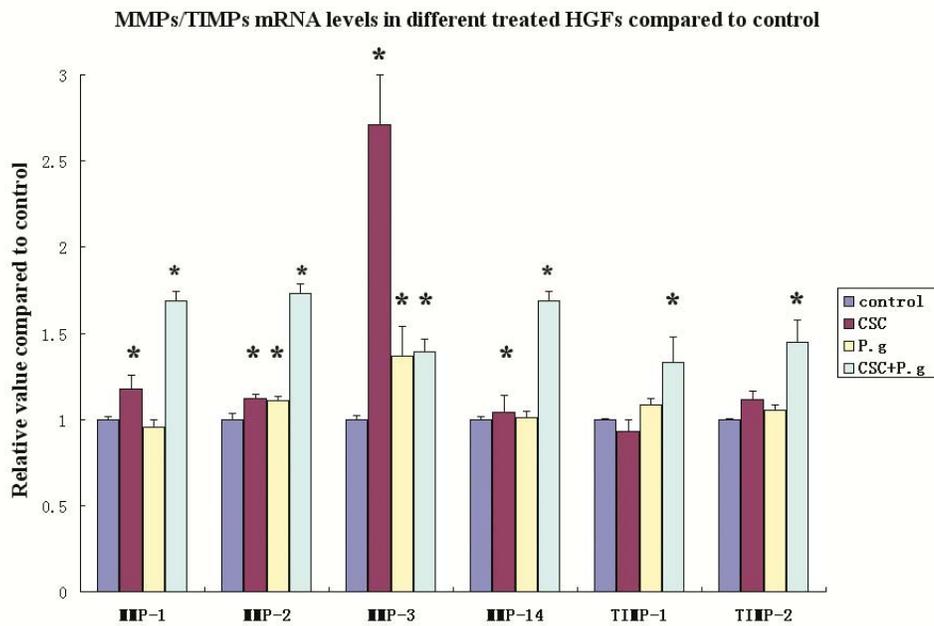


Figure 3.3.11. The graphic representation of 50 µg/ml of CSC, 10% (v/v) *P. gingivalis* supernatant, or 50 µg/ml of CSC plus 10% (v/v) *P. gingivalis* supernatant on MMP and TIMP mRNA expression. MMP and TIMP mRNA expression levels in different treated HGFs compared to control. The cyclophilin bands were utilized for standardization of the RT-PCR results. Results were presented as mean and standard deviation (SD) and N=3. Error bar represent SD. The level of significance was set at p<0.05. * denote a significant difference compared to the control.



CHAPTER FOUR

Discussion

Cigarette smoke is a complex mixture that contains more than 6,000 chemicals (Gao et al., 2005b). Nicotine is the addictive agent in tobacco. According to the manufacturer, CSC was prepared from a standard tobacco cigarette (Lee et al., 1976) that was smoked with a smoking machine under conditions so as to simulate as closely as possible the smoking habits of the average tobacco cigarette smoker. The cigarette smoke condensate (CSC) was captured and dissolved in dimethyl sulfoxide (DMSO) (Lee et al., 1976).

Many chemicals in CSC are cytotoxic. Cytotoxicity is an important factor in understanding the mechanisms of action of different chemicals on cells and tissues. Cytotoxicity is thought to play an important role in a number of pathological processes including inflammation and carcinogenesis. Cytotoxicity may also modulate the activity of other agents including free radicals, cytokines, and genotoxins (Bombick et al., 1998). In order to determine the effects of smoke condensate on HGF proliferation, the doses of CSC particulate matter ranged from 0-800 $\mu\text{g/ml}$. These concentrations were similar to human exposure according the following calculation. As previously reported (Gao et al., 2005a; Hellermann et al., 2002) each cigarette yields 26.1 mg of CSC. At a half pack to a full pack (10-20 cigarettes) smoked per day per person, the exposure of CSC amounts to 261-522 mg. Since the amount of saliva that one person secretes in one day is approximately 1-1.5 liter, the CSC exposure doses is 174-522 $\mu\text{g/ml}$. When the HGFs were exposed to CSC doses above 800 $\mu\text{g/ml}$ (1000 $\mu\text{g/ml}$, 2000 $\mu\text{g/ml}$, or 4000 $\mu\text{g/ml}$)

in preliminary experiments, almost no HGFs could be observed alive in the tissue culture plates (data not shown). This was the rationale for using the range of 0-800 $\mu\text{g/ml}$ of CSC in this study. Furthermore, the WST-1 and LDH results in project one showed that CSC is cytotoxic to HGFs at concentrations equal to or greater than 400 $\mu\text{g/ml}$ and affects the cell proliferation of HGFs at concentrations equal to or greater than 200 $\mu\text{g/ml}$.

In project one, the treatment time of CSC exposure to the HGFs was 72 hours. In the preliminary experiments, 24 or 48 hours was too short for the CSC to fully affect the HGFs (data not shown). Possible reasons include not enough time for the altered transcription to be seen at the translation level and for the MMPs to be activated. Another possible reason is that active MMP-2 has low collagenolytic activity and so higher amounts are needed to generate enough collagen degradation to be observed. Cell culture media is regularly changed every 3-4 days to provide the nutrients for HGFs. At 72 hours, the effects of CSC on collagen could be observed. This was the rationale for using 72 hours as the treatment time. In project one, since almost no HGFs were still alive when the concentration reached 800 $\mu\text{g/ml}$, there were no data for membrane extracts at CSC concentration of 800 $\mu\text{g/ml}$.

In project two, 2.4 $\mu\text{g/ml}$ nicotine and 100 $\mu\text{g/ml}$ CSC were used to compare their effects on the collagen degrading ability of HGFs. According to the project one, CSC (100 $\mu\text{g/ml}$) did not affect the proliferation of HGFs and was not cytotoxic to HGFs, as well as in assays routinely performed in the laboratory with CSC or nicotine (data not shown). However, when the concentration of CSC increased to 200 $\mu\text{g/ml}$, it had a negative effect

on HGF proliferation (data not shown). Therefore, 100 µg /ml of CSC was utilized in the study. 2.4 µg/ml nicotine was used in this study to examine the effects of nicotine on HGFs because 2.4 µg is the level found in 100 µg of CSC. A previous study showed that when the concentration of nicotine was less than 250 µg/ml, it did not produce any cytotoxic effects on the HGFs (Zhou et al., 2007). Thus, 2.4 µg/ml nicotine did not produce significant negative effects on the proliferation of HGFs.

In project three, 50 µg /ml of CSC was utilized as an experimental concentration. Based on a previous study, a concentration of CSC less than 200 µg/ml did not affect the proliferation of HGFs and had no toxic effects on the HGFs (Zhang et al., 2009). 100 µg /ml of CSC may have no negative effects on HGFs, but the combined effects of 100 µg/ml CSC and 10% (v/v) *P. gingivalis* supernatants reduced the cell viability of the HGFs. Since one cigarette can produced 26.1 mg CSC, 50 µg /ml of CSC is clearly very low compared to the uptake by any average smoker of several cigarettes per day (Nagaraj and Zacharias, 2007; Zhang et al., 2009). Furthermore, the results also demonstrated that 10% (v/v) *P. gingivalis* supernatants and 50 µg/ml CSC plus 10% (v/v) *P. gingivalis* supernatant did not affect the proliferation of HGFs and have no toxic effects on the HGFs. This was the rationale for using 50 µg /ml of CSC plus 10% (v/v) *P. gingivalis* supernatants.

