PHENYTOIN AND ITS METABOLITE, 5-(P-HYDROXYPHENYL-), 5-PHENYLHYDANTOIN, DECREASE SUPERNATANT LEVELS OF MATRIX-METALLOPROTEASES IN THE HUMAN MACROPHAGE: IMPLICATIONS FOR DRUG-INDUCED GINGIVAL OVERGROWTH

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A thesis submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Master of Science in the School of Dentistry (Periodontology)

Chapel Hill

2009

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ABSTRACT

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Phenytoin and its Metabolite, 5-(p-Hydroxyphenyl-), 5-Phenylhydantoin, Decrease Supernatant Levels of Matrix-Metalloproteases in the Human Macrophage: Implications for Drug-Induced Gingival Overgrowth
(Under the Direction of Salvador Nares)

OBJECTIVES: To evaluate the effects of Phenytoin (PHT) and its metabolite 5-(p-Hydroxyphenyl-), 5-Phenylhydantoin (HPPH) on LPS-induced macrophage expression of MMP, TIMP, TNF-α, and IL-6. METHODS: Human macrophages were pretreated with either: 1) 15µg/ml PHT, 2) 50µg/ml PHT, 3) 15µg/ml HPPH, or 4) 50µg/ml HPPH for 1 hour, then challenged with 100ng/ml *A. actinomycetemcomitans* (*A.a.*) LPS. Untreated cells served as control cultures. Total RNA was extracted at 4 hours, and supernatants were collected at 24 hours. RESULTS: A dose-dependent reduction of supernatant *A.a.*-induced MMP-1, MMP-3, MMP-9, and TIMP-1 was noted upon pretreatment with PHT and HPPH. A minimal effect was noted on gene expression for MMP-9 and TIMP-1. MMP2, MMP12, TIMP2-4 production does not appear to be influenced Aa LPS, PHT, or HPPH. CONCLUSIONS: *A.a.* LPS-induced macrophage expression of MMP-1, MMP-3, MMP-9, and TIMP-1 is blunted by exposure to PHT and HPPH.

ACKNOWLEDGEMENTS

"Next in importance to freedom and justice is popular education, without which neither freedom nor justice can be permanently maintained**."**

James A. Garfield

This work represents the combined efforts of, not only myself, but a tremendously gifted and generous group of people around me, without whom, I could not have learned nor accomplished so much. For this, I would like to express my sincere gratitude and appreciation for the time and effort that these people so generously gave on my behalf, so that I may succeed in my scholastic and professional investigations and endeavors.

To my mentor, Salvador Nares, I hope that you know that I am greatly appreciative your wisdom, guidance, and commitment to excellence, not only through clinical education, but also in the development of a kindred intellect within the lab. I learned a tremendous amount about this subject as well as the investigative process from your guidance, and will always respect you as a scientist, mentor, and colleague.

To the members of my committee, David Paquette and Sylvana Barros, for always making time to help guide me through this journey and lending your unique and poignant insights to this investigation.

To Abdel-ghany Al-Saidi, whose help in the lab culturing monocytes and macrophages, as well as running the RT-PCR and ELISA assays, was indispensable in conducting this experiment.

To the rest of the laboratory group for their support.

To my program director David Paquette and the chair of the Department of Periodontology, Ray Williams for accepting me into the program for advanced training in periodontology as well as the degree program for a Master of Science in Periodontology. Your guidance and mentorship through this challenging process has been invaluable.

To my Father and Jen, whose encouragement and support through this journey have been indispensable. Without a doubt, your espousal of my academic pursuits have certainly helped make this dream a reality.

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LIST OF ABBREVIATIONS

µg	Microgram
µL	Microliter
A.a.	*Aggregatibacter actinomycetemcomitans*
bFGF	Basic fibroblast growth factor
C	Celsius
CsA	Cyclosporine A
CD14+	Cluster of differentiation 14 positive
cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon dioxide
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1
DIGO	Drug induced gingival overgrowth
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay

FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FDA	United States Food and Drug Administration
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	Granulocyte-macrophage-colony stimulating factor
h	Hour
HPPH	Phenytoin metabolite (5-(p-hydroxyphenyl-), 5-phenylhydantoin)
ID	Identification
IL	Interleukin
INF-γ	Interferon-gamma
L	Liter
LPS	Lipopolysaccharide
MAC	Macrophage
mg	Milligram
mL	Milliliter
MMP	Matrix metalloproteinase
MON	Monocyte

MTT	3-[4,5- diethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
Na^+	Sodium
ng	Nanograms
nm	Nanometers
PDGF	Platelet-derived growth factor
pg	Picograms
PG	Prostaglandin
PHT	Phenytoin
RNA	Ribonucleic acid
RT-PCR	Real-time polymerase chain reaction
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha

A REVIEW OF PHENYTOIN INDUCED GINGIVAL OVERGROWTH AND THE ROLE OF THE MACROPHAGE

Drug-induced gingival overgrowth (DIGO) is widely recognized as a common unwanted sequelae associated with a broad variety of medications prescribed to treat a litany of medical ailments. The American Academy of Periodontology classifies this as a "Drug influenced gingival enlargement" covered under the "Gingival diseases modified by medications" family (Armitage G, 1999). Currently, over 20 classes of medications (Rees et al, 1995), prescribed to at least 5% of the elderly population (Lewis et al, 1993) are associated with DIGO. Among these, the most common classes of medications associated with DIGO include anti-epileptics [(carbamazepine (Tegretol), phenytoin (Dilantin), mephenytoin (Mesantoin)[, calcium channel blockers [amlodipine (Norvasc), diltiazem (Cardizem), nifedipine (Procardia)], and immunosuppressants [cyclosporine (Sandimmune)]. The prevalence of DIGO varies among the classes of medications, with anti-epileptics producing clinically identifiable overgrowth in approximately 50% of patients, and calcium channel blockers and immunosuppressants contributing to gingival overgrowth in 6 – 25% of patients (Academy Report on Drug Induced Gingival Enlargement 2004, Neville et al, 2002). In addition, some reports have linked DIGO with erythromycin and certain oral contraceptives (Seymour et al, 2000).

To date, the pathological mechanisms associated with DIGO are not completely understood. Further, the etiology appears to be multifactorial in origin involving drug, host and microbial factors. The main target cell appears to be the fibroblast although other resident and infiltrating cells including the macrophage will be exposed and respond to these agents. Macrophages are differentiated monocytes with pivotal roles in many defensive and homeostatic responses. These cells migrate to and take up residence in virtually every tissue in the body. However, recent studies indicate that the macrophage may play a pivotal role in tissue fibrosis and DIGO. Here the current understanding regarding the role of the macrophage in phenytoin (PHT) associated gingival overgrowth is reviewed.

Pharmacology

Phenytoin (5,5-diphenyl-2, 4-imidazolidinedione) is an antiepileptic medication, similar in chemical structure to barbiturates (Figure 1). First synthesized by the German chemist Heinrich Blitz in 1908, it was not introduced for public consumption until 1938 as an alternative to phenobarbital (Smith, 1992). This was, in large part, due to the fact that no practical application could be devised during that period of time. Functionally, it acts to stabilize voltage gated sodium channels by increasing efflux or decreasing influx of sodium ions across cell membranes in the motor cortex during generation of nerve impulses (Goodman and Gillman, 2001). This has the effect of reducing electrical conductance among brain cells, and cardiac muscles. Phenytoin is commonly prescribed for the management of seizures, neuralgias, and cardiac arrhythmias, as it helps to dampen unwanted, runaway brain activity (Smith, 1992).

Phenytoin is metabolized in the liver via aromatic hydroxylation by the cytochrome P450 (CYP2C9 and CYP2C19) enzymes primarily to 5-(p-hydroxyphenyl-), 5-phenylhydantoin (HPPH). This may be further metabolized to a catechol that spontaneously oxidizes to semiquinone and quinone species, which covalently modify proteins. The reactive intermediate, arene oxide is deactivated by either epoxide hydrolase to dihydrodiol or by the action of glutathione (GSH) and glutathione transferase (Cuttle et al, 2000). Phenytoin metabolism may also be affected by other drugs. For example, drugs that decrease serum levels of phenytoin include rifampin and phenobarbital while drugs that increase phenytoin levels include amiodarone, chloramphenicol, cimetidine, disulfiram, fluconazole, fluoxetine, isoniazid (INH), omeprazole, and paroxetine. The oral absorption of phenytoin can be reduced by antacids containing magnesium, calcium carbonate, or aluminum, calcium salts, or enteral feeding products (tube feedings) (Katzung, 2004). Thus, measuring serum levels of phenytoin may be necessary when patients begin or discontinue other medications.

Side Effects

In February 2008, the United States Food and Drug Administration (FDA) announced an increased risk (2.1%) of suicidal behavior associated with patients taking certain anti-epilipetics, such as phenytoin (Information for Healthcare Professionals Suicidality and Antiepileptic Drugs, FDA, 2008). This was based on 199 placebo-controlled studies involving 43,892 patients (27,863 treated patients versus 16,029 placebo patients). Furthermore, phenytoin use has been associated with certain blood dyscrasias, including neutropenia, leucopenia, thrombocytopenia, and anemia, as well as certain dermatologic

reactions, such as epidermal necrolysis and Stevens-Johnson syndromes. Limited oral side effects are known (Katzung, 2004, Lexicomp, 2009). Special care should be taken when sedating patients taking phenytoin, as the sedative effects may be potentiated. If discontinuation of phenytoin is indicated, this should not be done abruptly because of the possibility of increasing seizure frequency.

Clinical and Histological Presentation

In the oral cavity, phenytoin has long been recognized as one of the most identifiable precipitating medications for DIGO, as the first reported case was described by Kimball in 1939. Phenytoin associated DIGO typically appears within 1 to 3 months following initiation of pharmacotherapy, and typically begins with the enlargement of the anterior labial papillae (Cohen, 2007). This overgrowth often extends facially, lingually and coronally and may result in malpositioning of teeth, esthetic disfigurement and interference in hygiene, mastication and speech (Hallmon, 1999). Expansion of the gingival overgrowth may reach its maximum level after 12 – 18 months (Brown et al, 1991). Gingival lobulations may appear fibrotic or inflamed in nature depending on the amount of plaque induced gingival inflammation (Figure 2A and B). Clinically, most DIGOs appear to be indistinguishable from one drug to the other with the exception of phenobarbitone and cyclosporine. Phenobarbitone generally produces a uniform enlargement of the gingival tissue without lobulation, which tends to be greater in the posterior. Cyclosporine on the other hand, may produce lobulations that appear pebbly in texture (Gregoriou et al, 1996) (Figure 3). These tissues have also been associated with common fungal Candidal superinfections, and the tissue tends to be more

hyperemic in nature than phenytoin induced gingival overgrowth (Academy Report on Drug Induced Gingival Enlargement, 2004). DIGO may further be classified by its level of severity. Aas et al (1963) developed a semiquantitative index for describing DIGO based upon the papillary and gingival structures involved, as well as the amount of coronal tooth structure being covered. This index classifies no overgrowth as 0, blunting of the gingival margin (mild gingival overgrowth) as 1, moderate gingival overgrowth (less than one-third of the crown length) as 2, and severe gingival overgrowth (more than one-third of the crown length) as 3.

