การแสดงออกของอินโดลเอมีน 2,3-ไดออกซีจีเนส ของเซลล์ไฟโบรบลาสต์ในเนื้อเยื่อเหงือกของมนุษย์

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INDOLEAMINE 2,3-DIOXYGENASE EXPRESSION IN HUMAN GINGIVAL FIBROBLASTS

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for the Degree of Master of Science Program in Periodontics

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Field of study	Periodontics
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จิตติมา มะกรูดทอง : การแสดงออกของอินโดเอมีน 2,3-ไดออกซีจีเนส ของเซลล์ไฟโบรบลาสต์ในเนื้อเยื่อเหงือกของมนุษย์ (INDOLEAMINE 2,3-DIOXYGENASE EXPRESSION IN HUMAN GINGIVAL FIBROBLASTS) อ. ที่ปรึกษา: อ.ทพญ.ดร. กนกวรรณ นิสภกุลธร, 64 หน้า.

อินโดลเอมีน 2,3-ไดออกซีจีเนส (ไอดีโอ) เป็นเอนไซม์ซึ่งทำหน้าที่ย่อยสลายกรดอะมิโนทริปโตเฟนและมีคุณสมบัติในการยับยั้งการตอบสนองของเซลล์ในระบบภูมิคุ้มกัน โรคปริทันต์อักเสบเป็นโรคที่มีลักษณะการอักเสบเรื้อรังอันก่อให้เกิดการทำลายอวัยวะปริทันต์และการสูญเสียฟันตามมา เซลล์ไฟโบรบลาสต์เป็นเซลล์ซึ่งเป็นองค์ประกอบส่วนใหญ่ในเนื้อเยื่อปริทันต์ มีบทบาทสำคัญในการสร้างและการคงสภาพของเนื้อเยื่อยึดต่อและยังทำหน้าที่ควบคุมการตอบสนองการอักเสบที่เกิดขึ้นในเหงือก การวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาการควบคุมการแสดงออกของอินโดลเอมีน 2,3-ไดออกซีจีเนสของเซลล์ไฟโบรบลาสต์ในเนื้อเยื่อเหงือกปกติของมนุษย์ โดยการกระตุ้นด้วยไซโตไคน์และสารสื่อการอักเสบ ประเมินผลการกระตุ้นจากการผลิตอาร์เอ็นเอนำรหัส (mRNA) โดยวิธีปฏิกิริยาโพลิเมอเรสลูกโซ่แบบผันกลับ (Reverse transcriptase-polymerase chain reaction) และประเมินการทำงานของเอนไซม์ด้วยวิธีสเปกโตรโฟโตเมทริก (spectrophotometric) ผลการวิจัยพบว่า ในสภาวะปกติเซลล์ไฟโบรบลาสต์ไม่มีการผลิตเอนไซม์ไอดีโอ และอินเตอร์เฟียรอน-แกมม่า (IFN-γ) สามารถกระตุ้นเซลล์ไฟโบรบลาสต์ให้มีการผลิตอาร์เอ็นเอนำรหัสของเอนไซม์ไอดีโอได้อย่างเด่นชัด ขณะที่ อินเตอร์ลิวคิน-1เบต้า (IL-1β), ทูเมอร์เนโครซิสแฟคเตอร์-แอลฟา (TNF-α) รวมทั้งไลโปโปลิแซคคาไรด์ (lipopolysaccharides; LPS) จาก *P. gingivalis* สามารถกระตุ้นการผลิตอาร์เอ็นเอนำรหัสของเอนไซม์ไอดีโอในเซลล์ไฟโบรบลาสต์ได้ในระดับต่ำๆ และมีแนวโน้มว่าผลการกระตุ้นแปรผันตามความเข้มข้นของสารกระตุ้นที่เพิ่มขึ้น การกระตุ้นเซลล์ไฟโบรบลาสต์ด้วยอินเตอร์ลิวคิน-1เบต้า, ทูเมอร์เนโครซิสแฟคเตอร์-แอลฟา รวมทั้งไลโปโปลิแซคคาไรด์ จาก *P. gingivalis* ร่วมกับอินเตอร์เฟียรอน-แกมม่าพบว่าให้ผลกระตุ้นการผลิตอาร์เอ็นเอนำรหัสที่เพิ่มขึ้น เมื่อเปรียบเทียบกับการกระตุ้นด้วยอินเตอร์เฟียรอน-แกมม่าเพียงชนิดเดียว สำหรับผลการกระตุ้นการทำงานของเอนไซม์ พบว่าอินเตอร์เฟียรอน-แกมม่า สามารถกระตุ้นเซลล์ไฟโบรบลาสต์ให้มีการทำงานของเอนไซม์ได้อย่างมีนัยสำคัญ ขณะที่ไม่พบการเพิ่มขึ้นของการทำงานของเอนไซม์ เมื่อกระตุ้นด้วยอินเตอร์ลิวคิน-1เบต้า, ทูเมอร์เนโครซิสแฟคเตอร์-แอลฟา และไลโปโปลิแซคคาไรด์จาก *P. gingivalis* แต่อินเตอร์ลิวคิน-1เบต้า, ทูเมอร์เนโครซิสแฟคเตอร์-แอลฟา สามารถเพิ่มผลการทำงานของเอนไซม์ไอดีโอ เมื่อกระตุ้นร่วมกับอินเตอร์เฟียรอน-แกมม่า จากผลการวิจัยนี้สรุปได้ว่า การแสดงออกของเอนไซม์อินโดลเอมีน 2,3-ไดออกซีจีเนส จากเซลล์ไฟโบรบลาสต์นั้น สามารถถูกควบคุมจากไซโตไคน์หรือสารสื่อการอักเสบได้หลายชนิด โดยการเหนี่ยวนำให้มีการแสดงออกของเอนไซม์ดังกล่าว อาจมีบทบาทในกลไกการดำเนินโรคในโรคปริทันต์อักเสบ

ภาควิชา .....ปริทันตวิทยา..... ลายมือชื่อนิสิต.....

สาขาวิชา.....ปริทันตศาสตร์..... ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา.....2550..... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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KEY WORD: HUMAN GINGIVAL FIBROBLASTS / INDOLEAMINE 2,3-DIOXYGENASE / PERIODONTITIS / IMMUNOPATHOGENESIS

JITTIMA MAKRUDTHONG: INDOLEAMINE 2,3-DIOXYGENASE EXPRESSION IN HUMAN GINGIVAL FIBROBLASTS. THESIS ADVISOR: KANOKWAN NISPAKULTORN, Ph.D, 64 pp.

Indoleamine 2,3-dioxygenase (IDO) is an intracellular tryptophan-metabolizing enzyme with immunosuppressive characteristics. Periodontitis is the chronic inflammatory disease that destroys the tooth-supporting structures. Gingival fibroblasts are major cell types in periodontal tissues which play a crucial role in maintaining connective tissue integrity and regulating local inflammatory responses. The purpose of this research is to study the regulation of IDO expression in primary cultures of human gingival fibroblasts (HGF) from healthy gingival tissues. HGF cells were treated with inflammatory cytokines and mediators. IDO mRNA expression and enzymatic activity was determined by reverse transcription-polymerase chain reaction (RT-PCR) and by spectrophotometric method, respectively. It was shown that HGF cells did not constitutively express IDO. IFN-γ strongly induced IDO expression in HGF cells. IL-1β, TNF-α, as well as lipopolysaccharides (LPS) from *P.gingivalis* were able to induce IDO expression, however, at a lower extent. IFN-γ, TNF-α, and *P. gingivalis* LPS appeared to induce IDO expression in a dose-dependent manner. Stimulation of HGF cells with combination between IFN-γ and other agents resulted in increased expression of IDO, as compared to IFN-γ alone. IDO activity was significantly increased in HGF treated with IFN-γ. HGF cells treated with IL-1β, TNF-α, and *P. gingivalis* LPS did not show significant increased in IDO activity. Combinations of IFN-γ and IL-1β as well as IFN-γ and TNF-α significantly increased IDO activity, as compared to that of IFN-γ alone. In conclusion, we showed that IDO expression and activity in HGF cells was regulated by several inflammatory cytokines and mediators. Induction of IDO expression in HGF cells by these mediators may play a role in the pathogenesis of periodontal diseases.

Department .....Periodontology..... Student's signature.....

Field of study.....Periodontics..... Advisor's signature.....

Academic year....2007..... Co-advisor's signature.....

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LIST OF ABBREVIATIONS

A. actinomycetemcomitans	*Aggregatibacter actinomycetemcomitans*
CTLA4	cytotoxic T-lymphocyte-associated protein 4
C. Trachomatis	*Chlamydia Trachomatis*
C. psittaci	*Chlamydia psittaci*
C. pneumoniae	*Chlamydia pneumoniae*
DMEM	Dulbecco's modified Eagle's medium
DC	dendritic cells
FCS	fetal calf serum
GAPDH	glyceraldehyde-3–phosphate dehydrogenase
GAS	gamma-activated sequence
HGF	human gingival fibroblasts
HC	hydrocortisone
HPLC	high pressure liquid chromatography
IDO	Indoleamine 2,3-dioxygenase
IFN	interferon
IL	interleukin
IRF1	interferon-regulatory factor1
ISRE	interferon-stimulated response elements
KYN	kynurenine
LPS	lipopolysaccharide
MLR	mixed lymphocyte reaction
MMPs	matrix metalloproteinases
1-MT	1-methyl-D- tryptophan
NAD	nicotinamide adenine dinucleotide
P. gingivalis	*Porphyromonas gingivalis*
PBMC	peripheral blood mononuclear cells
PGE	prostaglandin
RT-PCR	reverse transcriptase–polymerase chain reaction
SEM	standard error of mean

STAT1	signal transducer and activator of transcription 1
T. forsythia	*Tanerella forsythia*
TDLNs	tumor-draining lymph nodes
TDO	L-tryptophan 2,3-dioxygenase
TGF	transforming growth factor
TNF	tumor necrosis factor

CHAPTER I

INTRODUCTION

Background and significance

Indoleamine 2,3–dioxygenase (IDO) is an enzyme that metabolized the amino acid tryptophan. Tryptophan is the least abundant essential amino acid for mammals. It is utilized for protein synthesis as well as the synthesis of a neurotransmitter, serotonin. The majority of tryptophan is metabolized along the kynurenine (Kyn) pathway, leading to the synthesis of nicotinamide adenine dinucleotide (NAD) or the complete oxidation of the amino acid (Takikawa, 2005). IDO was expressed in many cell types including monocytes, macrophages, dendritic cells, fibroblasts, epithelial cells, astrocytes, and several cancer cell lines (Carlin et al., 1989; Dai and Gupta, 1990; Hwu et al., 2000; Taylor and Feng, 1991). Early studies showed that IDO was involved in the interferon-γ (IFN-γ)-mediated host defense to intracellular pathogens. IFN-γ was capable of limiting the intracellular growth of several bacteria, viruses, and parasites including *Toxoplasma gondii* (Pfefferkorn et al., 1986), *Chlamydia trachomatis* (Shemer and Sarov, 1985), *Chlamydia psittaci* (Byrne et al., 1986), *Chlamydia pneumoniae* (Pantoja et al., 2000), *Mycobacterium avium* (Hayashi et al., 2001), *group B streptococci* (Mackenzie et al., 1998), *Staphylococcus aureus* (Schroten et al., 2001), cytomegalovirus (Bodaghi et al., 1999), and herpes simplex virus (Adams et al., 2004). This

antimicrobial effect was mainly mediated through IFN-γ induced IDO expression and activity. It was suggested that IDO may inhibit intracellular growth and proliferation of microbial pathogens through depletion of essential amino acid tryptophan.