The Negative Effects of CSC on the Proliferation of HGFs

The health of periodontal tissues depends on the normal functions of periodontal cells. The attachment, migration, growth, and differentiation of periodontal cells are critical

steps in the repair and regeneration of periodontal tissues. Human gingival fibroblasts (HGFs) are the main cellular component of periodontal connective tissues. Therefore, the normal functions of HGFs are very important to periodontal health. In periodontal disease development, HGF migration plays an important role in cell attachment to the extracellular matrix (Pitaru and Melcher, 1983). The control HGFs displayed a normal long spindle-shape with a parallel arrangement. However, the HGFs treated with CSC produced a disordered alignment and some of the HGFs detached.

In project one, the effects of the CSC increased with increasing concentrations of CSC gradually despite some variations. The negative effects of the CSC varied at low concentrations. This may be due to fact that there are many different components in the CSC and that each component has individual effects. Some of the components may promote the proliferation of the cells, whereas other components may inhibit the proliferation of the cells. Therefore, the net result is a balance of all the components. The results of this study showed the total effects of these components on the proliferation of HGFs. At high concentrations (≥ 200 $\mu\text{g/ml}$), cell proliferation decreased with CSC treatment. Luppi et al. (Luppi et al., 2005) reported that low concentrations of CSC increased proliferation of a bronchial epithelial cell line, whereas high concentrations were inhibitory as a result of cytotoxicity. In the current study, similar results were also observed. The cytotoxicity, as measured by LDH, increased with the increasing of concentrations of CSC; although at low concentrations, the cytotoxicity varied greatly. When the concentrations of CSC were more than 200 $\mu\text{g/ml}$, the toxic effects increase was dependent on the concentrations of the CSC. Alonso et al. (Alonso De La Pena et

al., 2007) reported that the LDH in whole saliva could be useful as a biochemical marker of periodontal status. In that study, the values of salivary LDH activity from individuals with periodontal disease were significantly higher than those obtained in people with a healthy periodontium.

CSC Increased Collagen Degrading Ability of HGFs

Periodontal disease is a very common inflammatory disease. During the development of periodontal disease, collagen degradation occurs. Collagen is the major extracellular component of gingiva. The collagen degradation assays demonstrated the ability of HGFs to degrade type I collagen. At low concentrations of CSC (≤ 100 $\mu\text{g/ml}$), the collagen degrading ability of the HGFs gradually increased with the increases in the CSC. Overall, an increase in MMP activity was observed in CSC treated HGFs. Although the family members involved differed from the current study, Nordskog et al. (Nordskog et al., 2005) showed that exposure of endothelial cells to CSC resulted in the upregulation of MMP-1, MMP-8, and MMP-9. Furthermore, Zhou et al. (Zhou et al., 2007) reported that nicotine, an active agent in CSC, could increase collagen degradation. Other studies have shown that nicotine can bind to the teeth root surfaces in smokers, and alter gingival and periodontal ligament fibroblast attachment and proliferation *in vivo* (Chang et al., 2002a; Gamal and Bayomy, 2002; Giannopoulou et al., 1999; James et al., 1999). Tipton and Dabbous (Tipton and Dabbous, 1995) also suggested that nicotine itself may augment the destruction of the gingival extracellular matrix (ECM) occurring during periodontal inflammation associated with smokeless tobacco use. However, the treatment of cardiac fibroblasts with nicotine leads to a decrease in collagenase activity

(Tomek et al., 1994). These different results may be due to the differences in the fibroblast type, the CSC concentrations, and the treatment periods, as well as the levels of MMP production. In this study, there was a considerable increase in TIMP-1 in the conditioned media. This may be due to an attempt to balance the increasing production of MMPs in the conditioned media.

The collagen degradation was limited to the area underneath the cell colonies, which suggests that the increased collagen cleavage was cell membrane associated. Active MMP-2, MMP-14 (membrane type-1 MMP), and TIMP-2 increased in the membrane extracts of CSC treated HGFs. These findings can be explained by the MMP-2 activation model proposed by Strongin et al. (Strongin et al., 1995). In this model, the TIMP-2 molecule serves as a bridging molecule for the binding of latent MMP-2 to active MMP-14 on the cell membrane and then another molecule of MMP-14 activates the latent MMP-2 by cleaving its propeptide domain. Therefore, more TIMP-2 may be recruited to the cell membrane to enhance complex formation and MMP-2 activation. Decreased TIMP-2 in the conditioned media resulted from relocalization of TIMP-2 to the cell membrane, thus leading to increased activation of MMP-2. However, it should be noted that higher concentrations of TIMP-2 can inhibit the membrane bound MMPs and block MMP-2 activation by inhibiting all the MMP-14. Balance between these membrane bound MMPs and TIMP-2 is critical in determining whether MMP-2 activation or inhibition occurs, but ratios can be challenging to understand because it is difficult to determine which MMPs and forms (active/latent) should be included (Strongin et al., 1995). MMP-

14 increased at the mRNA level and at the protein level in the cell membrane extracts. This also supported the MMP-14, TIMP-2 and MMP-2 activation model.

MMP-2 has weak collagenolytic activity (Hannas et al., 2007) and thus the increase of active MMP-2 could be responsible, in part, for increasing the collagen degradation mediated by HGFs. In addition, MMP-14 can also degrade type I collagen at a slow rate (Strongin et al., 1995). As mentioned previously, the level of MMP-2 increased in the conditioned medium from CSC treated HGFs. MMP-2 has limited ability to cleave type I collagen and is not concentrated at a central location in the media as it is on the cell membrane, where it may affect type I collagen turnover because of a higher localized level of active MMP-2 (Strongin et al., 1995).

The mechanisms by which CSC regulates the MMPs are currently unclear. Previous research has focused mainly on nicotine. Some studies have shown that nicotine can affect the production and distribution of gingival fibroblast cell surface proteins (Leonardi et al., 1999) and the activation of latent MMP-2 by MMP-14 at higher concentrations (Stanton et al., 1998). In this study, the HGFs exposed to CSC had more rounded edges. This may imply that CSC can also affect the cytoskeleton and/or cell attachment receptors (Stanton et al., 1998; Tomasek et al., 1997). Therefore, CSC might cause the redistribution of cell surface proteins such as the integrins and the membrane type-MMPs, which could subsequently affect the activation of latent MMP-2 (Zhou et al., 2007).

Other Effects of CSC on Cells

CSC affects the pro-inflammatory cytokines. Chujo et al. (Chujo et al., 2010) showed that CSC induce pro-inflammatory cytokine expression from synovial cells and exacerbate collagen-induced arthritis in mice. When human fibroblast-like synoviocytes were treated with CSC, the expression levels of interleukin (IL)-1alpha, IL-1beta, IL-6, IL-8, and CYP1A1 mRNAs were up-regulated in time- and dose-dependent manners (Shizu et al., 2008). Mahanonda et al. (Mahanonda et al., 2009) showed that cigarette smoke extract stimulated IL-8 expression from human gingival epithelial cells. Nicotine (162 µg/ml) showed similar effects but to a much lesser extent, thus implying that non-nicotine components in cigarette smoke extract may be primarily responsible for these immune-modulating effects (Mahanonda et al., 2009). Almasri et al. (Almasri et al., 2007) showed that nicotine significantly increased the expression levels of growth regulated oncogene (GRO)-alpha, IL-7, IL-10, and IL-15. Wendell et al. (Wendell and Stein, 2001) also showed that nicotine can stimulate human gingival fibroblast expression of IL-6 and IL-8. Johnson et al. (Johnson et al., 2010) showed that 162 µg/ml nicotine up-regulated the production of IL-1 alpha, but had no significant effect on IL-8 production in gingival keratinocytes. Cytokines can affect the expression of the MMPs (Mauviel, 1993; Ries and Petrides, 1995; Rooprai et al., 2000) and MMPs can lead to increased collagen degradation as seen in periodontal disease.