Histologically, DIGO appears as an excessive accumulation of extracellular matrix proteins with varying degrees of inflammatory infiltrate present. The epithelium of involved gingival tissues demonstrates stratified squamous epithelium with elongated rete ridges that extend deep into the connective tissue (Figure 4A and B). Interestingly, there is considerable similarity in the histologic presentation of phenytoin-induced gingival overgrowth and that associated with calcium channel blockers or immunosuppressants suggesting a common mechanism underlying the pathophysiology of DIGO (Miranda et al, 2001). The degree of inflammatory infiltrate and fibrosis appears to be affected by the quality of oral hygiene and individual susceptibility, stemming from genetic, behavioral and environmental factors. Indeed, the importance of plaque as a cofactor has been recognized by the American Academy of Periodontology (AAP) which classifies this as gingival inflammation being modified by a medication (Academy Report on Drug Induced Gingival Enlargement 2004, Ellis et al, 1999, Armitage, 1999). Furthermore, it has been demonstrated that patients with pre-existing gingival inflammation prior to drug administration are more likely to develop DIGO (Varga et al, 1998). Additional factors associated with DIGO include age (inverse

relationship), gender (males are 3 times more likely to develop DIGO), concomitant medication use, genetic factors, and other periodontal variables that pertain to the development and control of local inflammation (Seymour et al, 2000). Simultaneous administration of phenytoin with cyclosporine or calcium channel blockers does not appear to affect the degree of gingival inflammation.

Treatment of DIGO

The first phase of treatment generally involves discontinuation and substitution of the offending medication by the prescribing physician. Substitutes nonetheless, may induce DIGO to a different degree. For example, valproic acid, has been associated with reduced incidence but not elimination of DIGO (Tan et al, 2004). If the use of PHT is mandatory, professional scaling and root planing, frequent reevaluation, and impeccable home care is necessary to help minimize the severity of DIGO (Somacarrera et al, 1997). Furthermore, concomitant treatment with phenytoin and other anti-epileptics does not appear to influence the extent or incidence of DIGO. This suggests that chronic co-medication with other anti-convulsants known to induce phenytoin metabolism, does not affect plasma or saliva HPPH steady-state levels, nor the degree of gingival overgrowth in adult epileptic patients (Kamali et al, 1999). Pharmacologic substitution has also been noted to be a successful treatment modality for other medications, such as azothioprine or tacrolimus for cyclosporine (Nam et al, 2008).

Plaque scores and gingival inflammation appear to exacerbate the expression of drug-induced gingival overgrowth, irrespective of the initiating drug (Seymour et al, 2000). Such a

finding confirms plaque biofilm as a significant risk factor for the expression of DIGO (Ellis et al. 1999; King et al. 1993; Pernu et al. 1992; Somacarrera et al. 1994; Thomason et al. 1995; Thomason et al. 1996; Thomason et al. 1993). Accordingly, factors that impede oral hygiene measures such as orthodontic appliances, may negatively impact plaque control and exacerbate the prevalence and severity of overgrowth (Daley et al. 1991). Therefore, mechanical therapy such as scaling and root planning, combined with proper home maintenance is of paramount importance. Chemotherapeutic plaque control measures, including chlorhexidine mouthrinses are often beneficial in preventing plaque buildup while short term resolution has been reported following a course of metronidazole and azithromycin, although the mechanism behind this is not entirely understood. The overall duration of the effect of azithromycin treatment has been reported to range between 3 months and two years, even though this has not been substantiated through rigorous testing (Strachan, 2003). Topical anti-fungal medication has also been demonstrated to produce improvement in the clinical presentation of DIGO. However, resolution may take anywhere from 1 to 8 weeks (Khocht et al, 1997).

If non-surgical interventions are unsuccessful, gingival excess may be removed via a scalpel or laser. While surgical resection by an external bevel or internal bevel incision using a scalpel blade remains the standard in the treatment of exuberant cases of DIGO (Rostocket al. 1986), some studies have demonstrated advantages to the use of laser resection. These include sterilization of the surgical field, reduced hemorrhage during excision, and reduced postoperative discomfort (Pick & Colvard 1993, Romanos & Nentwig1996, Seymour et al, 2000). Furthermore, a decrease in the recurrence of DIGO in tissues treated with lasers compared to traditional external- and internal bevel approaches after 6 months has been

reported (Mavrogiannis et al, 2006). Since cellular mitotic activity starts from within the gingival connective tissue following flap surgery, one possible explanation for this finding may be that more time is required for gingival enlargement to manifest itself clinically as a result of the decrease in collagen production by gingival fibroblasts or by delayed wound healing capabilities of tissues (Abergel et al. 1984a, b). Unfortunately, recurrence of gingival overgrowth will often require additional surgical intervention. In the presence of adequate periodontal maintenance, recurrence of DIGO appears to be around 40% in patients taking cyclosporine with higher incidence being associated with younger age, poor hygiene and gingival inflammation. Of note, rinsing with chlorhexidine twice per day has been reported to reduce or eliminate recurrence (Academy Report on Drug-Induced Gingival Enlargement 2004).

The Pathophysiology of DIGO and the Macrophage

Although the pathogenesis of phenytoin-induced gingival overgrowth is not clearly understood, numerous theories have been proposed to account for the disruption of extracellular matrix (ECM) homeostasis. It is widely recognized that fibroblasts are largely responsible for the maintenance of connective tissue homeostasis in human gingival tissues (Everts et al, 1996; Yamada et al, 2000) via an intracellular pathway involving phagocytosis of collagen and by an extracellular pathway that includes secretion of matrix-degradating enzymes, the matrix metalloproteinases (MMPs) (Kelly et al, 2003). Matrix metalloproteinases, such as MMP-1, play an important role in normal physiologic processes, such as wound healing and cell migration but conversely, dysregulated expression has been implicated in the pathological processes associated with cancer, rheumatoid arthritis,

atherosclerosis and periodontitis (Saarialho-Kere, 1999; Reed, 2000; Egeblad, Werb, 2002; Vincenti, Brinckerhoff, 2002; Nikkari et al, 1995; Kubota, 1996).

In addition to resident fibroblasts, a dense infiltration of mononuclear cells, including monocyte-derived macrophage have been observed in periodontitis affected gingival tissues. These cells play a key role in host defense although exuberant macrophage activity has been implicated in periodontal pathology (Genco RJ, Slots J. 1984; Uden CE et al, 1998; Zappa U et al, 1991; Nares et al, 2009). First identified as a major component of host defense by Eli Metchnikoff approximately 100 years ago, monocytes and macrophages originate as pleuripotent stem cells that differentiate into monoblast precursors in the bone marrow. Adult peripheral blood monocyte counts in adults are normally between 1 and 6 percent of the total white blood cell count (300-700 cells/µl of blood) and rarely exceed 10 percent. Monocytes circulate in the bloodstream and eventually emigrate and take up residence in virtually every tissue in the body where they differentiate into morphologically and functionally heterogeneous effector cells such as macrophages, microglia, Kupffer cells, dendritic cells and osteoclasts (Hume et al, 2002). Macrophages are typically large cells measuring between 10-30 mm in diameter (Junqueira, Basic Histology, 1988) with a round or indented nucleus located eccentrically. In addition, most macrophages have a well-developed Golgi apparatus, abundant endocytotic vacuoles, lysosomes, and phagolysosomes, and a plasma membrane covered with ruffles or microvilli. Interestingly, I have noted that these pleomorphic cells adapt to culture conditions and attachment substrate *in-vitro* forming morphologically-distinct phenotypes such as small, dendritic-like cells to large and round cells (Figure 5). These findings may reflect upon their functional capacity *in-vivo* within different tissues and substrates.

Macrophages play a critical role in innate (phagocytosis, pinocytosis) and adaptive (antigen presentation, Th cytokine production) immunity (Nares and Wahl, 2005). In innate immunity to infectious pathogens, macrophages are among the earliest responders, recognizing pathogens through a family of toll-like (TLR) and scavenger receptors that engage signals driving host defense resulting in activation and secretion of proinflammatory mediators. Similarly, products from antigen-activated Th1 lymphocytes, most notably IFN-γ interact with its receptor on these cells and drive changes associated with an activation phenotype, referred to as M1 (classically activated) macrophages, whereas Th2 cytokines, including IL-4 and/or IL-10, alternatively activate macrophages (M2) and may dampen its function. Further, the proinflammatory macrophage phenotype identified by the surface antigen, 27E10, initiate the recruitment and degranulation of neutrophils, and facilitate immune mediated inflammatory tissue destruction (Clark, 1988; Andreesen, 1990). These cells are associated with the production of proinflammatory cytokines, such as IL-1, IL-6, and TNF-α commonly seen in gingival tissues of chronic periodontitis patients (Clark, 1988; Andreesen, 1990). A second phenotype, the reparative and proliferative macrophage, is identified by the antigen RM3/1 (CD163), and is associated with the production of polypeptide growth factors including TGF-β and PDGF-B. These cells are identified in tissues following the resolution of pathologic processes and during the reparative phase following periodontal treatment (Ross, 1986; Martin, 1992). Studies indicate that this phenotype is present at higher levels within gingival specimens from DIGO subjects compared to the 27E10 phenotype (Iacopino et al, 1997). Nurmenniemi (2002) further demonstrated that this macrophage phenotype was present in significantly higher numbers from overgrowth biopsies derived from immunosuppressine agents relative to healthy

controls. This same study also reported elevated numbers of 27E10-positive macrophages in CSA-, CSA + nifedipine and nifedipine-induced gingival overgrowth (Nurmenniemi et al 2002).

Macrophages are one of the few cell types that can migrate among and between body compartments. Consequently, their capacity to degrade basement membranes and other extracellular matrices is accomplished by the production of collagenolytic, elastinolytic and gelatinolytic hydrolases (Takemura and Werb, 1984). These cells are also widely recognized as the principle mediators of connective tissue turnover, maintenance and repair (Riches, 1988; Andreesen et al, 1990; Messadi and Bertolami, 1991; Martin et al, 1992; Kreutz et al, 1992; Wikesjo et al, 1992). Indeed, the macrophage receives, amplifies, and transmits signals to fibroblasts, endothelial cells, and vascular smooth muscle cells by producing proinflammatory cytokines, matrix mettaloprotienases (MMPs) and their inhibitor (TIMPs) along with peptide growth factors, such as platelet-derived growth factor (PDGF), transforming growth factor (TGF), and basic fibroblast growth factor (bFGF) (Shimokado, 1985).