Recently, it was shown that IDO also plays a role in immunoregulation and tolerance induction. Macrophages and dendritic cells expressing IDO can suppress T-cell responses and promote tolerance (Mellor et al., 2004). IDO–dependent T cell suppression appears to be mediated by depletion of tryptophan in the microenvironment. Excess tryptophan was able to reverse the inhibition of T cells (Munn et al., 1999; Munn et al., 2004b). Toxic metabolites of tryptophan may also mediate the immunosuppressive effects of IDO. Tryptophan metabolites, such as quinolinic acid and 3-hydroxy-anthranilic acid induced the selective apoptosis of murine thymocytes and of Th1, but not Th2 cells (Fallarino et al., 2002). Human T cells were also sensitive to the antiproliferative and cytotoxic effects of exogenously added kynurenine, picolinic acid and 3-hydroxy-anthranilic acid (Frumento et al., 2002; Terness et al., 2002).

IDO expression is induced by several inflammatory cytokines and mediators. IFN-γ is a potent inducer of IDO expression. IFN-α and IFN-β are also able to induce IDO expression, but at a lesser extent (Dai and Gupta, 1990; Werner-Felmayer et al., 1989). TNF-α, IL- 1, prostaglandin E2 (PGE2) and lipopolysaccharide (LPS) also induce IDO expression either alone or in combination with IFN-γ (Babcock and Carlin, 2000; Chaves et al., 2001;

Hissong et al., 1995). Since IDO expression may lead to suppression of T cell proliferation and function, induction of IDO expression by inflammatory cytokines and mediators may limit excessive T-cell activation at local sites of inflammation, thus, serve an anti-inflammatory role. Consistent with the above findings, cytokines with anti-inflammatory effect appears to down-regulate IDO expression. Transforming growth factor-β (TGF-β) caused a marked reduction in intracellular IDO protein levels and abrogated IDO activity in human skin and synovial fibroblasts induced by IFN-γ.

Periodontal diseases are a group of chronic inflammatory conditions that result in the destruction of the supporting structures of the teeth. Periodontitis is the severe form of periodontal diseases and is one of the most common causes of tooth loss (Papapanou, 1996). The etiology of periodontitis is specific gram-negative microorganisms in dental plaque biofilm, including *Porphyromonas gingivalis (P.gingivalis), Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans) and Tanerella forsythia (T. forsythia)* (Zambon, 1996). To date it is becoming clear that the pathogenesis of the disease is caused by the complex interaction between host defense mechanisms and bacterial plaque pathogens and their products (Kinane and Lappin, 2002; Offenbacher, 1996; Van Dyke and Serhan, 2003). This interaction leads to the release of pro-inflammatory cytokines and mediators *e.g.* IL-1β, IFN-γ, TNF-α, IL-6, IL-8, matrix metalloproteinases (MMPs) and PGE_2 (Gorska et al., 2003; Okada and Murakami, 1998). High levels of these mediators have been identified in periodontitis lesions and thought to play a crucial role in periodontal tissue destruction.

The majority of cells in the periodontal connective tissues are the fibroblasts (Hassell, 1993). The main function of gingival fibroblasts is to maintain connective tissue homeostasis through production and destruction of structural connective tissue proteins. However, it was shown recently that gingival fibroblasts were able to secrete various immunoregulatory cytokines, chemokines and chemical mediators. These findings suggest that gingival fibroblasts could play important roles not only in maintaining periodontal tissue integrity or wound healing, but also in regulating local inflammatory responses.

Various cytokines that regulate IDO expression and activity were detected in periodontal tissues. High levels of IFN-γ, a strong IDO inducer, were detectable in inflammed gingival tissues (Shimabukuro et al., 1996; Takeichi et al., 2000). IL-1 and TNF-α levels have been shown to be elevated in the gingiva of chronic periodontitis and from active periodontitis sites (Graves and Cochran, 2003).

To date the biological mechanism for the pathogenesis of periodontitis are not fully understood. We hypothesize that IDO may exert immunosuppressive effect within periodontal tissues. Gingival fibroblasts may express IDO in response to various inflammatory cytokine and mediators. This, in turn, leads to local suppression of T cell proliferation and activity. Therefore, IDO expression may be one of several mechanisms to down-regulate the inflammatory process in periodontitis. To explore this hypothesis, we

determined the IDO expression in gingival fibroblasts and investigated the regulation of IDO expression in these cells. The results from this study provided a basis for further studies on the immunosuppressive role of IDO in periodontitis.

Objective

The aim of this study is

1. To determine IDO expression and activity in unstimulated human gingival fibroblasts.

2. To determine IDO expression and activity in human gingival fibroblasts stimulated by IL-1β, TNF-α , IFN-γ and *P.gingivalis* LPS.

Hypothesis

IL-1β, TNF-α , IFN-γ and *P.gingivalis* LPS may induce IDO mRNA expression and IDO activity in human gingival fibroblasts.

Field of Research

This is an *in vitro* study using cell culture model to determine the effect of inflammatory cytokines and mediators on IDO expression and activity by human gingival fibroblasts.

Limitations of research

In this study, IDO expression and activity was determined in primary culture of human gingival fibroblasts. For cell culture study, primary cultures are better than cell lines as their functions and activities are closer to normal cells. However, heterogeneity of the samples obtained from different individuals needs to be considered. In addition, the results obtained from cell culture study may not be similar to those occur *in vivo.*

Application and Expectation of Research

This is the first study to determine the IDO expression and activity in human gingival fibroblasts and to investigate the regulation of IDO expression in these cells. The results from this study may provide a basis for further studies on the immunosuppressive role of IDO in periodontitis. Better understandings of the disease pathogenesis may lead to novel treatment approaches for periodontal diseases.

CHAPTER II

LITERATURE REVIEW

Indoleamine 2,3–dioxygenase

Indoleamine 2,3–dioxygenase (IDO) is an enzyme that metabolized the amino acid tryptophan. Tryptophan is the least abundant essential amino acid for mammals. It is utilized for protein synthesis as well as the synthesis of a neurotransmitter, serotonin. Only a small amount of tryptophan (about 1%) is converted to serotonin whereas the majority (more than 95%) is metabolized along the kynurenine (Kyn) pathway, leading to the synthesis of nicotinamide adenine dinucleotide (NAD) or the complete oxidation of the amino acid (Takikawa, 2005). The metabolic pathway of tryptophan in humans is shown in Figure1.

There are two enzymes that catalyze that first step of the Kyn pathway, L-tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO). Both enzymes are heme-containing dioxygenase. TDO is highly specific to the L-isomer of tryptophan. It is expressed in the liver but not in the other tissues. Conversely, IDO exhibits a broad substrate specificity. It oxidized not only L-tryptophan, but also D-tryptophan, D and L-OH-Kyn, tryptamine, and serotonin (Shimizu et al., 1978). IDO is expressed in a variety of human tissues. The highest

activity was found in the term placenta and relatively high activity were detected in the lungs and intestines (Yamazaki et al., 1985).

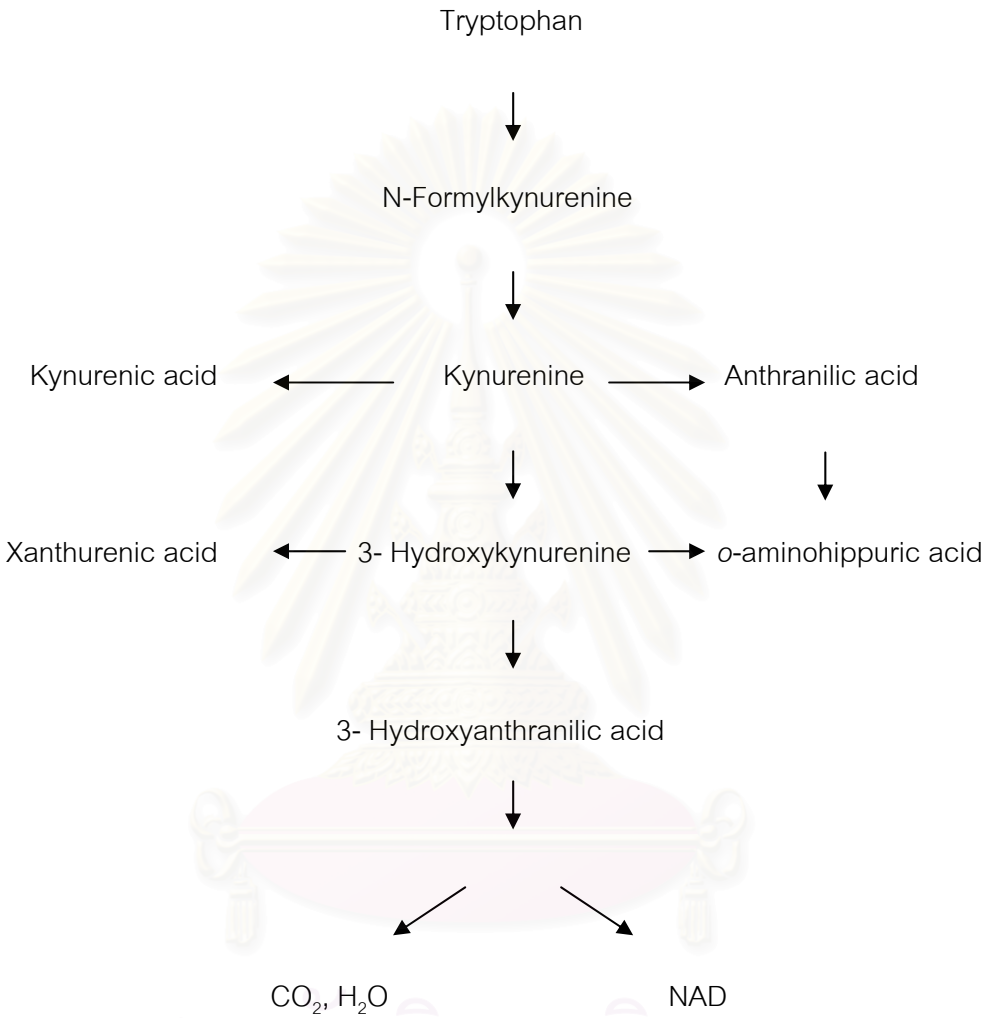

Figure 1. Tryptophan metabolism along the kynurenine pathway (Takikawa, 2005).

