THE EFFECT OF INFLAMMATION ON EXPRESSION OF THE RECEPTOR FOR ADVANCED GLYCATION END PRODUCTS (RAGE) AND HIGH MOBILITY GROUP BOX 1 (HMGB1) IN HUMAN PULP TISSUE

NUTTAVUN VECHVONGVAN

**A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE
(GENERAL DENTISTRY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY**

2008

COPYRIGHT OF MAHIDOL UNIVERSITY

Copyright by Mahidol University

Thesis
Entitled

THE EFFECT OF INFLAMMATION ON EXPRESSION OF THE RECEPTOR FOR ADVANCED GLYCATION END PRODUCTS (RAGE) AND HIGH MOBILITY GROUP BOX 1 (HMGB1) IN HUMAN PULP TISSUE

Nuttavun Vechvongvan
……………………………………
Miss.Nuttavun Vechvongvan
Candidate

Tasanee Tengrungsun
……………………………………
Assoc.Prof.Tassanee Tengrungsun
Grad. Dip. in Clin. Sc.(Endodontics)
Major-Advisor

……………………………………
Dr.Salunya Tancharoen
Ph.D. (Oral Biology and Endodontics)
Co- Advisor

B. Mahaisavariya
……………………………………
Prof.Banchong Mahaisavariya
M.D.
Dean
Faculty of Graduate Studies

……………………………………
Assist.Prof.Somchai Manopatanakul
Grad.Dip. (Periodontics),M.D.Sc.
(Orthodontics) ,Diplomate Australian
Board of Orthodontics
Chair
Master of Science Programme in
General Dentistry
Faculty of Dentistry

Copyright by Mahidol University

Thesis
Entitled

THE EFFECT OF INFLAMMATION ON EXPRESSION OF THE RECEPTOR FOR ADVANCED GLYCATION END PRODUCTS (RAGE) AND HIGH MOBILITY GROUP BOX 1 (HMGB1) IN HUMAN PULP TISSUE

was submitted to the faculty of Graduate Studies, Mahidol University
For the degree of Master of Science (General Dentistry)
on
10 April, 2008

Nuttavun Vechvongvan
....
Miss.Nuttavun Vechvongvan
Candidate

....
Dr.Kongkwan Mekaapiruk
Ph.D. (Dental science)
Chair

....
Assoc.Prof.Tassanee Tengrungsun
Grad. Dip. in Clin. Sc.(Endodontics)
Member

....
Dr.Salunya Tancharoen
Ph.D. (Oral Biology and Endodontics)
Member

....
Prof.Banchong Mahaisavariya
M.D.
Dean
Faculty of Graduate Studies
Mahidol University

....
Assoc. Prof. Theeralaksna Suddhasthira
B.Sc., D.D.S., Diplomate, American
Board of Oral and Maxillofacial Surgery,
Ph.D. (Biomedical Science)
Dean
Faculty of Dentistry, Mahidol University

Copyright by Mahidol University

ACKNOWLEDGEMENT

The success of this thesis can be attributed to the extensive support and assistance from my major advisor, Assoc.Prof. Tassanee Tengrungsun and my co-advisor, Dr.Salunya Tancharoen. I deeply thank them for their kindness, valuable guidance and helpful advice.

I would like to thank Dr.Kongkwan Mekaapiruk for kindness in examining the research and providing suggestions for improvement and who was the chair person of the thesis defense.

I would like to thank Prof. Ikuro Maruyama, for providing facilities and supportive valuable guidance.

I would like to thank the Laboratory of Vascular Medicine Cardiovascular and Respiratory Disorders Advanced Therapeutics, Kagoshima University Graduate School of Medical and Dental Sciences, Japan

I would like to thank the Research Unit and Department of Pharmacology, Faculty of Dentistry for providing the research instrument.

I am grateful to all the lecturers and staffs of the Department of Hospital Dentistry for their valuable advice and thank for their kind support.

Special thanks to my parents, my brother and my friends for their love, warmth and support.

Nuttavun Vechvongvan

Copyright by Mahidol University

THE EFFECT OF INFLAMMATION ON EXPRESSION OF THE RECEPTOR FOR ADVANCED GLYCATION END PRODUCTS (RAGE) AND HIGH MOBILITY GROUP BOX 1 (HMGB1) IN HUMAN PULP TISSUE

NUTTAVUN VECHVONGVAN 4937518 DTGD/M

M.Sc.(GENERAL DENTISTRY)

THESIS ADVISORS : TASSANEE TENGRUNGSUN, GRAD. DIP. IN CLIN.SC. (ENDODONTICS), SALUNYA TANCHAROEN, Ph.D. (ORAL BIOLOGY AND ENDODONTICS)

ABSTRACT

The aim of this study was to verify and compare RAGE and HMGB1 in healthy and inflamed human dental pulp tissues. 30 pulp samples were obtained from teeth having a clinical diagnosis of irreversible pulpitis (Inflamed pulp, N=15) and of impacted teeth or non-occluded teeth (Healthy pulp, N=15). Immunohistochemistry technique was used to detect expression of RAGE. Western blot analysis was used to detect both RAGE and HMGB1. Measurement of HMGB1 was by ELISA technique.

Immunostaining of specimens from inflamed pulp tissue showed that strong RAGE was primarily overexpressed and confined to the capillary endothelial cells, cell periphery of odontoblast, fibroblast, with occasional cytoplasmic staining from all subjects. In contrast, healthy pulp tissue showed only faint RAGE expression in the capillary endothelial cells and fibroblast-like cells but not in the odontoblast cell layer. By western blot analysis, RAGE and HMGB1 is significantly increased in the pulp inflammatory tissues compared to the healthy tissues. By ELISA technique, the concentration of HMGB1 in the inflamed group was significantly higher than the healthy group ($p<0.001$).

The findings imply that continued expression of RAGE and HMGB1 following inflammation can act, at least in part, as an important amplification signal for progressive tooth destruction.

KEY WORDS : RAGE / HMGB1 / Dental pulp / Inflammation

60 pp.

Copyright by Mahidol University

ผลของการอักเสบต่อการแสดงของตัวรับความรู้สึกชนิดแอดวานซ์ไกลเคชั่นเอนด์โปรดักซ์ หรือ เรจ และไฮโมบิลิตีกรุ๊ปบ๊อกซ์วัน หรือ เอชเอมจีบีวันในเนื้อเยื่อในโพรงฟัน (THE EFFECT OF INFLAMMATION ON EXPRESSION OF THE RECEPTOR FOR ADVANCED GLYCATION END PRODUCTS (RAGE) AND HIGH MOBILITY GROUP BOX 1 (HMGB1) IN HUMAN PULP TISSUE)

ณัฐวรรณ เวชวงศ์วาน 4937518 DTGD/M

วท.ม. (ทันตกรรมทั่วไป)

คณะกรรมการวิทยานิพนธ์ : ทัศนีย์ เต็งรังสรรค์ ท.บ., ป.บัณฑิต (วิทยาเอนโดดอนต์), ศรัณยา ต้นเจริญ, Ph.D. (Oral Biology and Endodontics)

บทคัดย่อ

การวิจัยครั้งนี้มีวัตถุประสงค์เพื่อแสดงตำแหน่งทางอิมมูโนวิทยาและ วัดปริมาณโปรตีนของ Receptor for Advance Glycation End Products (RAGE) ซึ่งเป็นตัวรับความรู้สึกของ High Mobility Group Box 1 (HMGB1) รวมทั้งเปรียบเทียบปริมาณ HMGB1 ในเนื้อเยื่อในโพรงฟันที่ปกติ 15 ตัวอย่างและที่มีการอักเสบ 15 ตัวอย่าง จากการศึกษา 3 วิธี 1) โดย immunohistochemistry พบว่า เนื้อเยื่อในโพรงฟันที่มีการอักเสบจะปรากฏ RAGE ในปริมาณมาก โดยจะพบที่เซลล์เยื่อบุหลอดเลือด เซลล์สร้างเส้นใย เซลล์สร้างเนื้อฟันในทุกตัวอย่าง ในทางตรงกันข้ามในเนื้อเยื่อที่ปกติจะมีการปรากฏของ RAGE เพียงเล็กน้อยในเซลล์เยื่อบุหลอดเลือด และเซลล์สร้างเส้นใย แต่จะไม่พบในชั้นเซลล์สร้างเนื้อฟัน 2) จากวิธี western blot analysis จะพบการเพิ่มขึ้นของ ปริมาณโปรตีน RAGE และ HMGB1 ในเนื้อเยื่อที่มีการอักเสบเมื่อเปรียบเทียบกับเนื้อเยื่อปกติ 3) โดย ELISA technique พบว่าความเข้มข้นของ HMGB1 ในเนื้อเยื่อที่มีการอักเสบมากกว่าเนื้อเยื่อปกติอย่างมีนัยสำคัญ ($p<0.001$)

เมื่อมีการอักเสบเกิดขึ้น จะมีสร้าง RAGE และ HMGB1 เพิ่มขึ้น จึงอาจกล่าวได้ว่า ทั้ง RAGE และ HMGB1 เป็นสัญญาณสำคัญที่แสดงให้เห็นถึงขบวนการที่ก่อให้เกิดการอักเสบในเนื้อเยื่อในโพรงฟัน

60 หน้า

Copyright by Mahidol University

CONTENTS

	Page
ACKNOWLEDGEMENT	iii
ABSTRACT	iv
LIST OF FIGURES	vii
LIST OF ABBREVATION	viii
CHAPTER	
I INTRODUCTION	1
II OBJECTIVE	5
III LITERATURE REVIEW	6
IV MATERIAL AND METHODS	34
V RESULTS	39
VI DISCUSSION	51
VII CONCLUSION	56
REFERENCES	57
APPENDIX	58
BIOGRAPHY	60

Copyright by Mahidol University

LIST OF FIGURES

	Page
Figure 1 RAGE binds to ligand at the cell membrane	15
Figure 2 RAGE converts acute cellular activation into a sustained cellular response	17
Figure 3 Antagonism of RAGE: Soluble RAGE (sRAGE)	20
Figure 4 Extracellular roles of HMGB1_	23
Figure 5 Immunohistochemical analysis of RAGE in pulpitis tissues (x100)	32
Figure 6 Immunohistochemical analysis of RAGE in pulpitis tissues (x200)	33
Figure 7 Immunohistochemical analysis of RAGE in pulpitis tissues (x400)	34
Figure 8 Immunohistochemical analysis of RAGE in healthy tissues (x100)	35
Figure 9 Immunohistochemical analysis of RAGE in healthy tissues (x400)	36
Figure 10 Immunohistochemical analysis of RAGE in pulpitis tissues (control)	37
Figure 11 Immunohistochemical analysis of RAGE in healthy tissues (control)	38
Figure 12 RAGE expression determined by Western blot analyses	39
Figure 13 HMGB1 expression determined by Western blot analyses	39
Figure 14 Cytosol HMGB1 levels as measured by ELISA	40
Figure 15 Graph show HMGB1 is increased in pulp inflammatory tissues compared with healthy tissues	41
Figure 16 The Postulate signaling pathway of inflammation by the binding of RAGE and HMGB1	45

Copyright by Mahidol University

LIST OF ABBREVATION

Ab	antibody
AGE-R3	AGE-receptor 3
AGEs	advanced glycation end products
BSA	bovine serum albumin
^{o}C	degrees Celsius
CGRP	calcitonin gene-related peptide
CLE	cutaneous lupus erythematosus
Cont.	continue
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinases
et al	et alii
Fig.	figure
h	hour
HLA	human leukocyte antigen
HMG	high mobility group
HMGB1	high mobility group box 1
ICAM-1	intercellular adhesion molecule-1
IgG	Immunoglobulin G
IgSF	immunoglobulin superfamilly
IL-1	interleukin-1
I/R	ischemia/reperfusion
IκBα.	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
K_d	Dissociate Constant (receptor affinity in pharmacology, chemistry)
kDa	kilodalton
L	liter

Copyright by Mahidol University

LIST OF ABBREVATION (Cont.)

LPS	lipopolysaccharide
MAP kinases	mitogen-activated protein kinase
min	minute
ml	milliliter
mol	mole
MHC III	major histocompatibility complex III
MIP	macrophage inflammatory protein
mM	millimole
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor-kappaB
ng	nanogram
NK1	neurokinin-1
nm	nanometer
NO	nitric oxide
PAF	platelet activating factor
PBS	phosphate-buffered saline
PMSF	phenylmethanesulphonylfluoride
RA	rheumatoid arthritis
RAGE	receptor for advanced glycation end products
RT-PCR	reverse transcription polymerase chain reaction
SAP	severe acute pancreatitis
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SP	substance P
sRAGE	soluble RAGE
SS	Sjögren's syndrome
TBS	Tris-buffered saline (Tris= trishydroxymethylaminomethane)
TNF	tumor necrosis factor

Copyright by Mahidol University

LIST OF ABBREVATION (Cont.)

TUNEL	Terminal uridine deoxynucleotidyl transferase biotin-dUTP nick end labeling
VCAM-1	vascular cell adhesion molecule-1
5HT	serotonin or 5-hydroxytryptamine
μg	microgram
μl	microliter
μm	micrometer

Copyright by Mahidol University

CHAPTER I
INTRODUCTION

Pain experience, normally conceived as a consequence of the disorders of the human body organs or systems, is considered a major public health problem (1). From an oral health perspective, untreated dental caries usually lead to a specific kind of pain, dental pain (2). It is known that such a phenomenon can affect people's daily lives (3), leading to sleep disorders, decreased work effectiveness, school absence, and avoidance of certain types of food (4, 5, 2). A large study was conducted to determine the reported incidence of orofacial pain. In Thailand, a cross sectional study of 12-year-old children, found that experience of pain during the previous 12 mo was reported by 53% of 12-year-old of children (6). An estimated prevalence and distribution reported of orofacial pain in the United States (7) found that 21.8% of adults in the United States experienced orofacial pain symptoms within 6 months before the study. The most common pain was toothache, which was estimated to occur in 12.3% of the population. In young male adults from southern Brazil, dental pain prevalence within 12 mo before the study was 21.2% (8). Similar results were found in Toronto, Canada, where dental pain prevalence reported in the previous four weeks was 18.0% among 14-20-year-olds of both sexes (9). The primary reason for avoiding dental treatment is fear, assessing by the need for anesthesia and sedation in the general population (10). Root canal treatment, in particular, is one of the most anxiety-producing procedures in dentistry (11).

The dental pulp is endowed with a rich neurovascular supply that promotes the effects of inflammation and may lead to rapid degeneration and necrosis. Pain is the evoked potential in the tooth that initiate signals to the brain. The tooth is innervated by a large number of myelinated and unmyelinated nerve fibers. Regardless of the nature of the sensory stimulus such as thermal change, almost all afferent impulses from the pulp results in the sensory of pain. Painful pulpitis is likely to be associated with nociceptive C fiber activity. Pain associated with an inflamed or degenerated pulp may be either provoked or spontaneous. The hyperalgesic pulp may respond to stimuli that usually do not evoke pain, or the pain may be exaggerated and persist longer (12).

Copyright by Mahidol University

Converging evidence indicated that movement of fluid in the dentinal tubules is the basic event in the arousal of pain (13). The severity of the clinical symptoms of acute pulpitis will vary as the inflammatory response increases. The degree of intensity of pain depends on the intensity of the intrapulpal pressure and the viability of peripheral sensory units. Unlike the pain of reversible pulpitis, this pain lingers after the external (primary) irritant has been removed. The pain of acute pulpitis is continuous or intermittent has periods. It is spontaneous pain because of the presence of necrotic tissue (the internal or secondary irritant), which further provokes and serves as a nidus for inflammation and the resultant 'suprathreshold' intrapulpal pressure increase. In the last few decades, studies have shown that numerous molecular mediators may act in synchrony to initiate, promote, or modulate the inflammatory response in the dental pulp. Many of these mediators tend to reduce the pain threshold, either directly by acting on peripheral nerve cells or through promoting the inflammatory process. Inflammatory mediators, such as histamines, bradykinin, prostaglandins, serotonins, substance P (SP), calcitonin gene-related peptides (CGRP), and leukotrienes can cause pain directly by activating or sensitizing pulpal nociceptors (14,15). They also cause pain indirectly by initiating a series of inflammation events that increase vascular permeability, edema and increase intrapulpal pressure (16). Prostaglandin (17), the vasoactive amine bradykinin (18), neuropeptides such as substance P (19) were shown to be elevated in pulp diagnosed with painful pulpitis.

