บทบาทของไลโพโปลีแซคคาไรด์ต่อการแสดงออกของเอชเอ็มจีบีวันในเซลล์ไฟโบรบลาสต์ของเอ็นยึดปริทันต์มนุษย์

บทคัดย่อ

ของ

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เสนอต่อบัณฑิตวิทยาลัย มหาวิทยาลัยศรีนครินวิโรฒ เพื่อเป็นส่วนหนึ่งของการศึกษา

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ยีนเอชเอ็มจีบีวันหรือแต่เดิมเรียกแอมโฟเทอริน ซึ่งเป็นโปรตีนที่อยู่ในนิวเคลียส ต่อมาพบว่าโปรตีนชนิดนี้สามารถถูกขับออกนอกเซลล์ได้ในเซลล์บางประเภท ส่วนของโปรตีนเอชเอ็มจีบีวันที่ขับออกมานอกเซลล์นั้น สามารถกระตุ้นให้เกิดการหลั่งของสารไซโตไคน์ซึ่งมีผลทำให้เกิดการอักเสบและมีบทบาทสำคัญต่อการเกิดโรคที่เกี่ยวข้องกับการอักเสบหลายโรค อย่างไรก็ตาม ยังไม่เคยมีผู้ทำการศึกษาถึงบทบาทของเอชเอ็มจีบีวันต่อการเกิดโรคปริทันต์อักเสบ ในการศึกษาครั้งนี้ ผู้วิจัยได้ศึกษาถึงการแสดงออกของเอชเอ็มจีบีวันในเซลล์ไฟโบรบลาสต์ที่ได้จากเอ็นยึดปริทันต์เปรียบเทียบกับเซลล์ที่ได้จากแหล่งอื่น ได้แก่ เซลล์ไฟโบรบลาสต์ที่ได้จากโพรงประสาทฟันและเนื้อเยื่อเหงือกของมนุษย์ รวมถึงเซลล์มะเร็งช่องปาก ผลการศึกษาพบว่ามีการแสดงออกของเอชเอ็มจีบีวันทั้งในระดับอาร์เอนเอและระดับโปรตีนภายในเซลล์เหล่านี้ แต่ไม่สามารถพบโปรตีนชนิดนี้ในอาหารเลี้ยงเซลล์ ซึ่งแสดงให้เห็นว่า เซลล์ที่นำมาศึกษาครั้งนี้ไม่สามารถขับโปรตีนเอชเอ็มจีบีวันออกมาภายนอกเซลล์ได้ นอกจากนี้ผู้วิจัยยังทำการศึกษาถึงการแสดงออกของเอชเอ็มจีบีวันในเซลล์ไฟโบรบลาสต์ของเอ็นยึดปริทันต์มนุษย์ภายหลังจากถูกกระตุ้นด้วยไลโพโปลีแซคคาไรด์ของเชื้ออีโคไล ผลที่ได้พบว่ามีการเพิ่มขึ้นของการแสดงออกของในระดับอาร์เอนเอ เฉพาะสภาวะที่ใช้อาหารเลี้ยงเซลล์ที่มีส่วนประกอบของซีรั่มเท่านั้น ผลที่ได้นี้น่าจะเป็นการแสดงถึงความต้องการโปรตีนที่มีอยู่ในซีรั่มของเซลล์ที่นำมาศึกษาในการทำหน้าที่ช่วยจับกับไลโพโปลีแซคคาไรด์ที่มากระตุ้น อย่างไรก็ตาม ไม่พบการเปลี่ยนแปลงของเอชเอ็มจีบีวันที่ระดับโปรตีนทั้งภายในและภายนอกเซลล์ แสดงให้เห็นว่า ภายใต้สภาวะของการวิจัย การเพิ่มขึ้นของยีนเอชเอ็มจีบีวันในระดับอาร์เอนเอนั้นไม่สามารถนำไปสู่การเพิ่มขึ้นของโปรตีนได้ ดังนั้นจากผลการศึกษาครั้งนี้ จึงยังไม่เพียงพอที่จะสรุปถึงบทบาทของเอชเอ็มจีบีวันในโรคปริทันต์อักเสบได้ ควรได้รับการศึกษาเพิ่มเติมถึงบทบาทของเอชเอ็มจีบีวันในโรคปริทันต์อักเสบต่อไป

EFFECT OF LPS ON HMGB1 EXPRESSION IN HUMAN PERIODONTAL LIGAMENT FIBROBLASTS

AN ABSTRACT

BY

KANIT NANTASENEE

Presented in partial fulfillment of the requirements for the

Master of Sciences degree in Periodontology

at Srinakharinwirot University

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High mobility group 1 (HMGB1), so called amphoterin, was originally known as a nuclear protein but later found to be secreted by some cell types. Extracellular HMGB1 activates proinflammatory cytokine and plays important roles in many inflammatory diseases. However, HMGB1 roles in periodontal disease are still limited. In this study, we investigated HMGB1 expression in HPDL as well as HPF, HGF and KB cell line. It was found that all cells expressed HMGB1 RNA and protein in cell lysates, but not in conditioned media excluding roles of HMGB1 as a secreted protein in these cells. We further looked at HMGB1 expression in HPDLF after E.Coli LPS treatment. Results showed that HMGB1 RNA was upregulated when treated with LPS, but only in the condition with fetal calf serum, confirming the requirement of LPS to LPS-binding protein (LBP) presence in serum. However, HMGB1 in cell lysate remained unchanged indicating that the increased RNA could not proceed through protein production in our experimental condition. Roles of HMGB1 in periodontitis required further investigation.

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The Thesis Committee and Oral Defense Committee have approved this thesis as partial fulfillment of the requirements of the Master of Sciences degree in Periodontology of Srinakharinwirot University.

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CHAPTER 1

Introduction

Background

High mobility group box protein 1 (HMGB1), a small protein (215 residues), was first identified 30 years ago[1]. It consists of two consecutive L-shaped basic domains (called HMG boxes) and a 30 amino acid-long acidic 'tail' connected by short linkers. HMGB1 is amongst the most ubiquitous, abundant and evolutionarily conserved protein in eukaryotes. It shares 100% amino acid sequence between mouse and rat, and 99% between rodent and human. Previously, HMGB1 was identified as a nuclear component[2]. Its role involved maintaining nucleosome structure and regulation of gene transcription[1]. Later studies showed extracellular role of HMGB1 as a proinflammatory cytokine[3]. This role has opened up a new field of research of HMGB1 role in inflammatory disease such as severe sepsis and arthritis.

