ROLE OF HMGB1 IN CELLS OF THE CIRCULATION

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LIST OF ORIGINAL PUBLICATIONS

This study is based on the following original publications, which are referred to in the text by their roman numerals:

I) Rouhiainen A, Imai S, Rauvala H, Parkkinen J. Occurrence of amphoterin (HMG1) as an endogenous protein of human platelets that is exported to the cell surface upon platelet activation. Thromb Haemost. 2000;84:1087-94.

II) Rouhiainen A, Kuja-Panula J, Wilkman E, Pakkanen J, Stenfors J, Tuominen RK, Lepäntalo M, Carpén O, Parkkinen J, Rauvala H. Regulation of monocyte migration by amphoterin (HMGB1). Blood. 2004;104:1174-82.

III) Sundén-Cullberg J, Norrby-Teglund A, Rouhiainen A, Rauvala H, Herman G, Tracey KJ, Lee ML, Andersson J, Tokics L, Treutiger CJ. Persistent elevation of high mobility group box-1 protein (HMGB1) in patients with severe sepsis and septic shock. Crit Care Med. 2005;33:564-73.

IV) Rouhiainen A, Tumova S, Valmu L, Kalkkinen N, Rauvala H. Pivotal advance: analysis of proinflammatory activity of highly purified eukaryotic recombinant HMGB1 (amphoterin). J Leukoc Biol. 2007;81:49-58.

ABBREVIATIONS

ABC = ATP-binding cassette transporter

ADP = adenosine diphosphate

ALCAM = activated leukocyte cell adhesion molecule

ALT = alanine aminotransferase

AMIGO = amphoterin-induced gene and open reading frame

AT-hook = adenine-thymine-hook

ATP = adenosine triphosphate

C5a = complement component C5a

C5L2 = second C5a receptor

CD = cluster of differentiation

CNS = central nervous system

CREB = cAMP response element binding

CRM1 = chromosome region maintenance 1

CRP = C-reactive protein

DEGA = differentially expressed in human gastric adenocarcinoma

DIC = disseminated intravascular coagulation

DIDS = 4,4’-diisothiocyanostilbene-2,2’-disulfonic acid

DNA = deoxyribonucleic acid

ELISA = enzyme-linked immunosorbent assay

ERK = extracellular signal-regulated kinase

FGF = fibroblast growth factor

g = relative centrifuge force

GP = glycoprotein

GPI = glycosylphosphatidylinositol

HIV = human immunodeficiency virus

HMGA = high-mobility group A

HMGB = high-mobility group B

HMGN = high-mobility group N

HSP = heat-shock protein

HUVEC = human umbilical vein endothelial cell

IκB = inhibitory kappa B

IL = interleukin

IL-R = interleukin receptor

JNK = Jun amino terminal kinase

Kb = kilobase

LBP = lipopolysaccharide binding protein

LDH = lactate dehydrogenase

LMG = low-mobility group

MD-2 = myeloid differentiation protein-2

mRNA = messenger ribonucleic acid

NF-κB = nuclear factor kappa B

NLS = nuclear localization signal

p53 = protein 53

PAMP = pathogen-associated molecular pattern

PMA = 4β-phorbol 12-myristate 13-acetate

RAGE = receptor for advanced glycation end-products

RNA = ribonucleic acid

RPTP = receptor protein tyrosine phosphatase

RT-PCR = reverse transcriptase polymerase chain reaction

sAMIGO = soluble amphoterin-induced gene and open reading frame

SBP = sulfatide binding protein

SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis

SOFA = sepsis-related organ failure assessment

sRAGE = soluble receptor for advanced glycation end-products

TLC = thin-layer chromatography

TLR = Toll-like receptor

TNF = tumor necrosis factor

t-PA = tissue plasminogen activator

vWF = von Willebrand factor

1 ABSTRACT

The matrix of blood is a liquid plasma that transports molecules and blood cells within vessels lined by endothelial cells. High-mobility group B1 (HMGB1) is a protein expressed in blood cells. Under normal circumstances, HMGB1 is virtually absent from plasma, but during inflammation or trauma its level in plasma is increased. In resting and quiescent cells, HMGB1 is usually localized in the intracellular compartment, with the exception of motile cells that express HMGB1 on their outer surface to mediate cell migration. During cell transformation or immune cell activation HMGB1 can be actively secreted outside of the cell. Further, when a cell is damaged, HMGB1 can passively leak into extracellular environment. Extracellular HMGB1 can then participate in regulation of the immune response and under some conditions it can mediate lethality in systemic inflammatory response.

The aim of this study was to evaluate the expression and functions of HMGB1 in cells of the vascular system and to investigate the prognostic value of circulating HMGB1 in severe sepsis and septic shock. HMGB1 was detected in platelets, leukocytes, and endothelial cells. HMGB1 was released from platelets and leukocytes, and it was found to mediate their adhesive and migratory functions. During severe infections the plasma levels of HMGB1 were elevated; however, no direct correlation with lethality was found. Further, the analysis of proinflammatory mechanisms suggested that HMGB1 forms complexes with other molecules to activate the immune system.

In conclusion, HMGB1 is expressed in the cells of the vascular system, and it participates in inflammatory mechanisms by activating platelets and leukocytes and by mediating monocyte migration.

2 REVIEW OF THE LITERATURE

2.1 HIGH-MOBILITY GROUP PROTEINS

2.1.1 High-mobility group protein family

The term “high-mobility group” (HMG) designates a 0.35 M NaCl-extractable chromatin protein fraction that is soluble in 2% trichloroacetic acid. Proteins of this fraction have a higher mobility in polyacrylamide gels than trichloroacetic acid-precipitated proteins, which in turn are called low-mobility group (LMG) –proteins [1, 2].

HMG proteins consist of three families: HMGA, HMGB, and HMGN.
They all have characteristic sequence motifs; HMGA family members have an ´AT hook´ motif, HMGB family members an ´HMG box`, and HMGN family members a ´nucleosomal binding domain´ [3].

All HMG proteins have intracellular, mainly nuclear localization. However, under certain conditions, some HMG proteins may have extranuclear localization [4, 5, 6]. HMG proteins bind to DNA and mediate various biochemical functions, such as cell cycle regulation and gene expression, through condensing or unfolding of chromatin [7].

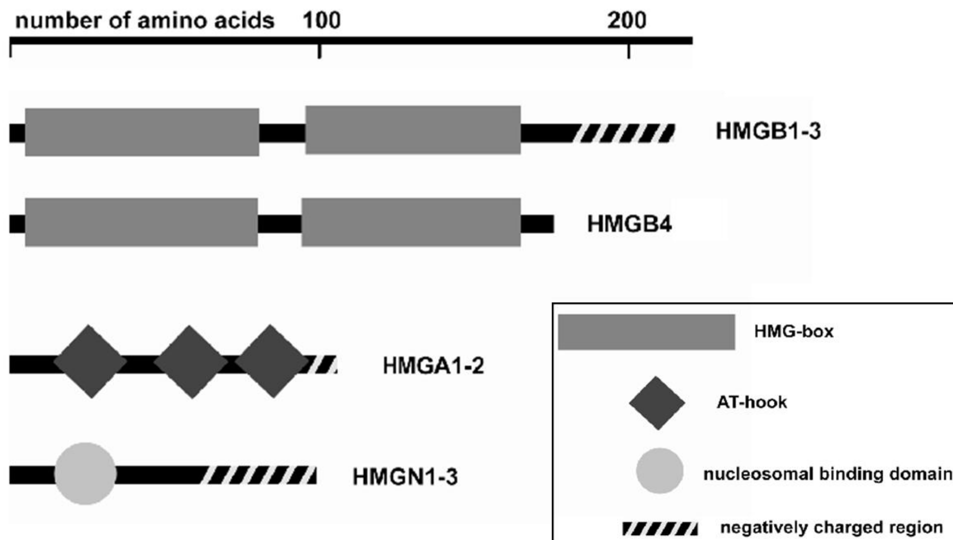

Figure 1. Schematic picture showing domain organization of HMG-proteins. HMG domains were analyzed using the ScanProsite program (ExPASy, Swiss Institute of Bioinformatics, Switzerland). Sequences are shown from amino terminus to the carboxy terminus. Negatively charged regions are indicated as described by Hock et al. [8]. Symbols representing specific protein regions are illustrated in the insert.

2.1.1.1 HMGB

Four members of the HMGB family are currently known in mammals: HMGB1-4 [9]. In addition to mammals, HMGB proteins are widely expressed in eukaryotic kingdoms [10].

The human HMGB1 gene encodes a protein of 214 amino acids, the HMGB2 gene encodes a protein of 208 amino acids, and the HMGB3 gene encodes a protein of 199 amino acids. They all have two HMGB boxes (A and B boxes), and an acidic carboxyl-terminal domain. HMGB4 has two HMGB boxes, but it lacks the acidic tail.

The HMGB boxes are involved in DNA binding, but the role of the acidic tail remains incompletely understood. The acidic tail is known to modulate poly(ADP)ribosylation-regulated nuclear localization of the protein, affect DNA-binding affinity of A- and B-boxes, and be involved in the pathology of polyglutamine diseases [11, 12, 13, 14, 15].

In mammals, HMGB1-3 proteins are coded by three intron-containing genes. HMGB4 is coded by an intronless gene. However, there is an HMGB4 splice variant that lacks the A box. Multiple HMGB pseudogenes are found in mammalian genomes, where HMGB3-originated pseudogenes seem to be most prominent [A. Rouhiainen and H. Rauvala, unpublished results]. The biological role of retroposed copies of HMGB genes is unknown [16].

2.1.1.1.1 HMGB1 (Amphoterin)

The HMGB1 gene is located in human chromosome 13 [17, 18]. It codes for a protein of 215 amino acids. HMGB1 is widely expressed in the adult organism, and during development it is expressed from the zygote stage [19]. HMGB1 is essential for normal development in mice since the knockout suffers from multiple organ failure and defects in bone formation, and the animals die soon after birth due to severe hypoglycemia [20, 21].

```
HMGB1           MGKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKF
HMGB2           MGKGDPNKPRGKMSSYAFFVQTCREEHKKKHPDSSVNFAEFSKKCSERWKTMSAKEKSKF
HMGB3           MAKGDPKKPKGKMSAYAFFVQTCREEHKKKNPEVPVNFAEFSKKCSERWKTMSSKEKSKF
HMGB4           MGEKDQLRPKVNVSSYIHFMLNFRNKFKEQQPNTYLGFKEFSRKCSEKWRSISKHEKAKY
                *.: *  :*: ::*:* .*: . *::.*:::*:  :.* ***:****:*:::* :**.*:

HMGB1           EDMAKADKARYEREMKTYIPPKGETKKKFKDPNAPKRPPSAFFLFCSEYRPKIKGEHPGL
HMGB2           EDLAKSDKARYDREMKNYVPPKGDKKGKKKDPNAPKRPPSAFFLFCSENRPKIKIEHPGL
HMGB3           DEMAKADKVRYDREMKDYGPAKGGKKK--KDPNAPKRPPSGFFLFCSEFRPKIKSTNPGI
HMGB4           EALAELDKARYQQEMMNYIGKR--RKRRKRDPKAPRKPPSSFLLFSRDHYAMLKQENPDW
                : :*: **.**::**  *   :   *   :**:**::***.*:**. :  . :*  :*.

HMGB1           SIGDVAKKLGEMWNNTAADDKQPYEKKAAKLKEKYEKDIAAYRAKGKPDAAKKGVVKAEK
HMGB2           SIGDTAKKLGEMWSEQSAKDKQPYEQKAAKLKEKYEKDIAAYRAKGKSEAGKKGPGRPTG
HMGB3           SIGDVAKKLGEMWNNLSDNEKQPYVTKAAKLKEKYEKDVADYKSKGKFDGAKG----PAK
HMGB4           TVVQVAKAAGKMWSTTDEAEKKPYEQKAALMRAKYFEEQEAYRNQCQ-------------
                :: :.**  *:**.     :*:**  *** :: ** ::   *: : :

HMGB1           SKKKKEEEDDEEDEEDEEEEEEEEEDEDEEEDDDDE
HMGB2           SKKKNEPEDEEEEEEEEEEEDDEEEEEDEE-----
HMGB3           VARKKVEEEEEEEEEEEEEEEEEEEDE---------
HMGB4           GRKGNFLESAKTSLKQ-------------------
                  : :  *. : . ::
```

Figure 2. An alignment of mouse HMGB proteins. Sequences were analyzed using ClustalW in EMBnet (ISREC Bioinformatics Groups, Epalinges, Switzerland). * = identical amino acids, : = similar amino acids.

There are multiple sizes of HMGB1 transcripts in different species and tissues. For example, rat HMGB1 gene is transcribed to at least three different types of mRNA. The variation is most likely due to differences in length of polyadenylation tails [22]. The conclusion that differences in poly-adenylation cause the changes in mRNA length and they are not derived from intronless pseudogenes is further supported by results from studies using chickens. Chickens, lacking HMGB1 pseudogenes have HMGB1 transcripts of multiple sizes, indicating that they are differently poly-adenylated forms of the HMGB1gene and are not derived from HMGB1 pseudogenes [23, 24].

Different forms of HMGB1 transcripts are differentially regulated, suggesting the existence of a regulatory mechanism for HMGB1 at the mRNA level [25].

HMGB1 mRNA can be localized to the cell periphery, where it may be translated [26, 27, 28]. However, no differences have been observed in localisation of different poly-adenylated HMGB1 mRNA forms [28].

Amino acid sequences of HMGB1 proteins are highly conserved between species; for example, human and rat HMGB1 differ by only two amino acids, which moreover are replacements of two similar amino acid residues. Although there is no known amino acid change causing mutations in human HMGB1, single nucleotide polymorphisms occur [29]. One HMGB1 protein isoform derived from alternatively spliced messenger has been detected from colon adenocarcinoma cells, suggesting the existence of HMGB1 polypeptides that vary in amino acid compositions [30].