Role of Nicotine in Collagen Degradation of HGFs

In project two, the level of nicotine (2.4 µg/ml) in CSC did not play a major role in altering the collagen degradation mediated by the HGFs, and it had little influence on the

protein and mRNA levels of MMP-2, MMP-14, and TIMP-2. Zhou et al. (Zhou et al., 2007) reported that nicotine at 250 µg/ml did increase HGF-mediated collagen degradation. Some studies have shown that nicotine can bind to teeth root surfaces and built up in smokers, and alter gingival and periodontal ligament fibroblast attachment and proliferation *in vivo* at concentrations of 250-10000 µg/ml (Chang et al., 2002a; Gamal and Bayomy, 2002; Giannopoulou et al., 1999; James et al., 1999). Tipton and Dabbous (Tipton and Dabbous, 1995) suggested that nicotine (≥ 250 µg/ml) may augment the destruction of the gingival extracellular matrix occurring during periodontal inflammation. The saliva samples from smokeless tobacco users contained as much as 1560 µg/ml of nicotine (Hoffmann and Adams, 1981) and smoking one cigarette can expose the oral cavity to 1000 µg of nicotine (Benowitz and Jacob, 1984) without being toxic to the user. According to a recent study from Robson et al. (Robson et al., 2010), the concentrations of nicotine in the smoker's saliva ranged from about 0.9 µg/ml to 4.6 µg/ml depending on the amount of tobacco use. Another recent report from Malhotra et al. (Malhotra et al., 2010) showed that the concentrations of nicotine in gingival crevicular fluid ranged up to 6 µg/ml. The concentration of nicotine (2.4 µg/ml) used in this study was within these ranges.

Combined Effects of CSC and *P. gingivalis* on the Collagen Degrading Ability of HGFs

Project three demonstrated that CSC can increase the *P. gingivalis* enhanced collagen degradation by HGFs. During this process, the balance of the MMPs and TIMPs may play a key role. In this study, active MMP-1 (47 kDa), active MMP-2 (67 kDa), and

active MMP-3 (44 kDa) increased in the 50 µg/ml of CSC plus 10% (v/v) *P. gingivalis* supernatant treated group when compared to the other groups. Active collagenase-1 (MMP-1) is capable of cleaving native fibrillar collagen type I at specific sites between Gly⁷⁷⁵-Ile⁷⁷⁶ of the α1 chains and Gly⁷⁷⁵-Leu⁷⁷⁶ residues of the α2 chain. Active gelatinase-A (MMP-2) also has weak catalytic activity towards the fibrillar collagens (Ala-aho and Kahari, 2005). MMP-14 (membrane type 1 MMP, MT1-MMP) also can cleave type I collagen (Aznavoorian et al., 2001). This suggests that several MMPs may be players in the increased collagen degradation seen in this study. At 5 days, the collagen coated in the wells was totally degraded by the HGFs treated with 50 µg/ml of CSC plus 10% (v/v) *P. gingivalis*. However, this degradation was inhibited by the MMP inhibitor MG6001. Furthermore, in zymography, the gelatin degradation was inhibited by another MMP inhibitor 1, 10-phenothroline. All these results demonstrated that the MMPs play a role in degrading the type I collagen. The levels of TIMP-1 and TIMP-2 in the conditioned media decreased in spite of the increase in the mRNA level. This may be because *P. gingivalis* supernatant can degrade the TIMP-1 and TIMP-2 proteins (Andrian et al., 2007; Grenier and Mayrand, 2001; Sato et al., 2009). In addition, the TIMP-2 level decreased in the conditioned media and increased in the membrane extracts probably due to relocalization. The balance between the MMPs and TIMPs were disturbed, which lead to increased collagen degradation.

The mechanisms of how CSC increased the effects of the *P. gingivalis* enhanced collagen degradation are not very clear. One of the possible mechanisms is that the increased mRNA levels of the MMPs in the 50 µg/ml of CSC plus 10% (v/v) *P. gingivalis*

treatment groups were higher than the increased mRNA levels of the TIMPs. In this study, the mRNA level of MMP-1, MMP-2, and MMP-14 increased in the 50 µg/ml of CSC plus 10% (v/v) *P. gingivalis* supernatant treated HGFs when compared to the other treatment groups and the control, which is consistent to the results at the protein levels. These results were consistent with previous studies. Exposure of endothelial cells to CSC resulted in the up-regulation of MMP-1, MMP-8, and MMP-9 mRNA levels (Nordskog et al., 2005) and *P. gingivalis* supernatant led to the up-regulation of MMP-1 and MMP-2 mRNA levels in HGFs (Zhou and Windsor, 2006).

Another possible mechanism may be interpreted by the MMP-1 activation model (Ala-aho and Kahari, 2005; Schuppan and Hahn, 2000). MMP-1 is secreted as 52 kDa and 57 kDa latent forms. MMP-3 can superactivate the proMMP-1 and convert them into the 42 and 47 kDa active forms (Ala-aho and Kahari, 2005; Beklen et al., 2007; DeCarlo et al., 1997). In this study, the increase of active MMP-3 (44 kDa) accompanied the increase of active MMP-1 (47/42 kDa), especially in 50 µg/ml of CSC plus 10% (v/v) *P. gingivalis* supernatant treated HGFs.

Furthermore, MMP-14 can activate proMMP-2 by forming a trimolecular complex of MMP-14/TIMP-2/proMMP-2 (Hotary et al., 2003) and then another molecule of MMP-14 mediates the cleavage of the propeptide domain of MMP-2. Upon the activation of MMP-2, the amino-terminus of MMP-14 is, in turn, cleaved off, which results in a smaller fragment of MMP-14 (Strongin et al., 1995). In the current study, the proMMP-2, MMP-2, MMP-14 and TIMP-2 in the cell membrane extracts supported the above

activation model. Furthermore, the existence of a low-molecular weight MMP-14 fragment (44 kDa) in the cell membrane extracts also supported this model. Overall, an increase in the MMPs was observed. MMPs can also act as initiators of other proMMP activation cascades (Ala-aho and Kahari, 2005). However, these are just some of possible explanations. Therefore, further studies should be undertaken.

CHAPTER FIVE

Summary and Conclusion

This chapter provides a general summary and conclusions from the three projects that were described in the previous chapters of this dissertation. This chapter also discusses the significance of these studies in general terms and suggests future directions for continuing the research.

The broad objective of these investigations was to increase the understanding of the causal association between periodontal disease and tobacco. The rationale for our research studies was to define the etiological roles of smoking on harmful health effects, especially periodontal disease.

These studies collectively were aimed at exploring the molecular and biological mechanisms underlying the possible etiological links between tobacco and periodontal disease. In general, it was hypothesized that tobacco can increase human gingival fibroblast mediated-collagen degradation as seen in periodontal disease, and that the combination of cigarette smoke condensate (CSC) and *P. gingivalis* supernatant can further increase the collagen degrading ability of HGFs. The possible causal relationships between induced tissue destruction (i.e., periodontal disease) and tobacco in three *in vitro* investigations were explored. These *in vitro* studies lay the ground work for future animal studies.