The IDO gene is a single gene with 10 exons spread over ~15 kbp of DNA located in a syntenic region of human and mouse chromosome 8 (Suzuki et al., 2003). A cDNA encoding human (Tone et al., 1990), mouse (Habara-Ohkubo et al., 1991), and rat (Pan et al.,

2000) IDO have been cloned. Human IDO cDNA encodes a protein of 403 amino acids with a molecular weight of about 45 kDa (Tone et al., 1990). The primary sequence of human IDO showed 57% and 58% identity to that of mouse IDO and rat IDO, respectively. Human IDO is a hemoprotein. However, there is no sequence similarity to other mammalian heme containing proteins such as hemoglobin, myoglobin, or cytochrome P-450. The mouse and human IDO gene promoters contain multiple sequence elements that confer responsiveness to type I interferon (IFN-α/β) and, more potently, type II interferon (IFN-γ) (Dai and Gupta, 1990; Hassanain et al., 1993). Signal transducer and activator of transcription 1 (STAT1) and IFN-regulatory factor 1 (IRF1) function cooperatively to mediate the induction of IDO expression by IFN-γ (Chon et al., 1996). Mice that lack either IFN-γ or IRF1 are deficient in IDO expression during infection (Silva et al., 2002).

IDO and innate host defense

Early studies showed that IDO was involved in the IFN-γ-mediated host defense to intracellular pathogens. IFN-γ was capable of limiting the intracellular growth of several bacteria, viruses, and parasites including *Toxoplasma gondii* (Pfefferkorn et al., 1986), *Chlamydia Trachomatis* (Shemer and Sarov, 1985), *Chlamydia psittaci* (Byrne et al., 1986), *Chlamydia pneumoniae* (Pantoja et al., 2000), *Mycobacterium avium* (Hayashi et al., 2001), *group B streptococci* (Mackenzie et al., 1998), *Staphylococcus aureus* (Schroten et al., 2001), cytomegalovirus (Bodaghi et al., 1999), and herpes simplex virus (Adams et al., 2004).

This antimicrobial effect was mainly mediated through IFN-γ induced IDO expression and activity since IDO-deficient mutant host cells can no longer inhibit intracellular microbial growth (Thomas et al., 1993). The IDO-mediated antimicrobial effect was observed in a variety of cell types including macrophages, fibroblasts, epithelial cells, endothelial cells and astrocytes. It was suggested that IDO may inhibit intracellular growth and proliferation of microbial pathogens through depletion of essential amino acid tryptophan. Addition of excess tryptophan in the environment was able to reverse the antimicrobial effect of IDO.

IDO and immunosuppression

Recently, it was shown that IDO plays a role in immunoregulation and tolerance induction. Cells expressing IDO can suppress T cell responses and promote tolerance. This immunoregulatory role of IDO, therefore, has a wide implication in several fields, including mammalian pregnancy, tumor resistance, chronic infections and autoimmune diseases (Mellor and Munn, 2004).

Dendritic cells are antigen-presenting cells (APCs) specialized to regulate immune responses. Dendritic cells not only control immunity, but also maintain tolerance to self-antigens. They are capable of expressing IDO, which allows them to downmodulate T cell activation and proliferation. In dendritic cells that express IDO, the activity of the enzyme is tightly regulated. Although the IDO protein is constitutively expressed, its functional activity

requires an additional set of triggering signals supplied during antigen presentation. Triggering of functional IDO obligately requires ligation of CD80/CD86 molecules on the dendritic cells by CTLA4 expressed on $CD4^+$T cells. When this interaction is disrupted, IDO remains in the inactive state. As a consequence, the dendritic cells are unable to inhibit T cell activation and proliferation (Boasso et al., 2005).

IDO-mediated T cell suppression acts in a dominant manner which means that IDO can suppress T cell responses to antigens that are presented by neighbouring APCs that do not express IDO (IDO^-APCs). This bystander suppression has been shown using defined IDO^+ and IDO^-APC populations, simultaneously presenting two different antigens in the same mixed lymphocyte reaction (MLR). Since only a small subset of dendritic cells is competent to express functional IDO, this bystander suppression effect can be dramatic *in vivo*. It will allow a small populations of IDO^+dendritic cells efficiently suppress all T cell responses to a particular antigen, despite the fact that the same antigen was presented by many other IDO^- APCs (Mellor et al., 2003; Munn et al., 2004a).

The expression of IDO protein in macrophages has also been shown to inhibit proliferation of $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes and natural killer (NK) cells. However, the proliferation of B lymphocytes was unaffected. Inhibition of cell proliferation induced by IDO activity was selective, applying only to cells undergoing activation. Resting cells were not affected and could be subsequently activated normally (Frumento et al., 2002).

IDO also regulates maternal T cell immunity during pregnancy. The high levels of IDO expression was observed at the maternal–fetal interface of human placenta, expressed by human extravillous trophoblast cells (Kudo et al., 2001; Kudo et al., 2004). These IDO-expressing cells prevented T cell cycle progression and enhanced activation-induced T cell death, thus protected the allogenic fetus from the mother's immune system. By blocking IDO expression and tryptophan catabolism during pregnancy, maternal T cells were allowed to trigger fetal allograft rejection (Munn et al., 1998).

Mechanisms of IDO-dependent T cell suppression

IDO–dependent T cell suppression appears to be mediated by depletion of tryptophan in the microenvironment. Excess tryptophan was able to reverse the inhibition of T cells (Munn et al., 1999; Munn et al., 2004b). Consistent with this possibility, *in vitro*-activated human and mouse T cells underwent cell-cycle arrest at mid G1 point when deprived of tryptophan and arrested cells were more sensitive to apoptosis (Lee et al., 2002; Munn et al., 1999). Toxic metabolites of tryptophan may also mediate the immunosuppressive effects of IDO. Tryptophan metabolites, such as quinolinic acid and 3-hydroxy-anthranilic acid induced the selective apoptosis *in vitro* of murine thymocytes and of Th1, but not Th2 cells (Fallarino et al., 2002). This raises the possibility that IDO might alter the Th1/Th2-cell balance. Human T cells were also sensitive to the antiproliferative and cytotoxic effects of exogenously added kynurenine, picolinic acid and 3-hydroxy-anthranilic acid (Frumento et al.,

2002; Terness et al., 2002). The effect of IDO-mediated T cell suppression by tryptophan depletion and toxic metabolites have been mainly shown *in vitro*. How these processes might function *in vivo* remains to be explored. IDO may also exert its effect on T cells indirectly through altered antigen-presenting cells functions. It was shown that pre-activation of $CD8^+$ dendritic cells *in vitro* with IFN-γ rendered them tolerogenic when subsequently injected *in vivo* (Grohmann et al., 2001). This effect required IDO expression during the *in vitro* pre-activation since the addition of 1-methyl-D-tryptophan (1-MT), an IDO inhibitor, during that time blocked the development of tolerogenic activity. IDO-induced tolerogenic dendritic cells may suppress T cell function and lead to immune tolerance.

IDO and cancer

Immune escape is crucial for cancer progression. Elevation of the IDO in tumor cells and presentation of tumor antigens by tolerogenic host APCs may facilitate immune escape. Mouse tumor-draining lymph nodes (TDLNs) contained a subset of plasmacytoid dendritic cells (pDCs) that constitutively expressed IDO. These pDCs, *in vitro*, potently suppressed T cell responses to antigens presented by the pDCs themselves and also suppressed T cell responses to third-party antigens presented by non-suppressive APCs. Adoptive transfer of dendritic cells from TDLNs into naive hosts created profound local T cell anergy which was

prevented by targeted disruption of the IDO gene in the dendritic cells or by administration of the IDO inhibitor drug, 1-MT, to recipient mice (Munn et al., 2004a).

Bin1 is a cancer suppressor gene which is attenuated in many human malignancies. Using a knockout mouse model, it was shown that IDO is under genetic control of Bin1. Bin1 loss elevated the STAT1- and NF-kappaB-dependent expression of IDO, promoting escape of tumor cells from T cell-dependent antitumor immunity (Muller et al., 2005). Clinically, increased expression of IDO is associated with poorer prognosis of several cancers including esophageal squamous cell carcinoma (Sakurai et al., 2004), hepatocellular carcinoma (Ishio et al., 2004), and ovarian cancer (Okamoto et al., 2005).

Regulation of IDO expression and activity

IDO plays a role in both innate and adaptive host defense. It helps to limit growth of intracellular pathogens as well as to downregulate the T cell response. Both functions appear to be paradoxical since one is to activate the host against pathogens whereas another is to activate an immunosuppressive mechanism. However, it is not uncommon in the immune system that inflammation frequently elicits counter-regulatory pathways. IDO expression is induced by several inflammatory cytokines and mediators, *i.e.* IFN-γ, TNF-α, IL-1, PGE2 and LPS. Induction of IDO expression may limit excessive T-cell activation at local sites of inflammation, thus, serve an anti-inflammatory role. In this context, IDO would

not need to be expressed by professional APCs. Consistent with this, inflammation-induced IDO expression has been described in many non-APCs, *i.e.* intestinal epithelial cells (Barcelo-Batllori et al., 2002), skin fibroblasts (Holmes, 1998), and resident lung cells (Hayashi et al., 2004). The regulation of IDO by various cytokines and mediators was summarized in Table 1.

Table 1. The effect of cytokines and mediators on IDO expression

Cells	Detection/ Method	Single agent		Combination with IFN			Authors
		Inducer	No effect	Additive effect	Inhibitory effect	No effect	
Human monocytes (primary culture)	RNA/ Northern blot Activity/HPLC	IFN-γ	IL-4	-	IFN-γ+ IL-4	-	Musso et al., 1994
Human monocyte-derived macrophage (primary culture)	Activity/HPLC	IFN-γ IFN-β	IL-1α	IFN-β+ IL-1α IFN-γ+ IL-1α IFN-γ+ IL-1β	-	-	Carlin and Weller ,1995
Human monocyte-derived macrophage (primary culture)	Activity/HPLC	IFN-γ IFN-β LPS	IL-1α	IFN-γ+ LPS IFN-β+ LPS IFN-γ+ IL-1α IFN-β+ IL-1α	-	-	Hissong et al., 1995
Epidermal langerhans cells (primary culture)	RNA/ RT-PCR Activity/ HPLC	IFN-γ	-	-	-	-	von Bubnoff et al., 2004
Human fibroblasts (cell line)	RNA/ RT-PCR Activity/HPLC	IFN-γ IFN-β TNF-α	IL-4 IL-10 IL-13	IFN-γ+ IFN-β IFN-γ+ TNF-α	IFN-γ+ IL-4 IFN-γ+ IL-13	IFN-γ+ IL-10	Chaves et al., 2001

RT-PCR, Reverse transcriptase-Polymerase chain reaction; HPLC, High pressure liquid chromatography