The structure of HMGB1 has been partially solved. Solution and crystal structures of the A box show that it forms three α helices that are folded into an L-shap [31, 32]. The B box forms four α helices in solution that are folded into an L-shape similar to the A box [33, 34].

In vivo, HMGB1 is often posttranslationally modified, yielding a protein that has altered affinity for its ligands [35]. An original acid extraction method has been shown to change in vitro biochemical properties of HMGB1 compared with native protein; for example, native HMGB1 forms homodimers and oligomers, whereas acid-extracted HMGB1 does not [36, 37]. In addition, DNA-binding capability and effects on plasminogen activation of HMGB1 are strongly affected by acid treatment of the protein [38, 39].

HMGB1 was originally considered a nuclear chromatin component. It binds to DNA in a sequence-independent manner and interacts with many different nuclear proteins [10]. However, its exact function in the nucleus remains obscure. Recent studies with HMGB1-/- cells suggest that HMGB1 is involved in maintenance of

genome stability, acting as a cofactor of base excision repair and possibly affecting telomerase functions [10, 40, 41, 42]. Microarray analysis revealed that cells lacking HMGB1 have altered gene expression of many genes involved in intracellular signaling, apoptosis, and cell cycle pathways. Further, knockout studies suggested that different cell types utilize nuclear HMGB1 in different ways [43].

In addition to the original finding that HMGB1 is a nuclear protein, it was later found also to be both a cytoplasmic [44] and extracellular protein [36, 45]. HMGB1 can be localized to the cell periphery and is actively secreted from some cells, although it lacks a classical signal sequence for the endoplasmic reticulum and Golgi-mediated secretion route [39, 46]. Thus, its secretion mechanism is unknown. The chemical structure of extracellularly secreted HMGB1 has not been fully determined, although a protein form methylated and/or acetylated in lysine appears to be secreted from lymphocytes and monocytes, respectively [47, 48, 49]. Further, acid-treated, secreted extracellular HMGB1 exists as a dimer after acid treatment [50].

In the literature, extracellular HMGB1 is mainly described as an inflammation-promoting protein [reviewed in 51]. However, there are studies showing that under certain circumstances HMGB1 can be an anti-inflammatory molecule [52, 53, 54]. Results of these studies indicate that the physiological role of HMGB1 is incompletely understood and further studies are needed.

Some studies have shown that the genuine HMGB1 polypeptide of eukaryotic origin is only weakly proinflammatory, whereas the bacterially produced recombinant protein is highly active. Part of this activity can be mediated by bacterial components that are carried by HMGB1 [IV, 55, 56, 57, 58, 59]. However, it has been suggested that eukaryotic HMGB1 released from necrotic cells can induce or enhance proinflammatory response, although there are controversial results [60, 61, 62]. In

addition, eukaryotic HMGB1 is able to induce expression of proinflammatory genes in neutrophils like chemokine ligands and members of interleukin family. These genes in neutrophils are similarly upregulated by LPS. There are, however, some genes that are upregulated by HMGB1, but not by LPS, suggesting that HMGB1 itself has some unique activities on neutrophils [63].

Circulating HMGB1 was originally isolated as a proinflammatory cytokine that mediates lethality in septic shock [64]. However, the role of circulating HMGB1 is not yet fully understood, and its role as a proinflammatory cytokine has not been clarified in detail [IV, 55]. Nor is the mechanism of HMGB1-mediated lethality fully explained. For example, high levels of HMGB1 expression in plasma are detected in liver transplantation patients who survive, suggesting that genuine HMGB1 polypeptide in circulation is not sufficient to mediate lethality, at least not in the short term [65]. However, there are animal studies that describe a protective effect of anti-HMGB1 antibodies against lethality in sepsis, suggesting that HMGB1 mediates lethality during prolonged complications [64, 66]. Further, anti-HMGB1 antibodies have a protective effect in other models of inflammatory diseases, e.g. pancreatitis [67].

In addition to inflammatory diseases, HMGB1 is often connected to cancer, and its expression is usually increased in transformed tissue and sometimes in blood plasma [68, reviewed in 69 and 70]. A role in anorexia nervosa is suggested for HMGB1, and higher plasma HMGB1 levels have been detected during the refeeding resistance period [71, 72]. HMGB1 levels are often increased in areas of ischemic insult, and this is thought to mediate ischemia-associated inflammatory response. In addition, HMGB1 has been suggested to play a role in regeneration of tissue after

injury. For example, after nerve damage the HMGB1 polypeptide is strongly upregulated in axons [27].

Nuclear and cytoplasmic HMGB1 have multiple interacting components, some bind directly to HMGB1 with high affinity, and others compose HMGB1-binding multicomplexes [7]. The exact effects of these proteins on functions of HMGB1 are poorly understood.

Extracellular HMGB1 has binding partners both at the cell surface and in the extracellular matrix. Cell surface binding sites encompass glycoproteins, proteoglycans, and phospho- and glycolipids. Extracellular matrix HMGB1-binding site proteins also encompass glycoproteins and proteoglycans (Table 1).

Since the expression of HMGB1 is increased in many diseases and HMGB1 mediates deleterious effects, there is a growing interest in finding small molecule inhibitors for HMGB1. One promising candidate molecule that has a well-documented anti-inflammatory effect and that binds to HMGB1 is glycyrrhizin. Sakamoto et al. [73] initially showed that glycyrrhizin binds to HMGB1 and affects DNA-binding properties of HMGB1. Glycyrrhizin has been subsequently shown to inhibit HMGB1-mediated inflammatory reactions and cell motility [74, 75].

Table 1. Extracellular binding partners for HMGB1.

Name	k_d (nM)	Role in HMGB1 biology	Physiological role
RAGE [76]	10.24±2.84	Colocalizes with HMGB1 in CNS	Mediates cell migration and inflammation
Phosphacan [77]	0.26	Colocalizes with HMGB1 in CNS	Coreceptor regulating cell growth
Neurocan [77]	7.6	-	Coreceptor regulating cell growth
TLR2/4 [78]	nd	Co-immunoprecipitates with HMGB1	Mediates inflammation
Glycolipids [79]	nd	Colocalizes with HMGB1 in CNS	Membrane components
Thrombomodulin [80]	nd	Inhibits proinflammatory activity of HMGB1	Blood coagulation
DNA [38]	2.5	HMGB1 binds to both nuclear and extracellular DNA	Carrier of genetic information
Phopsholipids [I, IV]	nd	HMGB1 inhibits effect of phosphatidylserine on macrophages	Blood coagulation and inflammation
Heparin [36]	nd	Blocks HMGB1 interactions with cells	Blood coagulation
Syndecan-1 [81]	nd	Colocalize in epithelial cells	Regulates cell migration
CD14 [82]	nd	Coreceptor in HMGB1/TLR4 interaction	LPS receptor

2.1.1.1.2 HMGB2

Human HMGB2 is coded by a gene located in chromosome 4 [83, 84]. It codes for a protein of 209 amino acids. HMGB2 is highly expressed during embryogenesis, and in adults it is mainly expressed in testicles and lymphoid organs [85]. Mice lacking HMGB2 gene are viable, but knockout males have reduced fertility. Analysis of the knockout mice revealed that in testis HMGB2 is expressed in primary spermatocytes and it is involved in germ cell differentiation. Further, HMGB2 is highly expressed in chondrocytes of the superficial zone in joints, and knockout mice develop osteoarthritis earlier than wild-type mice [86].

Like HMGB1, HMGB2 is released to extracellular space in various inflammatory states [87, 88]. HMGB2 binds to fucosylated sugars, including the Lewis X structure, present in infectious organisms, suggesting a role in immune defence [89]. Further, binding to RAGE has been suggested for HMGB2 [90]. Thus, HMGB1 and -2 may compensate for each other. In fact, studies with double HMGB1/HMGB2 knockouts suggest that these genes have overlapping functions that may help the single mutant to develop [91].

2.1.1.1.3 HMGB3

HMGB3 (previously known as HMG4 or HMG2b) is coded by a gene in human chromosome X [92]. Gene codes for protein of 200 amino acids. HMGB3 is expressed mainly in hematopoietic stem cells. Knockout mice are erythrocytemic having 10% more red cells than wild-type mice, and enforced HMGB3 expression in bone marrow inhibits myeloid and B-cell differentiation [93, 94].

2.1.1.1.4 HMGB4

HMGB4 is a new member of the HMGB-family. It has 186 amino acids, and it has both A and B boxes, but it lacks the acidic carboxyl terminal domain. Human HMGB4 gene is localized to chromosome 1. HMGB4 is a mammalian-specific gene probably originating from an intronless HMGB pseudogene. Since birds do not have HMGB pseudogenes, the occurrence of pseudogene-derived HMGB4 gene in mammals is consistent with this hypothesis. HMGB4 is much less conserved between species than other HMGB genes; its carboxyl terminal part in particular has hardly any conserved amino acids. Currently, little published data on HMGB4 exist. Affymetrix studies indicate that it is expressed in late and round spermatids [95, 96]. In situ studies reveal its expression in the mouse brain, especially in the hippocampus and cerebellum internal granule cell layer, and embryonal pancreatic epithelial cells [97, 98, 99]. Further, partial peptide sequences have been detected in mouse testicles [100].

2.1.1.2 HMGA

HMGA was previously known as HMGI/Y. According to the new nomenclature, it is now known as HMGA1 or HMGA2 [3]. These are widely expressed during development, but are missing in fully differentiated cells [101]. HMGA proteins have AT-hook domains and an acidic carboxyl terminal domain. The AT-hook domain functions as a DNA-binding region. The acidic carboxyl terminal domain can mediate protein-protein interactions [101].

The human HMGA1 gene contains eight exons and its mRNA has alternatively spliced forms that are translated [102]. These transcripts differ in 33 nucleotides coded by exon five, and code for 107 and 96 amino acid proteins in humans [103]. HMGA1 is also released from cells [104].

The human HMGA2 gene contains five exons [105]. Similarly to the HMGA1 mRNA, the HMGA2 messenger can be transcribed to alternatively spliced forms [101]. A full-length human HMGA2 has 109 amino acids [105].

2.1.1.3 HMGN

The HMGN proteins have been previously known as HMG-14 and HMG-17. Nowadays, there are at least three known members of HMGN proteins, HMGN1-3 [106]. They are involved in differentiation and are widely expressed in embryonic cells, but at lower levels in later developmental stages [101]. HMGN proteins have three domains: the nuclear localization signal domain, the nucleosome-binding domain, and the chromatin-unfolding domain [101]. HMGN proteins are associated with the nucleosome core histones and promote chromatin unfolding and transcription [107].

Human genes coding for HMGN proteins consist of six exons [108]. They code for proteins of 77-99 amino acids. As in other HMG families, there are alternatively spliced mRNAs since at least the HMGN3 transcript can be alternatively spliced [108].

Evidence indicates that HMGN2 has extracellular functions such as antimicrobial or tumor metastasis-mediating activities [109, 110, 111]. Thus, all HMG protein families appear to have members that are released from cells and to possess extracellular functions.

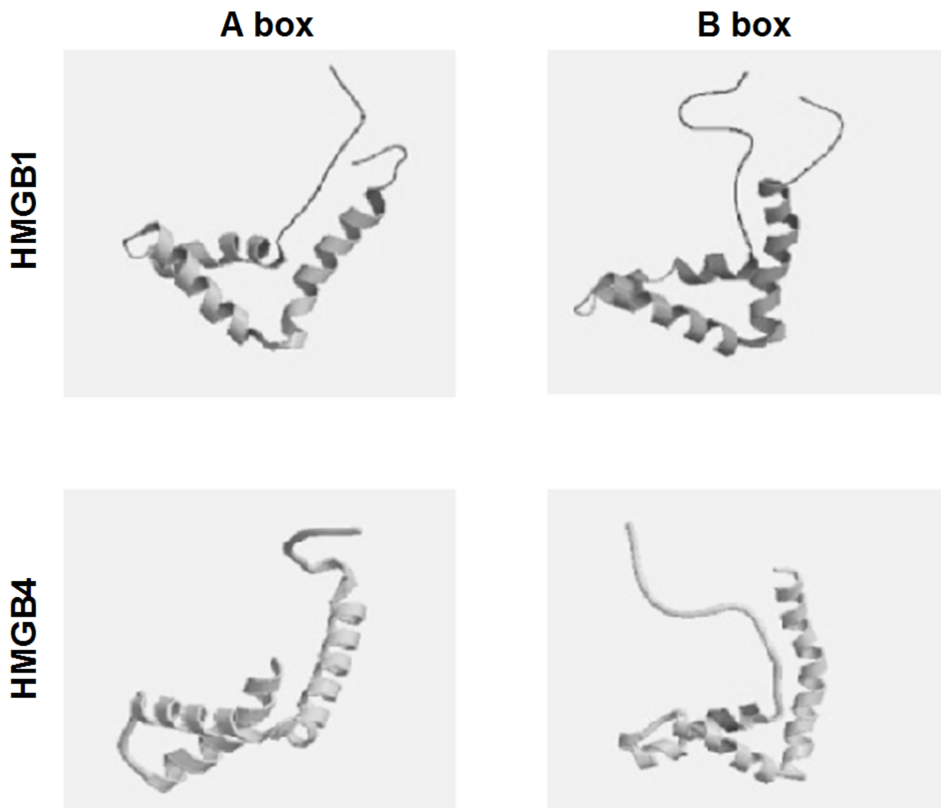

Figure 3. Similarity of predicted domain structures for HMG boxes of mouse HMGB1 and HMGB4. Structures are from amino acid sequences 1-77 (HMGB1 A box), 8-79 (HMGB4 A box), 75-165 (HMGB1 B box), and 67-149 (HMGB4 B box). Pictures were obtained from MODBASE [112].

