The function of high mobility group box 1 (HMGB1) in the immune system

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Promotor: Prof. Dr. B. Lambrecht

Scriptie voorgedragen in de 2de Master in het kader van de opleiding tot MASTER IN DE GENEESKUNDE
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“De auteur(s) en de promotor geven de toelating deze scriptie voor consultatie beschikbaar te stellen en delen ervan te kopiëren voor persoonlijk gebruik. Elk ander gebruik valt onder de beperkingen van het auteursrecht, in het bijzonder met betrekking tot de verplichting uitdrukkelijk de bron te vermelden bij het aantalen van resultaten uit deze scriptie.”

Datum
23/04/20

(handtekening student (en))

(Naam student)

(handtekening promotor)

(Naam promotor)
Foreword

The immune system represents a fundamental part of the human body. Therefore I suppose, in the future, I will be faced with many conditions, caused by problems in the immune system, no matter where I end up as a doctor. I am glad I have chosen this topic ("The function of HMGB1 in the immune system") as the subject of my thesis.

In the first place, I would like to thank my promoter for the help in the creation and realization of this thesis, for giving an answer to all my questions and for the time he saved for doing so. The time and patience he managed to fetch, are certainly appreciated. I would also like to thank my friends and family for other support.
Abstract

INTRODUCTION: The high mobility group (HMG) chromosomal proteins, which are common to all eukaryotes, bind DNA in a non-sequence-specific fashion to promote chromatin function and gene regulation. They directly interact with nucleosomes and are believed to be modulators of chromatin structure. High mobility group box 1 protein (HMGB1) is the human prototype of these non-histone nuclear proteins.

METHODOLOGY: The results were found in a severe selection of articles, using the search engine PubMed.

RESULTS: HMGB1 has a dual function. Inside the cell, HMGB1 binds DNA, regulating transcription and determining chromosomal architecture. Outside the cell, HMGB1 can serve as an alarmin to activate the innate immune system and mediate a wide range of physiological and pathological responses. To function as an alarmin and cytokine, HMGB1 translocates from the nucleus of the cell to the extra-cellular milieu, a process that can take place with cell activation as well as necrosis. Remarkably, apoptotic chromatin binds HMGB1 irreversibly, thereby ensuring that it will not diffuse away to activate responses from neighbouring cells. Thus, dying cells, necrotic and apoptotic, use their own chromatin to signal how they have died.

Cellular injury resulting in necrosis leads to passive HMGB1 release. Microbes, microbial particles, or proinflammatory cytokines may stimulate later active release from antigen-presenting cells. Proinflammatory HMGB1 may act as an adjuvant (immunological agent that modifies the effect of other agents) or assist in tissue repair. HMGB1 can interact with receptors that include RAGE (receptor for advanced glycation endproducts), as well as Toll-like receptor-2 (TLR-2), TLR-3, TLR-4, TLR-7, TLR-9. HMGB1 can function in a synergistic fashion with other proinflammatory mediators to induce responses.

DISCUSSION: The results suggest that HMGB1, when unregulated, may contribute to immune-related pathology. An overwhelming infection causing sepsis, or tissue damage caused by trauma, ischemia, or hemorrhage, may result in life-threatening out-of-control HMGB1 responses. HMGB1 has been shown to play a key role in the pathogenesis of several diseases (including autoimmune ones), i.e. rheumatoid arthritis, systemic lupus erythematosus, several types of cancer, cystic fibrosis, stroke, atherosclerosis,…
Nederlandse samenvatting:

INLEIDING: De high mobility group (HMG) chromosomale eiwitten, eigen aan alle eukaryoten, binden DNA op niet-sequentie-specifieke wijze om de functie van chromatinie, alsook genregulatie te bevorderen. Ze komen rechtstreeks in contact met nucleosomen en zijn modulatoren van de chromatinestructuur. High mobility group box 1 (HMGB1) vertegenwoordigt in het menselijk lichaam het prototype van deze niet-histone nucleaire eiwitten.