HMGB1 can be released by some cell types. It can be actively released by macrophages/ monocytes in response to stimulation with exogenous bacterial endotoxin e.g. lipopolysaccharide (LPS) or endogenous proinflammatory cytokines eg. tumour necrosis factor (TNF), interleukin 1β (IL-1β) and interferon gamma (IFN)-γ)[4,5,6].In addition to being actively released from activated innate immune cells, HMGB1 can also be passively released from necrotic or damaged cells. Once released, HMGB1 is able to activate several other cells involved in the immune response or inflammatory reaction. Therefore, HMGB1 might be a critical molecule that allows innate immune cells both to response to injury, and to further induce inflammation. HMGB1 has been termed as a late mediator of endotoxin lethality, because its releaseing is delayed by several hours compared with other proinflammatory cytokines that mediate shock and tissue injury[7].The cytokine activity of HMGB1 has been well-documented in many cell types and tissues such as macrophages/monocytes, endothelial cells, neutrophils, epithelial cells, dendritic cells, smooth muscle cells, brain, lung, joint and etc.[8]. However, most cells including

lymphocytes, adrenal or kidney cells, are unable to secrete HMGB1[4]. Also, HMGB1 is not released by apoptotic cells even after sequent secondary necrosis and partial autolysis. Thus, cells undergoing apoptosis fail to activate inflammatory responses[9].

HMGB1 has an important role in several acute and chronic inflammatory diseases such as sepsis, acute lung injury and arthritis. Periodontitis is an infection disease that leads to inflammation of the gingiva and destruction of periodontal tissues. Oral microbes are associated in the initiation and progression of periodontal disease that eventually cause tooth loss. Gram negative bacteria have become widely recognized as the periodontopathic bacteria[10]. The bacteria are capable of producing virulence substances that directly cause periodontal tissue breakdown and alveolar bone resorption[11].

LPS, a component of the bacterial outer membrane, is the pathogenic factor in a wide variety of periodontopathic bacteria. The LPS molecule consists of three parts: LIPID A, core polysaccharide, and O-specific chains (O antigens). When periodontopathic bacteria proliferate in periodontal pockets, it is suggested that LPS is amply released, causing fibroblasts as well as immune cells to steadily synthesize inflammatory cytokines including IL-1, IL-6 and TNF-α[12]. These cytokines, in turn, aggravate inflammation, destroy periodontal tissues, and induce alveolar bone resorption[13,14]. When derived from Escherichia coli (E.coli), LPS serves as polyclonal B-cell mitogens commonly used in immunology laboratory. Treatment of mice with LPS which cause liver injury, induces a three fold increase of HMGB1 protein in liver within 1-2 hours[15].

However evidence of HMGB1 role on oral diseases is still limited. Morimoto et al, 2006[16] showed expression of HMGB1 in gingival epithelium of patients with periodontitis as well as human gingival carcinoma cell line. The aim of this study is to determine the expression of HMGB1 in human periodontal ligament (HPDL) fibroblasts with or without stimulation by E.coli LPS. We hypothesized that the HPDL fibroblasts express HMGB1 and the level of expression is upregulated by LPS stimulation.

Objective of the Study

To examine the expression of HMGB1 in HPDL fibroblasts both in normal condition and after stimulation by E.coli LPS.

Significance of the Study

The outcome of this study will give sharpened understanding of the biologic basis of periodontal pathogenesis, which might lead to new strategies for the treatment of periodontitis.

Scope of the Study

The present study will characterize the mRNA and protein expression profile of HMGB1 in primary HPDL fibroblasts under normal condition and after treated with E.Coli LPS.

Definition of terms

-**High mobility group 1 (HMGB1)**: HMGB1 (formerly named HMG1 but also known as amphoterin was identified almost 30 years. It is a small protein (215 residues) that runs fast in SDS-polyacrylamide gels.

-**Lipopolysaccharide (LPS)**: Lipid-containing polysaccharides which are endotoxins and important group-specific antigens. They are often derived from the cell wall of gram-negative bacteria and induce immunoglobulin secretion. The lipopolysaccharide molecule consists of three parts: LIPID A, core polysaccharide, and O-specific chains (O antigens). When derived from Escherichia coli, lipopolysaccharides serve as polyclonal B-cell mitogens commonly used in laboratory immunology. (From Glossary)

-**Human Periodontal Ligament fibroblasts (HPDL fibroblasts)**: Periodontal ligament (PDL) cells reside between the cementum of the roots of teeth and the alveolar bone. In this location PDL cells are uniquely situated to maintain the overall integrity of the periodontal ligament.

CHAPTER 2

Review literature

Structure of HMGB1

The high mobility group (HMG) chromosomal proteins were discovered in mammalian cells more than 30 years ago and named according to their electrophoretic mobility in polyacrylamide gels[17]. The HMG proteins are subdivided into 3 superfamilies; HMGB, HMGN and HMGA. Each HMG superfamily has a characteristic functional sequence motif. The functional motif of the HMGB family is called the 'HMG-box'; that of the HMGN family is called the nucleosomal binding domain'; and that of the HMGA family is called the 'AT-hook'. Proteins containing any of these functional motifs embedded in their sequence are known as 'HMG-motif proteins'. The revisions, which have been applied to the canonical, mammalian HMG proteins, can be adapted to HMG-motif proteins from all organisms. The nomenclature of the HMG nuclear proteins has been revised to facilitate interactions between various laboratories, expedite literature searches and avoid confusion owing to similarity in the names of unrelated proteins. The main features of the revised nomenclature are summarized in Table 1.

Table 1[18] Revised nomenclature for the HMG chromosomal proteins

HMG motif protein	Functional motif	HMG proteins		
		Root symbol	New name (canonical HMGs)	Old name (canonical HMGs)
HMG-box proteins	HMG-box	HMGB	HMGB1,2,…n	HMG-1/HMG-2
NBD proteins	NBD	HMGN	HMGN1,2,…n	HMG-14/HMG-17
ATH proteins	ATH	HMGA	HMGA1,2,…n	HMG-I/HMG-Y/HMG-C

ATH; AT-hook, HMG; high mobility group, NBD; nucleosome binding domain

The structure of these three proteins is highly conserved (>80% amino acid identity), and their biochemical properties are so far indistinguishable. HMGBs are composed of three different domains. The two homologous DNA binding domains, HMG box A and B, are each approximately 75 amino acids in length. The C-terminal domain is highly negatively charged, consisting of a continuous stretch of glutamate or aspartate residues (as shown in fig.1).

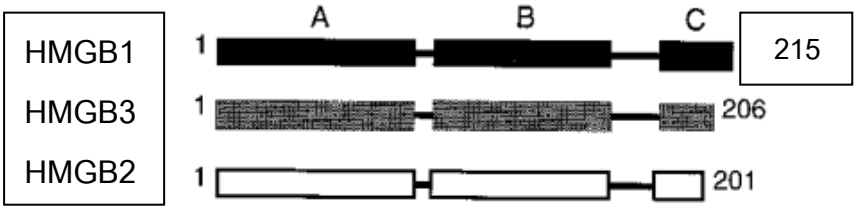

Fig.1[19,20] Human HMGBs family are composed of three domains: two homologous DNA-binding domains, A and B boxes, and a negatively charged C terminus (Acidic tail).