High mobility group box 1 (HMGB1) is an abundant and conserved nuclear protein that is released by necrotic cells and acts in the extracellular environment as a primary proinflammatory signal. Human microvascular endothelial cells also responded with up-regulation of adhesion molecules and production of proinflammatory cytokines upon HMGB1-stimulation (20). HMGB1 itself induces the production of inflammatory mediators by macrophages and neutrophils. HMGB1 is potent in stimulating the release of multiple proinflammatory cytokines, including tumor necrosis factor (TNF), interleukin-1alpha (IL-1α) , IL-1β, IL-6, IL-8, Macrophage Inflammatory Protein -1 alpha (MIP-1 α), and MIP-1β (21). In response to HMGB1 stimulation, human microvascular endothelial cells increase expression of adhesion molecules (such as RAGE, intercellular adhesion molecule-1 [ICAM-1] and vascular adhesion molecule [VCAM-1]) (20), suggesting that HMGB1 can propagate

Copyright by Mahidol University

an inflammatory response in the endothelium during infection or injury. Focal administration of HMGB1 in the region of the sciatic nerve induces dose-dependent unilateral and bilateral low threshold mechanical allodynia (22), suggesting a role in causing pathological pain during inflammation.

RAGE is a member of the immunoglobulin superfamily, and is expressed on mononuclear phagocyte, vascular smooth muscle cells and neurons (23, 24). RAGE interacts with a range of ligands, including advanced glycation end products (AGEs), HMGB1 and S100/calgranulins (25-27). RAGE converts acute cellular activation into a sustained cellular response. Activation of nuclear factor-kappaB (NF-κB) by a variety of stimuli results in increased gene expression of NF-κB regulated genes. The RAGE promoter itself is controlled by NF-κB, and upregulation of the receptor provides an increasing number of binding sites for RAGE ligands such as AGEs, HMGB1, and S100/calgranulins. Ligand binding results in RAGE-dependent sustained NF-κB activation, which perpetuates the cellular inflammatory response (28). RAGE is a counter-receptor for leukocyte integrins (29) and plays a key role in the inflammatory process. RAGE is expressed at low levels in normal tissues and in the vasculature and becomes up-regulated in the diabetic vasculature or at other sites where its ligands accumulate (24). Increased RAGE expression has been shown in both endothelium and vascular smooth muscle cells from diabetic patients (30,31) and in macrophages from patients with renal failure and dialysis-related amyloidosis (32). Although RAGE and HMGB1 have been located in other human inflamed tissues, the existence of RAGE and HMGB1 in human inflamed dental pulp tissue has not been elucidated. Thus, we investigated the expression of RAGE and HMGB1 associated with inflammation of human dental pulp tissues.

Copyright by Mahidol University

CHAPTER II

OBJECIVES

Although RAGE and HMGB1 have been located in other human inflamed tissue, the existence of RAGE and HMGB1 in human inflamed dental pulp tissue have not been elucidated. The objectives of this study are:

1. To verify RAGE and HMGB1 expression in dental pulp tissue.
2. To compare RAGE and HMGB1 expression levels between healthy and inflamed dental pulp tissues.

Copyright by Mahidol University

CHAPTER III
LITERATURE REVIEW
PART I
PULPAL INFLAMMATION

The dental pulp is a loose connective tissue, and when injured, it responds with an inflammatory reaction. Inflammation is the protective response of tissues to injury. The purpose of an inflammatory response is generally to neutralize, dilute, destroy, or wall off the injurious agent, to prepare the area for the reparative process.

Morphologic zones of the pulp (33)

Odontoblast Layer

The outermost stratum of cells of the healthy pulp is the odontoblast layer. This layer is located immediately subjacent to the predentin; the odontoblast process, however, passes through the predentin into the dentin. Consequently, the odontoblast layer is actually composed of the cell bodies of odontoblasts. In addition, capillaries, nerve fibers, and dendritic cells may be found among the odontoblasts.

Cell-Poor Zone

Immediately subjacent to the odontoblast layer in the coronal pulp is often a narrow zone approximately 40 μm in width relatively free of cells and is called the cell-free layer of Weil. It is traversed by blood capillaries, unmyelinated nerve fibers, and the slender cytoplasmic processes of fibroblasts. It may not be apparent in young pulps, where dentin forms rapidly, where reparative dentin is being produced.

Cell-Rich Zone

Usually conspicuous in the subodontoblastic area is a stratum containing a relatively high proportion of fibroblasts compared with the more central region of the pulp. It is much more prominent in the coronal pulp than in the radicular pulp. Besides

Copyright by Mahidol University

fibroblasts, the cell-rich zone may include a variable number of macrophages, dendritic cells, and undifferentiated mesenchymal or stem cells.

Pulp Proper

The pulp proper is the central mass of the pulp. It contains the larger blood vessels and nerves. The connective tissue cells in this zone consist of fibroblasts or pulpal cells.

Cells of the pulp (33,34)

Odontoblasts

Because it is responsible for dentinogenesis both during tooth development and in the mature tooth, the odontoblast is the most characteristic cell of the dentin-pulp complex. During dentinogenesis, the odontoblasts form the dentinal tubules, and their presence within the tubules makes dentin a living responsive tissue.

The odontoblasts are columnar cells that form a clearly defined layer adjacent to the dentin. Their appearance varies according to the rate of dentinogenesis. In general, odontoblast nuclei are placed basally in the cell but lie at different levels within the layer, giving a false appearance of stratification (pseudostratification). This arrangement is probably the result of crowding (as dentin formation continually decreases the pulpal surface area). It is much less evident in radicular pulp, where dentin formation is less extensive.

Once primary dentin has been formed, odontoblasts continue to produce dentin at a very slow rate throughout the life of the tooth. The ultrastructure of the odontoblast reflects this reduced level of activity in that the organelles responsible for protein synthesis (Golgi apparatus, rough endoplasmic reticulum and mitochondria) are still present in the supranuclear region but in relatively small amounts. One large process extends into the dentin but several smaller processes link odontoblasts to adjacent odontoblasts and to pulpal fibroblasts.

Copyright by Mahidol University

Pulp fibroblasts

Fibroblasts are the most numerous cells of the pulp. They appear to be tissue-specific cells that are capable of giving rise to cells that are committed to differentiation if given the proper signal. These cells synthesize types I and III collagen, as well as proteoglycans and glycosaminoglycans. Thus they produce and maintain the matrix proteins of extracellular matrix. Because they are also able to phagocytose and digest collagen, fibroblasts are responsible for collagen turnover in the pulp.

Most of the cells in the dental pulp have the appearance of the ubiquitous fibroblast. In its quiescent state, it is essentially ovoid in shape (with processes extending to connect with other cells), and it has relatively little cytoplasm around the nucleus. Consequently, few organelles exist and it is probably more accurate to describe the pulpal fibroblast as a fibrocyte. When activated, pulpal fibroblasts become markedly basophilic, their cytoplasm enlarges and a significant increase in organelles associated with protein synthesis is found. They then secret the precursors of both pulpal collagen and ground substance.

In the sub-odontoblastic region are cells that seem to retain much of their embryonic potential and can, when stimulated, differentiate into odontoblasts and produce dentine. At rest, these mesenchymal-type cells are indistinguishable from fibroblast.

Macrophages

Macrophages are monocytes that have left the bloodstream, entered the tissues, and differentiated into various subpopulations. Many are found in close proximity to blood vessels. A major subpopulation of macrophages is quite active in endocytosis and phagocytosis. Because of their mobility and phagocytic activity they are able to act as scavengers, removing extravasated red blood cells, dead cells, and foreign bodies from the tissue. Ingested material is destroyed by the action of lysosomal enzymes. When activated by the appropriate inflammatory stimuli, macrophages are capable of producing a large variety of soluble factors, including interleukin-1, tumor necrosis factor, growth factors, and other cytokines.

Copyright by Mahidol University

Dendritic Cells

Dendritic cells are accessory cells of the immune system. These cells are termed *antigen-presenting cells* and are characterized by dendritic cytoplasmic processes and the presence of cell surface class II antigens. Dendritic cells play a central role in the induction of T cell-dependent immunity. Like antigen-presenting macrophages, they engulf protein antigens and then present an assembly of peptide fragments of the antigens and class II molecules. Then the assembly binds to T cell receptor, and T cell activation occurs.

Mast Cells

Mast cells are widely distributed in connective tissues, where they occur in small groups in relation to blood vessels. The granules of mast cells contain heparin, anticoagulant, histamine and important inflammatory mediators as well as many other chemical factors.

Lymphocytes

A lymphocyte is a type of white blood cell in the vertebrate immune system. Lymphocytes play an important and integral role in the body's defenses. T cells are involved in cell-mediated immunity whereas B cells are primarily responsible for humoral immunity (relating to antibodies). T and B lymphocytes were observed in normal pulpal tissues with T8 lymphocytes being predominant (35).

Primary factors that initiate the acute inflammatory response (36)

The two primary factors responsible for pulpal and periapical inflammation are the nerve and the tissue injury factors. Both vascular and humoral responses are mediated by these two phenomena.

The nerve factor is the activation of neurologic responses by environmental irritants that injure the odontoblasts. Such primary irritants may cause the following:

1. Immediate and transitory pain perception as a result of pulpal intratubular pain fiber excitation (due to A-δ fiber activity);
2. Vasodilatation that persists (prolonged) if the injury is severe enough, leading to increased capillary (and small venule) permeability, fluid exudation

Copyright by Mahidol University

(edema), and leukocyte infiltration; This reaction marks the beginning of the exudative (acute) phase of inflammation;

3. Increased intrapulpal pressure in the affected pulpal region as a result of the increased blood volume (hyperemia) and tissue exudates;
4. Secondary (spontaneous) pain response if the intrapulpal pressure from the exudative (acute) response surpasses the threshold limits of the C nerve fibers in the affected area. The presence of necrotic tissue (secondary irritant) maintains the exudative response and allows the pain to persist.

The tissue injury factor is due to the release of mediators by the injured odontoblasts, the chemical substances, which set into motion the process of inflammation locally. These mediators produce the same local effects described for the nerve factor.

Pupal diseases

The diagnosis and classification of pulpal diseases are based on clinical signs and symptoms rather than on histologic findings. Pulpal conditions can be classified as reversible and irreversible pulpitis, hyperplastic pulpitis, and necrosis. Hard tissue responses include calcifications and resorption. In this study we focused on acute irreversible pulpitis.

Acute pulpitis (37)

This is a severely painful and irreversible acute inflammatory response characterized by exudative hyperactivity. The histopathologic features include vasodilation, fluid exudation (inflammatory edema), leukocyte infiltration, and ultimately, a pulpal abscess.

Etiology

Acute pulpitis may develop as an acute exacerbation of a previously existing, nonpainful, chronically inflamed pulp in which the exudative (acute) zones becomes hyperactive, as in the following situations:

1. Food impactions in a carious cavity with an exposed pulp may either block the

Copyright by Mahidol University

drainage from the exudative zones subjacent to the caries or force carious contaminants deeper into mobilized granulomatous tissue; The resultant pulpal pressure build up will produce painful symptoms. Indiscriminate excavation of caries may have the same effect;

2. A tooth that has become chronically inflamed either initially or as a sequela of acute inflammation may remain in an non-painful state for months or years; The stimulus of additional operative procedures may activate or intensify the exudative response. The intrapulpal pressure increases and pain results.

Histopathology

- The odontoblast layer was disrupted or destroyed.
- The predentin may be reduced in width or absent.
- The regional tissue cells die, producing small zones of necrosis.
- Neutrophilic leukocytes, released from the injured and dead cells, pass through the endothelial gaps of capillary walls to the area of injury and engulf bacteria and tissue agents.
- As the acute inflammation persists, blood-borne and tissue-borne macrophages make their appearance.

Chemical mediators (38)

Chemical mediators such as histamines, serotonin, plasma proteases and prostaglandins play a specific role in the inflammation process. Inflammation is controlled by the presence of a group of substances called chemical mediators. These mediators may be exogenous or endogenous in origin. In order to be immediately effective, this mediator is already present in the tissues before the damage occurs.

Histamines

The vasoactive amine histamine is important in the initiation of the early phases of acute inflammation as it mediates the monophasic response of increased vascular permeability. It is stored in the granules of mast cells and basophils as a heparin-protein-histamine complex. Mast cells are widely distributed in connective tissue and

Copyright by Mahidol University

are strategically located near venules and cutaneous and mucosal surfaces. Histamines are released when the mast cells degranulate.

Serotonins

Like histamines, serotonins (5-hydroxytryptamine, or 5HT) is a vasoactive amine, and it plays the same role as histamines. It is released from platelets by platelet activating factor (PAF), a minor component of platelets.

Plasma proteases

There are three interrelated enzyme systems from which chemical mediators of inflammation originate. These plasma protease systems are involved in the formation of peptides, which mediate vascular permeability. The systems are the kinin, fibrinolytic, and complement systems. Both the kinin and fibrinolytic systems are triggered by the activation of Hageman factor (factor XII).

The kinin system

Bradykinin is capable of 1) inducing arteriolar dilatation, 2) increasing the permeability of venules, and 3) causing pain. Like histamines, bradykinin increases gaps between endothelial cells and causes transient increase in venular permeability.

The fibrinolytic system

Following the formation of thrombus, the fibrinolytic system is activated to dissolve the fibrin deposit. When fibrin is degraded, fibrin ‘split products’ are formed, and these fibrinopeptides can induce increased vascular permeability.

The complement system

Once activated, the complement system is a potent effector mechanism for:

1. Mediating vascular responses
2. Recruiting phagocytic leukocytes
3. Opsonizing targets of phagocytic cells
4. Directly damaging target cells (or tissues).

Copyright by Mahidol University

Eicosanoids (arachidonic acid metabolites)

Eicosanoids are 20-carbon oxygenation products derived from arachidonic acid. Those involved in inflammation are generated by two major classes of intracellular enzymes:

1. Prostaglandins
2. Leukotrienes

Prostaglandins and leukotrienes are important in a number of physiologic and pathologic processes, and some play significant roles in inflammation. They are not stored in the tissues, but are synthesized within seconds in response to a variety of stimuli. Most cells can produce these compounds, but in the inflammatory process, neutrophils and macrophages, are particularly important sources.

Platelet activating factor (PAF)

PAF is generated by activated inflammatory endothelial and injured tissue cells. In addition to the induction of platelet aggregation and degranulation at the sites of tissue injury, PAF enhances the release of serotonin by platelets. PAF is a potent vasodilator and also increases vascular permeability. The action of PAF on phagocytes (neutrophils, monocytes/macrophages) is to enhance arachidonic acid metabolism, leading to increased motility, degranulation, and free radical formation.

Nitric oxide

Nitric oxide (NO), a short-lived, free radical gas produced by endothelial cells, relaxes vascular smooth muscle. Macrophages and other cells also produce nitric oxide. Vascular endothelial cells contain an enzyme nitric oxide synthase, that generates NO. NO promotes vasodilation.

Neuropeptides

The nerve fibers that contain the neuropeptides calcitonin gene-related peptide (CGRP) and substance P (SP) are capsaicin-sensitive, afferent, unmyelinated, C fibers. CGRP causes vasodilation, and SP is responsible for increased vascular permeability. These neuropeptides act directly on arterioles and venules and indirectly by

Copyright by Mahidol University

stimulating the release of histamines and eicosanoid production by mast cells. They also enhance neutrophil adhesion and chemotaxis and are involved in wound healing.

Copyright by Mahidol University

Part II
RECEPTOR FOR ADVANCED GLYCATION END PRODUCTS (RAGE)

Advanced glycation end products (AGEs) are a result of nonenzymatic protein glycation (glucosylation or glycosylation) by glucose or fructose. Glycation is the result of a sugar molecule, such as fructose or glucose, bonding to a protein or lipid molecule without the controlling action of an enzyme. Enzyme-controlled addition of sugars to protein or lipid molecules is termed glycosylation. Glycation is an accidental process that damages the functioning of biomolecules, while glycosylation occurs at defined sites on target molecules and is required for molecule to function.