METHODOLOGIE: De resultaten werden gevonden door het zoeken en selecteren van artikels, met behulp van de zoekfunctie van PubMed.

RESULTATEN: HMGB1 bezit een tweeledige functie. Binnen in de cel bindt het aan DNA en heeft het een functie in de transcriptieregulering en chromosomale architectuur. Buiten de cel kan HMGB1 optreden als een alarmin: het is in staat tot het activeren van het immuunsysteem, alsook een breed scala aan fysiologische en pathologische reacties. Om te kunnen functioneren als een alarmin en cytokine, moet HMGB1 zich verplaatsen vanuit de kern van de cel naar de extracellulaire omgeving. Dit proces kan in gang gezet worden door necrose enerzijds, en celactivering anderzijds. Er is aangetoond dat apoptotisch chromatine op irreversibele wijze bindt aan HMGB1, waardoor het onmogelijk uit de cel kan diffunderen om reacties uit nabijgelegen cellen te activeren. Op die manier gebruiken necrotische en apoptotische cellen hun eigen chromatine om aan te geven op welke wijze ze zijn doodgegaan.

Cellulaire schade als gevolg van necrose leidt tot passieve loslating van HMGB1 uit de cel. Microben, microbiële deeltjes of inflammatoire cytokines kunnen op een later tijdstip ook actieve vrijlating uit de antigenpresenterende cellen stimuleren. HMGB1 kan optreden als een adjuvans (immunologische stof die het effect van andere stoffen wijzigt) of helpen bij weefselherstel. HMGB1 interageert met receptoren, zoals RAGE (receptor for advanced glycation endproducts), Toll-like receptor-2 (TLR-2), TLR-3, TLR-4, TLR-7, TLR-9. HMGB1 kan op synergetische wijze functioneren met andere inflammatoire mediatoren om reacties te induceren.

DISCUSSIE: De resultaten suggereren dat HMGB1 kan bijdragen tot pathologie van het immuunsysteem, wanneer het ongereguleerd voorkomt. Overweldigende infecties die sepsis veroorzaken, alsook weefselbeschadiging als gevolg van trauma, ischemie of bloeding, kunnen leiden tot levensbedreigende, ongecontroleerde reacties van HMGB1. Het is aangetoond dat HMGB1 ook een belangrijke rol speelt in de pathogenese van talrijke ziekten (met inbegrip van auto-immuunziekten), zoals reumatoïde artritis, systemische lupus erythmatosus, verschillende vormen van kanker, mucoviscidose, beroertes, atherosclerose,...
2 Introduction

In this introduction, important background information will be given on the classification of the HMG proteins, to which HMGB1 belongs, and their function in interacting directly with DNA. Also, the differential features between apoptosis and necrosis will be discussed. This information is necessary to fully understand the chapter Results and further on, to be able to formulate an answer to the key question: “what is the function of HMGB1 in the immune system?”

2.1 The HMG proteins and DNA

Most DNA-related activities such as transcription, replication, recombination, and repair involve changes in the structure and organization of the DNA. Some of these structural changes are facilitated by a family of abundant non-histone nuclear proteins known as the high-mobility-group (HMG) proteins, typified by HMG-1/2, HMG-I/Y and HMG-14/17. With the exception of histone proteins, the high mobility group (HMG) proteins are among the most ubiquitous of the chromatin-associated proteins (1).

The functional motifs characteristic of the HMG-1 (2) and HMG-I/Y (3) subfamilies have been identified in numerous nuclear proteins that interact with DNA and chromatin. However, it is important to clearly distinguish the archetypal, or canonical HMG proteins from the proteins containing these HMG motifs embedded in their primary sequence.

The latter, the HMG motif proteins or transcription factors, typified by SRY (4-5) and lymphoid enhancer factor-1 (LEF-1) (6), are cell-type specific, are not abundant and bind to DNA in a sequence-specific fashion in promoter or enhancer regions of regulated genes (7). They usually contain a single HMG domain and additional, distinct non-HMG functional motifs.