Table 1(continued). The effect of cytokines and mediators on IDO expression

Cells	Detection/ Method	Single agent		Combination with IFN			Authors
		Inducer	No effect	Additive effect	Inhibitory effect	No effect	
Human skin and synovial fibroblasts	RNA/ Northern blot Activity/HPLC	IFN-γ	TGF-β IL-4	-	IFN-γ +TGF-β IFN-γ+ IL-4	-	Yuan et al.,1998
Human foreskin fibroblasts (cell line)	RNA/ RT-PCR Activity/ spectrophotometry	IFN-γ	-	IFN-γ +HC	-	-	Turck et al.,2005
Human foreskin fibroblasts (primary culture)	RNA/ Northern blot Activity/ Mass spectrometry	IFN-γ	-	-	-	-	Sarkosh et al., 2003a
Human foreskin keratinocytes (primary culture)	RNA/ Northern blot Activity/ Mass spectrometry	IFN-γ	-	-	-	-	Sarkosh et al., 2003b
Human epidermal keratinocytes (primary culture)	RNA/ quantitative real time PCR	IFN-γ	IL-4 IL-13	-	-	-	Ito et al., 2004

RT-PCR, Reverse transcriptase-Polymerase chain reaction; HPLC, High pressure liquid chromatography; HC, hydrocortisone

Table 1(continued). The effect of cytokines and mediators on IDO expression

Cells	Detection/ Method	Single agent		Combination with IFN			Authors
		Inducer	No effect	Additive effect	Inhibitory effect	No effect	
Human cervical epithelial cells (cell line)	Activity/ HPLC Promoter activity/ flow cytometry	IFN-γ	TNF-α IL-1β LPS	IFN-γ +TNF-α IFN-γ +IL-1β	-	IFN-γ+LPS	Babcock and Carlin, 2000
Human lung epithelial cells (cell line)	RNA/ Northern blot Activity/ spectrophotometry	IFN-γ	TNF-α LPS	-	-	IFN-γ+LPS	Van Wissen et al., 2002
Human uroepithelial cells (cell line)	Activity/ spectrophotometry	IFN-γ	-	-	IFN-γ +IL-1β IFN-γ +TNF-α	-	Daubener and Mac Kenzie, 1999
Human glioblastoma cells (cell line)	Activity/ spectrophotometry	IFN-γ	-	IFN-γ +TNF-α	-	IFN-γ +IL-1β	Daubener and Mac Kenzie, 1999
Human astrocytoma cells (cell line)	Activity/ spectrophotometry	IFN-γ	-	-	-	IFN-γ +IL-1β IFN-γ +TNF-α	Daubener and Mac Kenzie, 1999

RT-PCR, Reverse transcriptase-Polymerase chain reaction; HPLC, High pressure liquid chromatography

Cells	Detection/ Method	Single agent		Combination with IFN			Authors
		Inducer	No effect	Additive effect	Inhibitory effect	No effect	
Human astrocytes (primary culture)	RNA/ RT-PCR Activity/ spectrophotometry	IFN-γ	-	IFN-γ +HC	-	-	Turck et al., 2005
Human astrocytoma cells (cell line)	RNA/ RT-PCR Activity/ spectrophotometry	IFN-γ	-	IFN-γ +HC IFN-γ +TNF-α IFN-γ+TNF-α+HC	-	-	Turck et al., 2005
Human neurons , astrocytes, microglia (primary culture)	RNA/ RT-PCR Protein/immuno-cytochemistry	IFN-γ	-	-	-	-	Guillemin et al., 2005

RT-PCR, Reverse transcriptase-Polymerase chain reaction; HPLC, High pressure liquid chromatography; HC, hydrocortisone

IFN-γ a potent inducer of IDO expression in various cell types including macrophages, dendritic cells, fibroblasts, epithelial cells , and many cancer cell lines (Carlin et al., 1989; Dai and Gupta, 1990; Hwu et al., 2000; Taylor and Feng, 1991). IFN-α and IFN-β are also able to induce IDO expression, but at a lesser extent (Dai and Gupta, 1990; Werner-Felmayer et al., 1989). Several other pro-inflammatory cytokines and mediators also induce IDO expression either alone or in combination with IFN-γ.

TNF-α alone was ineffective in IDO induction of peripheral blood mononuclear cells, macrophages, epithelial cell line and an astrocytoma cell line (Adams et al., 2004; Carlin et al., 1987; Werner-Felmayer et al., 1989), but showed weak IDO induction in a fibroblast cell line (2C4)(Chaves et al., 2001). However, TNF-α consistently enhanced the effect of IFN-γ (Adams et al., 2004; Babcock and Carlin, 2000; Chaves et al., 2001; Robinson et al., 2003). The synergistic effect of TNF-α and IFN-γ on IDO induction was mediated at the level of transcription through an increase in IFN-γ receptor expression which enhanced the binding of STAT1 to gamma-activated sequences (GAS) and IRF-1 to IFN-stimulated response elements (ISRE) sites (Robinson et al., 2003). IL-1 alone was also unable to induce IDO-activity in macrophages (Carlin and Weller, 1995) and epithelial cells (Babcock and Carlin, 2000). Nonetheless, when used in combination with IFN-γ, it induced synergistic increase in IDO expression and activity.

Bacterial lipopolysaccharides (LPS) potentiates the IFN-γ induced IDO expression in human monocyte-derived macrophage (Hissong et al., 1995). Epithelial cell lines were not responsive to LPS, and no synergistic increase in IDO activity when IFN-γ was combined with LPS (Babcock and Carlin, 2000; van Wissen et al., 2002). Intraperitoneal injection of LPS resulted in marked IDO experssion mainly in the lung of mice *in vivo* (Hayaishi and Yoshida, 1978). The enzyme activity increased almost linearly during the first 24 hr and started to decrease after 48 hr and reached a normal value after about 6 days (Yoshida and Hayaishi, 1978). Immunohistochemistry showed that IDO was expressed mainly by the alveolar interstitial cells (Urade et al., 1983). IDO induction by pro-inflammatory cytokines including IL-1, TNF-α appears to be dependent on IFN-γ. Interestingly, IDO induction by LPS was not mediated through an IFN-γ dependent mechanism. Using IFN-γ or TNF-α gene-disrupted mice and IFN-γ antibody-treated mice, it was demonstrated that LPS-induced systemic IDO was largely dependent on TNF-α rather than IFN-γ. IDO induction by LPS was significantly attenuated in TNF-α-knockout mice when compared with that in genetically normal mice. In contrast, LPS induces IDO activity in both IFN-γ knockout mice and IFN-γ-antibody-treated mice at a level comparable to that of normal mice (Fujigaki et al., 2001). The significance of IDO induction in response to LPS is unclear.

Cytokines with anti-inflammatory effect appears to downregulate IDO expression. Transforming growth factor-β (TGF-β) caused a marked reduction in intracellular IDO protein

levels and abrogated IDO activity in human skin and synovial fibroblasts induced by IFN-γ (Yuan et al., 1998). TGF-β did not inhibit the induction of STAT1 and IRF-1 binding activity to their cognate DNA recognition sites in the IDO gene promoter. In contrast, the stability of IDO mRNA transcripts was reduced in fibroblasts treated with TGF-β. IL-4 and IL-13 alone did not affect IDO expression (Ito et al., 2004). However, it inhibited the IDO expression and activity induced by IFN-γ in human monocytes and cells from fibroblast lineages (Chaves et al., 2001; Musso et al., 1994). It is interesting that glucocorticoid, which has an anti-inflammatory action, induces IDO expression. Glucocorticoids such as hydrocortisone and dexamethasone enhanced IFN-γ-mediated IDO expression and activity in human peripheral blood monocytes (Ozaki et al., 1987), human astrocytoma cells and native human astrocytes (Turck et al., 2005). The synergistic effect of glucocorticoid on IDO expression may support the counter-inflammatory role of IDO.

The control of IDO activity appears to be complex and cell-type specific. Presence of the IDO mRNA and protein may not be correlated with its functional activity. Human dendritic cells constitutively expressed immunoreactive IDO protein as shown by flow cytometry and western blot, but it did not have functional enzymatic activity until these cells were activated by IFN-γ and CD80/CD86 ligation (Munn et al., 2004b). TNF-α enhanced IFN-γ induced IDO activity in human glioblastoma cells whereas this effect was blocked by TNF-α as well as by IL-1 in a human uroepithelial cell line. In human astrocytoma cells, IL-1 and TNF- α did not inhibit IDO activity (Daubener et al., 1999). Nitric oxide (NO) was shown to inhibit IDO activity

in the epithelial cells. This effect was not due to a decrease in IDO gene transcription or mRNA stability. However, NO production led to an accelerated degradation of IDO protein in the proteasome (Hucke et al., 2004).

Periodontal Disease

Periodontal diseases are a group of chronic inflammatory conditions that result in the destruction of the supporting structures of the teeth. Periodontitis is the severe form of periodontal diseases and is one of the most common causes of tooth loss (Papapanou, 1996). The etiology of periodontitis is specific gram-negative microorganisms in dental plaque biofilm, including *P. gingivalis, A. actinomycetemcomitans and T. forsythia* (Zambon, 1996). To date it is becoming clear that the pathogenesis of the disease is caused by the complex interaction between host defense mechanisms and bacterial plaque pathogens and their products (Kinane and Lappin, 2002; Offenbacher, 1996; Van Dyke and Serhan, 2003). This interaction leads to the release of pro-inflammatory cytokines and mediators *e.g.* IL-1β, IFN-γ, TNF-α, IL-6, IL-8, matrix metalloproteinases (MMPs) and PGE_2 (Gorska et al., 2003; Okada and Murakami, 1998). High levels of these mediators have been identified in periodontitis lesions and thought to play a crucial role in periodontal tissue destruction. However, the precise pathological mechanisms underlying tissue destruction are not well understood.

Various cytokines that regulate IDO expression and activity were detected in periodontal tissues. High levels of IFN-γ, a strong IDO inducer, were detectable in inflammed gingival tissues (Shimabukuro et al., 1996; Takeichi et al., 2000). In addition, IFN-γ could be detected in supernatant of gingival mononuclear cells from rapidly progressive periodontitis patients as well as in gingival crevicular fluid of chronic periodontitis patients (Salvi et al., 1998). IL-1 is the most potent known inducer of bone demineralization and synergizes with TNF-α in stimulating bone resorption (Stashenko et al., 1987). IL-1 levels have been shown to be elevated in the gingiva of chronic periodontitis (Honig et al., 1989; Tokoro et al., 1996) and from active periodontitis sites (Stashenko et al., 1991). Bacterial lipopolysaccharide (LPS), specifically LPS from *P. gingivalis* was detected in periodontitis lesions and was considered an important pathogenic component in the initiation and progression of periodontal disease (Wang and Ohura, 2002). At present, it is not known how these cytokines and mediators affect IDO expression and activity within periodontal tissues.

The majority of cells in the periodontal connective tissues are the fibroblasts (Hassell, 1993). Gingival fibroblasts have classically been described as engineers of the extracellular connective tissue matrix. However, recent observations have expanded their functions to include the synthesis of various mediators, including immunoregulatory cytokines, chemokines and chemical mediators. These findings suggest that gingival fibroblasts could play important roles not only in maintaining periodontal tissue integrity or wound healing, but also in regulating local inflammatory responses. In inflamed periodontal

tissues, dense accumulations of inflammatory cell infiltrates such as T lymphocytes, B lymphocytes, macrophages, and dendritic cells were detected histopathologically and so were their cell-derived cytokines. These locally infiltrated cells were located adjacent to the gingival fibroblasts (Murakami et al., 1999). It is, therefore, reasonable to speculate that these immune cells and their cytokines may have an opportunity to directly interact with the resident fibroblasts and are mutually affected through cell-cell interaction as well as cell-cytokine interaction.