The findings from the first project showed that HGF cell proliferation decreased and cytotoxicity of CSC increased when the concentrations of CSC increased. Cell proliferation decreased more than 50% when concentrations of CSC were above 200 $\mu\text{g/ml}$ and cytotoxicity of CSC increased more than 30% when the concentrations of CSC were above 400 $\mu\text{g/ml}$. CSC increased HGF collagen degrading ability, especially at the concentration of 100 $\mu\text{g/ml}$ when compared to the control. CSC increased the production of proMMP-1, proMMP-2, MMP-14, and TIMP-1, as well as decreased the production of TIMP-2, in the conditioned media. Furthermore compared to the control group, CSC increased MMP-2, MMP-14, and TIMP-2 in the membrane extracts, especially at concentrations of 50-100 $\mu\text{g/ml}$. However, this study did not identify which components in the CSC play key roles in these processes since CSC is a mixture. In brief, CSC affects HGF proliferation and is toxic at CSC concentrations of 400 $\mu\text{g/ml}$ and above. CSC can increase the collagen-degrading ability of HGFs by altering the production and localization of the MMPs and TIMPs.

The results of the second project confirmed the results of first study in that 100 $\mu\text{g/ml}$ CSC increased the collagen degradation by HGFs and enhanced the levels of TIMP-2, MMP-14 and active-MMP-2 in the membrane extracts of HGFs, as well as their mRNA levels. CSC also increased the level of active MMP-2 in the conditioned media. However, 2.4 $\mu\text{g/ml}$ nicotine, one of the major components and the most pharmacologically active agent in tobacco (2.4% nicotine in the CSC), had little influence on collagen degradation, as well as on the protein and mRNA levels of MMP-2, MMP-14, and TIMP-2 from the HGFs. Therefore, further studies examining other components

of CSC need to be taken. In brief, CSC may increase HGF-mediated collagen degradation by affecting membrane associated MMP-2, MMP-14, and TIMP-2, and the level of nicotine in the CSC may only play a limited role in this process.

The third project included a bacteria factor (*P. gingivalis* supernatant). *P. gingivalis* is one of the most abundant oral microorganisms associated with periodontal disease. The study illustrated that the combination of CSC and *P. gingivalis* supernatant increased collagen degradation, the protein levels of the active forms of MMP-1, MMP-2, MMP-3, and MMP-14 in the conditioned media, and the low molecular weight fragment of MMP-14 in the membrane extracts, as well as the mRNA levels of MMP-1, MMP-2, and MMP-14. In brief, the combined effects of CSC and *P. gingivalis* increased collagen degradation by HGFs by destroying the balance between the MMPs and TIMPs at multiple levels.

The cumulative evidence from these three studies supports the existence of biological mechanisms that could plausibly explain an etiological relationship between tobacco and tissue destruction (e.g., periodontal disease) and contribute to the risk of periodontal disease in susceptible individuals. These biological mechanisms mediating such a relationship are still not fully understood. However, scientists are successfully putting together the pieces of this puzzle. Future animal studies, as well as large and well controlled clinical trials, are necessary to develop the strategies to prevent and treat tobacco-related periodontal disease.

Significance of the Results

The results of the current studies provide data concerning the effects that CSC has on HGF-mediated collagen degradation in regards to affecting the expression of the MMPs/TIMPs, and may provide insights into the prevention and treatment options for tobacco-related periodontal disease that could be developed in the future.

Periodontal disease continues to be the leading cause of tooth loss in the U.S. and worldwide, and its impact on global health is immense. Traditional methods do not completely prevent and treat periodontal disease, and there is an increasing interest in studying the mechanisms of periodontal tissue destruction. The results of these studies can be considered as a step forward in determining how tobacco leads to periodontal tissue destruction. Thus, these results could be significant in providing new strategies for the prevention of periodontal disease through rebuilding the balance between the MMPs and TIMPs.

Future Directions

The studies described in this dissertation can be considered as preliminary investigations that possess great potential to develop more focused investigations. The fundamental objective of these studies was to assess the role of tobacco in cell-mediated collagen degradation. Thus, these findings can direct more specific and focused investigations in the future.

The first research project assessed CSC's cytotoxicity and its effects on cell proliferation, which provided suitable working concentrations of CSC for studies with the HGFs. Although collagen degradation might be the most important pathologic activity assay in the context of these studies, multiple assays examining additional aspects of the HGFs should be undertaken. Additional assays including those monitoring other proteases and cytokines should be employed in future studies.

The second project compared the effects of CSC and nicotine at the level in CSC on the collagen-degrading ability of HGFs. The results of this study showed that the level of nicotine in CSC may only play a limited role in this process. CSC is a complex mixture of components (Choi et al., 2008; Talhout et al., 2007) (e.g., benzo[α] pyrene, hydroquinone, acetaldehyde, acrolein, 2-naphthylamine, etc.) besides nicotine, which may be involved in the process of enhancing HGF-mediated collagen degradation and lead to periodontal disease. If the component or components responsible for the increased collagen degradation could be identified, the component or components could possibly be removed from the tobacco or interventions could be developed to block the ability of this component or components to enhance HGF-mediated collagen degradation in hopes to prevent tobacco related periodontal disease. Future studies on the other components in CSC should be undertaken.

In the third project, *P. gingivalis* supernatant was utilized to study the combined effects with CSC on human gingival fibroblasts. Future studies, similar to these, could be conducted utilizing other bacteria (e.g., *Actinobacillus actinomycetemcomitans*) with

cigarette smoke condensate in order to better understand bacterial and tobacco interactions with human gingival fibroblasts.

Each study in the dissertation investigated the effects of tobacco on HGFs, which came from one non-smoker patient. Individuals with the similar periodontal pathogens may experience different disease processes and/or severity (Newman, 1997; Phipps et al., 1997). Susceptibilities to periodontal disease have been demonstrated to be associated with certain genotypic and phenotypic characteristics (Lekic et al., 1997). For example, data from twin studies indicated that about half the population variance in periodontitis can be attributed to genetic factors (Corey et al., 1993; Michalowicz et al., 1991a; Michalowicz et al., 1991b; Michalowicz et al., 2000). Moreover, accumulating evidence shows that genetic variations in or near cytokine genes can affect the systemic inflammatory responses in people with periodontitis (D'Aiuto et al., 2004; Kornman et al., 1997). Heterogeneity exists in HGFs in regard to their collagenolytic activity in the presence of *P. gingivalis* (Zhou and Windsor, 2007). Heterogeneity of host immunological risk factors exist in patients with aggressive periodontitis (Takahashi et al., 2001). Furthermore, in the presence of *P. gingivalis*, collagen was more readily cleaved by gingival fibroblasts from inflamed tissues than from healthy tissues (Al-Shibani and Windsor, 2008). Biological diversity may also affect the HGF collagen degrading ability under the influence of environmental factors including tobacco use. For example, clinical evidence shows that some smokers even with very high dental plaque index still have good periodontal status and do not develop periodontal disease (Kondratiev et al., 2008). However, there is no data about the heterogeneity of the effects

of CSC on human gingival fibroblasts. Therefore, future studies should be conducted to assess the heterogeneity of different HGF cell lines in determining their CSC stimulated collagen-degrading ability.

As described earlier in this dissertation, traditional reverse transcript PCR techniques was used to detected the mRNA change of MMPs and TIMPs of tobacco treated HGFs. However, RT-PCR possesses some limitations including low sensitivity and no real-time detection. Further studies can be conducted using real-time PCR or other molecular techniques to detect the changes on mRNA levels.

Since all these studies were in vitro, future studies should use animal models to investigate the role of tobacco (with or without bacteria) in periodontal disease and its mechanisms. Furthermore, these studies just exposed the HGFs to CSC for a short period of time and focused on assessing the changes within this short time frame. Actually, patients are exposed to tobacco for a very long period of time. Future studies can explore the long-term effects such as on inflammatory cytokines. These studies should be more relevant to clinical applications.