Production of growth factors has been reported to be instrumental in facilitating neovascularization and synthesis of connective tissue proteins that comprise the ECM. Platelet derived growth factor (PDGF) is a dimeric polypeptide consisting of two related peptide chains, PDGF-A and PDGF-B, found in the alpha granules of platelets and is secreted by numerous cells including macrophages, endothelial cells and fibroblasts. Biological activity has been noted for the PDGF-AA, PDGF-BB and PDGF- AB isoforms. PDGF-B is a chemoattractant and mitogen for fibroblasts capable of inducing production of

glycosaminoglycans, proteoglycans, fibronectin and collagen (Raines, 1990, Pierce, 1991, Hosgood, 1993) and has also been shown to induce angiogenesis via growth and proliferation of endothelial and smooth muscle cells (Raines, 1990). MMP activity on the other hand is characteristic and instrumental in the maintenance of connective tissue homeostasis (Domeij et al, 2006). Therefore, it is possible that drugs inducing gingival overgrowth not only dysregulate expression of growth factors that trigger collagen production, but also dysregulate expression of matrix-degradating enzymes and/or inhibitors. Indeed numerous studies have identified upregulation of PDGF-B, fibroblast growth factor-2 (FGF-2), transforming growth factor-β (TGF-β), and connective tissue growth factor (CTGF) (Williamson et al, 1994; Nares et al, 1996; Plemons et al, 1996; Saito et al, 1996; Dill and Iacopino, 1997; Iacopino et al, 1997; Atilla and Kutukculer, 1998; Sasaki and Maita, 1998; Hong et al, 1999; Myrillas et al, 1999; Buduneli et al, 2001; Uzel et al, 2001; Cotrim et al, 2002), and while findings regarding MMP expression have yielded conflicting results (Tuter et al, 2002; Atilla et al, 2001; Emingil et al, 2008; Sonmez et al, 2008;), numerous studies have identified dysregulation of MMP expression in DIGO (Kanno et al, 2008; Sukkar et al, 2007; Sakagami et al, 2006; Dannewitz et al, 2006; Kato et al, 2006, 2005; Gagliano et al, 2005, 2004; Hyland et al, 2003; Cotrim 2002; Silva et al, 2001; Yamada et al, 2000; Bolzani et al, 2000; Kataoka et al, 2000; Sugano et al, 1998).

Justifiably, most of the *in-vitro* studies have been performed on gingival fibroblasts while very few have attempted to investigate growth factor and MMP/TIMP expression in immune cells. It has been previously demonstrated that a significant increase in PDGF-B production in the gingival tissues of both PHT- and CSA-induced gingival overgrowth compared with healthy individuals (Iacopino et al, 1997; Nares et al, 1996). The cellular

source of PDGF-B *in–vivo* was identified to be RM3/1-positive macrophages (Iacopino et al, 1997). More recently, we demonstrated that pretreatment of human macrophage cultures with phenytoin or its metabolite HPPH, prior to challenge with purified LPS from the periodontal patholgen, *Aggregatibacter actinomycetemcomitans*, blunted supernatant levels of MMP-1, MMP-2, MMP-3, MMP-9, and TIMP-1 compared with macrophages exposed to LPS alone suggesting that both phenytoin and its reactive metabolites may inhibit the ability of these cells to contribute to turnover of ECM in gingival tissues (Serra et al, in preparation). In support, Zhou (1996) reported that the cytochrome P450 enzymes, CYP1A1, CYP1A2, CYP2C9, CYP2E1 and CYP3A4 were abundantly present in gingival microsomes of biopsy samples taken from phenytoin-induced gingival overgrowth suggesting that reactive phenytoin metabolites such as HPPH may indeed form locally within gingival tissues. This is important given the prominent role of plaque and gingival inflammation that attract macrophages to affected tissues. Thus, these cells not only secrete growth factors instrumental in collagen synthesis upon exposure to CSA and PHT, their ability to degrade exuberant collagen production via MMP production is significantly blunted by PHT and its metabolite, HPPH (Serra et al, in preparation).

Tumor necrosis factor α (TNF-α), secreted by numerous cells including monocytes and macrophages has been shown to induce cellular proliferation (Sugarman et al, 1985), induce fibroblast production of ECM and inhibit phagocytosis of collagen by gingival fibroblasts resulting in collagen accumulation (Chou et al, 1996). Additionally, it has been shown that TNF-α induces IL-1β (Yucel-Lindberg T, 1995), IL-6, TGF and prostaglandin E_2 production in gingival fibroblasts (Modeer et al, 1992) all of which may positively impact ECM accumulation *in-vivo*. Conversely, TNF-α also has been reported to inhibit collagen

synthesis (Solis-Herruzo et al, 1988) and increase (MMP) synthesis by these same gingival fibroblasts (Domeij et al, 2002), which contributes to gingival breakdown. It remains unclear how these two contrasting functions are mediated by TNF-α (Kato et al, 2006), but the answer may lie in the relative concentration of TNF-α in the gingival tissues. TNF-α concentrations have been reported to range from 1 to 50 ng/ml in chronically inflamed sites (Stashenko et al, 1991), and it has been demonstrated that low levels of TNF-α (<10 ng/ml) induce collagen accumulation and fibrosis, whereas elevated levels result in collagen loss (Chou et al, 1996). Therefore, it appears that gingival fibroblasts exposed to lower levels of TNF-α may be at a higher risk for dysregulation than those exposed to higher levels. Further, spontaneous gingival overgrowth has been reported in patients with long standing low grade chronic gingival inflammation (Kato et al, 2006). Our *in-vitro* studies demonstrate that purified LPS from *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* stimulate human macrophages to produce significantly higher levels of TNF-α compared to untreated controls (Nares et al, 2009; Serra et al, in preparation). However, supernatant levels of TNF-α were significantly decreased in cultures pretreated with PHT, prior to challenge with LPS. This blunted production of TNF-α by inflammatory cells may tip the balance towards collagen accumulation and fibrosis rather than turnover.

IL-6 is a pleotrophic cytokine produced by numerous cells *in-vivo* known to enhance proliferation and regulation of collagen and glycosaminoglycan synthesis by fibroblasts (Wahl, 1985). Studies have reported elevated levels of IL-6 *in-vitro* (Chae et al, 2006; Modéer et al, 2000; Morton et al, 1999) and *in-vivo* (Myrillas et al, 1999; Williamson et al, 1994; Rostock et al, 1986) suggesting that dysregulated expression of IL-6 is associated with DIGO. Moreover, phenytoin has been reported to increase both IL-6 and IL-8 production by

gingival fibroblasts (Modéer et al, 2000). This effect was enhanced by IL-1β implying that the inflammatory milieu present in DIGO provides a positive feedback mechanism for IL-6 expression. Our studies demonstrate that unlike the fibroblast, phenytoin and its metabolite do not stimulate production of IL-6 compared to untreated controls in the absence of LPS. However, unlike TNF-α, supernatant IL-6 levels remain significantly elevated to levels comparable to LPS-only treated cells in the presence of these agents (Serra et al, in preparation). Thus gingival macrophages may contribute to elevated IL-6 levels *in-vivo* enhancing fibroblast proliferation and collagen synthesis.

Conclusion

Drug-induced gingival overgrowth is a multifactorial condition widely recognized clinically but not entirely understood pathophysiologically. DIGO occurs in approximately half of patients taking phenytoin and is closely correlated with a history of inflammation associated with inadequate plaque control. It is understood that gingival tissue homeostasis is maintained by a delicate balance of ECM production and degradation by a collective group of resident (fibroblasts) and transient cells (e.g. macrophages). As such, an increasing body of evidence now implicates the macrophage in the pathophysiology underlying DIGO. These cells differentiate from blood monocytes and are attracted to sites of inflammation such as affected gingival tissues. Further, the presence of cytochrome P450 enzymes implies that reactive metabolites of phenytoin such as HPPH, may form locally within gingival tissues exposing such cell to these toxic agents. Combined, these agents may steer extravasating monocytes to differentiate towards the reparative/proliferative RM3/1

macrophage phenotype with a concomitant increase in PDGF-B secretion and a decrease in MMP production and dysregulated cytokine expression locally. This functionally-altered macrophage state may tip the balance of ECM homeostasis towards one of collagen production and decreased turnover.

Phenytoin and its Metabolite, 5-(p-Hydroxyphenyl-), 5-Phenylhydantoin, Decrease Supernatant Levels of Matrix-Metalloproteases in the Human Macrophage: Implications for Drug-Induced Gingival Overgrowth

Introduction

Drug-induced gingival overgrowth (DIGO) is widely recognized as a common unwanted sequelae associated with a variety of medications. Among these, the antiepileptic agent, phenytoin (Dilantin), has been reported to induce DIGO in approximately 50% of patients taking this medication (Academy Report on Drug Induced Gingival Enlargement 2004, Neville et al,2002). Phenytoin (PHT) is a hydantoin-derivative anticonvulsant that exerts its anticonvulsant properties by stabilizing neuronal cell membranes to the action of sodium, potassium, and calcium. The drug also affects the transport of calcium across cell membranes and decreases the influx of calcium ions across membranes by decreasing membrane permeability and blocking intracellular uptake (Pincus, 1972). Further, one of the metabolites of phenytoin, 5-(4-hydroxyphenyl-), 5-phenylhydantoin (HPPH), may also contribute to the pathogenesis of DIGO (Lin et al, 2008; Ieiri et al, 1995). Zhou (1996) examined the metabolism of PHT by gingival microsomes and reported that the cytochrome P450 enzymes, CYP1A1, CYP1A2, CYP2C9, CYP2E1, and CYP3A4 were abundantly present within gingival specimens. This suggests that reactive PHT metabolites may form

locally within gingival tissues exposing resident and inflammatory cells to these toxic agents triggering and/or sustaining gingival overgrowth.

While most studies have focused on the role of the fibroblast (Seymour el, 2000, 1996; Abergel et al, 1984; Hassell et al, 1976), it is likely that other cells contribute to the pathogenesis of DIGO. In particular, tissue macrophages, present in elevated numbers within gingival tissues, possibly in response to accumulation of the plaque biofim (Penarrocha-Diago et al, 1990; Iacopino et al 1997), may play a role in pathogenesis. These long-lived multifaceted cells, strategically poised along portals of entry, perform numerous functions of vital importance to the host. Macrophages are TLR+ cells that respond rapidly to microbial infection as part of the innate arm of the immune system and aid in the transition to and the propagation of the adaptive immune response (Nares and Wahl, 2005). The macrophage is also recognized as the major mediator of connective tissue turnover, maintenance and repair (Riches, 1988; Andreesen et al, 1990; Messadi and Bertolami, 1991; Martin et al, 1992; Kreutz et al, 1992; Wikesjo et al, 1992), it receives, amplifies, and transmits signals to fibroblasts, endothelial cells, and vascular smooth muscle cells by producing pro-inflammatory cytokines, such as IL-1, IL-6 and TNF-α, as well as MMPs, TIMPs, and peptide growth factors, such as platelet-derived growth factor (PDGF), transforming growth factor (TGF), and basic fibroblast growth factor (bFGF) (Shimokado, 1985). However beyond these essential functions, the macrophage has been implicated in the evolution of pathological processes. Previous related studies indicate that the macrophage responds rapidly and aggressively to LPS from periodontal pathogens by generating chemokines, proteases, and cytokines capable of driving T-helper cell lineage polarization without evidence of corresponding immunosuppressive pathways (Nares et al, 2009, Moutsoupolous

et al, in preparation). Further studies have also demonstrated that the clinical presentation of PHT-induced gingival overgrowth is associated with specific macrophages phenotypes expressing IL-1β and PDGF-B (Iacopino et al, 1997; Nares et al, 1996) suggesting that these cells contribute to the pathogenesis of DIGO. These findings highlight their prominent role in host defense and conversely, progressive tissue pathogenesis evident in periodontal inflammation and gingival overgrowth.