2.2 EXPRESSION OF HMGB1

2.2.1 Expression during development and in adulthood

During development HMGB1 is expressed in many tissues at high levels and downregulated during differentiation. A good example of this is in the central nervous system [36]. However, in some cell types, HMGB1 levels remain high in adulthood, for example in the immune cells of the thymus and in circulating monocytes (II, 113).

The obvious importance of HMGB1 expression during development was shown in the study of HMGB1-knockout mice. Mice lacking the HMGB1 gene suffered from multiple organ failure and died soon after birth [20].

Expression of HMGB1 is usually upregulated in inflamed tissue. High HMGB1 expression is seen in mononuclear phagocytes present in inflamed areas. Further, cytoplasmic or extracellularly released HMGB1 can be frequently detected. The origin of extracellularly released HMGB1 can be either the inflamed tissue itself or infiltrated inflammatory cells [114, 115, 116, 117].

2.2.2 Intracellular expression of HMGB1

HMGB1 was initially described as a nuclear protein [2]. Later, it was found to be localized both in the nucleus and in the cytoplasm [44].

Nuclear proteins are translated in the cytoplasm, and must be transported to the nucleus for proper function. The classical nuclear localization signal occurs in many nuclear proteins and directs them to the nucleus. HMGB1, for instance, has two nuclear localization signals [49]. However, additional mechanisms for nuclear targeting of HMGB1 have been suggested. Proteins having an HMGB box can bind to calmodulin, which mediates their nuclear transport [118].

Export of HMGB1 from the nucleus to the cytoplasm is mediated by the chromosome region maintenance 1 protein (CRM1) [119]. Nucleus to cytoplasm transport is independent of protein synthesis [120]. Further, posttranslational modifications of HMGB1, including acetylation, serine phosphorylation, methylation, cysteine reduction/oxidation, and poly(ADP)ribosylation, affect HMGB1 localization within the cell [49, 120, 121, 122, 123].

HMGB1 moves rapidly within the cell, suggesting that HMGB1 binding to DNA/chromatin is moderately weak [124]. Photobleaching analyses using enhanced green fluorescent protein (EGFP) -tagged HMGB1 revealed fast movement within the nucleus and between the nucleus and the cytoplasm [124]. Although HMGB1 displays only a weak affinity to DNA/chromatin, this may be functionally sufficient because the intranuclear concentration is high, in the micromolar range [7].

In addition, cytoplasmic localization of HMGB1 can be controlled at the transcriptional level. mRNA coding for HMGB1 can be localized to the cell periphery and translated [26, 28]. This peripheral translation may minimize unnecessary nuclear transport in situations favoring cytoplasmic or extracellular targeting of HMGB1.

2.2.3 Extracellular expression of HMGB1

Although HMGB1 lacks a signal sequence for secretion, there are several studies on extracellular HMGB1, a form of HMGB1 first suggested to mediate neurite outgrowth by Pihlaskari and Rauvala in 1987 [36]. Later, other extracellular roles for HMGB1 were described, indicating roles as an erythroleukemia differentiation factor and as a RAGE ligand mediating neurite outgrowth [76, 125]. To date, many research groups have detected extracellular, actively secreted HMGB1 derived from various cell types [126, 127].

Several studies have examined the nature of extracellular HMGB1. In vivo, HMGB1 is expressed in extracellular space in humans with infectious and inflammatory diseases, cancer, and trauma [III, 128]. Extracellular HMGB1 can activate endothelial cells and mediate leukocyte diapedesis by mechanisms that involve RAGE [II, 129, 130]. HMGB1 has been detected in serum, and in cell culture HMGB1-free serum resulted in reduced myogenic differentiation [I, III, 131]. Some of the extracellular HMGB1 is released during necrosis, and it has been suggested to mediate inflammatory responses [132, 133]. In addition, apoptotic cells release HMGB1 [134].

2.2.3.1 Nonclassical secretion

A growing number of secreted proteins lack the amino terminal secretion signal sequence, but are, nevertheless, actively secreted to the extracellular space like HMGB1 [reviewed in 135]. Further, identical proteins may have different functions in different cellular compartments and in different tissues [136]. A great number of studies show that a single gene product may have alternative localizations and distinct functions. This suggests that these so-called moonlighting proteins are quite common in nature [137].

The secreted proteins lacking the secretion signal sequence have some shared characteristics, although it seems likely that there is no single common mechanism for nonclassical secretion. Nonclassically secreted proteins are usually small (<45 kDa) and lack N-glycosylation. Very often only a minor proportion of the intracellular pool of nonclassically secreted proteins is secreted [138]. Their secretion is unaffected, or enhanced, by the classical ER-Golgi secretion route inhibitors [139]. Further, protein synthesis is not required for secretion, at least in the case of HMGB1 and HSP70 [140, 141].

Some nonclassically secreted proteins share functional similarities with HMGB1. First, several other HMG proteins are released and possess some extracellular functions. HMGB2, which is released in vivo to plasma, has very similar nuclear functions to HMGB1 [3, 87]. HMGA1 has been described as an endothelial cell-secreted contact activation inhibitor, and HMGN2 as an antibacterial protein secreted from leukocytes [110, 142]. Second, nuclear proteins other than HMG proteins are also secreted from cells. DEK is a nuclear protein that serves as an autoantigen in autoimmune diseases, suggesting exists in the extracellular space. Further, DEK and another nuclear protein that is secreted from monocytes, the DNA repair protein Ku, mediate leukocyte migration [143, 144]. Further, histone H3 is a secreted nuclear protein. Sebocytes, a type of epithelial cells secrete histone H3 packed within microvesicles that are released to the extracellular space [145]. Unacetylated histone H2A in gastric gland cells is secreted and processed to antimicrobial peptide buforin I by pepsins, in contrast to the acetylated form, which is localized to the nucleus [146]. Histone H2A.X is another secreted histone type [147]. In addition to full-length histone molecules, a protein coded by alternatively spliced histone H4 mRNA is secreted [148]. Finally, some nuclear proteins in addition to HMGB1, e.g. IL-1α and IL-33, have been shown to mediate inflammation [149, 150].

Several mechanisms have been proposed to mediate nonclassical secretion of proteins. Some involve direct transport through the phospholipid membrane and others involve packing into and release from intracellular vesicles. A direct transport mechanism that does not require protein unfolding has been suggested to mediate FGF-2 export. FGF-2 secretion is dependent on cell surface proteoglycans that may act as a trap directing FGF-2 molecules to the extracellular space [151, 152, 153]. Vesicular mechanisms are known to mediate nonclassical secretion. One example is

in *Caenorhabditis elegans* spermatozoa, which lack components of the classical secretion route. The Major Sperm Protein is exported through a vesicle budding mechanism where the protein is packed between the inner and outer membranes of vesicles [154]. Another well-identified vesicular secretion route is known for IL-1β where secretory lysosomes mediate protein export [155]. A similar secretion route has suggested for HMGB1 [126]. Some proteins, such as Engrailed homeoprotein and FGF-9, have an internal signal sequence that directs them to secretion [156, 157].

The role of transporters in nonclassical protein secretion has been widely studied. One transporter relevant in the case of HMGB1 is ATP binding cassette transporter 1 [27, 158]. The ATP binding cassette transporters are a family of transmembrane proteins that have two ATP-binding domains and two transmembrane domains that both contain six membrane-spanning helices. There are almost 50 known ATP binding cassette transporter genes in the human genome. Inhibitors against ATP binding cassette transporter 1 inhibit both HMGB1 and IL-1β secretion from monocytes/macrophages [II, 159]. In addition, ATP binding cassette transporter 1 inhibitors decrease the release of other proteins lacking the secretion signal, annexin I, macrophage migration inhibitory protein, and Hsp70 [141, 160, 161]. Recently, a specific ABC transporter, ABCC1 (also called multi-drug resistance protein 1 (MRP-1)), was suggested to mediate HMGB1 secretion from macrophages [27, 162].

The importance of posttranslational modifications for nonclassical secretion has been recognized. A very well-known post translational control mechanism occurs in IL-1 secretion. Both IL-1α and –β are released after proteolysis of the pre-protein [163]. Extracellular HMGB1 released from neutrophils has a methylated lysine residue that is not found in HMGB1 released from lymphocytes [47]. Whether this methylation is required for secretion from neutrophils is not known. Further, HMGB1

acetylated in lysine has been detected in the extracellular space [47]. S100A12 released from neutrophils has an S-Sulfo-cysteine residue. The S-Sulfo-form of S100A12 was highly enriched in the extracellular pool, suggesting that S-Sulfo modification is associated with S100A12 secretion in neutrophils [164]. Finally, the importance of phosphorylation in the secretion of proteins lacking signal peptide has been shown. The secreted extracellular forms of annexin I and phosphohexose isomerase have phosphorylated serine residues [165, 166, 167]. Phosphorylation of serine residues was suggested to mediate secretion of HMGB1 from macrophages, and a mutant form linked to GFP protein mimicking serine-phosphorylated HMGB1 was secreted from cells [120].

2.3 HMGB1 RECEPTORS

In the literature, the major cell surface receptor for HMGB1 is the receptor for advanced glycation end-products (RAGE). However, there is evidence that RAGE is not the sole receptor for HMGB1 [27, 114, 168]. This is also the case for some other RAGE ligands, including members of the S100 protein family. Although RAGE seems to be the major receptor mediating inflammatory effects of S100 proteins, proteoglycans and ALCAM (CD166) can mediate some effects of the S100 proteins independently of RAGE [169, 170].

2.3.1 RAGE

RAGE was isolated as an advanced glycation end-product (AGE) binding protein from the bovine lung [171, 172]. Early studies revealed that RAGE has three immunoglobulin domains, a single transmembrane domain, and a short cytoplasmic domain. RAGE has been subsequently shown to have alternatively spliced forms. RAGE has a significant role in cell motility; transgenic dominant-negative RAGE or

RAGE-knockout leukocytes have a reduced ability to migrate to tissues. This leads to changes in inflammatory responses and regeneration, and during development, changes in bone morphology [173, 174, 175, 176, 177, 178]. The role of RAGE in innate immune response, using cecal ligation and a puncture-mediated sepsis model, was clarified in a recent study [179]. Further, RAGE has roles in adaptive immune responses [180, 181].

RAGE-HMGB1 interactions in the context of cell migration have been widely studied. RAGE as an HMGB1 receptor was originally described using protein binding studies, and in a neurite outgrowth model using primary neurons, RAGE was shown to mediate HMGB1-dependent neurite outgrowth [76]. Later RAGE-HMGB1 interaction has been described to mediate migration of various cell types, including smooth muscle cells, cancer cells, and immune cells [182, 183, 184, 185, 186]. However, in some cell types, the role of RAGE as an HMGB1-induced migration-mediating receptor is not unambiguous. Mesoangioplasts, a type of mesodermal stem cell that responds to HMGB1, migrate to dystrophic muscle independent of RAGE expression, and rhabdomyosarcoma cell migration was inversely correlated to RAGE expression and signaling [50, 114].

The ligand-binding site of RAGE was initially localised to the distal V1 domain [187]. However, recent studies have revealed that the C1 domain is an additional ligand-binding domain; it binds to S100 proteins [188, 189]. Further, antibodies against the C1 domain possess function-blocking properties both in vitro and in vivo, suggesting a functional role for the C1 domain in ligand-receptor interactions [II, 190]. In addition, the C2 domain of RAGE binds the soluble ligand S100A12 [189]. RAGE itself can form dimeric and oligomeric complexes. This suggests that RAGE may serve as a homophilic protein [191, 192, 193, 194, 195].

In addition to HMGB1, RAGE binds to various other ligands and is called a "pattern recognition" receptor that recognizes three-dimensional structures rather than specific amino acid sequences in its ligands. This kind of ligand recognition is common for receptors of the innate immune system [196]. Pattern recognition receptors recognize pathogen-associated molecular patterns (PAMPs) that are conserved microbial features not produced by the host itself [197].

Ligands in addition to HMGB1 and PAMPs include the amyloid-β peptide and members of the S100 protein family [Figure 4, 198, 199]. Further, RAGE binds to CD11b/CD18 integrin, and this mediates intercellular adhesion of leukocytes with endothelial and epithelial cells [178, 200].

The closest homolog of RAGE in humans is ALCAM (CD166) (Figure 5). ALCAM binds to S100b and its ligation activates NF-κB [170]. Further, changes in cytoplasmic localization of both ALCAM and RAGE have prognostic value in cancers [201, 202]. These data suggest that ALCAM and RAGE share some common functional characteristics.