In contrast, the archetypal HMG proteins, typified by HMG1/2 (8), are ubiquitous in all the cells of higher eukaryotes and are relatively abundant. They contain multiple HMG domains that bind to DNA in a sequence-independent fashion, to facilitate nucleosome function, DNA recombination and repair, as well as activation (9) and repression (10) of general transcription. They interact directly with nucleosomes (11) and are believed to be modulators of chromatin structure.(1) These HMG proteins
are also important in V(D)J recombination (12) and in activating regulators of gene expression (including p53, Hox transcription factors and steroid hormonereceptors), by increasing their affinity for DNA (13).

Both groups, the sequence-specific HMG motif proteins, and the non-sequence-specific chromosomal proteins, bind to DNA using the 80 residue HMG domain (14). Common properties of all these HMG domain proteins include interaction with the minor groove of the DNA helix, binding to irregular DNA structures, and the capacity to modulate DNA structure by bending. DNA bending induced by the HMG domain can facilitate the formation of higher-order nucleoprotein complexes, suggesting that HMG domain proteins may have an architectural role in assembling such complexes (14-15).

Figure 1: An HMG-domain protein (HMGB1; domain A shown as gray ribbon) inserts a phenyl group (yellow) into the groove created when cisplatin (platinum shown in red) forms a complex with DNA, causing it to bend (URL1, 69).
In the narrowest traditional sense, the archetypal HMG protein family consists of six proteins and is again subdivided into three subfamilies: the HMG-1/-2 subfamily, the HMG-I/Y subfamily and the HMG-14/-17 subfamily. These three HMG subfamilies are similar in several physical characteristics. However, each subfamily has a specific type of targets and induces characteristic changes in the structure of its binding site. Each of the subfamilies has a characteristic functional sequence motif. These functional motifs are the main site of interaction between the HMG proteins and the DNA or chromatin targets. The **HMG-1 domain** (often referred to as the HMG-1 box) is the functional motif of the largest HMG subfamily, the HMG-1/-2 proteins; the **AT hook** is the functional motif of the HMG-I/Y group, and the **nucleosomal binding domain** is the functional motif of the HMG-14/-17 subfamily (15). Significantly, all of these functional motifs bind to specific structures in DNA or in chromatin, with little if any specificity for the target DNA sequence, as mentioned above.

With the exception of HMG-I/Y, each of the HMG proteins is encoded by a unique gene and all the HMG proteins are found in most higher eukaryotic cells. The ubiquitous distribution of all the HMG proteins argues that each of the archetypal HMG proteins is involved in a distinct and important cellular function. It is not clear to what degree there is functional redundancy even among the members of an HMG subfamily, which structurally are very similar. Thus, HMG-4 (16) and HMGIC (17), which are extremely similar to HMG-1/2 and HMG-I/Y, respectively, are expressed specifically in early development or in certain types of neoplastic tissues. Depletion of HMG-2 by antisense technology affects the rate of cell proliferation (18). Clearly the closely related HMG-1 cannot substitute for the missing HMG-2. Likewise, deletion of the HMG-1 gene causes lethal hypoglycaemia. The HMG-2 gene does not compensate for the missing HMG-1 gene (19).

![Figure 2: HMGA = HMG1/Y; HMGB = HMG1/2; (HMGN = HMG14/17, not in this figure) (20).](image-url)
HMG1, or recently named HMGB1 (also known as amphoterin and sulfoglucuronyl carbohydrate binding protein, SBP-1) is part of the first subfamily and was identified about 30 years ago. It is a protein with a molecular mass of ~27 kilodaltons. Its extracellular activities qualify it as a potent cytokine and tissue damage signal (see below). HMGB1 is ubiquitous and only 10 times less abundant than core histones, at 106 molecules per typical mammalian cell.