HMGB1 is ubiquitous and only 10 times less abundant than core histones. Expression of the other two family members is more restricted: HMGB3 is only expressed to a significant amount during embryogenesis[21]; HMGB2 is widely expressed during embryonic development, but restricted mainly to lymphoid organs and testis in the adult mouse[22]. HMGB1 is extremely conserved, and has 99% identity amongst all mammals. As a highly conserved protein, HMGB1 shares 100% identity in amino acid sequence between mouse and rat, and a 99% amino acid identity between rodent and human[23,24,25]. The C-terminal portion of HMGB1 contains a continuous sequence between 35 and 40 aspartic and glutamic acid residues and the N-terminal portion of HMGB1 comprises two internal repeats of a positively charged domain of about 80 amino acids (as shown in fig.2).

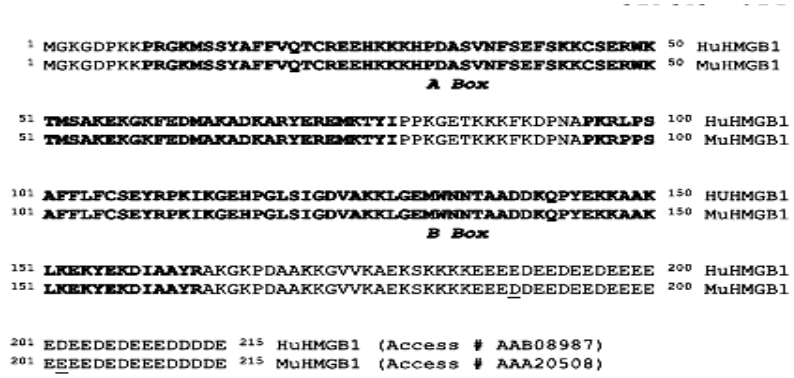

Fig.2[3] Amino acid sequence of murine HMGB1 (MuHMGB1) and human HMGB1 (HuHMGB1).

Expression of HMGB1

HMGB1 is produced by many cell types, and cellular levels vary in development and age[26]. The localization of these proteins in most cells is nuclear. Cellular localization studies have revealed that HMGB1 can migrate between the cytoplasm and nucleus in a cell cycle-dependent fashion. The expression and localization of HMGB1 are summarized in Table 2.

TABLE 2[27] High mobility group box protein 1 (HMGB1) expression and localization

Tissue/tumor/cell line	Expression level	Method	HMGB1 level	Subcellular location
Tissue				
Spleen	P	Cell fractionation	High	N, C
Thymus	P	Cell fractionation	High	N, C
Testis	P	Cell fractionation	High	N, C
Liver	P	Cell fractionation	Low	C
Adult brain	P	Cell fractionation	Low	C
Thymus	P	Prechloric acid, CM-Sephadex fractionation, acetic acid-urea polyacrylamide gel electrophoresis	High in young rat Low in old rat	nd
Mammary gland	R	Northern	High in pregnant and non pregnant mice, low in lactating mice and during gland involution	nd
Testis	P	Immunohistochemistry	High in spermatogonia, low in spermatocytes, absent in spermatids	N
Brain	P	Cell fractionation	High in perinatal brain, low in adult brain	C, M
Brain	P	Immunohistochemistry	Undetectable in most cells of adult brain, During development high in proliferating and migrating neurons	N, C, M
Brain cortex	R	mRNA profiling	High after damage	nd
Spinal cord	P	Immunohistochemistry	High in oligodendrocyte in young rats Infrequent positive oligodendrocytesin adult rat	N, C, M
Peripheral nervous system	P	Immunohistochemistry, Western	High in neuron, Schwann cells	N, C, M
Endothelial cells	P	Immunohistochemistry	High	N
Smooth muscle cells	P	Immunohistochemistry	Low	N, C
Monocytes, macrophages	P	Immunofluorescence	High	N in resting cells, C in activated cells
Tumours				
Human hepatocarcinoma	R	Northern	Higher in cancer tissue than in normal tissue	nd
Gastric mucosa and colon	R	Northern	Higher in cancer tissue than in normal tissue	nd
Breast carcinoma	P	Western	Higher in human breast carcinomas than in normal tissue	nd
Human breast cancer	R	Northern	Very variable	nd

TABLE 2[27] (continue)

Tissue/tumor/cell line	Expression level	Method	HMGB1 level	Subcellular location
Smooth muscle tissue of gastro-intestinal tract	P	2D gel analysis and mass spectrometry	Higher in tumours with KIT mutation	Mainly N
Mammary and pancreatic adenocarcinoma	R	substractive cDNA cloning, Northen	Higher in metastatic carcinomas	nd
Cell lines				
Neuroblastoma	P	Acetic acid/urea/PAGE	Low in differentiated, high in undifferentiated	nd
Friend erythroleukaemia	P	Acetic acid/urea/PAGE	High in differentiated, low in undifferentiated	nd
N18 Neuroblastoma	P	Immunofluorescence	-	C, M, filopodia
Muscle cell lines	R	Northern	Low in myotubes, high in myoblasts	nd
Leukaemia cell lines	P	Perchloric acid extraction and SDS-PAGE	Higher in myeloid than in lymphoid cells	nd
Melanocytic cells	P	Western, immunohistochemistry	Higher in melanoma than in melanocytes	N
U937 monocytic cells	P	Immunofluorescence	High	N in resting cells, C in activated cells

Expression level; R=RNA level, P=protein level

Subcellular location; N=nuclear localization; C=cytoplasmic localization, M=plasma membrane localization, nd=not determined

SDS-PAGE= polyacrylamide gel electrophoresis

HMGB1 function and secretion

HMGB1 has been implicated in diverse cellular functions, including determination of nucleosomal structure and stability, and binding of transcription factors to their cognate DNA sequences[28]. In the nucleus, HMGB1 binds to DNA with little or no sequence specificity. The HMG boxes provide the structure-specific binding sites for DNA with secondary structures. These observations suggested a possible role of HMGB1 in DNA recombination, repair, replication and gene transcription[18,28]. Hmgb1-deficient mice demonstrated the important role of this gene as a regulator of transcription. These animals died within a few hours after birth because they were unable to use the glycogen stored in the liver as a result of inefficient activation of glucocorticoid receptor-responsive genes[29].

HMGB1 does not contain a signal sequence and thus, does not traverse the endoplasmic reticulum or Golgi system, but it is released into the extracellular milieu. HMGB1 can be released actively and passively under inflammatory or injurious conditions.

-Active release of HMGB1; cultures of macrophages, monocytes and pituicytes actively release HMGB1 after stimulate with exogenous bacterial endotoxin (e.g. LPS) or endogenous proinflammatory cytokines (e.g. TNF, IL-1ß, IFN-γ) in a time- and dose-dependent manner[4,5,6].

-Passive release of HMGB1; HMGB1 can also be passively released by necrotic or damaged cells. HMGB1 is capable of inducing an inflammatory response, thereby transmitting the injury signal to neighbouring immune cells[7].