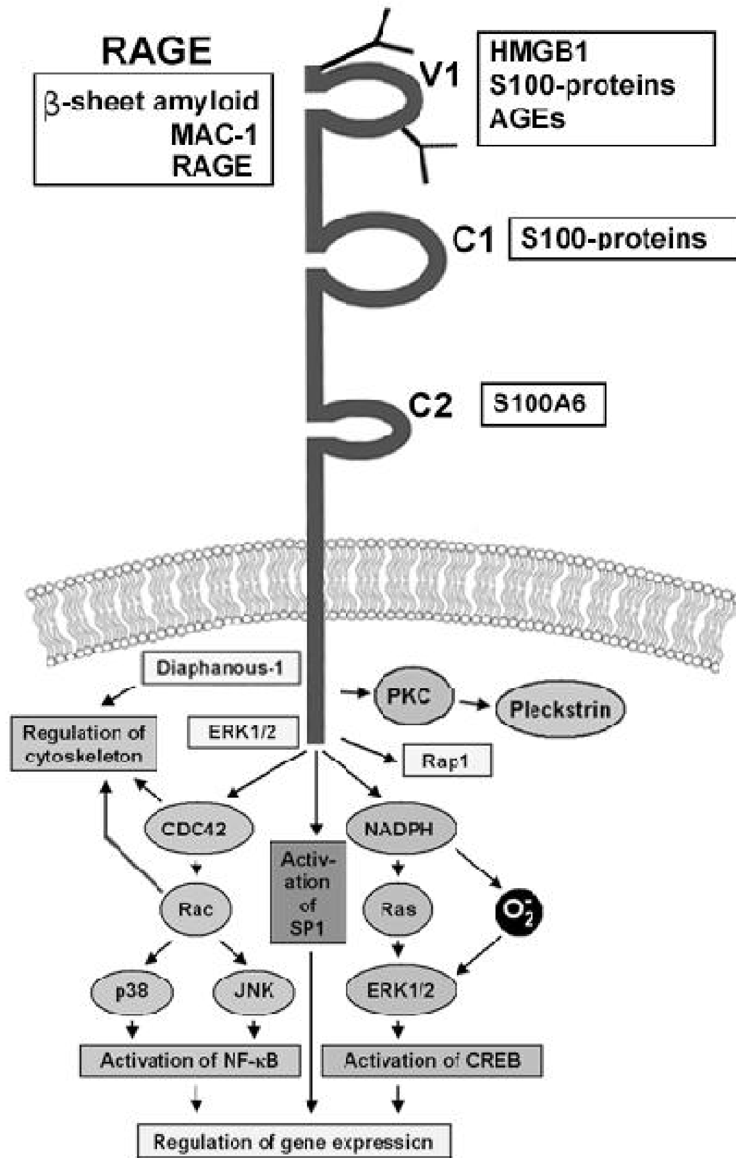

Figure 4. Schematic picture showing RAGE-interacting proteins and signaling pathways. Modified from Rauvala and Rouhiainen [233].

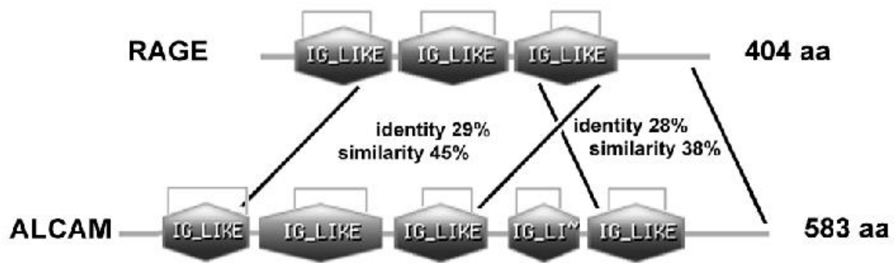

Figure 5. RAGE and ALCAM proteins show significant homology in structures. Homology and domain searches of human RAGE and ALCAM were done using tools in the website of National Center for Biotechnology Information (Rockville Pike, Bethesda, MD, USA).

RAGE ligation by either HMGB1 or other ligands induces some common pathways that result in cellular activation and cell motility [203]. Small GTPases Cdc42, Rac, and Rap1 are involved in RAGE-mediated cell motility regulation [204]. Further, RAGE ligation induces transcription of RAGE gene itself by an SP1-mediated mechanism [205]. RAGE ligands have both apoptotic and anti-apoptotic roles [206]. Anti-apoptotic Bcl-2 expression is regulated by HMGB1-RAGE interactions [207]. Thus far, the only signaling molecules known to bind directly to the cytosolic tail of RAGE are ERK 1/2 and diaphanous-1 [203, 208].

Activation of the transcription factor NF-κB is the most studied signaling route in RAGE biology. NF-κB expression is required for RAGE expression [209]. NF-κB activation by RAGE depends on the activation of the classical mitogen-activated protein kinase (MAPK) pathway that involves also p38 MAP kinase and the stress-activated protein kinase/c-Jun-NH_2-terminal kinase (SAPK/JNK). In addition,

HMGB1-RAGE ligation induces phosphorylation of the cyclic AMP response element-binding protein (CREB), leading to its nuclear localization. Further, the RAGE ligand S100b induces cytokine secretion from monocytes via a PKC-pleckstrin-mediated pathway, suggesting their role in RAGE signaling [210]. In endothelial cells, proinflammatory activity of S100 proteins seems to require preactivation of endothelial cells and a dimeric form of S100 proteins [211].

A role for PKC proteins in RAGE signaling has been suggested in several reports. In addition to monocytes, RAGE signaling activates PKC in muscle, myocytes and endothelial cells [210, 212, 213].

The role of RAGE in neurobiology has been intensively investigated. The original finding that RAGE is an HMGB1 receptor was made using brain neuron culture as a model [76]. Later, amyloid-β peptide was shown to mediate RAGE-dependent neuronal degeneration and to induce monocyte transendothelial migration through the blood-brain barrier by activating endothelial cells by a RAGE-dependent mechanism [198, 214]. The role of RAGE in diabetes-induced neuronal dysfunction was studied recently. RAGE was shown to be a key mediator of diabetes-induced loss of pain perception [215]. Further, RAGE ligation induces mitogenic signaling and cell survival phenomena in neurons [216]. RAGE and HMGB1 have a significant role in regeneration of peripheral nerves after nerve damage; blocking of either RAGE or HMGB1 reduced nerve regeneration in a mouse model [217]. Recently, the role for RAGE in endocytosis of S100b by Purkinje cells in spinocerebellar ataxia 1, a disease leading to the loss of cerebellar Purkinje cells and brainstem neurons, was suggested [218].

RAGE has several splicing isoforms that code for both transmembrane and soluble proteins. Further, a soluble form of RAGE can be generated by proteolysis

[219, 220]. The function of the soluble RAGE forms is poorly understood; a recent study showed a correlation of plasma sRAGE levels to sepsis severity [221]. Further, changes in plasma levels of sRAGE have been associated with many inflammatory diseases [222]. Systemic administration of recombinant sRAGE inhibited leukocyte adhesion to activated endothelium in a blood/retinal barrier model, and sRAGE has protective effects in many diseases [223, 224]. However, sRAGE has similar effects in RAGE-knockout mice, suggesting that it affects additional RAGE-independent mechanisms [225]. This suggests that sRAGE is involved in inflammatory processes.

RAGE is posttranslationally modified: it contains disulfide bonds within its Ig-domains, and its V1-domain is N-glycosylated. An amino terminal glycosylation is suggested to have di-antennary complex glycosylation with core fucosylation, whereas glycosylation in V1-Ig domain is more complex [226, 227]. Glycan moieties mediate RAGE binding to HMGB1, regulate neurite outgrowth on HMGB1, and mediate neutrophil migration through the endothelium [228, 229, 230]. In contrast, RAGE glycosylation inhibits binding of other ligands. EN-RAGE and AGE-protein binding to RAGE and signal transduction was enhanced by deglycosylation of RAGE [231, 232]. These results suggest that both ligand affinity and specificity of RAGE are regulated by glycosylation.

2.3.1.1 Gene expression induced by RAGE ligation

Ligation of RAGE leads to changes in gene expression. Most of the RAGE ligands induce signaling pathways that lead to NF-κB activation, which is the key regulator of many genes involved in inflammatory mechanisms [233]. NF-κB activation is regulated by an evolutionary conserved signaling pathway where signals derived from transmembrane receptors, like TLRs and IL-R1, activate NF-κB in a Myd88/IRAK and IκB degradation-dependent manner [234].

In addition to inflammatory response genes, some genes coding for extracellular proteins and peptide precursors are upregulated by HMGB1-RAGE interaction. These include members of the granin family and the immunoglubulin superfamily [235, 236].

The granin family consists of negatively charged proteins that are expressed in the nervous, endocrine, and immune systems. Granins are involved in secretory granule biogenesis [237, 238]. Chromogranin B is a member of the granin family found in the nucleus in some cells, and it appears to affect transcriptional control of several genes [239]. Further, granins are precursors of many biologically active peptides. For example, an antibacterial peptide called secretolytin is derived from the carboxy-terminal part of chromogranin B [240, 241].

In studies searching for HMGB1-inducible genes, Kuja-Panula et al. [235] found a novel gene that was named AMIGO (amphoterin-induced gene and ORF). In the same study, two other closely homologous genes were identified, AMIGO-2 and AMIGO-3. All AMIGOs mediate homophilic binding. AMIGO was shown to be involved in neurite faciculation. Later, Ono et al. [242] identified AMIGO-2 as an Alivin 1 that is a neuronal survival promoting molecule. In addition, Rabenau et al. [243] found that AMIGO-2 (called DEGA i.e. differentially expressed in human gastric adenocarcinomas) mediates tumor cell migration and tumorigenicity. The role of AMIGO-2 in cancer development was further supported by studies showing a strong upregulation of AMIGO-2 in pancreatic cancer and in a lung cancer cell line [244, 245]. The molecular mechanism of AMIGO-2 regulation in cancer was suggested to involve the insulin-like growth factor-II messenger RNA-binding protein 1 [245].

2.3.2 Proteoglycans

The original work that demonstrated HMGB1 to be an extracellular molecule utilized heparin-Sepharose chromatography to isolate neurite outgrowth-promoting proteins. The strong heparin binding capacity of HMGB1 is suggestive of its ability to bind cell surface proteoglycans. In fact, some proteoglycans, such as syndecan-1, neurocan, and phosphacan, are known to serve as HMGB1 receptors [77, 81].

Syndecans are transmembrane cell surface protoglycans. There are four members of syndecans in humans, syndecans 1-4, that are expressed widely in tissues. Syndecan-1 is present in the endothelium, pre-B-cells, plasma cells, monocytes, and macrophages [246, 247]. It inhibits leukocyte adhesion to the endothelium, and migration through the endothelium and epithelium [248, 249, 250, 251]. Further, it regulates angiogenesis in a glycosaminoglycan side-chain-dependent manner [252].

HMGB1 binds to syndecan-1 in a heparin and heparan sulphate-dependent manner [81]. HMGB1 and syndecan-1 colocalize to the plasma membrane of mouse epithelial cells. Binding of radiolabeled HMGB1 to epithelial cells is inhibited by trypsin treatment of cells and by soluble heparin implying that cells may use proteoglycan-type structures to bind HMGB1.

Neurocan is a matrix protein whereas phosphacan is a splicing variant of the transmembrane protein receptor protein tyrosine phosphatase β/ξ (RPTPβ/ξ) [77]. This suggests that the transmembrane form of RPTPβ/ξ may mediate HMGB1-induced signaling to the cytoplasm.

2.3.3 Lipids and carbohydrates

In addition to protein-type receptors, HMGB1 binds to lipids present on the cell surface (I, IV). However, the roles of HMGB1-lipid interactions have not been

extensively investigated. Further, binding of HMGB1 to different carbohydrates has been described. These issues are discussed in the following sections.

An early report by Mohan et al. [79] described HMGB1 as a sulfoglucuronyl glycolipid and sulfatide binding protein. Studies on HMGB1-platelet interactions suggested a similar binding [I]. In the brain, both HMGB1 and sulfoglucuronyl carbohydrates colocalize during development, but their possible cooperative function in the developing brain is unknown [253]. In addition, HMGB1 binds to cholesterol-3-sulfate, which modulates phosphorylation of HMGB1 [254].

The essential role of phosphatidylserine in phosphorylation of HMGB1 was initially described by Ramachandran et al. [255] in a study demonstrating the role of HMGB1 as a kinase substrate. Further, HMGB1 was shown to enhance transfection efficiency of phosphatidylserine containing erythrocyte membranes in a DNA transfection complex [256]. Later, HMGB1 was reported to bind directly to phosphatidylserine and modulate its functions in an immune cell model [I, IV].

Extracellular HMGB1 was originally isolated as a binding component of heparin, a carbohydrate [36]. HMGB1 was later found to bind carboxylated glycans present in the proteins on the surface of different mammalian cell types [257]. These glycans are recognized by monoclonal antibody GB3.1, which inhibits cell migration [258]. One of the major core proteins recognized by mAb GB3.1 in tumor cells is RAGE [257]. Glycosylation of RAGE enhances its binding to HMGB1 and modulates HMGB1-dependent signal transduction [228].

2.3.4 Toll-like receptors

Lemaitre et al [259] were the first to describe the role of toll receptors in immunity in 1996. This original finding was made using a fruit fly model. The role of

toll-like receptors in immunity of other animals has been subsequently widely studied. In mammals, ≥10 toll-like receptors are currently known [260].

HMGB1 has been described as a ligand of TLR2 and –4 and has been suggested to activate inflammatory responses through the TLRs [78, 261, 262]. However, recent studies suggest that the genuine HMGB1 polypeptide alone does not bind to the TLRs, and induction of an inflammatory response requires cofactors like lipids or CpG-A [IV, 263].

2.3.5 Thrombomodulin

Thrombomodulin is a transmembrane protein that has an extracellular amino terminal lectin-like domain, six epidermal growth factor domains, and an O-glycosylation site-rich domain [264]. It regulates enzymatic activities of thrombin by changing its function from a coagulation factor to an anticoagulant factor that activates protein C. In addition to hemostatic functions, thrombomodulin has roles in tumorgenesis, inflammation, and angiogenesis [265].

The amino terminal lectin-like domain binds to HMGB1 and inhibits its pro-inflammatory activities [80]. Further, HMGB1 inhibits protein C activation by the thrombin-thrombomodulin complex by a mechanism that does not involv the amino terminal lectin-like domain, suggesting that HMGB1 may bind directly to protein C and/or thrombin [266].

2.4. HMGB1 AND MECHANISMS OF INFLAMMATION

Inflammation is a host response to such harmful substances as pathogens, injured cells, or irritants. Components of the vascular system mediate inflammatory responses.

2.4.1 HMGB1 in cells of the vascular system

Blood cells circulate within vessels that are lined by endothelial cells. Vessels can be categorized according into their function to three different classes, arteries, veins, and capillaries. There are three major types of blood cells: red blood cells, platelets, and leukocytes. The predominant circulating cell type is the red blood cell (erythrocyte), numbering ~5 x 10^{12}/l [267]. Platelets are the second most common circulating cell type, at ~3 x 10^{11}/l [268]. The third group of circulating cells is leukocytes (or white blood cells), which consist of different sub-populations. Neutrophils are most common leukocytes in blood, numbering 1.8-7.7 x 10^9/l [269]. Lymphocytes are the second most common leukocyte type in circulation, at 1-4 x 10^9/l. About two-thirds of these are T-cells and one-third B-cells. A small fraction of lymphocytes belongs to the natural killer cell category. Monocytes comprise 3-8% of leukocytes, numbering 0.3 x 10^9/l. Eosinophils, basophils, and mast cells are smaller classes of leukocytes.