In all cells, including resting inflammatory cells, HMGB1 shuttles between nucleus and cytoplasm. Nuclear import is active, and the protein migrates back to the cytoplasm via passive diffusion and XPO1 (Exportin-1)-mediated active export. When HMGB1 is underacetylated, the rate of nuclear import exceeds that of rediffusion plus export, and the protein appears predominantly or solely nuclear (21).

HMGB1 is actually a small protein (215 residues). Structurally, it has a tripartite structure composed of three domains: two homologous, L-shaped DNA-binding motifs termed A and B boxes, each made up of approximately 80 amino acids, and a negatively charged 30 amino acid-long, highly acidic COOH-terminus (22). It binds to DNA segments at the entry/exit of nucleosomes, much in the same way as histone H1. But whereas H1 is believed to have a general repressing function, locking in place nucleosomes and rendering them less accessible and less mobile, HMGB1 can facilitate nucleosome sliding (23). For this to happen, HMGB1’s interaction with nucleosomes must be highly reversible: in fact, an HMGB1 truncation lacking the acidic tail binds to nucleosomes much more tightly and impedes their sliding.

In living cells, HMGB1 is indeed the most mobile nuclear protein (24). Photobleaching experiments established that the entire pool of HMGB1 roams the nucleus. Each individual nucleosome is visited by HMGB1 every 2 seconds on average, and the protein will stay there for a small fraction of a second. This hectic movement ensures that HMGB1 will simply “wander” where it is required within a reasonable time, do its job, and then leave. In a sense, HMGB1 can be regarded as the general lubricant of chromatin, or a “chromatin chaperone” that uses no energy besides Brownian motion (25).

The HMG-1 domain consists of 80 amino acids. As mentioned above, it has a characteristic, L-shaped fold, formed by three a-helical segments. The HMG-1 domain binds the DNA exclusively through the minor groove. A wedge of hydrophobic amino acids protruding from the concave surface of the protein partially intercalates between the DNA bases, expanding the minor groove, thereby significantly unwinding and bending the DNA. The various HMG-1 domains produce specific changes in the structure of the target DNA. The amino acid sequence in the helical regions of the L-shaped HMG-1 domain provides specificity in DNA binding, while the type of intercalating amino acids and the angle of the L-shaped fold affect the degree to which the DNA is unwound and bent. The interactions between the HMG-1 domain and its target are highly specific and affected by single point mutations.
Additional factors that determine the binding specificity of this motif are the amino acid sequences adjacent to the HMG-1 domain and the number of the domains in the protein. The ability of the HMG-1 domain to induce site-specific DNA deformations is an important aspect of its biological function. An additional fundamental property is the ability of this domain to recognize and bind to altered DNA conformations, such as stem-loops, four-way junctions, and specifically kinked or underwound DNA (15).

All these characteristics enable HMGB1 to act inside the cell and bind DNA, regulating transcription and determining chromosomal architecture.

Figure 3: An architectural and chaperone role for HMGB1 (green) in facilitation of transcription factor binding (left fork) and assembly of nucleoprotein complexes (right fork) (22).
2.2 Apoptosis and necrosis

There are many observable morphological and biochemical differences between necrosis and apoptosis (see Table 1).

Necrosis occurs when cells are exposed to extreme variance from physiological conditions (e.g., hypothermia, hypoxia) which may result in damage to the plasma membrane. Under physiological conditions direct damage to the plasma membrane is evoked by agents like complement and lytic viruses. Necrosis begins with an impairment of the cell’s ability to maintain homeostasis, leading to an influx of water and extracellular ions. Intracellular organelles, most notably the mitochondria, and the entire cell swell and rupture: cell lysis. Due to the ultimate breakdown of the plasma membrane, the cytoplasmic contents including lysosomal enzymes are released into the extracellular fluid. Therefore, in vivo, necrotic cell death is often associated with extensive tissue damage resulting in intense inflammatory responses.