Along with the production of growth factors, cytokines and chemokines, activated macrophages are also capable of producing MMPs and TIMPs. As tissue homeostasis requires the proper balance of metabolism and catabolism of the extracellular matrix, it is possible that macrophage-derived cytokines, MMPs and TIMP levels are altered in response to PHT and HPPH. Here I investigated the effects of these agents on expression of MMPs (MMP1, MMP2, MMP3, MMP9, MMP12), TIMPs (TIMP1, TIMP2, TIMP3, TIMP4) and proinflammatory cytokines (TNF-α, IL-6) in human monocyte-derived macrophages and report that indeed, these agents significantly impact macrophage function and their ability to regulate MMP and cytokine levels in response to periodontopathic LPS.

Materials and Methods

Monocyte Isolation and Differentiation into Macrophages

Peripheral blood mononuclear cells were obtained from buffy coats (Oklahoma Blood Institute, Oklahoma City, OK) derived from healthy donors by density gradient centrifugation using Ficoll-paque (Amersham, Uppsala, Sweden). Six independent cultures were obtained from 6 independent donors. While several studies have shown a gender

component to DIGO, with males being three times more likely to develop this than females (Academy Report on Drug Induced Gingival Enlargement 2004; Seymour et al, 2000; Ellis et al, 1999), limitations within this investigation did not allow me to discriminate between these. Monocytes were isolated using CD14 MicroBeads (Miltenyi Biotec, Auburn, CA) according to manufacturer's instructions and cultured as previously described (Nares et al, 2009; Peng et al, 2007). Briefly, isolated monocytes were plated onto duplicate 12-well tissue culture-treated plates (BD Biosciences, San Jose, CA) at a density of 5 x 10^5 cells/cm^2 in serum-free DMEM (Cellgro, Manassas, VA) containing 50 μg/mL gentamicin (Sigma, St. Louis, MO) at 37 C, 5% CO_2 to promote monocyte attachment. After 2 hours, heat-inactivated fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) was added to a final concentration of 10%. Cells were >95% CD14+ as determined by FACS analysis (data not shown) prior to culture.

Macrophage Stimulation

After 5 days, the media and non-adhered cells were removed and replaced with complete media (DMEM, L-glutamine, gentamycin, 10% FBS). Cultures were incubated at 37°C and 5% CO_2 with media replacement every 2 days. Experiments were initiated upon confirmation of macrophage differentiation after 7 days in culture (Nares, et al, 2009, Nares and Wahl, 2005, Peng et al, 2007). Macrophages were used between day 7 and 10 and pretreated for 1 hour with either: 1) 15μg/mL of PHT-Na+ (Sigma), (serum levels), (Vajda, 1970; Hvidberg and Dam, 1976; Eadie, 1976); 2) 50μg/mL phenytoin-Na+ (high dose); 3) 15μg/mL phenytoin metabolite (Sigma), [5-(4'-hydroxyphenyl), 5-phenylhydantoin, HPPH]; or 4) 50μg/mL HPPH. Untreated cells served as control cultures. Stock solutions of PHT-

Na+ (150 mg/mL) were prepared in sterile deionized water while HPPH (150mg/mL) solutions were prepared in dimethyl sulfoxide (DMSO). Each stock solution of each agent was further diluted prior to use. The total concentration of DMSO in cultures was always less than 0.05%. Concentrations less than 0.1% have been determined not to affect cellular viability and function (Chen et al, 2005; Rival et al, 2004).

To induce MMP expression, macrophages were challenged with 100ng/mL purified LPS in serum-free media from *Aggregatibacter actinomycetemcomitans* (*A.a.*), serotype b, strain Y4, a kind gift from K. Kirkwood (University of South Carolina), for either 4 hours (measure mRNA gene expression) or 24 hours (measure protein levels). Isolation and purification of *A.a.* LPS has been previously described (Rossa et al, 2007). *A.a.* was selected because it is known to, not only colonize periodontal pockets, but also gain access to the circulation through the gingival tissues (Spahr et al, 2006; Scheinkein et al, 2000). This pathogen has also been shown to produce LPS which induces macrophages to produce TNF-α and MMPs (Zadeh et al, 2000). This concentration of LPS was selected based upon results from our pilot study (data not shown), investigating TNF-α and MMP production in response to 1ng/mL, 10ng/mL, 100ng/mL, and 1000ng/mL of *A.a*. For both measures, an asymptotic response was observed at the 100ng/mL concentration, which corresponds with optimal dosage.

Real Time-PCR

After 4 hours of LPS stimulation (5 hours PHT or HPPH exposure), the media was removed and cells immediately lysed using 1 mL Tri Reagent (Invitrogen) and total RNA

isolated according to manufacturer's instructions. This time point was selected based upon previous findings by Nares (unpublished, data not shown) which demonstrate a general down regulation of gene expression at 4 hours with a rebound effect noted at 12 hours. Total RNA concentration was adjusted to 1µg/mL, and cDNA was synthesized using a random hexamer primer (Invitrogen) and Superscript II enzyme (Invitrogen) in a 25µL reaction mix at 42 C for 1 h. The cDNA was quantified using a NanoDrop, and 1µg of each RT product was amplified by real-time PCR in a 20µL reaction mix on an ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). Amplification was performed with TaqMan on-demand gene expression assays for MMP1 (Assay ID: Hs00233958 m1), MMP9 (Assay ID: Mm00600164 g1), TIMP1 (Assay ID: Hs00355335 g1) from Applied Biosystems. GAPDH was used for normalization control. Amplification parameters and conditions were set by the manufacturer. Data were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and results reported as fold changes.

MMP, TIMP Protein Assay

Quantification of supernatant MMP1, MMP2, MMP3, MMP9, MMP12 and TIMP1, TIMP2, TIMP3 and TIMP4 were assayed using the Fluorokine MAP Multiplex Human MMP Panel (R & D, Minneapolis, MN) and the Human TIMP Fluorokine MAP 4-plex Kit (R & D) respectively, on the Luminex 100 System (Luminex Co., Austin, TX). After 24 hours, the media was collected, spun at 12,000 x g, transferred to fresh tubes and stored at -80° C until further use. Six independent experiments were performed from cells derived from six different donors. The assays were performed in 96-well plates, as previously

described (Nares et al, 2009). For MMP determination, microsphere beads coated with monoclonal antibodies against the five different target analytes were added to the wells. For TIMP determination, microsphere beads coated with monoclonal antibodies against the four different target analytes were added to the wells of a separate plate. To remain below the upper level of quantitation, samples containing LPS were diluted 10-fold prior to analysis. This dilution factor was based on our previous pilot studies (data not shown). Samples and standards were pipetted into wells, incubated for 2 hours with the beads then washed using a vacuum manifold (Millipore Corporation). Biotinylated secondary antibodies were added and incubation for 1 h. The beads were then washed and incubated for an additional 30 minutes with streptavidin conjugated to the fluorescent protein, R-phycoerythrin (streptavidin/R-phycoerythrin). The beads were washed and analyzed (a minimum of 50 per analyte) using the Luminex 100 system. The Luminex 100 measures the amount of fluorescence associated with R-phycoerythrin, reported as median fluorescence intensity of each spectral-specific bead allowing it to distinguish the different analytes in each well. The concentrations of the unknown samples (antigens in macrophage supernatants) were estimated from the standard curve using a third-order polynomial equation and expressed as pg/ml after adjusting for the dilution factor. Samples below the detection limit of the assay were recorded as zero. The minimum detectable concentrations for the assays were as follows: MMP-1: 4.4 pg/ml, MMP-2: 25.4 pg/ml, MMP-3: 1.3 pg/ml, MMP-9: 7.4 pg/ml, TIMP-1: 1.54 pg/ml, TIMP-2: 14.7 pg/ml, TIMP-3: 86 pg/ml and TIMP-4: 1.29 pg/ml. All values were standardized for total protein using the Bradford assay (Pierce, Thermo Scientific, Rockford, IL) according to manufacturer's instructions. Briefly, culture supernatants were mixed with assay reagent and incubated for 10 minutes at room temperature in 96 well plates. BSA (Invitrogen) was used

as a standard. The absorbance at 595 nm was read using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA). Control values were arbitrarily used as a baseline measure. The ratio, (control)/(supernatant protein value) was used to normalize each sample based on total protein.

Cytokine Assay

After 24 hours, supernatants were collected and supernatant levels of TNF-α and IL-6 determined by ELISA (RayBiotech, Norcross GA) according to manufacturer instructions. The absorbance at 450 nm was read using a SpectraMax M2 microplate reader (Molecular Devices) with the wavelength correction set at 550 nm. The rated sensitivities of the commercial ELISA kits was 15 pg/mL for TNF-α and 6 pg/ml for IL-6. Values were standardized for total protein using the Bradford assay as described above.

Cell Viability

Viability of macrophages was evaluated using the CellTiter 96 AQueous One Solution Cell Proliferation Assay [3-(4,5- diethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium,inner salt, MTS] assay according to the manufacturer's protocol (Promega, Madison, WI). This colorimetric method can be used to determine the number of viable cells in proliferation or to evaluate cytotoxicity. Briefly, macrophages were cultured in triplicate in 96-well plates and treated with PHT, HPPH and LPS as described above. Unstimulated cells served as control cultures. After 24 h, the cells were incubated

with MTS for 2 h at 37 C, 5% CO_2. The absorbance was read at 490 nm using a SpectraMax M2 microplate reader.

Statistics

Data were analyzed using a hierarchical multiple regression approach relative to LPS, drug and dose. The first tier sought to confirm the biology and establish the validity of the positive control, LPS vs the negative control group. The second tier of this analysis is aimed at determining whether or not PHT or HPPH have an effect on this biologic response. Finally, the third tier sought to contrast dose response and compare one drug with another. Data were expressed as mean ± SEM and compared using a two-tailed Student's *t* test for correlated samples (GraphPad Prism, GraphPad Software, La Jolla, CA). Results were considered statistically significant at $p<0.05$.