The receptor for AGEs (RAGE) is a member of the immunoglobulin superfamilly (IgSF). IgSF is a large group of cell surface and soluble proteins that are involved in the recognition, binding, or adhesion processes of cells. Members of the IgSF include cell surface antigen receptors, co-receptors and co-stimulatory molecules of the immune system, molecules involved in antigen presentation to lymphocytes, cell adhesion molecules and certain cytokine receptors. They are commonly associated with roles in the immune system.

Structure and ligand recognition

The receptor for advanced glycation end products (RAGE) is localized on chromosome 6 near the human leukocyte antigen (HLA) locus in the vicinity of the major histocompatibility complex III (MHC III) in humans and mice, in close proximity to the homeobox gene HOX12 and the human counterpart of the mouse mammary tumor gene int-3 (39). The receptor is composed of three immunoglobulin-like regions; one “V”-type domain and two “C”-type domain, and a 43-amino acid cytoplasmic tail (25, 40,41). V-type domain is critical for ligand binding and the cytosolic tail is essential for RAGE-mediated intracellular signaling. A truncated form of RAGE, which lacks the cytosolic tail, remains firmly embedded in the membrane. Although this form (termed “DN-RAGE) of the receptor is capable of binding the complement of RAGE ligands, it not transmit RAGE-mediated cellular activation (27, 42). RAGE interacts with a range of ligands (Figure 1.), including advanced

Copyright by Mahidol University

glycation end products (AGEs), HMGB1 and S100/calgranulins (25-27). Structural analysis of RAGE and ligands interaction showed the receptor recognized three dimensional structures, such as β-sheets and fibrils, rather than specific amino acid sequences (43, 24).

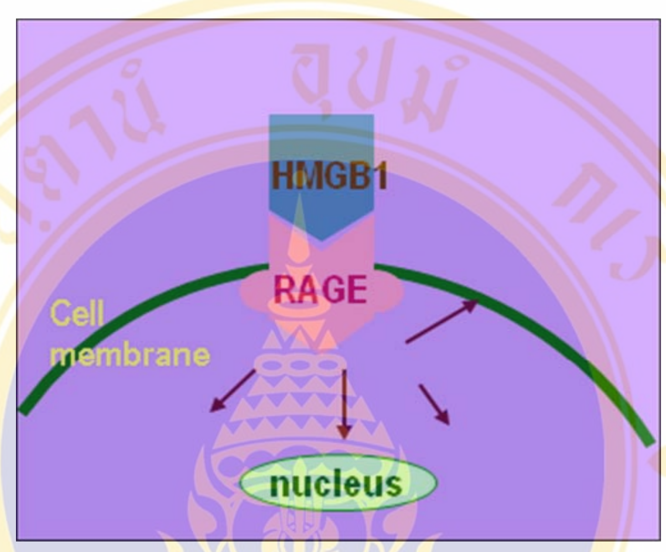


Figure 1. RAGE binds to ligand at the cell membrane.

RAGE and cellular response

Activation of mitogen-activated protein kinase (MAP kinases) is typically an early event in cellular activation. RAGE-AGEs interaction on smooth muscle cell is necessary for MAP kinase activation. RAGE-mediated induction of cellular oxidant stress triggers a cascade of intracellular signals involving $p21^{ras}$ and MAP kinase, culminating in transcription factor activation. The molecular mechanism that triggers this pathway likely involves oxidant modification and activation of $p21^{ras}$ (44).

Leukocyte recruitment is an integral part of inflammatory processes or vascular remodeling and requires multistep adhesive and signaling events, including selectin-mediated rolling, leukocyte activation, and integrin-mediated firm adhesion and diapedesis (45). The receptor for advanced glycation end products (RAGE) is a counter-receptor for leukocyte integrins that contributes to the recruitment of inflammatory cells, particularly under stress or pathological conditions such as in diabetes. RAGE can directly modulate leukocyte recruitment as it acts as an endothelial cell adhesive receptor attracting leukocytes (29).

Copyright by Mahidol University

Activation of the transcription factor NF-κB has been suggested to participate in chronic disorders. In resting cells, NF-κB resides in the cytoplasm in its inactive form bound to the inhibitor molecule nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα). Upon activation, IκBα is rapidly phosphorylated and degraded, resulting in release and translocation of NF-κB into the nucleus (46). To understand factors underlying RAGE expression, researchers cloned the 5′-flanking region of the RAGE gene and characterized putative regulatory motifs. Analysis of the putative promoter region revealed the presence of three potential NF-κB-like and two SP1 binding sites. Transient transfection of vascular endothelial and smooth muscle cells using chimeric 5′-deletion constructs linked to luciferase reporter revealed that the region −1543/−587 contributed importantly to both basal and stimulated expression of the RAGE gene. This region of the RAGE gene contained three putative NF-κB-like binding sites and was responsible for increased luciferase activity observed when endothelial or smooth muscle cells were stimulated with lipopolysaccharide. DNase I footprinting assays and electrophoretic mobility shift assay revealed that two of the three NF-κB-like binding sites (1 and 2) were likely functional and responsive to stimuli. Upon simultaneous mutation of NF-κB-like sites 1 and 2, both basal promoter expression and response to stimulation with lipopolysaccharide (LPS), as measured by relative luciferase activity, were significantly diminished. These results point to NF-κB-dependent mechanisms regulating cellular expression of RAGE and suggest a means of linking RAGE to the inflammatory response (47).

RAGE-ligand interaction results in intracellular signaling, leading to activation of the proinflammatory transcription factor NF-κB, the last rapidly activated as part of the first line of cellular defense (48). RAGE changes acute cellular activation into a maintained cellular response. Activation of NF-κB by a variety of stimuli results in increased gene expression of NF-κB regulated genes. The RAGE promoter itself is controlled by NF-κB, and upregulation of the receptor provides an increasing number of binding sites for RAGE ligands such as AGEs, HMGB1, and S100/calgranulins. Ligand binding results in RAGE-dependent maintained NF-κB activation, which perpetuates the cellular inflammatory response (Figure 1) (28).

Copyright by Mahidol University

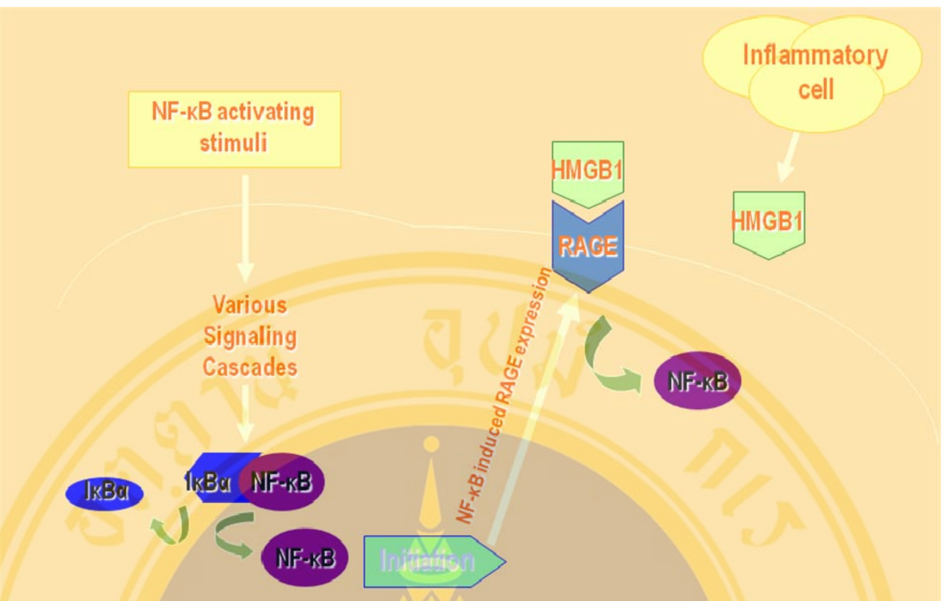


Figure 2. RAGE converts acute cellular activation into a sustained cellular response. Activation of NF-κB by a variety of stimuli results in increased gene expression of NF-κB regulated genes and this NF-κB induced RAGE expression. HMGB1, released from the inflammatory cell, can bind to RAGE receptor and induced NF-κB activation.

RAGE expression

Brain

Amyloid-beta peptide is central to the pathology of Alzheimer's Disease, because it is directly neurotoxic by inducing oxidant stress, and indirectly by activating microglia. A specific cell-surface acceptor site that could focus its effects on target cells has been postulated but not identified. Researchers have presented evidence that the 'receptor for advanced glycation end products' (RAGE) is such a receptor, and that it mediates effects of the peptide on neurons and microglia. Increased expression of RAGE in Alzheimer's Disease indicates that it is relevant to the pathogenesis of neuronal dysfunction and death. RAGE has been studied in relation to amyloid-beta peptide neurotoxicity in Alzheimer's Disease (49).

Heart

Expression of the AGE-receptor 3 (AGE-R3) and the receptor for AGEs (RAGE) was compared on the mRNA and protein level in the aging human heart. Western Blot

Copyright by Mahidol University

and Reverse Transcription Polymerase Chain Reaction (RT-PCR) analysis of the AGE receptors from the cardiac auricles and adult patients was performed and compared with young controls. Whereas the expressions of AGE-R3 as well as RAGE protein were significantly upregulated in the senescent population, only the upregulation of RAGE is associated with reduced heart function (50).

Skin

RAGE is highly expressed in skin and upregulated in sun-exposed skin. RAGE-positive cells are mainly keratinocytes, skin fibroblasts, dermal dendrocytes, and endothelial cells, but some expression was also observed in lymphocytes (51).

Labial salivary gland

Receptor for advanced glycation end product (RAGE) is a cell-surface receptor with ligands capable of inducing proinflammatory responses in autoimmunity. RAGE is present in the labial salivary glands of both normal and Sjögren's Syndrome (SS) patients, with preliminary data suggesting over-expression in SS tissues (52).

RAGE and diseases

RAGE is a multiligand receptor on vascular cells playing a key role in the inflammatory process. RAGE is expressed at low levels in normal tissues and in the vasculature and becomes up-regulated in the diabetic vasculature or at other sites where its ligands accumulate (24). Increased RAGE expression has been shown in both endothelium and vascular smooth muscle cells from diabetic patients (30,31) and in macrophages from patients with renal failure and dialysis-related amyloidosis (32).

RAGE modulates hepatic ischemia/reperfusion (I/R) injury, at least in part by activation of key signaling pathways linked to proinflammatory and cell death-promoting responses (53).

Advance glycation end products (AGEs) accumulate in the plasma and tissues of diabetic subjects. Evidence for the role of receptors of AGE (RAGE) in periodontal disease was verified in a murine model for type 2 diabetes. RAGE accelerated periodontal inflammation and alveolar bone loss in diabetic rodents. (54).

Copyright by Mahidol University

Some researchers demonstrated positive staining of RAGE in the endothelium and the basal and spinous layers of the inflamed gingival epithelium in chronic periodontitis patients with and without type 2 diabetes (55). Based on data from a variety of sources, including studies of RAGE-deficient mice, it appears that RAGE plays a central role in oral infection and exaggerated inflammatory host responses (55).

Antagonism of RAGE

Soluble RAGE (sRAGE), the extracellular ligand binding domain of RAGE that serves as a decoy, thereby suppressing ligand-induced stimulation of cell surface receptors (Figure 3) (27, 56). The blockade of RAGE, using pharmacological antagonists or transgenic mice in which a signaling-deficient RAGE mutant is expressed in cells of mononuclear phagocyte lineage, significantly increases survival after massive liver resection. In the first hours after massive resection, remnants retrieved from RAGE-blocked mice displayed increased activated NF-kappaB, principally in hepatocytes, and enhanced expression of regeneration-promoting cytokines, TNF-alpha and IL-6, and the antiinflammatory cytokine, IL-10. Hepatocyte proliferation was increased by RAGE blockade, in parallel with significantly reduced apoptosis. RAGE blockade is a strategy to promote regeneration in the massively injured liver (57). Hepatic I/R injury, associated with liver transplantation and hepatic resection, is characterized by hepatocellular damage and a deleterious inflammatory response. Animals treated with soluble RAGE (sRAGE) displayed increased survival after total hepatic I/R compared with vehicle treatment. TUNEL assay and histologic analysis revealed that blockade of RAGE was highly protective against hepatocellular death and necrosis on I/R; in parallel, proliferating cell nuclear antigen was enhanced in livers of mice treated with sRAGE. Rapid activation of p38, p44/42, stress-activated protein kinase and c-Jun N-terminal kinase mitogen-activated protein kinases, signal transducer and activator of transcription-3, and nuclear translocation of activator protein-1 was evident at early times on I/R. In the remnants of sRAGE-treated livers; however, activation of each of these signaling and transcription factor pathways was strikingly decreased. The sRAGE-treated remnants displayed enhanced activation of

Copyright by Mahidol University

nuclear factor kappaB, in parallel with increased transcripts for the proregenerative cytokines and tumor necrosis factor-alpha (53).

Blockade of RAGE results in suppression of both alveolar bone loss and markers of cellular activation/tissue-destructive properties in diabetic mice infected with *P. gingivalis*. sRAGE is the extracellular domain of the receptor, which binds ligand and blocks interaction with, and activation of, cell-surface RAGE. Blockade of RAGE diminished alveolar bone loss in a dose-dependent manner. Moreover, decreased generation of the proinflammatory cytokines TNF-α and IL-6 in gingival tissue, as well as decreased levels of matrix metalloproteinases was noted. Gingival AGEs were also reduced in mice treated with sRAGE, paralleling the observed suppression in alveolar bone loss. These findings link RAGE and exaggerated inflammatory responses to the pathogenesis of destructive periodontal disease in diabetes (54).

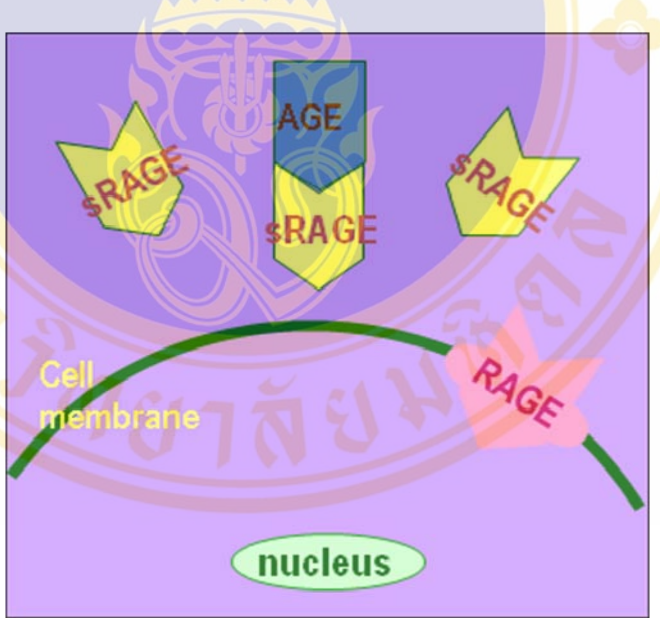


Figure 3. Soluble RAGE (sRAGE) is a truncated form of the receptor comprising the extracellular domain and thereby functions as a decoy that prevents ligands from interacting with cell surface receptor.

Copyright by Mahidol University

Part III

HIGH MOBILITY GROUP BOX 1 (HMGB1)

High mobility group box 1, a 30 kDa protein was first co-purified from nuclei with histones, and termed 'high mobility group 1' (HMG-1) protein because of its rapid mobility on electrophoresis gel (58). High mobility group box 1 protein (HMGB1; also called amphoterin) is a very abundant chromatin-binding protein residing in the eukaryotic cell nucleus and acting in the assembly of nucleoprotein complexes (59).

Structure and distribution

The human HMGB1 gene is located on chromosome 13q12 and encodes a protein of 216 amino acids (60-62). HMGB1 is composed of three domains: two internal repeats of positively charged domains, A box and B box (HMG boxes), and a negatively charged
C terminus (acidic tail) (63).

