INTERACTIONS OF Porphyromonas gingivalis WITH HUMAN ENDOTHELIAL CELLS

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2007
ACKNOWLEDGMENTS

I acknowledge my father, Donald L Totten, whose support over the years has guided and facilitated me to excel in my education efforts. My father and my stepmother, Reba, have been in my corner from the beginning of my graduate career through to its cessation. Their love and support have meant more to me than any words could ever say.

I thank my mother Babs Dinsmoor, and my stepfather David, for their love and support. I am truly blessed to have two such wonderful sets of people in my life.

In addition to my family, I acknowledge my mentors Dr. Ann Progulske-Fox and Dr. William Dunn Jr. for their advice and guidance throughout my graduate career. Their scientific knowledge has been very instrumental to my growth and success.

My path through graduate school has been one of the most fulfilling experiences of my life thus far. I know that I am destined to succeed in my career goals armed with the knowledge and techniques acquired during my time at the University of Florida.
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INTERACTIONS OF *Porphyromonas gingivalis* WITH HUMAN ENDOTHELIAL CELLS

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August 2007

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Over the past several years, evidence has been accumulating that strongly suggests a connection between specific periodontal pathogens and cardiovascular disease (CVD). One such pathogen, *Porphyromonas gingivalis*, has been isolated directly from diseased vascular tissue and has been shown in animal models to be directly involved in pathological changes associated with CVD. In addition, *P. gingivalis* has been shown to invade human cardiovascular endothelial cells. The aim of our study was to evaluate the ability of *P. gingivalis* to affect human endothelial cell monolayer destruction and/or cell death by using *in-vitro* models focused on the presence of the β-catenin protein of the endothelial adherens junctions and cell death markers. First, the secreted protein fractions (SPF) of *P. gingivalis* wild-type strains W83 and 381 were tested using human umbilical vein endothelial cells (HUVEC) for their ability to alter the endothelial adherens junction integrity. The results demonstrated that HUVEC monolayers were disrupted and the ability of the endothelial cells to remain adhered to the coverslip was hindered after treatment with SPF of either wild-type strain 381 or W83. Next, live cells of a triple gingipain knock out mutant, CW501, a fimA deficient mutant, YPF1, and *P. gingivalis* wild-type strains 381 and ATCC 33277 were examined using an invasion assay at an MOI of 100
for their ability to induce HUVE cell death. In addition, live, whole cell *P. gingivalis* strains 381, ATCC 33277 and W83 caused HUVEC monolayer disruption and cellular detachment after 20 hours whereas the triple gingipain mutant CW501 and *fimA* - YPF1 did not. As determined by electron microscopy, numerous 381 cells were observed inside the HUVECs after 5 and 20 hours in the invasion assay, whereas the CW501 cells were only seen in very low numbers, primarily at the cell periphery. Flow cytometry analysis of HUVECs after twenty four hours of exposure to wild-types 381 and ATCC 33277 demonstrated approximately 30% cell death, whereas exposure to YPF1 and CW501 resulted in only 13% cell death. When *P. gingivalis* internalization was inhibited with cytochalasin D, HUVEC death was not observed with the wild-type strains 381 and ATCC 33277. These data indicate that endothelial cell internalization of *P. gingivalis* and *P. gingivalis* gingipains play a significant role in the disruption of the adherens junctions as well as facilitating cell death in human endothelial cells.
CHAPTER 1
INTRODUCTION

Dental Plaque

The formation of dental plaque on oral surfaces is a highly complex process. The hard
tissues of the oral cavity (teeth) accrue plaque in the form of multi-component biofilms.
Contrary to early observations, dental plaque does not exist as a homogeneous or standardized
structure. In actuality, plaque exists in various forms depending on its location in the oral cavity
(Rosan and Lamont, 2000). Greater than 700 bacterial species have been identified in the oral
cavity. Typically, gram-positive organisms such as *Streptococcus gordonii*, *Actinomyces*, and
other related species are found at the gingival level of the tooth where they coexist in a balanced,
symbiotic relationship with the host (Liebana *et al.*, 2004; Rosan and Lamont, 2000; Marsh,
1994; Kolenbrander and London, 1993). If this balance is disturbed, the equilibrium between the
host gingival tissue and the plaque is lost. As the plaque thickens, the biofilm becomes more
diversified and forms areas with decreased oxygen reduction potential between the supragingival
and subgingival regions. In time, the gram-positive organisms are followed by a series of gram-
negative anaerobes which situate themselves within these newly developed, lower oxygen areas
(Socransky *et al.*, 2000). One such gram-negative anaerobe to inhabit the subgingival dental
plaque biofilm is *Porphyromonas gingivalis*. *P. gingivalis* is capable of attaching to a number of
oral bacteria including *S. gordonii* and *Fusobacterium nucleatum* (Kolenbrander and London,
1993; Lamont *et al.*, 1992) and *in-vivo* studies using healthy volunteers have demonstrated that
*P. gingivalis* locates predominately on the heavily streptococcal-laden areas of the subgingival
dental plaque (Slots and Gibbons, 1978). The colonization of multiple bacterial species within
periodontal pockets leads to the destruction of host gingival tissue, thus leading to periodontal
disease (Nakamura *et al.*, 1999). The migration of *P. gingivalis* into the dental biofilm is a vital
process in the conversion from a commensal plaque to a pathogenic unit (Listgarten, 1999; Whittaker et al., 1996).

*Porphyromonas gingivalis* and Host Interactions

Periodontal diseases are categorized as a collection of infections that ultimately lead to gingival inflammation, periodontal tissue destruction, alveolar bone loss, and eventually, tooth exfoliation (Socransky and Haffajee, 1992). Periodontal disease affects about 23% of adults’ ages 65-74, and 14% of adults between the ages of 45-54 (US Public Health Service, 2000). In the United States, it is estimated that fifty million people have some form of periodontal disease (Cutler et al., 1995).

There are two major types of periodontal diseases: gingivitis and periodontitis. Gingivitis is an acute infection involving the coronal plaque, and periodontitis is a chronic infection involving the subgingival plaque (Castillo and Alvarez, 2004; Liebana et al., 2004). Dilations of the vasculature and an increase of phagoctytic cells are common host tissue responses to growing and multiplying bacteria during acute infections such as gingivitis. Tissue infections of this kind usually stimulate swift, unsystematic inflammatory responses to the invading bacteria and bacterial products (Olsen and Dahlen, 2004; Dahlen et al., 1982). The viable and dividing bacteria within the tissue represent a vital stage of an acute infection. Symptoms commonly associated with these types of infections are tenderness and pain. The threat of spreading is brought on by the host response to polymorphonuclear leukocytes (PMNs) and tissue destruction (Dahlen et al., 1982).

Characteristically, anaerobic bacteria cooperate with multiple bacteria to promote acute infections. Anaerobic bacteria equipped with polysaccharide capsules, collagenases and other proteolytic enzymes that degrade tissues pose a significant threat. Characteristics of chronic infections include a slower tissue response and lymphocyte dominated tissue restructuring and
repair (Olsen and Dahlen, 2004; Dahlen et al., 1982). In healthy individuals, the subgingival plaque is located in the “virtual space” of the gingival sulcus and maintains a very low rate of colonizing bacteria (Haffajee et al., 2003; Socransky et al., 1998). Periodontitis is characterized by plaque induced chronic inflammation and lesion formation, leading to the formation of periodontal pockets and oral bone loss. Periodontitis can progress to several different manifestations including progressive, chronic or aggressive, and may also be localized or generalized (Slots, 2000; Slots and Jorgensen, 2000). Both gingivitis and periodontitis display gingival tissue inflammation in response to bacteria laden plaque. Unlike periodontitis, gingivitis is devoid of the detrimental effects of tissue destruction and can be eradicated by plaque treatments and proper dental health (Castillo and Alvarez, 2004; Liebana et al., 2004).

The oral pathogen, Porphyromonas gingivalis, is a vital microbe involved in initiating chronic periodontitis (CP). P. gingivalis is a highly adapted, black-pigmented, asaccharolytic, anaerobic, rod shaped bacterium that is equipped with an array of recognized virulence factors capable of causing disease (Genco CA, 1999; Cutler et al., 1995; Genco, 1992a; Genco, RJ 1992c). Examples of these virulence factors include fimbriae and lectin-type adhesion molecules, lipopolysaccharide (LPS), a capsular polysaccharide (CPS) (or K antigen), hemolysins and hemagglutinins (Progulske-Fox et al., 1993), toxic metabolic products, outer membrane vesicles (blebs), and cysteine proteinases (gingipains) (Aduse-Opoku et al., 2006; Cutler et al., 1995).

The host defense system mounts a counterattack to the challenge of this microbe’s surface-associated and extracellular virulence factors through both the innate and acquired immunities. The balance between the net equilibrium of the body against the development of destructive disease is dependent on the dynamics of this host-microbe interaction. A successful host
response manages the challenge with little inflammation to the gingiva and without any lasting tissue destruction. Alternatively, an aggravated host response, such as the response induced by *Porphyromonas gingivalis*, causes an increase in the amount of inflammation and can ultimately lead to permanent destruction of host tissue (Curtis *et al.*, 1999a).

*P. gingivalis* has also been shown to cross the barrier of the oral mucosal tissue and enter the bloodstream after eating, tooth brushing, and flossing and this entry into the bloodstream is hypothesized to be crucial in causing systemic infections (Darveau *et al.*, 1995). For example, *P. gingivalis* invasion of the endothelial cells of the arterial walls could initiate persistent injury leading to adverse cardiovascular events (Deshpande and Khan, 1999; Dorn *et al.*, 1999; Deshpande *et al.*, 1998b; Deshpande *et al.*, 1998a).

**Cardiovascular Disease (CVD)**

The development of atherosclerosis is minimized by high plasma levels of the antioxidant/anti-inflammatory, high-density lipoproteins (HDLs) (Castelli *et al.*, 1986; Gordon *et al.*, 1977). In the artery wall, HDL promotes the efflux of cholesterol, binds lipopolysaccharides, protects erythrocytes from developing procoagulant activity, stimulates endothelial migration, inhibits endothelial cell synthesis of the platelet activating factor, and inhibits endothelial cell adhesion molecules and monocyte chemotactic protein (MCP-1) (Zhang *et al.*, 2003; Barter *et al.*, 2002; Barter and Rye, 1996; Sugatani *et al.*, 1996; Epand *et al.*, 1994; Murugesan *et al.*, 1994; Levine *et al.*, 1993; Yui *et al.*, 1988; Fleisher *et al.*, 1982). HDLs are antithrombotic and likely to alter endothelial functions by stimulating the expression of endothelial nitric oxide (NO) (Viswambharan *et al.*, 2004; O'Connell and Genest, 2001; Zeiher and Schachinger, 1994). Nitric oxide (NO) mediates chemical anti-atherogenic properties such as blood pressure by acting as a vasodilator or vasorelaxer (Knowles *et al.*, 2000). Early atherosclerotic events include the
enrollment and build up of monocytes and lymphocytes, and the collection of oxidized low-density lipoproteins (LDLs) to the arterial wall (Hegele, 1999). The monocytes can traverse into the intima where they can replicate and differentiate into lipid and cholesterol accruing macrophages or foam cells (Ross, 1993). LDL then initiates the transport of cholesterol and phospholipids into the mammalian cells (Navab et al., 2000). To circumvent this process, macrophages release a 34-kDa glycoprotein, apolipoprotein (apoE), which helps prompt cholesterol efflux to HDL (Lusis, 2000).

Apolipoprotein is a structural constituent of LDL, VLDL (very low-density lipoprotein), and some HDLs (Krimbou et al., 1997; Mahley, 1988). Plasma apoE is manufactured mainly by the liver but also by a variety of organ associated macrophages (Williams et al., 1985; Zannis et al., 1985; Driscoll and Getz, 1984). Hypercholesterolemia and scattered atherosclerotic lesions occur from apoE deficiencies (apoE -/-), in humans and gene altered mice (Plump et al., 1992; Zhang et al., 1992; Ghiselli et al., 1981). Interestingly, Li et al. reported that heterozygous apolipoprotein E (apoE) +/- mice that received weekly intravenous injections of \textit{P. gingivalis} into the systemic circulation resulted in elevated lipid deposits and developed rapid atherosclerosis when compared to sham injected mice (Li et al., 2002). Oral infection with \textit{P. gingivalis} has also been shown to increase the progression of early atherosclerosis within apolipoprotein E-null mice (Lalla et al., 2003). Jain et al. reported that rabbits with experimentally induced periodontitis that were exposed to a high fat diet developed elevated levels of vascular lipid deposits compared to healthy controls fed the same high-fat diet (Jain et al., 2003).

**Periodontal Disease is Correlated with Cardiovascular Disease**

In the western world, CVD is by far the primary cause of death. Until recently, the most common identified risk factors associated with this disease were smoking, high blood pressure,
diabetes, and obesity. Interestingly, these common risk factors for CVD could not explain many coronary related deaths. According to the Framingham Heart Study risk scores, approximately 15% of women and 25% of men in the lowest two risk factor quintiles of this study died of CVD (Leaverton et al., 1987). Given this data, it is possible that CVD may be initiated by additional stimuli, possibly poor dental health (Leaverton et al., 1987). In fact, over the past several years, scientists have accumulated data which strongly associates a connection between periodontal pathogens and CVD (Mattila et al., 1989). Furthermore, it has been reported that the presence of periodontal pathogens in the host are not restricted to the periodontal tissues and in fact, can act systemically (Beck et al., 1996).

Entry into the circulation could provide this bacterium with direct access to the cells that make up the vessel walls (Carroll and Sebor, 1980; Silver et al., 1977; Sconyers et al., 1973). A report released in 1989 by Mattila et al, showed that patients with myocardial infarctions had an increased level of poor dental health compared to the control population (Mattila et al., 1989). Interesting information using the murine macrophage cell line J774 A1 was published by Kuramitsu et al., which reported that foam cells (an important indicator of CVD) were induced by the outer membrane vesicles (blebs) of P. gingivalis, as well as P. gingivalis alone, a response likely mediated by LPS (Kuramitsu et al., 2001). In addition, P. gingivalis has been identified directly from vascular tissue in the diseased state. A 1998 publication from a study by Haraszthy et al. reported that P. gingivalis had been identified, using specific PCR primers, in atheromatous plaques (Haraszthy et al., 2000). In addition, in 1999, Porphyromonas gingivalis was detected in atherosclerotic plaques using immunostaining techniques (Chiu, 1999). Cavrini et al. identified P. gingivalis via PCR and fluorescence in situ hybridization, in atheromatous plaques of two atherosclerotic patients, suggesting that P. gingivalis may be metabolically active within these
plaques (Cavrini et al., 2005). After incubating endothelial cells with atherosclerotic plaque homogenates, Kozarov et al. reported the presence of both *A. actinomycetemcomitans* and *P. gingivalis* (Kozarov et al., 2005). Since only viable bacteria are capable of invading host cells (Lamont et al., 1995; Meyer et al., 1991), this study confirmed the presence of live *P. gingivalis* within diseased atherosclerotic sites (Kozarov et al., 2005). Moreover, Desvarieux et al. conducted an “Oral Infections and Vascular Disease Epidemiology Study (INVEST)” which was designed to evaluate a possible link between periodontal disease and the development of myocardial infarctions, atherosclerosis, cardiovascular disease and stroke frequencies (Desvarieux et al., 2005). After adjusting for common cardiovascular disease risk factors such as age, gender, smoking, diabetes and high blood pressure, this group reported a direct correlation between the burden of periodontal pathogens, specifically, *P. gingivalis*, and the thickness of the intima-media of the carotid artery (Desvarieux et al., 2005).

Ulcerations of the gingiva and changes within the local vasculature caused by *P. gingivalis* infections can lead to severe bacteremias and increased levels of inflammation. (Slade et al., 2003) *P. gingivalis* induced local inflammation within arterial walls may lead to the acceleration of atherosclerotic events (Gibson et al., 2006). Elevated levels of C-reactive protein (CRP), markers for inflammation and CVD, have also been shown to be associated with *P. gingivalis* infections (Teragawa et al., 2004). In the study conducted by Grossi et al, serum and carotid endarterectomy samples taken from 16 subjects who presented periodontal disease showed an association between attachment loss of greater than or equal to 4 mm and CVD history (Grossi et al., 1994). This study also determined that the serum CRP levels were considerably elevated in 75% of these subjects (Glurich et al., 2002; Grossi et al., 1994). In addition, CVD has been shown to be associated with serum antibodies to *P. gingivalis* (Pussinen et al., 2003). Finally, as
mentioned above, studies using animal models have established accelerated atherosclerotic events following continual injections of *P. gingivalis* (Lalla *et al.*, 2003; Li *et al.*, 2002) and *P. gingivalis* orally challenged homozygous ApoE<sup>-/-</sup> mice were vulnerable to accelerated atherosclerosis (Gibson *et al.*, 2004; Lalla *et al.*, 2003). This collection of scientific evidence validates the conclusion that a strong correlation exists between CVD and *P. gingivalis* associated periodontal disease.

*P. gingivalis* may induce cardiovascular events by several different mechanisms. First, *P. gingivalis* endotoxins such as LPS, may antagonize monocytes and produce elevated numbers of chemokines and cytokines systemically, leading to increased lipid induction, endothelial cell proliferation, and the luring of PMNs, causing vascular wall thickening (Liebana *et al.*, 2004). An increase in the amount of proinflammatory cytokines could also result in an increase of foam cells. In murine macrophages, the uptake of LDL is elevated when exposed to *P. gingivalis* (Qi *et al.*, 2003) and this increased uptake of LDL has also been shown to induce the formation of foam cells in HUVECs (Kuramitsu *et al.*, 2003). In fact, the chemoattractant MCP-1 from HUVECs was increased 5-fold after exposure to *P. gingivalis* strain 381 (Kuramitsu *et al.*, 2001).

Second, the activation of complement by endotoxins and C reactive protein (CRP) leads to an influx of PMNs, and platelet aggregation induced by monocytes, which can lead to increased inflammation associated with cardiovascular disease events (Kinane, 1998). Specifically, *P. gingivalis* Arg-gingipains (Rgps) can increase the calcium concentration in human platelets and thereby, cause their aggregation (Imamura and Travis, *et al.*, 2003). *P. gingivalis* can also induce platelet aggregation by expressing collagen-like platelet aggregation-associated proteins (PAAP) (Beck and Offenbacher, 2001). Aggregation of platelets is a consequence of *P. gingivalis* present on the platelet surface. These aggregating platelets may play a part in
thrombus formation (Beck and Offenbacher, 2001; Beck et al., 2000). Also, as mentioned above, the existence of *P. gingivalis* DNA within atheromatous plaques provides at least circumstantial evidence of *P. gingivalis* involvement in CVD events (Garcia et al., 2001; Haraszthy et al., 2000).

*P. gingivalis* may also be directly involved in the formation of atherosclerotic plaques. During fibrous plaque development, lipid discharge and vessel narrowing may be induced by atheroma cells experiencing apoptosis, necrosis, and mineralization (Ross, 1999). Ischemia and myocardial infarctions are initially mediated by plaque fissures, and later by thrombosis of the vessel (Lowe, 1998; McGill, 1968). Matrix metalloproteinases (MMPs) induced by *P. gingivalis* may contribute to these plaque fractures, or “ruptures”, leading to the *P. gingivalis* induced degradation of human atheroma samples in vitro (Kuramitsu et al., 2001).

**P. gingivalis** Virulence Factors

**Capsule**

*P. gingivalis* contains virulence factors such as polysaccharide capsules (K antigen), outer membrane vesicles (blebs), toxic metabolic products, hemagglutinins, gingipains, and fimbriae. The polysaccharide capsules, characteristic of some strains of *P. gingivalis*, evade the host antimicrobial defenses by playing a role in inhibiting the attachment of complement, creation of pustules, and evasion from phagocytes (Brook, 1987; Dahlen and Nygren, 1982). Encapsulated *P. gingivalis* contain an important virulence factor, K antigen, which is associated with serum resistance, phagocyte resistance, and the recruitment of specific antibodies necessary for opsonization and complement-mediated killing (van Winkelhoff et al., 1993). Therefore, capsules provide protection from the host defense system (Olsen and Dahlen, 2004).
**Outer Membrane Vesicles (OMVs)**

During normal growth, many gram negative bacteria including *P. gingivalis* shed outer membrane vesicles (OMV) into the culture media (Beveridge and Kadurugamuwa, 1996; Grenier and Mayrand, 1987). Components of the bacterial cell surface including LPS, proteinases, and adhesins are contained within OMVs (Qi et al., 2003) and OMVs have been shown to express amounts of proteolytic activities equal to that of whole cell *P. gingivalis* (Grenier and Mayrand, 1987). OMVs are not only capable of aiding the attachment of parent strains to host cells, but are also capable of attaching to host cells directly (Grenier and Mayrand, 1987). During periodontitis, *P. gingivalis* and its OMVs may be secreted into the circulation through transient bacteremias that can occur after chewing food, tooth brushing, and/or flossing (Miyakawa et al., 2004; Carroll and Sebor, 1980). The presence of LDL with the heat stable OMVs or LPS of *P. gingivalis* 381 have been shown to induce foam cells (an essential CVD characteristic) in murine macrophages (Kuramitsu et al., 2001).

**Toxic Metabolic Products**

Catalase, peroxidase and superoxide dismutase (SOD) all play a role in neutralizing toxic oxygen metabolites and are expressed by both aerobic and anaerobic bacteria (Amano et al., 1998; Amano et al., 1988). *P. gingivalis* is known to express SOD in higher amounts than other anaerobic, gram-negative rods, such as *Porphyromonas levii*, *Fusobacterium nucleatum* and *Prevotella* species including *intermedia, denticola, melaninogencia, and loescheii* (Amano et al., 1998; Amano et al., 1988). An increased level of SOD activity would defend *P. gingivalis* against the O2 from prolonged air exposure and the neutrophil created bactericidal superoxide anion (O2-) (Amano et al., 1998; Amano et al., 1992). Because a gene-inactivated *sod* mutant rapidly lost viability after O2 exposure and the mRNA sod levels were increased in response to elevated temperatures characteristic of periodontal pockets, it has been proposed that SOD is a
contributing virulence factor in the pathogenicity of \textit{P. gingivalis} (Mayrand and Holt, 1988). Thus, increased SOD activity in-vivo may provide \textit{P. gingivalis} with protection against the high levels of superoxide induced by neutrophils within the irritated periodontal pockets (Amano et al., 1994).

\textbf{Gingipains}

Gingipains are cysteine proteinases and have been implicated in the activation of host proenzymes, degradation of host tissues, and the neutralization of the host immune response (Kuramitsu et al., 1995). The generation of nutritional requirements, host immune protein degradation, host tissue adhesion, and the unearthing of cell cryptitopes are all examples of the virulence traits that have been proposed or attributed to \textit{P. gingivalis} gingipains (Gibbons et al., 1990; Mayrand and Holt, 1988). For example, gingipains are responsible for the degradation of several host proteins including collagen, fibrinogen, and fibronectin (Abe et al., 1998; Okamoto et al., 1998; Kadowaki et al., 1994), as well as the cytokines interleukin-8 (IL-8), IL-6, and TNF-\(\alpha\) (Oido-Mori et al., 2001; Banbula et al., 1999; Calkins et al., 1998). Gingipains are also implicated in the degradation of immunoglobulins and the complement factors C3 and C5 (Abe et al., 1998; Kadowaki et al., 1994; Wingrove et al., 1992), interruption of polymorphonuclear leukocyte (PMN) bactericidal activity (Abe et al., 1998; Nakayama et al., 1995; Kadowaki et al., 1994) and as a potent inducer of human umbilical vein endothelial cell, HUVEC, and human fibroblast cell death (Baba et al., 2002; Baba et al., 2001). These gingipains also likely function in the progression of vascular permeability, cytokine and cytokine receptor inactivation, platelet aggregation, inhibition of blood coagulation, are cytotoxic to host cells, and are important for growth. The ability of \textit{P. gingivalis} to reproduce and survive within the periodontal pockets is also partially dependent on the gingipains (Chen et al., 2001b). \textit{P. gingivalis} also possesses an
endopeptidase, PepO. PepO has considerable homology to the endothelin-converting enzyme (ECE-1), a NEP family member, and has been shown to affect the kallikrein/kinin cascade by cleaving bradykinin (Awano et al., 1999).

The P. gingivalis gingipains include RgpA, RgpB, and Kgp. Rgp and Kgp cleave natural and synthetic substrates following arginine and lysine residues, respectively (Awano et al., 1999). Two related genes encode the arginine-specific gingipain while only one gene encodes the lysine-specific gingipain. Two forms of gingipains are produced, a membrane-associated form accounting for approximately 80% of the Rgp and Kgp activity, and secreted forms. Through the use of specific inhibitors, Potempa et al. determined that 85% of the bacterial trypsin-like activity was attributed to the gingipains and the concentration of Rgp is 3-fold higher than Kgp (Potempa et al., 1997). The gingipain genes \textit{rgpA} and \textit{kgp} encode a propeptide including both catalytic and an adhesion/hemagglutinin domain, which appears to be postranslationally processed. \textit{RgpB} encodes a propeptide and contains the catalytic domain but lacks the hemagglutinin/adhesion domain (Kuramitsu, 1998; Potempa et al., 1995). \textit{RgpA} and \textit{RgpB} are soluble, single-chain forms of Arg-gingipains with a mass of 44-50 kDa and are present in the growth media in varying amounts depending on the strain, age of culture, and media composition (Bedi and Williams, 1994; Kadowaki et al., 1994; Chen et al., 1992; Nishikata and Yoshimura, 1991; Fujimura and Nakamura, 1990; Tsutsui et al., 1987). \textit{RgpA} also exists as a non-covalent 95 kDa complex containing an adhesin/hemagglutinin domain and catalytic domain (HR\textit{gpA}) (Rangarajan \textit{et al.}, 1997; Pike \textit{et al.}, 1996; Pike \textit{et al.}, 1994). Most strains of \textit{P. gingivalis} maintain \textit{RgpB} on the cell surface, which is denoted as membrane-type \textit{RgpB} (mt-RgpB) with masses of 70-90 kDa, and secrete low levels of soluble \textit{RgpB} (Curtis \textit{et al.}, 1999a; Curtis \textit{et al.}, 1999b). \textit{Kgp} exists either as a single-chain or as a complex containing
non-covalently associated catalytic domain and adhesion/hemagglutinin domains (Fujimura et al., 1998; Pike et al., 1996; Ciborowski et al., 1994; Fujimura et al., 1993; Scott et al., 1993). The activity of Kgp occurs mainly on the bacterial cell surface and may exist as a complex of fully processed multi-domains (Bhogal et al., 1997). The membrane associated forms are proposed to be primarily accountable for the virulence associated with this bacterium (Rajapakse et al., 2002; Slakeski et al., 1998).

Both Rgp and Kgp are assumed to play a pivotal role in modulating host immune defenses, acquisition of nutrients, and tissue invasion (Genco et al., 1999; Genco CA, 1999; Lamont and Jenkinson, 1998). These enzymes can cause potentially damaging activities by activating the kallikrein/kinin cascade, degrading host proteinase inhibitors, inactivating complement proteins C3 and C5, inactivating fibrinogen’s clotting ability, modifying the antimicrobial activity of neutrophils, and degrading bactericidal proteins, immunoglobins, and iron transporting proteins. All of these properties indicate that the gingipains of P. gingivalis can account for many of the clinical manifestations seen in periodontitis (Curtis et al., 1999a; Curtis et al., 1999b; Mayrand and Holt, 1988). In addition, these enzymes can stimulate serotonin secreting platelets, which could potentially give rise to cardiovascular impediments because an increase in serotonin leads to an imbalance between the nitric oxide (NO)/serotonin levels, thus promoting endothelial dysfunction (Curtis et al., 1999a; Curtis et al., 1999b).

The structure of the rgp gene is highly conserved since cloning and sequencing of rgp from strains HG66, W83, W50, 381, and ATCC 33277 displayed very little coding sequence variations (Mikolajczyk-Pawlinska et al., 1998; Allaker et al., 1997). The organization of the genes suggests that either rgpA gave rise to rgpB by gene duplication minus the adhesin/hemagglutinin domain or that rgpB was copied and then introduced with an
adhesin/hemagglutinin domain (Potempa et al., 2003). It has been determined that widespread proteolytic processing of the emerging translation products is responsible for the production of the mature enzymes (Pavloff et al., 1997; Pike et al., 1996; Pavloff et al., 1995; Pike et al., 1994). The proteolytic activities of Rgp and Kgp are necessary for the creation of the 43 kDa and 47 kDa proteins from the precursor Rgp and Kgp proteins (Abe et al., 2004). Evidence for this was provided by the use of the rgpA-, rgpB-, kgp- and the rgpA-, kgp-, hagA- mutants as well as the combined use of Rgp and Kgp inhibitors which completely inhibited the coaggregation ability of *P. gingivalis* (Abe et al., 2004). Not only does Arg-gingipain contribute to their own maturation via autoproteolysis, they are also responsible for the accurate and well organized maturation of Kgp (Potempa et al., 2003; Kadowaki et al., 1998; Okamoto et al., 1996). Kgp has been shown to be important for hemoglobin adsorption through the processing of the 19-kDa hemoglobin receptor protein (HBr) encoded by the hagA, kgp and rgpA genes of *P. gingivalis* and hence, Kgp is likely very important as an energy source for *P. gingivalis* (Nakayama et al., 1998; Han et al., 1996; Okamoto et al., 1996; Pavloff et al., 1995).

Differences in amino acid binding specificities at prime sites exist between RgpB and HRgpA. The crystal structure of RgpB and HRgpA revealed identical active sites with the exception of four additional amino acid substitutions within the HRgpA. Ally et al. suggested it was the four additional active substitutions that were responsible for the observed differences between the binding specificities of RgpB and HRgpA, rather than the presence of the adhesin/hemagglutinin domains in HRgpA (Ally et al., 2003). It was also reported that the RgpAcat domain was unable to mirror HRgpA’s binding to fibrinogen, fibronectin, and laminin suggesting that the extra adhesin subunits of HRgpA were responsible for the observed binding to the above proteins (Ally et al., 2003; Pike et al., 1996). Also, when HRgpA, RgpAcat, and
RgpB were tested for the ability to degrade fibrinogen, all three were able to cleave the Aα-chain but HRgpA, followed by RgpA_cat and RgpB degraded the Bβ-chain most proficiently. The extra adhesin domains of HRgpA likely contributed to the observed differences in the degradation of the Bβ-chain of fibrinogen. Since both forms of RgpA were more efficient than RgpB, the active site differences may play a significant role (Ally et al., 2003).