Acknowledgments

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REFERENCES

- Al-Shibani N, Windsor LJ (2008). Effects of *Porphyromonas gingivalis* on human gingival fibroblasts from healthy and inflamed tissues. *J Periodontol Res* 43(4):465-470.
- Ala-aho R, Kahari VM (2005). Collagenases in cancer. *Biochimie* 87(3-4):273-86.
- Almasri A, Wisithphrom K, Windsor LJ, Olson B (2007). Nicotine and lipopolysaccharide affect cytokine expression from gingival fibroblasts. *J Periodontol* 78(3):533-41.
- Alonso De La Pena V, Diz Dios P, Tojo Sierra R (2007). Relationship between lactate dehydrogenase activity in saliva and oral health status. *Arch Oral Biol* 25(10):911-5.
- Andrian E, Grenier D, Rouabhia M (2006). *Porphyromonas gingivalis*-epithelial cell interactions in periodontitis. *J Dent Res* 85(5):392-403.
- Andrian E, Mostefaoui Y, Rouabhia M, Grenier D (2007). Regulation of matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases by *Porphyromonas gingivalis* in an engineered human oral mucosa model. *J Cell Physiol* 211(1):56-62.
- Aznavoorian S, Moore BA, Alexander-Lister LD, Hallit SL, Windsor LJ, Engler JA (2001). Membrane type I-matrix metalloproteinase-mediated degradation of type I collagen by oral squamous cell carcinoma cells. *Cancer Res* 61(16):6264-75.
- Barbour SE, Nakashima K, Zhang JB, Tangada S, Hahn CL, Schenkein HA, et al. (1997). Tobacco and smoking: environmental factors that modify the host response (immune system) and have an impact on periodontal health. *Crit Rev Oral Biol Med* 8(4):437-60.
- Beklen A, Ainola M, Hukkanen M, Gurgan C, Sorsa T, Kontinen YT (2007). MMPs, IL-1, and TNF are regulated by IL-17 in periodontitis. *J Dent Res* 86(4):347-51.
- Benowitz NL, Jacob P, 3rd (1984). Daily intake of nicotine during cigarette smoking. *Clin Pharmacol Ther* 35(4):499-504.
- Bergstrom J (2004). Tobacco smoking and chronic destructive periodontal disease. *Odontology* 92(1):1-8.
- Birkedal-Hansen H, Wells BR, Lin HY, Caufield PW, Taylor RE (1984). Activation of keratinocyte-mediated collagen (type I) breakdown by suspected human periodontopathogen. Evidence of a novel mechanism of connective tissue breakdown. *J Periodontal Res* 19(6):645-50.

- Birkedal-Hansen H, Moore WG, Bodden MK, Windsor LJ, Birkedal-Hansen B, DeCarlo A, et al. (1993). Matrix metalloproteinases: a review. *Crit Rev Oral Biol Med* 4(2):197-250.
- Bombick BR, Avalos JT, Nelson PR, Conrad FW, Doolittle DJ (1998). Comparative studies of the mutagenicity of environmental tobacco smoke from cigarettes that burn or primarily heat tobacco. *Environ Mol Mutagen* 31(2):169-75.
- Bosco AF, Bonfante S, de Almeida JM, Luize DS, Nagata MJ, Garcia VG (2007). A histologic and histometric assessment of the influence of nicotine on alveolar bone loss in rats. *J Periodontol* 78(3):527-32.
- Chamson A, Frey J, Hivert M (1982). Effects of tobacco smoke extracts on collagen biosynthesis by fibroblast cell cultures. *J Toxicol Environ Health* 9(5-6):921-32.
- Chang YC, Huang FM, Tai KW, Yang LC, Chou MY (2002a). Mechanisms of cytotoxicity of nicotine in human periodontal ligament fibroblast cultures in vitro. *J Periodontol* 37(4):279-85.
- Chang YC, Lai CC, Yang SF, Chan Y, Hsieh YS (2002b). Stimulation of matrix metalloproteinases by black-pigmented Bacteroides in human pulp and periodontal ligament cell cultures. *J Endod* 28(2):90-3.
- Chaussain-Miller C, Fioretti F, Goldberg M, Menashi S (2006). The role of matrix metalloproteinases (MMPs) in human caries. *J Dent Res* 85(1):22-32.
- Choi JM, Cho YC, Cho WJ, Kim TS, Kang BY (2008). Hydroquinone, a major component in cigarette smoke, reduces IFN-gamma production in antigen-primed lymphocytes. *Arch Pharm Res* 31(3):337-41.
- Chujo S, Okamoto S, Sunahara R, Adachi M, Yamada K, Hayashi H, et al. (2010). Cigarette smoke condensate extracts augment collagen-induced arthritis in mice. *Int Immunopharmacol* 10(10):1194-1199.
- Corey LA, Nance WE, Hofstede P, Schenkein HA (1993). Self-reported periodontal disease in a Virginia twin population. *J Periodontol* 64(12):1205-8.
- D'Aiuto F, Parkar M, Brett PM, Ready D, Tonetti MS (2004). Gene polymorphisms in pro-inflammatory cytokines are associated with systemic inflammation in patients with severe periodontal infections. *Cytokine* 28(1):29-34.
- DeCarlo AA, Grenett HE, Harber GJ, Windsor LJ, Bodden MK, Birkedal-Hansen B, et al. (1998). Induction of matrix metalloproteinases and a collagen-degrading phenotype in fibroblasts and epithelial cells by secreted Porphyromonas gingivalis proteinase. *J Periodontol* 33(7):408-20.

- DeCarlo AA, Jr., Windsor LJ, Bodden MK, Harber GJ, Birkedal-Hansen B, Birkedal-Hansen H (1997). Activation and novel processing of matrix metalloproteinases by a thiol-proteinase from the oral anaerobe *Porphyromonas gingivalis*. *J Dent Res* 76(6):1260-70.
- Fang Y, Svoboda KK (2005). Nicotine inhibits myofibroblast differentiation in human gingival fibroblasts. *J Cell Biochem* 95(6):1108-19.
- Fortino V, Maioli E, Torricelli C, Davis P, Valacchi G (2007). Cutaneous MMPs are differently modulated by environmental stressors in old and young mice. *Toxicol Lett* 173(2):73-9.
- Gamal AY, Bayomy MM (2002). Effect of cigarette smoking on human PDL fibroblasts attachment to periodontally involved root surfaces in vitro. *J Clin Periodontol* 29(8):763-70.
- Gao L, Tucker KL, Andreadis A (2005a). Transcriptional regulation of the mouse microtubule-associated protein tau. *Biochim Biophys Acta* 1681(2-3):175-81.
- Gao S, Chen K, Zhao Y, Rich CB, Chen L, Li SJ, et al. (2005b). Transcriptional and posttranscriptional inhibition of lysyl oxidase expression by cigarette smoke condensate in cultured rat fetal lung fibroblasts. *Toxicol Sci* 87(1):197-203.
- Giannobile WV (2008). Host-response therapeutics for periodontal diseases. *J Periodontol* 79(8 Suppl):1592-600.
- Giannopoulou C, Geinoz A, Cimasoni G (1999). Effects of nicotine on periodontal ligament fibroblasts in vitro. *J Clin Periodontol* 26(1):49-55.
- Grayson R, Douglas CW, Heath J, Rawlinson A, Evans GS (2003). Activation of human matrix metalloproteinase 2 by gingival crevicular fluid and *Porphyromonas gingivalis*. *J Clin Periodontol* 30(6):542-50.
- Grenier D, Mayrand D (2001). Inactivation of tissue inhibitor of metalloproteinases-1 (TIMP-1) by *Porphyromonas gingivalis*. *FEMS Microbiol Lett* 203(2):161-4.
- Guthrie EH (1966). What's happened since the Surgeon General's report on smoking and health? *Am J Public Health Nations Health* 56(12):Suppl 56:1-6.
- Hannas AR, Pereira JC, Granjeiro JM, Tjaderhane L (2007). The role of matrix metalloproteinases in the oral environment. *Acta Odontol Scand* 65(1):1-13.
- Hellermann GR, Nagy SB, Kong X, Lockey RF, Mohapatra SS (2002). Mechanism of cigarette smoke condensate-induced acute inflammatory response in human bronchial epithelial cells. *Respir Res* 3(1):22.