Cells undergoing apoptosis do not release HMGB1, even when the integrity of their membranes are lost. Thus, cells undergoing apoptosis fail to activate inflammatory responses, whereas cells undergoing necrosis release HMGB1 and activate inflammatory responses during tissue injury and trauma[7].

Binding of HMGB1

Interestingly, HMGB1 is secreted into the extracellular medium upon exposure to the inflammatory mediators such as LPS, IL-1ß and TNF-α[4,5,6]. The secretory pathway for HMGB1 is a mystery since the protein lacks a conventional signal sequence for secretion. The previous study identified receptor for advanced glycation end products (RAGE) as a cellular binding site for HMGB1, based on binding and functional data[30]. This receptor is a member of the

immunoglobulin superfamily and is expressed on mononuclear phagocytes, vascular smooth muscle cells and neurons[31,32]. Secreted HMGB1 stimulates the synthesis and release of proinflammatory cytokines by monocytes[33]. This, in turn, induces transcription of RAGE by a mechanism that is not yet clear, and stimulates further TNF-α release. The previous studies of structure/function of HMGB1 revealed that a motif in the C-terminus is responsible for RAGE binding[34].

The role of HMGB1 in the inflammatory regulations

The cytokine activity of HMGB1 has been well-documented in many cell types (Table 3)[35]. *In vitro* study, macrophages, monocytes, and pituicytes stimulated by LPS, IL-1, or TNF secrete HMGB1[4,5]. Stimulation with HMGB1 increased expression of TNF mRNA and induced the release of proinflammatory cytokines from monocytes.[32,36]

TABLE 3[34] Actions of HMGB1 in vitro and in vivo

Cells	HMGB1	Effects
Macrophages/monocytes	1) Increase TNF mRNA and protein release; increase IL-1α,β, IL-1RA, IL-6, IL-8, MIP-1α and β release. 2) Release after LPS stimulation.	Inflammation
Endothelial cells	Induces expression of adhesion molecules (ICAM-1, VCAM-1) and RAGE; induces cytokine release (TNF and IL-8) and expression of MCP-1, tPA, and PAI-1.	Increase neutrophil adhesion, inflammation, regulation of fibronolysis
Neutrophils	Increase TNF, IL-1β and IL-8 gene expression.	Inflammation
Epithelial cells	Increase enterocyte permeability.	Increase bacterial translocation
Dendritic cells	1) Increase TNF, IL-1α, IL-6, IL-8, and IL-12 release. 2) Increase CD40, CD54, CD58, CD80, and CD83 expression.	Dendritic cell maturation
Smooth muscle cells	Cause cell migration and cytoskeleton reorganization.	Chemotaxis
Tissue	**Effects**	
Brain	Induces fever, anorexia, taste aversion, and weight loss; induces brain cytokine expression (TNF, IL-1, and IL-6).	
Lung	Cause acute lung injury, increased pulmonary levels of TNF, IL-1β and MIP-2, lung edema, and neutrophil accumulation.	
Intestine	Cause intestinal barrier of dysfunction and bacterial translocation.	
Joints	Induces arthritis and inflammation	
Heart	Arrhythmia	
Others	Bactericidal activity	

These results show that HMGB1 acts as a potent, proinflammatory cytokine, orchestrates a cascade of injurious, inflammatory responses, and induces a broad spectrum of systemic changes in various systems in vitro and in vivo.

Previous study indicated the important role of HMGB1 in chronic inflammatory diseases such as Rheumatoid arthritis. HMGB1 were elevated in synovial fluid of patients with rheumatoid arthritis as well as in that experimental arthritis in animal models[37]. Administration of HMGB1 into mice joints also induced arthritis changes[38] and stimulated the synovial macrophages to release proinflammatory cytokines including TNF, IL-1ß, IL-6[39]. These data indicate that HMGB1 plays a role in the inflammatory and destructive processes in the pathogenesis of arthritis.

The kinetics of HMGB1 accumulation in vivo has been studied in murine models of endotoxaemia and sepsis. Serum HMGB1 was first detectable 8 hours after administration of an LD50 dose of endotoxin, and increased to a prolonged plateau level from 16 to 32 hours after endotoxin infusion[4]. LPS induced the appearance of HMGB1 protein in the conditioned medium that was not apparent at earlier time points. Serum HMGB1 remained significantly elevated for at least 72 hours, a time course similar to the delayed kinetics in endotoxaemia as shown in fig.3.

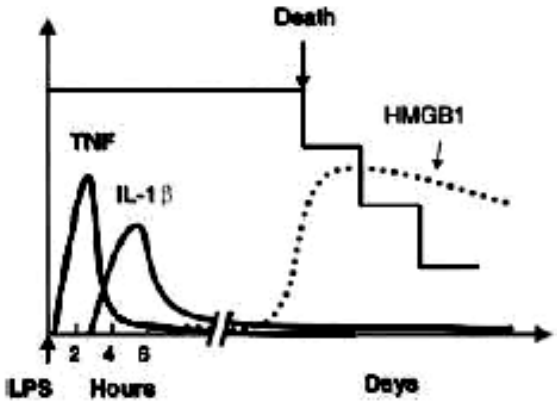

Figure.3[40] Early versus late mediators of endotoxin lethality. Vertebrates treated with lethal doses of LPS succumb at latencies of up to several days, long after serum TNF and IL-1ß have returned to basal levels. The kinetics of HMGB1 release is delayed, and parallels the onset of lethality.

The release of HMGB1 by activated macrophages, its causative role in lethal endotoxemia, and its activity as a macrophage-stimulating agent (Table 4) reveal that HMGB1 is a late cytokine mediator of systemic inflammation.

TABLE 4[40] Pathophysiology of HMGB1 release

	Cell Culture	Mice	Humans
Release of HMGB1	Increased levels in pituicyte cultures 8-10 h after TNF stimulation and in macrophage cultures 8 h after LPS stimulation	Increase in serum 16 h after LPS administration, and remains elevated for>36 h	Elevated levels of HMGB1 detectable in serum of patients with surgical sepsis and hemorrhagic shock

Hemorrhagic shock, like septic shock, is characterized by activation of inflammatory cytokines, even in the absence of bacterial products. Serum HMGB1 levels were increased significantly within 24 hours after the onset of hemorrhagic shock and returned toward basal level as the clinical conditions improved[41].