Gingipains share regions of homologous sequences with each other and have also been reported to have sequence homology with several P. gingivalis hemagglutinin activity genes (Potempa et al., 1995; Progulske-Fox et al., 1993). The proteolytic activities of Rgp and Kgp are necessary for hemagglutination (Shi et al., 1999) and the colonization and invasion of host tissues by P. gingivalis is at least partially dependent on RgpA and Kgp adhesin/hemagglutinin activity (Kelly et al., 1997). The requirement of RgpA and Kgp proteolytic activities for adhesion/hemagglutination are likely because they process the proproteins involved. RgpA and Kgp participate in the production of the 15 kDa adhesin/hemagglutinin domain, HA2, also referred to as the hemoglobin receptor (HbR), which participates in the binding of hemoglobin and the acquisition of heme from erythrocytes (Kelly et al., 1997). The activity of Kgp is vital for the discharge of HbR from the HagA protein and progingipains (Okamoto et al., 1998). The downstream processing of HA2 (HbR) is accomplished with the contribution of Kgp, since both HA2 processing and pigmentation were severely hindered in the kgp- mutant (Shi et al., 1999; Nakayama et al., 1998). The binding of hemoglobin also makes Kgp a significant player in the acquisition of iron (Travis et al., 1997).

**Fimbriae**

Fimbriae play a major role in P. gingivalis attachment and invasion (Njoroge et al., 1997). Fimbriae also demonstrate a variety of biological activities such as stimulating the production of
cytokines and bone resorption (Hamada et al., 1998; Yoshimura et al., 1984). Fimbriae were first identified in 1984 by Yoshimura et al. using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). There are currently two known types of fimbriae, major, discovered by Yoshimura et al, and minor fimbriae, discovered independently by Ogawa et al. in 1995 and Hamada et al. in 1996. The major subunit fimbrillin was determined to be 43 kDa in size and was recognized as the bacterium’s first step in disease induction and development (Hamada et al., 1998; Yoshimura et al., 1984). The major fimbriae are composed of a subunit protein, fimbrillin (FimA), and function by mediating the bacterial adhesion and the colonization of host tissue (Amano, 2003; Lamont and Yilmaz, 2002). The filamentous components of major fimbriae are up to 3μm long and 5nm in width, and are located on the cell surface. They are able to specifically bind to, enter, and activate a number of host cells including human endothelial and epithelial cells, peripheral blood monocytes, fibroblasts, peritoneal macrophages, THP-1 cells and spleen cells (Lamont and Yilmaz, 2002; Deshpande et al., 1998b).

The minor fimbriae were determined to be 67 kDa and were uncovered by use of a fimA (major fimbria-deficient) mutant of wild-type ATCC 33277 and shown to possess short fimbria-like additions (Umemoto and Hamada, 2003; Hamada et al., 1996; Kokeguchi et al., 1994). These minor fimbriae range in length from 0.1μm to 0.5μm, width, ca. 6.5nm, and are distinct from the long, major fimbriae, both genetically and antigenically (Hamada et al., 1996; Ogawa et al., 1995; Yoshimura et al., 1984). In vitro preparations of broken cells showed that the short fimbriae were located in both the membrane fractions and the soluble fractions (Park et al., 2005). The minor fimbriae are composed primarily of the subunit protein (Mfa1), which encodes the mfa1 gene. The Mfa1 molecule has been shown by Yoshimura et al., (1989), to be
equivalent to the 75 kDa outer membrane protein as detailed in several reports and Pg-II (a 72 kDa cell surface protein) in wild-type ATCC 33277 (Ogawa et al., 1995).

Several researchers have proposed the possibility that more than one mechanism for fimA regulation exists and that both environmental cues and endogenous factors are involved in its expression (Nishikawa et al., 2004; Xie et al., 2004). Inflamed subgingival pockets have a characteristically higher temperature (39°C) than normal gingival tissue. As mentioned above, high temperatures influence fimA regulation by causing a decrease in both fimA mRNA and protein expression, while a lower temperature of (34°C), produced higher fimA transcriptional activity (Xie et al., 1997; Amano et al., 1994). Studies using standard growth conditions and the anti-fimbrillin antibody showed that FimA from wild-type ATCC 33277 was present in both the prefimbrillin and mature fimbrillin protein forms and was produced throughout the growth cycle (Nishikawa et al., 2004).

The maturation of the fimbrial 43kDa and 75kDa proteins were determined to be dependent on the precise cleavages of the amino (NH-2) terminal Arg46-Ala47 bond and the Arg49-Ala50 bond of the precursor proteins, respectively (Hamada et al., 1994; Ogawa et al., 1994; Watanabe et al., 1992; Lee et al., 1991; Dickinson et al., 1988). Using electron microscopy, Kadowaki et al. showed that the Kgp-null mutant and wild-type ATCC 33277 had numerous cell surface associated curly fimbriae and the Rgp-null mutant revealed little, if any, fimbriation, which suggests that fimbriation of P. gingivalis is regulated by Rgp proprotein processing but not by Kgp (Kadowaki et al., 1998). In fact, the prefimbrillin 45-kDa protein vanished and a 43-kDa protein emerged after treatment with Rgp and this action was inhibited when the Rgp inhibitors leupeptin and EDTA were used, suggesting adhesion of P. gingivalis to host tissue is partially controlled by Rgp (Kadowaki et al., 1998). To further confirm this theory,
western blotting was conducted to compare the FimA levels of the 381 parent strain and G102, *rgpA*- gingipain mutant. The G102 mutant is likely deficient in fimbrial expression (Tokuda *et al.*, 1996) because the results revealed that the mutant contained lower FimA levels than the 381 parent strain. The expressed FimA in the G102 mutant was appropriately processed in size, however the mutant expressed lower *fimA* mRNA levels than the parent strain, thus suggesting a defect in the *fimA* gene transcription due to the lack of RgpA (Tokuda *et al.*, 1996). The G102 (*rgpA*-), mutant also had an increased level of extracellular vesicles. Cultures of *P. gingivalis* grown in low levels of iron have been shown to induce such increases in surface blebs. The increase of these protease enriched extracellular vesicles may be an effect of the amino acid deprivation caused from low iron stress (McKee *et al.*, 1986).

To further characterize major and minor fimbriae with respect to bacterial virulence, the oral cavities of rats were inoculated with isogenic mutants of *P. gingivalis* ATCC 33277 including MPG1, (*fimA*-), MPG67, (*mfa1*-) and MPG4167 (*fimA-, mfa1-*) (Umemoto and Hamada, 2003). The ATCC 33277 parent strain adhered by auto-aggregation in clumps to the ATCC CCL17 (KBs) originally thought to be oral epithelial cells and now known as Hela cells, and the MPG67, which contained numerous long fimbriae on its surface, formed even larger clumps than did the parent strain. The MPG1 mutant showed no apparent auto-aggregation and a decrease in adherence to single cells, while MPG4167, which lacked both fimbrial structures, lost adhesive abilities entirely. This data suggests that adherence of *P. gingivalis* to epithelial cells is partially dependent on the production of both the major and minor fimbriae. Invasion studies revealed that all three fimbriae-deficient mutants had a reduction in invasion in excess of eight times that of ATCC 33277. The results by Umemoto and Hamada also demonstrated that MPG1 caused a larger amount of oral bone loss as compared MPG67 but considerably less than
the periodontal bone loss induced by the wild-type strain. MPG4167’s ability to induce oral bone loss was markedly suppressed, suggesting the expression of both major and minor fimbriae are necessary for the virulence associated with *P. gingivalis* in this model (Umemoto and Hamada, 2003).

In agreement with MPG1, DPG3, a *fimA*-(fimbrillin) mutant of strain 381, exhibited no apparent autoaggregation, lower binding to gram-positive bacteria, and reduced binding to epithelial cells (Tokuda *et al.*, 1996). From this data, fimbriae appear to also be required for autoaggregation. Since reduced autoaggregation would be mirrored by reduced adhesion, the fimbriae may be a vital participant in colonization in vivo (Tokuda *et al.*, 1996; Genco *et al.*, 1994). Also, another *fimA*- mutant of ATCC 33277, YPF1, is unable to express the FimA protein due to an insertional inactivation of the *fimA* gene (Yilmaz *et al.*, 2003; Tokuda *et al.*, 1996; Genco *et al.*, 1994). Data from assays using YPF1 and gingival epithelial cells (GECs) revealed a 10-fold decrease in internalization, yet still retained some invasion potential and maintained non-fimbrial dependent interactions with GECs (Yilmaz *et al.*, 2003). The levels of FimA were quickly decreased upon the interaction of *P. gingivalis* with GECs, suggesting that internalized wild-type *P. gingivalis* may be only sparingly fimbriated (Wang *et al.*, 2002).

Extended periods of treatment, e.g., 24 hours, revealed both the wild-type parent strain and the YPF1 mutant colocalized with paxillin and FAK, a common outcome of internalization, suggesting that the presence of fimbriae was not required (Yilmaz *et al.*, 2003).

Taken together, these data establish that the FimA structural subunit protein contains specific peptide domains which are responsible for much of the adherence activity of *P. gingivalis* (Lamont and Jenkinson, 2000). Wild-type *P. gingivalis* is capable of activating paxillin, a major player in the integrin-associated cell signaling pathway, whereas the YPF1
mutant was unable to induce this pathway (Yilmaz et al., 2002). Fimbriae-independent mechanisms of invasion may be important for P. gingivalis in addition to or when the fimbriae are downregulated (Yilmaz et al., 2002).

**Epithelial and Endothelial Cells**

The cells found lining body cavities, such as stomach, intestines, and skin are known as epithelial cells. Epithelial cells are polarized and their plasma membrane contains two separate regions, apical and basolateral that are made up of different sorting and transport proteins. Also, epithelial cells contain multiple specialized regions known as cell junctions, within their plasma membranes (Bazzoni and Dejana, 2004). These cell junctions function to seal off body cavities by acting as a barrier and restricting the transport of plasma-membrane molecules from the apical to the basolateral surfaces. In addition to their barrier function, cell junctions provide rigidity and strength to the tissues. Examples of these epithelial cell junctions are tight junctions, adherens junctions, desmosomes and gap junctions, which function to maintain the integrity of the epithelium (Bazzoni and Dejana, 2004; Stevens et al., 2000). Tight junctions are composed of both intracellular, zonula occludens (ZO-1, ZO-2, ZO-3), and transmembrane molecules occludin and claudin (Bazzoni and Dejana, 2004; Morita et al., 1999; Furuse et al., 1993). The adherens junctions are composed of cadherins and catenins (Bazzoni and Dejana, 2004; Angst et al., 2001a; Angst et al., 2001b; Aberle et al., 1997; Takeichi, 1993). Although different molecules form the tight junctions and adherens junctions, both junctions function to promote homophilic interactions and form a pericellular “zipper-like” pattern along the cell boundary (Tsukita et al., 2001; Chitaev and Troyanovsky, 1998).

Blood vessels primarily function to partition blood from underlying tissues. One of the main cellular components of blood vessels are endothelial cells. This “sheet-like” layer of endothelial cells lines the lumen of blood vessels and functions as a barrier, thereby controlling
the influx of cells and blood proteins into the walls of the vasculature (Dejana, 2004; Lodish et al., 2000). This endothelial barrier function is also carried out by tight junctions and adherens junctions, which function to maintain the integrity of the endothelium by defending the vessels from fluctuations in cell permeability and increases of inflammation and platelet aggregation, or thrombotic events (Stevens et al., 2000). Unlike epithelial cells where the tight junctions are primarily located on the apical surface along the intercellular cleft, endothelial cells contain tight junctions that are more integrated within the adherens junctions (Lodish et al., 2000). These endothelial junctional complexes are highly defined but unlike the junctional complexes of epithelial cells, they not as organized and rigid. Another difference between endothelial and epithelial cells is that human endothelial cells lack the junctional structures known as desmosomes (Lodish et al., 2000; Dejana et al., 1995).

The intricate complex of transmembrane adhesion proteins that make up the adherens junctions are known as cadherins. Cadherins function to maintain different aspects of vascular homeostasis by mediating cell-cell adhesions and anchorage to the actin cytoskeleton via specific linkages to defined intracellular molecules, catenins (Dejana et al., 1999a). In addition, the proteins of the adherens junctions can modulate endothelial cell growth, positioning, motility, and apoptosis through the transmission of intracellular signals (Lampugnani and Dejana, 1997). This intracellular signaling can be direct by employing growth-factor receptors or signaling proteins, or indirect, by anchoring and maintaining transcription factors at the cell membrane, thereby decreasing their translocation to the nucleus (Matter and Balda, 2003; Wheelock and Johnson, 2003; Braga, 2002; Bazzoni et al., 1999). Ultimately, adherens junctions play a role in regulating permeability of plasma solutes. They also regulate the opening and closing of their...
cell-to-cell linkages and in turn control the efflux and influx of leukocytes into areas of inflammation (Muller, 2003; Johnson-Leger et al., 2000).

**Cell-Cell Adhesion Proteins**

Cell-cell adhesion is predominately arbitrated by the Ca\(^{2+}\) dependent, homophilic binding, classical cadherin family containing E-, N-, and P-cadherin as well as other members (Angst et al., 2001a; Ranscht, 1994). Endothelial cells found within all vessels express a specific cadherin called VE cadherin. VE-cadherin first becomes expressed during development when the cells become committed to become endothelial cells (Breier et al., 1996; Dejana et al., 1995). The cadherins consists of a highly conserved cytoplasmic domain and group of repeating Ca\(^{2+}\) binding subdomains (Kepler, 1992). The cadherin cytoplasmic domain connects to the cytoskeleton by interacting with plakoglobin, \(\alpha\)-catenin, \(\beta\)-catenin, and p120 (Reynolds et al., 1994; Keeler, 1993; Keeler, 1992; Ozawa et al., 1989). Cadherin-mediated cell adhesion is dependent on the catenin family of cytoplasmic polypeptides, since aggregation assays showed the removal of the catenin binding site within the cytoplasmic tail of cadherins eliminates the functionality of the cadherins (Ozawa et al., 1989; Nagafuchi and Takeichi, 1988). In addition to the classical cadherins, desmosome-associated cadherins are a group of cadherins associated with tight junctions that form intracellular connections with intermediate filaments instead of components of the actin cytoskeleton (Hynes, 1999). Widespread sequence similarities exist between \(\beta\)-catenin and plakoglobin (\(\gamma\) catenin) as well as between \(\alpha\)-catenin and the actin binding protein, vinculin (Butz et al., 1992; Herrenknecht et al., 1991; Nagafuchi et al., 1991). Plakoglobin communicates with the classical and desmosomal cadherins, while the \(\beta\)-catenin interacts primarily with the classical cadherins of the adherens junctions (Gumbiner, 1995; Gumbiner and Yamada, 1995).
Classical cadherins participate in the development and protection of tissues during gastrulation, neurulation and organogenesis, and are important in the organization of *Xenopus* embryos (Aberle *et al.*, 1996; Redies and Takeichi, 1996; Takeichi, 1995). Many routes of epithelial development, growth and phenotype are influenced by cadherin mediated adhesion (Marrs *et al.*, 1995). The formation of invasive and metastatic tumors is associated with the loss of E-cadherin, thus implying that E-cadherin is crucial for the development and maintenance of the epithelia (Bracke *et al.*, 1996; Larue *et al.*, 1994). Intestinal cells containing overexpressed E- or N-cadherin displayed alterations in their proliferation, migration, and observed apoptosis (Hermiston *et al.*, 1996; Hermiston and Gordon, 1995a; Hermiston and Gordon, 1995b). Through contact-induced inhibition, cadherins have also been suggested to be responsible for reduced cell growth. This reduction in cell growth occurs through cell-cycle arrest at the G1 phase and is at least partially due to a decrease in the level of cyclin-D1 (Venkiteswaran *et al.*, 2002; Gottardi *et al.*, 2001; Mueller *et al.*, 2000; St Croix *et al.*, 1998). Cadherin could limit cell growth by occupying β-catenin at the cell membrane thereby limiting β-catenin translocation to the nucleus where it would ultimately upregulate the transcription of cyclin D1 (van de Wetering *et al.*, 2002; Gottardi *et al.*, 2001; Ben-Ze'ev and Geiger, 1998). Collectively, cadherins are involved in signaling pathways that control adhesion and may individually launch regulation signals for central cellular processes such as proliferation, migration, cell differentiation, and apoptosis (Bracke *et al.*, 1996; Marrs *et al.*, 1995; Larue *et al.*, 1994; McCrea *et al.*, 1991).

*P. gingivalis* has been shown to exhibit a highly elevated amount of proteolytic activity for the proteins of the epithelial junctions. For example, *P. gingivalis* gingipains have been shown to degrade E-cadherin in experiments using MDCK cells (Katz *et al.*, 2002; Katz *et al.*, 2000). Loss of cell adhesion favoring bacterial invasion and infection of the underlying tissues could be
a consequence of the loss of E-cadherin molecules from epithelial cells (Katz et al., 2002; Gottardi et al., 2001). In addition, the loss of E-cadherin induces cell proliferation by prompting a change in gene expression through β-catenin-LEF/TCF upregulation (Sadot et al., 1998; Simcha et al., 1998).

_P. gingivalis_ has also been shown to exhibit a highly elevated amount of proteolytic activity for the proteins of the endothelial cell junctions. Sheets _et al._ found that the gingipains downregulated the endothelial intercellular junctional cadherin in bovine coronary artery endothelial cells (BCAEC) and human microvascular endothelial cells (HMVEC) leading to increased vascular permeability and apoptosis (Sheets _et al._, 2005). This increased permeability is caused by a disruption in the endothelial junctional barrier, specifically the severing of the linkage between cadherin and β-catenin within the adherens junctions, and has also been shown to increase inflammation by allowing the migration of leukocytes (Yun _et al._, 2005; Hordijk, 2003; Schenkel _et al._, 2002; Muller _et al._, 1993). A transient bacteremia and subsequent systemic dissemination of _P. gingivalis_ after dental treatments or flossing could potentially make endothelial cells prospective targets of _P. gingivalis_.

β-catenin is a proto-oncogene and vertebrate homolog to _armadillo_ in _Drosophila_ (McCrea _et al._, 1991). In addition to directly linking cadherin to the actin cytoskeleton, β-catenin is also a signal transduction molecule that is known to mediate signaling through the Wnt signaling pathway (Moon _et al._, 2002). Wnts are a family of highly conserved signaling molecules that are essential for the maintenance of adult tissues (Goichberg _et al._, 2001). Normally, when the Wnt ligand is absent, the cytosolic β-catenin (not bound to cadherin at the membrane) forms a complex with the adenomatous polyposis coli (APC) protein and is phosphorylated and targeted for degradation by an assembly of proteins, ensuring low β-catenin levels. In contrast, when the
Wnt ligand is present, Wnt signaling occurs through its receptor frizzled and inhibits the degradation of β-catenin by inhibiting its phosphorylation. This inhibition of β-catenin degradation allows β-catenin to build up in the cytosol where it can then enter the nucleus and interact with the transcription factor T-cell factor (TCF) or leukocyte enhancing factor (LEF) and induce the expression of target genes (Liebner et al., 2006; Gumbiner, 2005; Bazzoni and Dejana, 2004). This Wnt signaling enables cells within multicellular organisms to communicate with each other in order to organize a significant array of cellular processes such as cell performance and migration during morphogenesis, cell propagation versus delineation, and cell survival versus apoptosis (Logan and Nusse, 2004).

β-catenin is stabilized and maintained at the cell membrane when it is bound to cadherin. Both the Wnt/Wg pathway and the cadherin-mediated cell adhesion complex compete for the availability of β-catenin. Overexpression of cadherins represses the signaling associated with β-catenin suggesting that the signaling actions of β-catenin are physiologically controlled by cadherins (Heasman et al., 1994). If there is a mutation or decrease in cadherin expression, the pool of free β-catenin may increase, thereby increasing β-catenin signaling, possibly leading to endothelial cell growth and transformation (Gottardi et al., 2001; St Croix et al., 1998). For example, if the Xenopus embryo overexpresses cadherin, the cytosolic and/or nuclear β-catenin pools are directed to the plasma membrane, thereby causing a parade of developmental anomalies due to the lack of nuclear communication with β-catenin (Fagotto and Gumbiner, 1996; Heasman et al., 1994). Mutations of β-catenin have been linked with malignant cell transformations and truncation of β-catenin in a carcinoma cell line has been shown to induce cadherin dysfunction (Polakis, 2000; Kawanishi et al., 1994). An embryonic lethal phenotype caused by an endothelial-specific β-catenin deletion in mice and the phenotype of an
adenomatous polyposis coli (APC) mutant in zebrafish demonstrates that β-catenin signaling participates in endothelial transformations into other cell lineages such as mesenchymal cells during development (Liebner et al., 2004; Cattelino et al., 2003; Hurlstone et al., 2003).

In summary, β-catenin can be found bound to cadherins at the cell membrane where it acts to physically link cadherin to the actin cytoskeleton (Juliano, 2002). β-catenin is also responsible for intracellular signaling. For example, cell growth and apoptosis are controlled through the signaling at these intercellular contacts. Adherens junction proteins also regulate vascular permeability by controlling the passage of solutes and circulating cells. Some signals may be necessary for endothelial stability while others may primarily function to maintain cellular permeability or the passage of leukocytes through the endothelial junctions. It is also possible that the junctional proteins indirectly modulate cell function by communicating directly with growth factor receptors (Liebner et al., 2006; Logan and Nusse, 2004). Collectively, the proteins of the intercellular adherens junctions are responsible for cell-to-cell adhesion and intracellular signaling.

**Cell-Matrix Adhesion Proteins**

Animal epithelia and organized assemblies of cells, e.g., muscle tissue, are either surrounded by or underlined with components of the extracellular matrix, such as collagen fibers, proteoglycans, and adhesive matrix proteins. Functions of the extracellular matrix (ECM) include the organization and coordination of tissues, routes for migrating cells, and stimulation of cell growth, proliferation, and gene expression through classic signal-transduction pathways (Haynes and Webb, 1992). Members of the adhesion receptor family include: selectins, the immunoglobulin cell adhesion superfamily (IgCAMs) and integrins. The selectin family, L-, E-, and P-selectin, are composed of lectin-like adhesion receptors that bind carbohydrate moieties on
mucin-like CAMs and negotiate calcium-dependent, heterotypic cell-cell interactions. P-selectins are physiologically important during the inflammatory process because they participate in the tightly regulated adherence of leukocytes and platelets to endothelial cells (Springer, 1995). Selectins, along with their glycoprotein counterreceptors, regulate the trafficking of leukocytes between the bloodstream and tissues and are involved in the activation of β2 integrins (Hu et al., 2000; Hartwell and Wagner, 1999; Vestweber and Blanks, 1999; Lorenzon et al., 1998).

IgCAMs are adhesive receptors that play very important roles in development (Murase and Schuman, 1999). Some examples of IgCAMs include NCAM, ICAM, and PECAM-1. NCAMs function as calcium-independent, homotypic, cell-cell adhesion receptors, while ICAM functions by directly linking specific membrane receptors to the actin cytoskeleton (Crossin and Krushel, 2000; Bretscher, 1999). PECAM-1 is a homotypic receptor found on platelets, endothelial cells, and various leukocytes, and engages in the development of endothelial junctions and the release of leukocytes (Aplin et al., 1998; Newman, 1997). PECAM-1 also has the ability to recruit phosphatases that potentially could counteract the tyrosine kinases recruited by other cell surface receptors (Newman, 1999). The ligation of PECAM-1 can also stimulate the αvβ3 integrin demonstrating this IgCAM is also proficient in signal transduction.

The *P. gingivalis* major fimbria interacts with the host cell and causes the induction of expression of vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E- and P-selectins (Khlgatian et al., 2002; Nassar et al., 2002). Confocal microscopy and FACS analysis determined that *P. gingivalis A7436* infection of HUVECs induced VCAM-1, ICAM-1, P- and E-selectin expression (Khlgatian et al., 2002). Evidence to support the involvement of *P. gingivalis* invasion with adhesion molecule expression was
obtained with the use of cytochalasin D, an actin polymerization inhibitor. *P. gingivalis* invasion of HUVEC was prevented with the addition of 1ug/ml cytochalasin D (Deshpande *et al.*, 1998b). Furthermore, pretreatment of HUVECs with cytochalasin D, preceding infection with *P. gingivalis*, resulted in a considerable reduction of ICAM-1 and VCAM-1 expression. The stimulation of these cell adhesion molecules was later determined to be a direct outcome of major fimbriae-mediated attachment (Khlgatian *et al.*, 2002). Further evidence singling out the importance of bacterial attachment was confirmed when the use of DPG3, a nonadhering fimA deficient strain, and antibodies against the fimbrillin peptide, failed to yield stimulation of these specific adhesion molecules (Khlgatian *et al.*, 2002).

Integrins are chiefly involved in cell-matrix interactions and bind to fibronectin, laminin, collagen and various other matrix proteins to form intricate networks called focal adhesions or focal contacts. Collagens are composed of many diverse proteins and are also the most profuse structural components making up the extracellular matrix within all tissues (Gumbiner and Yamada, 1995). Integrins are heterodimers composed of noncovalently associated subunits (α and β) and contain a large extracellular domain, short cytoplasmic domain, and a single membrane spanning region that undergo conformational domain changes or subunit rearrangements during the ligand binding process (Hynes, 1999; Aplin *et al.*, 1998). The organization of the cytoskeleton, signal transduction, and cell motility are examples of integrin functions carried out, in part, by the cytoplasmic domains of the α and β subunits (Juliano, 2002). Integrins have also been shown to directly activate aspects of intracellular signaling. The gathering of integrins bound to groups of radicals, ions, or molecules (ligands), can direct various structural and signaling constituents and stimulate essential signal transduction cascades.
such as the mitogen-activated MAP kinases (Aplin and Juliano, 1999; Giancotti and Ruoslahti, 1999; Aplin et al., 1998).

Previous reports have shown that *P. gingivalis* has the ability to direct their entry into both endothelial and epithelial cells by utilizing the host cells signaling pathways (Yilmaz et al., 2002; Deshpande et al., 1998b; Lamont et al., 1995). Periodontal tissues infected with periodontal pathogens are repaired and maintained in part by the interactions between the ECM proteins and the host cells (Kapila et al., 1998; Rautemaa and Meri, 1996). Signals overseeing cell function, migration, and anchorage abilities are provided by the adhesion interaction between tissue cells and the proteoglycans and/or the integrins of (ECM) proteins (Kapila et al., 1998). Integrin mediated adherence of GEC is commonly associated with temporary, low levels of cytoskeletal rearrangements (Young et al., 1992). The invasion dependent trigger necessary for the reorganization of microtubules as seen for other bacteria like urophathogenic *E. coli*, is supplied by the activation of integrin and it is possible that *P. gingivalis* is also equipped with this mechanism (Guignot et al., 2001).

**P. gingivalis Invasion of Epithelial and Endothelial Cells**

In response to bacterial challenge, macrophages, neutrophils and dendritic cells engulf, or phagocytose bacteria and internalize them into phagosomes which later transport the bacteria to the lysosome for degradation (Stossel et al., 1999). Several highly pathogenic pathogens are capable of circumventing this process, thus leading to disease events (Pieters, 2001). The concept of bacteria possessing the ability to invade gingival tissue was first proposed by Goadby in the early twentieth century (Goadby, 1907). Bacterial uptake is now known to occur by the utilization of various pathways depending on the bacterial strain and cell type involved (Lamont and Yilmaz, 2002; Lamont and Jenkinson, 2000). Bacterial invasion of host cells is likely stimulated by specific signal transduction pathways given that recent reports have provided
evidence to support the involvement of protein kinases (Collaco et al., 1995; Rosenshine et al., 1994; Rosenshine et al., 1992). Other investigators have reported that a kinase-mediated phosphorylation of a specific 68-kDa protein (pp68) in mouse peritoneal macrophages was inducible by *P. gingivalis* fimbriae and this inducible activity was repressed after the use of staurosporine, a specific inhibitor to protein kinase C (PKC) (Murakami et al., 1994).

*P. gingivalis* utilizes its major fimbriae, FimA, for adherence to KB cells and GECs and appear to also require gingipain activity for optimal invasion (Lamont and Yilmaz, 2002; Chen et al., 2001b; Lamont et al., 1995), since treatment of these cells with a combination of various protease inhibitors: aprotinin, leupeptin, pepstatin and benzanidime, drastically reduced invasion (Lamont et al., 1995). KB and GEC cells accomplish bacterial uptake through phagocytosis, allowing the bacteria to be either restricted to a membrane bound vacuole or free in the cytoplasm (Houalet-Jeanne et al., 2001; Njoroge et al., 1997; Sandros et al., 1996; Sandros et al., 1993). In addition, *Legionella pneumophilia* and *Brucella abortus* utilize lipid rafts for entrance into the host cells and it has been suggested that *P. gingivalis* also utilizes this mechanism (Belanger et al., 2006; Tsuda et al., 2005; Duncan et al., 2004; Watarai, 2004; Duncan et al., 2002). Also, several bacteria, for example *Yersinia enterocolitica, Salmonella choleraeuis, Actinobacillus actinomycetemcomitans*, and *Shigella flexneri*, depend on the action of microfilaments for invasion (Finlay and Falkow, 1997; Sreenivasan et al., 1993; Finlay and Falkow, 1989), while the action of both the microfilaments and microtubules are required for *Neisseria gonorrhoeae, Citrobacter freundii*, and *Haemophilus influenzae*, invasion (Oelschlaeger et al., 1993; Donnenberg et al., 1990; Richardson and Sadoff, 1988). Cytoskeletal rearrangements were also determined to be important for the invasion GEC by *P. gingivalis* since nocodazole, a microtubule assembly inhibitor, and cytochalasin D, an actin polymerization
inhibitor, severely retarded invasion (Houalet-Jeanne et al., 2001; Lamont et al., 1995). Further evidence to support this invasion inhibition was provided by electron microscopy, which showed a lack of internalized P. gingivalis present within the nocodazole and cytochalasin D treated GECs (Houalet-Jeanne et al., 2001; Lamont et al., 1995).