Apoptosis, in contrast, is a mode of cell death that occurs under normal physiological conditions and the cell is an active participant in its own demise (“cellular suicide”). It is most often found during normal cell turnover and tissue homeostasis, embryogenesis, induction and maintenance of immune tolerance, development of the nervous system and endocrine-dependent tissue atrophy. Cells undergoing apoptosis show characteristic morphological and biochemical features. These features include chromatin aggregation, nuclear and cytoplasmic condensation, partition of cytoplasm and nucleus into membrane bound-vesicles (apoptotic bodies) which contain ribosomes, morphologically intact mitochondria and nuclear material. In vivo, these apoptotic bodies are rapidly recognized and phagocytized by either macrophages or adjacent epithelial cells. Due to this efficient mechanism for the removal of apoptotic cells in vivo no inflammatory response is elicited. In vitro, the apoptotic bodies as well as the remaining cell fragments ultimately swell and finally lyse. This terminal phase of in vitro cell death has been termed “secondary necrosis”.
<table>
<thead>
<tr>
<th>Necrosis</th>
<th>Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Morphological features</strong></td>
<td></td>
</tr>
<tr>
<td>Loss of membrane integrity</td>
<td>Membrane blebbing, but no loss of integrity</td>
</tr>
<tr>
<td>Begins with swelling of cytoplasma and mitochondria</td>
<td>Aggregation of chromatin at the nuclear membrane</td>
</tr>
<tr>
<td>Ends with total Lysis</td>
<td>Begins with shrinking of cytoplasma and condensation of nucleus</td>
</tr>
<tr>
<td>No vesicle formation, complete lysis</td>
<td>Ends with fragmentation of cell in smaller bodies</td>
</tr>
<tr>
<td>Desintegration (swelling) of organelles</td>
<td>Formation of membrane bound vesicles (apoptotic bodies)</td>
</tr>
<tr>
<td></td>
<td>Mitochondria become leaky due to pore formation involving proteins of the bcl-2-family</td>
</tr>
<tr>
<td><strong>Biochemical features</strong></td>
<td></td>
</tr>
<tr>
<td>Loss of regulation of ion homeostasis</td>
<td>Tightly regulated process involving activation and enzymatic steps</td>
</tr>
<tr>
<td>No energy requirement (passive process, also occurs at 4°C)</td>
<td>Energy (ATP)-dependent process (active process, does not occur at 4°C)</td>
</tr>
<tr>
<td>Random digestion of DNA (smear of DNA after agarose gel electrophoresis)</td>
<td>Non-random mono- and oligonucleosomal length fragmentation of DNA (Ladder pattern after agarose gel electrophoresis)</td>
</tr>
<tr>
<td>Postlytic DNA fragmentation (= late event of death)</td>
<td>Prelytic DNA fragmentation</td>
</tr>
<tr>
<td></td>
<td>Release of various factors (cytochrome C, AIF) into cytoplasm by mitochondria</td>
</tr>
<tr>
<td></td>
<td>Activation of caspase cascade</td>
</tr>
<tr>
<td></td>
<td>Alterations in membrane asymmetry (i.e. translocation of phosphatidylserine from the cytoplasmic to the extracellular side of the membrane)</td>
</tr>
<tr>
<td><strong>Physiological significance</strong></td>
<td></td>
</tr>
<tr>
<td>Affects groups of contiguous cells</td>
<td>Affects individual cells</td>
</tr>
<tr>
<td>Evoked by non-physiological disturbances (complement attack, lytic viruses, hypothermia, hypoxia, ischemia, metabolic poisons)</td>
<td>Induced by physiological stimuli (lack of growth factors, changes in hormonal environment)</td>
</tr>
<tr>
<td>Phagocytosis by macrophages</td>
<td>Phagocytosis by adjacent cells or macrophages</td>
</tr>
<tr>
<td>Significant inflammatory response</td>
<td>No inflammatory response</td>
</tr>
</tbody>
</table>

Table 1: Differences between necrosis and apoptosis.
2.2.1 HMGB1 modifications during apoptosis?