- Hilgers KK, Kinane DF (2004). Smoking, periodontal disease and the role of the dental profession. *Int J Dent Hyg* 2(2):56-63.
- Hoffmann D, Adams JD (1981). Carcinogenic tobacco-specific N-nitrosamines in snuff and in the saliva of snuff dippers. *Cancer Res* 41(11 Pt 1):4305-8.
- Hotary KB, Allen ED, Brooks PC, Datta NS, Long MW, Weiss SJ (2003). Membrane type I matrix metalloproteinase usurps tumor growth control imposed by the three-dimensional extracellular matrix. *Cell* 114(1):33-45.
- Hujoel PP, del Aguila MA, DeRouen TA, Bergstrom J (2003). A hidden periodontitis epidemic during the 20th century? *Community Dent Oral Epidemiol* 31(1):1-6.
- James JA, Sayers NM, Drucker DB, Hull PS (1999). Effects of tobacco products on the attachment and growth of periodontal ligament fibroblasts. *J Periodontol* 70(5):518-25.
- Johnson GK, Todd GL, Johnson WT, Fung YK, Dubois LM (1991). Effects of topical and systemic nicotine on gingival blood flow in dogs. *J Dent Res* 70(5):906-9.
- Johnson GK, Guthmiller JM (2007). The impact of cigarette smoking on periodontal disease and treatment. *Periodontol* 2000 44(1):178-94.
- Johnson GK, Guthmiller JM, Joly S, Organ CC, Dawson DV (2010). Interleukin-1 and interleukin-8 in nicotine- and lipopolysaccharide-exposed gingival keratinocyte cultures. *J Periodontal Res* 45(4):583-8.
- Kinane DF, Galicia JC, Gorr SU, Stathopoulou PG, Benakanakere M (2008). P. gingivalis interactions with epithelial cells. *Front Biosci* 13(1):966-84.
- Kondratiev S, Gnepp DR, Yakirevich E, Sabo E, Annino DJ, Rebeiz E, et al. (2008). Expression and prognostic role of MMP2, MMP9, MMP13, and MMP14 matrix metalloproteinases in sinonasal and oral malignant melanomas. *Hum Pathol* 39(3):337-43.
- Kornman KS, Crane A, Wang HY, di Giovine FS, Newman MG, Pirk FW, et al. (1997). The interleukin-1 genotype as a severity factor in adult periodontal disease. *J Clin Periodontol* 24(1):72-7.
- Lee ML, Novotny M, Bartle KD (1976). Gas chromatography/mass spectrometric and nuclear magnetic resonance spectrometric studies of carcinogenic polynuclear aromatic hydrocarbons in tobacco and marijuana smoke condensates. *Anal Chem* 48(2):405-16.
- Lekic PC, Pender N, McCulloch CA (1997). Is fibroblast heterogeneity relevant to the health, diseases, and treatments of periodontal tissues? *Crit Rev Oral Biol Med* 8(3):253-68.

- Leonardi R, Lanteri E, Stivala F, Caltabiano M, Fenga C, Travali S (1999). Alteration in alpha 2 integrin immunocytochemical expression on cultured human gingival fibroblasts following nicotine exposure. *Minerva Stomatol* 48(11):495-9.
- Luppi F, Aarbiou J, van Wetering S, Rahman I, de Boer WI, Rabe KF, et al. (2005). Effects of cigarette smoke condensate on proliferation and wound closure of bronchial epithelial cells in vitro: role of glutathione. *Respir Res* 6(1):140.
- Mahanonda R, Sa-Ard-Iam N, Eksomtramate M, Rerkyen P, Phairat B, Schaecher KE, et al. (2009). Cigarette smoke extract modulates human beta-defensin-2 and interleukin-8 expression in human gingival epithelial cells. *J Periodontol Res* 44(4):557-64.
- Makela M, Salo T, Uitto VJ, Larjava H (1994). Matrix metalloproteinases (MMP-2 and MMP-9) of the oral cavity: cellular origin and relationship to periodontal status. *J Dent Res* 73(8):1397-406.
- Makino A, Yamada S, Okuda K, Kato T (2008). Nicotine involved in periodontal disease through influence on cytokine levels. *FEMS Immunol Med Microbiol* 52(2):282-6.
- Malhotra R, Kapoor A, Grover V, Kaushal S (2010). Nicotine and periodontal tissues. *J Indian Soc Periodontol* 14(1):72-9.
- Mauviel A (1993). Cytokine regulation of metalloproteinase gene expression. *J Cell Biochem* 53(4):288-95.
- Michalowicz BS, Aeppli D, Virag JG, Klump DG, Hinrichs JE, Segal NL, et al. (1991a). Periodontal findings in adult twins. *J Periodontol* 62(5):293-9.
- Michalowicz BS, Aeppli DP, Kuba RK, Bereuter JE, Conry JP, Segal NL, et al. (1991b). A twin study of genetic variation in proportional radiographic alveolar bone height. *J Dent Res* 70(11):1431-5.
- Michalowicz BS, Diehl SR, Gunsolley JC, Sparks BS, Brooks CN, Koertge TE, et al. (2000). Evidence of a substantial genetic basis for risk of adult periodontitis. *J Periodontol* 71(11):1699-707.
- Mineoka T, Awano S, Rikimaru T, Kurata H, Yoshida A, Ansai T, et al. (2008). Site-specific development of periodontal disease is associated with increased levels of *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* in subgingival plaque. *J Periodontol* 79(4):670-6.
- Morozumi T, Kubota T, Sato T, Okuda K, Yoshie H (2004). Smoking cessation increases gingival blood flow and gingival crevicular fluid. *J Clin Periodontol* 31(4):267-72.
- Nagaraj NS, Zacharias W (2007). Cigarette smoke condensate increases cathepsin-mediated invasiveness of oral carcinoma cells. *Toxicol Lett* 170(2):134-45.