Possible role of HMGB1 in periodontal pathogenesis

Periodontitis is a chronic inflammatory disease that leads to loss of tooth supporting structures and eventually the tooth itself. The pathogenesis of periodontitis is complicated. Evidences strongly supported that bacteria is involved in initiate and progression of the disease both directly and indirectly. The bacteria are capable of producing virulence substances directly damage periodontal tissue breakdown and alveolar bone resorption[14]. Periodontal disease is caused by bacteria in dental plaque, with evidence that specific periodontal pathogens are responsible for the progressive form of the disease. However, some individuals harbor these specific microorganisms, but do not appear to show evidence of disease progression. However, the periodontal tissue destruction mainly results from host immune and inflammatory response to the microbial challenge. It is now accepted that the host responds to bacteria and their products entering the tissues, activating inflammatory and immune processes by increasing the secretion of inflammatory cytokines and host proteolytic enzymes. This is the major cause of the periodontal destruction[42,43,44]. Patient susceptibility is of utmost importance to the outcome of periodontal disease, and although periodontal bacteria are the major

etiological agents, the host immune response to these bacteria is of fundamental importance[45]. Although patient susceptibility is of major importance in determining the outcome of periodontal disease, the problems in detecting susceptible individuals have not been solved. The study of cytokines in periodontal disease lesions may throw some light on this problem and may also suggest future lines of therapy.

Cytokines are recognized as being vital in the immunopathology of an ever-increasing number of diseases, and the production of appropriate cytokines is essential for the development of protective immunity. If inappropriate cytokines are elicited, destructive or progressive disease can result[46] . Determination of the features of both the host and pathogen that direct how and where the organism is presented to cytokine-producing cells is necessary if to understand the pathogenesis of not only periodontal but all infectious diseases. Cytokines are low-molecular-weight proteins involved in the initiation and effector stages of immunity and inflammation, in which they regulate the amplitude and duration of the response. They interact in a network: first by inducing each other, second by transmodulating cell surface receptors and third by synergistic, additive or antagonistic interactions on cell function[47]. There appears to be a very complex network of interactions within the immune system. During disease progression, pathogenic bacteria can invade host cells by shedding vesicles containing microbial toxins, proteases, and LPS. These molecules penetrate the tissues, and stimulate monocytes to secrete mediators of inflammation[48,49].

LPS or endotoxin are major constituents of the outer membrane of most Gram-negative bacteria[14,50]. Systemically, LPS is capable of inducing all the symptoms of septic shock, including fever, vascular collapse and death. Locally, LPS can stimulate various cell types including monocytes, macrophages, polymorphonuclear leukocytes (PMN), B-lymphocyte, vascular cells, epithelial cell and fibroblasts. These cells react to LPS in several ways such as mediator production, phagocytosis, proliferation and differentiation. LPS can trigger monocytes to release inflammatory mediators such as IL-1,-6,-8, and TNF that increase local destruction of the connective tissues structure elements[11,51,52]. Host responses can also be regulated by cytokines produced by several cell types in response to stimulation with LPS. In this regard, the macrophage plays an important role in regulating local inflammatory responses to bacterial

insult by the production of cytokines, particularly proinflammatory IL-1b, TNF- α, the anti-inflammatory IL-1 receptor antagonist (IL-1ra) [11,51,53] and HMGB1[4].

Apart from the inflammatory cells, PDL fibroblasts themselves also play important roles in pathogenesis of periodontitis by participating in synthesis and degradation of tissue matrix components. PDL fibroblasts located in the periodontal ligament space. Not only did they form and maintain periodontal ligament but also repair, remodel and regenerate of the adjacent alveolar bone and cementum [54,55,56,57,58]. HPDL fibroblasts can express proinflammatory cytokines e.g. IL-6 and IL-8 after induced by LPS[12]. However, there has been no study on the expression of HMGB1 in HPDL fibroblasts. Thus, to observe cellular response to periodontitis, this study was taken on HPDL fibroblasts with or without stimulation by LPS.

CHAPTER 3

METHODOLOGY

Cell culture and activation by LPS

Human Periodontal Ligament Fibroblasts (HPDLF), Human Gingival Fibroblast s (HGF), Human Pulpal Fibroblasts (HPF) were cultured from the explants obtained from the non-carious, freshly extracted third molars, or teeth removed for orthodontic reason with inform consent[59]. The periodontal ligament (PDL) was scraped from the middle third of the root. Pulpal tissue was obtained from the pulp chamber after separated the tooth. Gingival tissue was obtained from healthy gingiva attached to extracted tooth which was removed for orthodontic reason. At least 2 sets for each tissues were used in this study. All of the tissue were washed with sterile phosphate buffer saline (PBS) and cut into small pieces, then plated into 60-mm tissue cultured plates (Nunc, Napervile, IL, USA). All explants as well as KB cells line (originally from ATCC NO.CCL-17: oral epidermal carcinoma cell) were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal calf serum (5%FCS-DMEM), 2mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin at 37 °c in humidified atmosphere of 5% CO_2. Media and the supplements were from Gibco BRL (Carlsbad, CA, USA). Cells from the third to eighth passages were used in this study. Lipopolysaccharide (LPS) from Escherichia coli (E.coli) (0111:B4 serotype) (Sigma) was dissolved in distilled water and stock prepared at 10 µM.

Cells were seeded in 6-well plates ($2x10^5$cells/well) (Nunc, Napervile, IL, USA) or in 96-well plates (15,000 cells/well) in 5% FCS –DMEM and allowed to attach overnight. After attachment, cells were washed 3 times with DMEM and media changed to serum free DMEM (SF-DMEM) as well as 2.5% fetal calf serum (2.5%FCS-DMEM). In LPS treatment group, LPS was added to a final concentration of 25 µg/ml and 50 µg/ml and sterile distilled water was used as a control. Durations of LPS treatment were 24 hours for RNA extraction, 48 and 72 hours for cell lysate extraction and conditioned media collection. The experiments were performed twice with duplication in each experiment.

HMGB1 expression determination

To determine HMGB1 expression, total RNA analysis was performed by RT-PCR and protein analysis was investigated both in cell lysate and cultured media by Western analysis.

-RNA analysis

-Total RNA extraction

Total RNA was extracted using TRIzol reagent (Life Technologies), as recommended by the manufacturer. Briefly, 1 ml of TRIzol reagent was added to each well for 5 mins. Then 200 µl of chloroform were added and centrifuged at 10,000 rpm, 4°c, for 15 mins. The upper aqueous part was collected, 500 µl of isopropanol was then added and centrifuge at 10,000 rpm, 4°c, for 10 mins. The cell pellet was collected and washed with 75% ethanol, mixed and spun down. The pellet was air dried at room temperature. Total RNA was dissolved by adding 15 µl of RNase-free water, warmed at 55°c for 10 mins and stored at -70 °c until used.

-First strand cDNA synthesis

First strand cDNA was generated according to RevertAid™ first strand cDNA synthesis kit (Fermentas, Life sciences, USA). The RNA was first DNase-treated in a 20 µl reaction containing 10 µl of RNA, 2 µl PCR buffer, 10 U DNase, and 40 U RNase inhibitor. Reverse transcription was conducted in a 25 µl reaction of 2 µg of DNase-treated RNA, 0.5 µg of oligo d(T) 12-18 and DEPC-treated water. The reaction was heated to 70 °c for 10 mins then chilled on ice for 5 mins. The content of 5X reaction buffer, 40 U of ribonuclease inhibitor and dNTP was added into the mixture and incubated at 37 °c for 5 mins before the addition of 1 µl of RevertAid™ M-MuLV reverse transcriptase. The mixture was mixed and incubated at 42 °c for 1 hour. The mixture was then collected and stored at -20 °C until used.