Deshpande et al. and Dorn et al. have demonstrated that P. gingivalis is also able to actively invade and replicate within endothelial cells (ECs). Using an MOI of 1:100 of P. gingivalis strains A7436 and 381 and bovine aortic endothelial cells (BAEC), Deshpande et al. determined that the greatest invasion was observed after 2 hours with no additional increase in invasion reported up to four hours (Deshpande et al., 1998b; Deshpande et al., 1998a; Njoroge et al., 1997). Scanning electron microscopy showed the attachment of P. gingivalis wild-type strain A7436 to BAEC was mirrored by alterations in the normal architecture of the EC surface and the presence of protruding microvilli from the EC engulfing the attached bacteria. This EC engulfment is likely the stimulus for the preliminary formation of a cytoplasmic vacuole. In fact, BAEC, human umbilical endothelial cells (HUVEC), and fetal bovine heart endothelial cells (FBHEC), all appeared to contain P. gingivalis A7436 within apparent cytoplasmic vacuoles (Deshpande et al., 1998b; Deshpande et al., 1998a; Njoroge et al., 1997). Further studies investigating the metabolic requirements for invasion showed that the de novo protein synthesis inhibitors, chloramphenicol and cycloheximide, and nalidixic acid and rifampin, inhibitors of bacterial DNA and RNA synthesis, were unable to reduce the invasion of BAEC by P. gingivalis (Deshpande et al., 1998b; Deshpande et al., 1998a; Njoroge et al., 1997). However, in agreement with data from GECs, invasion was significantly reduced by the use of cytochalasin D and nocodazole (Lamont et al., 1995). These results indicate that bacterial and BAEC protein synthesis and bacterial DNA and RNA synthesis are not required for P. gingivalis invasion of
endothelial cells. However, the role of actin polymerization and microtubule formation are important for invasion in both cell types (Deshpande et al., 1998b; Lamont et al., 1995). In addition to cytoskeletal reorganization, protein phosphorylation is also likely involved since the use of the broad spectrum protein kinase inhibitor, staurosporine, inhibited *P. gingivalis* invasion of ECs (Deshpande et al., 1998b; Lamont et al., 1995). Sodium azide, an inhibitor of cytochrome oxidase and proton motive forces, also repressed invasion providing evidence for the involvement of both *P. gingivalis* and EC energy metabolism (Deshpande et al., 1998b; Deshpande et al., 1998a).

It appears that adherence to and invasion of ECs is also partially dependent on the expression of the major fimbriae (Dorn et al., 2000; Dorn et al., 1999; Deshpande et al., 1998b; Deshpande et al., 1998a). The *fimA*- DPG3 mutant was unable to adhere to BAEC or FBHEC or induce changes to the normal endothelial cell surface architecture, providing further evidence to support the pivotal role for major fimbriae in invasion of endothelial cells (Deshpande et al., 1998b; Deshpande et al., 1998a; Njoroge et al., 1997). The major fimbriae may be responsible for generating a cascade of events leading to the long, microvilli characteristic of cytoskeletal reorganization of the ECs (Deshpande et al., 1998b; Deshpande et al., 1998a).

Bacterial attachment to heart and aortic endothelial cells (HCAEC) most likely requires the action of additional adhesins because the major fimbriae alone are not sufficient for optimal invasion (Dorn et al., 2000; Dorn et al., 1999; Deshpande et al., 1998a). Like GECs, cytoskeletal rearrangements are necessary for *P. gingivalis* invasion of the HCAEC, although once internalized, the bacteria appear to be localized within multimembranous, autophagosome like vacuoles instead of within the cytoplasm as observed with epithelial cells (Dorn et al., 2001; Dorn et al., 1999).
Autophagosomes are multimembranous vacuoles formed by invaginations of the rough endoplasmic reticulum (RER) that are devoid of ribosomes (Dunn, 1994). Specifically, autophagosomes are discrete structures involved in autophagy, a cellular response to nutrient deprivation whereby the organelles and cytosolic components are gathered and degraded by lysosomes (Dunn, 1990). Under normal conditions, the autophagosome fuses with a lysosome, forming a hydrolase filled autolysosome capable of degrading cellular contents. Early after *P. gingivalis* invasion, the vacuoles containing *P. gingivalis* contain Rab5 and HsAtg7, both markers of early endosomes. Subsequent to this, *P. gingivalis* traffics to a late autophagosome deficient of cathepsin L, but containing both the lysosomal protein LGP120, and the endoplasmic reticulum protein, BIP (Dorn et al., 2001). Other pathogens evade the host immune system through a similar process. For example, pathogens such as *Legionella pneumophilia* and *Brucella abortus* localize and replicate within vacuoles containing proteins of the endoplasmic reticulum (Pizarro-Cerda et al., 1998b; Pizarro-Cerda et al., 1998a). Like *L. pneumophila* and *B. abortus*, the autophagic like vacuoles containing *P. gingivalis* do not mature into a autolysosome containing lysosomal hydrolases (Dorn et al., 2001; Pizarro-Cerda et al., 1998b; Pizarro-Cerda et al., 1998a; Swanson and Isberg, 1995). *P. gingivalis* appears to have refined its survival within endothelial cells by adapting a mechanism that enables its evasion from lysosomes of the endocytic pathway, and trafficking to the autophagocytic pathway (Dorn et al., 2001). Bacterial entry into the host cell would provide a protected niche and a generous supply of amino acids for metabolic energy, as well as essential nutrients such as iron, which is required for *P. gingivalis* growth and survival (O'Brien-Simpson et al., 2000). Bacterial invasion of HCAEC could result in chronic injury to endothelial layer of the arterial wall, thus either promoting the initiation or the progression of atherosclerotic development (Dorn et al., 1999).
**P. gingivalis Persistence in Epithelial and Endothelial Cells**

*P. gingivalis* is able to replicate and persist within a number of epithelial and endothelial cell lines (Dorn et al., 2000; Dorn et al., 1999; Deshpande et al., 1998a). It undergoes quick and efficient internalization (15 minutes) within GECs. Invasion of GEC by *P. gingivalis* ATCC 33277 was determined to be optimal when *P. gingivalis* was grown to the mid-log phase, followed by the early stationary phase and the late stationary phase (Lamont et al., 1995). High numbers of the active and viable intracellular *P. gingivalis* accumulated perinuclearly (Houalet-Jeanne et al., 2001; Belton et al., 1999). In GECs, this organism is not restricted to a vacuole and is capable of replicating intracellularly, which suggests that *P. gingivalis* has adapted a mechanism for survival within these cells (Lamont and Jenkinson, 1998; Lamont et al., 1995). In fact, it has been reported that *P. gingivalis* remains viable within these cells with no appearance of necrosis or apoptotic cell death for extended periods (Lamont and Yilmaz, 2002; Houalet-Jeanne et al., 2001; Belton et al., 1999). In addition, *P. gingivalis* can be found both within single-membrane vacuoles or free in the cytosol of KB cells. However, KB cells have been shown to undergo cell death after exposure to *P. gingivalis* (Wang et al., 1999; Chen et al., 2001), but, Madianos et al. reported that *P. gingivalis* is capable of surviving within KB cells for up to eight days (Madianos et al., 1996). The fact that *P. gingivalis* survives for extended periods of time within these epithelial cells suggests that the host cells are capable of adapting to this bacterial challenge of *P. gingivalis* and/or *P. gingivalis* modulates its intracellular behavior so as not to profoundly affect its host cell. This persisting internal survival of *P. gingivalis* within these cells provides a possible explanation for the dormancy and aggravating characteristics associated with periodontal disease (Weinberg et al., 1997). *P. gingivalis* may, in fact, be capable of regulating the expression of both genes and proteins in relation to their existing epithelial cell surroundings (Zhang et al., 2005; Nelson et al., 2003).
Many pathogens have also acquired mechanisms for survival within endothelial cells. *Brucella abortus* and *Legionella pneumophila* have been shown to traffic intracellularly so to achieve entry into multimembranous vacuoles that resembles autophagosomes. These bacteria are capable of replicating and persisting within these autophagosome like vacuoles (Dunn, 1994; (Meresse *et al*., 1999); Pizarro-Cerda *et al*., 1998a; Pizarro-Cerda *et al*., 1998b). In Human Coronary Artery Endothelial cells (HCAE) cells, *P. gingivalis* has also been shown to be contained within vacuoles resembling autophagosomes and has been shown to stimulate autophagy (Dorn *et al*., 1999). The stimulation of autophagy may promote the survival of *P. gingivalis* by supplying a pool of free amino acids, which could be consumed for their own metabolism or to inhibit host protein synthesis (Sinai and Joiner, 1997). In addition to promoting cell survival, autophagy has been shown to be important for cell death (Codogno and Meijer, 2005). Therefore, the stimulation of autophagy may be a requirement for cellular homeostasis since it is essential for cell survival as well as cell damage and cell death (Codogno and Meijer, 2005; Shintani and Klionsky, 2004).

**Apoptosis of Epithelial and Endothelial Cells**

In vitro studies have shown that the gingipains of *P. gingivalis* are capable of inducing cell death in several cell types including epithelial cells, fibroblasts, and endothelial cells (Baba *et al*., 2001; Graves *et al*., 2001; Wang *et al*., 1999; Johansson and Kalfas, 1998; Graves and Jiang, 1995; Morioka *et al*., 1993; Shah *et al*., 1992). A physiological type of cell death called programmed cell death, or apoptosis, has numerous biochemical episodes and characteristic, physical cellular outcomes. Periodontal lesions contain these apoptotic cells and cells containing the apoptotic regulating molecule p53, (Gamonal *et al*., 2001; Koulouri *et al*., 1999; Sawa *et al*., 1999; Tonetti *et al*., 1998) mirrored by a reduction in cells positive for the apoptotic suppression molecule, Bcl-2 (Sawa *et al*., 1999; Tonetti *et al*., 1998). Chen *et al*.

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treated with gingipain-active W83 extracts underwent apoptosis subsequent to cleavage of the neural cadherin (N-cadherin) and integrin β1 induced loss of adhesion to the culture surfaces (Chen et al., 2001a). W83 extract pretreated with the Rgp and Kgp inhibitor, Nα-p-tosyl-L-lysine chloromethylketone (TLCK) failed to demonstrate any considerable rounding or detachment of the KB cells in contrast to cells treated with the extract in the absence of the inhibitor (Chen et al., 2001a; Abaibou et al., 2000; Pike et al., 1996; Fletcher et al., 1994). In addition to gingipains, *P. gingivalis* promotes MMP secretion from macrophages which can promote detachment from the cell surface and may subsequently induce anoikis (Grayson et al., 2003; Kuramitsu, 1998; DeCarlo and Harber, 1997; Arribas et al., 1996; Ding et al., 1995; Ehlers and Riordan, 1991). The *P. gingivalis* induced MMPs are capable of both activating cadherin and cleaving a number of cell surface proteins including growth factors and cytokine precursors (Grayson et al., 2003; Kuramitsu, 1998; Arribas et al., 1996).

In contrast to the apoptosis observed with gingipains and MMPs, many investigators have reported that epithelial cells with internalized *P. gingivalis* experience no apparent negative side effects (Nakhjiri et al., 2001; Katz et al., 2000; Fives-Taylor et al., 1999; Madianos et al., 1996). Therefore, *P. gingivalis* may also act to inhibit cell death. The interaction of *P. gingivalis* with integrin may actually alter the naturally occurring rate of apoptosis. Support for this theory was provided by the reports from Yilmaz et al., which showed that the effects of the apoptosis inducer, camptothecin, were blocked by *P. gingivalis* invasion of GEC (Yilmaz et al., 2002; Nakhjiri et al., 2001). These short-lived, primary gingival epithelial cells were able to withstand a 24 hour exposure to high levels of *P. gingivalis* (Nakhjiri et al., 2001; Belton et al., 1999). In fact, the levels of the anti-apoptotic Bcl-2 mRNA and protein were upregulated and BAX, a characteristic early step in the apoptotic pathway, was downregulated after *P. gingivalis* invasion.
of GEC (Yilmaz et al., 2002; Nakhjiri et al., 2001). Other investigators have shown the swift cytosolic to mitochondrial translocation of a pro-apoptotic molecule, BAX, after the loss of integrin adhesion in primary mouse mammary cells isolated from pregnant ICR mice (Gilmore et al., 2000). Taken together, the upregulation of Bcl-2 and the down regulation of Bax likely inhibits apoptosis in P. gingivalis infected GEC, thus promoting the life of the cell (Nakhjiri et al., 2001).

The role of P. gingivalis in endothelial cell apoptosis may also directly involve the gingipains. Endothelial cells of connective tissue and all vessel types continually express integrin β1 (Mechtersheimer et al., 1994; Hormia et al., 1990). It has been suggested that the P. gingivalis gingipains participate in endothelial apoptosis/anoikis by disrupting integrin signaling through the rapid degradation of integrin β1 (Aoudjit and Vuori, 2001; Matter and Ruoslahti, 2001; Oguey et al., 2000; Lin et al., 1997). The P. gingivalis gingipains have also been shown to directly cleave the adherens junction protein, VE-cadherin (Sheets et al., 2005). As mentioned previously, VE-cadherin is connected to the actin cytoskeleton through the direct interactions with β-catenin, plakoglobin and p120 (Dejana et al., 1999b). VE-cadherin is specific to endothelial cells and functions by forming an intracellular seal within the junctional complex. Thus, the cleavage of CAMS by the P. gingivalis gingipains may induce apoptosis and hence cause damage to the tissues by disrupting the integrity of the endothelial monolayer. In addition, endothelial cells maintain vascular homeostasis by participating in a number of vascular processes such as the regulation of vascular permeability, angiogenesis and blood pressure (Rubanyi, 1993). The disturbance of the common endothelial functions may modify the endothelial state from healthy to diseased and as a result lead to an influx of inflammatory molecules characteristic of atherosclerosis (Takahashi et al., 2006). Thus, in addition to the
reported gingipain associated damage to the tissues within the periodontal pocket (Curtis et al., 1999a; Curtis et al., 1999b), gingipain activity with endothelial cells may have a significant role in causing the tissue damage associated with cardiovascular disease (Sheets et al., 2005).

Recent studies by Sheets et al. have shown that Human microvascular endothelial cells (HMVECs) and bovine coronary artery endothelial cells (BCAEC) treated with gingipain-active extracts undergo apoptosis after rounding and detaching from the culture surface (Sheets et al., 2005). However, these endothelial cells exhibited minimal rounding and detachment from the culture dish and minimal apoptosis in the presence of TLCK or the recA defective mutant which has considerably less gingipain activity compared to its parent strain W83 (Sheets et al., 2005; Abaibou et al., 2000). Interestingly, the HMVEC were more sensitive to the W83 extract than were the BCAECs, indicating that either the TLCK was more toxic to these cells than the BCAECs or these cells were more stressed during culturing (Sheets et al., 2005). The endothelial cell responses to stimuli may be ligand and/or cell-type specific (Marschang et al., 2006; Takahashi et al., 2006). For example, venous and arterial endothelial cells have been reported to have diverse gene expression patterns and therefore likely respond differently to stimuli (Takahashi et al., 2006).

In addition, BCAECs treated with the gingipain-active W83 extracts have been reported to have an increased level of caspase 3 activity (Sheets et al., 2005). This increase in caspase 3 activity was strongly inhibited in the presence of TLCK, suggesting that this activity was induced by the P. gingivalis gingipains (Sheets et al., 2005). Thus, these results suggest that the gingipains are directly responsible for the observed rounding, detachment, and subsequent cell death of BCAEC endothelial cells. Taken together, this evidence combined with the accelerated atheroma development induced by P. gingivalis invasion of aortic tissue within the ApoE −/−
mouse, suggests that the gingipains may stimulate endothelial dysfunction, leading to apoptosis within the cells of the periodontal pocket and the cardiovascular system (Sheets et al., 2005; Gibson et al., 2004).

**Chapter Summary**

*P. gingivalis* is equipped with an array of recognized virulence factors capable of causing disease (Cutler et al., 1995; Genco, 1992a; Genco, 1992b). Examples of these virulence factors include fimbriae and lectin-type adhesions, a polysaccharide capsule, hemolysins and hemagglutinins, toxic metabolic products, outer membrane vesicles (blebs), and cysteine proteinases (gingipains) (Cutler et al., 1995). The LPS from *P. gingivalis* can cause macrophages, fibroblasts, and monocytes to release cytokines such as IL-1, TNF-α, and/or C-reactive proteins (CRP) (Darveau et al., 1998; Valtonen, 1991). *P. gingivalis* expresses cysteine proteinases or gingipains, called Rgp and Kgp, as well as PepO, an endopeptidase that participates in the degradation of proteins, peptides, and glycopeptides (Ansai et al., 2003). *P. gingivalis* gingipains Rgp and Kgp cleave natural and synthetic substrates following arginine and lysine residues, respectively (Awano et al., 1999). Two related genes encode the arginine-specific gingipain while only one gene encodes the lysine-specific gingipain. A catalytic and hemagglutinin domain and a propeptide are encoded in *rgpA* and *kgp* (Genco et al., 1999; Genco CA, 1999). Both Rgp and Kgp likely play a pivotal role in modulating host immune defenses, acquisition of nutrients, and tissue invasion (Genco et al., 1999; Lamont and Jenkinson, 1998). These enzymes can cause potentially damaging activities by degrading host cell adhesion molecules and proteinase inhibitors, modifying the antimicrobial activity of neutrophils, and degrading bactericidal proteins, immunoglobins, and iron transporting proteins. All of these properties signify that the gingipains of *P. gingivalis* can account for much of the clinical
manifestations seen in periodontitis such as elevated levels of gingival crevicular fluid, bleeding, and oral bone loss (Curtis et al., 1999a; Mayrand and Holt, 1988). In addition, these enzymes can stimulate serotonin secreting platelets, which could potentially give rise to cardiovascular impediments because an increase in serotonin leads to an imbalance between the nitric oxide (NO)/serotonin levels, thus promoting endothelial dysfunction (Curtis et al., 1999a). Gingipain induced cleavage and/or degradation of adhesion molecules may provoke detrimental damage to the endothelial monolayer.

Gingipain extracts have been shown to induce the detachment of Human Microvascular Endothelial Cells (HMVEC) from the monolayer in and this detachment was inhibited by the presence of the cysteine proteinase inhibitor TCLK (Kobayashi-Sakamoto et al., 2006). Previous data have shown that gingipains can facilitate the cleavage of a number of cell surface proteins such as growth factors, cytokine precursors and receptors, and cell adhesion molecules, including integrin β1 and cadherins, by proteolytically activating host MMPs (Herren et al., 1998; Arribas et al., 1996; McGeehan et al., 1994). The cleavage of the cell adhesion molecules would allow the cells to detach from one another, causing disruption of the monolayer (Garton et al., 2003; Herren et al., 1998; Arribas et al., 1996; McGeehan et al., 1994; Ehlers and Riordan, 1991). Gingipain active extracts have also been shown to induce apoptosis in KB epithelial cells and the endothelial cell lines HMVEC and BCAEC (Kobayashi-Sakamoto et al., 2006; Sheets et al., 2005; Chen et al., 2001b). Therefore, the gingipains may participate in vascular tissue damage by degrading a number of adhesion molecules, leading to increased endothelial cell detachment and cell death (Sheets et al., 2005; Bazzoni and Dejana, 2001; Dejana et al., 2001).
Objectives

*P. gingivalis* has been identified directly from diseased tissue and support for a role of infectious bacteria, e.g., *P. gingivalis*, with the initiation of atherosclerotic plaques has been provided (Garcia et al., 2001; Haraszthy et al., 2000). Specifically, the proteolytic activities of the *P. gingivalis* gingipains and the induction of inflammatory molecules by the gingipains have been suggested to play a significant role in the development of atherosclerotic events. However, definitive proof of the direct involvement of *P. gingivalis* in the development and progression of CVD is still lacking. The concept of whether the gingipains are primarily acting from the exterior of the cell or perhaps, instead from the interior of the cell, has not been confirmed. In fact, *P. gingivalis* gingipains may have multiple and different functions depending on their location. For example, the intracellular bacteria may either promote cell survival or induce cell death, while extracellular bacteria may degrade CAMs in order to penetrate the tissue, infiltrate the vasculature and cause disease.

This project examined the interactions of *P. gingivalis* with endothelial cells. The first objective was to investigate the effects of *P. gingivalis* gingipains on the proteins of the adherens endothelial junctions of human endothelial cells using wild-type strains of *P. gingivalis* and gingipain knock-out mutants. The second objective was to examine the level of importance of the concept of adhesion versus invasion of *P. gingivalis* in causing the disruption of the integrity of the endothelial cell monolayer. The third objective was to determine if cell death is stimulated or hindered by *P. gingivalis* and whether *P. gingivalis* is likely contributing to these effects from the exterior of the cell or from within the cell.
CHAPTER 2
MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

P. gingivalis Subculture

P. gingivalis strains (see Table 1 for the list of strains used) were grown on tryptic soy agar (Difco Laboratories, Detroit, MI) supplemented with 0.5% yeast extract (Fisher Chemicals, Fair Lawn, NJ), 5% sheep blood (Quad Five, Ryegate, MT), vitamin K (5μg/ml), and hemin (5μg/ml). Overnight cultures were grown in tryptic soy broth (Difco) supplemented with 0.5% yeast extract, haemin (5μg/mL), and vitamin K (5μg/mL) under anaerobic conditions [37°C in a Coy anaerobic chamber (Ann Arbor, MI) with an atmosphere of 5% CO₂, 10% H₂, and 85% N₂].

Cell Culture Conditions

Human umbilical vein endothelial cells (HUVEC, Cambrex, Baltimore, MD) and Human hepatic epithelial cells (HuH7, Michael Kilberg, University of Florida) were used for this study. The HUVEC were maintained using Microvascular Endothelial Growth Medium-2 (EGM-2; Clonetics, Inc., San Diego, CA), which contains endothelial cell basal medium-2 supplemented with hydrocortisone, human recombinant fibroblast growth factor, 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), ascorbic acid, human recombinant epidermal growth factor, gentamicin (Sigma, St. Louis, MO), recombinant insulin growth factor-1, and amphotericin B as well as 10,000 units/ml of streptomycin and 10,000 units/ml of penicillin G, and 200 mM L-glutamine. The HuH7 cells were maintained in Eagle’s minimum essential medium (Mediatech, Herndon, VA) supplemented with 10% FBS (Invitrogen) 10,000 units/ml of streptomycin, 10,000 units/ml of penicillin G, and 200 mM L-glutamine (Sigma). Cells were cultured in 75-cm² flasks at 37°C in a 5% CO₂ humidified atmosphere.
Bacterial Preparations

*P. gingivalis* Lysate

For the preparation of *P. gingivalis* lysates, *P. gingivalis* W83 was inoculated into 5 mls of Blood Heart Infusion Broth (BHI) (Difco) and allowed to grow at 37°C in a Coy anaerobic chamber (Ann Arbor, MI) with an atmosphere of 5% CO₂, 10% H₂, and 85% N₂, for 5 to 7 days until the BHI broth was very opaque. The 5 mls of the bacterial broth was then transferred to a larger volume (1 liter) of BHI broth and incubated anaerobically for an additional 3 to 5 days. When the bacterial solution was very opaque, the liter of bacterial cells was centrifuged using a Beckman J2-21 centrifuge with a JA-10 rotor at 11,315 X g at 4°C for 30 minutes to 1 hour. The resulting cell pellet was resuspended in 5 mls of 50 mM Tris, pH 7.5 and sonicated for 20 minutes to lyse the bacterial cells. The lysed bacterial cells were then concentrated using a Centriprep 10,000 MWCO (Millipore Corp., Bedford, MA) and a Sorvall RC-5B centrifuge with a HB-4 rotor at 16,320 X g for 30 min. The final volume was approximately 6 mls.

Secreted Protein Factor

*P. gingivalis* bacterial strains W83 and 381 were inoculated into 0.5 mls of Blood Heart Infusion Broth (BHI) (Difco) and allowed to grow at 37°C in a Coy anaerobic chamber (Ann Arbor, MI) with an atmosphere of 5% CO₂, 10% H₂, and 85% N₂ for 2 days. Next, 0.5 mls of each bacterial culture was transferred to 100 ml of BHI and allowed to grow overnight. Following growth, 100 ml of the bacterial cultures were transferred to 1 liter of BHI and allowed to grow for 24-48 hours. The bacterial cells were spun down using a Beckman J2-21 centrifuge with a JA-10 rotor at 11,315 X g at 4°C for 30 minutes to 1 hour and the supernatant was collected and filtered through a 0.2 micron filter (Whatman, Mobile, AL). Then, 36.1g ammonium sulfate (Sigma-Aldrich) was added to each 100 mls of filtered supernatant and allowed to dissolve by stirring in a 4°C cold room overnight. The supernatant was then spun
down using an Eppendorf Centrifuge 5801 with an A4-62 rotor at 3,166 X g and 4°C for 30 minutes, the resulting supernatant was decanted, and the pellet was collected and dissolved in 50 mM Tris (Fisher) pH 7.5. This solution was then dialyzed against 50mM Tris, pH 7.5, for 48 hours exchanging with fresh Tris buffer pH 7.5, twice, followed by concentration using a Centriprep 10,000 MWCO (Millipore Corp., Bedford, MA) and a Sorvall RC-5B centrifuge with a HB-4 rotor at 16,320 X g to a final volume of 1 ml.

**Microassay Procedure for Proteins**

The Bio Rad protein assay dye reagent concentrate was used for the colorimetric detection and quantitation of total protein. Each test tube contained 800μl of the sample. Then, 200μl of the dye concentrate reagent was added to each tube and the tubes were vortexed for 30 seconds. The test tubes were then incubated at room temperature for five minutes and the absorbance was measured at 595 nm using a Spectronic 1001 spectrophotometer (Milton Roy Company, Rochester, NY). A standard curve of BSA at concentrations ranging from 5 to 20μg/ml was used to determine protein concentration. The protein solutions were assayed in duplicate. The data was interpreted using Sigma Plot 2000 (Systat, Point Richmond, CA).

**Enzymatic Assay**

Arginine-specific cysteine proteinase activity was determined using 5 mM N-α-benzoy-DL-arginine p-nitroanilide (BAPNA) (Sigma, St. Louis MO) in 125 mM Tris-HCL, pH 8.0, containing 12.5 mM L-cysteine and 12.5 mM CaCl₂ in a total volume of 1 ml at room temperature (Shoji et al., 2002; Chen et al., 2001). After the sample was added, the absorbance (A₄₀₅) was continuously recorded using a Spectronic 1001 spectrophotometer (Milton Roy Company, Rochester, NY). The proteinase activity of the secreted protein fractions was determined by the increase in absorbance/min/ml culture. Kinetic analysis was interpreted using Sigma Plot 2000 (Systat, Point Richmond, CA).
Inhibitors

To determine if the effects observed with the cell extract of *P. gingivalis* W83 were due to the gingipains, HuH7 cells were incubated for twelve and twenty four hours at 37°C in the presence of a 1 mM concentration of the cysteine protease inhibitor Na-α-tosyl-L-lysine chloromethyl ketone (TLCK) (Sigma), a 2mM concentration of a serine protease inhibitor PMSF (Sigma), and a 2mM concentration of a carboxypeptidase, serine and cysteine proteinase inhibitor (PIC) (Sigma).

Microscopy

Antibodies

Mouse anti-β-catenin (Zymed Laboratories Inc., South San Francisco, CA), rabbit polyclonal anti-β-catenin (Santa Cruz Biotech Inc., Santa Cruz, California), anti-pan cadherin (Sigma-Aldrich), and *P. gingivalis* rHagB polyclonal (Kohler, J. Song, H.) antibody were used in this study. The purified rHagB was obtained and prepared as described previously (Song et al., 2005). The *P. gingivalis* rHagB polyclonal antisera, A7986, was raised against the purified rHagB protein (Strategic Biosolutions, Newark, DE). Pre-immune rabbit serum was obtained prior to the first immunization and used as a negative control. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Sigma-Aldrich) and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit antibody (Sigma-Aldrich) were used as secondary antibodies.

Immunofluorescence Microscopy

HUVECs were allowed to grow to confluency on glass coverslips in six-well tissue culture plates (Corning Inc., Corning, NY). The HUVECs were then washed with antibiotic-free EGM-2 media three times prior to infection with the *P. gingivalis* strains listed, at various lengths of time, at a multiplicity of infection (MOI) of approximately 100. The media was removed from
each well and the cells were then fixed in 4% paraformaldehyde in PBS for 30 minutes at room temperature. This was followed by washing three times with PBS and quenching in 50 mM NH₄Cl and 0.2% Triton X 100-PBS for 10 minutes at room temperature. The quenching solution was removed and the HUVECs were again washed three times with PBS. The primary antibodies, diluted 1:50 for host proteins or 1:200 for bacteria in PBS with 0.5% normal goat serum and 0.2% Triton X-100, were applied at room temperature for 2 hours. The HUVECs were then washed three times with PBS for 5 minutes each. The bacteria were detected with TRITC-conjugated goat anti-rabbit secondary antibody while the host protein markers were detected using FITC-conjugated goat anti-mouse antibody or TRITC-conjugated goat anti-rabbit, depending on labeling techniques. The secondary antibodies, diluted 1:200 dilution in PBS with 5% normal goat serum and 0.2% Triton X-100, were applied for 1 hour at room temperature. The HUVECs were then washed three times with PBS before mounting with Vectashield with DAPI (H-1200, (Vector Labs, Burlingame, CA) onto glass microscope slides. A Zeiss Axiophot fluorescence photomicroscope with a Cool Snap (Photometrics) camera and IP Lab imagining software (Scanalytics Inc., Rockville, MD) was used to view the images. All immunofluorescence experiments were repeated at least twice.

Transmission Electron Microscopy

HUVECs were allowed to grow to confluency on ACLAR (Kingsley RE) layered in 6-well dishes with EGM-2 supplemented media. After a 5 and 20 hour incubation of the HUVECs in antibiotic free EGM-2 media with bacteria, the cells were fixed in 2% glutaraldehyde in 0.1M sodium cacodylate (Sigma-Aldrich, St. Louis, MO) buffer, pH 7.4, with 1 mM calcium chloride (Sigma-Aldrich), for 1 hour. Next, the cells were rinsed 3 times for 5-10 minutes each in 0.1M sodium cacodylate buffer and then postfixied with 1% osmium tetroxide (Electron Microscopy Sciences, Fort Washington, PA) in 0.1M sodium cacodylate for 1 hour. They were rinsed in
deionized H₂O three times for 10 minutes each and dehydrated in a graded ethanol series (50, 70, 95, 100%) for 10 minutes each, followed by dehydration in 100% acetone for 15 minutes. The cells were then infiltrated with Spurr’s resin (Spurr, A.R.) and acetone 1:1 for 1 hour, followed by 100% resin overnight. The samples were cured in flat embedding molds overnight at 60°C. Then, 60-80 nm sections were obtained using Formvar-coated copper grids, and were stained with 2% uranyl acetate (Sigma-Aldrich) and 0.1% lead citrate (Venable JH) or for acid phosphatase. Samples were examined at 60 KV on JEOL 100CX electron microscope.