Gingival fibroblasts could secrete various soluble mediators of inflammation including PGE_2, IL-1, IL-6 and IL-8 in response to extrinsic environmental factors such as dental plaque bacteria and their products and cytokines (Kida et al., 2005; Takada et al., 1991; Tamura et al., 1992; Wang and Ohura, 2002). These fibroblast-derived mediators are thought to play an important role in inflammatory response in local periodontal lesions. Many plaque bacterial products *e.g.* LPS derived from *P. gingivalis, A. actinomycetemcomitans* and *Prevotella intermedia* (*P. intermedia*) were shown to enhance IL-6 and IL-8 production from gingival fibroblasts (Dongari-Bagtzoglou and Ebersole, 1996a; Dongari-Bagtzoglou and Ebersole, 1996b; Imatani et al., 2001; Takada et al., 1991). In periodontitis, gingival fibroblasts are considered to be major sources of IL-6 and IL-8 cytokines which are secreted in high amount both constitutively (Bartold and Haynes, 1991; Dongari-Bagtzoglou and Ebersole, 1996b) and in response to periodontal bacteria, IL-1, TNF-α (Takashiba et al., 1992) and IFN-γ (Daghigh et al., 2002; Sakuta et al., 1998). At present, it is not known whether gingival fibroblasts could

express IDO and how various inflammatory cytokines and mediators in periodontitis tissues affects IDO expression of gingival fibroblast cells.

To date the biologic mechanism for the pathogenesis of periodontitis are not fully understood. IDO may have an immunoregulatory role in periodontitis via its effect on T cell suppression. Therefore, it is the aim of this study to investigate the IDO expression in periodontitis lesions and to determine the effect of inflammatory cytokines and immunomodulating agents on IDO expression of human gingival fibroblasts. The results from this study provided a basis for further studies on the interaction between T cells and gingival fibroblasts. Better understandings of the disease pathogenesis may lead to novel treatment approaches for periodontal diseases.

CHAPTER III

MATERIAL AND METHODS

Gingival tissue sample collection

Gingival tissue samples were collected from dental patients at the faculty of Dentistry, Chulalongkorn University. Informed consent was obtained prior to tissue collection. The protocol was approved by the ethics committee of Faculty of Medicine, Chulalongkorn University. Healthy gingival samples were obtained from healthy gingiva overlying the impacted third molars or marginal gingival excised during periodontal surgery. Tissue samples were washed briefly in normal saline solution and placed immediately into tissue culture media. Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), gentamycin (50 μg/ml), penicillin G (50U/ml), streptomycin (50 μg/ml),and fungizone (2.5 μg/ml) (Gibco, Grand Island, NY) were used for tissue collection and cell culture. The samples were kept on ice and processed within a few hours.

Primary culture of human gingival fibroblasts

The method to obtain human gingival fibroblast (HGF) cells from the gingival tissue was that described by Murakami (Murakami et al., 1999). Briefly, the biopsy was washed twice with DMEM to remove blood clots and adherent erythrocytes. Then, it was cut into fragments of 1-3 mm^3 with a sterile scapel. These tissues were transferred to a 35 mm tissue

culture dish containing 2 ml of culture media and incubated at 37 °C in a humidified 5% CO_2-air atmosphere. Culture medium was changed twice weekly. When the fibroblast cells surrounding the tissue explants were confluent, they were subcultured into a larger tissue culture dish. HGF at the passages 3 to 6 were used in this study.

Detection of IDO mRNA expression by RT-PCR

HGF ($2.5x10^5$ cells/ well) were seeded into a 6-well tissue culture plate (Costar®, USA) overnight. The cells were stimulated with the following agents: IFN-γ (10-1,000 U/mL) (R&D systems, USA), TNF-α (0.5-50 ng/mL) (R&D systems, USA), IL-1β (0.5-50 ng/mL) (R&D systems, USA), LPS from *P. gingivalis* strain 381 (100-10,000 ng/mL) (Schifferle et al., 1998; Preshaw et al., 1999). In addition, the combination of IFN-γ and each reagent (TNF-α, IL-1β and LPS) were used. Un-stimulated HGF were served as a control. After 24 h, HGF were harvested to determine IDO expression.

Total RNA was extracted with TRIzol Reagent (Invitrogen, Life technology, USA), according to the standard protocol. 1 µg of total RNA was used for reverse transcription with random hexamer and Improm-II reverse transcriptase (Improm-IITM, Promega Corp, USA), following the manufacturer's instruction. The cDNA was used for detection of IDO mRNA by polymerase chain reaction (PCR). PCR was carried out with PCR mixture contained 15mM of $MgCl_2$, 20µM of IDO primer, 10 mM of dNTP, 5 unit/µl of Taq polymerase (QIAGEN, USA) and the DNA template. The PCR condition consists of a first heating step (95°C for 5 min), 30

amplification cycles(95°C for 15 s, 60°C for 30s, 70 °C for 30 s) and one final extension step (72°C for 7 min). Amplification was performed on Master cycler gradient (Eppendorf, Germany). Specific primer sequences for the gene were as follows: human GAPDH: forward 5'-TCATCTCTGCCCCCTCTGCTG-3' and reverse 5'-GCCTGCTTCACCACCTTCTTG -3' (approximate size of 400 bp); human IDO: forward 5' CTTCCTGGTCTCTCTATTGG 3' and reverse 5' GAAGTTCCTGTGAGCTGGT 3'; (approximate size of 430 bp) (von Bubnoff et al., 2004). PCR products were separated by electrophoresis in a 1.2% agarose gel containing ethidium bromide. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. As a negative control, a PCR reaction was performed without an RT sample. The target bands were visualized with an UV illuminator and analyzed with image analysis software (Gel Imaging Analysis, Bio Imaging system, Inc., Gene Genius, UK). The experiments were repeated using human gingival fibroblasts from four different individuals.

Detection of IDO enzymatic activity

HGF (3 x10^4 cells/ well) were seeded into a 96-well microtiter plate (Costar®, USA) overnight. The cells were stimulated with the following agents: IFN-γ, TNF-α, IL-1β, *P.gingivalis* LPS or their combinations. Un-stimulated HGF were served as a control. After 24 and 48 h, culture supernatants were harvested and assayed for the presence of kynurenine, the first stable catabolite of tryptophan in the kynurenine pathway. Kynurenine was detected by using a modified spectophotometric assay (Braun et al., 2005). The amount of 50 µL of 30%

trichloroacetic acid was added to 100 µL culture supernatant, vortexed, and centrifuged at 8000 g for 5 minutes. Volume (75 µL) of the supernatant was then added to an equal volume of Ehrlich reagent (100 mg P-dimethylbenzaldehyde, 5 mL glacial acetic acid) in a microtiter plate well (96-well format). Optical density was measured at 492 nm, using an Anthos Zenyth 200 monocromator microplate reader (Jencon, UK). A standard curve of defined kynurenine concentration (0-100 µM) permitted analysis of unknown.

Statistical Analysis

The one sample t-test was used to compare the level of IDO activity between samples. Results were expressed as mean $\pm$ standard error of the mean (SEM). Data were analyzed by using SPSS version12.0 software. Statistical differences with a p-value < 0.05 were considered significant.

CHAPTER IV

RESULT

HGF cells expressed IDO mRNA upon stimulation with inflammatory cytokines and mediators

To investigate whether inflammatory cytokines and mediators induce IDO mRNA expression in HGF cells, RT-PCR analysis was performed. Figure 2 depicts a representative RT-PCR result. HGF cells do not normally express detectable levels of IDO mRNA. However, upon treatment with IFN-γ (100U/ ml), IL-1β (5 ng/ml), TNF-α (5 ng/ml), or *P. gingivalis* LPS (PgLPS) (1000 ng/ ml), IDO expression was induced. IFN-γ appears to be the strongest inducer for IDO expression. IL-1β, TNF-α, and PgLPS induced IDO mRNA expression at lower levels.

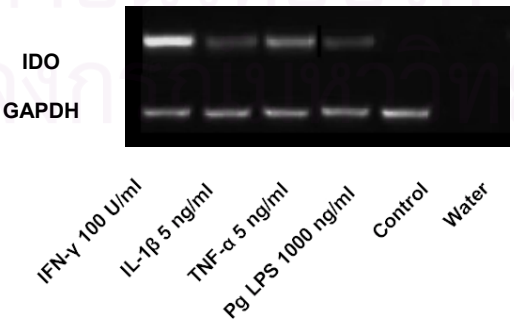

Figure 2. IDO mRNA expression in HGF cells treated with IFN-γ, IL-1β, TNF-α, or PgLPS.

Dose-dependent expression of IDO mRNA upon stimulation with IFN-γ, TNF-α, or *P. gingivalis* LPS

IFN-γ, TNF-α, and *P. gingivalis* LPS appeared to induce IDO mRNA expression in a dose-dependent manner. Stimulation with IL-1β, however, did not show the dose-dependent effect. Figure 3 depicts a representative RT-PCR result. Relative IDO mRNA expression, as determined by the ratios of IDO mRNA and GAPDH mRNA, from 4 separate experiments was shown in Figure 4.

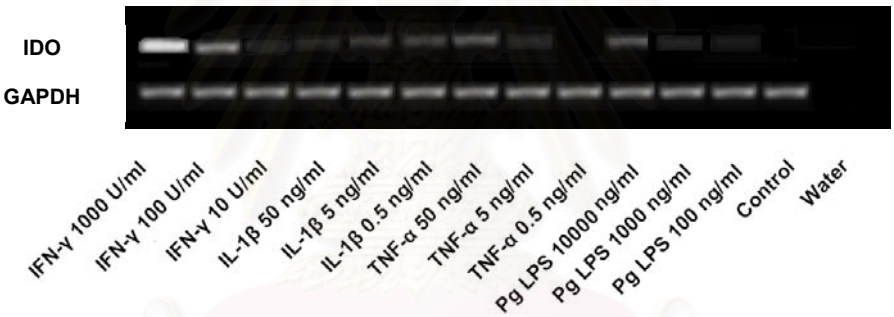

Figure 3. IDO mRNA expression in HGF cells treated with different concentrations of IFN-γ, IL-1β, TNF-α, or PgLPS.