-Polymerase chain reaction (PCR) condition

The PCR was performed using Taq polymerase with a PCR volume of 25 µl. The mixtures contained 10 pg-1µg of cDNA template, 0.2 mM of each dNTPs, 10x PCR buffer, $MgCl_2$ concentration between 1-5 mM, 0.6 U of Taq DNA polymerase, 0.2-0.3 µM of HMGB1 and GAPDH primers. The PCR working conditions were set for initial activation at 95 °c, for 5 mins, a denaturation at 94 °c, for 15 sec, primer annealing at 53 °c, for 1 min, chain elongation at 72 °c, for 1 min and final extention at 72 °c for 10 mins on a DNA thermal cycler (ICycler, BioRad) The amplified DNA was then electrophoresis on a 1.6% agarose gel and visualized by ethidium bromide staining.

-Semi-quantification and agarose gel electrophoresis

-Primer selection and determination of Tm

HMGB1 primers were synthesized by OPERON (A Qiagen company). The sequence of primers were determined using the clustalx 1.6b. Primers were length between 18 to 25 bases and Tm comprised between 50 to 65 °c (oligo v.3.4). To determine specificity, all sequences were compared with the Genbank using the program Blast available at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.

The sequences of primers are as follows:

HMGB1; forward; 5'-CTCTTCTGCTCTGAGTATCGC-3'

reverse; 5'-CAACTGAAGATGAAAAAACTACC-3'

GAPDH; forward; 5'-TGAAGGTCGGAGTCAACGGAT-3'

reverse 5'-TCACACCCATGACGAACATGG-3'.

The PCR reaction was performed in 25 µl of TaqMan universal PCR master mix (PE Applied Biosystems) containing 25 pmole of each primer, 200 nM of the TaqMan probe and 0.8 ng of sample DNA. The PCR

conditions were initially 95 °c for 5 mins followed by 22 to 26 cycles of 94 °c for 15 sec, annealing at 53 °c for 1 min chain elongation at 72 °c, 1 min, and final extension at 72 °c for 10 min.

-Cycle numbers (Determination of cycling parameters)

The PCR amplifications were carried out at different cycle numbers including 18, 20, 22, 24, 26, 28 and 30. After amplifications, PCR products were run on an agarose gel electrophoresis. In order to compare between control and LPS treated group, appropriate cycle number, between 22-26 cycles, was selected.

-Control for competition between primer sets

To determine the competition between primer sets, cDNAs were amplified at 3 conditions; 1) amplification with the control primer set, 2) amplification with the interesting gene primer set, and 3) amplification with both primer sets. PCR products were run on the agarose gel. The band intensity of each primer set was compared. Decreasing of band intensity indicated competition between two primer sets. When competition was detected, different reaction condition were tested (e.g. adjusting the concentration of $MgCl_2$).

The ratio between the interesting gene and the internal control products was determined by electrophoresis on 1.6% agarose gels. PCR products were combined with the gel-loading dye before loading to the agarose gel. A 100 bp DNA ladder was used as a standard DNA marker. After electrophoresis at 100 volts, the gel was stained with 2.5 µg/ml ethidium bromide (EtBr) for 10 minutes and destained in distilled water for 15 minutes.

- Protein analysis

Conditioned media were collected for extracellular protein measurement. Then, cells were washed with PBS and protein extracted by 100 µl of 1% SDS for cell lysate analysis. The total proteins were measured by Bradford reagent. All samples were fractionated by 10% SDS-PAGE. Amount of samples loading were calibrated base on amount of total protein in cell lysate. The proteins were then transferred to a (poly) vinylidene difluoride

immunoblot membrane (PVDF) (Bio-Rad Laboratories, Hercules, CA). The membrane was blocked for 1 hour with blocking solution (5% skim milk in TBS-T) and then incubated with HMGB1 antibodies (Monoclonal Anti-HMG-1 Clone HAP46.5 , Sigma, USA) for 1 hour, at 1:1,000 dilution . The membrane was washed for 10 minutes 5 times in washing solution and then incubated with ECL Anti-Mouse IgG, Horseradish Peroxidase-Linked Species-Specific whole Antibody (from sheep) (Amersham Bioscience, UK) as the secondary antibody, dilution 1:50,000 for 1 hour at room temperature. Signals were developed with an enhanced chemiluminescence kit (ECL plus) (Amersham Pharmacia Biotech) according to manufacturer's protocol. For detection of the chemiluminescent signal, the membrane should be exposed to standard Hyperfilm™ ECL (Amersham Bioscience, UK). Adjust the exposure time to the signal strength. The several exposures may be required in order to generate the appropriate signal strength.

CHAPTER 4

FINDINGS

Expression of HMGB1 in HPDL, HPF, HGF and KB cell line

HMGB1 RNA, protein expression in HPDLF and their responses by LPS activation were investigated in this study. In our initial study, it was of interest to determine the expression of HMGB1 in primary HPDLF compare to other oral cells including primary HPF, HGF as well as KB cell line. We performed RT-PCR and western analysis for RNA and protein detection respectively. It was found that all cells expressed HMGB1 RNA (fig.1A) as well as HMGB1 protein in cell lysate (fig.1B). HMGB1 was not found in conditioned media for all cells in this study.

HMGB1 RNA expression after LPS activation

E.coli LPS has been known to cause cell injury in many studies[15,60,61,62]. We hypothesized that the activation by LPS may affect HMGB1 mRNA and protein expression in HPDLF. Previous study has suggested that activation by LPS requires specific plasma protein such as LPS-binding protein (LBP) in order to mediate attachment to receptors on cells [49]. Thus, we treated HPDLF with E.Coli LPS in both serum-free and in 2.5%FCS media condition.

After 24 hours incubation, expression of HMGB1 RNA by semi-quantitative RT-PCR was analysed. GAPDH as a house keeping gene was used for an internal control. Appropriate cycle number was initially tested and the cycle between 18 to 30 were selected. The data showed that the expression of HMGB1 RNA has been upregulated by 25 and 50 µg/ml of LPS at 24 cycles only in 2.5% FCS-DMEM condition(fig 2A), but not clearly upregulated in serum free condition (fig 2B).

HMGB1 protein expression and secretion after LPS activation

In this study, we investigated HMGB1 protein expression both in cell lysate and in conditioned media. It was found that cellular HMGB1 was unchanged after LPS treatment for 48-72 hours, as shown in fig.3. We also investigated HMGB1 secretion in conditioned media since there has been studies showed induction of HMGB1 secretion with LPS, IL-1ß and TNF-α stimulation[4,5,6]. However, after 48, 72 and 96 hour-LPS treatment, HMGB1 was still undetectable in conditioned media as shown in figure 3C for 96 hour incubation. KB cell lysate was used as a control.