Cell Death Assay

Quantification of Endothelial Cells by Flow Cytometry

Endothelial cells were seeded and allowed to grow to confluency in 6 well dishes. All of the wells were washed twice with antibiotic free EGM-2 media prior to the addition of *P. gingivalis*. After the indicated incubations, the antibiotic-free media plus free-floating *P. gingivalis* were transferred using a pipette, to 1.5 ml microcentrifuge tubes (USA Scientific, Ocala, FL) and centrifuged using a Fisher Microcentrifuge model 59A at 5800 X g for 5 minutes. 0.5ml of 0.25% trypsin-EDTA was added to each well and the dishes were then placed back into the 37°C incubator for 5 minutes. The supernatant from the 1.5 ml centrifuge tubes was decanted and the trypsin-EDTA/cell mixture from each well was added to the corresponding tubes and centrifuged for 5 minutes at 5800 X g. The supernatant was decanted and the cell pellet was resuspended in 300 µl PBS prior to transfer to a 5 ml polystyrene round bottom tube (Falcon, Bedford, MA). Propidium iodide (Sigma-Aldrich) at 1 µg/ml final concentration was then added to each tube. Flow cytometry was performed using a FACSort flow cytometer (BD Biosciences, San Jose, CA). The instrument illuminates individual cells in flow using an argon-ion laser emitting 15 milliwatts of 488 nm of light. Forward and side light scatter and orange fluorescence (585 nm +/- 21 nm) for each cell was measured. Data for 10,000 particles were
collected per sample. Cell Quest software (Version 3.3; BD Biosciences) was used for data collection and subsequent analysis. Intact cells were defined based on their forward and side scatter and their orange fluorescence (propidium iodide emission) was displayed. Dead cells (orange fluorescence above background) were counted and expressed as a percentage of total cells in each sample (Fig. 2-1).

**Antibiotic Protection Assay**

Approximately $10^5$ HUVECs were seeded per well in 6-well tissue culture plates (Corning Inc, Corning, NY) followed by washing three times with antibiotic-free EGM-2 media. The wells were then infected with an overnight liquid culture of $10^7$ bacteria (MOI 100) in 1.0 ml of $37^\circ$C EGM-2 with 200 $\mu$g ml$^{-1}$, metronidazole (Sigma) and 300 $\mu$g ml$^{-1}$, gentamicin (Sigma) antibiotics. After 0.5, 2.5, 12, and/or 24 hours of incubation, the media were collected and centrifuged as described above. Cell only wells were used as controls.

**Inhibitors**

To determine the effects of the actin polymerization inhibitor Cytochalasin D (Sigma) on bacterial invasion, HUVECs were preincubated for 0.5 hrs with cytochalasin D [5 $\mu$g ml$^{-1}$ in dimethly sulfoxide (DMSO)] prior to the addition of the overnight culture preparations of *P. gingivalis* strains.

**Caspase 3 Activity Assay**

The use of both apoptotic and non-apoptotic cells were required for this assay. Confluent monolayers of HUVEC were treated for 2.5, 12, and 24 hours with *P. gingivalis* wild-type strains 381 and ATCC 33277 (MOI 100) with and without antibiotics. An inducer of apoptosis, staurosporine (STS) at a concentration of 0.5 $\mu$m, was used as a positive control. At each time-point, the media were collected on ice, and the adherent cells were scraped from each well and also transferred into 1.5 ml microcentrifuge tubes on ice. After centrifugation of the tubes at
1500g for 5 minutes, the medium was decanted and the pellets carefully resuspended in 100 μl of sterile filtered cell lysis buffer (1X) (10 mM Tris-HCL, 10 mM NaH$_2$PO$_4$/NaPO$_4$, pH 7.5, 130 mM NaCl, 1% TritonX-100, 10 mM NaPP$_i$ (sodium pyrophosphate). Following 30 minutes on ice, the lysed cell solution was centrifuged at 1500g for 10 minutes at 4°C. The liquid was then separated from the pellet and collected on ice into fresh 500 μl microcentrifuge tubes. A 96 well plate was used to conduct the Caspase 3 protease assay. A 20 μM final concentration of Ac-DEVD-AMC Fluorogenic Substrate (BD Biosciences Cat. No. 66081U) was added to 170 μl of freshly made Protease Assay Buffer (1X): 20 mM HEPES (pH 7.5), 10% glycerol, 2 mM DTT plus 20 μl of each sample. The amount of AMC liberated from A-DEVD-AMC was measured using a spectrofluorometer (Perkin-Elmer Victor 1420-011, Wallac, Turku, Finland) with an excitation wavelength of 380 nm and a 430-460 nm emission wavelength.

**Statistical Analysis**

Significant differences between the means (+/- standard deviations) for the cell death, transepithelial resistance, and caspase 3 activity assays were determined using a two-tailed Student’s $t$ test. Differences in $P$ values of <0.05 were considered significant.

**Transepithelial Resistance**

TER was measured across HuH7 monolayers grown on standard 24 well cell culture transwell 6.5 mm, 3.0 μm pore size inserts (Corning Incorporated, Acton, MA) and collagen coated Corning transwell-COL inserts (Corning Incorporated ). Transepithelial resistance (TER) was measured using an EndOhm chamber (World Precision Instrument, Inc., Sarasota, FL) and the EVOM electrical resistance system (World Precision Instruments, New Haven, CT). Cell-free transwell inserts were used to obtain baseline levels. The baseline monolayer resistance was measured on the third day after seeding. The tight-junctioned HuH7 monolayers were incubated at 37°C in EMEM medium supplemented with 10,000 units/ml of streptomycin and 10,000
units/ml of penicillin G, 5% FBS and 2 mM glutamine. Freshly harvested *P. gingivalis* at an MOI of 100 in 0.4 ml antibiotic-free EMEM medium were added to the upper chamber. Antibiotic-free supplemented EMEM medium (1.0 ml) was then added into the lower chamber. The TER of the HuH7 monolayers was measured at each time point. The resistance from each well was corrected for the resistance of a cell-free insert, yielding the resistance of the monolayer expressed in Ohms.
Figure 2-1. Flow cytometry scatter plot of HUVECs showing live cells and dead cells. (A) Twelve hour scatter plot of untreated HUVECs and (B) scatter plot of HUVECs exposed to strain 381 at an MOI of 100 for 12 hours. Live and dead cells are labeled in each plot.
### Table 2-1. Bacterial strains and plasmids used and constructed in this study

<table>
<thead>
<tr>
<th>Wild-type Strains</th>
<th>Mutated gene</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>381</td>
<td>None</td>
<td>SUNY-Buffalo&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>W83</td>
<td>None</td>
<td>SUNY-Buffalo&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>a</sup>Abbreviations: ATCC = American Type Culture Collection  
<sup>b</sup>SUNY-Buffalo = State University of New York, Buffalo, NY, USA
CHAPTER 3
RESULTS

Monolayer Integrity

Effects of \textit{P. gingivalis} Lysate on the Cellular Distribution of the Junctional Proteins in \textit{HuH}_7 and \textit{HUVEC} Cells

Initial experiments were performed using the Human Hepatic Epithelial Cell line (\textit{HuH}_7), and Human Umbilical Vein Endothelial Cells (\textit{HUVEC}) to observe whether the lysate (cell extract) of \textit{P. gingivalis} W83 had the ability to cause the alteration of the location of the proteins within the adherens junctional complex from the cell border to a more cytoplasmic location, subsequently disrupting integrity of the monolayer. Monolayer integrity is defined as a layer of continuous, tightly apposed cells. Confluent monolayers of both cell types were treated with varying concentrations of the \textit{P. gingivalis} W83 lysate and the effects on the integrity of the intact monolayer were compared to a negative control (\textit{E. coli} lysate) strain using immunofluorescence microscopy (IMF). \textit{HuH}_7 cells were treated with 0.2 and 0.4 mg/ml concentrations of the W83 lysate and 0.4 mg/ml of the \textit{E. coli} lysate (Fig. 3-1). The \textit{HuH}_7 cells were labeled with antibodies against two proteins located in the adherens junctional complex, cadherin and $\beta$-catenin. The results from the untreated \textit{HuH}_7 cells showed that the majority of these proteins were concentrated at the cell surface, between adjacent cells (arrows) (Fig. 3-1). $\beta$-catenin could also be seen sparsely scattered throughout the cytoplasm. After 24 hours of incubation, the W83 lysate, but not the \textit{E. coli} lysate, was able to affect the distribution of these proteins within these cells (Fig. 3-1). Treatment of \textit{HuH}_7 cell monolayers with 0.2 mg/ml of the W83 lysate resulted in a loss of monolayer integrity, specifically a loss of cadherin and $\beta$-catenin from the cell surface. Concurrently, both of these proteins were in punctate areas throughout the cytoplasm and $\beta$-catenin was also detected in the proximity of the nucleus (Fig. 3-1). Exposing
HuH7 monolayers to a 0.4 mg/ml concentration of the W83 lysate for 24 hours resulted in no detectable cells remaining on the coverslip. Thus, exposure of the HuH7 cells to the W83 lysate but not the *E. coli* lysate resulted in an alteration of location of two adherens junction proteins, cadherin and β-catenin, and disruption of the monolayers’ integrity.

We next sought to examine if the *P. gingivalis* W83 lysate was capable of stimulating similar effects on the endothelial cell line, HUVEC. For these experiments, confluent HUVEC monolayers were treated with 0.2 mg/ml and 0.4 mg/ml of the W83 lysate for 24 hours. As with the HuH7 cells, HUVECs treated with 0.4 mg/ml of the W83 lysate resulted in no detectable cells remaining on the coverslip. Exposure of 0.2 mg/ml of the W83 lysate resulted in the alteration of the cadherin and β-catenin proteins from the cell surface to punctate areas within the cytoplasm. However, very few cells remained adhered to the coverslip (data not shown). Given that the HUVECs were very sensitive to the W83 lysate, lower concentrations (0.05 and 0.1 mg/ml) of the W83 lysate were also examined. The 0.05 mg/ml concentration of the W83 lysate was unable to alter the location of cadherin (data not shown) and β-catenin from the cell surface, demonstrating that this concentration was not high enough to disrupt the integrity of the HUVEC monolayer. However, exposure of 0.1 mg/ml of the W83 lysate to the HUVEC monolayers resulted in alteration of the location of the cadherin (data not shown) and β-catenin proteins (arrows), shifting these proteins from a cell surface location to punctate areas within the cytoplasm (Fig. 3-2). In addition, there was a reduction in the amount of cells adhering to the coverslip compared to the untreated cells (Fig. 3-2). This data suggests *P. gingivalis* contains proteins that are capable of affecting the cell-cell adhesion proteins cadherin and β-catenin and the cell-matrix adhesion protein, integrin, in both epithelial and endothelial cells.
Effects of Heat and Protease Inhibitors on the Cellular Distribution of the Cadherin and β-catenin Proteins in HuH₇ Cells Treated with *P. gingivalis* W83 Lysates.

*P. gingivalis* is an asaccharolytic anaerobe that expresses arginine (Rgp) and lysine (Kgp) cysteine proteinases (gingipains). A primary function of these proteinases is the breakdown of proteins and peptides within its environment, which provide this bacterium with energy and carbon. As discussed in chapter 1, these proteinases have also been shown to be major factors influencing the virulence of this pathogen (O’Brien-Simpson et al., 2003). The loss of the HuH₇ and HUVEC monolayer integrities observed after exposure of these intact monolayers to the W83 lysate suggests that proteins from *P. gingivalis* are responsible for this disruptive effect. The following experiments were done to determine the identity of the active component(s).

We first sought to establish if temperature affected the activities of the *P. gingivalis* lysate. The W83 lysate was thus heated for 20 minutes at 60°C (Yumoto et al., 2005; Nassar et al., 2002) to determine if heat treatment had any inhibitory effects on the efficacy of the W83 lysate to stimulate epithelial monolayer disruption and the alteration of the location of the adherens junction proteins in HuH₇ cells. Confluent HuH₇ monolayers treated with the heated W83 lysate resulted in no detectable differences in the localization of cadherin (Fig. 3-3) and β-catenin (Fig. 3-4) proteins in these cells compared with the untreated cells. Thus, the failure of the heat-treated W83 lysate to disrupt the junctional complexes is likely due to the denaturation of specific proteins associated with the activity.

We next sought to determine whether the proteins involved in stimulating endothelial and epithelial monolayer disruption were cysteine proteinases (e.g., gingipains). To this end, specific proteinase inhibitors were tested to determine their inhibitory effects on the active proteins in the W83 lysates. Three proteinase inhibitors including PMSF, a serine proteinase inhibitor, TLCK, a cysteine proteinase inhibitor, and PIC, a cocktail of inhibitors of carboxypeptidases, serine and
cysteine proteinases, were tested. Of these inhibitors, TLCK was the only one shown previously to efficiently block *P. gingivalis* cysteine proteinase activity (Hintermann *et al.*, 2002). After 22 hours of incubation, neither PMSF nor PIC at a concentration of 2 mM inhibited the destructive effects of the 0.4 mg/ml W83 lysate on the HuH7 monolayers since there were no detectable cells remaining after treatment. However, TLCK at a concentration of 1 mM inhibited the effects of the W83 lysate on the cell-matrix adhesions by preventing the detachment of cells from the monolayer (Figs. 3-3, 3-4). The results from the TLCK treated HuH7 cells were similar to control cells in that the cell monolayer was almost intact. However, unlike the control cells, the distribution of cadherin (Fig. 3-3) and β-catenin (Fig. 3-4) changed from a cell surface location to punctate areas within the cytoplasm. The redistribution of cadherin and β-catenin ultimately led to the disruption of the adherens junctions as observed by the loss of the continuous monolayer. The reduced capacity of the W83 lysate in the presence of TLCK to disrupt the cell monolayer suggests that the active component(s) responsible for disrupting cell-matrix and cell-cell adhesion are cysteine proteinases.

**Proteinase Inhibitor Treatment of *P. gingivalis* W83 on HuH7 Adherens Junctional Complexes**

The previous experiments using the *P. gingivalis* W83 lysate suggested that the cysteine proteinases (gingipains) were most likely responsible for disrupting junctional proteins since the cysteine proteinase inhibitor TLCK was capable of inhibiting this activity. The next step was to determine whether the effects of the gingipains were also mediated from within the cell. Since strain W83 is known to adhere to and invade host cells, we determined if the gingipains from live, intact *P. gingivalis* W83 were capable of stimulating the HuH7 monolayer disruption we observed with the W83 lysate. After strain W83 was co-cultured with confluent HuH7 monolayers at an MOI of 1000 for 24 hours, there were no detectable HuH7 cells remaining on
the coverslip. In order to determine if the cysteine proteinase inhibitor, TLCK, would suppress these effects, live W83 cells were co-cultured with confluent HuH7 monolayers and 1 mM TLCK (Figs. 3-3, 3-4). After 24 hours, many HuH7 cells remained on the glass coverslip. However, no cadherin or β-catenin could be detected at the cell membranes of the remaining cells (Figs. 3-3, 3-4). Taken together, these results suggest that the disruption of the epithelial and endothelial cell-cell and cell-matrix adhesions and subsequent loss of monolayer integrity are stimulated by the cysteine proteinases (gingipains) associated with live *P. gingivalis* W83 and the W83 lysate. These data do not prove but suggest that the gingipains may act both from within the epithelial and endothelial cells as well as from the cells’ exterior. Treatment with TLCK was capable of inhibiting the *P. gingivalis* cysteine proteinases Rgp and Kgp from stimulating the detachment of HuH7 cells, but was unable to inhibit the alteration of the location of β-catenin from the cell surface to within the HuH7 cytoplasm. Since TLCK has been shown to inhibit Kgp activity more than Rgp activity (Pike *et al.*, 1994), it is possible that the activity of Rgp is largely accountable for the observed alteration of location of cadherin and β-catenin within the epithelial and endothelial monolayers examined.

**Effects of *P. gingivalis* Secreted Protein Fraction (SPF) on the Adherens Junctional Complex Proteins of HUVECs**

Experiments using the cysteine proteinase inhibitor TLCK suggested that the gingipains from both live *P. gingivalis* W83 and the W83 lysate are likely responsible for the disruption of the HuH7 monolayer, specifically the junctional proteins cadherin and β-catenin. Since the gingipains are known to exist in both cell membrane associated and secretory forms (Kadowaki and Yamamoto, 2003; Potempa *et al.*, 2003; Kadowaki *et al.*, 2000; Potempa *et al.*, 1998; Potempa *et al.*, 1997; Travis *et al.*, 1997), we next sought to determine if *P. gingivalis* surface as well as secreted proteins were capable of stimulating endothelial monolayer disruption.
To investigate this, the activities of the *P. gingivalis* soluble surface and/or secreted proteins (SPF) on HUVEC monolayers were studied to determine their levels of proteolytic activity relative to the junctional complexes. The SPF was prepared using ammonium sulfate to precipitate out the secreted and cell surface associated proteins from *P. gingivalis*. The protein concentration of the SPF from *P. gingivalis* was determined to be 23 mg/ml for strain W83 and 0.2 mg/ml for strain 381. Confluent HUVEC monolayers were then treated with various concentrations of these SPFs for 24 hours. Twenty-two μg/ml of the W83 SPF and 1 μg/ml of the 381 SPF resulted in a loss of both β-catenin (Fig. 3-5) and cadherin (Fig. 3-6) from the cell surface of HUVECs. Approximately half of the HUVECs treated with the SPFs remained adhered to the coverslip compared with the untreated cells. The results showed that exposure of the HUVECs to 0.02 μg/ml of the 381 SPF and 0.2 μg/ml of the W83 SPF resulted in an altered distribution of cadherin and β-catenin (Fig. 3-5, 3-6). However, a considerably greater number of cells remained on the coverslip in comparison to the number of cells exposed to the higher concentrations of the SPFs (22 μg/ml of the W83 SPF and 1 μg/ml of the 381 SPF). In addition, the disruption in the HUVEC monolayer was more pronounced after treatment with the 381 SPF (0.02 μg/ml) than with the W83 SPF (0.2 μg/ml) (Fig. 3-5; 3-6). One explanation for this difference is that the level of arginine protease activity of the strain 381 SPF, measured as activity per μg of protein using an enzymatic assay with 5 mM N-α-benzoyl-DL-arginine p-nitroanilide (BAPNA) was approximately two-fold greater than that of the W83 SPF. This data indicates that the deleterious effects to the monolayers associated with *P. gingivalis* are at least in part due to surface associated and/or secreted proteins, (e.g., gingipains), since the SPFs alone were capable of inducing such monolayer disruption.
Effects of *P. gingivalis* on Confluent HUVEC Monolayers

We have shown that the surface associated/secreted proteins (e.g., gingipains) from *P. gingivalis* W83 affect the HUVE cells so as to alter the distribution of both cadherin and β-catenin, as well as to stimulate disruption of the epithelial and endothelial monolayers. We next sought to test whether live *P. gingivalis* was capable of inducing the same effects to the HUVEC monolayer as observed with the *P. gingivalis* lysate and SPF. IMF was used to detect the loss of or changes in the cellular location of the junctional protein, β-catenin, within the confluent HUVEC monolayer after treatment with live *P. gingivalis* wild-type strains W83, 381, ATCC 33277, and AJW4 (Table 3-1). Data published by others previously reported that strains W83, 381 and ATCC 33277 are all, with different efficiencies, capable of adhering to, invading and persisting within a number of cell types including human coronary artery endothelial cells, HCAEC, gingival epithelial cells, GECs, and HUVECs (Yilmaz *et al.*, 2003; Dorn *et al.*, 1999; Deshpande *et al.*, 1998). In contrast, AJW4 has been shown to adhere to but not invade or persist within HCAEC or HUVECs (Dorn *et al.*, 2000). These data are summarized in table 3-1.

In our study, HUVEC monolayers co-cultured with these strains at an MOI of 100 appeared unaffected after 1 hour (data not shown) and 5 hours of incubation (Figs. 3-8 - 3-11). The majority of β-catenin was concentrated at the cell surface, between adjacent cells (white arrows) (Figs. 3-8 - 3-11). Yet, at both times, *P. gingivalis* W83, 381 and ATCC 33277 appeared to be located on the cell surface as well as internalized with the HUVECs (yellow arrows). In contrast, the HUVEC monolayers exposed to AJW4 for 1 and 5 hours showed very few *P. gingivalis* attached to the monolayer. After 10 hours of co-culture with strains W83 and 381, there was an observable loss of β-catenin at the cell surface (white arrows) (Figs. 3-8, 3-9). Strains AJW4 and ATCC 33277 did not stimulate the HUVECs to alter the localization of β-
catenin within the confluent monolayers until 15 and 20 hours, respectively (Figs. 3-10, 3-11). Treatment of HUVEC monolayers for 20 hours with all four strains resulted in disruption to the monolayers as evident by the loss of cells and less β-catenin (white arrows) at the cell surface (Figs. 3-8 - 3-11). Except for AJW4, each strain of *P. gingivalis* was internally localized or associated with the HUVEC surface at each of the time points tested (yellow arrows) (Figs. 3-8, - 3-11). Strain AJW4 did not appear to be internalized or associated with the HUVEC cell surface until 10 hours of exposure.

Increasing the MOIs from 100 to 1000 caused more dramatic effects on the HUVEC monolayer. HUVEC monolayers exposed to strain W83 at an MOI of 1000 remained intact and retained β-catenin at the cell surface through 5 hours of co-culture (Fig. 3-12). However, 5 hours of co-culture of HUVECs with strains 381, ATCC 33277 and AJW4 at this concentration resulted in a deleterious effect on the monolayers (Figs. 3-13 - 3-15). At this time, notably reduced amounts of β-catenin were detected at the cell surface (white arrows). After 10 hours of exposure to all four strains, the HUVEC monolayers were markedly disrupted and no longer intact. In addition, a higher number of each strain of *P. gingivalis*, including AJW4 (yellow arrows) were intracellularly localized and/or associated with the cell surface at this time point, relative to Time 0 (Figs. 3-12 - 3-15). Treatment for 20 hours with all four strains at an MOI of 1000 resulted in considerable disruption to the monolayer, causing the majority of the HUVECs to elongate and/or detach (Figs. 3-12 -3-15). At this time point, high numbers of all four strains of *P. gingivalis*, including AJW4, were detected both associated with the HUVEC surfaces and internalized within the cytoplasm (yellow arrows) (Figs. 3-12 - 3-15). In addition, HUVEC monolayers treated for 20 hours with each *P. gingivalis* strain resulted in a lack of junctional complexes and β-catenin was no longer detected at the cell surface.
These data presented here indicate that 10 hours of treatment with the *P. gingivalis* strains W83 and 381 at an MOI of 100, which have been shown to adhere, invade and persist within a number of different cell types including HUVEC (Table 3-1), is sufficient for inducing HUVEC monolayer disruption and the alteration of the location of β-catenin from the cell surfaces, to a location within the cytoplasm (Figs. 3-8, 3-9). The results with strain ATCC 33277 at an MOI of 100, which has also been shown to adhere, invade and persist within HUVEC, showed that this bacterium was also capable of inducing HUVEC monolayer disruption, but only after 20 hours of co-culture (Fig. 3-10). Interestingly, AJW4 at an MOI of 100, which has been shown to adhere to but not invade or persist within a number of different cell types including HUVEC, was capable of inducing HUVEC monolayer disruption after 15 hours of co-culture (Fig. 3-11).

Since invasion efficiencies are characteristically determined after 2.5 hours of *P. gingivalis* exposure with the cell of interest, it is possible that the extended interaction times of *P. gingivalis* with the HUVE cells were sufficient for AJW4 invasion. Barring this possibility, these data suggest that internalized *P. gingivalis* play a role in stimulating HUVEC monolayer disruption and that the gingipains associated with the internalized *P. gingivalis* are perhaps inducing their deleterious effects from within these cells. However, since the results with the *P. gingivalis* SPFs showed monolayer disruption, we can’t rule out the possibility that the gingipains interacting with the HUVE cells’ exterior and/or gingipains associated with the *P. gingivalis* that adhered to the cell surface also participate in this activity.

**Effects of Gingipain Mutants on the Junctional Complex Protein β-catenin in Confluent HUVEC Monolayers**

The data using specific proteinase inhibitors indicated that the cysteine proteinases, (gingipains) from *P. gingivalis* are likely responsible for the observed HUVEC monolayer disruption. The goal of the next set of experiments was to determine which of the gingipains is
most active in HUVEC monolayer disruption. Gingipain knock-out (KO) mutants of *P.*
*gingivalis* were used in this set of experiments. All gingipain mutants (Table 3-2) were
constructed from *P. gingivalis* strain 381 (Chen and Kuramitsu, 1999) except for YPP2 (*kgp-*),
which was constructed in strain ATCC 33277 (Park and Lamont, 1998).

Confluent HUVEC monolayers were co-cultured with live gingipain mutants including
MT10 (*rgpA*-*), MT10W (*rgpA-* *kgp-*), G102 (*rgpB-*), G102W (*rgpB-* *kgp-*), CW401 (*rgpA-*
*rgpB-*), CW501 (*rgpA-* *rgpB-* *kgp-* and YPP2 (*kgp-* at an MOI of 100 (Figs. 3-16 - 3-22).
Through 10 hours, the *P. gingivalis* gingipain mutants were detected at the cell surface and/or
within the cell (yellow arrows). However, unlike the monolayers exposed to strain 381 (Fig. 3-9),
the monolayers exposed to each *P. gingivalis* gingipain mutant remained intact at this time
point. Furthermore, the HUVEC monolayers remained intact with β-catenin at the cell surface
(white arrows) even after 15 hours of co-culture with G102 (*rgpB-* (Fig. 3-18), CW401 (*rgpA-*
*rgpB-* (Fig. 3-21) and CW501 (*rgpA-* *rgpB-* *kgp-* (Fig. 3-22). In contrast, HUVEC
monolayers exposed to MT10 (*rgpA-* (Fig. 3-16), MT10W (*rgpA-* *kgp-* (Fig. 3-17) and G102W
(*rgpB-* *kgp-* (Fig. 3-19) displayed a loss of β-catenin staining at the cell surface (white arrows)
and a disruption of the confluent monolayers, suggesting some cells within the monolayers had
detached. The HUVEC monolayers co-cultured with MT10, MT10W, G102, G102W, and YPP2
for 20 hours (Figs. 3-16 - 3-20) resulted in numerous *P. gingivalis* (yellow arrows) attached to
and localized within the cells and a lack of β-catenin staining at the cell surface (white arrows)
(Fig. 3-43). In addition, these monolayers were no longer continuous since some cells had
broken free and detached from the monolayer. In contrast, the HUVEC monolayers exposed to
CW401 (*rgpA-* *rgpB-* and CW501 (*rgpA-* *rgpB-* *kgp-* for 20 hours resulted in only a few *P.*
*gingivalis* attached to or internalized with the cells (Figs. 3-21, 3-22) and no alteration of
location of the junctional protein, β-catenin. Thus, the HUVEC monolayers treated with CW401 and CW501 maintained monolayer integrity and resembled the untreated cells at each time point. A comparison of the HUVEC monolayers treated for 20 hours with all of the gingipain mutants and their parent strains can be viewed in figure 3-43.

To summarize these results, the HUVE cell monolayers exposed to the parent strains 381 and ATCC 33277 resulted in obvious monolayer disruption after 20 hours of exposure. HUVE cell monolayers exposed to the gingipain mutants containing RgpB (MT10W) or RgpA (G102W) only, resulted in partial monolayer disruption after 20 hours. In addition, the gingipain mutants containing Kgp and RgpB (MT10) or Kgp and RgpA (G102) also resulted in partial monolayer disruption after 20 hours of exposure. In contrast, HUVEC monolayers exposed to the gingipain-null (CW501) and Rgp-null (CW401) mutants resulted in no monolayer disruption and resembled the untreated cell monolayers. Interestingly, the gingipain mutant containing both RgpA and RgpB but lacking Kgp resulted in only minimal monolayer disruption after 20 hours of exposure. These data suggest that to some extent, each gingipain is involved in stimulating HUVE cell monolayer disruption. However, it is possible that other factors (e.g., LPS) associated with P. gingivalis are involved in the disruption of the HUVEC monolayer.

Next, particularly high concentrations (MOIs of 1000) of the P. gingivalis gingipain mutants were used to determine if increasing the concentration would disrupt the HUVEC monolayers more quickly. Similar to the results observed with the parent strains 381 and ATCC 33277, the HUVEC monolayers co-cultured with MT10 (rgpA-), MT-10W (rgpA-, kgp-), G102 (rgpB-), G102W (rgpB-, kgp-), and YPP2 (kgp-) at an MOI of 1000 for 5 hours resulted in partial disruption to the monolayer (Figs. 3-23 - 3-27). However, at this time, HUVEC monolayers treated with P. gingivalis CW401 (rgpA-, rgpB-) and CW501 (rgpA-, rgpB-, kgp-) remained
intact (Figs. 3-28, 3-29). In addition, all of the gingipain mutants except for CW501 were observed, in varying degrees, in clusters or aggregates, and appeared to be attached to the cell surface and/or internalized within the HUVEC cytoplasm at this time point (yellow arrows) (Figs. 3-23 - 3-28). HUVEC monolayers exposed to MT10, MT-10W, G102, G102W, and YPP2 for 10, 15, and 20 hours resulted many *P. gingivalis* attached to the cell surface and/or internalized within the cell, the loss of β-catenin at the cell membrane (white arrows) and a loss of adherent cells (Figs. 3-23 - 3-27). In contrast, monolayers co-cultured with CW401 and CW501 remained intact through 15 hours with minimal *P. gingivalis* attached to or internalized within the HUVECs (Figs. 3-28, 3-29). After 20 hours, HUVEC monolayers treated with CW401 (*rgpA-, rgpB-*) (Fig. 3-28) resulted in minimal monolayer disruption and a nominal loss of β-catenin from the cell surface (white arrow). However, the monolayers co-cultured with CW501 (*rgpA-, rgpB- kgp-*) (Fig. 3-29) for 20 hours maintained monolayer integrity, as well as β-catenin at the cell surface (white arrow) and were comparable to the untreated HUVEC. This data suggests that Kgp is capable of inducing HUVE cell monolayer disruption but only at a high MOI.