- Newman MG (1997). Genetic risk for severe periodontal disease. *Compend Contin Educ Dent* 18(9):881-4, 886, 888 passim; quiz 894.
- Noiri Y, Li L, Yoshimura F, Ebisu S (2004). Localization of Porphyromonas gingivalis-carrying fimbriae in situ in human periodontal pockets. *J Dent Res* 83(12):941-5.
- Nordskog BK, Fields WR, Hellmann GM (2005). Kinetic analysis of cytokine response to cigarette smoke condensate by human endothelial and monocytic cells. *Toxicology* 212(2-3):87-97.
- Office-of-Applied-Studies (2005). Substance Abuse and Mental Health Services Administration. Results from the 2004 National Survey on Drug Use and Health: National Findings. *DHHS Pub. No. SMA 05-4062*.
- Peacock ME, Sutherland DE, Schuster GS, Brennan WA, O'Neal RB, Strong SL, et al. (1993). The effect of nicotine on reproduction and attachment of human gingival fibroblasts in vitro. *J Periodontol* 64(7):658-65.
- Phipps RP, Borrello MA, Blieden TM (1997). Fibroblast heterogeneity in the periodontium and other tissues. *J Periodontal Res* 32(1 Pt 2):159-65.
- Pihlstrom BL, Michalowicz BS, Johnson NW (2005). Periodontal diseases. *Lancet* 366(9499):1809-20.
- Pitaru S, Melcher AH (1983). Orientation of gingival fibroblasts and newly-synthesized collagen fibers in vitro. Resemblance to transeptal and dento-gingival fibers. *J Periodontal Res* 18(5):483-500.
- Ra HJ, Parks WC (2007). Control of matrix metalloproteinase catalytic activity. *Matrix Biol* 26(8):587-96.
- Ries C, Petrides PE (1995). Cytokine regulation of matrix metalloproteinase activity and its regulatory dysfunction in disease. *Biol Chem Hoppe Seyler* 376(6):345-55.
- Rivera-Hidalgo F (2003). Smoking and periodontal disease. *Periodontol 2000* 32(1):50-8.
- Robertson PB, Walsh M, Greene J, Ernster V, Grady D, Hauck W (1990). Periodontal effects associated with the use of smokeless tobacco. *J Periodontol* 61(7):438-43.
- Robson N, Bond A, Wolff K (2010). Salivary nicotine and cotinine concentrations in unstimulated and stimulated saliva. *Afr J Pharm and Pharmacol* 4(2):061-065.
- Rooprai HK, Rucklidge GJ, Panou C, Pilkington GJ (2000). The effects of exogenous growth factors on matrix metalloproteinase secretion by human brain tumour cells. *Br J Cancer* 82(1):52-5.

- Ryder MI (2007). The influence of smoking on host responses in periodontal infections. *Periodontol 2000* 43(1):267-77.
- Sato Y, Kishi J, Suzuki K, Nakamura H, Hayakawa T (2009). Sonic extracts from a bacterium related to periapical disease activate gelatinase A and inactivate tissue inhibitor of metalloproteinases TIMP-1 and TIMP-2. *Int Endod J* 42(12):1104-11.
- Schuppan D, Hahn EG (2000). MMPs in the gut: inflammation hits the matrix. *Gut* 47(1):12-4.
- Shizu M, Itoh Y, Sunahara R, Chujo S, Hayashi H, Ide Y, et al. (2008). Cigarette smoke condensate upregulates the gene and protein expression of proinflammatory cytokines in human fibroblast-like synoviocyte line. *J Interferon Cytokine Res* 28(8):509-21.
- Sorsa T, Tjaderhane L, Salo T (2004). Matrix metalloproteinases (MMPs) in oral diseases. *Oral Dis* 10(6):311-8.
- Stanton H, Gavrilovic J, Atkinson SJ, d'Ortho MP, Yamada KM, Zardi L, et al. (1998). The activation of ProMMP-2 (gelatinase A) by HT1080 fibrosarcoma cells is promoted by culture on a fibronectin substrate and is concomitant with an increase in processing of MT1-MMP (MMP-14) to a 45 kDa form. *J Cell Sci* 111(Pt 18):2789-98.
- Strongin AY, Collier I, Bannikov G, Marmer BL, Grant GA, Goldberg GI (1995). Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloprotease. *J Biol Chem* 270(10):5331-8.
- Takahashi K, Ohyama H, Kitanaka M, Sawa T, Mineshiba J, Nishimura F, et al. (2001). Heterogeneity of host immunological risk factors in patients with aggressive periodontitis. *J Periodontol* 72(4):425-37.
- Talhout R, Opperhuizen A, van Amsterdam JG (2007). Role of acetaldehyde in tobacco smoke addiction. *Eur Neuropsychopharmacol* 17(10):627-36.
- Tervahartiala T, Pirila E, Ceponis A, Maisi P, Salo T, Tuter G, et al. (2000). The in vivo expression of the collagenolytic matrix metalloproteinases (MMP-2, -8, -13, and -14) and matrilysin (MMP-7) in adult and localized juvenile periodontitis. *J Dent Res* 79(12):1969-77.
- Tipton DA, Dabbous MK (1995). Effects of nicotine on proliferation and extracellular matrix production of human gingival fibroblasts in vitro. *J Periodontol* 66(12):1056-64.
- Tomar SL, Asma S (2000). Smoking-attributable periodontitis in the United States: findings from NHANES III. National Health and Nutrition Examination Survey. *J Periodontol* 71(5):743-51.

Tomasek JJ, Halliday NL, Updike DL, Ahern-Moore JS, Vu TK, Liu RW, et al. (1997). Gelatinase A activation is regulated by the organization of the polymerized actin cytoskeleton. *J Biol Chem* 272(11):7482-7.

Tomek RJ, Rimar S, Eghbali-Webb M (1994). Nicotine regulates collagen gene expression, collagenase activity, and DNA synthesis in cultured cardiac fibroblasts. *Mol Cell Biochem* 136(2):97-103.

Verstappen J, Von den Hoff JW (2006). Tissue inhibitors of metalloproteinases (TIMPs): their biological functions and involvement in oral disease. *J Dent Res* 85(12):1074-84.

Wahlgren J, Maisi P, Sorsa T, Sutinen M, Tervahartiala T, Pirila E, et al. (2001). Expression and induction of collagenases (MMP-8 and -13) in plasma cells associated with bone-destructive lesions. *J Pathol* 194(2):217-24.

Wendell KJ, Stein SH (2001). Regulation of cytokine production in human gingival fibroblasts following treatment with nicotine and lipopolysaccharide. *J Periodontol* 72(8):1038-44.

Winn DM (2001). Tobacco use and oral disease. *J Dent Educ* 65(4):306-12.

Zhang W, Song F, Windsor LJ (2009). Cigarette smoke condensate affects the collagen-degrading ability of human gingival fibroblasts. *J Periodontal Res* 44(6):704-13.

Zhou J, Windsor LJ (2006). Porphyromonas gingivalis affects host collagen degradation by affecting expression, activation, and inhibition of matrix metalloproteinases. *J Periodontal Res* 41(1):47-54.

Zhou J, Olson BL, Windsor LJ (2007). Nicotine increases the collagen-degrading ability of human gingival fibroblasts. *J Periodontal Res* 42(3):228-35.

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□ **AD HOC REVIEWERS FOR INTERNATIONAL JOURNAL**

- ◆ Journal of Periodontal Research
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- ◆ International Journal of Dermatology

□ SELECTED PEER REVIEW PUBLICATIONS

1. Effects of Cigarette Smoke Condensate and Nicotine on Human Gingival Fibroblast-mediated Collagen Degradation. Zhang W, Fang M, Song F, Windsor L. Journal of Periodontology 2010 Dec 13. [Epub ahead of print]
2. Matrix metalloproteinase expression during blastema formation in regeneration-competent versus regeneration-deficient amphibian limbs. Santosh N, Windsor LJ, Mahmoudi BS, Li B, Zhang W, Chernoff EA, Rao N, Stocum DL, Song F. Developmental Dynamics 2010 Dec 2. [Epub ahead of print]
3. Tobacco and *P. gingivalis* Effects on Gingival Fibroblasts. W. Zhang, F. Song, L. J. Windsor. Journal of Dental Research 2010 May; 89(5):527-31.
4. Multifocal myopericytoma in the maxillofacial region: a case report. Xia L, Chen Y, Geng N, Jiang J, Yang M, Zhang W. Oral Surg, Oral Medicine, Oral Pathology, Oral Radiology, Endodontics 2010 Feb; 109(2):e59-62.
5. Cigarette Smoke Condensate Affects the Collagen-Degrading Ability of Human Gingival Fibroblasts. Zhang W, Song F, Windsor L. Journal of Periodontal Research 2009 Dec; 44(6):704-13.
6. Malignant transformation of Oral lichen planus: a report of 23 cases. Fang M, Zhang W, Chen Y, He Z. Quintessence International 2009; 40(3): 235-242.
7. MMPs, TIMP-2, and TGF- β 1 in the Cancerization of Oral Lichen Planus. Chen Y, Zhang W, Geng N, Tian K, Windsor L. Head & Neck 2008; 30(9):1237-45.
8. A case report of chondroma in the soft tissue of tongue. Bao DM, Chen Y, Yang MZ, Geng N, Zhang WP. Chinese Journal of Stomatology 2007; 42(4):248.