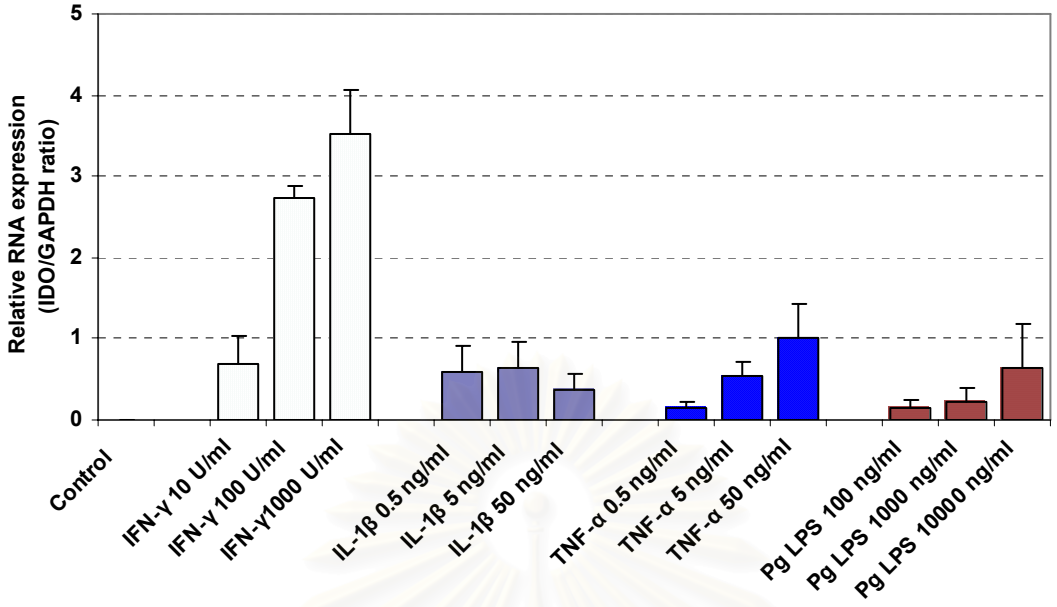

Figure 4. Relative IDO mRNA expression in HGF cells treated with different concentrations of IFN-γ, IL-1β, TNF-α, or PgLPS. Data were shown as mean $\pm$ SE from 4 separate experiments.

Increased IDO mRNA expression in HGF cells treated with combinations of IFN-γ and other agents

HGF cells treated with combinations of IFN-γ and IL-1β, TNF-α, or *P.gingivalis* LPS showed increased IDO expression compared to IFN-γ alone. Figure 5 showed a representative RT-PCR result. Relative IDO mRNA expression from 4 separate experiments was shown in Figure 6.

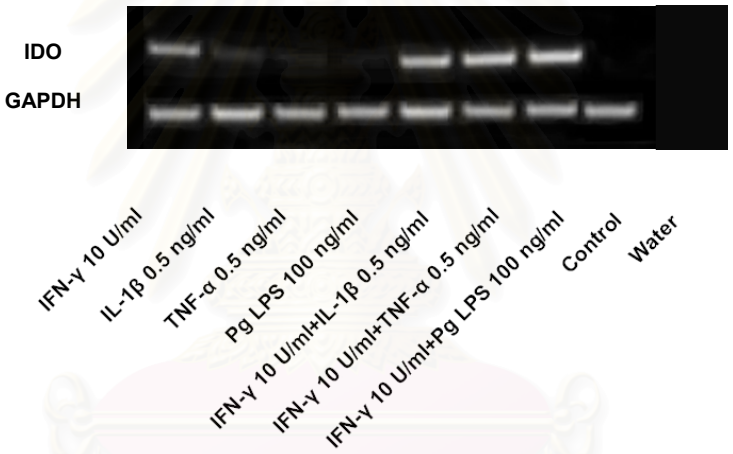

Figure 5. IDO mRNA expression in HGF cells treated with combinations of IFN-γ and IL-1β, TNF-α or PgLPS.

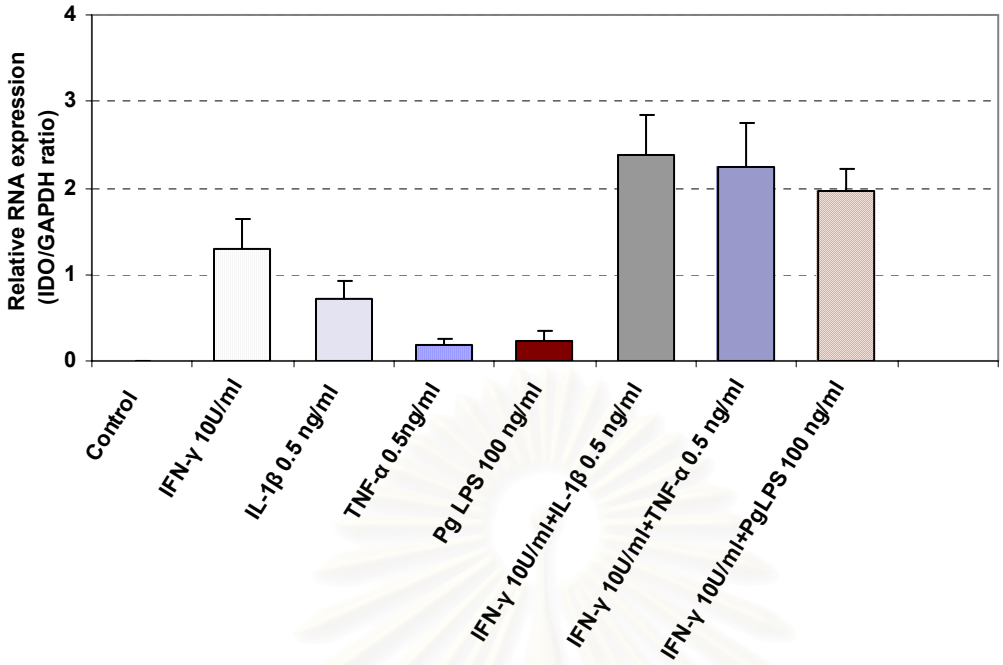

Figure 6. Relative IDO mRNA expression in HGF cells treated with combinations of IFN-γ and IL-1β, TNF-α, or PgLPS. Data were shown as mean $\pm$ SE from 4 separate experiments.

Increased IDO activity in HGF cells treated with IFN-γ and their combinations.

To evaluate whether HGF cells produced functional IDO, we detected IDO enzymatic activity by measuring kynurenine level which is the first stable catabolite in the metabolic pathway of tryptophan. Significant increased IDO activity was only detected in HGF cells treated with IFN-γ. IDO activity in cells treated with IL-1β, TNF-α, and *P.gingivalis* LPS was not significantly different from that of the control (Figure 7). IDO activity appeared to be increased over time. At 48 h, HGF cells treated with the combination between IFN-γ and IL-1β as well as IFN-γ and TNF-α showed significantly higher IDO activity as compared to, that of IFN-γ alone (Figure 8).

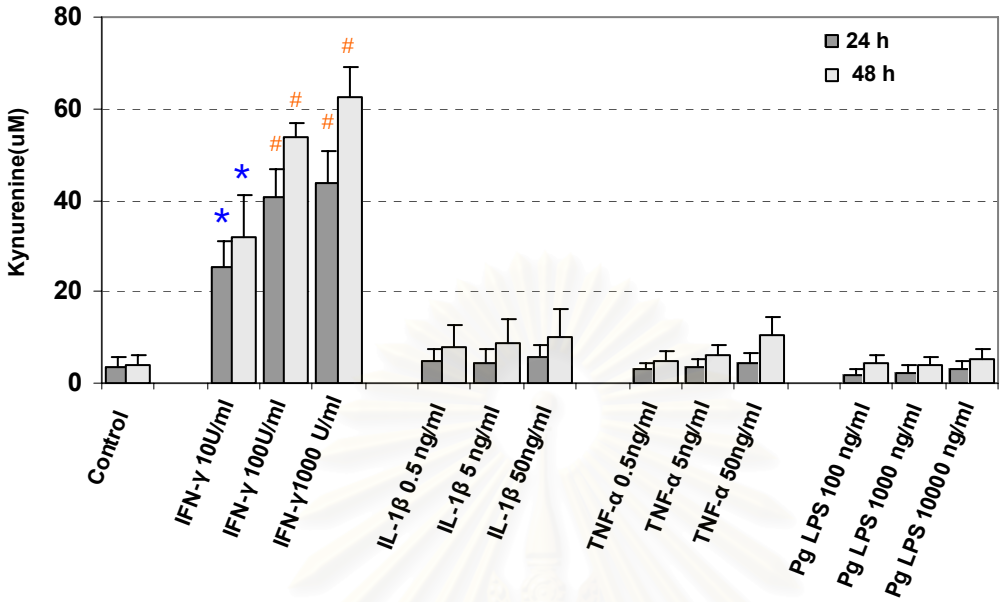

Figure 7. IDO activity in HGF cells treated with different concentration of IFN-γ, IL-1β, TNF-α, or PgLPS. Data were shown as mean $\pm$ SE from 4 separate experiments. *p<0.05 and #p<0.01 significant different from the control (untreated).

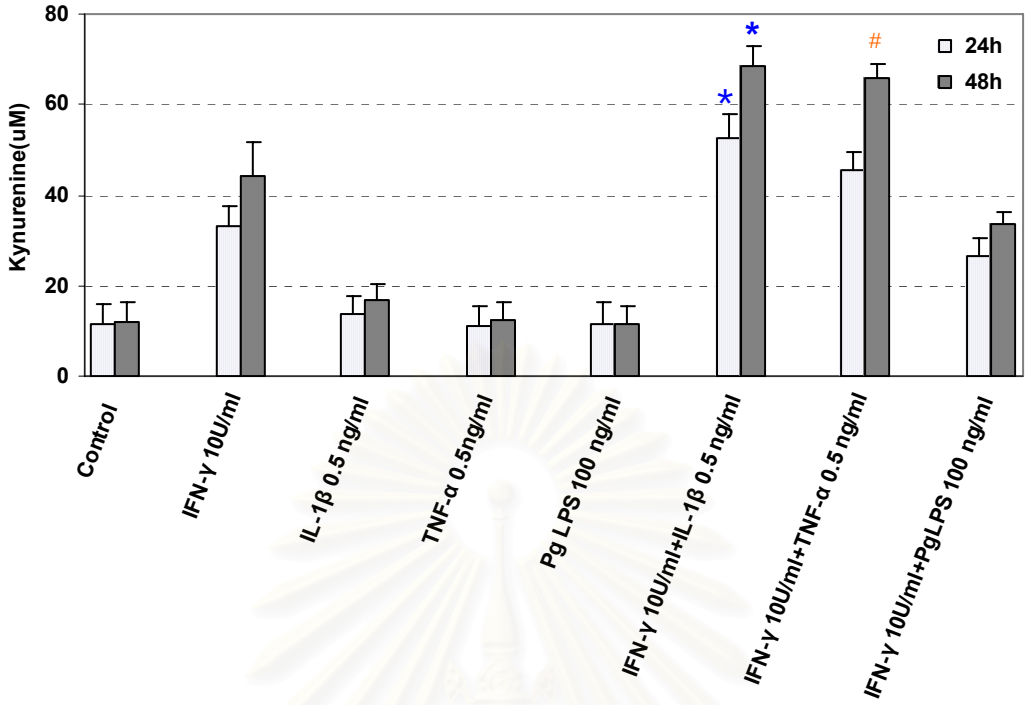

Figure 8. IDO activity in HGF cells treated with combinations of IFN-γ and IL-1β, TNF-α, or *P.gingivalis* LPS (PgLPS). Data is shown as mean $\pm$ SE from 4 separate experiments.