Taken together, these results establish that the gingipains are responsible for the disruption of the HUVEC monolayers. The result that the HUVEC monolayers co-cultured with CW401 (*rgpA-, rgpB-*) at an MOI 1000 for 20 hours were only minimally disrupted suggests that Rgp, but not Kgp, is essential for this activity. Moreover, the lack of HUVEC monolayer disruption observed after treatment with CW501 further supports the involvement of gingipains. However, it cannot be ruled out that the minimal or lack of monolayer disruption observed after co-culture with the CW401 and CW501 respectively, may be due to the decreased invasion abilities of these
mutants and that some factor other than the gingipains may also play a role in monolayer disruption.

**Effects of Adherence Mutants of *P. gingivalis* from Strains W83 or ATCC 33277 on Confluent HUVEC Monolayers**

Data from the above experiments strongly establish that the gingipains are required for the disruption of the HUVEC monolayer and the loss of β-catenin from the cell surface. We next sought to study whether adherence of *P. gingivalis* to host cells was also a requirement of *P. gingivalis* to effect HUVEC monolayer disruption. To investigate this, *P. gingivalis* mutants with defective adherence abilities were examined. Adhesion mutants (Table 3-3) constructed from the wild-type strain W83, including PG1683 (conserved hypothetical protein), PG0242 (conserved hypothetical protein), and PG1118 (clpB), in addition to an adhesion mutant constructed from the wild-type strain ATCC 33277, YPF1 (*fimA*-) (Love *et al.*, 2000; Xie *et al.*, 2000) were used for these studies. In work done by Love *et al.* and others in our laboratory, these mutants were tested for their adhesion abilities with endothelial (HCAEC) and/or epithelial (GEC or KB) cells. The conserved hypothetical proteins PG0242 and PG1683 displayed 11.7 fold and 2.1 fold decreases in adhesion to HCAEC, respectively. The *clpB*- mutant, PG1118, and the *fimA*- mutant, YPF1, showed 2.8 fold and 10-fold decreases in adhesion to GECs, respectively.

HUVEC monolayers co-cultured for 10 hours with all four adhesion mutants at an MOI of 100 resulted in very few *P. gingivalis* attached to or localized within the monolayers (yellow arrows) (Figs. 3-30 - 3-33). These HUVEC monolayers remained visibly intact with β-catenin concentrated at the cell surface (white arrows). At 15 hours, the attachment and internalization of YPF1 (Fig. 3-33) and PG0242 (Fig. 3-31) with HUVECs was minimal (yellow arrows). In contrast, numerous PG1118 (Fig. 3-32) and PG1683 (Fig. 3-30) could be detected attached to
and internalized within the HUVEC monolayer after 15 hours (yellow arrows). Interestingly, the HUVEC monolayers co-cultured with each of these mutants for 15 hours remained intact with β-catenin (white arrows) at the cell surface (Figs. 3-30 - 3-33).

A comparison of the HUVEC monolayers treated for 20 hours with all four adhesion mutants (Table 3-3) and their parent strains can be viewed in figure 3-44. At 20 hours, monolayers treated with YPF1 (Fig. 3-33) resulted in fewer *P. gingivalis* (yellow arrows) attached to or internalized within the HUVECs than the monolayers treated with PG1683 (Fig. 3-30), PG0242 (Fig. 3-31) and PG1118 (Fig. 3-32). However, HUVEC monolayers co-cultured with all of the aforementioned *P. gingivalis* mutants, except for PG0242 (Fig. 3-31) retained β-catenin staining at the cell surface and generally intact monolayers (white arrows). The integrity of HUVEC monolayers co-cultured with PG0242 was disrupted and the concentration of β-catenin (white arrow) shifted from the cell surface to sparsely distribute throughout the cytoplasm (Fig. 3-31). The results with PG1118, PG1683 and YPF1 suggest that adhesion of *P. gingivalis* to the HUVEC monolayer is necessary for *P. gingivalis* to cause monolayer disruption. However, the HUVEC monolayer disruption observed after co-culture with PG0242 refutes this and suggests that adhesion alone is not directly correlated to monolayer disruption.

**Effects of Invasion/Persistence Mutants of *P. gingivalis* from Strains W83 or ATCC 33277 on Confluent HUVEC Monolayers**

To determine the importance of *P. gingivalis* invasion of HUVECs to the disruption of the monolayers, several *P. gingivalis* mutants with decreased invasive capabilities (Table 3-4) were tested for their ability to effect disruption of the monolayers. YPEP (*pepO*), PG1118 (*clpB*), PG0717 (putative lipoprotein) and PG1286 (ferritin) were co-cultured at an MOI of 100 with confluent HUVEC monolayers to determine their effects on β-catenin localization and monolayer disruption. The YPEP (*pepO*) mutant resulted in a 25% decrease in invasion of
GECs compared to its parent strain ATCC 33277 (Park et al., 2004; Park and Lamont, 1998). Work done by others in our laboratory reported that PG1286 and PG0717 showed a 2.0 and 2.7-fold decrease in invasion of human coronary artery endothelial cells (HCAECs), respectively, while PG1118 had a 15-fold decrease in invasion of HCAECs (unpublished data). The ferritin mutant PG1286 was also determined to be a persistence mutant since it demonstrated a 33-fold decrease in persistence compared to its parent strain W83 through 48 hours.

HUVEC monolayers co-cultured with the *P. gingivalis* mutants PG1286 (Fig. 3-35) and PG1118 (Fig. 3-32) for 5 hours resulted in very few *P. gingivalis* (yellow arrows) attached to or localized within the intact monolayer. In contrast, HUVEC monolayers co-cultured with the mutants PG0717 (Fig. 3-34) and YPEP (*pepO-*) (Fig. 3-36) resulted in numerous *P. gingivalis* (yellow arrows) attached to the cell surface and/or internalized, although the monolayers remained intact. HUVEC monolayers treated with PG1286 (Fig. 3-35) did not show numerous *P. gingivalis* (yellow arrows) attached to and/or localized within the cells until after 15 hours of treatment. In addition, the *P. gingivalis clpB* mutant, PG1118, resulted in only minimal attachment and invasion of the HUVEC monolayers through 20 hours of treatment (yellow arrows) (Fig. 3-32). However, the HUVEC monolayers co-cultured with each of these mutants for 20 hours remained intact with β-catenin at the cell surface (white arrows) (Figs. 3-32, 3-34 - 3-36). A comparison of the HUVEC monolayers treated for 20 hours with all four invasion mutants and their parent strains can be viewed in figure 3-45. After 20 hours of exposure, there were no detectable disruptions of the HUVEC monolayers and β-catenin remained at the cell surface. These results with the invasion mutants thus suggest that adhesion of *P. gingivalis* to the HUVEC monolayers is not as instrumental in causing monolayer disruption, as is *P.*
*P. gingivalis* invasion. In addition, these data support the hypothesis that the *P. gingivalis* gingipains act from within the HUVE cell as well as from the outside.

**Effects of Additional *P. gingivalis* Mutants from Strain W83 on Confluent HUVEC Monolayers**

Additional mutants (Table 3-6) constructed from *P. gingivalis* W83 were examined at an MOI of 100 to determine their effects on the HUVEC monolayer. These mutants showed either no difference in invasion of HCAEC through 2.5 hours, PG0293 (putative secretion activator protein) and PG0686 (conserved hypothetical protein), or increased invasion of HCAEC, PG1788 (putative cysteine peptidase). After 5 hours, HUVEC monolayers treated with all three mutants resulted in numerous *P. gingivalis* attached to and localized within the cells (yellow arrows), yet the monolayers remained intact (Figs. 3-37 - 3-39). At 10 hours, HUVEC monolayers exposed to PG0293 resulted in monolayer disruption and a decrease of β-catenin at the cell surface (white arrow) (Fig. 3-38). However, HUVEC monolayers treated with PG1788 which resulted in a 2-fold increase in invasion of HCAECs compared to its parent strain W83 also demonstrated numerous *P. gingivalis* (yellow arrows) attached to and internalized within the cells yet remained intact at 5 and 10 hours of exposure. However, at 15 hours there was a decrease of β-catenin at the cell surface (white arrow) as well as a disrupted monolayer (Fig. 3-37). In contrast, HUVEC monolayers treated for up to 20 hours with PG0686, which showed no difference in invasion compared to its parent strain W83, resulted in *P. gingivalis* (yellow arrows) attached to and/or internalized within the cells but maintained an intact monolayer with β-catenin at the cell surface (white arrow) (Fig. 3-39; 3-46). A comparison of the HUVEC monolayers treated for 20 hours with these additional mutants and their parent strain can be viewed in figure 3-46. The results with PG1788 and PG0293 suggest that these proteins are not necessary for monolayer disruption. In contrast, the lack of HUVEC monolayer disruption
observed with PG0686, which adheres and invades as well as its wild-type parental strain, indicates that this conserved hypothetical protein is critical for monolayer disruption.

**Effects of Additional *P. gingivalis* Mutants at Higher MOIs**

The data reported above suggests that adhesion and invasion are involved in one possible mechanism that *P. gingivalis* utilizes to induce HUVEC monolayer disruption and β-catenin re-localization. At an MOI of 100, the adhesion mutant, YPF1 (fimA-) (Table 3-3), the invasion mutant, YPEP (pepO-) (Table 3-4), and PG0686 (Table 3-6) did not cause a disruption of the HUVEC monolayers. Therefore, MOIs of 1000 were examined to observe if increasing the concentration of *P. gingivalis* could induce monolayer disruption. Treatment of HUVEC monolayers with YPF1, YPEP and PG0686 for 5 hours resulted in numerous *P. gingivalis* (yellow arrows) attached to and internalized within the cells (Figs. 3-40 - 3-42). However, the HUVEC monolayers remained intact after treatment for 5 hours with YPF1 (fimA-) (Fig. 3-40) and PG0686 (Fig. 3-42) and β-catenin remained concentrated at the cell surface (white arrows). In contrast, HUVEC monolayers treated with YPEP (pepO-) at this time point were partially disrupted and maintained minimal β-catenin (white arrow) at the cell surface (Fig. 3-41). Additional treatments for 15 hours with all three *P. gingivalis* mutants demonstrated that the integrity of the HUVEC monolayer was altered in that β-catenin was no longer visualized at the cell surface. Also, numerous *P. gingivalis* (yellow arrows) were observed attached to the HUVE cell surface and internalized within the cytoplasm (Figs. 3-40 - 3-42). After 20 hours, the HUVEC monolayers treated with YPEP and YPF1 were no longer intact and β-catenin was no longer associated with the cell surface. These monolayers were severely disrupted suggesting that numerous cells had become detached. *P. gingivalis* (yellow arrows) was also observed attached to the HUVE cell surface as well as internalized with the cytoplasm (Figs. 3-40, 3-41).
Similarly, the HUVEC monolayers treated with PG0686 for 20 hours also demonstrated numerous *P. gingivalis* (yellow arrow) attached to the cell surface and internalized within the cells (Fig. 3-42). Most significantly, however, the HUVEC monolayer was only partially disrupted.

The effects observed with YPF1 (*fimA*- ) and YPEP (*pepO*- ) at an MOI of 1000 after 20 hours were comparable to their parent strain ATCC 33277 (Fig. 3-40, 3-41). These results suggest that both adhesion and invasion impaired *P. gingivalis* mutants at high enough concentrations (MOI of 1000) are capable of altering the location of β-catenin from the HUVE cell surface to within the cytoplasm, as well as inducing monolayer disruption. However, HUVEC monolayers co-cultured with PG0686 for 20 hours remained mostly intact, although β-catenin did not remain concentrated at the cell surface (Fig. 3-42). The effects observed with the PG0686 conserved hypothetical protein mutant at an MOI of 1000 further suggest that this protein is critical for *P. gingivalis* to induce HUVEC monolayer disruption.

**Invasion**

The purpose of the following experiments was to determine the effects on the ultrastructure of HUVECs exposed to strain 381 containing gingipains, compared to strain CW501, lacking gingipains. To investigate this, confluent HUVEC monolayers were co-cultured with CW501 (gingipain-null) mutant and its parent strain 381, at an MOI of 100 for 5 and 20 hours. Transmission electron microscopy (TEM) was used to observe the interactions of *P. gingivalis* with HUVECs. The untreated HUVECs demonstrated normal morphology, in that they displayed an intact cell membrane and nucleus as well as a diffuse distribution of organelles (Figs. 3-47, 3-48). Exposure of HUVEC monolayers to *P. gingivalis* CW501 for 20 hours was comparable to the untreated cells with the exception of the presence of a few empty vacuoles
(Fig. 3-48). Also, very few CW501 bacteria were observed at the cell surface (white arrows). In contrast, co-culture of HUVEC monolayers with strain 381 demonstrated considerably different results. After 5 and 20 hours, many *P. gingivalis* were observed at the HUVEC surface (white arrows) and internalized within these cells (yellow arrows) (Fig. 3-47). Strain 381 appeared to be primarily localized within vacuoles, possibly autophagic vacuoles, in the HUVEC cytoplasm (yellow arrows) (Fig. 3-47). Thus, *P. gingivalis* 381 invasion of HUVECs resulted in morphological alterations in the host cell, specifically the appearance of vacuoles containing *P. gingivalis* (yellow arrows). In contrast, with the exception of a few additional empty vacuoles, there was no apparent disruption of HUVEC morphology and no internalization of CW501 through 20 hours of co-culture (Fig. 3-48). Taken together, the morphological alterations observed with 381 and not with CW501 suggest that *P. gingivalis* gingipains play a role in this activity.

Increasing the MOI of both *P. gingivalis* 381 and CW501 to 1:1000 had an affect on the morphology of the HUVECs (Fig. 3-49). HUVECs co-cultured with strain 381 for 20 hours showed numerous *P. gingivalis* 381 located around the periphery (white arrows) of the cells. However, most of the bacteria were internalized within cytoplasmic vacuoles (yellow arrows) (Fig. 3-49). Compared to the untreated cells, the HUVECs co-cultured with strain 381 exhibited cellular distortions including the presence of numerous empty vacuoles (black arrows) and vacuoles containing *P. gingivalis* (yellow arrows), as well as invaginations of the cell membrane. In contrast, HUVECs co-cultured with CW501, demonstrated very few *P. gingivalis* located around the cell periphery (white arrows) or internalized with the cells (yellow arrows) (Fig. 3-49). With the exception of an increased number of empty vacuoles (black arrows) within the HUVECs co-cultured with CW501, the cellular morphology resembled the untreated cells.
through 20 hours of co-culture (Fig. 3-49). These results suggest that the gingipains are likely involved in effecting invaginations of the cell membrane since the increased concentration (MOI of 1000) of the gingipain-null mutant, CW501, did not have the same effect. However, this concentration of CW501 did result in the appearance of additional vacuoles, most of which were devoid of *P. gingivalis*. In addition, since CW501 does not invade, we cannot rule out that *P. gingivalis* invasion is a requirement for the observed alterations in HUVEC morphology.

In previous experiments, the conserved hypothetical protein mutant PG0686 showed no difference in adhesion or invasion abilities compared to its parent strain and demonstrated no disruption to the HUVEC monolayer at an MOI of 100 through 20 hours. Therefore, this mutant was also tested to determine its effects on the HUVEC morphology. After 20 hours of co-culture of HUVEC monolayers and PG0686 at an MOI of 1000, numerous *P. gingivalis* were observed both around the cell periphery (white arrows), as well as internalized (yellow arrows) within the cells (Fig. 3-49). Elevated numbers of PG0686 were localized within multiple vacuoles (yellow arrows) throughout the HUVECs cytoplasm (Fig. 3-49). However, the cell membrane of the PG0686 treated HUVECs remained intact, not invaginated like the cells treated with strain 381. In addition, with the exception of increased cytoplasmic vacuoles containing *P. gingivalis* (yellow arrows), the HUVECs exposed to PG0686 for 20 hours appeared to resemble the untreated cells (Fig. 3-49).

These results show that *P. gingivalis* 381 and PG0686 are capable of internalizing and persisting within the HUVECs for extended times. These strains appear to be primarily localized within intracellular vacuoles in the cytoplasm. In addition, previous experiments suggested that *P. gingivalis* invasion may be necessary for monolayer disruption since CW501 and the invasion mutants of *P. gingivalis*, YPEP, PG1118, PG0717, and PG1286, demonstrated no disruption of
the HUVEC monolayer and very little internalization within the cell. Taken together, these results suggest that the \textit{P. gingivalis} gingipains may be acting upon the HUVEC monolayer from within the cell. However, HUVEC monolayers treated with the conserved hypothetical protein mutant PG0686 at an MOI of 100, which showed no difference in adhesion or invasion when compared to its parent strain W83, also resulted in a lack of monolayer disruption. This data indicates that the gingipains and the PG0686 protein are both critical for inducing disruption of the HUVEC monolayers.

**Cell Death**

**Cell Death of HUVECs after Exposure to \textit{P. gingivalis}**

The previous experiments demonstrated that (1) exposure of HUVEC monolayers to certain \textit{P. gingivalis} strains caused disruption of the monolayers and cell detachment and (2) that the lysate and SPF of \textit{P. gingivalis} induced epithelial and endothelial cell detachment from monolayers. These data suggest that a soluble protein or proteins secreted from \textit{P. gingivalis} (e.g., gingipains) are responsible for this observed activity. Thus, we next wanted to examine the fate of the detached cells from the HUVEC monolayer. We sought to determine if \textit{P. gingivalis} was capable of inducing HUVE cell death and if so, was this death a result of \textit{P. gingivalis} components acting from outside or from within the cells.

To investigate this, HUVEC monolayers were co-cultured with different strains of \textit{P. gingivalis} at an MOI of 100 for 24 hours. Included among the \textit{P. gingivalis} strains tested were mutants CW501 (gingipain-null) and YPF1 (\textit{fimA-}). Both of these mutants showed reduced abilities to attach to the HUVECs and were unable to induce HUVEC monolayer disruption at an MOI of 100. In addition, their parent strains, 381 and ATCC 33277, respectively, were also used in these experiments. Cell death was assessed using propidium iodide, which stains the DNA of “leaky” or permeable cells, and quantified using flow cytometry.
The results through 2.5 hours revealed no significant differences in cell death of the HUVECs co-cultured with any of the *P. gingivalis* strains compared to the untreated cells (Fig. 3-50). However, by 12 hours, HUVECs treated with ATCC 33277 and 381 resulted in a 12% and 13% (p<0.05) increase in cell death, respectively. HUVECs exposed to CW501 and YPF1 resulted in approximately 5% cell death, a level approximating that of the untreated cells.

Exposure of HUVEC monolayers to strains ATCC 33277 and 381 for 24 hours resulted in an 18% and 21% increase in cell death, respectively (Fig. 3-50). The increase in cell death after 24 hours of HUVEC exposure to strains 381 and ATCC 33277 was significant (p<0.001) and (p<0.01) respectively, relative to the 13% cell death observed after exposure to strains CW501 and YPF1, and the 11% cell death of untreated cells (Fig. 3-50). The results with the gingipain negative mutant, CW501, suggest that the gingipains are involved in inducing HUVEC cell death. In addition, the results with CW501 and YPF1, which are known to have decreased adhesion and invasion abilities, likely due to their lack of the major attachment protein FimA, suggest that *P. gingivalis* adhesion and/or subsequent invasion of HUVEC cell monolayers are also necessary for *P. gingivalis* stimulated cell death.

Given that adherence of *P. gingivalis* to HUVECs has been shown to be necessary for *P. gingivalis* invasion, we next wanted to determine if invasion was a requirement for *P. gingivalis* induced cell death. Internalized *P. gingivalis* were examined for their ability to induce HUVEC cell death levels as reported with the previous assay. For these experiments, HUVEC monolayers were again treated with the gingipain-null mutant CW501, the major fimbriae (FimA) mutant YPF1, and their parent strains 381 and ATCC 33277, respectively, at an MOI of 100. After 1.5 hours of treatment, the bacterial cells were removed and the HUVECs were washed and subjected to antibiotics (refer to materials and methods) in order to kill any
remaining extracellular *P. gingivalis*. Cells were then incubated in the presence of the antibiotics for the remainder of the 2.5, 12, and 24-hour time points. Consistent with the results obtained previously, there were no statistical differences in cell death between the untreated HUVEC monolayers and any of the monolayers exposed to *P. gingivalis* for 2.5 hours (Fig. 3-51). However, at 12 hours of treatment with 381 and ATCC 33277, 24% and 23% cell death was observed, an increase of 8% (p<0.05) and 7%, respectively, compared to the untreated cells (Fig. 3-51). HUVEC monolayers treated with CW501 and YPF1 followed the same pattern of cell death (approximately 16%) as the untreated cells through 24 hours. In contrast, HUVECs exposed to 381 and ATCC 33277 for 24 hours resulted in approximately 36% cell death (p<0.001) relative to the 16% death observed with the untreated cells and the two mutant strains (Fig. 3-51). This 20% increase in cell death observed with strains 381 and ATCC 33277 is consistent with those levels reported in the previous assay. These results suggest that the cell death observed at 24 hours of co-culture is most likely stimulated by the internalized *P. gingivalis*.

After repeating this experiment with strain 381 and fresh HUVE cells, there was an insignificant difference between the levels of cell death observed after exposure to strain 381 with and without antibiotics (Fig. 3-54). These results suggest that the interaction of *P. gingivalis* 381 with the HUVECs before antibiotic protection was not sufficient to induce/cause cell death equal to that observed with pulsed *P. gingivalis* co-culture.

In addition to the above data, HUVECs were exposed to strain CW501 at an MOI of 1000 to investigate whether the increased concentration could affect cell death. Compared to the untreated cells, there was no significant difference in the numbers of dead cells upon co-culture with strain CW501 at an MOI of 1000 for 2.5 and 12 hours (Fig. 3-54). However, 24 hours of
exposure of HUVECs to CW501 at this concentration resulted in a 6% increase in cell death compared to the untreated cells (p<0.001) (Fig. 3-54). These results suggest that *P. gingivalis* may be equipped with mechanisms in addition to the gingipains for inducing HUVE cell death since the gingipain-null mutant, CW501, caused cell death albeit only after 24 hours of exposure at an MOI of 1000.

**Inhibition of *P. gingivalis* Internalization of HUVEC**

The next sets of experiments were designed to determine if the internalized *P. gingivalis* were responsible for inducing the observed HUVE cell death. To test this, *P. gingivalis* invasion of HUVECs was inhibited using a known inhibitor of *P. gingivalis* internalization, cytochalasin D. HUVECs were pretreated with 5 μg/ml cytochalasin D for 30 minutes prior to exposure to *P. gingivalis* 381 and ATCC 33277 at an MOI of 100. Since it is possible that HUVE cells may react adversely in response to the presence of antibiotics, this experiment was conducted with and without antibiotics. The results from these experiments showed there were no significant differences in the levels of cell death observed between the untreated cells and cytochalasin D treated cells exposed to strains 381 and ATCC 33277 through 24 hours (Fig. 3-52, 3-53). Thus, exposure to cytochalasin D completely inhibited the *P. gingivalis* induced cell death (Fig. 3-52, 3-53). HUVE cells treated with antibiotics produced the same results as those treated without antibiotics, suggesting that the presence of antibiotics did not cause any adverse affects on the cells. Unless cell death requires factors inhibited by cytochalasin D, these data establish that inhibition of *P. gingivalis* internalization suppressed its ability to induce cell death of HUVECs.

**P. gingivalis** Secreted Protein Fraction (SPF) and HUVE cell death

As described earlier, proteins secreted from *P. gingivalis* (SPFs) are capable of disrupting HUVEC monolayers. Thus the following experiments were designed to determine if the *P. gingivalis* SPF was also capable of stimulating HUVEC death. A 0.02 μg/ml concentration of
the 381 SPF was used for these experiments because this concentration was shown in previous experiments to cause disruption of the HUVEC monolayer proportional to that of an MOI of 100. Exposure of HUVE cells to 0.02 μg/ml of the 381 SPF at 2.5 and 12 hours resulted in cell death levels similar to the untreated cells. However, 24 hours of HUVECs exposure to 0.02 μg/ml of the 381 SPF resulted in a 3% increase in cell death compared to the untreated cells (p<0.05) (Fig. 3-54). Thus, 0.02 μg/ml of the 381 SPF was not able to induce the same level of cell death as observed with the live strain 381. These results suggest that the gingipains are involved in HUVE cell death but that invasion of *P. gingivalis* is a requirement for *P. gingivalis* to induce optimal levels of cell death. However, since gingipain inhibitors were not used to confirm this, it is possible that some other component of the SPF is responsible for this activity. Thus, these data suggest that *P. gingivalis* is inducing its activity from within the HUVE cell.

**HUVEC Exposure to *P. gingivalis* Results in Caspase 3 Activity**

The previous experiments were done using propidium iodide and flow cytometry to quantitate total HUVE cell death. In order to better characterize the cell death occurring within the HUVEC monolayers, caspase 3 activity, a marker for apoptosis, was quantitated. Staurosporine (STS), an apoptosis inducer that functions by inhibiting protein kinase C (PKC), at a concentration of 0.5 μm, was used as a positive control. Compared to the untreated cells, no significant differences were observed when HUVEC monolayers were exposed to *P. gingivalis* 381 or ATCC 33277 at an MOI of 100 with or without antibiotic treatment for 2.5 hours (Fig. 3-55). The STS-treated HUVEC control showed a 6.7-fold and 6.2-fold increase (p<0.001) in caspase 3 activity, respectively, compared to the untreated cells at 12 and 24 hours. After 12 and 24 hours, there were no significant increases in caspase 3 activity in HUVECs treated with 381 and ATCC 33277 in the presence of antibiotics. In contrast, after 12 hours, caspase 3 activity of HUVECs exposed to strains 381 and ATCC 33277 without antibiotics increased approximately
25% (p <0.05) compared to the untreated cells. After 24 hours, HUVECs exposed to strains 381 and ATCC 33277 without antibiotics resulted in a 2.7-fold (p<0.001) and 2.4-fold (p<0.01) increase in caspase 3 activity respectively, compared to the untreated cells. Therefore, it appears that the cell death observed in the flow cytometry experiments was coincident with caspase-related apoptosis.

Given the results presented here with the HUVE cells co-cultured with strains 381 and ATCC 33277 at an MOI of 100 with and without antibiotic exposure (to kill any remaining extracellular \textit{P. gingivalis}), it is possible that this endothelial cell undergoes more than one type of cell death. The results reported using propidium iodide and flow cytometry showed that there were significant increases in cell death compared to the untreated cells when HUVECs were exposed to strains 381 and ATCC 33277 with and without antibiotic treatment. However, only the HUVECs treated with strains 381 and ATCC 33277 without antibiotics caused a significant increase in caspase 3 activity compared to the untreated cells. Therefore, the \textit{P. gingivalis} internalized within the cells before antibiotic exposure may induce a caspase independent form of cell death while the constant exposure of \textit{P. gingivalis} to HUVECs appears to stimulate caspase 3 dependent apoptosis.

\textbf{Transepithelial Resistance}

Bacterial pathogens must first surpass the epithelial barrier, the hosts’ first line of defense, before they are able to penetrate deeper tissues and cause disease (Kazmierczak \textit{et al.}, 2001). The paracellular permeability or the transepithelial resistance (TER) of epithelial cell monolayers has been shown to be modified by several pathogens (Sears, 2000). Thus, the evaluation of the TER of endothelial cells after incubation with \textit{P. gingivalis} would provide additional information concerning the ability of this pathogen to alter the endothelial cell barrier.
These experiments were designed to determine whether the effects of adherent/invasive strains of *P. gingivalis*, 381 and W83, on TER were different than the effects caused by the non adhesive/invasive gingipain-null mutant, CW501. Initial experiments using HUVE cell monolayers resulted in inconsistent TER readings. To determine if the HUVECs were producing tight junctions, IMF experiments using HUVEC monolayers and an antibody against the tight junction protein occludin were conducted. These IMF experiments resulted in no apparent occludin staining, suggesting no tight junctions were present. After determining the endothelial HUVEC were not suitable cells on which to conduct TER experiments, an epithelial cell line, HuH7, which displayed occludin staining, was utilized as an alternative cell type (Fig 3-1).

HuH7 cells were allowed to seed on Corning transwell filters with a pore size of 0.3 μm. The HuH7 monolayers were then treated for various times with *P. gingivalis* strains 381, W83, and CW501 at an MOI of 100 from either the apical (Fig. 3-56) or basolateral side (Fig. 3-57). The TER of the cell free insert (baseline TER) was subtracted from each time point to determine the true level of the TER.

Apical exposure of HuH7 cell monolayers to all three *P. gingivalis* strains for 12 hours resulted in no significant differences in the TER of the HuH7 cells relative to the untreated cells. In contrast, at 24 hours, the TER increased after apical exposure of the HuH7 cell monolayers to each of the *P. gingivalis* strains. However, only the 43% increase in the TER of the HuH7 monolayers apically exposed to strain W83 was significant (p<0.05) relative to the untreated HuH7 cells (Fig. 3-56). At 48 hours, there was a slight decrease in the TER levels of the apically exposed HuH7 cell monolayers treated with strains W83 and CW501 compared to the untreated cells. This decrease in TER was followed by an additional decrease in TERs after 72 hours (Fig. 3-56). However, these observed decreases in the TERs with strains W83 and CW501 were not
statistically significant. Similarly, after 48 hours of apical exposure of HuH7 cell monolayers to strain 381, there was a moderate, but not significant, decrease in the TER relative to the untreated cells. After 72 hours, in contrast to the TER results with strains W83 and CW501, apical exposure of HuH7 cells to strain 381, resulted in a sharp 61% decrease in the TER. This decrease was highly significant (p<0.01) compared with the untreated HuH7 cells (Fig. 3-56).