Nuclear macromolecules, including HMGB1, can translocate to the extracellular space, where they can activate immunity. HMGB1 acts as a potent cytokine and tissue damage signal (see below), and therefore its release must be tightly controlled. From this point of view, it is obvious that apoptotic cells should not release any HMGB1, otherwise they activate the same inflammatory responses of necrotic cells. Apparently, also secondarily necrotic cells do not broadcast HMGB1, and do not trigger any inflammation (24).

Several proteins are cleaved by caspases in apoptotic cells, but HMGB1 is not cut, nor it undergoes other posttranslational modifications. Rather, HMGB1 binds tightly and irreversibly to the condensed chromatin of apoptotic cells. Analysis shows that the mobility of HMGB1 in apoptotic cells is
essentially zero: HMGB1 gets unable to diffuse away from the remnants of the dead cell. Apoptotic nuclei represent a sink for extracellular HMGB1: incubation of permeabilised apoptotic cells with soluble recombinant HMGB1 results in efficient binding of HMGB1 to chromatin (25). Since HMGB1 is not chemically altered during apoptosis, it must be chromatin itself that gets modified. Indeed, a generalized underacetylation of histone during apoptosis has been demonstrated. This will be further explained in the chapter Results.
3 Methodology

The aim of this thesis about HMGB1 (a protein that recently has come into full research), is to provide an understanding of the function(s) it has in the immune system. Of course, that is what the literature had to be about.

First of all, some general information on HMGB1 was researched. After that, the search for relevant literature began in the PubMed database. The search in PubMed was limited to English literature. Terms like HMGB1, DNA, chromatin, necrosis, apoptosis, high mobility group (HMG) chromosomal proteins, alarmin, dendritic cells, pathogenesis, rheumatic disease,… were entered in PubMed. However, this way an abundant amount of available literature was obtained. It was therefore decided to address the limited issue, by combining these terms in numerous ways to obtain the best possible outcomes. Also, closer study of the obtained articles made it clear that several articles generally did not provide any further information on the subject. Thus, the number of studies on the subject, became the more and more limited.

However, to make a further selection in the plethora of articles in a structured and scientific way, the following pattern was applied. By the examination of the titles of the articles, a great shifting could be done already. Also, in reading the abstracts a number of articles was found not to be relevant for the content of the thesis. A further selection was performed on the impact factor of the journals and the number of citations of the articles. Subsequently, the availability of the sources was examined. The full version of the article was always retrieved, online if possible. Where no electronic version available, it was checked in the Aleph catalog whether the relevant journal in the library of the Ghent University was to be found. This way, sometimes an electronic version could still be found. Finally, some useful references were found in the reference lists of the selected articles, as well as some interesting links in the section “related articles” in PubMed.

While writing, the search for articles continued, where appropriate, to determine whether certain statements were supported or even invalidated by other authors. Of the relevant articles, features such as the kind of study and methodology, were taken into consideration. What inclusion criteria were used? Was the study population compared with a control population? These features played a role in the creation of a balanced view on the outcomes.
Then a table was created with a substantive overview of the information of the selected articles, which was intended to be a tool for later writing.

In the chapter *Results* some parameters are systematically discussed. There is a split between the actual functions of HMGB1 in the immune system (*Results*) and the role HMGB1 plays in the pathogenesis of several diseases (*Discussion*). Such classification was created because, after thorough study of the literature, it seemed most appropriate to include all data in a clear way. In the *Discussion* the collected results are also summarized and commented. Certain diseases are further examined.
“What is the function of HMGB1 in the immune system?” To get an answer to this key question, first of all, an analysis of the main functions of HMGB1 (and its mechanisms) will be given, based on the study of literature. After that, a conclusion will be formulated, at the end of the chapter Discussion.