9. Reactive gingival lesions: A retrospective study of 2,439 cases. Zhang W, Chen Y, An Z, Geng N, Bao D. Quintessence International 2007; 38(2):103-10.
10. A case report of a hybrid odontogenic tumour: Ameloblastoma and adenomatoid odontogenic tumour in calcifying cystic odontogenic tumour. Weiping Zhang, Yu Chen, Ning Geng, Dongmei Bao and Mingzhong Yang. Oral Oncology Extra 2006; 42(9): 287-290.
11. The role of matrix metalloproteinases and their tissue inhibitors in oral lichen planus. Zhang WP, Chen Y, Geng N, etc. Chinese Journal of Stomatology 2006; 41(7): 420-1.
12. Relationship of the disturbed balance between matrix metalloproteinases and their tissue inhibitors and the invasion of malignant salivary gland tumours. Tian K, Chen Y, Geng N, Yang MZ, Zhang WP. West China Journal of Stomatology 2005; 23(4):273-6, 279.
13. The expression of matrix metalloproteinases and their tissue inhibitors in pleomorphic adenoma. Chen Y, Tian K, Geng N, Yang MZ, Zhang WP. Chinese Journal of Stomatology 2005; 40(1):58-61.
14. Matrix metalloproteinases and their tissue inhibitors in the oral precancerous lesions. (A review) Zhang Weiping, Yang Mingzhong, Chen Yu. Foreign Medicine of Stomatology 2005; 32(1): 28-29.

□ CONFERENCES AND ABSTRACTS

1. Heterogeneity of Human Gingival Fibroblasts in Tobacco-stimulated Collagen Degradation. Zhang W, Fang M, Song F, Windsor L. Indiana Section American

Association for Dental Research /Indiana University School of Dentistry Research Day, Indianapolis, IN, April 24, 2010.

2. Combined Effects of Tobacco and Porphyromonas gingivalis on Gingival Fibroblasts. W. Zhang, F. Song, L. J. Windsor. Indiana Section American Association for Dental Research /Indiana University School of Dentistry Research Day, Indianapolis, IN, April 24, 2009.
3. The effects of Cigarette smoking condensate on cancer cells. Stephanie Flaig, Jun Sun, Fengyu Song, Weiping Zhang, L. Jack Windsor. Indiana University Purdue University Indianapolis Life-Health Science Internships Spring 2009 Poster Session, April 9, 2009.
4. Cigarette smoking condensate and nicotine affect human gingival fibroblast. W. Zhang, F. Song, L. J. Windsor. International Association for Dental Research (IADR)/American Association for Dental Research (AADR) 38th Annual Meeting, Miami, FL. April 4, 2009.
5. The effect of nicotine on osteoblast cell proliferation and viability. Elizabeth F. Smith, Jun Sun, Chialei Ang, Weiping Zhang, Fengyu Song and L. Jack Windsor. Committee on Institutional Cooperation (CIC) meeting, Michigan State University, East Lansing, MI, July 25, 2008.
6. Effects of dental cements on the expression of cytokines and growth factors by human pulp cells. Kellen Knowles, Jun Sun, Weiping Zhang and L. Jack Windsor. Committee on Institutional Cooperation (CIC) meeting, Michigan State University, East Lansing, MI, July 25, 2008.

7. Nicotine effects on cancer cells. Chialei Ang, Jun Sun, Elizabeth F. Smith, Weiping Zhang, Fengyu Song and L. Jack Windsor. Committee on Institutional Cooperation (CIC) meeting, Michigan State University, East Lansing, MI, July 25, 2008.
8. Effect of cigarette smoking condensate on human gingival fibroblast' proliferation and viability. W. Zhang, L. J. Windsor. American Association for Dental Research (AADR) 37th Annual Meeting, Dallas, Texas. April 3, 2008.
9. Effect of cigarette smoking condensate on human gingival fibroblast' proliferation. W. Zhang, F. Song, L. J. Windsor. Indiana Section American Association for Dental Research /Indiana University School of Dentistry Research Day, Indianapolis, IN, April 11, 2008.
10. Differential protein expression by Streptococcus mutans planktonic and biofilm cells. W. Zhang, T. Sanui, R. L Gregory. Indiana Section American Association for Dental Research /Indiana University School of Dentistry Research Day, Indianapolis, IN, April 11, 2007.
11. Expression of matrix metalloproteinases and its inhibitors in the odontogenic ghost cell carcinoma and odontogenic ghost cell tumor. Geng Ning, Chen Yu, Ban Yu, Bao Dongmei, Tian Kun, Zhang Weiping. The 7th Chinese Congress on Oral Pathology, Shenyang, China, 2006.
12. Expression of cytokeratin in the calcified cyst odontogenic tumor, odontogenic host cell carcinoma and odontogenic ghost cell tumor. Geng Ning, Ban Yu, Chen Yu, Bao Dongmei, Tian Kun, Zhang Weiping. The 7th Chinese Congress on Oral Pathology, Shenyang, China, 2006.

□ **HONORS AND AWARDS**

- ♦ Trident Award for Innovation in Oral Care Research, Indiana Section American Association for Dental Research /Indiana University School of Dentistry Research Day, Indianapolis, IN, 2009
- ♦ Educational Enhancement Grant, Graduate School, Indiana University Indianapolis, IN, 2008
- ♦ Travel Fellowship Award, Graduate School, Indiana University Indianapolis, IN, 2008
- ♦ Research Investment Fund (RIF) Fellowship for Ph.D. studies, IUSD, Indianapolis, Indiana (2006-2011)
- ♦ Kwang-Hua Fellowship (first prize) School of Graduate, Sichuan University (2004, 2005)
- ♦ Colgate Medicine Prize School of Stomatology, Tianjin Medicine University (2000, 2002)
- ♦ Medical Education Scholarship of the United Laboratories, Tianjin Medicine University (1998)

□ **CERTIFICATIONS**

1. Certificate of Dentistry license, China (active currently) 2003
2. Certificate for CPR & AED, U.S.A. 2009

□ **TEACHING EXPERIENCES**

- ♦ Teaching Assistant for Blood Pressure Competency Examination for Systems Approach to Biomedical Sciences (SABS) I course, Indiana University School of Dentistry, spring semester, 2009
- ♦ Teaching Assistant for Hematology lab in SABSII course, Indiana University School of Dentistry, spring semester, 2009
- ♦ Teaching Assistant for Enzyme-linked Immunosorbent Assay (ELISA) to determine the Level of Salivary IgA antibodies to streptococcus Mutans for SABS I course, Indiana University School of Dentistry, spring semester, 2008 and 2009
- ♦ Teaching Assistant for musculoskeletal module for SABS I course, Indiana University School of Dentistry, spring semester, 2009
- ♦ Bench Instructor for Oral Pathology course in West China College of Stomatology, Sichuan University, China, fall semester, 2004 and 2005

□ **SERVICE ACTIVITIES**

- Indiana University School of Dentistry Graduate Student Council (GSC), 2007-2008
- Indiana University School of Dentistry Graduate Student Professional Conduct Committee (GSPCC), 2009-2011