*$p<0.05$ and #$p<0.01$ significant different from the IFN-γ (10U/ml) group.

CHAPTER V

DISCUSSION

The aim of this study is to determine the effect of inflammatory cytokines and mediators on IDO expression and activity in human gingival fibroblasts. Here, we showed that gingival fibroblasts are able to express IDO in response to several cytokines and mediators. Gingival fibroblasts are the major cell type within periodontal tissues and may participate directly with several inflammatory mediators during periodontitis. We utilized IFN-γ, IL-1β, TNF-α, and LPS of *P.gingivalis* as stimulating agents since these agents were detected at high levels in periodontitis tissues and were involved in pathogenesis of periodontal diseases. Gingival fibroblasts did not constitutively express IDO. However, IDO expression was inducible in these cells. IFN-γ is a potent inducer for IDO expression in human gingival fibroblasts. Lower levels of IDO expression was detected upon stimulation with IL-1β, TNF-α, and *P. gingivalis* LPS. IFN-γ has been shown to be a strong inducer of IDO expression in many cell types, including dendritic cells, macrophages, epithelial cells, skin fibroblasts, and many cancer cell lines and many cancer cell lines (Carlin et al., 1989; Dai and Gupta, 1990; Hwu et al., 2000; Taylor and Feng, 1991). Consistent with the finding, it was shown that the IDO promoter contains the interferon-stimulated response element (ISRE) and gamma activated sequence (GAS). These sequences were the binding site for the

transcription factor IFN-regulatory factor-1 (IRF-1) and signal transducer and activator of transcription1 (STAT1), which allows activation of IDO gene in response to IFN-γ (Robinson et al., 2003).

We showed that IL-1β and TNF-α induced low levels of IDO expression in human gingival fibroblasts. TNF-α was ineffective in IDO induction of peripheral blood mononuclear cells, macrophages, epithelial cell line and an astrocytoma cell line (Adams et al., 2004; Carlin et al., 1987; Werner-Felmayer et al., 1989), but showed weak IDO induction in a fibroblast cell line (Chaves et al., 2001). IL-1 was also unable to induce IDO expression in macrophages (Carlin and Weller, 1995) and epithelial cells (Babcock and Carlin, 2000). Therefore, IDO expression in response IL-1β and TNF-α appeared to be cell type-specific.

In this study, LPS from *P. gingivalis* induced low level of IDO expression in human gingival fibroblasts. It has been shown that LPS from *P. gingivalis* also stimulates human gingival fibroblasts to produce several inflammatory cytokines including IL-1, IL-6, and IL-8 (Takada et al., 1991; Tamura et al., 1992). Whether these secreted cytokines also indirectly involve in the induction of IDO expression by LPS from *P. gingivalis* is unknown. Previous studies showed that bacterial LPS did not induce IDO expression in human epithelial cell line from cervix (Babcock and Carlin, 2000) and lung (van Wissen et al., 2002). These studies utilized widely used LPS from enteric bacteria, *Escherichia coli*. *P. gingivalis* is a key

periodontal pathogen and LPS from *P. gingivalis* was detected at high level in periodontitis tissues. Therefore, it appears to be more relevant to study the effect of LPS from *P. gingivalis* on gingival fibroblasts. The LPS of *P. gingivalis* has many characteristics that are distinct from that of other gram-negative bacteria. The protein structure of *P. gingivalis* lacks heptose and 2-keto-3-deoxyoctonate and its lipid A contains tri-, tetra-, and penta-acetylated components. *P. gingivalis* LPS showed very little endotoxic activity compared to that of the LPS isolated from enteric bacteria. However, *P. gingivalis* LPS is a potent inducer of various biological responses such as bone resorption, polyclonal B cell activation, and inhibition of bone formation and fibroblast proliferation (Ogawa et al., 2007; Wang and Ohura, 2002; Wang et al., 2002).

Combinations between IFN-γ and IL-1β, TNF-α, or *P. gingivalis* LPS also augmented the level of IDO expression in human gingival fibroblasts as compared to that of IFN-γ alone. Combinations of IFN-γ and IL-1β as well as IFN-γ and TNF-α increased IDO expression in human monocyte derived macrophages and human cervical epithelial cells (Babcock and Carlin, 2000; Carlin and Weller, 1995). The synergistic effect of TNF-α and IFN-γ on IDO induction has been shown to be mediated at the level of transcription through an increase in IFN-γ receptor expression which enhanced the binding of STAT1 to GAS and IRF-1 to ISRE sites (Robinson et al., 2003). Epithelial cell lines were not responsive to LPS, and no synergistic increase in IDO activity when IFN- γ was combined with LPS (Babcock and

Carlin, 2000; van Wissen et al., 2002). The combination effect of several inflammatory cytokines and mediators on IDO expression may be important for enhancing regulatory function of IDO *in vivo*.

The control of IDO activity appears to be complex and cell-type specific. Presence of the IDO mRNA and protein may not be correlated with its functional activity. Human dendritic cells constitutively express IDO protein, but it does not have functional enzymatic activity until these cells are activated by IFN-γ and CD80/CD86 ligation (Munn et al., 2004). In this study, we showed that increased IDO mRNA expression in human gingival fibroblast upon treatment with IFN-γ was positively correlated with increased IDO activity. In addition, the IDO activity was increased over time. However, IDO activity in human gingival fibroblasts treated with IL-1β, TNF-α, or *P. gingivalis* LPS was not significantly different from that of untreated controls. This may be due to the low level of IDO mRNA expression in these cells and the wide variation in the level of response between primary cell lines. We also showed that IL-1β and TNF-α augmented the IDO activity in IFN-γ treated-HGF cells. IL-1β and TNF-α has been shown to enhance the IDO activity induced by IFN-γ in human monocyte-derived macrophages (Carlin and Weller, 1995; Hissong et al., 1995) and human cervical epithelial cells (Babcock and Carlin, 2000). In contrast, the presence of IL-1β and TNF-α decreased the IDO activity induced by IFN-γ in uroepithelial cell line (Daubener and MacKenzie, 1999) and showed no effect on the IDO activity induced by IFN-γ in astrocytoma cell line (Daubener

and MacKenzie, 1999). An *in vivo* study in mice showed that LPS induced a significant increase in systemic IDO activity, as indirectly measured by the increased level of plasma kyneurenine (Fujigaki et al., 2001). Using IFN-γ or TNF-α gene-disrupted mice and IFN-γ antibody-treated mice, it was demonstrated that LPS-induced systemic IDO is largely dependent on TNF-α rather than IFN-γ. IDO induction by LPS was significantly attenuated in TNF-α-knockout mice when compared with that in genetically normal mice. In contrast, LPS induced IDO activity in both IFN-γ knockout mice and IFN-γ-antibody-treated mice at a level comparable to that of normal mice. It appeared that human gingival fibroblasts were able to produce functional IDO in response to several inflammatory cytokines and mediators. These agents may work together or regulate each other to control IDO expression and activity *in vivo*.

Cells expressing IDO have been shown to down-regulate immune response through inhibition of immune cell proliferation and induction of cell apoptosis (Ghahary et al., 2004; Munn et al., 1998). In periodontitis, increased level of inflammatory cytokines IFN-γ, IL-1β, TNF-α, and *P. gingivalis* LPS may induce IDO expression by gingival fibroblasts. Since gingival fibroblasts were in close proximity with infiltrated T cells, IDO expression by gingival fibroblasts may directly affect T cells proliferation and functions. This could lead to down-regulation of immune response. Therefore, it is possible that IDO expression in periodontal tissue may act as a negative feedback to control excessive inflammation. If it was true,

increased IDO expression may be beneficial for controlling periodontal destruction and disease progression. Understanding IDO function and regulation within periodontal tissue may provide a better understanding for disease pathogenesis and new approach for periodontal treatment.

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APPENDICES

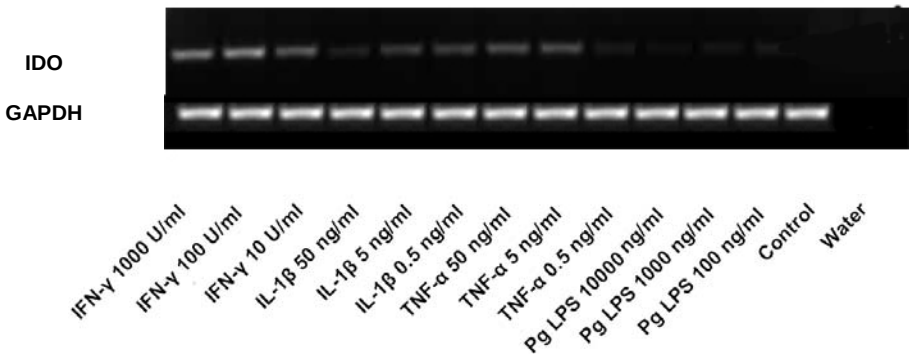

Figure 9. IDO mRNA expression in HGF cells (HGF1P6) treated with different concentrations of IFN-γ, IL-1β, TNF-α, or PgLPS.

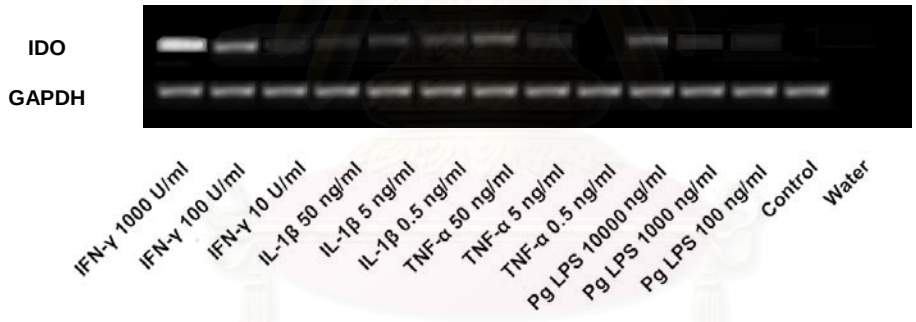

Figure 10. IDO mRNA expression in HGF cells (HGF2P3) treated with different concentrations of IFN-γ, IL-1β, TNF-α, or PgLPS.

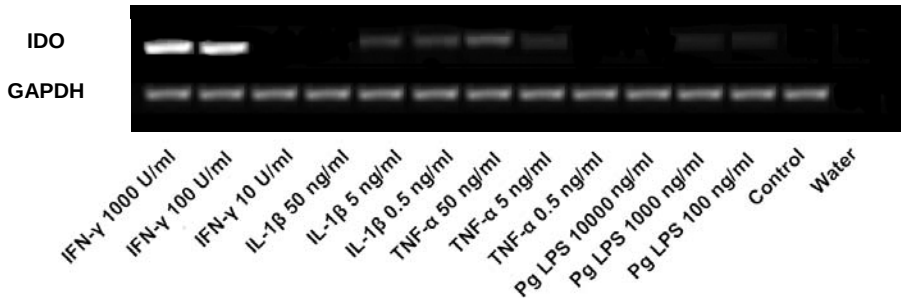

Figure 11. IDO mRNA expression in HGF cells (HGF7P5) treated with different concentrations of IFN-γ, IL-1β, TNF-α, or PgLPS.