2.4.1.1 Platelets

Platelets are anucleated cells circulating in the vascular system. Their main function is to prevent blood loss during endothelial and subendothelial damage.

Platelets are derived from megakaryocytes, myeloidic stem cells, in bone marrow. Platelets are formed from filopodia extending through the endothelium between the bone marrow and blood vessels directly to the circulation [270, 271]. In circulation, when endothelial damage occurs or blood flow is perturbed, platelets start to adhere to the subendothelial matrix or to each other [272].

Platelets contain only a small number of RNA and ribosomes that partly originated from mitochondria [273]. However, platelets are capable of translation, which can be induced by platelet activation [274, 275].

Platelets contain four types of secretory granules in their cytoplasm: α-granules, dense bodies, lysozymes, and peroxisomes. The granule content can be released to the extracellular space during platelet activation.

Platelets have adhesion receptors on their surface that can bind to the subendothelial matrix at the site of endothelial damage. The two most important receptors in primary adhesion are the collagen receptor glycoprotein IaIIa (GPIaIIa, $\alpha_2\beta_1$, CD49b/CD29) and the von Willebrand –factor (vWF) receptor CD42. These receptors are able to bind ligands even when the platelet is still at an unactivated state, in contrast to CD41, which can bind fibrinogen and vWF ligands only after platelet activation. Platelets can interact with the subendothelial matrix directly or via vWF, fibronectin (Fn), laminin, or thrombospondin (TSP) [276]. Direct binding to collagen usually occurs under low shear force and is mediated by GPIaIIa. Under high shear stress, platelets bind to collagen via vWF and glycoprotein Ib-V-IX (GPIb/V/IX, CD42) [277, 278, 279, 280].

Although primary adhesion to the subendothelial matrix does not require platelet activation, adhesion induces signal transduction to the cytoplasm that may result in cell activation. When the platelet is activated, it releases its contents of secretory granules to the extracellular space, exposes phosphatidylserine to the outer leaflet of the plasma membrane bilayer, and activates its adhesion receptors [281].

Platelets adhering to the endothelial damage site form a cell layer that can bind more platelets to the same site via activated CD41 and fibrinogen [282]. This forms a loose platelet plug. Activated platelets can induce aggregation of other platelets by releasing activation factors such as ADP [283], gelatinase A [284], and thromboxane A_2 [276]. The phosphatidylserine surface, Ca^{2+}, Factor Va, and Factor Xa form a reaction complex that catalyzes thrombin formation from prothrombin

[285]. Thrombin is a major serine protease enzyme of the blood clotting cascade [286]. It cleaves fibrinogen to fibrin, forming fibrin polymers which together with platelets form a fibrin clot [285, 287, 288, 289]. Further, it is a potent platelet activator. Fibrin formed due to proteolytic activity of thrombin are covalently linked to each other by Factor XIIIa. Platelets in fibrin clots rearrange their cytoskeleton and induce clot retraction, rendering the fibrin clot more resistant to shear stress [276].

Rapidly after its formation, the fibrin clot starts to be dissolved by plasmin. Plasminogen (a plasmin zymogen) and t-PA released from the endothelium bind to fibrin, forming a complex that catalyzes plasmin formation.

In addition to the role in thrombosis, platelets modulate immune responses directly by expressing molecules involved in immunity, e.g. platelets can release bactericidial peptides [268, 290, 291].

The HMGB1 mRNA and protein are expressed in platelets; however, the function in platelets is not fully understood [I]. Of the suggested HMGB1 transmembrane receptors, platelets have been shown to express TLR-2, -4, -9 and thrombomodulin [292, 293, 294]. The role of these receptors in platelet-HMGB1 interactions has not been elucitated.

Platelets have an important role in regulation of proteolytic cascades in the circulation. Activated platelets express phosphatidyl serine on their surface, which serves as a platform for coagulation factor activation complexes. Whether HMGB1 that binds to phosphatidylserine is able to affect coagulation factor activation on the lipid surface is unknown.

Blood coagulation is regulated by both pro- and anticoagulant factors [295]. Abnormalities in the proteolytic cascades of the circulatory system are often deleterious in sepsis and septic shock. For example, disseminated intravascular

coagulation (DIC) causes ischemic necrosis in tissues. The role of HMGB1 in plasma proteolytic systems has been examined and there are some hints suggesting that HMGB1 is capable of enhancing proteolytic systems [266]. For instance, plasma HMGB1 levels correlatinf with DIC [128]. Recently, HMGB1 was shown to bind thrombomodulin and interfere with its functions [80, 266]. Thrombomodulin is a protein that catalyzes thrombin-mediated protein C activation. Activated protein C is an anti-inflammatory molecule that protects against septic shock. Its effect is enhanced by heparin [296]. The in vivo effects of thrombin are potentiated by HMGB1. These results suggest that HMGB1-mediated organ dysfunction may involve increased thrombosis [266].

However, in another proteolytic system, the plasmin/plasminogen system, a direct effect of HMGB1 has also been shown. HMGB1 enhances plasminogen activation; however, HMGB1 itself is rapidly degraded by plasmin [39, 297]. Maruyama et al. [298] recently suggested a role for plasmin in the clearance of systemic HMGB1 and Ali et al. [299] proposed that HMGB1 would inhibit transendothelial transport of t-PA. Since HMGB1 may activate the plasmin/plasminogen system, HMGB1 may also have anti-thrombotic functions.

Maruyama et al. [298], profiled a degradative activity on the endothelium, protein “X”, which may mediate HMGB1 disappearance from the circulation. This suggests that there may be other than proteolytic activities other than the plasminogen system in the circulation that degrade HMGB1. Another study by Hagiwara et al. [300] showed that the serine protease inhibitor nafamostat mesilate is able to decrease plasma levels of HMGB1, probably via inhibition of IκB, suggesting indirect effects of serine proteases on HMGB1.

Complement is another proteolytic cascade occurring in plasma, and it has a role in both innate and adaptive immunity. The role of C5a receptor C5L2 in HMGB1 release during sepsis was recently shown [301].

2.4.1.2 Monocytes and macrophages

Monocytes and macrophages compose the monocytic cell lineage. Monocytes are derived from bone marrow myeloid precursor cells and circulate in blood. They are large cells that have a folded nucleus. When they emigrate to tissue, they start to differentiate into macrophages. The main functions of monocytic cells are phagocytosis, antigen presentation, and production of immune modulator molecules. Phagocytosis involves removal of debris and foreign material. This leads to localization of phagocytosed material inside the cells that may result in activation of macrophages. Activation stimulates macrophage metabolism, motility, and phagocytosis, and can lead to secretion of immune modulators like cytokines. Cytokines are small (≤30 kDa) extracellular proteins that mediate signals to cells. Cytokines bind to receptors on target cells and induce signal transduction to the cytoplasm.

Monocytic cell surface receptors include scavenger receptors, adhesion receptors, complement receptors, and recognition receptors. All of these receptors can function individually or in concert to modulate monocytic cell responses to trauma, cancer, or infection. Further, monocytic cells have a role in tissue modeling and regeneration.

One of the current research interests is elucidating the role of HMGB1 in regulation of dendritic cell maturation [181, 185, 302, 303]. Dendritic cells are antigen-presenting cells. Monocytes are precursors of myeloid dendritic cells whereas plasmacytoid dendritic cells, originate from cells of lymphoid lineage. Immature

dendritic cells in tissue, when challenged by antigens, start to mature and move towards lymph nodes where they can regulate adaptive immune responses [304]. HMGB1 has been shown to either enhance or suppress dendritic cell maturation; thus its status in the dendritic cell maturation process remains poorly understood [54, 55, 359].

A very recent study indicated that HMGB1 mediates adaptive immune responses against tumor cells. The response was TLR4-dependent and a naturally occurring polymorphism in TLR4 was shown to affect HMGB1 interactions with TLR4 [305].

2.4.1.3 Endothelial cells

The endothelium comprises the inner surface of blood vessels. It consists of 1-6 x 10^{13} endothelial cells that are firmly associated together with tight junctions. These tight junctions prevent accidental leakage of cells and macromolecules into tissue. In normal conditions, the endothelium regulates transport of molecules and cells to tissue in a highly sophisticated manner. When pathological events occur in tissue, the endothelium can become activated and recruit inflammatory cells to the damaged area. This usually occurs in the vessel walls of capillaries [306, 307].

2.4.2 Acute inflammation

In addition to sepsis and systemic inflammatory response, plasma HMGB1 levels are upregulated in other diseases. Suda et al. [308] reported that serum HMGB1 levels in surgery patients correlated with pre- and postoperative complications, and Yasude et al. [309] detected high HMGB1 levels in serum, correlating with severity of acute pancreatitis. Similarly, in cerebral malaria nonsurvivors had elevated levels

of HMGB1 in plasma [310]. Further, elevated levels of HMGB1 in cerebrospinal fluid were detected in bacterial meningitis [311].

The role of solid tissue HMGB1 in inflammatory diseases has been evaluated in detail. In stroke, HMGB1 is upregulated and it has a significant role in mediating inflammation and tissue injury [312, 313, 314]. A study by Liu et al. [315] revealed a significant protective effect of intravenous anti-HMGB1 antibodies in rat ischemic stoke. In ischemia-reperfusion of the liver, HMGB1 is upregulated and released, and it mediates tissue injury that can be inhibited by anti-HMGB1 antibodies [316]. In a kidney ischemia-reperfusion model, similar HMGB1 upregulation is seen [317, 318].

A recent study suggested a role for HMGB1 in skin "first-line defence" rapidly after birth [319]. This suggests a role for HMGB1 in innate immunity in a situation where adaptive immune system is still undeveloped. Similarly, involvement in innate immune has been proposed for another secreted protein lacking signal sequence, IL-1α. This implies common functions for nuclear cytokine-like molecules IL-1α and HMGB1 [149].

2.4.3 Chronic inflammation

Another mechanism exploring the proinflammatory role of HMGB1 has been tendered; an HMGB1-mediated inflammation induced by DNA. Extracellular DNA has a potent role in the immune system. Eukaryotic methylated DNA activates complement, induces macrophage activation in SLE, and forms antibacterial neutrophil extracellular traps [55, 320, 321]. Unmethylated bacterial DNA is recognized by TLRs and it is a highly proinflammatory material. HMGB1 binds to both eukaryotic and prokaryotic DNA, and it can act as a carrier of DNA molecules to immune cells to enhance proinflammatory responses [IV, 322]. Further, helper T-cell-

mediated adaptive immune response in lupus is often directed against HMGB-chromatin complexes [323, 324].

Plasma HMGB1 levels are upregulated in some chronic diseases studied. Nowak et al. [325] described elevated plasma levels of HMGB1 in HIV patients with clinical complications, and Yamada et al. [326] found elevated serum HMGB1 levels in nonsurviving acute coronary syndrome patients. Further, in atherosclerosis; HMGB1 expression in the plaque area is upregulated, and HMGB1 mediates cell migration into lesions [327, 328, 329]. In arthritis, HMGB1 is upregulated and HMGB1 blockade reduces disease severity [330].

RAGE has been linked to chronic inflammation in several diseases. Ligation of RAGE activates NF-κB and induces formation of oxygen radicals; both of which are hallmarks of inflammation. Several inflammation-associated transcription factors, i.e. NF-κB and SP1, upregulate RAGE expression. These transcription factors are further activated by RAGE ligation, leading to amplification of RAGE transcription and resulting in a positive feedback loop that enhances inflammation. In addition, accumulation of RAGE ligands in the matrix can cause chronic RAGE activation and inflammation in diabetes and atherosclerosis [224, 331].

2.4.4 Sepsis and septic shock

Sepsis is one of most common causes of death. Sepsis may result from either the immune response to severe infection or the response to endogenous factors [296]. The immunological response may in turn cause a systemic inflammatory response and/or multiple organ dysfunction syndromes. The central role of TLR receptors and their exo- or endogenous ligands in development of sepsis is currently accepted [296].

Blood plasma is a strongly anti-bacterial environment; it has several bacteria-inhibiting mechanisms, including antibacterial peptides, phospholipases, and other

microbicidal proteins, and lack of free iron [332, 333]. One indicator of sepsis is the existence of living bacteria in circulation. Other indicators of sepsis comprice a clinical assessment of infection together with systemic inflammatory reaction that can include fever or hypothermia, increased heart or respiratory rate, and increased leukocyte count [III]. In 1999 Wang et al. [64] showed that HMGB1 is upregulated in plasma during sepsis and that HMGB1 can mediate lethality in sepsis. Later, up-regulation of HMGB1 mRNA in blood cells was associated with poor outcome in septic shock [334]. However, the relationship of HMGB1 protein levels in plasma to sepsis severity, and especially to lethality, is currently controversial [IV, 335, 336, 337, 338, 339, 340]. Further, controversial results exist on about plasma HMGB1-mediated lethality, questioning whether HMGB1 polypeptide alone would be lethal [266, 341].

Some proteins, such as pancreatic elsatase, α1-antitrypsin, C-reactive protein, and heat-shock proteins, express similar proinflammatory activities as HMGB1 when bound to enhancing factors. Therefore, there are contradictory results in the literature considering pro-inflammatory activity of these proteins [342, 343, 344, 345, 346, 347, 348]. Further, natural purified TLR ligands may be contaminated by other TLR ligands, causing misleading experimental results [349]. Whether proinflammatory activity of HMGB1 is mainly mediated by associated factors is currently under investigation.