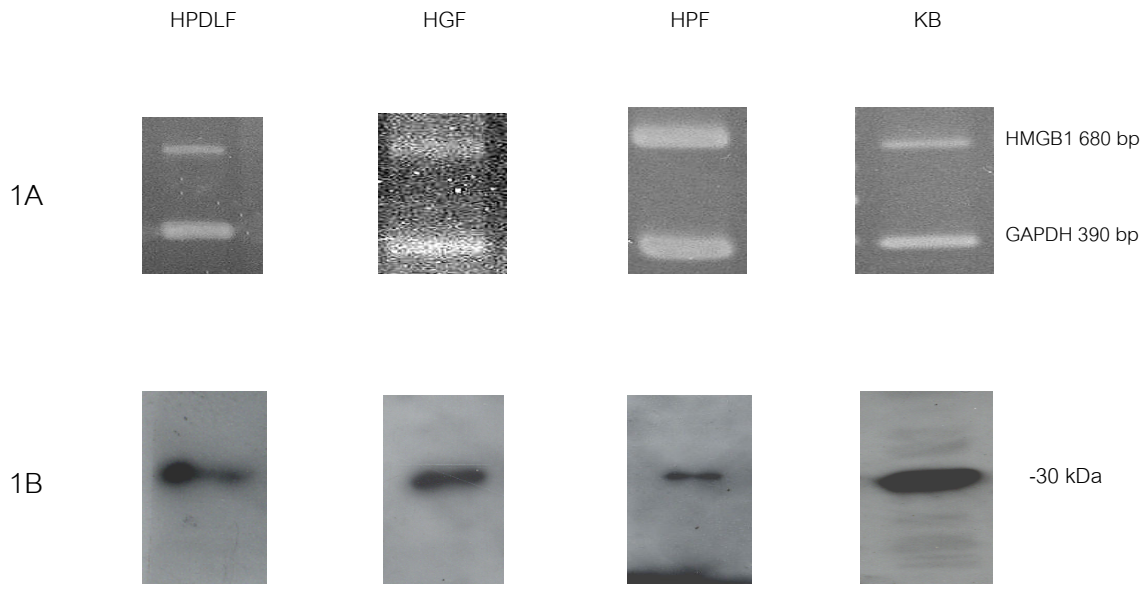

Fig.1 Expression of HMGB1 in various oral fibroblasts and KB cells. Cells ($2x10^5$ cells/well) were plated in 6-well-plates. After attachment, media were changed to DMEM with 2.5% FCS. At 24 and 48 hours, RNA and cell lysate were collected respectively. HMGB1 RNA were shown by RT-PCR along with GAPDH (fig.1A). The level of cellular HMGB1 contained in the cell lysate were determined by western anlysis of the cell lysate (fig.1B).

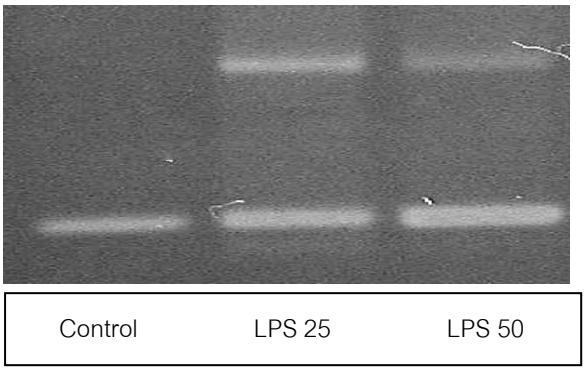

Fig.2A

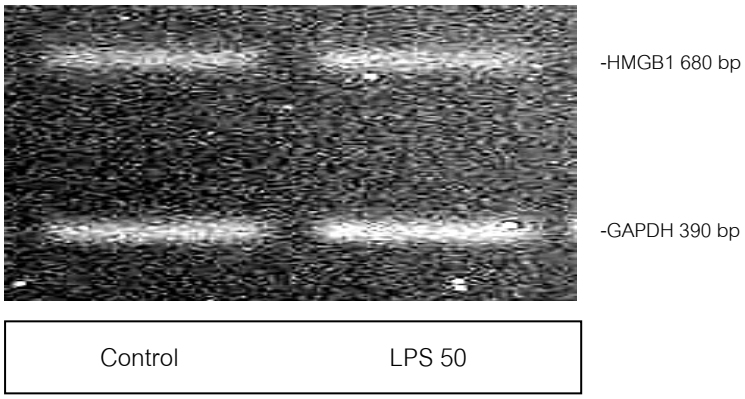

Fig.2B

Fig.2 Upregulation of HMGB1 RNA after LPS treatment. HPDLF ($2x10^5$ cells/well) were plated in 6-well-plate and treated with 25 and 50 µg/ml of E.Coli LPS in 2.5% FCS-DMEM (fig.2A) and SF-DMEM (fig.2B) for 24 hours. Sterile dH_2O was used as a control. After RNA extraction, RT-PCR was performed to analyse HMGB1 (680 bp) and GAPDH (390 bp), internal control. Only in media with 2.5% FCS that upregulation of HMGB1 RNA could be seen.