RESULTS

PHT and HPPH Inhibit LPS-Induced Supernatant Levels of MMP-1, MMP-2, MMP-3, MMP-9, and TIMP-1 in a Dose-Dependent Manner

Previous studies have determined that PHT plasma levels of 10-20mg/mL are usually necessary to effectively maintain effective seizure control (Vajda, 1970; Hvidberg and Dam, 1976; Eadie, 1976). Thus, the concentrations used in our study represent therapeutic as well as elevated levels of PHT permitting the evaluation of dose on MMP and TIMP production. To rule out the possibility that differences in supernatant levels of these readouts were due to decreased cell viability, I performed a viability assay on cells cultured in each condition. No significant differences were noted in viability in cells exposed to LPS and either dose of PHT

and HPPH or LPS/PHT and LPS/HPPH as determined by MTS assay (data not shown). Further, I standardized the results of each analyte to total protein concentration for each condition using a Bradford assay. As expected, *A.a.* LPS induced a marked increase in supernatant MMP-1, MMP-3, MMP-9, and TIMP-1 levels in each of the six independent cultures after a 24-hour exposure (Figure 6 – 9). In contrast, neither PHT nor HPPH induced any significant change in supernatant levels of these proteins compared to untreated cultures. However, pretreatment of macrophages with PHT or HPPH prior to LPS challenge was associated with reduced supernatant levels of MMP-1, MMP-3, MMP-9 and TIMP-1 when compared with macrophages exposed to LPS alone. A dose-dependent effect was noted as pretreatment with 15µg/mL of PHT prior to LPS challenge reduced MMP-1 and MMP-3 ($p<0.01$) levels to a lesser extent than that noted for 50µg/mL (MMP-1, $p<0.05$; MMP-3, $p<0.001$) (Figure 6 – 9). At this concentration, MMP-9 and TIMP-1 levels were also significantly reduced ($p<0.01$; $p<0.05$, respectively). At 50µg/mL of HPPH, MMP-9 ($p<0.05$) and TIMP-1 ($p<0.05$) were significantly reduced compared to LPS only. Interestingly, HPPH but not PHT was associated with reduced levels of MMP-2 compared to LPS only, but this relationship was not significant. MMP-12 and TIMPs-2-4 remained below levels of detection in all groups and cultures (data not shown).

Supernatant Levels of TNF-α but not IL-6 are Decreased in Response to PHT

At 24 hours, supernatant levels of TNF-α and IL-6 were significantly increased by *A.a.* LPS compared to untreated controls. Similar to MMP and TIMP levels, no significant increases in TNF-α and IL-6 was noted in culture supernatants exposed to either 15 or 50µg/mL PHT and HPPH compared to untreated cells. However, macrophage cultures

pretreated with 50μg/mL PHT then challenged with *A.a.* LPS showed a significant ($p<0.05$) decrease in TNF-α levels compared to LPS only treated cultures. No difference was noted for 15μg/mL of PHT or HPPH at either concentration (Figure 13). Regardless of dosage, pretreatment with PHT and HPPH prior to LPS challenge had no significant effect ($p>0.05$) on supernatant IL-6 levels compared to LPS only treated cultures.

PHT Inhibit LPS-Induced MMP-9 and TIMP-1 mRNA in a Dose-Dependent Manner at 4 hours

At 4 hours, MMP-9 mRNA was markedly upregulated in macrophage cultures challenged with *A.a.* LPS. Exposure of cells to 15μg/mL of PHT or HPPH increased MMP-9 mRNA levels relative to controls. However at 50μg/mL of PHT or HPPH, MMP-9 mRNA levels remained near control levels. Strikingly, pretreatment of cells with either PHT or HPPH prior to LPS challenge resulted in downregulation of MMP-9 mRNA levels to that comparable to control cultures (Figure 11). TIMP-1 mRNA was downregulated in LPS-only cultures as well as PHT and HPPH at either dose. When pre-incubated with these agents then challenged with LPS, TIMP-1 mRNA levels returned to near control levels. Interestingly, the combination of 15μg/ml HPPH plus LPS upregulated TIMP-1 mRNA two-fold compared to controls (Figure 12).

DISCUSSION

Macrophages are involved in a remarkably diverse array of homeostatic processes of vital importance to the host. In addition to their critical role in innate immunity, these cells help orchestrate immune responses within tissues at sites of antigen deposition and/or

pathogen invasion by processing and presenting antigen in the context of MHC class II molecules to effector cells, as well as amplifying the immune response via the production of proinflammatory mediators, cytokines and chemokines (Nares and Wahl, 2005). Macrophages are also widely recognized as ubiquitous mediators of cellular turnover and maintenance of extracellular homeostasis (Riches, 1988; Andreesen et al, 1990; Messadi and Bertolami, 1991; Martin et al, 1992; Kreutz et al, 1992; Wikesjo et al, 1992). However, beyond their essentiality in immunity and tissue homeostasis, the macrophage has also been implicated in the evolution of periodontal pathological processes including periodontal disease and DIGO (Nares et al, 2009; Mousopolous et al, in preparation; Trackman et al, 2004; Nurmenniemi PK et al, 2002; Iacopino et al, 1997; Nares et al, 1996). This investigation posited that macrophage-derived expression of pro-inflammatory cytokines, MMPs and/or TIMP expression is blunted upon exposure to PHT and/or HPPH hindering the ability of these cells to contribute to the fibroblast-mediated degradation of exuberant ECM proteins seen in DIGO. Since plaque-induced gingival inflammation exacerbates the manifestations of PHT-induced DIGO (Majola et al, 2000), I exposed macrophage cultures to LPS from the periodontal pathogen *Aggregatibacter actinomycetemcomitans* and examined levels of proinflammatory cytokines, MMPs and TIMPs. As expected, *A.a.* LPS challenge markedly increased supernatant levels of TNF-α, IL-6, MMP-1, MMP-3, MMP-9, as well as TIMP-1, in agreement with previous studies (Bodet et al, 2006; Woo et al, 2004; Zhang et al, 1998). This highlights the responsiveness of these cells to toxins from periodontopathic organisms and confirms the capability of these cells to produce matrix-degradating enzymes.

MMP-1 is recognized as an important mediator of connective tissue remodeling reported to be present at high concentrations in inflamed gingiva (Ryan and Golub, 2000).

Sonmez (2008) recently compared MMP-1 levels between gingival fibroblasts derived from patients with cyclosporine-A (CSA)-induced gingival overgrowth to those derived from patients taking CSA but without overgrowth. This study found no difference in MMP-1 expression between the groups although no comparisons were made between gingival biopsies derived from periodontally-healthy subjects not taking CSA. Sukkar (2007) reported that fibroblasts derived from CSA-induced gingival overgrowth produced significantly lower levels of MMP-1 than fibroblasts derived from healthy subjects. Further, when fibroblast cultures were challenged with interleukin-1 and oncostatin M, a significant increase in the upregulation of MMP-1 was noted which was blunted when cyclosporin or nifedipine were added to the cell cultures (Sukkar et al, 2007). In our study, supernatant MMP levels, including MMP-1 from human macrophages did not demonstrate any difference in response to PHT and HPPH at either dose compared to untreated cultures. However, when cultures were pretreated with these agents prior to LPS challenge, the extent of the drug-induced blunting of MMP levels became evident. After 24 hours, supernatant levels of several MMPs were significantly decreased relative to LPS-only cultures in a dose-dependent manner suggesting that local elevations of PHT and HPPH concentrations within gingival tissues mitigate the macrophage's ability to degrade ECM proteins by limiting its natural response to produce metalloproteinases. Such a dose response is consistent with other studies which have demonstrated, not only a similar effect on MMP-1 and MMP-3 at the protein and mRNA level (Sugano et al. 1998, Thomason et al. 1998, Bolzani et al. 2000, Kataoka et al. 2000, Yamada et al. 2000, Hyland et al. 2003), but also that a threshold of serum concentration of CSA helps to govern this mechanism (McGaw et al. 1987; Pan et al. 1992; Pernu et al. 1992a; King et al. 1993; O'Valle et al. 1994; Thomason et al. 1995). For cyclosporine, this

threshold is generally regarded as 200µg/L (Thomason et al, 2005). Further, expression of MMP-9 mRNA at 4 hours was dramatically elevated upon LPS exposure but severely blunted when cells were pretreated with PHT or HPPH prior to LPS challenge. Increased supernatant levels of MMPs at 24 hours may result from a rebound effect relative to 4 hours as our microarray data has demonstrated wholesale downregulation of gene expression at 4 hours but rebounding of mRNA levels at 12 hours albeit at a suppressed level relative to untreated controls (Nares, unpublished findings).

MMP activity is counteracted by the actions of TIMPs. Here I report that exposure of macrophages to *A.a.* LPS was associated with an increase in TIMP-1 production while exposure to high concentration (50µg/mL) of PHT and HPPH, on the other hand, significantly reduced TIMP-1 levels. This finding is consistent with *in-vivo* and *in-vitro* evaluation of gingival fibroblasts which reported a relative reduction in MMP-1 and MMP-8/TIMP-1 serum and GCF concentration in CSA- associated gingival overgrowth subjects compared with healthy controls (Eminigil et al, 2008; Gagliano et al, 2005). This reflects more a decrease in MMP production rather than an increase in TIMP. In fact, this corresponds with our findings in that supernatant levels of TIMP-1 in samples treated with both *A.a.* LPS and 50µg/mL PHT or HPPH were not significantly different relative to untreated controls. This demonstrates that, much like MMP expression, the exposure of macrophages to these agents blunts the LPS-induced expression of TIMP-1 implying that the effects of PHT and HPPH are not necessary protein or gene specific, but merely affect a common pathway associated with either gene expression or post-transcriptional regulation associated with both MMP and TIMP production. Regardless, the net effect is based on the relative ratios of MMP and TIMP. When MMP levels decrease or TIMP levels increase, the

turnover of ECM diminishes, potentially leading to an exuberant accumulation of these proteins which may affect the macrophage's synergistic relationship with the fibroblast in this pathologic process.

The overall upregulation of TNF-α observed in all samples stimulated with LPS corresponds with observations by Zhang et al (1998) and Saren et al (1996), who noted a transition to an inflammatory phenotype when provoked with similar stimuli. This proinflammatory cytokine has been shown to induce interstitial MMP-1 (Domeij et al, 2002; Birkedal-Hansen et al, 1993) and MMP-9 (Saren et al, 1996) production by fibroblasts. Similar to supernatant MMP levels, neither PHT nor HPPH at 15μg/mL induced TNF-α or IL-6 levels beyond that seen in control cultures. I did however, note a significant decrease in TNF-α ($p<0.05$) when macrophages were also exposed to high concentrations (50μg/mL) of PHT but not HPPH. It is interesting to note that high concentrations of PHT suppressed TNF-α production in our study, whereas HPPH appeared to have no effect. Metabolism of PHT to HPPH by gingival cytochrome P450 enzymes may override suppression of TNF-α locally resulting in elevated levels of this cytokine. This is of particular relevance, as TNF-α has been shown to stimulate fibroblast and macrophage production of MMPs, collagenases, gellatenases, and stromolysins as part of the mechanism for inducing increased ECM turnover (Zhou et al, 2009; Wang et al, 2009). This inflammatory response may play a role in multiple mechanisms, including maintenance of ECM homeostasis, inflammatory cell migration, and tumor cell motility and invasion (Kim et al, 2009; Leber et al, 1998; Rosen et al, 1991). This mechanism has been corroborated, as the addition of enteracept, a TNF-α inhibitor, has been shown to cause a downregulation of MMP expression (Kim et al, 2009).