Although HMGB1 polypeptide itself may not mediate a strong pro-inflammatory response, some studies clearly show protective effects of anti-HMGB1 antibodies in sepsis and systemic inflammatory response [64, 350, 351]. These studies indicate that there is a mechanism that mediates deleterious effects mediated by HMGB1. Disturbance of the epithelial cell layer by HMGB1 in a RAGE–dependent

manner is one such mechanism [352, 353, 354]. In this model, exposure to HMGB1 leads to formation of nitric oxide and peroxynitrite within epithelial cells and results in impaired epithelial function. A similar epithelial disturbance is seen in the lung, where HMGB1 induces “an epithelial-mesenchymal transition” in a RAGE-dependent manner that leads to pathological situations [355]. The role of RAGE in systemic infection was further clarified in a study that, in addition to showing a protective effect of RAGE blockade in severe sepsis, revealed RAGE independency in the bacterial clearance within tissues [356].

Monocytes have a high concentration of HMGB1 and when monocytes are activated they localize HMGB1 from the nucleus to the cytoplasm [II, 117]. This cytoplasmic localization is suggested to be a prerequisite for secretion of monocyte HMGB1. Recently, a role for intracellular HMGB1 in inflammation was suggested; HMGB1 was speculated to act as a sensor for intracellular LPS or cytosolic DNA [357, 358, 359].

Cytosolic DNA is a potent immune cell activator. This activation can be mediated by the DNA-dependent activator of IFN regulatory factors, the TANK-binding kinase 1, or by components of inflammasome [360 ,361, 362]. Inflammatory cytosolic DNA and its binding factors are considered to be exogenous, and endogenous DNA is protected by the nuclear envelope. However, during cell division the nuclear envelope becomes disrupted and nuclear DNA can be exposed to cytosolic molecules. Therefore, nuclear DNA must be protected, possibly by the chromatin structure [363].

2.4.5 Cholinergic anti-inflammatory pathway

The cholinergic anti-inflammatory pathway encompasses downregulation of inflammation by a neural mechanism [364]. One major molecular mechanism that

controls immune cells by neurons involves the α7 nicotinic acetylcholine receptor in macrophages [365]. α7 nicotinic acetylcholine receptor-mediated effects lead to decreased inflammation by reducing the levels of inflammatory mediators, including HMGB1. Further, the control of associated atherothrombotic risk factors, increased heart rate, and microinflammaton is suggested to be mediated by sympathetic or vagal neural systems similarly as in the cholinergic anti-inflammatory pathway [366]. In addition, nicotine, which acts via the α7 nicotinic acetylcholine receptors, has a protective effect against immune system-mediated organ damage [317].

However, the cholinergic anti-inflammatory pathway is not the only neuronal mechanism that interacts with the immune system. Infections in the periphery affect neurotransmitter levels in the brain by a mechanism not mediated by the vagus nerve [367]. Further, cytokines are known to alter emotional behavior [368]. For example, HMGB1 has been suggested have a role in anorexia nervosa, and higher plasma HMGB1 levels were detected during the refeeding resistance period [369, 370].

All of these data suggest that the nervous and immune systems cooperate in a highly sophisticated manner, and they both have afferent and efferent effects on each other.

Stimulation of the vagus nerve using either chemical or electrical activation inhibits HMGB1-mediated inflammation [371]. Activation of the vagus nerve leads to release of acetylcholine, which binds to the α7 nicotinic receptors and can inhibit tissue macrophages by downregulating the NF-κB pathway [371]. Acetylcholine can be mimicked by nicotine or other α7 acetylcholine receptor agonists, and these molecules can decrease serum HMGB1 levels during inflammation [372, 373].

Table 2. HMGB1 in human pathological states. Examples of changes in HMGB1 expression and correlations with clinical outcome.

Pathophysiological state	HMGB1 expression	Correlates with	Reference
Anorexia	upregulated	daily energy intake and body weight	72
Arthritis	upregulated	-	374
Atherosclerosis	upregulated	secretory phenotype of macro-phages in fibrofatty lesions	327
Cancer	upregulated	tumor progression	68, 375, 376
Liver transplantation	upregulated	ALT	65
Myocardial ischemia, stroke	upregulated	more prolonged symptoms	377
HIV infection	upregulated	deteriorated immune status	378
Malaria	upregulated	lethality	310
Pancreatitis	upregulated	CRP, LDH, total bilirubin	309
Sepsis/septic shock	upregulated	DIC, SOFA, lethality	128
Sjögren’s syndrome	upregulated	immunologic aberrations in salivary gland biopsies	379
Surgery	upregulated	complications	308

2.5. ANGIOGENESIS

Angiogenesis is a process leading to formation of new blood vessels during development, growth, tissue repair, and tumor growth. For example, hypoxia induces formation of new blood vessels [reviewed in 380]. The endothelial cell layer of a new vessel is formed by either dividing existing endothelial cells or through recruited endothelial precursor stem cells called angioblasts.

Expression of HMGB1 is upregulated in hypoxic tissues, and HMGB1 was found to be an angiogenetic switch molecule that promotes vessel formation [381, 382, 383, 384]. In addition, RAGE is upregulated in hypoxia and is involved in angiogenesis, especially in formation of microvessels within tumors [206, 385]. HMGB1 and other sRAGE ligands upregulate vascular endothelial growth factor (VEGF) and regulate angiogenesis [232, 386, 387, 388]. Although VEGF is upregulated by HMGB1 in tumors, it does not appear to have a role in HMGB1-dependent vessel formation in developing bone [21, 386].

In addition to RAGE, other HMGB1 receptors, syndecan-1, and thrombomodulin, are involved in angiogenesis [252, 389]. Further, the angiogenic mechanism involves HMGB1-mediated homing of endothelial precursor cells [390].

Results of these studies indicate that HMGB1 and its receptors have a crucial role in controlling new vessel formation.

3. AIMS OF THE STUDY

The aim of this study was to investigate the functions of HMGB1 in cells and plasma of the circulatory system under physiological and pathophysiological conditions.

Specific aims were as follows:

1) To investigate the expression and functions of HMGB1 in anucleated cells to reveal other functions besides those associated with nuclear DNA binding. The study was done using platelets as model cells.
2) To evaluate the role of extracellular HMGB1 in mononuclear cells. Specifically, the role of HMGB1 in monocyte adhesion and migration and in macrophage activation was investigated. Further, the specific HMGB1 secretion mechanism was examined in detail.
3) To elucidate the mechanism of HMGB1-mediated inflammation using both in vitro and in vivo models. Both primary and transformed mononuclear cells were used in in vitro cell culture studies. In vivo studies were carried out using arterial thrombi samples and serum samples derived from hospitalized patients. The aim was to clarify the status of HMGB1 as a proinflammatory cytokine.

4. EXPERIMENTAL PROCEDURES

Experimental procedure	**Publication**			
Subcellular fractionation	I			
Electron microscopy	I			
Thin-layer chromatography	I			
Immunofluorescence microscopy	I	II		
Cell adhesion assay	I	II		
Enzyme activity measurement	I	II		
Western blotting	I	II	III	
Enzyme-linked immunoabsorption assay	I	II	III	IV
Affinity and ion exchange chromatographies	I	II		IV
DNA quantification	I	II		IV
Reverse transcriptase PCR	I	II		IV
Antibody production		II		
Endotoxin analysis		II		
Immunohistochemistry		II		
Cell migration assay		II		
Statistical analysis		II	III	IV
Recombinant protein production		II		IV
Cell culture		II		IV
Mass analysis				IV
Database searches				IV
Solid-phase binding assay				IV
Phospholipid vesicle production				IV
Nitric oxide assay				IV

Table 3. Experimental procedures used in studies I-IV. Detailed descriptions of materials and methods can be found in the original publications at the end of this thesis. The original publications are referred to by their Roman numerals.

4.1 Additional exprimental procedures

4.1.1 Northern blot of platelet RNA

Platelet RNA was isolated from two units (120 ml) of one-day-old leukocyte-free platelet concentrates (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland). RNA was electrophoresed in 1.2% agarose gel and blotted to nylon membrane. HMGB1 mRNA was detected with dioxigenin-labeled rat HMGB1 anti-sense RNA-probe (a kind gift from Dr. R. Nolo) and anti-dioxigenin alkaline phosphatase conjugated Fab′ antibody (Boehringer-Mannheim, Mannheim, Germany). Color was developed with 5-bromo, 4-chloro, 3-indolylphosphate/nitro-bluetetrazolium staining [28].

5. RESULTS

5.1 Expression of HMGB1 (I, II, IV)

Western blotting of platelet and mononuclear cell lysates with affinity-purified anti-HMGB1 antibodies revealed a 30-kD band that migrated with recombinant HMGB1. Further, anti-HMGB1 detected a 30-kDa band from microglial and endothelial cell lysates in Western blotting analysis. Biochemical characterization revealed that a similar 30-kDa protein from platelet or monocyte lysates bound to heparin-Sepharose and was eluted with 0.6-0.75 M NaCl, in a similar manner to rat brain HMGB1, and it was recognized by anti-HMGB1 antibodies.

The levels of HMGB1 in platelets and monocytes were assessed by measuring the intensity of the immunoreactive 30-kDa band in Western blotting. The amount of HMGB1 was estimated to be 0.01‰ of total platelet protein, and 0.5% of total monocyte protein. Thus, in monocytes, the HMGB1 concentration was approximately

50 times higher that in platelets. Quantitative estimation of HMGB1 polypeptide in platelets revealed that 0.01% of total protein corresponds to about 85 ng protein/10^9 platelets. This corresponds to approximately $2x10^3$ HMGB1 polypeptides/single platelet.

5.2 Structural analysis of HMGB1 (IV)

Structures of both recombinant and tissue-derived HMGB1 were studied. Recombinant proteins were produced in S9 baculovirus and purified with heparin-Sepharose and ion exchange chromatography. Tissue HMGB1 was isolated from young rat brain with a two-step chromatography method utilizing heparin-Sepharose and Affi Gel Blue chromatography. SDS-PAGE and mass spectrometric analyses showed that the recombinant HMGB1 has one intrachain disulfide bond in it's a box. Mass spectrometric analyses of tissue HMGB1 indicated that its major form lacks the carboxyl terminal glutamic acid residue, and the minor form is the full-length protein. Further, SDS-PAGE analysis revealed a similar oxidation stage in both recombinant and tissue-derived HMGB1 (IV). In silico analyses suggested that the lack of the glutamic acid residue is not due to changes in the HMGB1 transcript.

5.3 Identification of HMGB1 mRNA in platelets and monocytes (I)

Analysis of HMGB1 mRNA in platelets using RT-PCR resulted in a strong amplification of a band of expected size. Amplification of monocyte mRNA under identical conditions resulted in an intensive band of the same size. To exclude leukocyte contamination in the platelet preparation, amplification of the cDNA encoding CD18 was assayed. No amplification was observed in the platelet cDNA

preparation by using primers specific for the CD18, whereas amplification of monocyte cDNA under the identical conditions resulted in a strong 350-bp band.

Northern blotting revealed the existence of three major forms of HMGB1 mRNA in platelets (Figure 6).

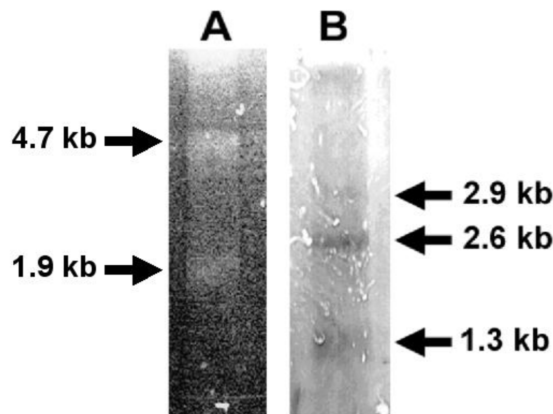

Figure 6. Northern blot of platelet HMGB1 mRNA. A) Ethidium bromide staining of ribosomal RNA. B) HMGB1 antisense staining. kb=kilobase.

5.4 Subcellular localization of HMGB1 in platelets and monocytes (I, II)

Immunostaining and biochemical methods were used for in vitro studies of resting and activated platelets and mononuclear cells. In immunofluorescence microscopy studies, staining of nonpermeabilized thrombin-activated platelets or resting permeabilized platelets with anti-HMGB1 antibodies revealed staining within platelets and at the surface of activated platelets. No staining was observed in resting nonpermeabilized platelets. In monocytes, immunofluorescence microscopy studies revealed adhesion-dependent surface expression of HMGB1. Characteristic for the staining was the occurrence of HMGB1 as intense patches at the cell surface.

Immunoreactivity of HMGB1 in pre-embedding electron microscopy studies was observed at the cell surface of the collagen-activated platelets, whereas there was no immunoreactivity on the resting platelets. Post-embedding cryosection electron microscopy in turn indicated intracellular localization of HMGB1 in platelets. In both collagen-activated and resting platelets, numerous gold particles immunoreactive to HMGB1 were localized in the cytoplasm. The activated platelets displayed a significant number of gold particles at the cell surface, in contrast to the resting platelets.

Most of the HMGB1 was found in the cytoplasmic fraction of resting platelets after subcellular fractionation. Some of HMGB1 was also present in the membrane fraction, whereas a negligible amount was detected in the granular fraction. Partitioning of HMGB1 to the 100 000 g soluble fraction of resting platelets indicates its localization to nonvesicular structures in the platelet cytoplasm. Further, HMGB1 was not associated with the platelet cytoskeleton.

Expression of HMGB1 was studied in vivo using frozen sections of arterial thrombi containing adherent monocytes and platelets. Strong HMGB1 immunoreactivity was seen within mononuclear cells, and a fainter extracellular reactivity was present in regions populated by mononuclear cells.