Basolateral exposure of HuH7 cell monolayers to *P. gingivalis* W83, 381 and CW501 at an MOI of 100 resulted in no significant differences in TERs through 3 hours. Although the TERs of the basolateral exposed HuH7 monolayers with W83 were slightly lower than with strains 381 and CW501, they were not significant, compared to the untreated cells (Fig. 3-57). After 12 and 24 hours, basolateral exposed HuH7 cell monolayers to W83 and CW501 had a TER similar to the untreated cells. There was a slight, though non-significant increase in the TER of the monolayers exposed to *P. gingivalis* 381. After 48 and 72 hours, basolateral exposure of HuH7 cell monolayers to all three *P. gingivalis* strains resulted in decreased TERs (Fig. 3-57). However, only after 72 hours of basolateral exposure of HuH7 cells with strains 381 and W83, but not CW501, were the observed decreases in TER significant. Basolateral exposure of HuH7 cell monolayers to strains W83 and 381 resulted in 40% (p<0.01) and 52% (p<0.001) decreases in TER respectively, compared to the untreated cells (Fig. 3-57).

These data indicate that *P. gingivalis* disrupts the epithelial barrier function. Unexpectedly, the HuH7 basolateral surfaces appear to be more vulnerable to this deterioration than the apical surface. The effects on the TERs of basolateral exposure of HuH7 cell monolayers to *P. gingivalis* strains suggest that an intimate interaction between the bacterium and the HuH7 cells is not required for the observed effects, since the membrane is located between the basolateral side and the bacteria. Furthermore, these results suggest that the
contributions of bacterial soluble factors, perhaps gingipains, which are able to disseminate through the membrane pores to elicit these TER effects, are the active component. The lack of significant differences in the TERs of apical or basolateral exposed HuH7 cell monolayers to strain CW501 (gingipain-null) further suggests that the gingipains are responsible for the epithelial and endothelial cell monolayer disruption observed with IMF. The drop in TER after 48 and 72 hours of apical (Fig. 3-56) and basolateral (Fig. 3-57) treated HuH7 cell monolayers could be the result of HuH7 cell death caused by *P. gingivalis* exposure.
Table 3-1. Comparison of wild-type *P. gingivalis* strains and their effects on the HUVEC monolayer.

<table>
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<th>Wild-type strain</th>
<th>Adhesion</th>
<th>Invasion</th>
<th>Persistence</th>
<th>Cell-type Assayed</th>
<th>Monolayer disruption (HUVEC) MOI 100</th>
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<td>381</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>HUVEC GEC HCAEC KB</td>
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<td>No</td>
<td>No</td>
<td>HUVEC GEC HCAEC KB</td>
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</tbody>
</table>

Table 3-2. Comparison of *P. gingivalis* gingipain mutants and their effects on the HUVEC monolayer.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutated genes</th>
<th>Parent strain</th>
<th>Monolayer disruption (HUVEC) MOI 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT10</td>
<td><em>rgpA</em></td>
<td>381</td>
<td>Yes 15 hours</td>
</tr>
<tr>
<td>MT10W</td>
<td><em>rgpA, kgp</em></td>
<td>381</td>
<td>Yes 15 hours</td>
</tr>
<tr>
<td>G102</td>
<td><em>rgpB</em></td>
<td>381</td>
<td>Yes 20 hours</td>
</tr>
<tr>
<td>G102W</td>
<td><em>rgpB, kgp</em></td>
<td>381</td>
<td>Yes 15 hours</td>
</tr>
<tr>
<td>YPP2</td>
<td><em>kgp</em></td>
<td>ATCC 33277</td>
<td>Yes 20 hours</td>
</tr>
<tr>
<td>CW401</td>
<td><em>rgpA, rgpB</em></td>
<td>381</td>
<td>No</td>
</tr>
<tr>
<td>CW501</td>
<td><em>rgpA, rgpB, kgp</em></td>
<td>381</td>
<td>No</td>
</tr>
</tbody>
</table>
Table 3-3. Comparison of *P. gingivalis* adhesion mutants and their effects on the HUVEC monolayer.

<table>
<thead>
<tr>
<th>TR ID /mutant notation</th>
<th>Gene name</th>
<th>Parent strain</th>
<th>Adhesion</th>
<th>Cell-type assayed</th>
<th>Monolayer disruption (HUVEC) MOI 100</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG0242(^b)</td>
<td>Conserved hypothetical protein</td>
<td>W83</td>
<td>11.7-fold decrease</td>
<td>HCAEC</td>
<td>Yes 20 hours</td>
<td>81% similar to tetapyrrole methylases. Likely involved in protoporphyrin and protohaem metabolism.</td>
</tr>
<tr>
<td>PG1118(^c)</td>
<td>clpB</td>
<td>W83</td>
<td>2.1-fold decrease</td>
<td>HCAEC</td>
<td>No(^a)</td>
<td>Chaperone belonging to the AAA+ protein superfamily. Generally drives the assembly and disassembly of protein cplxs by ATP-dep remodeling of protein substrate. ClpB mutants of <em>L. monocytogenes</em> and <em>S. typhimurium</em> were found to have decreased virulence.</td>
</tr>
<tr>
<td>PG1683(^b)</td>
<td>Conserved hypothetical protein</td>
<td>W83</td>
<td>2.1-fold decrease</td>
<td>HCAEC</td>
<td>No(^a)</td>
<td>In an operon with PG1682. Shows 68% homology with (\alpha)-amylases. (\alpha)-amylases are involved in coaggregation of <em>P.g.</em> with other oral bacteria.</td>
</tr>
<tr>
<td>YPF1(^d)</td>
<td>Major fimbriae protein FimA</td>
<td>ATCC 33277</td>
<td>2-fold decrease</td>
<td>GEC</td>
<td>No(^a)</td>
<td>FimA is the major fimbrial protein of <em>P.g.</em>, primary function is attachment.</td>
</tr>
</tbody>
</table>

\(^a\) No observed monolayer disruption after 20 hours.
\(^b\) Paulo Rodrigues, unpublished data.
\(^c\) Lihui Yuon, unpublished data.
Table 3-4. Comparison of *P. gingivalis* invasion mutants and their effects on the HUVEC monolayer.

<table>
<thead>
<tr>
<th>TGIR ID/mutant notation</th>
<th>Gene name</th>
<th>Parent strain</th>
<th>Invasion (2.5h)</th>
<th>Cell-type assayed</th>
<th>Monolayer disruption (HUVEC) MOI 100</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG0717&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Putative lipoprotein</td>
<td>W83</td>
<td>2.7-fold decrease</td>
<td>HCAEC</td>
<td>No&lt;sup&gt;a&lt;/sup&gt;</td>
<td>No homology</td>
</tr>
<tr>
<td>PG1286&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Ferritin</td>
<td>W83</td>
<td>2.0-fold decrease</td>
<td>HCAEC</td>
<td>No&lt;sup&gt;a&lt;/sup&gt;</td>
<td>One of the intracellular iron storage proteins and may also contribute to the protection of organisms against oxidative stresses.</td>
</tr>
<tr>
<td>PG1118&lt;sup&gt;c&lt;/sup&gt;</td>
<td>clpB</td>
<td>W83</td>
<td>51.4-fold decrease</td>
<td>KB</td>
<td></td>
<td>Chaperone which belongs to the AAA+ protein superfamily. Generally drives the assembly and disassembly of protein complexes by ATP-dependent remodeling of protein substrate. ClpB mutants of <em>L. monocytogenes</em> and <em>S. typhimurium</em> were found to have decreased virulence.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>15-fold decrease</td>
<td>HCAEC</td>
<td>No&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>197-fold decrease</td>
<td>GEC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YPEP&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Endopeptidase PepO</td>
<td>ATCC 33277</td>
<td>25-fold decrease</td>
<td>GEC</td>
<td>No&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Homology to the endothelin converting enzyme ECE-1 which converts Big endothelin in the potent vasoconstrictor ET-1.</td>
</tr>
</tbody>
</table>

<sup>a</sup>No observed monolayer disruption after 20 hours.
<sup>b</sup>Paulo Rodrigues, unpublished data.
<sup>c</sup>Lihui Yuon, unpublished data.
<sup>d</sup>Park et al., 2004; Park and Lamont, 1998.
Table 3-5. *P. gingivalis* persistence mutant and its effects on the HUVEC monolayer.

<table>
<thead>
<tr>
<th>TIGR ID/mutant notation</th>
<th>Gene name</th>
<th>Parent strain</th>
<th>Persistence (24h)</th>
<th>Persistence (48h)</th>
<th>Cell-type assayed</th>
<th>Monolayer disruption HUVEC MOI 100</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG1286(^b) Ferritin W83</td>
<td>10-fold decrease</td>
<td>33.3-fold decrease</td>
<td>HCAEC</td>
<td>No(^a)</td>
<td>One of the intracellular iron storage proteins and may also contribute to the protection of organisms against oxidative stresses.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)No observed monolayer disruption after 20 hours.

\(^b\) Paulo Rodrigues, unpublished data.
Table 3-6. Comparison of other mutants of *P. gingivalis* and their effects on the HUVEC monolayer.

<table>
<thead>
<tr>
<th>Other Mutants</th>
<th>Gene Name</th>
<th>Parent strain</th>
<th>Invasion (2.5 h)</th>
<th>Persistence (48 h)</th>
<th>Cell-type assayed</th>
<th>Monolayer disruption (HUVEC) MOI 100</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG1788c</td>
<td>Putative cysteine peptidase</td>
<td>W83</td>
<td>2-fold increase</td>
<td>Not tested</td>
<td>HCAEC</td>
<td>Yes 15 hours</td>
<td>Proteases play important roles in nutrient acquisition, tissue invasion and modulation of host immune defense.</td>
</tr>
<tr>
<td>PG0293c</td>
<td>Putative secretion activator protein</td>
<td>W83</td>
<td>No difference</td>
<td>Not tested</td>
<td>HCAEC</td>
<td>Yes 10 hours</td>
<td>Homologous to <em>B. melitensis</em> secretion activator protein. Secreted proteins may play an important role in virulence.</td>
</tr>
<tr>
<td>PG0686b</td>
<td>Conserved hypothetical protein</td>
<td>W83</td>
<td>No difference</td>
<td>No difference</td>
<td>HCAEC</td>
<td>Noa</td>
<td>Located in an operon with two genes involved in the metabolism of succinyl-CoA. This gene could be important in acid neutralization of the autophagosomes.</td>
</tr>
</tbody>
</table>

*a* No observed monolayer disruption after 20 hours.

*b* Paulo Rodrigues, unpublished data.

*c* Sheila Walters, unpublished data.
Figure 3-1. Effects of *P. gingivalis* W83 lysate on the HuH7 junctional proteins. HuH7 cells incubated for 24 hours with anti-Pan-cadherin (A) anti-β-catenin (B), anti-occludin (C) untreated controls and 0.4 mg/ml *E. coli* lysate (negative control) (D). HuH7 cells incubated with anti-Pan-cadherin (red) or anti-β-catenin (red) after treatment with 0.2 mg/ml of the *P.g.* lysate for 24 hours (E & F, respectively). Arrows indicate the cellular junctions. Magnification: A-E 340x.
Figure 3-2. Effects of \textit{P. gingivalis} W83 lysates on HUVEC junctional proteins. (A) untreated control incubated with anti-\(\beta\)-catenin, (B) HUVEC cells treated with 0.05 mg/ml of the \textit{P.g.} W83 lysate and (C) 0.1 mg/ml of the \textit{P.g.}W83 lysate for 24 hours. Arrows indicate the cellular junctions (A). Magnification: A-C 440x.

Figure 3-3. Effects of temperature and TLCK treatment on the proteolytic activity of live \textit{P. gingivalis} and the \textit{P.g.} lysate. HuH\textsubscript{7} cell monolayers were treated with the \textit{P.g.} W83 lysate or live \textit{P.g.}, and incubated with an antibody against Pan-cadherin. (A) untreated HuH\textsubscript{7} control, (B) HuH\textsubscript{7} cells treated with 0.4 mg/ml \textit{P.g.} W83 lysate that had been heated for 20 minutes at 60\(^\circ\)C, (C) HuH\textsubscript{7} cells treated with W83 lysate incubated with 1 mM of the cysteine proteinase inhibitor TLCK for 24 hours. (D) HuH\textsubscript{7} cells incubated with anti-Pan-cadherin after treatment with live \textit{P. gingivalis} W83 (MOI of 1000) + 1 mM TLCK for 24 hours. Arrows indicating cell junctions (A, B) and the absence of cell junction staining (C). Magnification: A-D 340x.
Figure 3-4. Effects of temperature and TLCK treatment on the proteinase activity of live *P. gingivalis* and *P. g.* lysate on HuH7 cells. All cells were incubated with an antibody against β-catenin. (A) untreated HuH7 control, (B) HuH7 cells treated with 0.4 mg/ml *P.g.* W83 lysate that had been heated for 20 minutes at 60°C, (C) HuH7 cells treated with 0.4 mg/ml W83 lysate incubated with 1 mM of the cysteine proteinase inhibitor TLCK for 24 hours. (D) HuH7 cells incubated with anti-Pan-cadherin after treatment with live *P. gingivalis* W83 (MOI of 1000) + 1 mM TLCK for 24 hours. Arrows indicate cell junctions (A, B) and the absence of cell junction staining (C). Magnification: A-D 340x.
Figure 3-5. Effects of varying concentrations of SPF from *P. gingivalis* W83 and 381 on HUVE cell adhesion. HUVECs were treated with the secreted protein factor (SPFs) of *P. gingivalis* W83 and 381 for 24 hours and stained using antibodies against β-catenin (red). (A) untreated cells, (B) 0.2 μg/ml of the *P. gingivalis* W83 SPF, (C) 2 μg/ml of the *P.g.* W83 SPF, (D) 22 μg/ml of the *P.g.* W83 SPF, (E) 0.02 μg/ml of the *P.g.* 381 SPF, and (F) 1 μg/ml of the *P.g.* 381 SPF. Arrows indicate cell junctions (A, B, E) and a lack of cell junction staining (C, E). Magnification: A-F 440x.
Figure 3-6. Effects of varying concentrations of SPF from *P. gingivalis* W83 and 381 on HUVE cell adhesion. HUVEC cells were treated with the secreted protein factor (SPF) from strains W83 and 381 for 24 hours and stained using antibodies against Pan-cadherin (red). (A) untreated cells, (B) 0.2 μg/ml of the *P. gingivalis* W83 SPF, (C) 22 μg/ml of the *P.g.* W83 SPF, (D) 0.02 μg/ml of the *P.g.* 381 SPF, and (E) 1 μg/ml of the *P.g.* 381 SPF. Arrows indicate cell junctions (A) and a lack of cell junction staining (B, D). Magnification: A-F 440x.
Figure 3-7. Junctional complexes of untreated HUVEC monolayers remain intact through 20 hours. HUVEC monolayers were stained with antibodies against the junctional protein β-catenin (green) and DAPI (blue) (nuclear labeling) after (A) 5 hours, (B) 10 hours, (C) 15 hours, and (D) 20 hours. Arrows indicate the junctions between the cells. Magnification: A-C, 440x; D, 580x.
Figure 3-8. Junctional labeling of HUVEC monolayers after treatment with the *P. gingivalis* strain W83 at an MOI of 100. The untreated HUVEC monolayer as shown in (A) was labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P. g.* HagB (red), DAPI, a nuclear stain (blue) and viewed by IMF. HUVEC monolayers treated with *P. g.* for 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A-C, 440x; D-E, 580x.
Figure 3-9. Junctional labeling of HUVEC monolayers after treatment with the *P. gingivalis* strain 381 at an MOI of 100. The untreated HUVEC monolayer as shown in (A) was labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P. g.* HagB (red), DAPI, a nuclear stain (blue) and viewed by IMF. HUVEC monolayers treated with *P. g.* for 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A, 440x; B-C 480x; D, 580x; E, 440x.
Figure 3-10. Junctional labeling of HUVEC monolayers after treatment with the *P. gingivalis* strain ATCC 33277 at an MOI of 100. The untreated HUVEC monolayer as shown in (A) was labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P. g*. HagB (red), DAPI, a nuclear stain (blue) and viewed by IMF. HUVEC monolayers treated with *P. g*. for 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A, 500x; B, 340x; C, 440x, D, 340x; -E, 440x.
Figure 3-11. Junctional labeling of HUVEC monolayers after treatment with the *P. gingivalis* strain AJW4 at an MOI of 100. The untreated HUVEC monolayer as shown in (A) was labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P.g*. HagB (red), DAPI, a nuclear stain (blue) and viewed by IMF. HUVEC monolayers treated with *P.g*. for 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A, 440x; B-C, 480x; D-E, 500x.
Figure 3-12. Junctional labeling of HUVEC monolayers after treatment with the P. gingivalis wild-type strain W83 at an MOI of 1000. The untreated HUVEC monolayer as shown in (A) was labeled with antibodies against the junctional protein β-catenin (green), antibodies against P.g. HagB (red), DAPI, a nuclear stain (blue) and viewed by IMF. HUVEC monolayers treated with P.g. for 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). Yellow arrows indicate P. gingivalis either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A-B, 580x; C-E, 440x.
Figure 3-13. Junctional labeling of HUVEC monolayers after treatment with the *P. gingivalis* strain 381 at an MOI of 1000. The untreated HUVEC monolayer as shown in (A) was labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P.g.* HagB (red), DAPI, a nuclear stain (blue) and viewed by IMF. HUVEC monolayers treated with *P.g.* for 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A; 440x; B, 580x; C, 440x; D-E, 580x.
Figure 3-14. Junctional labeling of HUVEC monolayers after treatment with the *P. gingivalis* strain ATCC 33277 at an MOI of 1000. The untreated HUVEC monolayer as shown in (A) was labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P. g.* HagB (red), DAPI, a nuclear stain (blue) and viewed by IMF. HUVEC monolayers treated with *P. g.* for 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A, 580x; B-C, 440x; D, 580x; E, 340x.
Figure 3-15. Junctional labeling of HUVEC monolayers after treatment with the *P. gingivalis* strain AJW4 at an MOI of 1000. The untreated HUVEC monolayer as shown in (A) was labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P.g.* HagB (red), DAPI, a nuclear stain (blue) and viewed by IMF. HUVEC monolayers treated with *P.g.* for 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A, 500x; B-E, 440x.
Figure 3-16. Junctional labeling of HUVEC monolayers after treatment with *P. gingivalis* MT10 (*rgpA*) at an MOI of 100. The HUVEC monolayers were labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P. g*. HagB (red), and DAPI, a nuclear stain (blue) and observed by IMF after 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). An untreated HUVEC monolayer (A) and a HUVEC monolayer treated with the wild-type parent strain 381 at an MOI of 100 for 20 hours (positive control) (F) are shown for comparison. Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A, 580x; B, 500x; C, 440x; D-E, 500x; F, 580x.
Figure 3-17. Junctional labeling of HUVEC monolayers after treatment with *P. gingivalis* MT10W (*rgpA*-,*kgp*) at an MOI of 100. The HUVEC monolayers were labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P.g.* HagB (red), and DAPI, a nuclear stain (blue) and observed by IMF after 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). An untreated HUVEC monolayer (A) and a HUVEC monolayer treated with the wild-type parent strain 381 at an MOI of 100 for 20 hours (positive control) (F) are shown for comparison. Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A, 500x; B-C, 480x; D-F, 500x.
Figure 3-18. Junctional labeling of HUVEC monolayers after treatment with *P. gingivalis* G102 (*rgpB*) at an MOI of 100. The HUVEC monolayers were labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P. g.* HagB (red), and DAPI, a nuclear stain (blue) and observed by IMF after 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). An untreated HUVEC monolayer (A) and a HUVEC monolayer treated with the wild-type parent strain 381 at an MOI of 100 for 20 hours (positive control) (F) are shown for comparison. Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A-F, 580x.
Figure 3-19. Junctional labeling of HUVEC monolayers after treatment with *P. gingivalis* G102W (*rgpB*, *kgp*) at an MOI of 100. The HUVEC monolayers were labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P. g.* HagB (red), and DAPI, a nuclear stain (blue) and observed by IMF after 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). An untreated HUVEC monolayer (A) and a HUVEC monolayer treated with the wild-type parent strain 381 at an MOI of 100 for 20 hours (positive control) (F) are shown for comparison. Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A, 580x; B-D 440x; E, 500x; F, 440x.
Figure 3-20. Junctional labeling of HUVEC monolayers after treatment with *P. gingivalis* YPP2 (*kgp-*) at an MOI of 100. The HUVEC monolayers were labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P. g.* HagB (red), and DAPI, a nuclear stain (blue) and observed by IMF after 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). An untreated HUVEC monolayer (A) and a HUVEC monolayer treated with the wild-type parent strain ATCC 33277 at an MOI of 100 for 20 hours (positive control) (F) are shown for comparison. Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A-D, 580x; E-F, 480x.
Figure 3-21. Junctional labeling of HUVEC monolayers after treatment with *P. gingivalis* CW401 (rgpA-, rgpB-) at an MOI of 100. The HUVEC monolayers were labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P. g.* HagB (red), and DAPI, a nuclear stain (blue) and observed by IMF after 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). An untreated HUVEC monolayer (A) and a HUVEC monolayer treated with the wild-type parent strain 381 at an MOI of 100 for 20 hours (positive control) (F) are shown for comparison. Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A-B 440x; C, 480x; D, 580x; E-F, 480x.
Figure 3-22. Junctional labeling of HUVEC monolayers after treatment with *P. gingivalis* CW501 (rgpA-, rgpB-, kgp-) at an MOI of 100. The HUVEC monolayers were labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P.g.* HagB (red), and DAPI, a nuclear stain (blue) and observed by IMF after 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). An untreated HUVEC monolayer (A) and a HUVEC monolayer treated with the wild-type parent strain 381 at an MOI of 100 for 20 hours (positive control) (F) are shown for comparison. Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A, 720x; B, 500x; C, 440x; D, 580x; E-F, 480x.
Figure 3-23. Junctional labeling of HUVEC monolayers after treatment with *P. gingivalis* MT10 (*rgpA*-) at an MOI of 1000. The HUVEC monolayers were labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P.g.* HagB (red), and DAPI, a nuclear stain (blue) and observed by IMF after 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). An untreated HUVEC monolayer (A) and a HUVEC monolayer treated with the wild-type parent strain 381 at an MOI of 1000 for 20 hours (positive control) (F) are shown for comparison. Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A-D, 440x; E-F, 580x.
Figure 3-24. Junctional labeling of HUVEC monolayers after treatment with *P. gingivalis* MT10W (*rgpA-, kgp-*) at an MOI of 1000. The HUVEC monolayers were labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P.g.* HagB (red), and DAPI, a nuclear stain (blue) and observed by IMF after 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). An untreated HUVEC monolayer (A) and a HUVEC monolayer treated with the wild-type parent strain 381 at an MOI of 1000 for 20 hours (positive control) (F) are shown for comparison. Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A; 720x; B, 440x; C, 580x; D, 500x, E-F 580x.
Figure 3-25. Junctional labeling of HUVEC monolayers after treatment with *P. gingivalis* G-102 (*rgpB*- ) at an MOI of 1000. The HUVEC monolayers were labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P. g.* HagB (red), and DAPI, a nuclear stain (blue) and observed by IMF after 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). An untreated HUVEC monolayer (A) and a HUVEC monolayer treated with the wild-type parent strain 381 at an MOI of 1000 for 20 hours (positive control) (F) are shown for comparison. Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A; 720x, B-C, 440x; D-F, 500x.
Figure 3-26. Junctional labeling of HUVEC monolayers after treatment with *P. gingivalis* G102W (*rgpB-, kgp-*) at an MOI of 1000. The HUVEC monolayers were labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P.g.* HagB (red), and DAPI, a nuclear stain (blue) and observed by IMF after 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). An untreated HUVEC monolayer (A) and a HUVEC monolayer treated with the wild-type parent strain 381 at an MOI of 1000 for 20 hours (positive control) (F) are shown for comparison. Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A, 720x; B, 440x; C, 580x; D-E, 440x; F, 580x.
Figure 3-27. Junctional labeling of HUVEC monolayers after treatment with *P. gingivalis* YPP2 (*kgp*) at an MOI of 1000. The HUVEC monolayers were labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P. g.* HagB (red), and DAPI, a nuclear stain (blue) and observed by IMF after 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). An untreated HUVEC monolayer (A) and a HUVEC monolayer treated with the wild-type parent strain ATCC 33277 at an MOI of 1000 for 20 hours (positive control) (F) are shown for comparison. Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A-C, 440x; D, 580x, E, 500x; F, 440x.
Figure 3-28. Junctional labeling of HUVEC monolayers after treatment with *P. gingivalis* CW401 (*rgpA*-*,rgpB*-*) at an MOI of 1000. The HUVEC monolayers were labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P. g*. HagB (red), and DAPI, a nuclear stain (blue) and observed by IMF after 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). An untreated HUVEC monolayer (A) and a HUVEC monolayer treated with the wild-type parent strain 381 at an MOI of 1000 for 20 hours (positive control) (F) are shown for comparison. Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A-C, 440x; D-E, 500x; F, 580x.
Figure 3-29. Junctional labeling of HUVEC monolayers after treatment with *P. gingivalis* CW501 (*rgpA*-*, rgpB*-*, kgp*-*) at an MOI of 1000. The HUVEC monolayers were labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P. g.* HagB (red), and DAPI, a nuclear stain (blue) and observed by IMF after 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). An untreated HUVEC monolayer (A) and a HUVEC monolayer treated with the wild-type parent strain 381 at an MOI of 1000 for 20 hours (positive control) (F) are shown for comparison. Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A, 580x; B-D, 440x; E-F, 580x.
Figure 3-30. Junctional labeling of HUVEC monolayers after treatment with a *P. gingivalis* adhesion mutant PG1683 (conserved hypothetical protein) at an MOI of 100. The HUVEC monolayers were labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P. g.* HagB (red), and DAPI, a nuclear stain (blue) and observed by IMF after 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). An untreated HUVEC monolayer (A) and a HUVEC monolayer treated with the wild-type parent strain W83 at an MOI of 100 for 20 hours (positive control) (F) are shown for comparison. Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A-C, 500x; D, 580x; E, 480x; F, 720x.
Figure 3-31. Junctional labeling of HUVEC monolayers after treatment with a *P. gingivalis* adhesion mutant PG0242 (conserved hypothetical protein) at an MOI of 100. The HUVEC monolayers were labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P.g*. HagB (red), and DAPI, a nuclear stain (blue) and observed by IMF after 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). An untreated HUVEC monolayer (A) and a HUVEC monolayer treated with the wild-type parent strain W83 at an MOI of 100 for 20 hours (positive control) (F) are shown for comparison. Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A, 720x; B-C, 480x; D, 500x; E-F, 440x.
Figure 3-32. Junctional labeling of HUVEC monolayers after treatment with a *P. gingivalis* adhesion and invasion mutant PG1118 (*clpB*) at an MOI of 100. The HUVEC monolayers were labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P. g.* HagB (red), and DAPI, a nuclear stain (blue) and observed by IMF after 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). An untreated HUVEC monolayer (A) and a HUVEC monolayer treated with the wild-type parent strain W83 at an MOI of 100 for 20 hours (positive control) (F) are shown for comparison. Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A, 720x; B, 500x; C, 440x; D, 500x; E, 480x; F, 720x.
Figure 3-33. Junctional labeling of HUVEC monolayers after treatment with a *P. gingivalis* adhesion mutant YPF1 (*fimA*- ) at an MOI of 100. The HUVEC monolayers were labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P. g*. HagB (red), and DAPI, a nuclear stain (blue) and observed by IMF after 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). An untreated HUVEC monolayer (A) and a HUVEC monolayer treated with the wild-type parent strain ATCC 33277 at an MOI of 100 for 20 hours (positive control) (F) are shown for comparison. Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A, 440x; B-C, 500x; D, 440x; E-F, 580x.
Figure 3-34. Junctional labeling of HUVEC monolayers after treatment with a *P. gingivalis* invasion mutant PG0717 (putative lipoprotein) at an MOI of 100. The HUVEC monolayers were labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P. g*. HagB (red), and DAPI, a nuclear stain (blue) and observed by IMF after 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). An untreated HUVEC monolayer (A) and a HUVEC monolayer treated with the wild-type parent strain W83 at an MOI of 100 for 20 hours (positive control) (F) are shown for comparison. Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A, 580x; B, 440x; C-E, 500x; F, 440x.
Figure 3-35. Junctional labeling of HUVEC monolayers after treatment with a *P. gingivalis* invasion mutant PG1286 (ferritin) at an MOI of 100. The HUVEC monolayers were labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P.g.* HagB (red), and DAPI, a nuclear stain (blue) and observed by IMF after 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). An untreated HUVEC monolayer (A) and a HUVEC monolayer treated with the wild-type parent strain W83 at an MOI of 100 for 20 hours (positive control) (F) are shown for comparison. Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A, 580x; B, 440x; C-E, 500x; F, 580x.
Figure 3-36. Junctional labeling of HUVEC monolayers after treatment with a *P. gingivalis* invasion mutant YPEP (*pepO*) at an MOI of 100. The HUVEC monolayers were labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P. g*. HagB (red), and DAPI, a nuclear stain (blue) and observed by IMF after 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). An untreated HUVEC monolayer (A) and a HUVEC monolayer treated with the wild-type parent strain ATCC 33277 at an MOI of 100 for 20 hours (positive control) (F) are shown for comparison. Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A, 500x; B, 440x; C, 580x; D, 500x; E, 440x; F, 580x.
Figure 3-37. Junctional labeling of HUVEC monolayers after treatment with a *P. gingivalis* putative cysteine peptidase mutant PG1788 at an MOI of 100. The HUVEC monolayers were labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P. g.* HagB (red), and DAPI, a nuclear stain (blue) and observed by IMF after 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). An untreated HUVEC monolayer (A) and a HUVEC monolayer treated with the wild-type parent strain W83 at an MOI of 100 for 20 hours (positive control) (F) are shown for comparison. Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A, 720x; B-D, 480x; E-F, 500x.
Figure 3-38. Junctional labeling of HUVEC monolayers after treatment with a *P. gingivalis* putative secretion activator protein mutant PG0293 at an MOI of 100. The HUVEC monolayers were labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P.g.* HagB (red), and DAPI, a nuclear stain (blue) and observed by IMF after 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). An untreated HUVEC monolayer (A) and a HUVEC monolayer treated with the wild-type parent strain W83 at an MOI of 100 for 20 hours (positive control) (F) are shown for comparison. Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A, 720x; B, 440x; C-E, 500x; F, 580x.
Figure 3-39. Junctional labeling of HUVEC monolayers after treatment with a *P. gingivalis* conserved hypothetical protein mutant PG0686 at an MOI of 100. The HUVEC monolayers were labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P. g*. HagB (red), and DAPI, a nuclear stain (blue) and observed by IMF after 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). An untreated HUVEC monolayer (A) and a HUVEC monolayer treated with the wild-type parent strain W83 at an MOI of 100 for 20 hours (positive control) (F) are shown for comparison. Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A, 580x; B-C, 440x; D, 580x; E, 440x; F, 500x.
Figure 3-40. Junctional labeling of HUVEC monolayers after treatment with a *P. gingivalis* adhesion mutant YPF1 (*fimA*) at an MOI of 1000. The HUVEC monolayers were labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P. g*. HagB (red), and DAPI, a nuclear stain (blue) and observed by IMF after 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). An untreated HUVEC monolayer (A) and a HUVEC monolayer treated with the wild-type parent strain ATCC 33277 at an MOI of 1000 for 20 hours (positive control) (F) are shown for comparison. Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A-B, 580x; C-D, 500x; E, 580x; F, 440x.
Figure 3-41. Junctional labeling of HUVEC monolayers after treatment with a *P. gingivalis* invasion mutant YPEP (*pepO*) at an MOI of 1000. The HUVEC monolayers were labeled with antibodies against the junctional protein β-catenin (green), antibodies against *P.g. HagB* (red), and DAPI, a nuclear stain (blue) and observed by IMF after 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). An untreated HUVEC monolayer (A) and a HUVEC monolayer treated with the wild-type parent strain ATCC 33277 at an MOI of 1000 for 20 hours (positive control) (F) are shown for comparison. Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A-E, 440x.
Figure 3-42. Junctional labeling of HUVEC monolayers after treatment with a \textit{P. gingivalis} conserved hypothetical protein mutant PG0686 at an MOI of 1000. The HUVEC monolayers were labeled with antibodies against the junctional protein $\beta$-catenin (green), antibodies against \textit{P.g}. HagB (red), and DAPI, a nuclear stain (blue) and observed by IMF after 5 hours (B), 10 hours (C), 15 hours (D), and 20 hours (E). An untreated HUVEC monolayer (A) and a HUVEC monolayer treated with the wild-type parent strain W83 at an MOI of 1000 for 20 hours (positive control) (F) are shown for comparison. Yellow arrows indicate \textit{P. gingivalis} either attached to or localized within the HUVECs. White arrows indicate the junctions between the cells. Magnification: A, 500x; B-F 440x.
Figure 3-43. Comparisons of the HUVEC monolayers after treatment with the gingipain mutants and their parent strains at an MOI of 100 for 20 hours. HUVECs were labeled with anti-β-catenin (green), anti *P. gingivalis* HgB (red), and DAPI, a nuclear stain (blue) and observed by IMF. (A) Untreated control, (B) 381, (C) MT10 (*rgp*A-), (D) MT10W (*rgp*A-, *kgp*), (E) G102 (*rgp*B-), (F) G102W (*rgp*B-, *kgp*) (G) YPP2 (*kgp*) ATCC 33277 mutant, (H) CW401 (*rgp*A-, *rgp*B-), (I) CW501 (*rgp*A-, *rgp*B-, *kgp*), and (J) ATCC 33277. Yellow arrows indicate *P. gingivalis* either attached to or localized within the HUVECs and white arrows indicate the junctions between the cells. Magnification: A-B, 440x; C-F, 500x, G-I, 480x; J, 580x.
Figure 3-44. Comparisons of the HUVEC monolayers after treatment with *P. gingivalis* adhesion mutants and their parent strains at an MOI of 100 for 20 hours. The HUVEC monolayers were labeled with anti-β-catenin (green), anti-*P. gingivalis* HagB (red), and DAPI, a nuclear stain (blue) and observed by IMF. (A) Untreated control, (B) YPF1 (*fimA*-), (C) PG1683 (conserved hypothetical protein), (D) PG1118 (*clpB*-), (E) PG0242 (conserved hypothetical protein), (F) W83 and (G) ATCC 33277. Magnification: A, 440x; B, 500x; C-D, 480x; E-F, 440x; G, 580x.
Figure 3-45. Comparisons of the HUVEC monolayers after treatment with *P. gingivalis* invasion mutants and their parent strains at an MOI of 100 for 20 hours. The HUVEC monolayers were labeled with anti-β-catenin (green), anti-*P. gingivalis* HagB (red), and DAPI, a nuclear stain (blue) and observed by IMF. (A) Untreated control, (B) PG1118 (*clpB*), (C) PG0717 (putative lipoprotein), (D) PG1286 (ferritin) (E) YPEP (*pepO*), (F) W83 and (G) ATCC 33277. Magnification: A, 440x; B, 480x; C-D, 500x; E-F, 440x; G, 580x.
Figure 3-46. Comparisons of the HUVEC monolayers after treatment with additional *P. gingivalis* mutants and their parent strain at an MOI of 100 for 20 hours. The HUVEC monolayers were labeled with anti-β-catenin (green), anti-*P. gingivalis* HagB (red), and DAPI, a nuclear stain (blue) and observed by IMF. (A) Untreated control, (B) PG0293 (putative secretion activator protein), (C) PG1788 (putative cysteine peptidase), (D) PG0686 (conserved hypothetical protein) and (E) W83. Magnification: A, 440x; B-C, 500x; D-E, 440x.
Figure 3-47. Transmission electron microscopy of HUVEC monolayers internalized with *P. gingivalis* 381 at an MOI of 100 and stained for acid phosphatase. (A) Untreated HUVEC monolayer. (B) HUVEC monolayer after treatment with *P. gingivalis* strain 381 for 5 hours and (C) 20 hours. Black arrows indicate empty vacuoles, white arrows designate *P. gingivalis* and yellow arrows indicate internalized *P. gingivalis* within a cytoplasmic vacuole.
Figure 3-48. Transmission electron microscopy of HUVEC monolayers internalized with *P. gingivalis* CW501 (rgpA-, rgpB-, kgp-) at an MOI of 100 and stained for acid phosphatase. (A) Untreated HUVEC monolayer. (B) HUVEC monolayer after treatment with *P. gingivalis* CW501 for 5 hours, and (C) 20 hours. Black arrows indicate empty vacuoles and white arrow designates *P. gingivalis*.
Figure 3-49. Transmission electron microscopy of HUVEC monolayers internalized with *P. gingivalis* wild-type 381, CW501 (*rgpA-, rgpB-, kgp-*), and a conserved hypothetical protein mutant PG0686 at an MOI of 1000 and stained with uranyl acetate (A) Untreated HUVEC monolayer. (B) HUVEC monolayer after treatment with *P. gingivalis* 381 for 20 hours, (C) incubated with the gingipain null mutant CW501 for 20 hours (C) and PG0686 for 20 hours. Black arrows indicate empty vacuoles, white arrows designate *P. gingivalis*, yellow arrows indicate internalized *P. gingivalis* contained within a cytoplasmic vacuole.
Figure 3-50. Effects of *P. gingivalis* on HUVE cell death. HUVEC monolayers were treated for 30 min, 2.5, 12, and 24 hours with *P. gingivalis* strains 381, ATCC 33277, CW501 and YPF1 (fimA-) at an MOI of 100. HUVECs were stained with propidium iodide and quantified for cell death using flow cytometry. P-values are represented as follows: (*p<0.05), (**p<0.01), and (***)p<0.001).
Figure 3-51. *P. gingivalis* interactions with HUVEC monolayers induces HUVE cell death after antibiotic treatment. HUVEC monolayers were treated for 2.5, 12, and 24 hours with *P. gingivalis* 381, ATCC 33277, CW501 and YPF1 (*fimA*-), at an MOI of 100 with the addition of the antibiotics metronidazole 200 μg ml⁻¹ and gentamicin 300 μg ml⁻¹ after 1.5 hours of co-culture. HUVECs were stained with propidium iodide and quantified for cell death using flow cytometry. P-values are represented as follows: (*)p<0.05) and (***)p<0.001).
Figure 3-52. Inhibition of *P. gingivalis* internalization of HUVECs with cytochalasin D inhibits HUVE cell death in the presence of antibiotics. HUVEC monolayers were pretreated with 5 μg/ml cytochalasin D for 0.5 hours prior to exposure to *P. gingivalis*. HUVEC monolayers were treated with *P. gingivalis* 381, or ATCC 33277 at an MOI of 100 for 1.5 hours at which time the unattached bacteria were washed off and the cells were then incubated for the remainder of the 2.5, 12, and 24 hour time points with fresh EGM-2 media plus the antibiotics metronidazole 200 μg ml⁻¹ and gentamicin 300 μg ml⁻¹. HUVEC were stained with propidium iodide and quantified for cell death using flow cytometry. There are no statistical differences between any of the time-points tested.
Figure 3-53. Inhibition of *P. gingivalis* internalization of HUVEC with cytochalasin D inhibits HUVE cell death. HUVEC monolayers were pretreated with 5 μg/ml cytochalasin D for 0.5 hours prior to exposure to *P. gingivalis*. HUVEC monolayers were then co-cultured with *P. gingivalis* 381 or ATCC 33277 at an MOI of 100 for 1.5 hours at which time the unattached bacteria were washed off and the cells were then incubated for the remainder of the 2.5, 12, and 24 hour time points with fresh EGM-2 media. HUVEC were stained with propidium iodide and quantified for cell death using flow cytometry. There are no statistical differences between any of the time-points tested.
Figure 3-54. Cell death of HUVE cells after treatment with live *P. gingivalis* 381 with and without cytochalasin D, 0.02 μg/ml of the 381 SPF and the CW501 (gingipain-null) mutant. HUVEC monolayers were incubated for 2.5, 12, and 24 hours with 381 at an MOI of 100 with and without antibiotics (metronidazole 200 μg ml⁻¹ and gentamicin 300 μg ml⁻¹) and *P. gingivalis* CW501 at an MOI of 1000. HUVEC monolayers were also exposed to 0.02 μg/ml of the 381 SPF. In addition, HUVEC monolayers were pretreated with 5 μg/ml cytochalasin D for 0.5 hours prior to exposure to *P. gingivalis* 381 at an MOI of 100 and 0.02 μg/ml of the 381 SPF for 1.5 hours. Cells were then washed and incubated for the remainder of the 2.5, 12, and 24 hour time points with fresh EGM-2. HUVECs were stained with propidium iodide and quantified for cell death using flow cytometry. P-values are represented as follows: (*p<0.05) and (**p<0.001).
Figure 3-55. HUVEC monolayers treated with *P. gingivalis* 381 and ATCC 33277 exhibit caspase 3 activity. HUVEC monolayers were treated for 2.5, 12, and 24 hours with *P. gingivalis* 381 and ATCC 33277 at an MOI of 100 with and without the antibiotics (ABs) (metronidazole 200 μg ml\(^{-1}\) and gentamicin 300 μg ml\(^{-1}\)). An apoptosis inducer, Staurosporine (STS), at a concentration of 0.5 μm was used as a positive control. P-values are represented as follows: (*p<0.05), (**)p<0.01), and (***)p<0.001).
Figure 3-56. Membrane resistance of HuH7 monolayers after apical exposure to *P. gingivalis* strains W83, 381, and the gingipain-null mutant, CW501, at an MOI of 100. HuH7 monolayers were seeded on collagen coated Corning transwell-COL inserts. Transepithelial Resistance (TER) was measured using an EndOhm chamber and the EVOM electrical resistance system. Cell-free transwell inserts were used to obtain baseline levels which were then subtracted from each time point measured. P-values are represented as follows: (*p<0.05) and (**p<0.01).
Figure 3-57. Membrane resistance of HuH7 monolayers after basolateral exposure to *P. gingivalis* strains W83, 381, and the gingipain-null mutant, CW501, at an MOI of 100. HuH7 monolayers were seeded on collagen coated Corning transwell-COL inserts. Transepithelial Resistance (TER) was measured using an EndOhm chamber and the EVOM electrical resistance system. Cell-free transwell inserts were used to obtain baseline levels which were then subtracted from each time point measured. P-values are represented as follows: (**p<0.01) and (***)p<0.001).
CHAPTER 4
DISCUSSION