These findings are in agreement with studies by Kato (2005) which documented a synergistic effect of TNF-α and PHT on decreasing cellular endocytosis and degredation of collagen by MMPs in human gingival fibroblasts. Studies by Meikle (1989) and Brew (2000) have also demonstrated that PHT or TNF-α can decrease MMP but increase TIMP production within human gingival tissues. When combined, there was a synergistic effect as well as a suppression of collagen degredation.

IL-6 is a proinflammatory cytokine secreted by numerous cells including T cells and macrophages in response to inflammatory or traumatic stimuli. IL-6 appears to target connective tissue cells such as fibroblasts by enhancing proliferation and by exerting a positive effect on collagen and glycosaminoglycan synthesis (Ramsden et al, 1992; Snow et al, 1987**)**. I note that *A.a.* LPS significantly ($p < 0.0001$) increased IL-6 supernatant levels compared to control cultures. These levels remained elevated despite pretreated with PHT or HPPH suggesting that IL-6 primarily responds to proinflammatory stimuli but not necessarily to these agents. This increase in IL-6 corresponds with previous observations within gingival samples taken from CSA-induced (Drozdzik et al, 2004; Myrillas et al, 1999) and PHT induced gingival overgrowth patients (Bunduneli N et al, 2000). Atilla (1998), further noted that GCF concentrations of IL-6 corresponded more with the level of gingival inflammation of CsA induced DIGO patients, compared with the presence of the drug itself. They noted that IL-6 levels were significantly higher in inflamed CsA treated patients, not exhibiting DIGO compared to uninflamed CsA treated patients that did exhibit DIGO. Furthermore, the presence or absence of CsA appeared not to affect the overall IL-6 GCF concentration. This corroborates our findings, indicating that MAC production of IL-6 is most likely linked more

to an inflammatory response, and has very little to do with the presence or absence of PHT or CsA.

In conclusion, the present results demonstrate that not only PHT but its metabolite, HPPH inhibit *A.a.* LPS-induced levels of MMPs in a dose-dependent manner and that a high concentration of PHT decrease TNF-α but not IL-6 levels in the human macrophage. Given that the degree of inflammatory infiltrate and fibrosis appears to be influenced by the quality of plaque control and the presence of macrophages in affected tissues, our data reveals a mechanism whereby PHT and HPPH preferentially dysregulate production of macrophage-derived TNF-α but not IL-6 in response to the plaque biofilm enhancing collagen production by fibroblasts but without the concomitant production of MMPs in response to bacterial stimulation.

FIGURE 1

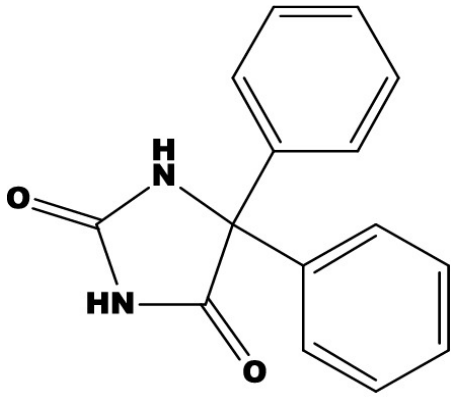

Figure 1 – Structure of PHT

Page reference – 2

FIGURE 2

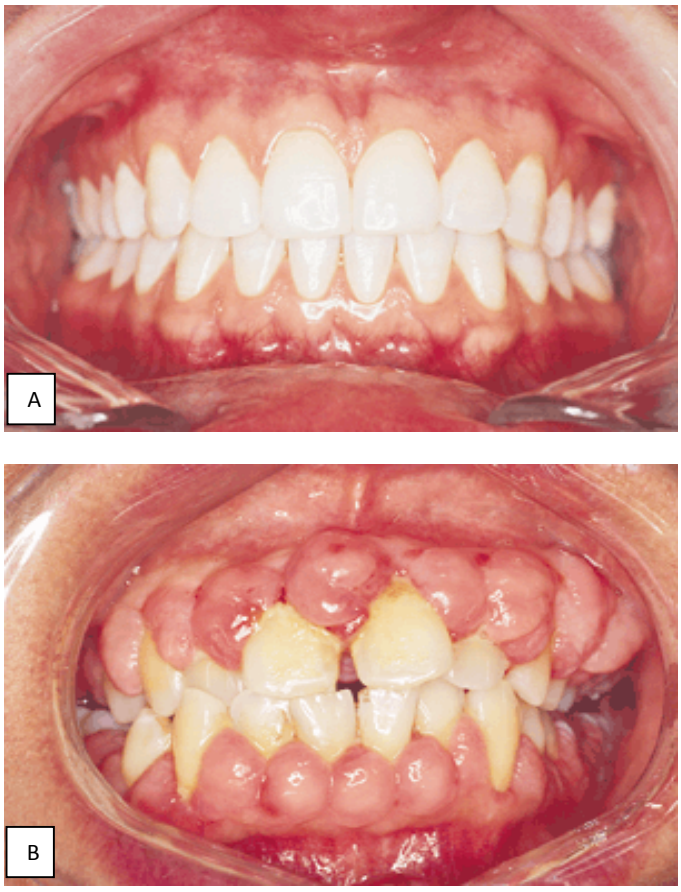

Figure 2 – A. Normal gingival contour characterized by scalloped, knife-edged contour, pink color, and stippling B. PHT-induced gingival overgrowth, characterized by marginal erythema, lobulated papillae, and plaque accumulation.

Page reference – 4

FIGURE 3

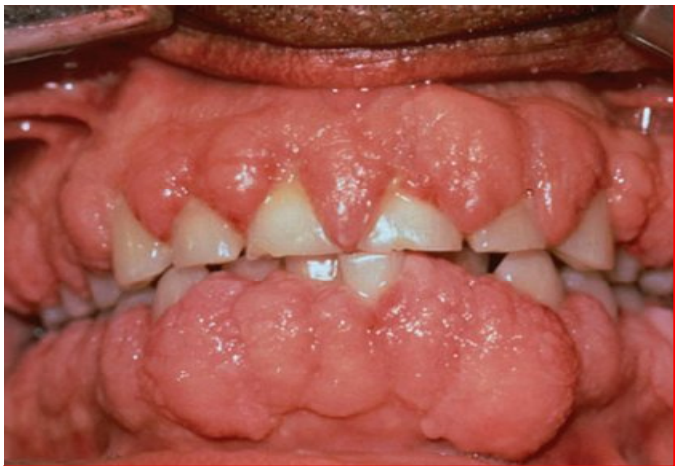

Figure 3 – CSA-induced gingival overgrowth demonstrating lobulations with a pebbly-like appearance

Page reference – 4

FIGURE 4

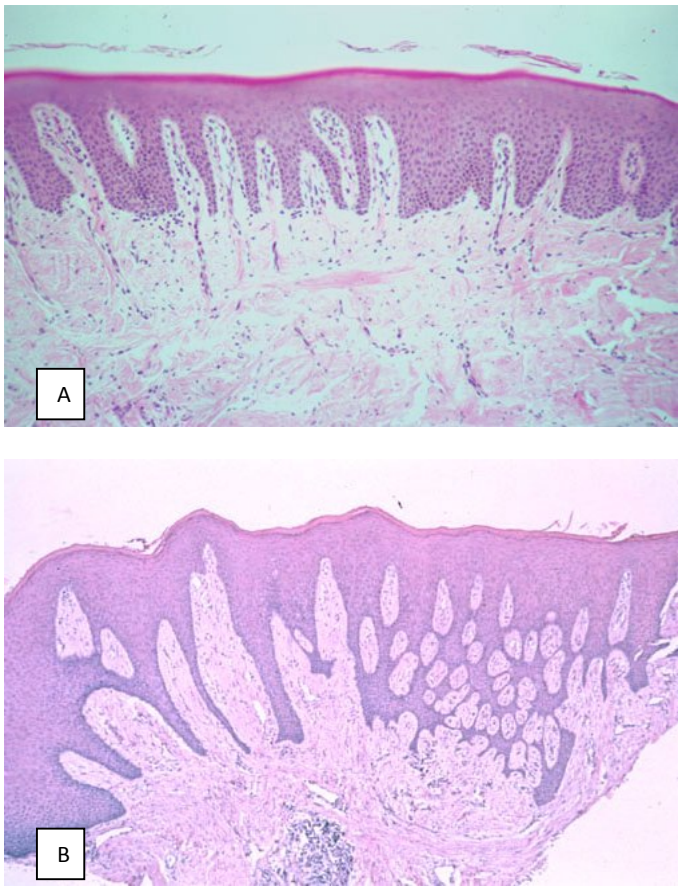

Figure 4 –A. Histological appearance of normal gingiva is characterized by uniform, shallow rete ridge formations, mild inflammatory infiltrate, and normal ECM accumulations B. Gingiva from drug-induced overgrowth is characterized by elongated rete ridges, abundant inflammatory infiltrate, and increased accumulations of ECM.

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FIGURE 5

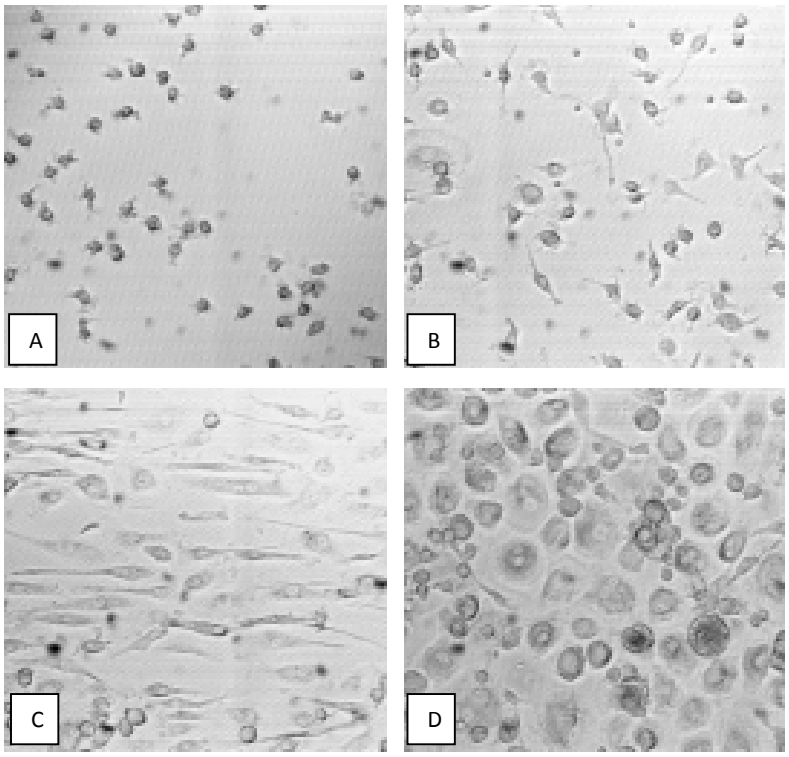

Figure 5 – Macrophages display morphologically distinct characteristics after 13 days in culture depending on plating surface. Peripheral blood monocytes were isolated and plated onto culture dishes coated with either: A. collagen IV, B. fibronectin, C. collagen I, and D. untreated plastic. (Nares, unpublished findings)