HMGB1 is produced by nearly all cell types, but cellular levels vary with development and age (64). HMGB1 can migrate between the cytoplasm and nucleus in a cell cycle-dependent fashion. Lymphoid cells contain HMGB1 in both cytoplasm and nucleus (63). The cellular localization of HMGB1 is tissue-specific with high levels found in the thymus, lymphoid tissues, testis, and neonatal livers. Intracellularly, HMGB1 is more concentrated in the cytoplasm of liver and brain cells, and is concentrated in the nuclei in most other tissues (65).

Nuclear HMGB1 as a DNA-binding protein

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 (66). The two HMG boxes are 70 to 80 amino acid L-shaped domains formed by 3 α-helical segments that are important for DNA binding (67). HMGB1 binds to the minor groove of DNA through hydrophobic amino acids that expand the groove and facilitate the unwinding and bending of DNA, allowing formation of nucleoprotein complexes that enhance the activity of several

Copyright by Mahidol University

transcription factors (67). HMG boxes provide the structure-specific binding sites for DNA with secondary structures such as supercoided DNA and cruciform (21). HMGB1 plays a possible role in DNA recombination, repair, replication, gene transcription (68) and modulating the activity of steroid hormone receptors (69,70).

HMGB1 and its receptor (RAGE)

HMGB1 can bind to receptors for advanced glycation end products (RAGE). Binding of HMGB1 to RAGE was specific, saturable, and of higher affinity than AGEs (K_d of ~ 6 nM for HMGB1 *versus* K_d of ~50 nM for AGE albumin). Domains in HMGB1 mediating interaction with RAGE appear to be unrelated to AGE-like epitopes, as enzyme-linked immunosorebent assay of HMGB1 praparations showed no detectable AGE antigen and anti-AGE IgG has no effect on HMGB1 binding to RAGE (26). A motif (amino acids 150-183) in the C-terminus of HMGB1 is responsible for RAGE binding (71). Engagement of RAGE and ligands (such as HMGB1 or AGEs), activates the NF-κB signaling pathway (72-74), and the MAPK pathways (75). HMGB1-mediated chemotaxis and cytokine production can be partially inhibited by antibodies directed against RAGE, implicating RAGE as a receptor mediating the HMGB1-dependent migratory responses (75,76).

Extracellular roles of HMGB1 in inflammation (Figure 4.)

HMGB1 itself induces the production of inflammatory mediators by macrophages and neutrophils. HMGB1 is potent in stimulating the release of multiple proinflammatory cytokines, including TNF, IL-1α , IL-1β, IL-6, IL-8, MIP-1 α, and MIP-1β (21). HMGB1 activated mitogen-activated protein kinases (such as p38 and ERK 1/2) and enhances the expression of proinflammatory cytokines in human neutrophils (77). Human microvascular endothelial cells also responded with up-regulation of adhesion molecules and production of proinflammatory cytokines upon HMGB1-stimulation. In response to HMGB1 stimulation, human microvascular endothelial cells increase expression of adhesion molecules (such as RAGE, ICAM-1 and VCAM-1), suggesting that HMGB1 can propagate an inflammatory response in the endothelium during infection or injury (20). HMGB1 and B box increase the permeability in cultured enterocytes via a nitric oxide (NO)-dependent pathway (74).

Copyright by Mahidol University

HMGB1 acts as a chemotatic agent for smooth muscle cells, causing their migration from tunica to intima, implicating HMGB1-induced pathology in vascular diseases such as atherosclerosis and restenosis (75,78).

Focal administration of HMGB1 in the region of the sciatic nerve induces dose-dependent unilateral and bilateral low threshold mechanical allodynia (22), suggesting a role in causing pathological pain during inflammation.

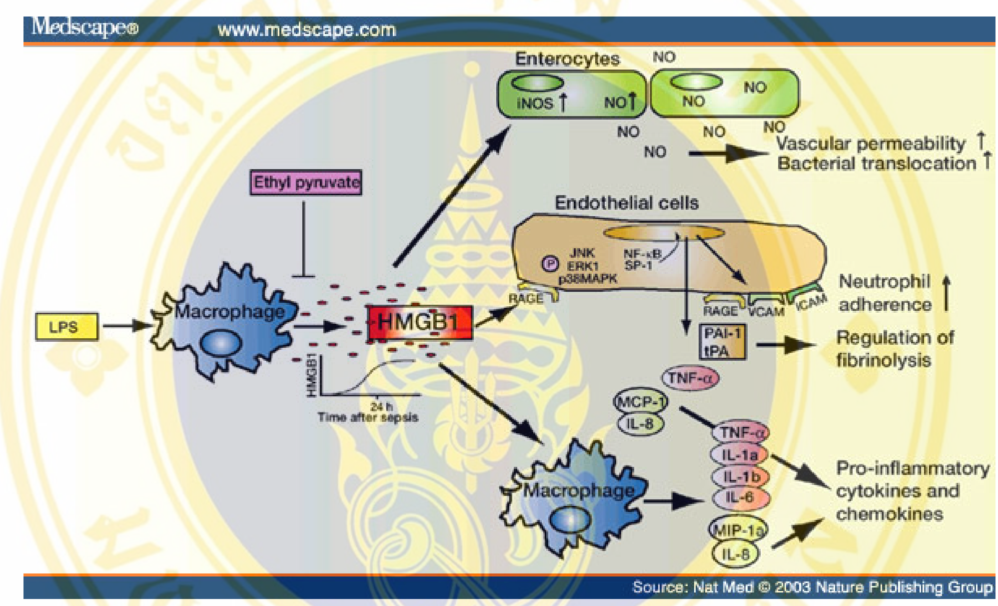


Figure 4. HMGB1 is produced by macrophages in response to inflammatory stimuli such as Lipopolysaccharide. In enterocytes, HMGB1 increases the activity of intrinsic nitric oxide synthase, leading to heightened nitric oxide production and augmented permeability, resulting in enhanced bacterial translocation through the gut barrier. HMGB1 binds to RAGE on endothelial cells and evokes intracellular signaling through kinases, leading to nuclear translocation of transcription factors. In response, endothelial cells express RAGE, adhesion molecules (VCAM-1 and ICAM-1), TNF- , chemokines, PAI-1 and tissue plasminogen activator . HMGB1 may thereby contribute to regulation of fibrinolysis. HMGB1 is not only released in response to proinflammatory stimuli , but itself induces the production of inflammatory mediators by macrophages and neutrophils

Copyright by Mahidol University

Release of HMGB1

High mobility group box 1 (HMGB1) is released by necrotic cells and acts in the extracellular environment as a primary proinflammatory signal. In response to stimulation with exogenous bacterial endotoxin or endogenous proinflammatory cytokines, cultures of macrophages and monocytes actively release HMGB1 (79, 80). HMGB1 is time-dependently released from endotoxin-stimulated macrophage cultures, with significant HMGB1 accumulation first detectable 8 hours after stimulation (79).

HMGB1 is secreted by activated monocytes and macrophages, and is passively released by necrotic or damaged cells. Apoptotic cells do not release HMGB1 even after undergoing secondary necrosis and partial autolysis, and thus fail to promote inflammation even if not cleared promptly by phagocytic cells. In apoptotic cells, HMGB1 is bound firmly to chromatin because of generalized underacetylation of histone and is released in the extracellular medium (promoting inflammation) if chromatin deacetylation is prevented. Thus, cells undergoing apoptosis are programmed to withhold the signal that is broadcast by cells that have been damaged or killed by trauma (81). But another study showed by confocal microscopy that HMGB1 and DNA change their nuclear location in Jurkat cells undergoing apoptosis suggesting that the release process may vary with cell type (82).

Expression

HMGB1 is upregulated in liver, lung and blood samples from mice tissues at 24 h postburn, which remained markedly elevated up to 72 h after thermal injury. Tissue samples from liver and lungs were collected to measure tissue endotoxin levels and HMGB 1 mRNA expression. In addition, blood samples were obtained to measure organ function parameters. Taken together, these findings indicate that thermal injury per se can markedly enhance HMG-1 gene expression in various organs. Up-regulation of HMG-1 expression may be involved in the pathogenesis of endogenous endotoxin-mediated multiple organ damage secondary to major burns (83).

HMGB1 was abundantly expressed as a nuclear, cytoplasmic, and extracellular component in synovial tissues from rheumatoid arthritis (RA) patients and from rats with experimental arthritis. Immunostaining of specimens from normal rats showed

Copyright by Mahidol University

that HMGB-1 was primarily confined to the nucleus of synoviocytes and chondrocytes, with occasional cytoplasmic staining and no extracellular matrix deposition. In contrast, inflammatory synovial tissue from rats with experimental arthritis as well as from humans with RA showed a distinctly different HMGB1 staining pattern. Nuclear HMGB1 expression was accompanied by a cytoplasmic staining in many mononuclear cells, with a macrophage-like appearance and an extracellular matrix deposition. Analysis of synovial fluid samples from RA patients further confirmed the extracellular presence of HMGB1 (84).

An altered expression of HMGB1 was observed both in the epidermis and in the dermal infiltrates of cutaneous lupus erythematosus (CLE) skin. In comparison with the unaffected skin, expression of HMGB-1 in the epidermis and dermis was increased and translocation to the cytoplasm as well as the extracellular presence of secreted HMGB1 were found. Increased levels of TNFalpha and IL-1beta were also observed in the dermal infiltrates of lesional skin (85).

Antagonism of HMGB1

Protective effects were observed with anti-HMGB1 antibody, anti-HMGB1 treatment by A box or ethyl pyruvate, a nontoxic food additive and an experimental anti-inflammatory agent (79, 86, 87). Passive immunization with anti-HMGB1 antibodies significantly protects against lethal endotoxemia in mice, even when treatment was delayed 2 h after LPS exposure (79, 86). Delayed treatment with anti-HMGB1 antibodies or other antagonists (A box or ethyl pyruvate) dose-dependently rescued mice from lethal sepsis induced by cecal perforation, and treatment was effective even when the first dose was given at 24 h after the cecal ligation and puncture surgery (86, 87).

HMGB1-targeted intervention with either neutralizing antibodies or the antagonistic A box domain of HMGB1 ameliorates collagen-induced arthritis both in mice and rats, and inhibits the local overexpression of IL-1beta in the joints. It is thus conceivable that therapeutic HMGB1 blockade may contribute to future treatment of human chronic arthritis (88).

In endotoxin-induced acute lung inflammation, administration of anti-HMGB1 Abs either before or after endotoxin exposure decreased the migration of neutrophils

Copyright by Mahidol University

to the lungs as well as lung edema. These protective effects of anti-HMGB1 were specific, because pulmonary levels of IL-1beta, TNF-alpha, or macrophage-inflammatory protein-2 were not decreased after therapy with anti-HMGB1 (89).

To examine the effects of anti-high mobility group box 1 (HMGB1) neutralizing antibody in experimental severe acute pancreatitis (SAP), SAP was induced immediately after intraperitoneal injection of anti-HMGB1 neutralizing antibody. The severity of pancreatitis, organ injury (liver, kidney and lung), and bacterial translocation to pancreas was examined 12 h after induction of SAP. Anti-HMGB1 neutralizing antibody significantly improved the elevation of the serum amylase level and the histological alterations of the pancreas and lung in SAP. Anti-HMGB1 antibody also significantly ameliorated the elevations of serum alanineaminotransferase and creatinine in SAP. Blockade of HMGB1 attenuated the development of SAP and associated organ dysfunction (90).

Copyright by Mahidol University

CHAPTER IV
MATERIALS AND METHODS

Human

This study was conducted with approval by the Committee on Human Rights Related to Human Experimentation, Mahidol University. Participants were informed of risks, benefits and signed an approved informed consent document prior to enrollment. Written informed consent was obtained from each patient. Pulp samples were obtained from patients $<$ 35 years old of both sexes, who did not take antibiotics for three weeks, without systemic diseases, no periapical lesions and no moderate to severe loss of periodontal attachment. Fifteen pulp samples were collected from teeth having a clinical diagnosis of irreversible pulpitis. These patients were suffering spontaneous pain of approximately 24 h duration. Fifteen pulp samples were collected from teeth extracted for orthodontic reason or third molar having a clinical diagnosis of non-occluded teeth.

Preparation of pulp tissues

Both groups were anesthetized and extracted. Immediately after extraction, the teeth were then sectioned using a cylindrical diamond bur in a high speed handpiece. Pulp tissues were collected using a sterile endodontic excavator.

Antibody (Ab)

RAGE Ab was purchased from RDI division of Fitzgerald Industries Intl (MA, USA). HMGB1 Ab was obtained from SHINO-TEST (Tokyo, Japan). All other reagents were supplied by Sigma-Aldrich Inc. (St. Louis, MO, USA).

Immunohistochemistry to detect RAGE expression

Five healthy and five inflamed pulp tissue samples were placed into 10% buffer formalin overnight and kept at 4 ^{o}C. The tissues were sliced to 5 µm sections. Sections were embedded in paraffin. Immunohistochemistry stainings for RAGE were

Copyright by Mahidol University

performed using the DAKO System, HRP (DakoCytomation). Paraffin-embedded sections were deparaffinized in xylene and rehydrated though a series of decreasing concentrations of ethanol. After blocking endogenous peroxidase activity with 0.3% H_2O_2. The non-specific background was reduced by treating with a blocking buffer containing 10% rabbit serum for 30 min and then incubating with rabbit polyclonal serum anti-RAGE antibody (dilution 1:1000) overnight at 4°C. The sections were washed three times with Tris-buffered saline (TBS) for five minutes and incubated with secondary antibody at room temperature for 1 hour. After rinses in TBS, bound antibody was detected using the substrate diaminobenzidine tetra-hydrochloride (DAB; DAKO), rinsed in double-distilled water and background stained with hematoxylin for one min. All sections were mounted, coverslipped and examined under a light microscope.

Western blot analysis for RAGE expression

Ten healthy and ten inflamed dental pulp tissues were prepared as described previously. Briefly, tissues lysates were obtained by adding 120 μl of SDS sample buffer containing 50 mM DTT, 1 mM PMSF and 0.5 mM Na_2VO_3. The supernatants and lysates were assayed for their protein concentrations using a Protein Assay Kit (Bio-Rad, Hercules, CA, USA) and subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples containing 10 μg of total protein and a protein size marker were electrophoresed on 12% SDS-PAGE gel and transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). To prevent non-specific binding, the membrane was blocked in a blocking solution containing 5%(w/v) nonfat dry milk/1% (v/v) Tween-20 in PBS for one h at room temperature. The membrane was probed with the RAGE antiserum (dilute in blocking solution at 2 μg/ml) over night at 4°C. Unbound antiserum was removed by washing the membrane in 0.01 M PBS containing 0.2% Tween-20 (five min, three times). The membrane was subsequently incubated with the secondary antibody (diluted in blocking solution at 2 μg/ml) for one h at room temperature. Again the membrane was washed as described above. Bound antibodies were detected by detection reagent.

Copyright by Mahidol University

Sample preparation for HMGB1 detection

Ten healthy and ten inflamed pulp tissues were prepared as described previously. Since cytoplasmic HMGB1 acts in the extracellular environment as a primary proinflammatory signal. Preparation of cytoplasmic fractions by compartment protein extraction kit (Chemicon, USA) was performed. The cytosol proteins were assayed for their protein concentrations using a Protein Assay Kit.

Western blot analysis for HMGB1 expression

Cytosol proteins were subjected to 12% SDS-PAGE. Samples containing 10 μg of total protein and a protein size marker were electrophoresed on 12% SDS-PAGE gel and transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The membrane was incubated with blocking solution and probed with the HMGB1 antibody. The membrane was subsequently incubated with the secondary antibody and bound antibodies were detected.