*P. gingivalis* has the ability to invade, persist and replicate in many endothelial and epithelial cell lines including primary cultures of human endothelial cells (Dorn *et al.*, 1999; Lamont *et al.*, 1995). Endothelial cells infected with *P. gingivalis* display a number of phenotypic traits including elevated levels of coagulation and secretion of inflammatory cytokines (Ross, 1993). Vascular modifications and gingival ulcerations stimulated by the local inflammation caused by *P. gingivalis* may promote the occurrence and severity of septic bacterial infections after injury to junctional barriers of the gingival tissue. The persistent challenge with *P. gingivalis* could obstruct the proliferation and adjustment of cell-matrix adhesion and impede the remodeling of the cell-matrix (Yilmaz *et al.*, 2003). These interactions likely play a key role in the development of periodontal disease by *P. gingivalis* by providing the cells with signals for regulating cell function, cell migration and adherence (Nakamura *et al.*, 1999). The integrity of the oral mucosal basement membrane, specifically the gingival sulcus, is crucial for the prevention of the introduction of oral bacteria into the bloodstream (Daly *et al.*, 1997). In addition, similar interactions may also be of significance in the endothelial cell layer of cardiovascular tissues.

**Epithelial Barrier Function**

The junctional complexes of the cell-cell and cell-matrix associations initiate the development of the strong barrier function of the polarized epithelium. Epithelial cells are held together by both tight and adherens junctions. Tight junctions function in the paracellular pathway by creating a major barrier to macromolecule, fluid, electrolyte and pathogen diffusion (Gumbiner, 1993; Gumbiner and McCrea, 1993). Tight junctions are located on the apical portion of the cell and form a periphery between the surfaces of the apical and basolateral
membranes. They are essential for the construction and preservation of cell surface polarity because they create a barrier against the diffusion of proteins and/or lipids in the plasma membrane (van Meer et al., 1986). Tight junctions maintain this barrier function by communicating with the structured cytoskeletal actin filaments (Fanning et al., 1999). The epithelial structure is also dependent on the integrin mediated cell-matrix adhesion, the ability of the epithelial cell layer to attach to the basement membrane (Balkovetz and Katz, 2003). Bacterial pathogens must first surpass the epithelial barrier, the hosts’ first line of defense, before they are able to penetrate deeper tissues and cause disease (Kazmierczak et al., 2001).

The paracellular permeability or the transepithelial resistance (TER) of epithelial cell monolayers has been shown to be modified by several pathogens (Sears, 2000). A number of bacteria can infiltrate the host tissues and/or secrete toxins into the host via the paracellular route by surpassing the cell-cell junctions, leaving the subepithelial connective tissue susceptible to bacterial invasion (Balkovetz and Katz, 2003).

The evaluation of the TERs of HuH7 cell monolayers after incubation with P. gingivalis provided information concerning the ability of this pathogen to alter the epithelial cell barrier. The HuH7 basolateral surfaces appeared to be more vulnerable to this result than the apical surface. The effects on the TERs of basolateral exposure of HuH7 cell monolayers to P. gingivalis strains suggest that an intimate interaction between the bacterium and the HuH7 cells is not required for the observed effects, since the membrane is located between the basolateral side and the bacteria. Furthermore, these results suggest that the contributions of bacterial soluble factors, perhaps gingipains, which are able to disseminate through the membrane pores to elicit these TER effect, are the active component. The lack of significant differences in the TERs of apical or basolateral exposed HuH7 cell monolayers to strain CW501 (gingipain-null) suggests
that the gingipains are intimately involved in this activity. The drop in TER after 48 and 72 hours of apical and basolateral treated HuH7 cell monolayers could be the result of disruption of the HuH7 cell junctions or cell death caused by *P. gingivalis* exposure.

**Monolayer Integrity**

*P. gingivalis* W83 and 381 SPF and W83 Lysate Adversely Affect Endothelial and Epithelial Adherens Proteins

An array of events such as differentiation and survival are controlled by the interactions of cells with adjacent cells and with the ECM (Bazzoni and Dejana, 2004; Juliano, 2002). Intercellular adherens junctions hold these adjoining epithelial and endothelial cells together. Adherens junctions are primarily composed of cadherins and catenins, which together provide stability by anchoring the cells to the actin cytoskeleton (Bazzoni and Dejana, 2004). These cell-cell adherens junctions are also essential for the proper organization of vessels during angiogenesis and are involved in the preservation of specific cell properties including contact inhibition of cell growth and the permeability to inflammatory cells or other solutes (Bazzoni and Dejana, 2004; Juliano, 2002). In addition to cell-cell adhesion, the adherence of cells to the ECM is crucial for proper organization, development, and function of cells (Wu *et al.*, 2001).

Substances responsible for increasing vascular endothelial permeability disturb the protective function of the intracellular junctions (Carmeliet *et al.*, 1999; Dejana *et al.*, 1999). In order to gain access to the underlying connective tissues, bacterial pathogens must first infringe upon these intracellular adherens junctions (Wu *et al.*, 2001). There are multiple mechanisms by which bacterial infections can occur including direct trauma and epithelial and endothelial cell damage (Wu *et al.*, 2001). Thus, the loss of cell adhesion and subsequent infection of the underlying tissues could be a consequence of the loss of junctional protein molecules from
within the intracellular junctions of epithelial and endothelial cells (Katz et al., 2002; Gottardi et al., 2001).

The results using the lysate of *P. gingivalis* W83 on both the HuH7 epithelial and HUVEC endothelial cell lines suggest that proteins from *P. gingivalis* are capable of disrupting the integrity of the cell monolayers. The HuH7 and HUVEC untreated control cells demonstrated that the majority of the β-catenin and cadherin proteins were concentrated at the cell surfaces. β-catenin could also be seen sparsely punctate throughout the cytoplasm. In contrast, the HuH7 and HUVE cell monolayers exposed to the *P. gingivalis* W83 lysate displayed monolayer disruption and resulted in a loss of cadherin from the cell surface concomitant with an alteration in the localization of β-catenin from the cell surface to cytoplasmic structures in the proximity of the nucleus. In addition, several cells had detached from the monolayer compared to the untreated control. Thus, the loss of β-catenin and cadherin from the cell surface appeared to considerably reduce these cells’ ability to maintain their intercellular contacts, leading to cell detachment.

Treatment of the HuH7 cell monolayers with heat-killed W83 lysate completely inhibited the active component(s) within this lysate from disrupting the HuH7 monolayer. Since lipopolysaccharide, LPS, is particularly heat stable, it is likely LPS does not participate in disrupting the cell monolayer (Kuramitsu et al., 2003). This suggests that other protein component(s) from *P. gingivalis* are responsible for this activity.

Exposure of HuH7 cell monolayers to either live *P. gingivalis* W83 or the W83 lysate in the presence of the cysteine proteinase inhibitor, Na-α-tosyl-L-lysine chloromethyl ketone (TLCK), resulted in a reduction of monolayer disruption and few detached cells. Treatment with TLCK inhibited the *P. gingivalis* cysteine proteinases from causing detachment of the HuH7 cells but was unable to fully inhibit the alteration of the location of cadherin or β-catenin from the cell
surface to within the HuH7 cytoplasm. Other proteinase inhibitors such as PMSF and PIC had no observed inhibitory effect on the W83 lysate. Given that the PIC cocktail contains a cysteine proteinase inhibitor, the inability of PIC to inhibit the activity of the W83 lysate could be because the concentration used was not adequate to inhibit the cysteine proteinase activity. Since TLCK has been shown to inhibit Kgp activity more significantly than Rgp activity (Pike et al., 1994), it is possible that Kgp is more actively involved in affecting cell-matrix adhesions, whereas the activity of Rgp is largely accountable for the observed alteration of location of cadherin and β-catenin within the epithelial and endothelial monolayers examined. The data establish that the P. gingivalis cysteine proteinases, gingipains, are the active components effecting both cell-cell and cell-matrix adhesion in both epithelial and endothelial cells.

The adhesive properties of endothelial cells are interrupted upon treatment with secreted protein fractions (SPFs). Confluent HUVEC monolayers treated with W83 and 381 SPFs resulted in a loss of cadherin and β-catenin at the cell surface. The disruption in the HUVEC monolayer was more pronounced after treatment with the 381 SPF than with the W83 SPF. One explanation for this difference is that the level of P. gingivalis 381 Rgp enzymatic activity was 2-fold higher than that of strain W83. HUVECs exposed to the SPFs of both strains yielded similar results concerning the location of β-catenin and cadherin distribution as those observed with the W83 lysate. However, a much lower concentration of the SPF was needed to yield results equal to those observed with the W83 lysate, suggesting that the majority of the damaging effects associated with P. gingivalis are surface associated and/or secreted.

The results of this study establish that the proteolytically active extracellular protein fractions from P. gingivalis W83 and 381 were capable of stimulating epithelial and endothelial cell rounding as well as the detachment of cells from the culture dish and from each other. Thus,
*P. gingivalis* appears to exhibit copious amount of proteolytic activity that affects the location of the cadherin and β-catenin proteins of both epithelial and endothelial junctions. Furthermore, the data suggest that the active component(s) responsible for cell-cell and cell-matrix adhesions are cysteine proteinases, gingipains.

The collection of data using HUVEC and HuH7 cells suggests that periodontal tissue destruction characteristic of chronic periodontitis could therefore be a consequence of the *P. gingivalis* surface associated and/or secreted proteins, e.g., gingipains. Data from other laboratories support this conclusion. For example, oral keratinocytes exposed to *P. gingivalis* displayed proteolysis of adherens junction proteins (e.g., catenins) and this result is likely due to the gingipains since TLCK was capable of inhibiting this activity (Hintermann *et al.*, 2002).

Katz *et al.* provided further evidence to support the involvement of the gingipains in the proteolysis of the proteins of the adherens junctions. This group also showed that incubation of *P. gingivalis* with MDCK cell monolayers and immunoprecipitated E-cadherin caused the degradation of E-cadherin and preincubation with the Rgp and Kgp specific inhibitors leupeptin and acetyl-Leu-Val-Lys-aldehyde respectively, abrogated this activity (Katz *et al.*, 2002). The loss of cell adhesion and subsequent infection of the underlying tissues could be a consequence of the loss of E-cadherin molecules from epithelial cells (Katz *et al.*, 2002; Gottardi *et al.*, 2001).

In addition, Sheets *et al.* reported that the gingipain active extracts were responsible for the cleavage of N and VE-cadherin and integrin β1 and the loss of cell adhesion of bovine coronary artery endothelial cells (Sheets *et al.*, 2005). In agreement with Hintermann *et al.*, this activity could be inhibited or considerably delayed by pre-incubation of these cells with TLCK, further suggesting the direct involvement of the gingipains (Sheets *et al.*, 2005; Hintermann *et al.*, 2002).
In addition to cell surface and/or secreted proteins of *P. gingivalis*, live *P. gingivalis* could also induce disruption of the epithelial and endothelial monolayers. Four different wild type strains of *P. gingivalis* including, W83, 381, ATCC 33277 and AJW4 were tested for their abilities to disrupt the junctional protein, β-catenin, and thus, the endothelial monolayer. Each strain of *P. gingivalis* except for AJW4, which has wild-type levels of adherence but a significantly reduced ability to invade, has been shown to adhere, invade and persist within HUVE cells. Interestingly, by 10 hours of exposure, all four strains were detected both associated with the cell surface and internalized within the cytoplasm of the HUVECs. Of these strains, W83 and 381 demonstrated the most activity on the HUVEC endothelial monolayers followed by AJW4 and ATCC 33277, respectively. Our data suggests that *P. gingivalis* is working from within the HUVECs since the results with AJW4 was shown to adhere to but not to invade the HUVE cells (Dorn *et al.*, 2000). Invasion studies are characteristically determined after the cell line of interest is exposed to the desired strain for 2.5 hours. Therefore, it is possible that the extended interaction times of HUVECs with AJW4 were adequate for its invasion of HUVEC monolayers. These data suggest that adhesion and invasion of live *P. gingivalis* may also play a role in the disruption of the cell monolayers and re-localization of the β-catenin and cadherin proteins. Given that adhesion and invasion efficiencies were strain dependent, there could be a connection between the ability of one strain to adhere and invade host cells and its ability to breakdown host proteins (Hintermann *et al.*, 2002).

**P. gingivalis** Gingipain Mutants Demonstrate Different Effects on HUVE Cell Monolayers

The proteolysis of signaling molecules that regulate adhesion, survival, differentiation, proliferation, and migration are likely accomplished by *P. gingivalis* gingipains (Hintermann *et al.*, 2002). Gingipains are capable of activating the host cell metalloproteinases to breakdown
components of their extracellular matrix in addition to degrading cell adhesion molecules from within the periodontal tissue (Katz et al., 2000; Kuramitsu, 1998). Thus, the relocation of the cadherins and catenins by *P. gingivalis* may potentiate the cellular and subsequent tissue invasion characteristic of periodontal disease.

The Rgp-null mutant CW401, expressing only Kgp, was unable to alter the location of β-catenin from the HUVE cell surface and induce monolayer disruption. This result is consistent with the W83 lysate and TLCK experiments which showed a lack of junctional complexes but minimal cell detachment. Since TLCK has been shown to inhibit Kgp more than Rgp (Pike et al., 1994) this result suggests that Rgp is more effective in causing the disruption of the cell-cell contacts and subsequent disruption to the HUVEC monolayers than is Kgp. HUVEC monolayers co-cultured with the gingipain mutants that express RgpB, MT10 (*rgpA*-), and MT10W (*rgpA-, kgp-*) and the mutant that expresses RgpA, G102W (*rgpB-, kgp-*) resulted in monolayer disruption and a loss of β-catenin staining at the cell surface after 15 hours. HUVECs exposed to the *P. gingivalis* strain that expresses only RgpA and Kgp, G102 (*rgpB-), also resulted in monolayer disruption and a loss of β-catenin staining at the cell surface but only at 20 hours of exposure. These data indicate that both RgpA and RgpB are active in HUVEC monolayer disruption. However, the gingipain mutant G102W that expresses only RgpA caused a slightly greater disruption of the HUVEC monolayer after 15 hours than MT10W that expresses only RgpB. Therefore, it is likely that RgpA is more active than RgpB in this regard.

It is important to note that Rgp is involved in the proteolytic activation of the major *P. gingivalis* fimbrial protein, FimA, that has been shown to be important for the appropriate attachment and invasion of *P. gingivalis* of host cells (Kadowaki et al., 2000; Potempa et al., 2000; Kadowaki et al., 1998; Xie et al., 1997; Tokuda et al., 1996). In addition, Arg-gingipains
have a role in a number of pathways including contributing to their own post-translational processing, expression of the mature 75-kDa cell surface protein, profimbrillin, and pro-Kgp. (Kadowaki et al., 2000; Potempa et al., 2000; Kadowaki et al., 1998; Xie et al., 1997; Tokuda et al., 1996). Thus, the loss of Rgp results in decreased attachment and invasion of \( P. \text{gingivalis} \) to host cells due to the improper processing of FimA (Kadowaki et al., 2000; Potempa et al., 2000; Kadowaki et al., 1998; Xie et al., 1997; Tokuda et al., 1996).

In addition to its lack of fimbriae, the decrease in attachment and invasion of the gingipain-null mutant, CW501, to HUVEC monolayers is likely a result of its lack of gingipains. The lack of monolayer disruption or redistribution of \( \beta \)-catenin observed with HUVECs co-cultured with CW501 provides additional evidence that the gingipains play a role in this activity. In addition, exposure of HUVECs to the Rgp-null mutant, CW401, which is also devoid of fimbriae, suggests that the Arg- gingipains may disrupt the endothelial HUVEC monolayers. These data also suggest that there may be cooperation between the gingipains. It is possible that Kgp acts as a tertiary effector and may work in concert with RgpA and/or RgpB since HUVEC monolayers exposed to the gingipain mutants containing Kgp in addition to RgpA or RgpB, showed a higher level of monolayer disruption than the mutants expressing RgpA or RgpB alone. In contrast, HUVEC monolayers exposed to the YPP2 (\( \text{kgp}^- \)) mutant, that retains both RgpA and RgpB activity, only caused disruption to the monolayer after 20 hours of treatment, similar to its parent strain. A possible explanation for these results is a difference between the wild-type strains. YPP2 was constructed in ATCC 33277, a different parental strain than the rest of the gingipain mutants, which were constructed in \( P. \text{gingivalis} \) 381. In support of this explanation, HUVEC monolayers treated with strain 381 at an MOI of 100 induced monolayer disruption after 10 hours of co-culture, while strain ATCC 33277 at the same MOI, did not induce HUVEC
monolayer disruption until 20 hours of co-culture. These observed differences could be a result
of differentially regulated gingipain expression between strains and/or from diverse experimental
procedures such as the construction of the mutants, single versus double crossover (Tokuda et
al., 1998). The variation among strains may also be the consequence of a number of factors such
as lower metabolic or enzymatic activity of the bacteria.

**Effects of *P. gingivalis* on Adhesion and Invasion of HUVEC Monolayers**

*P. gingivalis* coaggregation with other bacteria as well as adherence to and colonization of
host tissues is mediated by adhesion factors such as hemagglutinins, vesicles and fimbriae
(Hamada et al., 1998). The results with HUVEC monolayers exposed to the adhesion mutant
YPF1 (*fimA*) showed minimal adhesion of this mutant to the cell monolayers and no monolayer
disruption through 20 hours of co-culture. The adhesion mutants PG1683 (conserved
hypothetical protein) and PG1118 (*clpB*) showed adhesion to the HUVEC monolayers after 10
and 15 hours of co-culture, respectively. However, the monolayers remained intact after
treatment with each of these adhesion mutants. This data suggests that adhesion alone may not
sufficient for monolayer disruption. In contrast, although PG0242 (conserved hypothetical
protein) demonstrated minimal attachment to the HUVEC monolayer through 15 hours of co-
culture, the attachment of PG0242 to HUVEC monolayers after 20 hours of co-culture was
markedly increased. In addition to the increased attachment, PG0242 also demonstrated
HUVEC monolayer disruption, suggesting that adhesion plays a role in this activity.

PG1683 has significant homology with \( \alpha \)-amylases, which participate in the coaggregation
of *P. gingivalis* with other oral bacteria and in the attachment of *P. gingivalis* to epithelial cells
(Belanger et al., 2006; Ellen et al., 1997; Kamaguchi et al., 1994). ClpB is a heat shock protein
that participates in many infections induced by bacterial pathogens by acting as a chaperone,
inhibiting the aggregation of proteins and aiding in the proper folding of proteins (Weibezahn et al., 2005; Goulhen et al., 2003; Lu and McBride, 1994). The adhesion of PG1118 (clpB-) to the HUVEC monolayer suggests that this protein is important for \textit{P. gingivalis} to be able to regulate and control its own aggregation and the binding of specific proteins in response to environmental stress.