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FIGURE 6

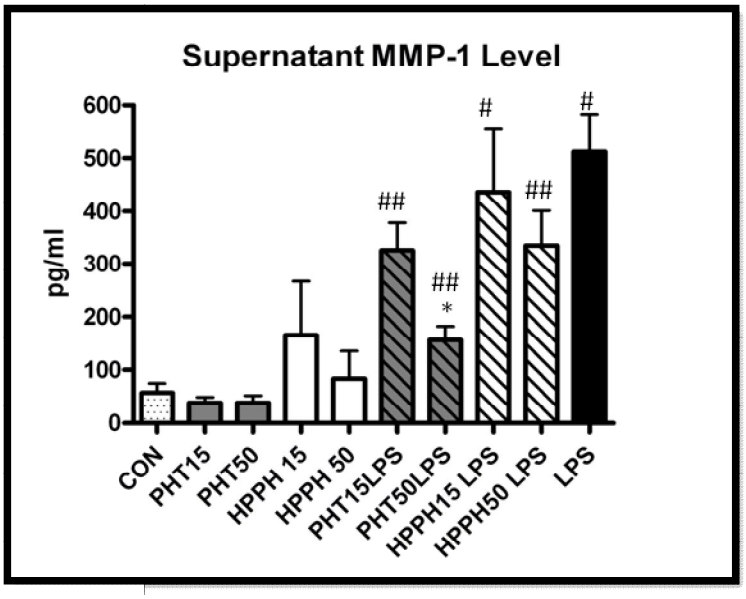

Figure 6 – Analysis of MMP supernatant protein level was completed using Fluorokine MAP Multiplex Human MMP Panel (R & D, Minneapolis, MN) and the Luminex 100 System (Luminex Co., Austin, TX). The expression was compared relative to the negative control (no treatment), and a positive control (*A.a.* LPS challenge alone). Samples were pretreated with PHT or HPPH at indicated concentrations (μg/mL) then challenged with *A.a.* LPS where indicated. Samples were collected at 24 hours.

Legend

Compared to LPS, * $p<0.05$, ** $p<0.01$, *** $p<0.001$
Compared to CON, # $p<0.05$, ## $p<0.01$, ### $p<0.001$
Student *t*-test, N=6 independent donors

FIGURE 7

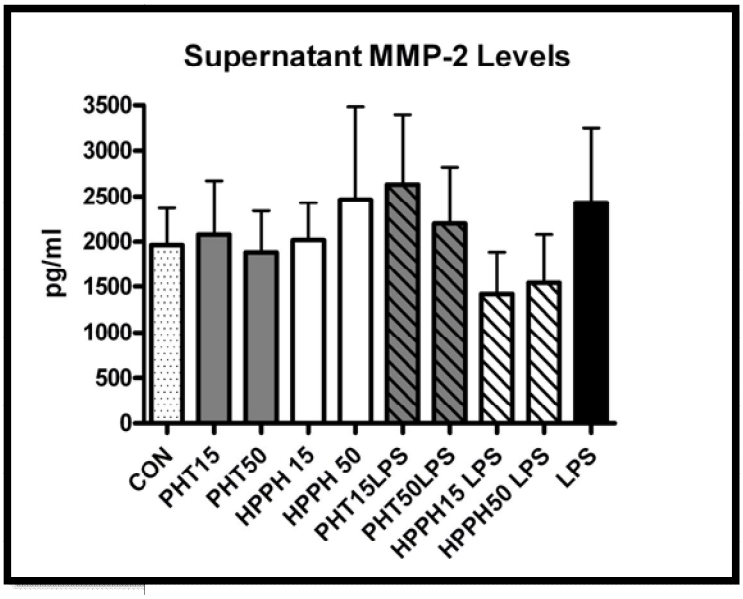

Figure 7 – LPS produces no significant effect on MMP-2 expression when compared with Control. Furthermore, the concomitant exposure to PHT or HPPH had no significant effect on MMP-2 expression. Analysis of MMP supernatant protein level was completed using Fluorokine MAP Multiplex Human MMP Panel (R & D, Minneapolis, MN) and the Luminex 100 System (Luminex Co., Austin, TX). The expression was compared relative to the negative control (no treatment), and a positive control (*A.a.* LPS challenge alone). Samples were pretreated with PHT or HPPH at indicated concentrations (µg/mL) then challenged with *A.a.* LPS where indicated. Samples were collected at 24 hours.

Legend

Compared to LPS, * $p<0.05$, ** $p<0.01$, *** $p<0.001$
Compared to CON, # $p<0.05$, ## $p<0.01$, ### $p<0.001$
Student *t*-test, N=6 independent donors

FIGURE 8

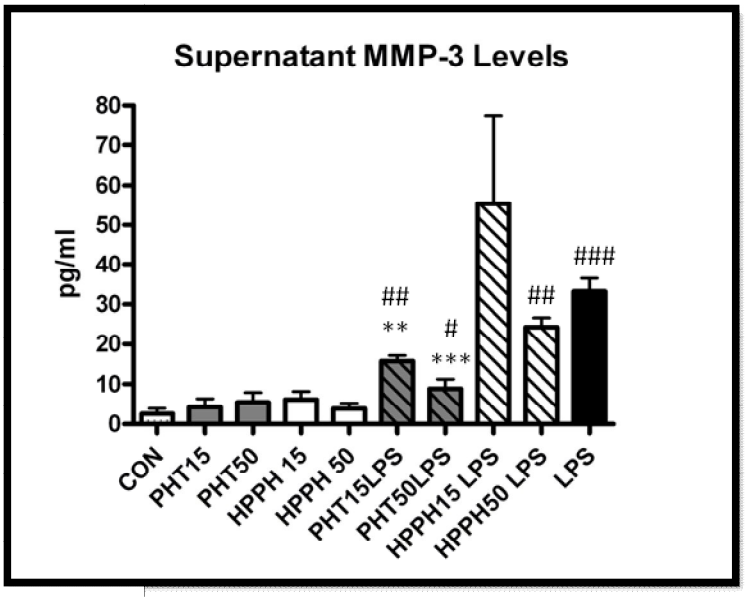

Figure 8 – LPS significantly increased MMP-3 expression. However, pretreatment of MACs with 50µg/mL and 15µg/mL of PHT prior to LPS challenge decreased supernatant MMP-3 levels compared to LPS-only treated cells. The addition of 50µg/mL HPPH demonstrated a similar trend. Analysis of MMP supernatant protein level was completed using Fluorokine MAP Multiplex Human MMP Panel (R & D, Minneapolis, MN) and the Luminex 100 System (Luminex Co., Austin, TX). The expression was compared relative to the negative control (no treatment), and a positive control (*A.a.* LPS challenge alone). Samples were pretreated with PHT or HPPH at indicated concentrations (µg/mL) then challenged with *A.a.* LPS where indicated. Samples were collected at 24 hours.

Legend

Compared to LPS, * $p<0.05$, ** $p<0.01$, *** $p<0.001$
Compared to CON, # $p<0.05$, ## $p<0.01$, ### $p<0.001$
Student *t*-test, N=6 independent donors

FIGURE 9

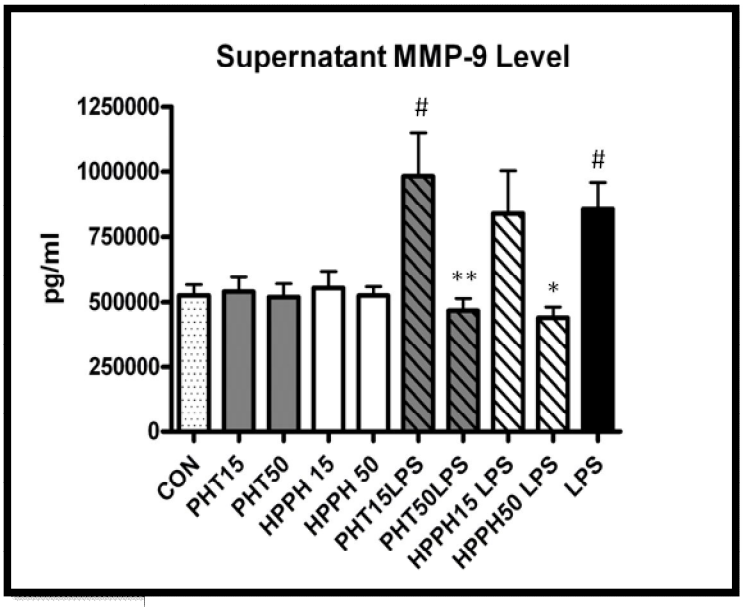

Figure 9 – LPS significantly increased MMP-9 expression. This increase was not observed in MACs pretreated with 50µg/mL of PHT and 50µg/mL of HPPH prior to LPS challenge. Analysis of MMP supernatant protein level was completed using Fluorokine MAP Multiplex Human MMP Panel (R & D, Minneapolis, MN) and the Luminex 100 System (Luminex Co., Austin, TX). The expression was compared relative to the negative control (no treatment), and a positive control (*A.a.* LPS challenge alone). Samples were pretreated with PHT or HPPH at indicated concentrations (µg/mL) then challenged with *A.a.* LPS where indicated. Samples were collected at 24 hours.