Measurement of HMGB1 by ELISA technique

Cytosol proteins were measured protein levels. Enzyme-linked immunosorbent assay (ELISA) for HMGB1 was performed with the use of monoclonal antibodies to HMGB1 and with standardization to a curve of recombinant human HMGB1. A polystyrene microtiter plate was coated with 100 ml of 3 mg/l anti-HMGB1 polyclonal antibody (Shino-TEST, Kanagawa, Japan) and incubated at 37 °C overnight. The unbound antibodies were removed by washing plates three times with PBS containing 0. 05% Tween 20 (washing buffer) and the remaining binding sites in the wells were blocked by incubating the plates for 2 h with 400 ml/well PBS containing 1% BSA. After washing, 50 μL of each dilution of the standards and samples was added to the wells. The microtiter plates were incubated for 24 h at room temperature. After washing, 100 μL/well of antihuman HMGB1 peroxidase-conjugated monoclonal antibody (Shino-TEST) was added and the plate was incubated at room temperature for two h after washing, 3,3'5,5'- tetramethylbenzide was added to the well. The enzyme reaction was allowed to proceed for 30 min at room temperature. The

Copyright by Mahidol University

chromogenic substrate reaction was stopped by addition of stop solution (0.35 mol/L Na_2SO_4) and the absorbance was read at 450 nm.

Data analysis of HMGB1 level by ELISA technique

The levels of HMGB1 in cytosol proteins were analyzed using Mann-Whitney U test. An α-level of $p < .05$ was taken as an indication of statistic significance.

Copyright by Mahidol University

CHAPTER V

RESULTS

Immunohistochemistry to detect RAGE expression

In pulpitis tissues, strong expression (brown stain) of RAGE is noted in the capillary endothelial cells around blood vessels and inflammatory cells, cell peripheries and odontoblast and fibroblast-like cells (Figures 5, 6 and 7). In healthy pulp tissue, faint RAGE expression is evident in the capillary endothelial cells around the blood vessels and fibroblast-like cells (Figures 8, 9). Histology of odontoblast cells shows no RAGE localization (Figure 7, arrow head). Control isotype Immunoglobulin G (IgG) was employed at the same time (Figures 10, 11).

Western blot analysis for RAGE expression

Protein extracts from pulpitis and healthy human pulp tissue were blotted onto nitrocellulose membranes and incubated with anti-RAGE antibody. A strong band at ~42 kDa was detected in pulpitis tissues and faint band at ~42 kDa was detected in healthy tissues (Figure 12).

Western blot analysis for HMGB1 expression

Cytosol protein extracts from inflamed and healthy dental pulp tissues were blotted onto nitrocellulose membranes and incubated with HMGB1 antibody. HMGB1, which has molecular weight ~27 kDa, has been found to be increased in inflamed pulp tissues compared with a healthy group (Figure 13).

Measurement of HMGB1 by ELISA technique (Figures 14, 15)

HMGB1 has been found to be increased in cytoplasm of inflamed pulp tissues compare with a healthy tissues. Cytosol HMGB1 concentrations were 3.73 ± 0.69 ng/ml in the healthy group and 12.08 ± 0.82 ng/ml in the inflamed group. The concentration of HMGB1 in the inflamed group was significantly higher than the healthy group ($p<0.001$).

Copyright by Mahidol University

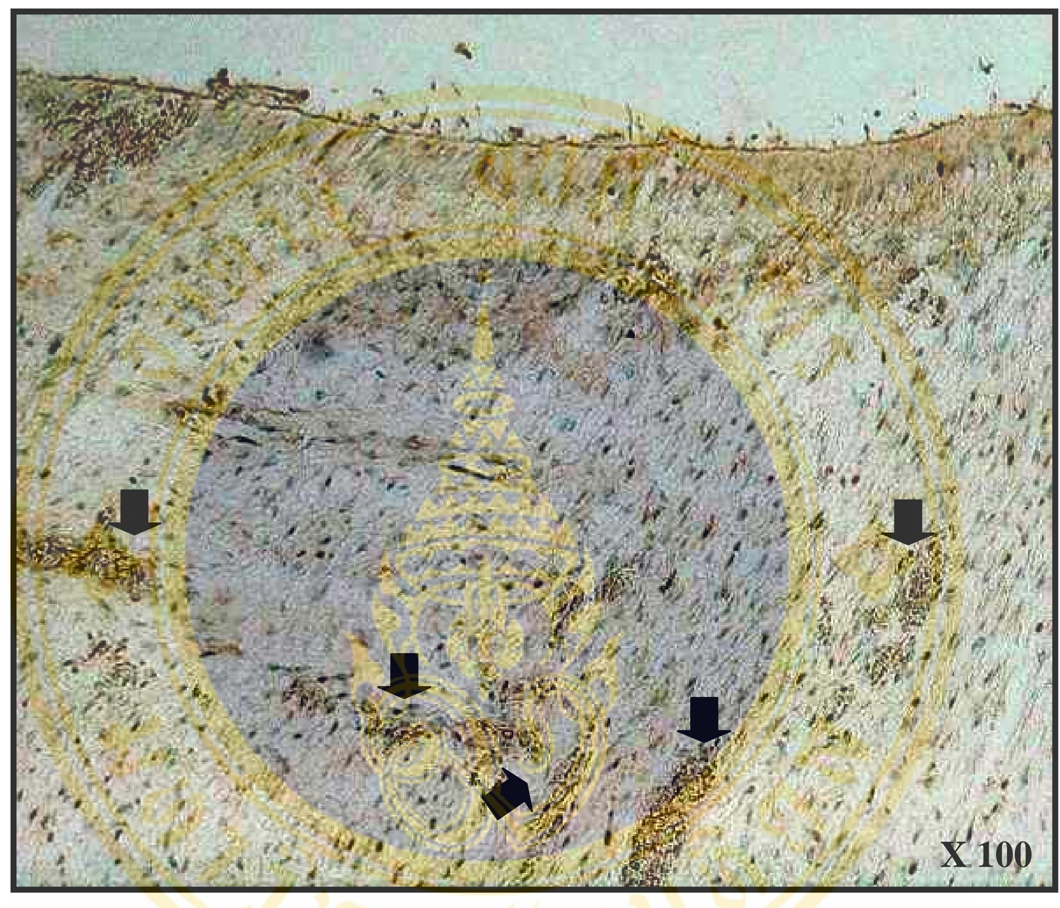


Figure 5. Immunohistochemical analysis of RAGE. In pulpitis tissues, strong expression of RAGE is noted in the capillary endothelial cells around blood vessels (arrow) and inflammatory cells.

Copyright by Mahidol University

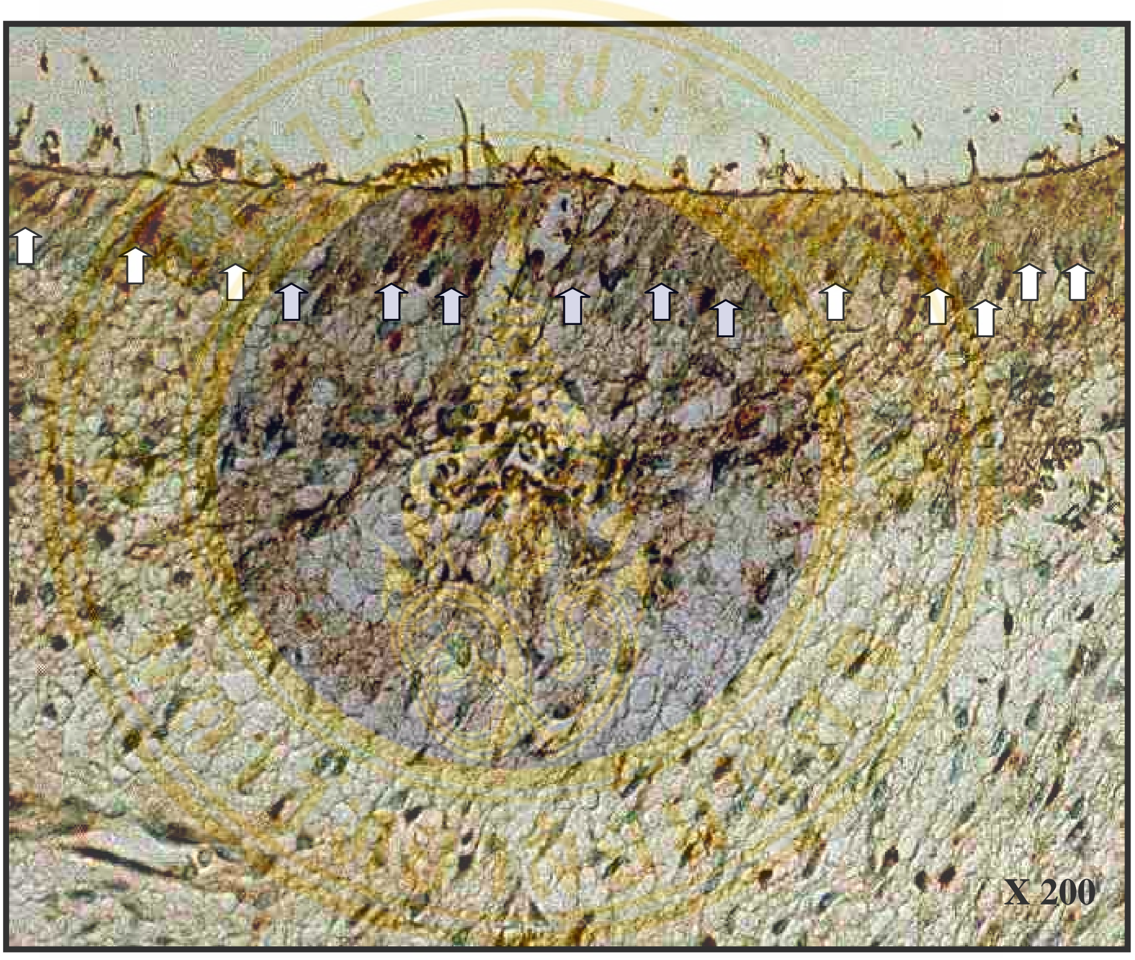


Figure 6. Immunohistochemical analysis of RAGE. In pulpitis tissues, strong expression of RAGE is noted in the cell peripheries and cytoplasm of odontoblast-like cells.

Copyright by Mahidol University

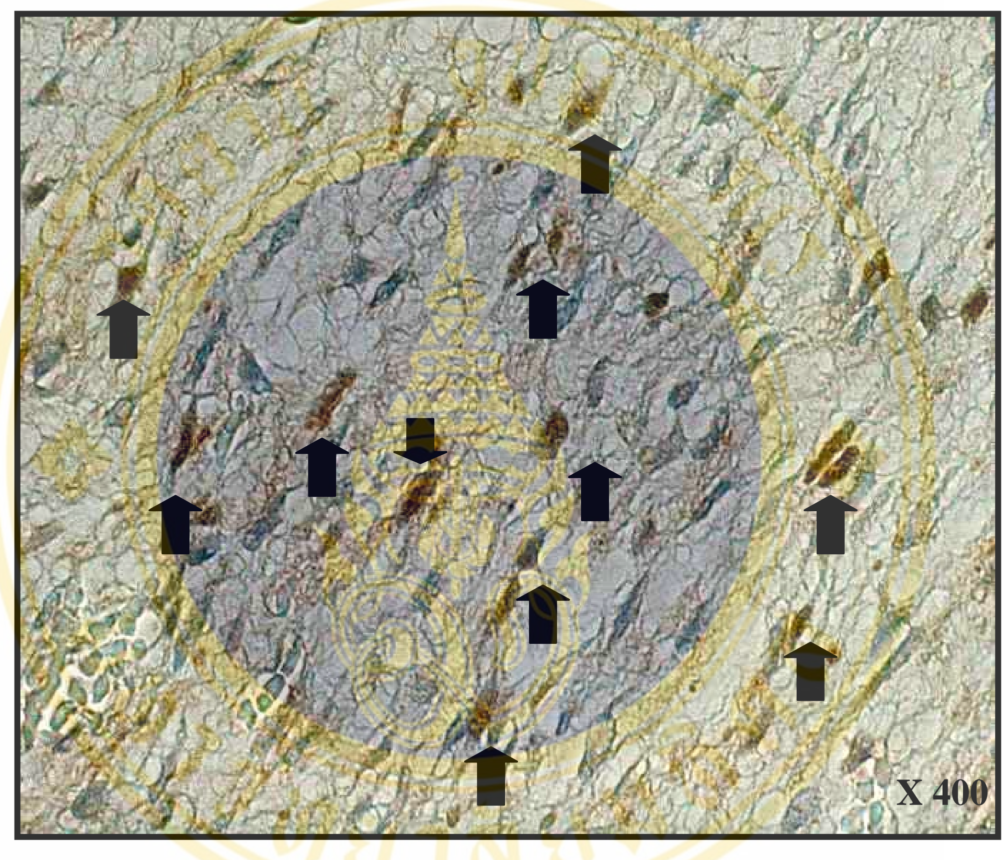


Figure 7. Immunohistochemical analysis of RAGE. In pulpitis tissues, strong expression of RAGE is noted in the fibroblast-like cells.

Copyright by Mahidol University

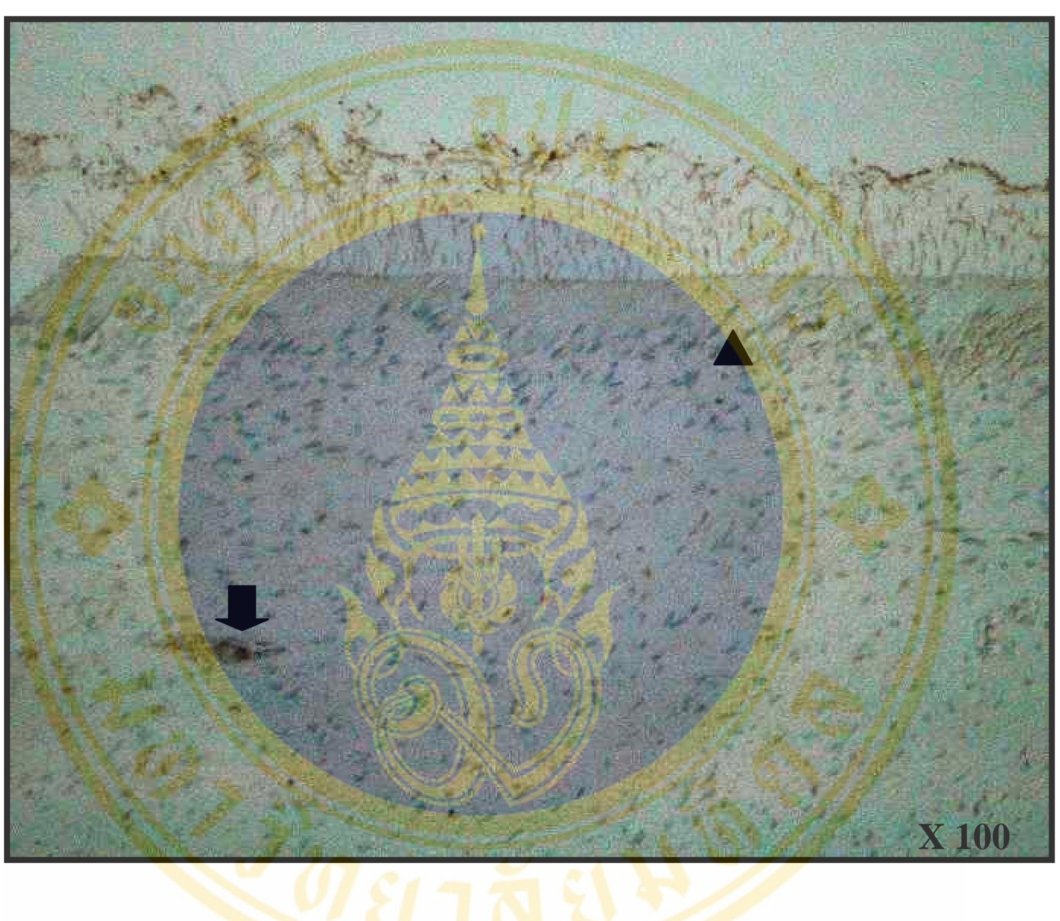


Figure 8. Immunohistochemical analysis of RAGE. In healthy tissues, faint expression of RAGE in the capillary endothelial cells around blood vessels (arrow). Histology of odontoblast cells shows no RAGE localization (arrow head).