The YPF1 mutant, which lacks the major fimbrial protein important for adhesion, FimA, showed a low level of attachment to the HUVEC monolayer and no disruption of the cell monolayer after 20 hours of co-culture. In contrast, HUVEC monolayers treated with the PG0242 (conserved hypothetical protein) mutant for 20 hours resulted in numerous \textit{P. gingivalis} attached to the cell monolayer and monolayer disruption. A blast search revealed that PG0242 has 81% similarity with tetapyrrole methylases and is likely involved in the metabolism of protoporphyrin and protohaem. Work done in our laboratory found that the loss of this protein, which would likely decrease the ability of this \textit{P. gingivalis} mutant to utilize haem, resulted in decreased adhesion and invasion of \textit{P. gingivalis} of HCAECs. The availability of hemin or iron has been shown to regulate several factors connected to the virulence of \textit{P. gingivalis} including growth, survival and the presence of gingipains (Kesavalu et al., 2003). \textit{P. gingivalis} grown under iron limited conditions has been shown to increase the production of outer membrane vesicles (OMVs), LPS and gingipain expression (Kesavalu et al., 2003). Therefore, the reduced ability of \textit{P. gingivalis} to breakdown iron storage molecules would likely result in the upregulation of other virulence factors, which may account for the monolayer disruption observed with PG0242.

Taken together, these data suggest that adhesion of \textit{P. gingivalis} to host cells is not sufficient but facilitates \textit{P. gingivalis} to stimulate the alteration of location of \(\beta\)-catenin as well as
monolayer disruption. However, since the adhesion mutant PG0242 was capable of altering the location of β-catenin and disrupting the HUVEC monolayer, it is possible that attachment is not an absolute requirement for \textit{P. gingivalis} to induce monolayer disruption. There may be additional mechanisms that \textit{P. gingivalis} utilizes to stimulate monolayer disruption.

As mentioned previously, \textit{P. gingivalis} has been shown to invade a number of cell types including epithelial and endothelial cells (Dorn \textit{et al.}, 1999; Lamont \textit{et al.}, 1995). The ability of \textit{P. gingivalis} to internalize within various cell types may prolong the survival of this bacterium by providing a protective niche from the host immune system within a nutritionally abundant environment (Dorn \textit{et al.}, 1999; Lamont \textit{et al.}, 1995). Invasion of endothelial cells by \textit{P. gingivalis} has been shown to be required for the stimulation of various inflammatory IgCAMs such as ICAM-1, VCAM-1 and E-selectin by \textit{P. gingivalis} (Khlgatian \textit{et al.}, 2002). In addition, invasive, but not non-invasive \textit{P. gingivalis} has been shown to “activate” the endothelium leading to atherosclerotic events in apoE-/- mice by accelerating the local inflammatory responses within the aortic arch (Chou \textit{et al.}, 2005; Gibson \textit{et al.}, 2004). Therefore, the alteration of the junctional proteins cadherin and catenin by invasive \textit{P. gingivalis} would affect the integrity of the tissue, thereby enabling \textit{P. gingivalis} entry into the vasculature (Chen \textit{et al.}, 2001b).

\textit{P. gingivalis} invasion of HUVEC monolayers was determined to be instrumental in the induction of monolayer disruption. HUVECs co-cultured with mutants with reduced invasive capabilities, PG1118 (\textit{clpB}-), PG0717 (putative lipoprotein), PG1286 (ferritin) and YPEP (\textit{pepO}-) appeared to be attached to the HUVEC surface after 20 hours of exposure. However, none of these invasion mutants had any effects suggesting that invasion is necessary for HUVEC monolayer disruption.
P. gingivalis PepO is an endopeptidase with significant homology to the mammalian endothelin converting enzyme ECE-1, which is responsible for converting big-endothelin into the most potent vasoconstrictor, endothelin-1 (ET-1) (Ansai et al., 2003; Ansai et al., 2002). PepO has been suggested to be a key player in the interaction of P. gingivalis with host cell membranes during invasion (Ansai et al., 2003; Ansai et al., 2002). Thus, the decreased monolayer disruptive abilities of the invasion pepO- mutant may be due to the inability of this mutant to appropriately interact with the HUVEC membrane. In addition, since the pepO-mutant retains the FimA protein, the decreased adhesion and invasion observed with this mutant is not due to the lack of fimbriae (Ansai et al., 2003; Ansai et al., 2002) and suggests that this endopeptidase is necessary for P. gingivalis to cause monolayer disruption. The decreased invasion of the lipoprotein mutant, PG0717, is perhaps due to the decreased ability of this P. gingivalis mutant to assemble onto lipid rafts. Lipid rafts are present on the mammalian cell surface and are important for the transport of cholesterol, signal transduction and invasion (Giacona et al., 2004). Lipid rafts are utilized by other bacterial species such as Legionella pneumophilia and Brucella abortus for entrance into the host cells (Duncan et al., 2004; Watarai, 2004; Duncan et al., 2002). Recently, P. gingivalis has also been demonstrated to utilize lipid rafts for internalization into host cells (Belanger et al., 2006; Tsuda et al., 2005). Thus, the presence of this lipoprotein appears to be important for P. gingivalis to induce HUVEC monolayer disruption.

P. gingivalis 1286 is an intracellular protein encoding a ferritin gene that participates in iron storage and may also play a role in defending P. gingivalis against oxidative stress (Andrews et al., 1998). In addition, the heat shock protein (HSP), ClpB, is an immuno-dominant antigen widely expressed by P. gingivalis that helps the bacterial cell to adapt to environmental
stressors such as elevated temperatures (Shelburne et al., 2005; Goulhen et al., 2003). The lack of HUVEC monolayer disruption and re-localization of β-catenin observed with the \( P. \text{gingivalis} \) invasion mutants PG1286 and PG1118 (clpB-) may be due to a reduced ability of these mutants to properly process and/or secrete proteins responsible for this effect in response to environmental stress.

Collectively, these data with the invasion mutants suggests that adhesion of \( P. \text{gingivalis} \) to the HUVEC monolayers is not as fundamental in causing monolayer disruption, as is \( P. \text{gingivalis} \) invasion. In fact, the effects of internalized bacteria may be characteristically different than external bacteria. The proteins of the junctional complexes (cadherins and integrins) may be degraded by the OMVs secreted by the internalized \( P. \text{gingivalis} \). Since OMVs contain hemagglutinins, they may be able to escape the vacuole through the lipid membrane into the cytosol and act on the junctional proteins directly (Qi et al., 2003; Beveridge and Kadurugamuwa, 1996; Grenier and Mayrand, 1987). These data support the hypothesis that the gingipains of live \( P. \text{gingivalis} \) induce the majority of their activity from within the HUVE cells, and less from the outside.

HUVEC monolayers exposed to mutants of \( P. \text{gingivalis} \) that displayed no difference in adhesion or invasion of HCAECs compared to the parent strain W83, PG0293 (putative secretion activator protein), or an increase in invasion, PG1788 (putative cysteine peptidase), resulted in the alteration of location of β-catenin and monolayer disruption. A blast search revealed that PG0293 displays homology to the secretion activator protein of \( Brucella \) melitensis and may contribute to the virulence of \( P. \text{gingivalis} \). The ability of the PG0293 mutant to cause monolayer disruption suggests that this protein does not play a crucial role in \( P. \text{gingivalis} \) ability to cause monolayer disruption. PG1788 is a putative cysteine peptidase that likely plays
important roles in the acquisition of nutrients, tissue invasion and host immune defense modulation (Lamont and Jenkinson, 1998). The ability of this mutant to disrupt the HUVEC monolayer and alter the location of β-catenin suggests that this protein is also not essential for this activity.

In contrast, HUVEC monolayers exposed to PG0686 (conserved hypothetical protein), which also showed no difference in adhesion or invasion of HCAECs compared to the parent strain W83, resulted in no monolayer disruption even though numerous P. gingivalis were attached to or internalized within the HUVECs. The increased attachment of PG0686 to HUVECs is in agreement with reports that P. gingivalis invasion of epithelial and endothelial cells coincides with an up-regulation of the gene corresponding to PG0686 (Belanger et al., 2006; Hosogi and Duncan, 2005). A blast search revealed that PG0686 has significant homology to hemerythrin, an oxygen transporting protein found in many eukaryotic invertebrates (Isaza et al., 2006) and to a PAS/PAC sensor protein, which senses oxygen in many varieties of prokaryotes including E. coli and Rhizobium meliloti (Buck et al., 2001). Shelburne et al., reported that the expression of the P. gingivalis gingipains rgpA and rgpB were upregulated in response to oxidative stress (Shelburne et al., 2005). It is possible that the loss of the 0686 gene disrupts the ability of P. gingivalis to sense and respond to environmental stress e.g., oxygen, which could result in the failure of P. gingivalis to upregulate rgpA and rgpB. This possibility could account for the decreased ability of PG0686 to induce HUVEC monolayer disruption, due to its inability to upregulate the gingipains rgpA and rgpB. The data from mutants PG0293 and PG1788 suggests that invasion of HUVECs is necessary for P. gingivalis to fully induce the re-distribution of β-catenin and monolayer disruption. However, invasion of HUVECs by the PG0686 mutant did not induce monolayer disruption or the alteration of location of β-catenin.
This data suggests that *P. gingivalis* invasion of host cells also requires PG0686 to induce the disruption to the HUVEC monolayers.

To summarize the conclusions from this collection of data, adhesion and invasion together are a mechanism that *P. gingivalis* utilizes to induce HUVEC monolayer disruption and β-catenin re-localization from the cell surface to cytoplasmic. At an MOI of 100, the adhesion mutant YPF1 (*fimA-*), the invasion mutant YPEP (*pepO-*), and the PG0686 mutant do not cause a disruption of the HUVEC monolayers. However, co-culture with all three *P. gingivalis* mutants at an MOI of 1000 demonstrated that the integrity of the HUVEC monolayer was altered in that β-catenin was no longer visualized at the cell surface. In addition, numerous *P. gingivalis* were observed attached to the HUVE cell surface and internalized within the HUVEC cytoplasm. The effects observed with the adhesion mutant YPF1 and the invasion mutant YPEP were comparable to their parent strain ATCC 33277. These results suggest that both adhesion and invasion impaired *P. gingivalis* mutants at high enough concentrations (MOI of 1000) are capable of altering the location of β-catenin from the HUVE cell surface to within the cytoplasm, as well as inducing HUVEC monolayer disruption. However, HUVEC monolayers co-cultured with PG0686 at this concentration for 20 hours remained mostly intact although β-catenin did not remain concentrated at the cell surface. The effects observed with the PG0686 conserved hypothetical protein mutant further suggest that this protein is critical for *P. gingivalis* to induce HUVEC monolayer disruption. Taken together, these data suggest that HUVEC monolayer disruption may be a consequence of *P. gingivalis* internalization. These data do not prove but suggest that the gingipains of live *P. gingivalis* may act primarily from within the endothelial cells, and less from the cells’ exterior.
In vitro experiments have shown that *P. gingivalis* has the ability to invade, persist, and replicate in many endothelial (EC) and epithelial cell lines including primary cultures of human endothelial cells (Dorn *et al.*, 1999; Lamont *et al.*, 1995). The ability of *P. gingivalis* to invade and persist establishes that this bacterium is equipped with a mechanism capable of evading host defenses, ultimately leading to pathogenicity (Finlay and Falkow, 1997). This data suggests that invasion, following adhesion is more important for *P. gingivalis* derived disruption of the HUVEC monolayer, than is adhesion alone.

Dorn *et al.* showed that invasive *P. gingivalis* localizes inside autophagic vacuoles within HCAECs (Dorn *et al.*, 2001). Results from this study using transmission electron microscopy (TEM) showed that HUVEC monolayers exposed to the invasive *P. gingivalis* 381 and PG0686 at an MOI of 100 resulted in the localization of these strains within intracellular vacuoles, possibly autophagic vacuoles. In contrast, HUVEC monolayers treated with the non-invasive strain, CW501, at the same MOI resulted in very few *P. gingivalis* at the HUVE cell surface. In addition, the morphology of the HUVE cell was very similar to the untreated control cells. In addition to its lack of gingipain activity, CW501 likely does not invade because it does not correctly process and express FimA, a protein that facilitates *P. gingivalis* adhesion and invasion (Kadowaki *et al.*, 2000; Potempa *et al.*, 2000; Kadowaki *et al.*, 1998; Xie *et al.*, 1997; Tokuda *et al.*, 1996). Altogether, the results with the gingipain-null mutant, CW501, suggest that *P. gingivalis* gingipains and/or fimbriae are necessary for both adhesion and invasion.

Increasing the MOI of strains 381, PG0686 and CW501 to 1000 affected the morphology of the HUVECs. HUVECs co-cultured with strains 381 and PG0686 showed numerous *P. gingivalis* located around the periphery of the HUVECs, as well as internalized within intracellular vacuoles. Compared to the untreated control cells, the HUVECs co-cultured with
strain 381 exhibited gross cellular distortions, including an invaginated cell membrane and the presence of numerous empty intracellular vacuoles and vacuoles containing *P. gingivalis*. High numbers of PG0686 were localized within multiple intracellular vacuoles throughout the HUVECs. However, the HUVE cell membrane remained intact in contrast to the HUVECs treated with strain 381. HUVEC's exposed to CW501 demonstrated very few *P. gingivalis* located around the cell periphery or internalized within the HUVECs. With the exception of an increased number of empty vacuoles within the HUVECs co-cultured with CW501, the cellular morphology resembled the untreated control cells. These data suggest that the gingipains are likely involved in affecting the HUVEC morphology (invagination of the cell membrane) since the increased concentration (MOI of 1000) of the gingipain-null mutant did not have the same effect on the membrane morphology as did strain 381. However, incubation of the HUVECs with CW501 at this concentration did result in the appearance of additional intracellular vacuoles, most of which were devoid of *P. gingivalis*. These results support my previous data that the disruption of the HUVEC monolayer caused by exposure to strain 381 but not to CW501 is the result of the *P. gingivalis* gingipains.

In addition to the gingipains, *P. gingivalis* invasion of HUVECs may also be necessary or a second mechanism for this bacteria to cause monolayer disruption since CW501 and the invasion mutants of *P. gingivalis*, YPEP, PG1118, PG0717, and PG1286, demonstrated very little internalization within the HUVECs and no monolayer disruption. However, HUVEC monolayers treated with the conserved hypothetical protein mutant PG0686 at an MOI of 100, which showed no difference in adhesion or invasion when compared to its parent strain W83, also resulted in a lack of HUVEC monolayer disruption. Taken together, the results from the
IMF and TEM experiments suggest that the gingipains and the 0686 protein in addition to \( P. \) \textit{gingivalis} invasion are critical for the disruption of the HUVEC monolayers.

**Cell Death**

Our previous experiments revealed that exposure of HUVEC monolayers to certain \( P. \) \textit{gingivalis} strains caused disruption of the monolayers and cell detachment. We have also shown that the lysate and SPF of \( P. \) \textit{gingivalis} caused epithelial and endothelial cell monolayer disruption and the detachment of cells from these monolayers. This collection of data suggests that a secreted protein or proteins from \( P. \) \textit{gingivalis} (e.g., gingipains) are responsible for this observed activity.

Using flow cytometry, the detached cells from the intact HUVEC monolayers exposed to \( P. \) \textit{gingivalis} 381 and ATCC 33277 were determined to be dead, suggesting that these strains were capable of inducing HUVE cell death. In contrast, the \( P. \) \textit{gingivalis} mutant strains, CW501 and YPF1, were unable to induce such cell death and instead produced cell death levels comparable to the untreated cells. In addition to its lack of gingipains, it is plausible that CW501 was unable to induce cell death because of its lack of fimbriae and subsequent reduced ability to attach to and invade the HUVECs. Interestingly, YPF1 does contain the gingipains (Love \textit{et al.}, 2000). However, like CW501, this mutant was unable to induce HUVE cell death.

As mentioned previously, adherence and invasion of host cells have been shown to be hindered by the lack of the FimA protein (Hamada \textit{et al.}, 1994). However, it is possible that both mutant strains CW501 and YPF1 could utilize a different mechanism for attachment since other non-fimbrial adhesions associated with the outer membrane have been isolated from \( P. \) \textit{gingivalis} (Lepine \textit{et al.}, 1996; Lamont \textit{et al.}, 1994; Grenier and Mayrand, 1987; Okuda \textit{et al.}, 1986; Boyd and McBride, 1984). The existence of other adhesions is anticipated due to the findings that the \textit{fimA}- mutant, DPG3, retained the ability to adhere to normal human gingival
epithelial cells, NHGEC, though 50% less, and to GEC, 65% less (Weinberg et al., 1997; Hamada et al., 1994). In addition to the adhesin domains of RgpA and Kgp, adherence of \textit{P. gingivalis} to host cells has been shown to be mediated by the hemagglutinins, specifically, Hag A and Hag B, which are large surface proteins with considerable homology to the adhesin domains of the gingipains (Song et al., 2005; Ally et al., 2003; Grenier et al., 2003; Chen et al., 2001a; Dorn et al., 2000; Progulske-Fox et al., 1999; Progulske-Fox et al., 1993). \textit{P. gingivalis} also possesses a minor fimbrial protein (Mfa1), which has been shown to be highly immunogenic, and directly participates in the coadhesion with \textit{Streptococcus gordonii} (Park et al., 2005; Hiramine et al., 2003). Taken together, the reduced adhesion and invasion ability of CW501 and YPF1 suggests that \textit{P. gingivalis} adhesion and/or subsequent invasion of HUVE cell monolayers as well as \textit{P. gingivalis} gingipains are necessary for \textit{P. gingivalis} induced cell death.

Given that adherence of \textit{P. gingivalis} to HUVECs has been shown to be necessary for \textit{P. gingivalis} invasion, we determined if invasion was a requirement for \textit{P. gingivalis} induced HUVE cell death. HUVEC monolayers were exposed to \textit{P. gingivalis} 381, ATCC 33277, YPF1 and CW501 for 1.5 hours before being subjected to the antibiotic protection assay. Thus, internalized \textit{P. gingivalis} were examined for their ability to induce HUVE cell death. HUVEC control cells treated with antibiotics produced the same results as the untreated cells, suggesting that the presence of antibiotics did not cause any adverse affects on the cells. HUVECs exposed to CW501 and YPF1 followed the same pattern of cell death (approximately 16%) as the untreated HUVECs through 24 hours. However, consistent with the previous data, HUVECs exposed to both strains 381 and ATCC 33277 for 24 hours showed significantly higher levels of cell death relative to the untreated control cells and the two mutant strains. This data indicates
that the internalized *P. gingivalis* are capable of stimulating the HUVE cell death observed at 24 hours of co-culture.

One aspect of this experiment was the observation of 16% HUVE cell death at 2.5 hours with the untreated cells. This level of cell death was approximately 3 times the untreated control cell death level reported previously, and this level remained consistent through 24 hours. The HUVECs used in this experiment were older, passaged 10-14 times compared to the 4-8 passages of cells used with the pulsed *P. gingivalis* cell death assay. Since it is impossible to precisely standardize every experiment, an explanation for the discrepancy between the continuous exposure assay and the antibiotic protection assay could be experimenter error or a difference in the cell culturing techniques. In addition, bacteria that were passaged fewer times were more active in cell death compared to cultures from multiple passages. Because invasion is dependent on both bacterial and cellular metabolism, discrepancies between different experimental results are most likely a consequence of the age of the bacterial and/or endothelial cells.

We have shown that the proteins secreted from *P. gingivalis* (SPFs) are capable of disrupting the HUVEC monolayer. Therefore, the *P. gingivalis* 381 SPF was tested for its ability to stimulate HUVE cell death. Interestingly, HUVECs treated with the 381 SPF resulted in a significant increase in cell death compared to the untreated control cells, but only after 24 hours of co-culture. In addition, the exposure of HUVECs to CW501 at an MOI of 1000 also resulted in a significant increase in cell death compared to the untreated cells after 24 hours. However, the levels of cell death observed with CW501 and the 381 SPF were dramatically less than the cell death observed with the live strain 381 after the same times of exposure. The induction of HUVE cell death after exposure to the 381 SPF suggests that the surface and/or secreted proteins
of *P. gingivalis*, e.g., gingipains, play a role in this regard. Conversely, it is likely that *P. gingivalis* is equipped with additional mechanisms other than the gingipains for inducing cell death since the gingipain-null mutant CW501 was able to induce HUVE cell death.

Typically, bacteria are capable of invading host cells by evoking reassembly of cytoskeletal components interceded by actin polymerization (Rosenshine and Finlay, 1993). This is likely true for *P. gingivalis* because invasion of this pathogen into HCAEC and MDDC cells was profoundly repressed when cytochalasin D, an inhibitor of actin polymerization, was added to the experiment (Jotwani and Cutler, 2004; Dorn *et al.*, 1999).

To further determine the importance of invasion on HUVE cell death, we decided to suppress *P. gingivalis* invasion of HUVECs. In this study, inhibiting the invasion of strains 381 and ATCC 33277 into HUVECs with cytochalasin D, completely inhibited HUVE cell death observed with these strains in previous experiments. Overall, *P. gingivalis* 381 and ATCC 33277 were capable of inducing HUVE cell death. This cell death was likely the result of *P. gingivalis* components, e.g., gingipains, acting from within the HUVE cells instead of from the outside since the inhibition of *P. gingivalis* internalization appeared to suppress its ability to induce cell death of HUVECs. Thus, this data provides further support for the hypothesis that *P. gingivalis* gingipains and invasion of HUVECs are both important for *P. gingivalis* to induce cell death.

The flow cytometry results previously reported using propidium iodide showed that there were significant increases in cell death compared to the untreated cells when HUVECs were exposed to strains 381 and ATCC 33277 with antibiotics (to kill any remaining extracellular bacteria) and without antibiotic treatment. However, the quantification of the activation of caspase 3 activity showed only the HUVEC monolayers exposed to strain 381 and ATCC 33277
without antibiotics had significant increases in caspase 3 activity relative to the untreated HUVEC control. One explanation for this result is that there is a difference in the number of intracellular bacteria present at each time point. Other bacterial species such as *A. actinomycetemcomitans* and *Salmonella spp.*, can replicate intracellularly and multiple researchers have proposed that *P. gingivalis* is capable of this as well (Lamont *et al.*, 1995; Blix *et al.*, 1992; Finlay and Falkow, 1989). In fact, a 90 minute incubation with GEC with antibiotics, which destroys the exterior bacteria, produced a noticeable increase in the number of internal bacteria after four hours (Lamont *et al.*, 1995). Given that HUVE cells co-cultured with strains 381 and ATCC 33277 with and without antibiotic exposure yielded different caspase 3 activity levels, it is possible that this endothelial cell undergoes more than one type of cell death in response to *P. gingivalis* exposure levels. Therefore, the *P. gingivalis* internalized within the HUVE cells treated with antibiotics may induce a caspase independent form of HUVE cell death while the constant exposure of *P. gingivalis* to HUVE cells appears to stimulate caspase 3 dependent apoptosis.

In summary, this study has provided evidence that the *P. gingivalis* conserved hypothetical protein, 0686, is crucial for this bacterium to induce HUVEC monolayer disruption. In addition, the *P. gingivalis* gingipains are also important in the disruption of the HUVEC monolayer and the re-localization of β-catenin from the cell surface as well as in HUVE cell death. The *P. gingivalis* gingipains are responsible for the breakdown of the proteins (e.g., catenins and VE-cadherin) within endothelial (HMVEC, BAEC and HUVEC) and (catenins and E-cadherin) within epithelial (KB, MDCK, GEC and HuH7) cell-cell junctional complexes (Chen *et al.*, 2001a; Katz *et al.*, 2000; Wang *et al.*, 1999). The loss of cell-cell adhesion due to the disruption of the HUVEC monolayer and the adherens junction protein, β-catenin, as well as the loss of
cell-matrix adhesions (detachment of cells), may play a role in *P. gingivalis* induced cell death. The HUVE cell death induced by the internalized *P. gingivalis* after the administration of antibiotics might be directly linked to the cleavage of the junctional proteins. The cleavage of the adherens junction proteins and subsequent cellular detachment raises the possibility that a unique form of apoptosis called anoikis, which has previously been suggested to be important in pathological progressions such as CVD, is induced by *P. gingivalis* gingipains (Michel, 2003; Frisch and Francis, 1994). In addition, Sheets *et al.* also suggested that the gingipains may induce cell death via anoikis, since N-cadherin, vascular endothelial cadherin (VE-cadherin) and integrin β1, were cleaved in bovine coronary artery endothelial cells (BCAEC) and human microvascular endothelial cells (HMVEC), causing detachment from the culture surface (Sheets *et al.*, 2005). Both anoikis and apoptosis can be induced through the loss of integrin (cell-matrix) signaling (Michel, 2003; Frisch and Francis, 1994). As previously mentioned, integrins are members of non-covalently linked transmembrane, heterodimeric glycoproteins. In addition to regulating attachment to the ECM, integrins are also inducers of signal transduction pathways that are responsible for cell transformation, differentiation, migration, proliferation, and inflammatory responses (Schoenwaelder and Burridge, 1999; Sheppard, 1996; Haynes and Webb, 1992). The increased tissue permeability induced by damage to the adherens junctions and extracellular matrix could potentially contribute to systemic infections by allowing the entry of *P. gingivalis* into the vascular system (Chen *et al.*, 2001b).

Results from the caspase 3 activity assay also demonstrated the possibility that *P. gingivalis* is capable of utilizing at least two different mechanisms for initiating HUVE cell death. Flow cytometry experiments using propidium iodide and HUVECs co-cultured with *P. gingivalis* with (to kill any remaining extracellular bacteria) and without antibiotics resulted in
significantly higher levels of cell death compared to the control cells. In contrast, the caspase 3 activity levels of the HUVECs co-cultured with *P. gingivalis* with and without antibiotics were different. Although the levels of caspase 3 activity for the HUVECs co-cultured with *P. gingivalis* and antibiotics were higher than the untreated cells, the difference level was insignificant. Only the continuous exposure of *P. gingivalis* with HUVECs yielded significantly higher levels of caspase 3 activity compared to the untreated cells. Since the antibiotics would have killed any extracellular *P. gingivalis* present within the wells, the death associated with increased caspase 3 levels of the HUVECs co-cultured with *P. gingivalis* and antibiotics must be due to the internalized *P. gingivalis*. This data suggests *P. gingivalis* located external to the cells may initiate one form of cell death while the internalized *P. gingivalis* initiate another.

Therefore, in addition to the caspase 3 dependent apoptosis observed after continuous exposure of *P. gingivalis* with HUVECs, *P. gingivalis* may also play a role in caspase independent apoptosis.

The results from the PG0686 mutant suggested that this protein was crucial for *P. gingivalis* to induce HUVEC monolayer disruption. Future directions for this project could be to analyze and characterize the PG0686 protein. Also, it would be interesting to determine if this mutant is capable of inducing HUVE cell death comparable to wild-type *P. gingivalis* or to the gingipain-null mutant, CW501. In addition, the use of caspase inhibitors such as z-VAD-FMK (general caspase inhibitor), z-YVAD-FMK (caspase-1 and -4 inhibitor), z-D(OMe)-E(OMe)-VD(OMe)-CH₂F (caspase-3 inhibitor) and z-VEID-FMK (caspase-6 inhibitor) would be beneficial in determining if *P. gingivalis* is capable of inducing caspase independent apoptosis in HUVE cells. Finally, further characterization of the effects of the gingipains on the HUVECs
would be greatly enhanced by the production of a \textit{P. gingivalis} mutant that expresses the gingipains, adheres, but does not invade.

The results reported here also provide support for the involvement of \textit{P. gingivalis} in the initiation of cardiovascular disease. Alterations within the vessel wall caused from an inflammatory response to chronic infections are essential for the production of adhesion molecules, proinflammatory cytokines, chemokines, inflammatory cell transmigration, and the formation of atheromas (Giacona \textit{et al.}, 2004). The development of inflammatory diseases such as periodontitis and CVD may depend on the severity of the inflammatory response to insult or injury. Myocardial infarctions may develop from the presence of severe inflammation within the coronary arteries (Kuramitsu \textit{et al.}, 2003). Atherosclerosis, which is likely a continual process, occurs as a consequence of arterial cell injury, increased leukocyte adhesion, and is arbitrated by inflammation (Ross, 1999). Several pathogens have been detected in human atheromas and suggested to be involved in the pathogenesis of atherosclerosis including \textit{Helicobacter pylori}, \textit{Cytomegalovirus}, \textit{Herpes simplex virus}, \textit{Streptococcus sanguinis}, \textit{Chalmydia pneumonias}, and \textit{P. gingivalis} (Haraszthy \textit{et al.}, 2000; Chiu \textit{et al.}, 1997). Interestingly, PCR experiments identified at least one periodontal pathogen in 44\% of the atheromas from the biopsied carotid endarterectomy specimens tested and 26\% of the observed pathogens was \textit{P. gingivalis} (Haraszthy \textit{et al.}, 2000). Therefore, infection of coronary cells with oral pathogens may aggravate the pathogenesis of cardiovascular disease (CVD) (Dorn \textit{et al.}, 1999). The confirmation of this hypothesis would be significant in that the damage to the vessel walls associated with atherosclerotic plaque formation could be minimized and/or possibly corrected by the direct control of periodontal pathogens through immunological or antibacterial mechanisms (Desvarieux \textit{et al.}, 2005).


BIOGRAPHICAL SKETCH

Kristen L Totten was born in Riverside, California on July 17th 1974. Kristen has had the opportunity of living in many interesting places throughout her life. When she was very young, her family moved to Hong Kong. At the age of three her family moved back to the United States and settled for three years in Columbia, South Carolina, before taking up residence in Marietta, Georgia. Kristen graduated from George Walton High School in June of 1992. That following August, she enrolled at the University of Alabama in Tuscaloosa, Alabama. Kristen graduated four years later in May of 1996 with a BS in Biology. She decided to take a few years off before entering graduate school. During those years she held several jobs in the hospitality industry before settling down in Gainesville, Florida with a technician position in the pharmacology laboratory of Dr. David Silverman at the University of Florida. Kristen was a technician in Dr. Silverman’s lab for two years before she entered the Interdisciplinary Program (IDP) at the University of Florida in August 2000. Kristen decided to pursue a project and a joint mentorship with Dr. Ann Progulske-Fox in Oral Biology and Dr. William Dunn Jr. in Cell Biology. Upon graduation, she plans on pursuing a career with the government.