5.5 Secretion of HMGB1 (I-III)

Immunostainings of activated platelets and monocytes revealed that they secrete HMGB1. Since mononuclear cells had a much higher expression level of HMGB1, we further studied the secretion using the transformed murine macrophage cell line RAW 264.7 as a model.

RAW 264.7 cells secreted HMGB1 after treatment with 20 ng/ml IFN-γ or 10 nM PMA. The kinetics of secretion was slow, taking several hours. Secretion did not

correlate with LDH-leakage, suggesting an active secretion process. Secretion of HMGB1 was dose-dependently inhibited by the ABC1 inhibitor DIDS, and 100 μM DIDS completely inhibited amphoterin secretion. IL-1β secretion was, as reported earlier, also inhibited by DIDS. An ABC1 inhibitor, glyburide, inhibited both HMGB1 and IL-1β release. However, even at high concentrations of glyburide, the inhibition was only partial. Another ABC-1 inhibitor, BSP, inhibited IL-1β secretion, but not HMGB1 secretion.

In vivo release of HMGB1 from mononuclear cells was observed within thrombi in immunofluorescence studies. Further, in vivo release of HMGB1 under different clinical circumstances, namely in sepsis and septic shock, also occurred.

5.6 HMGB1 as an adhesive and migration-promoting molecule (I, II, IV)

Both resting and activated platelets bound to the HMGB1-coated surface, and platelet activation increased the binding approximately threefold. The binding of resting platelets to fibrinogen was about three times higher than to HMGB1, whereas activation reduced the difference. Platelet binding to HMGB1 was divalent cation-independent. A difference in adhesion mechanism was found between resting and activated platelets using heparin in the culture medium. Soluble heparin inhibited binding of resting platelets to HMGB1, while it had no effect on activated platelet binding. Further, other glycosaminoglycans inhibited resting platelet binding to HMGB1, but less efficiently than heparin.

Most of the platelets bound to HMGB1 in the presence of activation inhibitors exhibited the spiculated morphology, with a round cell body and long thin filopodia. Lamellipodia were absent in most of the cells. Only about 30% of the platelets bound

to HMGB1 were spread and flattened. Most of the platelets bound to fibrinogen in the presence of activation inhibitors had lamellipodia and numerous filopodia.

When peripheral blood leukocytes were allowed to adhere to immobilized HMGB1, fibronectin, or albumin, CD14–positive cells bound to a similar extent to microwells coated with HMGB1 or fibronectin, whereas only low binding was observed on wells coated with albumin. The total number of adhered leukocytes was two- to threefold higher on fibronectin than on HMGB1. Interestingly, almost all cells adhering to HMGB1 were CD14-positive, whereas half of the cells adhering to fibronectin were CD14-negative.

Morphology of the CD14-positive cells adhering to the HMGB1 surface was strikingly different then that of the cells adhering to the fibronectin or vitronectin surface. Cells adhering to HMGB1 demonstrated remarkably flattened morphology and large lamellipodia compared with fibronectin or vitronectin-adherent cells. The surface area of HMGB1-adherent cells was clearly larger than that of fibronectin-adherent cells. In contrast, rat brain migroglial cells did not display differential spreading on HMGB1 and fibronectin.

Since HMGB1 was strongly adhesive for monocytes and adhesion induced drastic morphological changes in monocytes, we tested whether HMGB1 has any role in monocyte migration. Migration of monocytes across porous filters was studied under two different conditions, either porous membrane was used alone as a barrier or it was layered with endothelial cells. HMGB1 was not chemotactic in migration assays across uncoated or endothelium-coated polycarbonate filters. In transendothelial migration assay, TNFα-activated endothelial cells were used. Migration was significantly and dose-dependently inhibited by anti-HMGB1

antibodies. Further, the role of RAGE in transendothelial migration was suggested in studies utilizing anti-RAGE or soluble RAGE fragments.

5.7 Role of RAGE as an HMGB1 receptor (II)

RAGE was detected in human and rat monocytes by Western blotting of HMGB1-adherent cells, and the role of RAGE as an HMGB1 receptor in mononuclear cells was studied. RAGE-mediated gene expression and cell adhesion and migration were assayed. Upregulation of chromogranins has been identified as a hallmark of HMGB1-RAGE interactions. We investigated whether mononuclear cells express, in addition to chromogranin B, chromogranin C and whether the expression of chromogranins is upregulated by HMGB1. RT-PCR revealed a band of expected size for chromogranins B and C in both monocytes and microglial cells. Chromogranin B and C expression in HMGB1-adherent monocytes was not altered after culture for one hour when compared to fibronectin adherent cells. However, chromogranin B was strongly upregulated after adhesion to HMGB1 for 20 hours in both monocytes and microglia compared with fibronectin-adherent cells. Chromogranin C mRNA was not upregulated in monocytes or microglia during adhesion.

The role of RAGE in adhesion and spreading of monocytes to immobilized HMGB1 was evaluated using sRAGE as a spreading inhibitory agent. sRAGE inhibited spreading on HMGB1, whereas the control immunoglobulin superfamily protein, sAMIGO, had no effect.

5.8 Lipids as HMGB1-binding components (I, IV)

HMGB1 binds to sulfatide and to other glycolipids. Thus, the binding of HMGB1 to other lipids was also assayed. In a 2D-TLC overlay assay, iodinated

amphoterin bound to two spots of platelet lipid extract. The spots were identified as phosphatidylserine and phosphatidylethanolamine due to their R^F values and ninhydrin staining. In ELISA assay, HMGB1 bound strongly to phosphatidic acid and phosphatidylserine. Binding of HMGB1 to lipids was divalent cation-independent.

Further, recombinant HMGB1 produced in *E. Coli* carried lipids that were partly identified as phospholipids. In addition, organic solvent-soluble proinflammatory substances could be extracted from bacterially produced HMGB1. Whether these active organic solvent substances were lipids was not further analyzed.

5.9 HMGB1 in diseases and in inflammation (II-IV)

Serum samples of patients with severe sepsis and septic shock were analysed for concentration of HMGB1. HMGB1 was upregulated in serum during sepsis and septic shock, as were other proinflammatory cytokines, including IL-6, IL-8, IL-10, and TNF-α.

Both tissue-derived and recombinant eukaryotic HMGB1 exhibited only a relatively low proinflammatory activity compared with bacterially produced HMGB1. Bacterially produced recombinant HMGB1 was found to contain organic solvent soluble material that possessed proinflammatory activity. In addition, HMGB1 bound to bacterial DNA in an affinity chromatography assay.

Two different in vivo models were used to explore expression of HMGB1 during inflammatory conditions in humans. First, expression of HMGB1 was detected in arterial thrombi, which contained adherent monocytes, platelets, and fibrin. The presence of HMGB1 in mononuclear cells in vivo was detected. Second, the expression of HMGB1 was studied in serum samples of patients with severe sepsis and septic shock, and high levels of HMGB1 expression were measured compared with healthy controls.

The serum levels of HMGB1 did not correlate with disease severity, nor did they correlate with the other cytokines tested. Serum HMGB1 concentration remained high during the test period (0-144 hours), in contrast to other cytokines, in which serum concentration decreased.

6. DISCUSSION AND CONCLUSIONS

We investigated the role of HMGB1 in cells of circulation and in inflammatory cells. HMGB1 is expressed in platelets, mononuclear, and endothelial cells, and it acts as a mediator of immune cell adhesion and migration. The genuine HMGB1 polypeptide is only a weak proinflammatory cytokine, and it appears to act as an activator when complexed to DNA and lipids.

6.1 HMGB1 in platelets

Since platelets are anucleated cells we investigated whether they express HMGB1, which would have then some other function than nuclear DNA binding. Platelets were found to contain both HMGB1 mRNA and protein [I]. Others have later confirmed the expression of HMGB1 mRNA in platelets using microarray analysis [391].

The amount of HMGB1 in platelets was low when compared with the amount found in leukocytes. However, studies with IL-1β RNA have shown that platelets have the capacity for RNA splicing and translation, phenomena occurring after platelet activation [392]. A similar splicing phenomenon was suggested to play a role in platelet HMGB1 RNA processing, suggesting that platelets may synthesize HMGB1, yielding more significant HMGB1 concentrations within platelets [392].

Activated platelets expressed HMGB1 on their surface. Surface HMGB1 most probably originated from the platelet cytoplasm since in both electron microscopy and subcellular fractionation analyses of resting platelets intracellular HMGB1 was diffusively associated with the cytoplasm, and not with secretory vesicles. Thus, the secretion mechanism seems to differ from the mechanism suggested for monocyte HMGB1 secretion, which involves a vesicular mechanism [126]. Further, the HMGB1 secretion route in platelets differs from the nucleated cell secretion route since it bypasses the nucleus to cytoplasm transport, which is suggested to have a role in active HMGB1 secretion in some cells [126].

Surfaces coated with exogenous HMGB1 induced platelet adhesion and shape change to a spiculated form. Shape change is a one marker of platelet activation, suggesting that HMGB1 is a platelet-activating agent. The platelet cell surface binding receptor for HMGB1 is currently unclear, and it is unknown how HMGB1 signals in platelets. Platelet lipids, phosphatidyl serine and phosphatidyl ethanolamine, can bind to HMGB1 but protein-like platelet receptors remain to be clarified [I]. Platelets do not possess RAGE, but they express TLR2/4, suggesting a possible role of TLRs in platelet-HMGB1 interactions [292, 293, 393].

Spiculation of HMGB1-bound platelets resembles the migratory phenotype of cells. Induction of migration is one of the well-known functions of extracellular HMGB1. This suggests that HMGB1 may mediate platelet migration. Migration of platelets has been studied little, but chemotaxis and transendothelial migration of platelets have been described [394, 395]. Whether exogenous HMGB1 would be chemotactic for platelets is unknown. HMGB1 is not chemotactic for monocytes, probably due to their high endogenous HMGB1 content [II, 396]. HMGB1 might mediate chemotaxis of platelets that have only low levels of the endogenous protein.

HMGB1-induced platelet activation may have a role in sepsis and septic shock, where DIC, which involves platelet activation and coagulation, often occurs. Indeed, HMGB1 levels in plasma correlate with DIC, and systemic HMGB1 has been shown to promote coagulation [128, 266]. Again, further studies are required to clarify the role of HMGB1 in platelet biology in vivo.

6.2 HMGB1 in monocytes

In addition to platelets, leukocytes and endothelial cells were found to express HMGB1. Especially monocytes express high levels of HMGB1 and secrete it after activation. Extracellular HMGB1 mediates monocyte migration and promotes inflammatory responses of mononuclear cells.

The role of HMGB1 in transendothelial migration of cancer cells has been previously demonstrated [397]. In our study, the essential role of HMGB1 in transendothelial migration of the monocytes was revealed for the first time [II]. The results show that blocking either HMGB1 or RAGE inhibits transendothelial migration. This indicates that these proteins play a critical role in monocyte diapedesis.

HMGB1-mediated cell migration most likely occurs with an autocrine mechanism or with a mechanism involving matrix-bound HMGB1 since soluble exogenous HMGB1 is not chemotactic for monocytes [II, 396]. High endogenous monocyte HMGB1 concentration may cause inability of monocytes to respond in a chemotactic manner to exogenous soluble HMGB1. However, substrate-bound HMGB1 induces rapid and extensive monocyte spreading, suggesting a role for exogenous HMGB1 in monocyte motility. In contrast to monocytes, tissue macrophages do not spread on the HMGB1 surface, suggesting that peripheral blood

monocytes respond to HMGB1 using a mechanism that differs from tissue macrophage response.

Chromogranins B and C are neuroendocrine secretory granule proteins that are members of the granin family. In this study, chromogranin C expression was described for the first time in monocytes. Chromogranins B and C are precursors of various biologically active peptides, some affecting the immune system [398]. For example, the concentration of secretolytin, a chromogranin B-derived antibacterial peptide, is increased during surgery, suggesting secretolytin´s role as an innate immunity molecule [399]. Secretoneurin is a peptide derived from chromogranin C that induces leukocyte migration [398]. Both chromogranin B and C and the peptides derived from them are detected in human plasma [398]. However, the roles of chromogranin B and C in mononuclear cells and in circulation remain poorly understood.

RAGE is the major HMGB1 receptor in macrophages, and it mediates inflammatory and migratory responses of HMGB1 [III, 400]. In the literature, the majority of studies considering HMGB1 and its receptors focus on HMGB1/RAGE interactions. This should not diminish the importance of other cell surface molecules as HMGB1 receptors functioning in vivo. In fact, the clear differences in phenotypes of HMGB1 and RAGE knockouts — HMGB1 knockouts are lethal- whereas RAGE knockouts are alive and fertile — suggest that cells utilize RAGE-independent mechanisms to interact with HMGB1. Further, the RAGE binding site in HMGB1 is localized to the carboxyl terminal part of B box [397]. However, the recombinant A box of HMGB1 is able to antagonize the effects of full-length HMGB1 in cells of the circulatory system, suggesting that cells have receptors other than RAGE to bind to the HMGB1 A box [129].

Mononuclear cells express other HMGB1 receptors, e.g. TLR2/4, syndecan-1, and CD14 may interact with HMGB1 [II, 247, 260]. The role of TLR2 in cell migration has recently been studied. TLR2 can induce cell migration via both inside-out and outside-in signaling [401, 402]. Whether HMGB1 can affect cell motility via TLR2 is unknown.

TLR4 does not bind to genuine HMGB1, but is precipitated with HMGB1 from cell lysates, suggesting that HMGB1 containing TLR4 binding complexes exist [78, 262]. Currently, it is well accepted that many TLRs recognize their ligands presented in complexes with endogenous proteins. These include CD14, LBP, MD-2, glycosphingolipids, and dectin-1 [403, 404]. In this study, we found that all peripheral blood leukocytes that adhere to HMGB1 were CD14-positive. Very recently, HMGB1 was reported to bind to both CD14 and MD2, and HMGB1 signaling is dependent on CD14 expression. This suggests that CD14 serves as a monocyte HMGB1 receptor [82, 405, 406]. Another report showed that HMGB1 binds to LPS and mediates its binding to CD14 [357].