Copyright by Mahidol University

Figure 9. Immunohistochemical analysis of RAGE. In healthy pulp tissues, faint expression of RAGE is noted in the fibroblast-like cells.

Copyright by Mahidol University

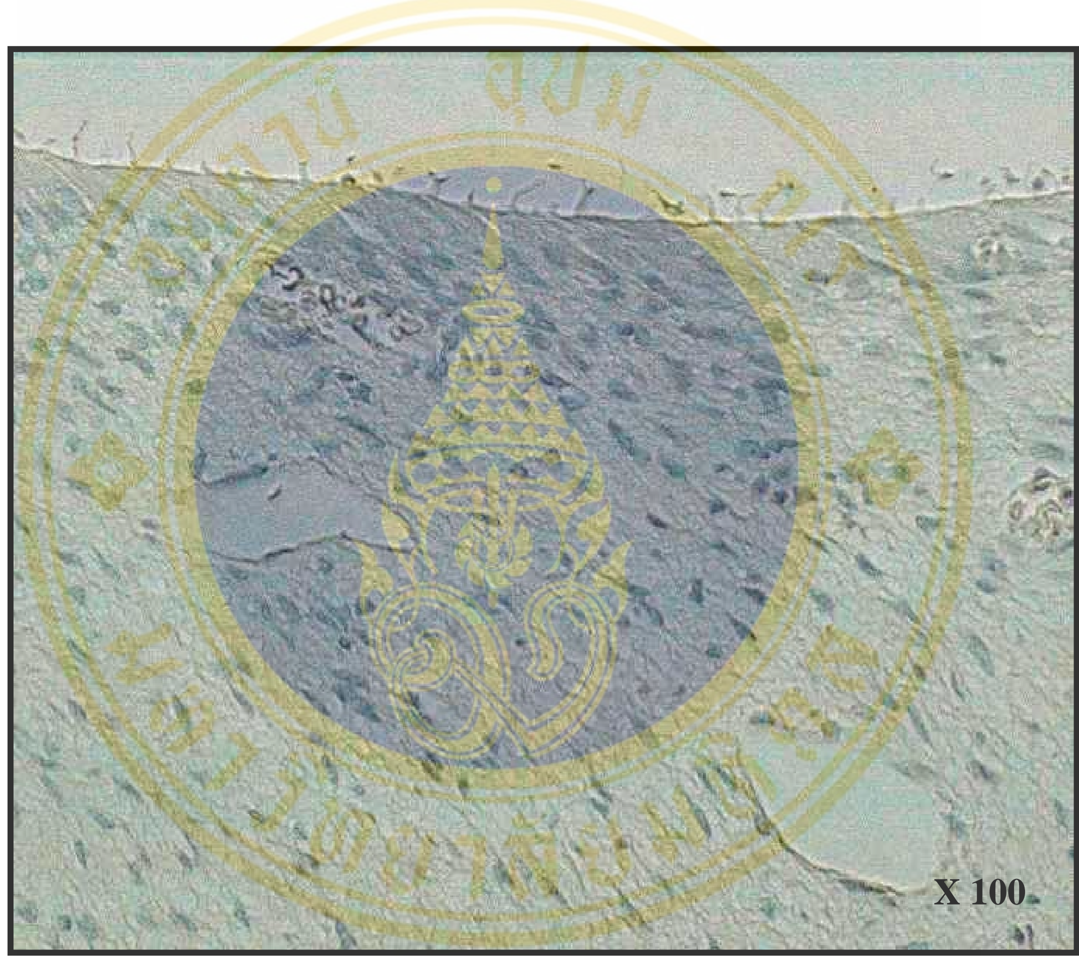


Figure 10. Immunohistochemical analysis of RAGE. In pulpitis tissues, control isotype IgG was employed at the same time.

Copyright by Mahidol University

Figure 11. Immunohistochemical analysis of RAGE. In healthy tissues, control isotype IgG was employed at the same time.

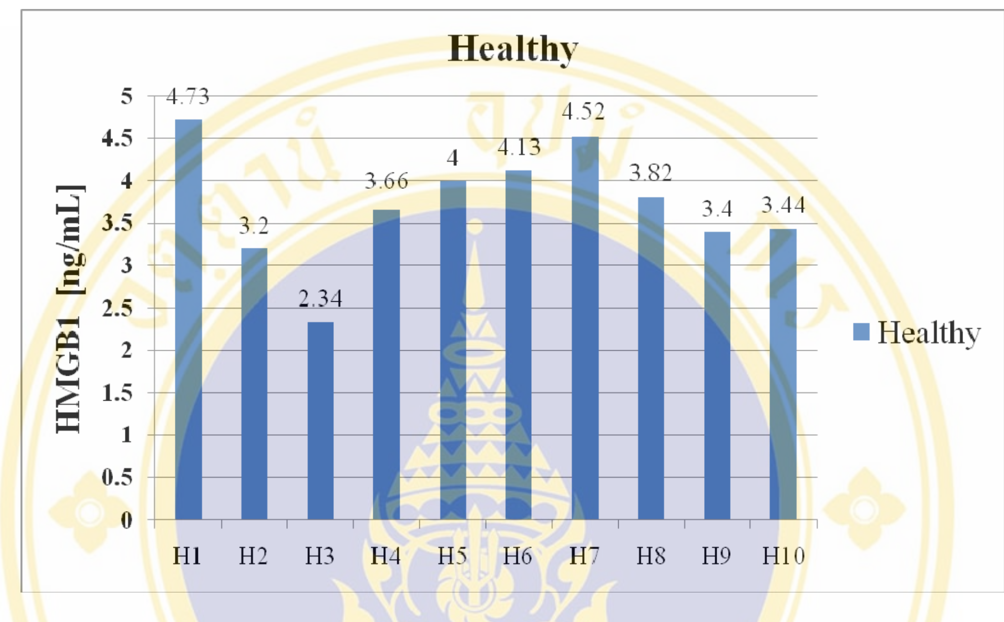
Copyright by Mahidol University

Figure 12. RAGE expression determined by Western blot analyses with RAGE Ab in the pulp tissue samples from three separate patients with pulpitis (left panels, *lanes 1-3*) and three healthy control subjects (right panels, *lanes 4-6*). Abundant RAGE (42 kDa) is present in tissues from pulpitis patients, but low signals have been found in control subjects.

Figure 13. HMGB1 expression determined by Western blot analyses with HMGB1 Ab in pulp tissue samples from three separate patients with pulpitis (left panels, *lane 1-3*) and three healthy control subjects (right panels, *lanes 4-6*). Abundant HMGB1 (27 kDa) is present in tissues from pulpitis patients, but low signals have been found in control subjects.

Copyright by Mahidol University

a.



b.

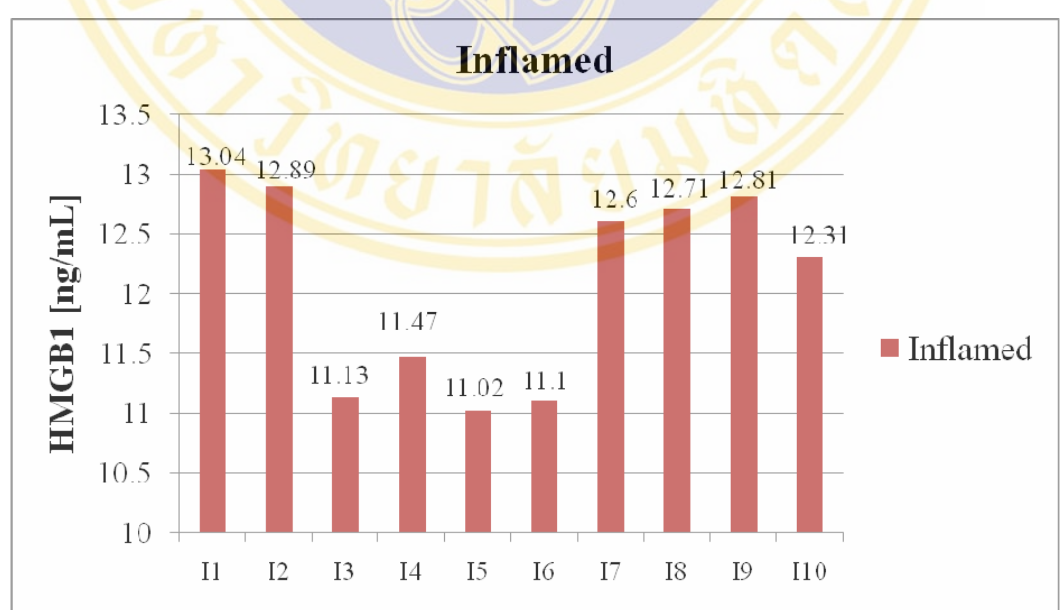


Figure 14. Cytosol HMGB1 levels as measured by ELISA. Each experimental group consisted of ten tissues.

Copyright by Mahidol University

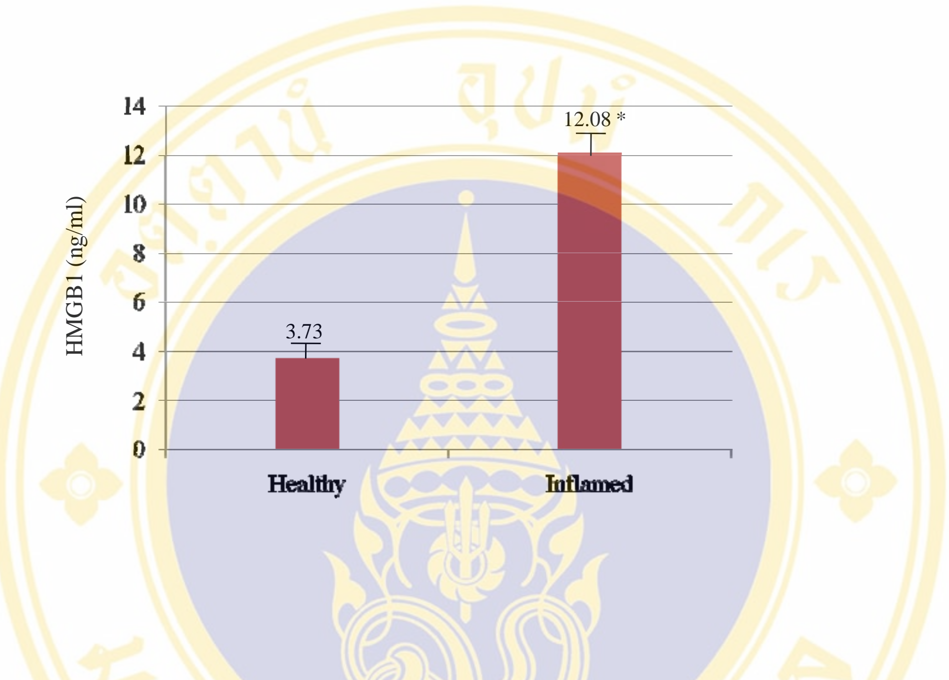


Figure 15. Cytosol HMGB1 concentrations were 3.73 ± 0.69 ng/ml in the healthy group and 12.08 ± 0.82 ng/ml in the inflamed group. Values are shown means ± SD $*p < 0.001$

Copyright by Mahidol University

CHAPTER VI

DISCUSSION

Advance glycation end products (AGEs) accumulate in the plasma and tissues of diabetic subjects. Evidence for the role of receptors of AGE (RAGE) in periodontal disease was verified in a murine model for type 2 diabetes (54). Some researchers demonstrated positive staining of RAGE in endothelium and the basal and spinous layer of the inflamed gingival epithelium in chronic periodontitis patients with and without type 2 diabetes (55). The expression of RAGE and HMGB1 was found during the late fetal and early postnatal stages in rats. RT-PCR demonstrated both HMGB1 and RAGE in human dental pulp cells in vitro, indicating that RAGE and HMGB1 participates in tooth mineralization and an autocrine/paracrine HMGB1 signaling axis in odontoblasts (91). However, the presence of RAGE and HMGB1 in inflamed pulp tissues has not been demonstrated. On the best of our knowledge, we are the first to report on RAGE and HMGB1 expression in inflammatory pulp tissues from all patients without diabetes, corresponding to a previous study in human periodontitis (55).

The early inflammatory response to caries is characterized by the focal accumulation of chronic inflammatory cells. This is most likely mediated initially by odontoblasts and later by dendritic cells. The odontoblast is positioned to encounter foreign antigens first. It is likely that these cells herald the influx of foreign material by the elaboration of chemokines that in turn attract an array of immune effector cells to the infection front (92).

As the carious lesion progresses, the density of the chronic inflammatory infiltrate, as well as that of dendritic cells in the odontoblast region, increases. Pulpal dendritic cells are responsible for antigen presentation and stimulation of T lymphocytes. In the uninflamed pulp, they are scattered throughout the pulp. With caries progression they aggregate initially in the pulp and subodontoblastic regions, then extend into the odontoblast layer and eventually migrate into the entrance to tubules beside the odontoblast process (93).

Copyright by Mahidol University

Evidence suggests that odontoblasts also play a role in humoral immune response to caries. Immunoglobulin G (IgG), IgM, and IgA have been localized in the cytoplasm and cell processes of odontoblasts in human carious dentin, suggesting that these cells actively transport antibodies to the infection front (94). In the incipient lesion, antibodies accumulate in the odontoblast layer and with lesion progression can be seen in the dentinal tubules. Finally, this leads to a focal concentration of antibodies beneath the advancing lesion (95). In this study, inflamed pulp tissues showed strong expression of RAGE in the capillary endothelial cells around blood vessels and inflammatory cells, cell peripheries and odontoblast and fibroblast-like cells. RAGE expression may be involved in the pathogenesis of pulpal inflammation.

HMGB1 is potent in stimulating the release of multiple proinflammatory cytokines, including TNF, IL-1α , IL-1β, IL-6, IL-8, MIP-1 α, and MIP-1β (21). Pulpal diseases are characterized by inflammation. Many cytokines were found in pulpal inflammation such as TNF-α, IL-6 and IL-8 (96-98). In this study, we demonstrated that HMGB1 is increased in pulp inflammatory tissues compared with healthy tissues and may play a role in the occurrence and development of human pulpitis.

Classic proinflammatory cytokines (such as IL-1 and TNF) are synergistic, redundant, and pleiotropic molecules produced by a variety of cell types including phagocytic cells such as monocytes/macrophages and the central nervous system (CNS) cells. Bacterial lipopolysaccharide, a component of the cell wall of Gram-negative bacteria, activates these cells to produce and secrete proinflammatory cytokines. Proinflammatory cytokines are involved in a variety of immune and inflammatory responses, most notably, the initiation of an adaptive local inflammatory response that helps to contain and eliminate invading pathogens. HMGB1 has been discovered to have many characteristics similar to classic proinflammatory cytokines. Phatological amounts of LPS increase levels of IL-1 and TNF in serum and peripheral immune tissue (such as lung and liver) and the pharmacological inhibition of any of the mediators significantly improves survival after a lethal dose of endotoxin (99-101). The finding of HMGB1 as a general proinflammatory mediator has opened up a new venue for anti-inflammatory intervention. In comparison with previously investigated TNF-α and IL-1 blocking therapy in acute inflammatory conditions, HMGB1-targeted therapies have a wider temporal treatment window. It is theoretically possible that

Copyright by Mahidol University

HMGB1-blocking therapy may provide additional clinical benefit to TNF-α and IL-1 blocking therapy in chronic disease (102). HMGB1-targeted intervention with either neutralizing antibodies or the antagonistic A box domain of HMGB1 ameliorates collagen-induced arthritis both in mice and rats, and inhibits the local overexpression of IL-1beta in the joints. It is thus conceivable that therapeutic HMGB1 blockade may contribute to future treatment of human chronic arthritis (103). Therefore, a continued expression of HMGB1 over time following inflammation can act, at least in part, as an important amplification signal for progressive pulpal destruction.