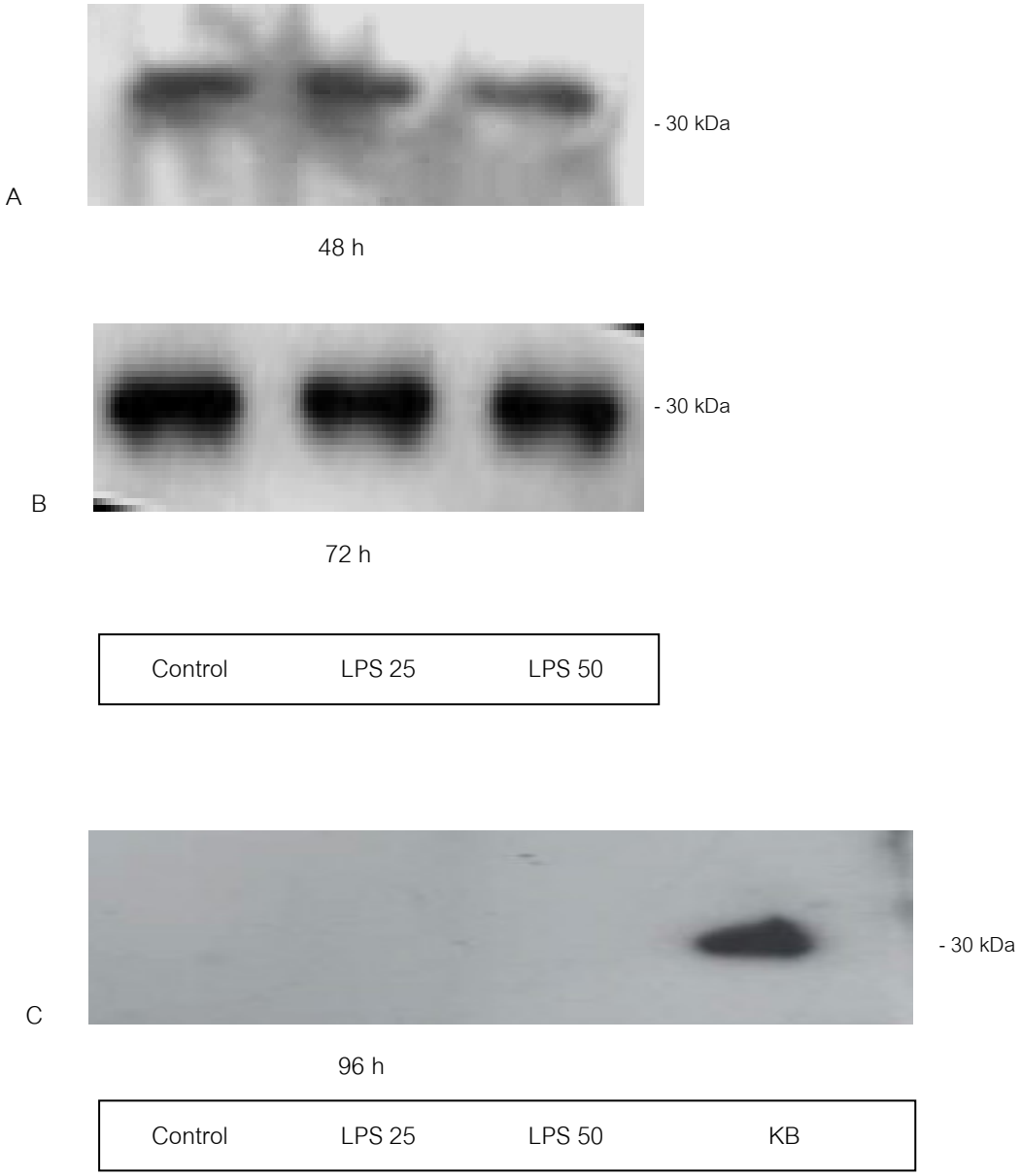

Fig.3

Fig.3 Western analysis of HMGB1 protein in cell lysate and conditioned media. HPDLF ($2x10^5$ cells/well) were plated in 6-well-plate and treated with 25 and 50 µg/ml of E.Coli LPS in 2.5%FCS-DMEM. Sterile dH_2O was used as a control. Cellular HMGB1 was not upregulated after LPS treatment for 48 and 72 hour (A and B respectively). In figure C HMGB1 was undetectable in conditioned media after 96 hour LPS treatment. KB cell lysate was used as a control for western analysis.

CHAPTER 5

CONCLUSION AND DISCUSSION

Many basic and clinical studies support the concept that human periodontitis is a disease caused by several types of Gram-negative bacteria that mainly exist in dental plaque [63]. It has also been clarified that periodontal disease is the end result of the host immune response to bacterial product such as capsular polysaccharide, outer membrane protein, enzymes and LPS, rather than the result of one sided invasion of a periodontopathic bacterium. HPDLF may interact directly with bacteria and their products and it is suggested that these cells play important roles in the host responses of patients. Previous studies showed that, upon stimulation by LPS, HPDLF produced various inflammatory cytokines including IL-6 and IL-8 [15]. These cytokines may then initiate and exacerbate periodontal inflammatory diseases.

HMGB1, a small protein discovered 30 years ago, was first described as a nuclear protein but later found to be extracellular in some cells types [28]. Previous study showed that, HMGB1 is produced in many cell types including monocytes, macrophages, endothelial cells, dendritic cells and smooth muscle cells etc. [64,65] Lymphoid cells contain HMGB1 in both cytoplasm and nucleus[66], whereas cells in liver and brain tissues contain HMGB1 predominantly in the cytoplasm[53]. Under inflammatory or injurious conditions, HMGB1 can be actively released by innate immune cells such as monocytes, macrophages and passively released by necrotic or damaged cells[7]. This extracellular HMGB1 triggers an inflammatory response [7]. Extracellular roles of HMGB1 include stimulation of cytokine release in monocyte/macrophage[22,39], and endothelial cells[67]. It also induced chemotaxis and cytoskeleton reorganization in rat smooth muscle cells[65]. However, most cell including kidney and adrenal cells are not able to secrete HMGB1[4].

There has been no report on HMGB1 expression in human fibroblasts and studies on HMGB1 in oral cells are still limited. Recently, Morimoto et al., found the expressions of

HMGB1 in the gingival epithelial tissue of the patients with periodontitis[16]. In this study, we found the expression of HMGB1 RNA and cellular protein from primary oral fibroblast cells and KB cells. However, we were unable to detect secreted HMGB1 either in normal HPDLF or stimulated with LPS, suggesting that HPDLF might be one of the cells lacking extracellular HMGB1.

Previous research showed upregulation of HMGB1 in inflamed cells, tissues or inflammatory diseases[4,5,6]. HMGB1 is secreted from necrotic or damaged cells[7]. Here, we used E.Coli LPS to induce cell injury in HPDLF. Results showed upregulation of HMGB1 RNA after 24- hour-incubation. This stimulation only worked in condition with media containing fetal calf serum, confirming the requirement of LPS on LPS-binding protein (LBP) presence in serum[49,68].

From previous knowledge, HMGB1 has a very strong TATA-less promotor, and 18-fold more active than the SV40 promotor[69]. *Hmgb1* gene is transcriptionally controlled by steroid hormone and *Hmgb1* upregulation in rodent are likely to be cytokine-dependent, and channelled through the JAK/STAT signalling pathway[27]. However, in our present study, the increased HMGB1 RNA was not accompanied by increased in protein production, either cellular or secreted. The reason for this discrepancy is still not known. Possible explanation might be the limitation of translation or protein degradation process in our particular *in vitro* condition. There has been no previous study on HMGB1 mRNA stability. However, HMGB1 protein appears very stable: with half-life of more than two-cell generations[70]. Also, no data exist on post-translationally modified of HMGB1. There has been a few study of LPS role on HMGB1. Wang et al.,[4] reported HMG-1 mRNA levels were unaffected by LPS treatment. However, they found increased HMGB1 protein cellularly and extracellularly. Sass et al demonstrated upregulation of HMGB1 protein after LPS treatment in liver of experimental mice [15].

In conclusion, we found primary oral fibroblasts and KB cell line to contain HMGB1 RNA and cellular protein, but they are unable to secrete HMGB1. In the presence of serum, LPS is able to upregulate HMGB1 transcription but not protein production in HPDLF. Roles of HMGB1 in periodontitis is still unclear and require further investigation. The LPS-induced increase in HMGB1 protein was markedly smaller than the increment in HMGB1 RNA. The

reason for this apparent discrepancy between RNA and protein levels for HMGB1 is not known. These data may suggest that LPS impaired the translation of HMGB1. The overall physiological relevance of these data remains to be determined.

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