6.3 Nonclassical secretion

Activated mononuclear cells secrete HMGB1, and the secretion is not due to cell necrosis [II]. Our results suggested a role for PKC activation in macrophage HMGB1 secretion since cell activation with PMA induced HMGB1 secretion. Later Oh et al. [407] have subsequently confirmed the role of activated PKC in HMGB1 secretion from macrophages. Similar activated PCK-mediated HMGB1 secretion occurs in erythroleukemia cells [408].

Secretion in monocytes utilizes a mechanism that is inhibited by DIDS [III]. This strongly suggests that secretion is an active process and that secretion involves

either DIDS-inhibitable ABC transporters or HCO_3^-/Cl^- channels. Indeed, mouse cells lacking ABCC1 have a decreased capacity to secrete HMGB1 [27, 162].

Some mechanisms involved in nonclassical secretion have been further clarified. Recent studies have indicated that when the secreted protein in the cytoplasm is localized to the cytoplasmic side of the plasma membrane it may be passively translocated (diffused) to the extracellular space [409]. Close localization to the membrane may involve membrane lipid binding and membrane perturbation. Since passive diffusion is bidirectional, the protein secreted to the extracellular space should be trapped on the extracellular side to prevent diffusion back into the cell. This is in fact the case in the secretion of FGF-2. The secretion of FGF-2 occurs in an energy-independent manner and does not require protein unfolding, but is dependent on extracellular trapping of FGF-2 by cell surface heparan sulfate proteoglycans. A similar secretion mechanism has been suggested for the lectin CGL-2, which binds to both glycolipids and glycoproteins. HMGB1 binds to membrane lipids and HSPGs, and its secretion most probably does not require protein unfolding [I, II, IV, 81, 120]. Whether it is secreted similarly to FGF-2 remains to be clarified.

6.4 Inflammation

It is now clear that HMGB1 is released from the cells in various types of inflammatory diseases and that extracellular levels of soluble HMGB1 often correlate with disease severity. In addition, the ability of anti-HMGB1 antibodies to protect against tissue injury and death in various animal models is unambiguous. However, there are contradictory results, for how plasma/serum HMGB1 levels correlate with lethality in inflammatory diseases. This suggests that the protective mechanism of anti-HMGB1 antibodies may involve blocking of HMGB1 in solid tissues or at the cell surface rather than blocking of the circulating plasma HMGB1.

Results in this study clarified for the first time that genuine HMGB1 has only a weak proinflammatory activity and that HMGB1 levels in serum do not correlate with lethality in sepsis and septic shock [III, IV]. Our results further demonstrate that HMGB1 can mediate proinflammatory reactions by binding adjuvant molecules, lipids, and DNA. Others have later shown that HMGB1 complexed with DNA mediates proinflammatory reactions via TLR9, and HMGB1 complexed with IL-1β induces proinflammatory reactions in an IL-1 receptor-dependent manner [55, 410].

6.4.1 Brain ischemia/reperfusion

Within the past three years, several studies on the role of HMGB1 in brain inflammation have been published. Brain ischemia induces massive injury that is associated with inflammation. The inflammatory response may have both harmful and protective roles. The effects of the inflammatory response are different at the different time-points after the insult. The role of HMGB1 has been studied using animal models, and its serum levels have been measured in human diseases.

Soon (within the first hours) after experimental ischemia-reperfusion, HMGB1 is translocated from the nucleus of neurons to the cytoplasm. Translocation occurs to a lesser degree in astrocytes and oligodendrocytes [314, 315, 411]. The overall expression level of HMGB1 protein is unchanged [411]. At the same time, HMGB1 is found in cerebrospinal fluid [411]. Elevated levels of HMGB1 in serum are seen in both experimental ischemia-reperfusion models and in human patients with cerebral ischemia [313, 377, 411]. After one day, HMGB1 disappears from the nuclei of neurons, but can be seen in degenerating neurons in ischemic areas [314].

Blocking of HMGB1 using antibodies or RNA inhibition protects against brain injury in a rat experimental ischemia-reperfusion model. RNA inhibition studies have indicated that blocking of just brain-derived HMGB1 is sufficient to explain the

protective effects seen in HMGB1-antagonized animals [313]. One proposed mechanism is blockade of the microglia-mediated inflammatory response that occurs several hours or days after the insult [313, 315].

The molecular mechanism that can connect neuronal HMGB1 release during early events of ischemia and late microglial infiltration and activation has been clarified by recent studies. Exogenous HMGB1 upregulates gene expression in target cells, leukocytes, and astrocytes, and especially the expression of chemokines seems to be strongly enhanced by HMGB1 [63, 396]. Chemokines are molecules that attract leukocytes to the injured tissue. Thus, ischemia-induced neuronal HMGB1 release may activate astrocytes to release chemokines, then astrocytes in turn attract activated macrophages to the area. These activated immune cells may then worsen tissue damage.

6.4.2 Liver ischemia/reperfusion

In liver ischemia-reperfusion, the role of HMGB1 has been studied in detail. In a mouse model, hepatic HMGB1 levels increase after insult during the first 12-24 hours, then decreasing [316, 412]. Similarly, plasma levels of HMGB1 are upregulated [412]. As in the brain ischemia-reperfusion model, anti-HMGB1 antibodies protect against tissue injury [316, 412].

The behavior of HMGB1 in human liver transplantation resembles that behavior of HMGB1 in the mouse ischemia-reperfusion model [65]. HMGB1 is massively released after reperfusion of transplanted liver, but the release rapidly declines, probably due to milder ischemia-reperfusion injury in human liver than in the experimental mouse model. Further, plasma HMGB1 levels correlate with alanine aminotransferase, suggesting that HMGB1 is released because of the liver injury [65].

6.5 Conclusions

In this work, the role of HMGB1 in circulatory cells has been investigated. Results obtained from in vitro studies were utilized to extend this work to in vivo.

HMGB1 is expressed in platelets that are anucleated cells, indicating that HMGB1 has functions other than nuclear chromatin binding. Platelet adhesive function is suggested for HMGB1. Further, adhesion to HMGB1 activates platelets, indicating that HMGB1 may be called a platelet activating factor. These results show for the first time that HMGB1 is expressed and has some functions in anucleated cells.

In addition to platelet adhesion and activation, HMGB1 mediates monocyte adhesion, migration, and activation. The results showing adhesive functions for monocytes are in parallel with findings of previous studies where HMGB1 has mediated adhesion and motility of various cell types.

Elevated HMGB1 levels in circulation during sepsis and septic shock are in parallel with previous reports. However, the correlation of HMGB1 levels with lethality in an earlier report could not be confirmed here. Currently, it is unclear how HMGB1 levels correlate with lethality in sepsis and septic shock.

Finally, our results are the first to show that the genuine HMGB1 polypeptide only displays a weak proinflammatory activity, but it may mediate proinflammatory reactions in complex with other molecules such as lipids and DNA. Others recently have reported similar results.

In conclusion, HMGB1 is released from immune cells both in vivo and in vitro, and it mediates cell migration and activation. The in vitro immune cell activation is enhanced with HMGB1-bound adjuvant molecules. HMGB1 may act in vivo as an

immune activator that mediates cell motility and recognizes infectious or damage-associated molecular patterns.

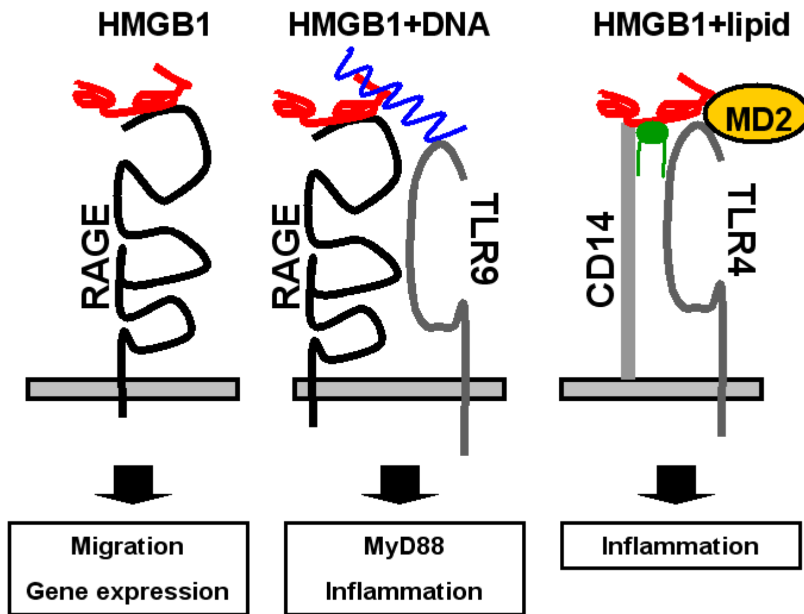

Figure 7. HMGB1 signaling to the cytoplasm is mediated by transmembrane receptors. Direct HMGB1 signaling from the cell surface to the cytoplasm is mediated by RAGE (A). HMGB1 in complex with DNA signals via RAGE and TLR9 in lysosomal vesicles (B). A lipid-bound HMGB1 may bind to CD14 and/or MD2 on the cell surface and induce signaling via TLR4 (C).

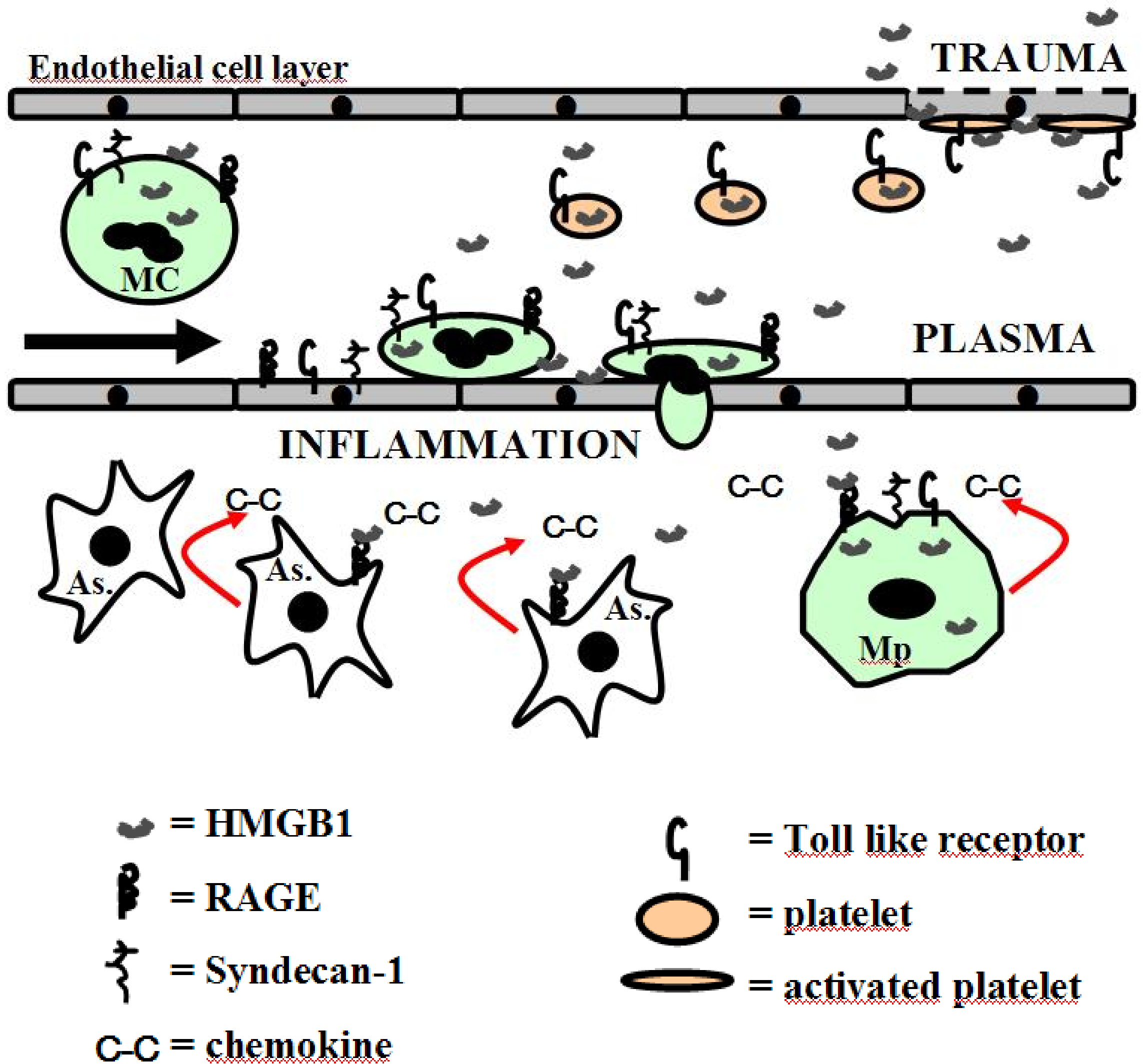

Figure 8. Proposed functions of HMGB1 and its cell surface receptors in a vessel of inflamed or necrotic tissue. The endothelium is activated in inflamed tissue, and it starts to express adhesion molecules to leukocytes, which then migrate through the endothelium to the inflamed area. Migrated monocytes mature to tissue macrophages. HMGB1 induces chemokine release from macrophages and other tissue cells, e.g. astrocytes. Chemokines attract more immune cells to the inflamed area. In trauma, the

endothelial cell layer is damaged and platelets adhere to the subendothelial matrix. Adhesion activates platelets, which then release HMGB1, further activating the platelets. In addition, platelet-derived HMGB1 can mediate platelet/monocyte interactions. MC = monocyte, Mp = macrophage, As. = astrocyte or other tissue cell. Black arrow indicates the direction of blood flow, red arrows indicate chemokine secretion.

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