Substance P, found in tooth pulp samples, having a clinical diagnosis of acute irreversible pulpitis (104), exerts its biological actions by binding to neurokinin-1 (NK1), located on most inflammatory and neuronal cells in pulp tissues (105). However, no evidence of the behavior of NK1 receptor expression during normal and inflamed stages in human dental pulp cells has been reported. In comparison with HMGB1 and engagement of RAGE on the pulp cells (odontoblasts and fibroblasts), both of these mechanisms can induce IL-8 production and activate NF-κB and p38 MAP kinase pathways (20). HMGB1 may produce proinflammatory cytokine-like effects directly by activating signaling cascades utilized by NF-κB and p38 MAP kinase pathways or indirectly by inducing proinflammatory cytokines (21). In this circumstance, a discovery of RAGE and HMGB1 in excessive inflammatory pulp may help to clarify the acceptably “novel mechanism” in pulp disease. In comparison with those previously investigated, it is possible that antagonist of RAGE and HMGB1 might have a valuable therapeutic role for the management of irreversible pulpitis. The postulate signaling pathway of inflammation by HMGB1 suggests that HMGB1 binds to RAGE on pulp cells leading to translocation of transcription factors. In response, pulp cells express RAGE and many proinflammatory cytokines lead to inflammatory response, transmigration of leukocytes, neutrophils recruitment and hyperalgesia (Figure 16.). Further experiments are underway to investigate this pathway. Accordingly, *in vitro* and *in vivo* studies of more subjects are required to examine the roles of RAGE and HMGB1 in the pulp pathology.

Copyright by Mahidol University

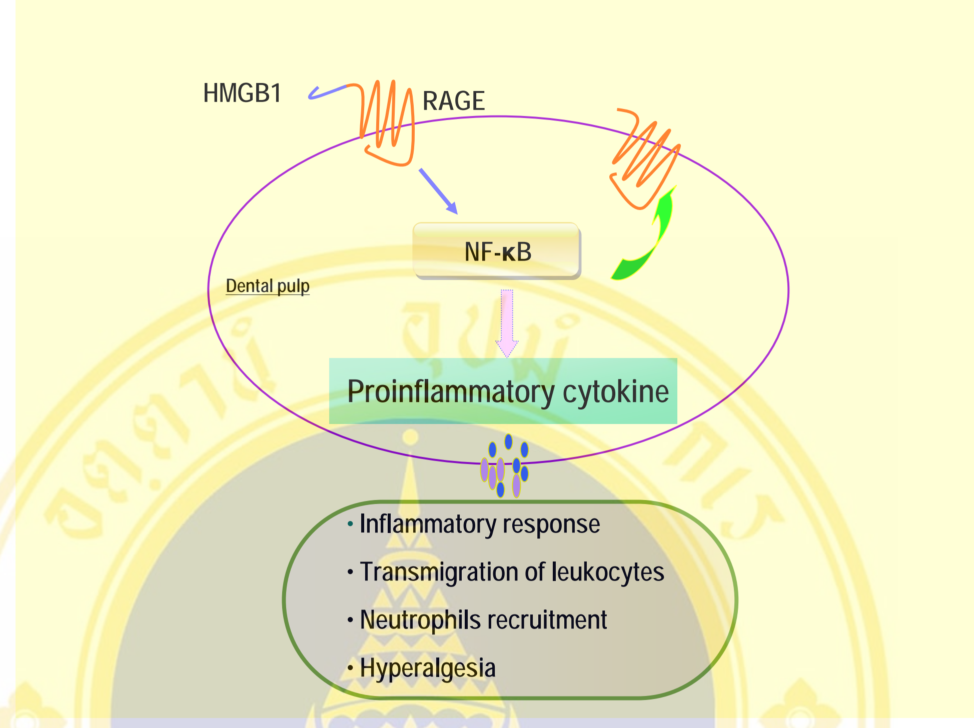


Figure 16. The postulate signaling pathway of inflammation by the binding of RAGE and HMGB1.

Copyright by Mahidol University

CHAPTER VII
CONCLUSION

From this study, it can be concluded that:

- RAGE is overexpressed in human inflammatory pulpal disease tissues and localized on the capillary endothelial cells, odontoblast and fibroblast-like cells.
- Healthy pulp tissues show RAGE expression in capillary endothelial cells and fibroblast-like cells.
- RAGE ligand, HMGB1 is also increased in pulp inflammatory tissues compared with healthy tissues.

Our findings imply that understanding the mechanisms of pro-inflammatory mediator HMGB1 may lead to novel therapeutic approaches in pulpal diseases.

Copyright by Mahidol University

REFERENCES

1. Osterweis M, Kleinman A, Mechanic D. Pain and disability - clinical, behavioral, and public policy perspectives. Washington: National Academy Press; 1987.
2. Slade GD. Epidemiology of dental pain and dental caries among children and adolescents. Community Dent Health 2001;18:219-27.
3. Locker D, Grushka M. The impact of dental and facial pain. J Dent Res 1987;66:1414-7.
4. Shepherd MA, Nadanovsky P, Sheiham A. The prevalence and impact of dental pain in 8-year-old school children in Harrow, England. Br Dent J 1999;187(1):38-41.
5. MacFarlane TV, Blinkhorn AS, Davies RM, Kincey J, Worthington HV. Oro-facial pain in the community: prevalence and associated impact. Community Dent Oral Epidemiol 2002; 30:52-60.
6. Lipton JA, Ship JA, Larach-Robinson D. J Am Dent Assoc. 1993 Oct;124(10): 115-21.
7. Bastos JL, Nomura LH, Peres MA Dental pain, socioeconomic status, and dental caries in young male adults from Southern Brazil.Cad Saude Publica. 2005 Sep-Oct;21(5):1416-23.
8. Clarke M, Locker D, Murray H, Payne B. The oral health of disadvantaged adolescents in North York, Ontario. Can J Public Health 1996; 4:261-3.
9. Petersen PE, Hoerup N, Poomviset N, Prommajan J, Watanapa A. Oral health status and oral health behaviour of urban and rural schoolchildren in Southern Thailand. Int Dent J. 2001 Apr;51(2):95-102.
10. Dionne RA, Gordon SM, McCullagh LM, Phero JC.J Am Dent Assoc. 1998 Feb;129(2):167-73.
11. Eli I, Bar-Tal Y, Fuss Z, Silberg A. Effect of intended treatment on anxiety and on reaction to electric pulp stimulation in dental patients. J Endod. 1997 Nov;23(11):694-7.

Copyright by Mahidol University

12. Cohen AS, Brown DC. Structure and Functions of the Dentin and Pulp Complex In: Cohen S, Burns RC, editors. Pathway of the Pulp. 8th ed: St Louis:Mosby; 2002. p. 430-437.
13. Trowbridge HO: Intradental sensory units: physiological and clinical aspects, J Endod. 1985 Nov;11(11):489-98.
14. Kim S. Neurovascular interactions in the dental pulp in health and inflammation. Endod. 1990 Feb;16(2):48-53.
15. Torabinejad M. Mediators of pulpal and periapical pathosis.CDA J. 1986 Dec;14(12):21-5.
16. Cohen AS, Brown DC.Orofacial Sental Pain Emergencies:Endodontic Diagnosed and management. In: Cohen S, Burns RC, editors. Pathway of the Pulp. 8th ed: St Louis:Mosby; 2002. p. 35-37
17. Cohen JS, Reader A, Fertel R, Beck M, Meyers WJ. A radioimmunoassay determination of the concentrations of prostaglandins E2 and F2alpha in painful and asymptomatic human dental pulps. J Endod. 1985 Aug;11(8):330-5.
18. Lepinski AM, Hargreaves KM, Goodis HE, Bowles WR. Bradykinin levels in dental pulp by microdialysis. J Endod. 2000 Dec;26(12):744-7.
19. Bowles WR, Withrow JC, Lepinski AM, Hargreaves KM. Tissue levels of immunoreactive substance P are increased in patients with irreversible pulpitis. J Endod. 2003 Apr;29(4):265-7.
20. Fiuza C, Bustin M, Talwar S et al. Inflammation-promoting activity of HMGB1 on human microvascular endothelial cells. Blood. 2003 Apr;101(7):2652-60.
21. Andersson U, Wang H, Palmblad K, Aveberger AC, Bloom O, Erlandsson-Harris H et al. High mobility group 1 (HMG-1) Stimulates Proinflammatory Cytokine Synthesis in Human Monocytes. J Exp Med. 2000 Aug ;192(4):565-70.
22. Chacur, Marucia et al. A new model of sciatic inflammatory neuritis (SIN): induction of unilateral and bilateral mechanical allodynia following acute unilateral peri-sciatic immune activation in rats. Pain. 2001 Dec;94(3): 231-44.

Copyright by Mahidol University

23. Schmidt AM, Hofmann M, Taguchi A, Yan SD, Stern DM RAGE: a multiligand receptor contributing to the cellular response in diabetic vasculopathy and inflammation. Semin Thromb Hemost. 2000;26(5): 485-93.
24. Schmidt AM, Yan SD, Yan SF, Stern DM. The multiligand receptor RAGE as a progression factor amplifying immune and inflammatory responses. J Clin Invest. 2001 Oct;108(7): 949-55.
25. Neeper M, Schmidt AM, Brett J, Yan SD, Wang F, Pan YC et al. Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins. J Biol Chem. 1992 Jul ;267(21): 14998-5004.
26. Hori, Osamu et al. The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphoterin. Mediation of neurite outgrowth and co-expression of rage and amphoterin in the developing nervous system. J Biol Chem. 1995 Oct ;270(43): 25752-61.
27. Hofmann MA, Drury S, Fu C, Qu W, Taguchi A, Lu Y et al. RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. Cell. 1999 Jun ;97(7): 889-901.
28. Bierhaus A, Humpert PM, Morcos M, Wendt T, Chavakis T, Arnold B, et al. Understanding RAGE, the receptor for advanced glycation end products. J Mol Med. 2005 Nov ;83(4): 876-86.
29. Chavakis T BA, Al-Fakhri N, Schneider D, Witte S, Linn T, et al. The pattern recognition receptor (RAGE) is a counterreceptor for leukocyte integrins: a novel pathway for inflammatory cell recruitment. J Exp Med. 2003 Nov 17;198(10): 1507-15.
30. Schmidt, Ann M. et al. The dark side of glucose. Nat Med. 1995 Oct :1(10): 1002-4.
31. Schmidt, Ann M. et al. Activation of receptor for advanced glycation end products: a mechanism for chronic vascular dysfunction in diabetic vasculopathy and atherosclerosis. Circ Res. 1999 Mar ;84(5): 489-97.
32. Miyata, Toshio et al. The receptor for advanced glycation end products (RAGE) is a central mediator of the interaction of AGE-beta2microglobulin with human mononuclear phagocytes via an oxidant-sensitive pathway. Implications for the pathogenesis of dialysis-related amyloidosis. J Clin Invest. 1996 Sep;98(5): 1088-94.

Copyright by Mahidol University

33. Pashley HD, Frederick RL. Structure and Functions of the Dentin-Pulp Complex. In:Cohan S, Hargreaves MK editors. Pathways of the Pulp. 9th ed: St Louis:Mosby; 2006. p. 472-478
34. Berkovitz BKB, Holland GR, Moxham BJ. A Color Atlas and Textbook of Oral Anatomy Histology and Embryology. 2nd ed: London;Wolfe Publishing Ltd;1992. p.146-148.
35. Hahn CL, Falkler WA Jr, Siegel MA. A study of T and B cells in pulpal pathosis. J Endod. 1989 Jan;15(1):20-6.
36. Kerdvongbundit V. Inflammation. 1st ed: Bangkok:Faculty of Dentistry Mahidol University; 2004. P. 7-9
37. Kerdvongbundit V. Inflammation 1st ed: Bangkok:Faculty of Dentistry Mahidol University; 2004. p.26-28
38. Kerdvongbundit V. Inflammation 1st ed: Bangkok:Faculty of Dentistry Mahidol University; 2004. p.94-104
39. Malherbe P, Richards JG, Gaillard H, Thompson A, Diener C, Schuler A, Huber G. cDNA cloning of a novel secreted isoform of the human receptor for advanced glycation end products and characterization of cells co-expressing cell-surface scavenger receptors and Swedish mutant amyloid precursor protein. Brain Res Mol Brain Res. 1999 Aug 25;71 (2):159-70.
40. Schmidt AM, Vianna M, Gerlach M, Brett J, Ryan J, Kao J et al. Isolation and characterization of two binding proteins for advanced glycosylation end products from bovine lung which are present on the endothelial cell surface. J Biol Chem.1992 Jul 25;267(21):14987-97.
41. Schmidt AM, Mora R, Cao R, Yan SD, Brett J, Ramakrishnan R et al. The endothelial cell binding site for advanced glycation end products consists of a complex: an integral membrane protein and a lactoferrin-like polypeptide. J Biol Chem. 1994 Apr 1;269(13):9882-8.
42. Taguchi A, Blood DC, del Toro G, Canet A, Lee DC, Qu W, Blockade of RAGE-amphoterin signalling suppresses tumour growth and metastases.Nature. 2000 May 18;405(6784):354-60.
43. Bucciarelli LG, Wendt T, Rong L, Lalla E, Hofmann MA, Goova MT et al. RAGE is a multiligand receptor of the immunoglobulin superfamily:

Copyright by Mahidol University

implications for homeostasis and chronic disease. Cell Mol Life Sci. 2002 Jul;59(7):1117-28.

44. Lander HM, Tauras JM, Ogiste JS, Hori O, Moss RA, Schmidt AM. Activation of the receptor for advanced glycation end products triggers a p21(ras)-dependent mitogen-activated protein kinase pathway regulated by oxidant stress. J Biol Chem. 1997 Jul 11;272(28):17810-4.
45. Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: The multistep Paradigm. Cell 1994 Jan 28;76(2):301-314.
46. Barnes PJ and Karin M. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med. 1997 Apr 10;336(15): 1066-71.
47. Li J, Schmidt AM. Characterization and functional analysis of the promoter of RAGE. J Biol Chem. 1997;272:16498–16506.
48. Bierhaus A, Schiekofer S, Schwaninger M, Andrassy M, Humpert PM, Chen J et al. Diabetes-associated sustained activation of the transcription factor nuclear factor-kappaB. Diabetes. 2001 Dec;50(12):2792-808.
49. Yan SD, Chen X, Fu J, Chen M, Zhu H, Roher A, et al. RAGE and amyloid-beta peptide neurotoxicity in Alzheimer's disease. Nature. 1996 Aug 22;382(6593):685-91.
50. Simm A, Casselmann C, Schubert A, Hofmann S, Reimann A, Silber RE. Age associated changes of AGE-receptor expression: RAGE upregulation is associated with human heart dysfunction. Exp Gerontol. 2004 Mar;39(3):407-13.
51. Lohwasser C, Neureiter D, Weigle B, Kirchner T, Schuppan D. The receptor for advanced glycation end products is highly expressed in the skin and upregulated by advanced glycation end products and tumor necrosis factor-alpha. J Invest Dermatol. 2006 Feb;126(2):291-9.
52. Katz J, Stavropoulos F, Bhattacharyya I, Stewart C, Perez FM, Caudle RM. Receptor of advanced glycation end product (RAGE) expression in the minor salivary glands of patients with Sjögren's syndrome: a preliminary study. Scand J Rheumatol. 2004;33(3):174-8.

Copyright by Mahidol University

53. Zeng S, Feirt N, Goldstein M, Guarrera J, Ippagunta N, Ekong U et al. Blockade of receptor for advanced glycation end product (RAGE) attenuates ischemia and reperfusion injury to the liver in mice. Hepatology. 2004 Feb;39(2):422-32.

54. Lalla E, Lamster IB, Feit M, Huang L, Spessot A, Qu W et al. Blockade of RAGE suppresses periodontitis-associated bone loss in diabetic mice. J ClinInvest. 2000 Apr;105(8):1117-24.

55. Katz J, Bhattacharyya I, Farkhondeh-Kish F, Perez FM, Caudle RM, Heft MW Expression of the receptor of advanced glycation end products in gingival tissues of type 2 diabetes patients with chronic periodontal disease: a study utilizing immunohistochemistry and RT-PCR. J Clin Periodontol. 2005 Jan;32(1):40-4.

56. Park L, Raman KG, Lee KJ, Lu Y, Ferran LJ Jr, Chow WS et al. Suppression of accelerated diabetic atherosclerosis by the soluble receptor for advanced glycation end products. Nat Med. 1998 Sep;4(9):1025-31.