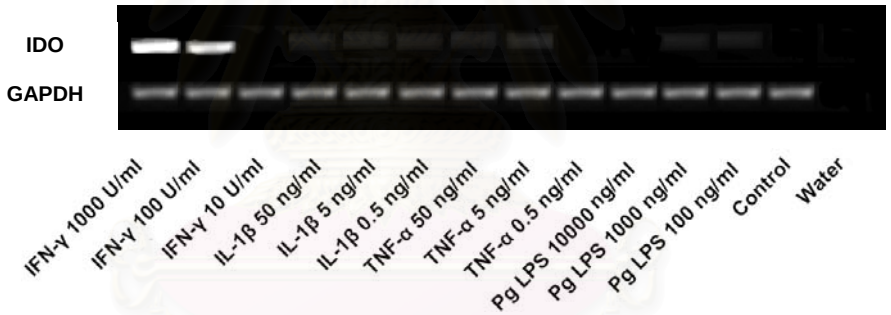

Figure 12. IDO mRNA expression in HGF cells (HGF8P5) treated with different concentrations of IFN-γ, IL-1β, TNF-α, or PgLPS.

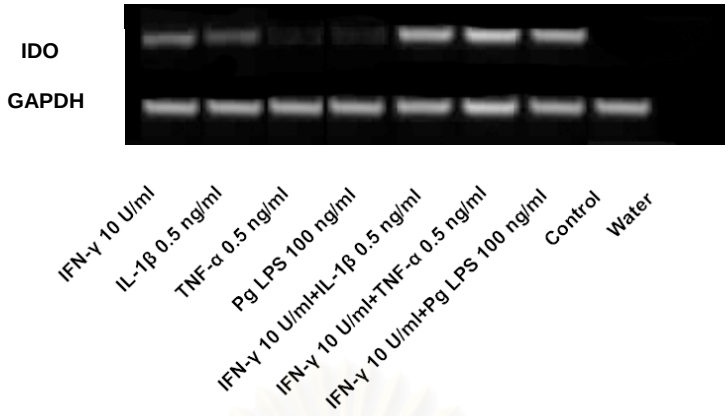

Figure 13. IDO mRNA expression in HGF cells (HGF1P6) treated with the combinations of IFN-γ and IL-1β, TNF-α, or PgLPS.

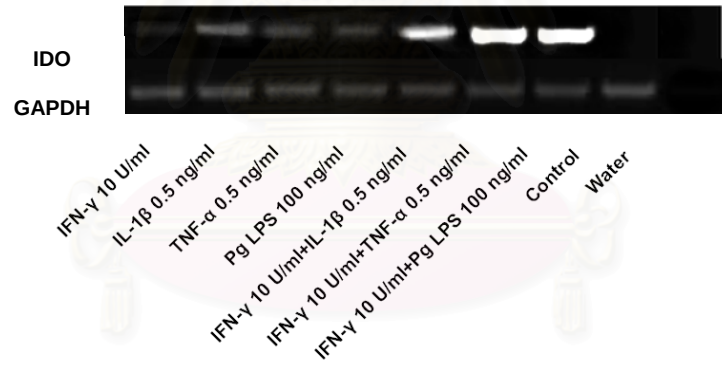

Figure 14. IDO mRNA expression in HGF cells (HGF2P3) treated with the combinations of IFN-γ and IL-1β, TNF-α, or PgLPS.

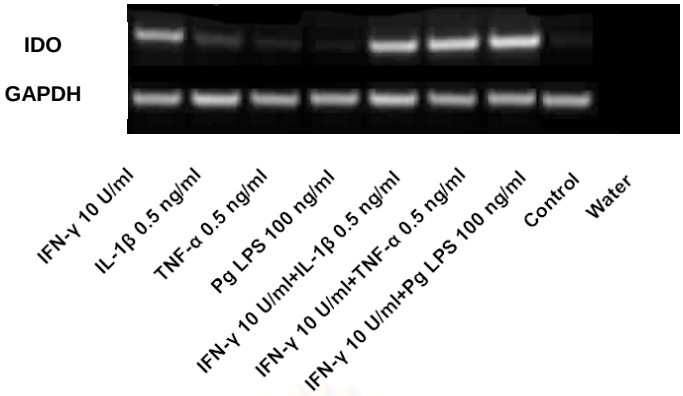

Figure 15. IDO mRNA expression in HGF cells (HGF7P6) treated with the combinations of IFN-γ and IL-1β, TNF-α, or PgLPS.

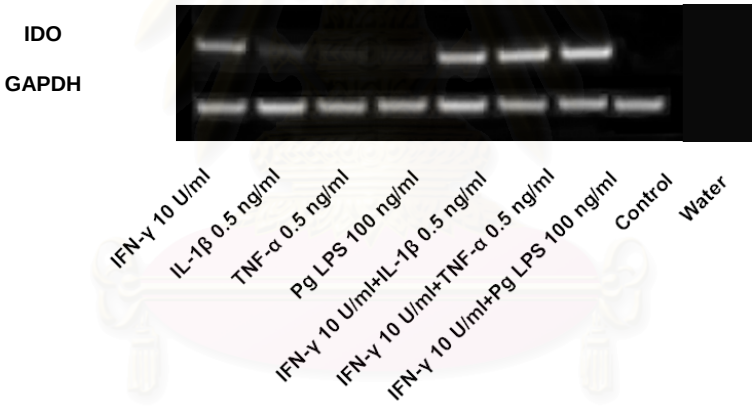

Figure 16. IDO mRNA expression in HGF cells (HGF8P6) treated with the combinations of IFN-γ and IL-1β, TNF-α, or PgLPS.

Table 2. Relative level of IDO mRNA expression from 4 different human gingival fibroblast cell lines stimulated with the single stimulator.

Cytokine and mediators	Relative level of IDO mRNA			
	HGF1P6	HGF2P3	HGF7P5	HGF8P5
IFN-γ 1000 U/ml	2.26	3.84	3.22	4.81
IFN- γ 100 U/ml	2.73	3.10	2.47	2.65
IFN- γ 10 U/ml	1.70	0.31	0.12	0.66
IL-1β 50 ng/ml	0.41	0.89	0.14	0.06
IL-1β 5 ng/ml	0.84	1.43	0.22	0.06
IL-1β 0.5 ng/ml	0.76	1.40	0.19	0.07
TNF-α 50 ng/ml	1.05	2.15	0.62	0.28
TNF-α 5 ng/ml	1.04	0.53	0.39	0.18
TNF-α 0.5 ng/ml	0.40	0	0.21	0.08
PgLPS 10000 ng/ml	0.21	2.24	0	0.07
PgLPS 1000 ng/ml	0.27	0.66	0	0
PgLPS 100 ng/ml	0.33	0.26	0.06	0
control	0.17	0	0.06	0

Table 3. Relative level of IDO mRNA expression from 4 different human gingival fibroblast cell lines stimulated with the combinations of IFN-γ and IL-1β, TNF-α, or PgLPS.

Cytokine and mediators	Relative level of IDO mRNA			
	HGF1P6	HGF2P3	HGF7P6	HGF8P6
IFN-γ 10 U/ml	1.70	0.31	1.40	1.78
IL-1β 0.5 ng/ml	0.76	1.40	0.28	0.43
TNF-α 0.5 ng/ml	0.31	0	0.18	0.22
PgLPS 100 ng/ml	0.33	0.26	0.13	0.17
IFN-γ10U/ml+IL-1β 0.5 ng/ml	1.97	3.72	1.79	2.08
IFN-γ10U/ml+TNF-α 0.5 ng/ml	2.21	3.66	1.32	1.78
IFN-γ10U/ml+PgLPS100 ng/ml	1.87	2.72	1.50	1.74
control	0.17	0	0.15	0.11

Table 4. IDO activity from 4 different HGF cell lines stimulated with the single stimulator.

Cytokine and mediators	IDO activity(μM)							
	HGF1P6		HGF2P3		HGF7P5		HGF8P5	
	24h	48h	24h	48h	24h	48h	24h	48h
IFN-γ 1000 U/ml	55.52	82.50	54.86	54.99	30.3	52.14	34.82	59.65
IFN- γ 100 U/ml	52.44	60.35	49.12	51.45	28.64	47.39	32.27	56.25
IFN- γ 10 U/ml	19.90	22.72	41.92	49.62	17.90	10.00	21.78	45.01
IL-1β 50 ng/ml	13.07	28.45	2.67	5.44	6.23	6.39	0	0
IL-1β 5 ng/ml	12.68	24.29	1.69	4.49	3.53	5.91	0	0
IL-1β 0.5 ng/ml	12.64	21.70	3.01	6.02	3.17	3.85	0	0
TNF-α 50 ng/ml	10.25	18.33	2.92	9.83	3.97	14.29	0	0
TNF-α 5 ng/ml	8.34	11.23	2.83	4.82	3.13	8.13	0	0
TNF-α 0.5 ng/ml	6.60	10.44	1.47	2.27	3.57	6.07	0	0
PgLPS10000 ng/ml	7.65	9.95	1.35	2.95	3.29	7.66	0	0
PgLPS1000 ng/ml	6.37	8.54	0.83	2.30	2.30	4.40	0	0
PgLPS 100 ng/ml	5.94	6.11	0	3.90	1.19	7.42	0	0
control	7.78	9.75	0	0	6.75	4.96	0	0

Table 5. IDO activity from 4 different HGF cell lines stimulated with the combinations of IFN-γ and IL-1β, TNF-α, or PgLPS.

Cytokine and mediators	IDO activity(μM)							
	HGF1P6		HGF2P3		HGF7P6		HGF8P6	
	24h	48h	24h	48h	24h	48h	24h	48h
IFN-γ 10U/ml	19.9	22.72	41.92	49.62	34.06	55.27	35.88	49.52
IL-1β 0.5 ng/ml	12.64	21.70	3.01	6.02	21.03	19.21	18.30	19.52
TNF-α 0.5 ng/ml	6.60	10.44	1.47	2.27	18.91	18.91	18.00	18.30
PgLPS 100 ng/ml	5.94	6.11	0	3.90	20.73	19.21	18.61	17.09
IFN-γ10U/ml+ IL-1β 0.5 ng/ml	43.55	61.09	46.78	61.04	51.94	73.76	67.39	78.61
IFN-γ10U/ml+ TNF-α 0.5 ng/ml	39.11	65.74	42.9	57.26	41.64	67.39	57.70	72.55
IFN-γ10U/ml+ PgLPS100 ng/ml	22.12	38.09	38.68	34.04	24.06	36.48	21.33	25.58
control	7.78	9.75	0	0	18.91	18.91	18.91	18.91

BIOGRAPHY

Mrs. Jittima Makrudthong was born on 6th of September 1977 in Angthong province. She graduated with D.D.S. (Doctor of Dental Surgery) from the Faculty of Dentistry, Srinakharinwirot University in 2001, and became a staff member of the General Dentistry department of the Faculty of Dentistry, Srinakharinwirot University. She studied in a Master degree program in Periodontology at Graduate School, Chulalongkorn University in 2004.