Legend

Compared to LPS, * $p<0.05$, ** $p<0.01$, *** $p<0.001$
Compared to CON, # $p<0.05$, ## $p<0.01$, ### $p<0.001$
Student *t*-test, N=6 independent donors

FIGURE 10

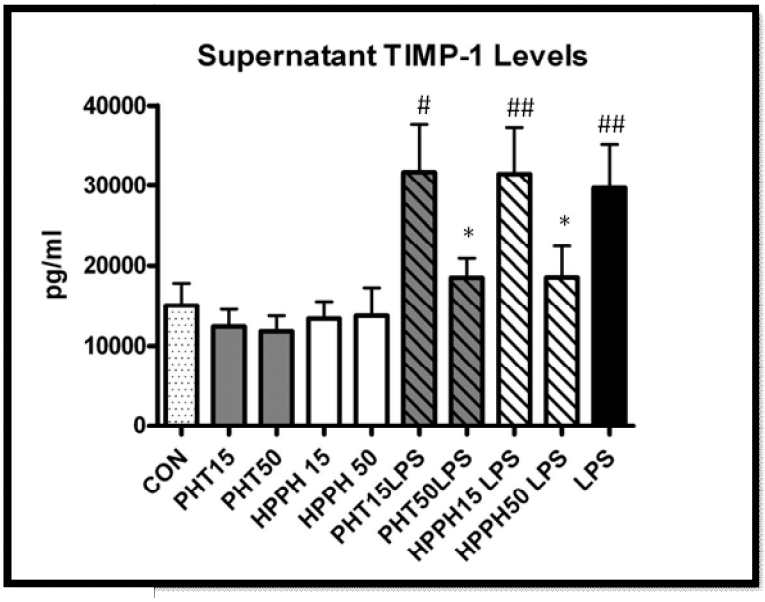

Figure 10 – LPS significantly increased TIMP-1 expression. This increase was not observed in MACs pretreated with 50µg/mL of PHT and 50µg/mL of HPPH prior to LPS challenge. Analysis of TIMP-1 supernatant protein level was completed using Fluorokine MAP Multiplex Human MMP Panel (R & D, Minneapolis, MN) and the Luminex 100 System (Luminex Co., Austin, TX). The expression was compared relative to the negative control (no treatment), and a positive control (*A.a.* LPS challenge alone). Samples were pretreated with PHT or HPPH at indicated concentrations (µg/mL) then challenged with *A.a.* LPS where indicated. Samples were collected at 24 hours.

Legend

Compared to LPS, * $p<0.05$, ** $p<0.01$, *** $p<0.001$
Compared to CON, # $p<0.05$, ## $p<0.01$, ### $p<0.001$
Student *t*-test, N=6 independent donors

FIGURE 11

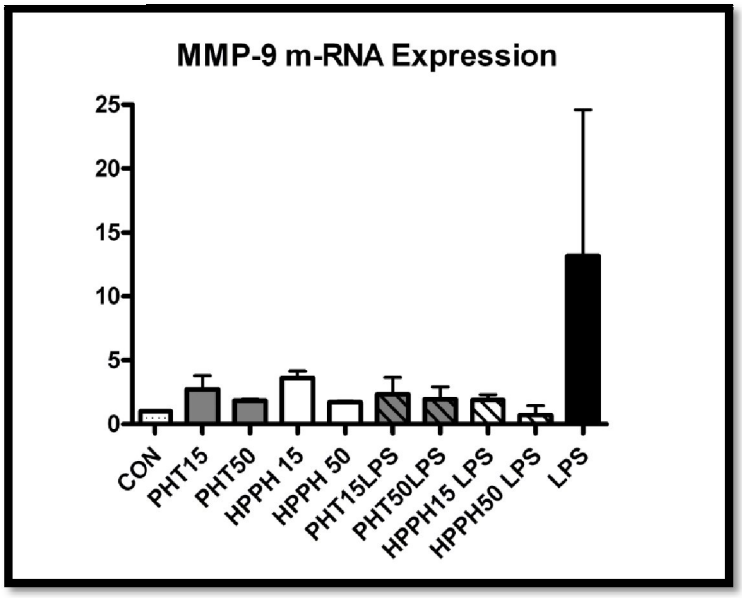

Figure 11 – LPS produced a marked increase in MMP-9 expression. This expression, however, appeared to be mitigated by the addition of PHT and HPPH, returning MMP-9 mRNA expression to CON levels. Analysis of MMP mRNA was completed by Real-Time PCR (Taqman, Applied Biosciences, Foster City, CA). The expression was compared relative to the negative control (no treatment), and a positive control (*A.a.* LPS challenge alone). Samples were pretreated with PHT or HPPH at indicated concentrations (µg/mL) then challenged with *A.a.* LPS where indicated. Samples were collected at 4 hours.

Legend

Compared to LPS, * $p<0.05$, ** $p<0.01$, *** $p<0.001$
Compared to CON, # $p<0.05$, ## $p<0.01$, ### $p<0.001$
Student *t*-test, N=6 independent donors

FIGURE 12

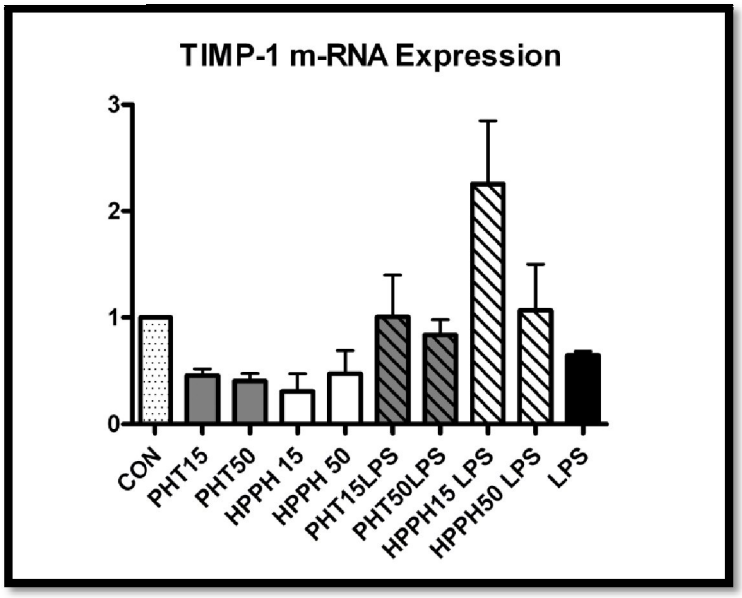

Figure 12 – LPS appeared not to have a profound impact on TIMP-1 mRNA expression. However, the combination of PHT/HPPH with LPS appeared to produce a greater level of TIMP-1 mRNA expression. Analysis of MMP mRNA was completed by Real-Time PCR (Taqman, Applied Biosciences, Foster City, CA). The expression was compared relative to the negative control (no treatment), and a positive control (*A.a.* LPS challenge alone). Samples were pretreated with PHT or HPPH at indicated concentrations (μg/mL) then challenged with *A.a.* LPS where indicated. Samples were collected at 4 hours.

Legend

Compared to LPS, * $p<0.05$, ** $p<0.01$, *** $p<0.001$
Compared to CON, # $p<0.05$, ## $p<0.01$, ### $p<0.001$
Student *t*-test, N=6 independent donors

FIGURE 13

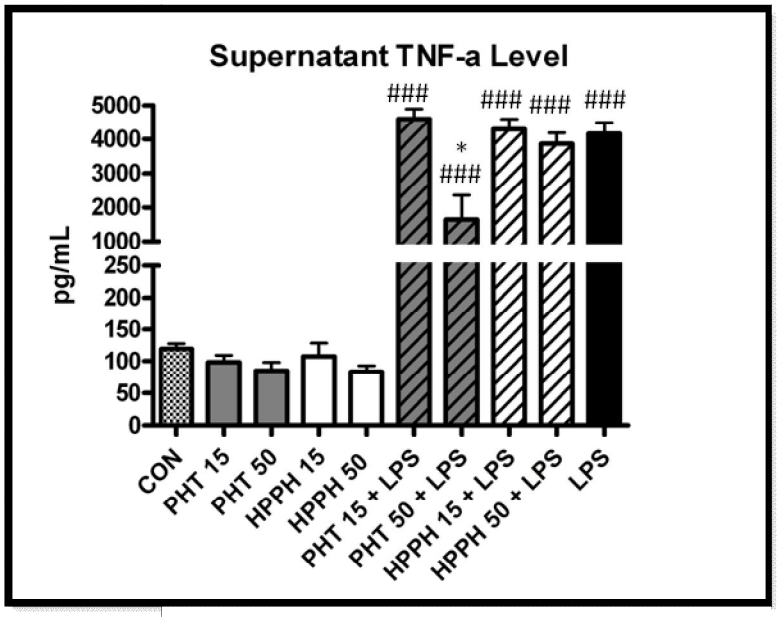

Figure 13 – LPS significantly increased TNF-α expression. This increase was mitigated by the addition of PHT 50μg/mL. HPPH had a minimal apparent effect. Supernatant levels of TNF-α was determined by enzyme-linked immunosorbent assay (ELISA) (RayBiotech, Norcross GA) after 24 hour incubation. Values were standardized for total protein using the Bradford assay. The expression was compared relative to the negative control (no treatment), and a positive control (*A.a.* LPS challenge alone). Samples were pretreated with PHT or HPPH at indicated concentrations (μg/mL) then challenged with *A.a.* LPS where indicated. Samples were collected at 24 hours.

Legend

Compared to LPS, * $p<0.05$, ** $p<0.01$, *** $p<0.001$
Compared to CON, # $p<0.05$, ## $p<0.01$, ### $p<0.001$
Student *t*-test, N=6 independent donors

FIGURE 14

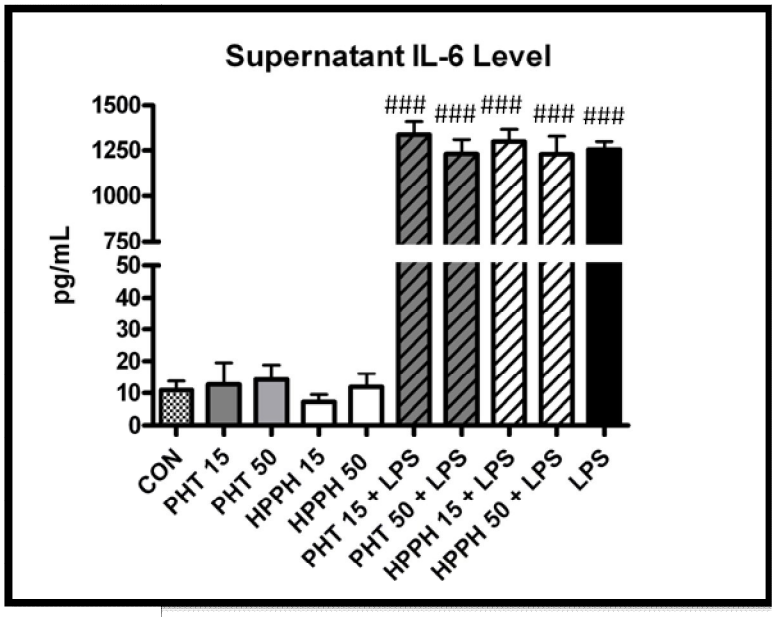

Figure 14 – LPS significantly increased TNF-α expression. The addition of PHT and HPPH had a minimal apparent effect. Supernatant levels of IL-6 was determined by enzyme-linked immunosorbent assay (ELISA) (RayBiotech, Norcross GA) after 24 hour incubation. Values were standardized for total protein using the Bradford assay. The expression was compared relative to the negative control (no treatment), and a positive control (*A.a.* LPS challenge alone). Samples were pretreated with PHT or HPPH at indicated concentrations (μg/mL) then challenged with *A.a.* LPS where indicated. Samples were collected at 24 hours.

Legend

Compared to LPS, * $p<0.05$, ** $p<0.01$, *** $p<0.001$
Compared to CON, # $p<0.05$, ## $p<0.01$, ### $p<0.001$
Student *t*-test, N=6 independent donors

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