57. Cataldegirmen G, Zeng S, Feirt N et al. RAGE limits regeneration after massive liver injury by coordinated suppression of TNF-alpha and NF-kappaB. J Exp Med. 2005 Feb 7;201(3):473-84.

58. Johns EW. History, definitions and problems. In: Johns EW, 1st ed. The HMG Chromosomal Proteins. London: Academic Press Inc. (London) Ltd., 1982; 1-8.

59. Bustin M. Regulation of DNA-dependent activities by the functional motifs of the high-mobility-group chromosomal proteins. Mol Cell Biol. 1999 Aug;19(8):5237-46.

60. Ferrari S, Finelli P, Rocchi M, Bianchi ME. The active gene that encodes human high mobility group 1 protein (HMG1) contains introns and maps to chromosome 13.Genomics. 1996 Jul 15;35(2):367-71.

61. Stros M, Dixon GH.A retropseudogene for non-histone chromosomal protein HMG-1.Biochim Biophys Acta. 1993 Feb 20;1172(1-2):231-5.

62. Wen L, Huang JK, Johnson BH, Reeck GR. A human placental cdna clone that encodes non histone chromosomal protein HMG-1. Nucleic Acids Res. 1989 17:1197-1214.

Copyright by Mahidol University

63. Landsman D, Bustin M.A signature for the HMG-1 box DNA-binding proteins. Bioessays. 1993 Aug;15(8):539-46.
64. Prasad S, Thakur MK. Age-dependent effects of sodium butyrate and hydrocortisone on acetylation of high mobility group proteins of rat liver. Biochem Int. 1988 Feb;16(2):375-82.
65. Mosevitsky MI, Novitskaya VA, Iogannsen MG, Zabezhinsky MA. Tissue specificity of nucleo-cytoplasmic distribution of HMG1 and HMG2 proteins and their probable functions. Eur J Biochem. 1989 Nov 6;185(2):303-10.
66. Wang H, Vishnubhakat JM, Bloom O, Zhang M, Ombrellino M, Sama A et al. Proinflammatory cytokines (tumor necrosis factor and interleukin 1) stimulate release of high mobility group protein-1 by pituicytes. Surgery. 1999 Aug;126(2):389-92.
67. Weir HM, et al. Structure of the HMG box motif in the B-domain of HMG1. EMBO J. 1993;12:1311–9., Hardman CH, et al. Structure of the A-domain of HMG1 and its interaction with DNA as studied by heteronuclear 3- and 4-dimensional NMR spectroscopy. Biochemistry. 1995;34:16596–607.
68. Bustin M. Regulation of DNA-dependent activities by the functional motifs of the high-mobility-group chromosomal proteins. Mol Cell Biol. 1999 Aug;19(8):5237-46.
69. Boonyaratanakornkit V, Melvin V,Prendergast P,Altmann M, RonfaniL, Bianchi ME et al. High-mobility group chromatin proteins 1 and 2 functionally interact with steroid hormone receptors to enhance their DNA binding in vitro and transcriptional activity in mammalian cells. Mol Cell Biol. 1998 Aug;18(8):4471-87.
70. Melvin VS, Roemer SC, Churchill ME, Edwards DP. Coregulatory proteins in steroid hormone receptor action: the role of chromatin high mobility group proteins HMG-1 and -2. Steroids. 1999 Sep;64(9):576-86.
71. Huttunen HJ, Fages C, Kuja-Panula J, Ridley AJ, Rauvala H. Receptor for advanced glycation end products-binding COOH-terminal motif of amphoterin inhibits invasive migration and metastasis. Cancer Res. 2002 Aug 15;62(16): 4805-11.

Copyright by Mahidol University

72. Sousa MM, Yan SD, Stern D, Saraiva MJ Interaction of the receptor for advanced glycation end products (RAGE) with transthyretin triggers nuclear transcription factor kB (NF-kB) activation. Lab Invest. 2000 Jul;80(7): 1101-10.
73. Thornalley PJ. Cell activation by glycated proteins. AGE receptors, receptor recognition factors and functional classification of AGEs. Cell Mol Biol (Noisy-le-grand). 1998 Nov;44(7):1013-23.
74. Sappington PL, Yang R, Yang H, Tracey KJ, Delude RL, Fink MP HMGB1 B box increases the permeability of Caco-2 enterocytic monolayers and impairs intestinal barrier function in mice. Gastroenterology. 2002 Sep;123(3):790-802.
75. Degryse B, Bonaldi T, Scaffidi P, Müller S, Resnati M, Sanvito F et al. The high mobility group (HMG) boxes of the nuclear protein HMG1 induce chemotaxis and cytoskeleton reorganization in rat smooth muscle cells. J Cell Biol. 2001 Mar 19;152(6):1197-206.
76. Li J, Kokkola R, Tabibzadeh S, Yang R, Ochani M, Qiang X et al. Structural basis for the proinflammatory cytokine activity of high mobility group box 1.Mol Med. 2003 Jan-Feb;9(1-2):37-45.
77. Park JS, Arcaroli J, Yum HK, Yang H, Wang H, Yang KY Activation of gene expression in human neutrophils by high mobility group box 1 protein. Am J Physiol Cell Physiol. 2003 Apr;284(4):C870-9.
78. Degryse B, de Virgilio M The nuclear protein HMGB1, a new kind of chemokine? FEBS Lett. 2003 Oct 9;553(1-2):11-7
79. Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J et al. HMG-1 as a late mediator of endotoxin lethality in mice. Science. 1999 Jul 9;285(5425):248-51.
80. Rendon-Mitchell B, Ochani M, Li J, Han J, Wang H, Yang H et al. IFN-gamma induces high mobility group box 1 protein release partly through a TNF-dependent mechanism. J Immunol. 2003 Apr 1;170(7):3890-7.
81. Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature. 2002 Jul 11;418(6894):191-5.

Copyright by Mahidol University

82. Charles W. Bell, Weiwen Jiang, Charles F. Reich, III, and David S. Pisetsky. The extracellular release of HMGB1 during apoptotic cell death. Am J Physiol Cell Physiol. 2006 Dec;291(6):C1318-25.
83. Fang WH, Yao YM, Shi ZG, Yu Y, Wu Y, Lu LR, Sheng ZY. The significance of changes in high mobility group-1 protein mRNA expression in rats after thermal injury. Shock. 2002 Apr;17(4):329-33.
84. Kokkola R, Sundberg E, Ulfgren AK, Palmblad K, Li J, Wang H et al. High mobility group box chromosomal protein 1: a novel proinflammatory mediator in synovitis. Arthritis Rheum. 2002 Oct;46(10):2598-603.
85. Popovic K, Ek M, Espinosa A, Padyukov L, Harris HE, Wahren-Herlenius M, Nyberg F. Increased expression of the novel proinflammatory cytokine high mobility group box chromosomal protein 1 in skin lesions of patients with lupus erythematosus. Arthritis Rheum. 2005 Nov;52(11) :3639-45.
86. Yang H, Ochani M, Li J, Qiang X, Tanovic M, Harris HE et al. Reversing established sepsis with antagonists of endogenous high-mobility group box 1. Proc Natl Acad Sci U S A. 2004 Jan 6;101(1):296-301.
87. Ulloa L, Ochani M, Yang H, Tanovic M, Halperin D, Yang R et al. Ethyl pyruvate prevents lethality in mice with established lethal sepsis and systemic inflammation. Proc Natl Acad Sci U S A. 2002 Sep 17;99(19):12351-6.
88. Andersson U, Erlandsson-Harris H. HMGB1 is a potent trigger of arthritis. J Intern Med. 2004 Mar;255(3):344-50.
89. Abraham E, Arcaroli J, Carmody A, Wang H, Tracey KJ. HMG-1 as a mediator of acute lung inflammation. J Immunol. 2000 Sep 15;165(6):2950-4.
90. Sawa H, Ueda T, Takeyama Y, Yasuda T, Shinzeki M, Nakajima T, Kuroda Y. Blockade of high mobility group box-1 protein attenuates experimental severe acute pancreatitis.World J Gastroenterol. 2006 Dec 21;12(47): 7666-70.
91. Kokkola R, Andersson Å, Mullins G, Östberg T, Treutiger CJ, Arnold B et al. RAGE is the major receptor for the proinflammatory activity of HMGB1 in rodent macrophages. Scand J Immunol. 2005 Jan;61(1):1-9.

Copyright by Mahidol University

92. Fouad A, Levin L. Pulpal reactions to caries and dental procedures. In:Cohan S, Hargreaves MK editors. Pathways of the Pulp. 9th ed: St Louis:Mosby; 2006. p.517.

93. Yoshiba N, Yoshiba K, Nakamura H, Iwaku M, Ozawa H. Immunohistochemical localization of HLA-DR-positive cells in unerupted and erupted normal and carious human teeth. J Dent Res. 1996 Aug;75(8):1585-9.

94. Okamura K, Maeda M, Nishikawa T, Tsutsui M Dentinal response against carious invasion: localization of antibodies in odontoblastic body and process. J Dent Res. 1980 Aug;59(8):1368-73.

95. Okamura K Histological study on the origin of dentinal immunoglobulins and the change in their localization during caries. J Oral Pathol. 1985 Oct;14(9): 680-9.

96. Pezelj-Ribaric S, Anic I, Brekalo I, Miletic I, Hasan M, Simunovic-Soskic M. Detection of tumor necrosis factor alpha in normal and inflamed human dental pulps. Arch Med Res. 2002 Sep-Oct;33(5):482-4.

97. Barkhordar RA, Hayashi C, Hussain MZ. Detection of interleukin-6 in human dental pulp and periapical lesions. Endod Dent Traumatol. 1999 Feb;15(1):26-7.

98. Guo X, Niu Z, Xiao M, Yue L, Lu H. Detection of interleukin-8 in exudates from normal and inflamed human dental pulp tissues. Chin J Dent Res. 2000 May;3(1):63-6.

99. Tracey KJ, Fong Y, Hesse DG, Manogue KR, Lee AT, Kuo GC, et al. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. Nature. 1987 Dec 17-23;330(6149):662-4.

101. Alexander HR, Doherty GM, Venzon DJ, Merino MJ, Fraker DL, Norton JA. Recombinant interleukin-1 receptor antagonist (IL-1ra): effective therapy against gram-negative sepsis in rats. Surgery. 1992 Aug;112(2):188-93

102. Libert C, Vink A, Coulie P, Brouckaert P, Everaerdt B, Van Snick J, Fiers W. Limited involvement of interleukin-6 in the pathogenesis of lethal septic shock as revealed by the effect of monoclonal antibodies against interleukin-6 or its receptor in various murine models. Eur J Immunol. 1992 Oct;22(10):2625-30.

Copyright by Mahidol University

103. Kokkola R, Li J, Sundberg E, Aveberger AC, Palmblad K, Yang H et al. Successful treatment of collagen-induced arthritis in mice and rats by targeting extracellular high mobility group box chromosomal protein 1 activity. Arthritis Rheum. 2003 Jul;48(7):2052-8.

104. Caviedes-Bucheli J, Gutierrez-Guerra JE, Salazar F et al., Substance P receptor expression in healthy and inflamed human pulp tissue. Int Endod J. 2007 Feb;40(2):106-11.

105. Takahashi K, Tanaka A, Hara M, Nakanishi S. The primary structure and gene organization of human substance P and neuromedin K receptors. European Journal of Biochemistry. 1992 Mar 15;204(3):1025-33

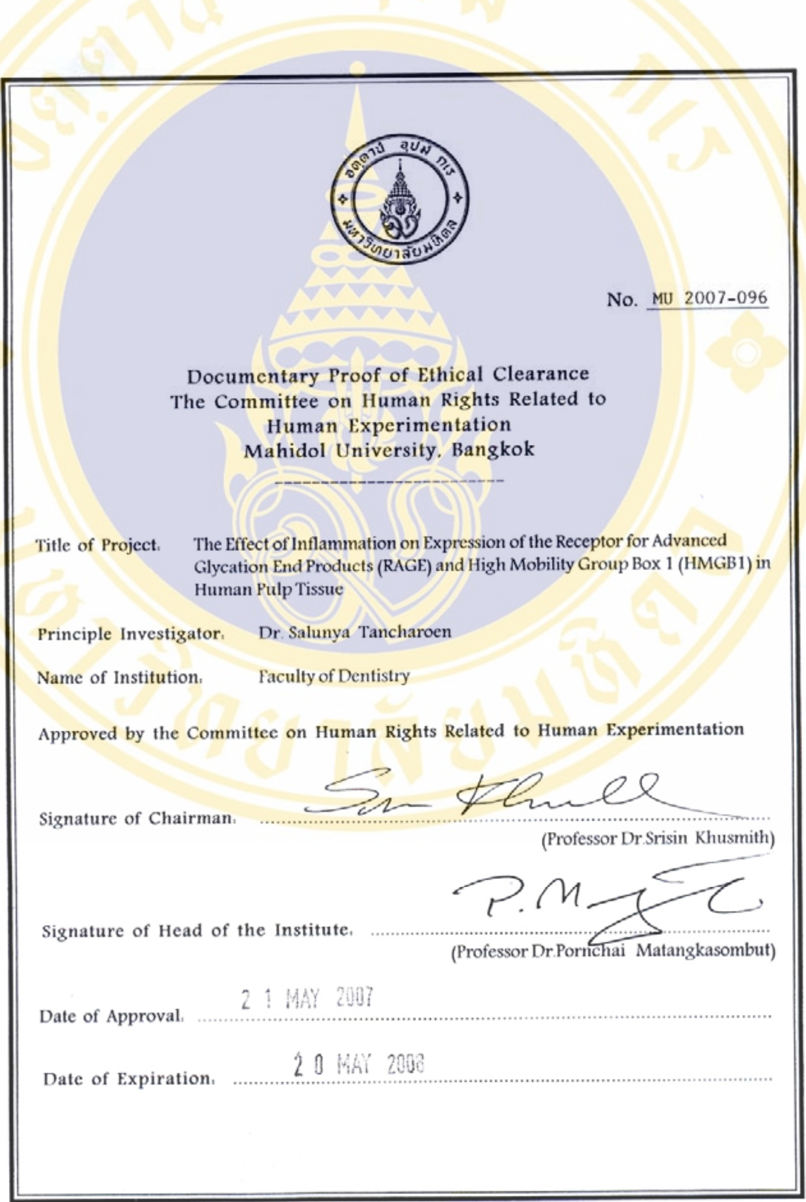
Copyright by Mahidol University

APPENDIX

Copyright by Mahidol University

APPENDIX A

Documentary Proof of Ethical Clearance



No. MU 2007-096

Documentary Proof of Ethical Clearance
The Committee on Human Rights Related to
Human Experimentation
Mahidol University, Bangkok

Title of Project: The Effect of Inflammation on Expression of the Receptor for Advanced Glycation End Products (RAGE) and High Mobility Group Box 1 (HMGB1) in Human Pulp Tissue

Principle Investigator: Dr. Salunya Tancharoen

Name of Institution: Faculty of Dentistry

Approved by the Committee on Human Rights Related to Human Experimentation

Signature of Chairman: ....
(Professor Dr.Srisin Khusmith)

Signature of Head of the Institute: ....
(Professor Dr.Pornchai Matangkasombut)

Date of Approval: 21 MAY 2007

Date of Expiration: 20 MAY 2008

Copyright by Mahidol University

BIOGRAPHY

NAME	Miss Nuttavun Vechvongvan
DATE OF BIRTH	16 November 1980
PLACE OF BIRTH	Nakhon Pathom, Thailand
INSTITUTIONS ATTENDED	Mahidol University, 1998-2005:
	Doctor of Dental Surgery
	Mahidol University, 2006-2008:
	Master of Science
	(General Dentistry)
RESEARCH GRANT	Partially supportted by the graduate studies of Mahidol university alumni association.
POSITION&OFFICE	Nakhon Pathom Municipality,
	Nakhon Pathom, Thailand
	Position: Dentist
	Tel. 0-3425-3850
HOME ADDRESS	676/8 Thahanbok Rd., Muang
	Nakhon Pathom, Thailand.73000

Copyright by Mahidol University