### 4.1 Nuclear protein

As pointed out in the *Introduction*, High mobility group 1 (HMGB1) protein is both a nuclear factor and a secreted protein. It was originally described as a nuclear protein only. Later on, the role of HMGB1 in inflammation was investigated. In the cell nucleus it acts as an architectural chromatin-binding factor that bends DNA, stabilizes nucleosomes and facilitates transcription by promoting protein assembly on specific DNA targets.

HMGB1 can interact via the HMG boxes with a broad range of nuclear proteins. Interaction with HMGB1 has been described for several transcription factors (p53, Hox, Pou, Oct, steroid hormone receptors and TATA-binding protein) and the recombination activation gene proteins (RAG1 and 2). It has been reported that HMGB1 has a function in the nucleus as a specific enhancer of p53 activity. In general, HMGB1 increases the DNA binding affinity of those factors and shows either negative or positive effect on transcription.

Summarized, as a nuclear protein, HMGB1 is multifunctional, with roles in regulating cell function, chromatin structure, transcriptional regulation and V(D)J recombination (27).
Figure 5: Model for HMGB protein function. (A) HMGB proteins and linker histone H1 similarly bind to chromatin, and HMGB may replace H1 facilitating transcription factor binding. (B) HMGB proteins can assist the binding of transcription factors (indicated by ovals) to their DNA target sites, and promote the formation of higher order complexes regulating transcription. HMGB proteins may be recruited to their sites of action by direct interaction with certain transcription factors. (C) In other cases, HMGB proteins are recruited (without direct protein interaction) by a structural trapping mechanism, since HMGB proteins bind with high affinity to DNA sites displaying a (protein-induced) bent. The DNA-bending activity of HMGB proteins is the prominent feature in the assembly of nucleoprotein complexes (20).
4.2 Tissue damage and cell death

4.2.1 DNA

As mentioned, nuclear macromolecules, in addition to their intracellular role in regulating cell function, can translocate into the extracellular space where they can activate innate immunity. The translocation of HMGB1 to the extracellular milieu can occur in various settings and reflects the dynamic nature of nuclear structure. Of nuclear molecules, DNA and the DNA-binding protein, HMGB1, display distinct patterns of immune activity. For DNA, immune activity depends on sequence, base methylation, and context. While bacterial DNA is an immune activator, mammalian DNA is either inert or inhibitory when free. In contrast, mammalian DNA in the form of immune complexes can induce inflammation and antimicrobial immunity, by triggering immune cell activation (28-29). This activation of innate immune responses is crucial to protective and pathological immunities and is mediated by the transmembrane Toll-like receptors (TLRs) and cytosolic receptors (30).

Thus, DNA can be detected in different cellular compartments and can induce a range of cellular responses, such as an antiviral response and pyroptosis (a form of programmed cell death with antimicrobial responses during inflammation) (29). As shown in in vivo and in vitro studies, DNA can exit cells during apoptotic as well as necrotic cell death in a process that may depend on the presence of macrophages (28).

The release of HMGB1, which is of another kind, will be discussed further.

4.2.2 Cell death

When tissues are damaged, they usually heal. The cellular responses towards healing require the prior recognition that damage has occurred. HMGB1 is passively released by cells that have died in a traumatic, non-programmed way: necrosis (31). The nuclear protein HMGB1 takes on proinflammatory properties when released during cell death. Other intercellular mediators of inflammation generated during cell death are microparticles. These are small, membrane-bound structures that extrude from cells when they die and contain cell surface proteins and nuclear material from their parent cells. Microparticles circulate widely throughout the vasculature and mediate long-distance communication between cells. Both HMGB1 and microparticles have been implicated in the pathogenesis of a broad spectrum of inflammatory diseases, including autoimmune conditions (32), as will be explained in the Discussion. Next, the focus will be on HMGB1, not the microparticles.
A necrosis-style release of lytic enzymes in the surrounding medium could harm nearby cells. However, such pollution could be easily avoided by sealing the plasma membrane and exposing “eat me” signals to accelerate physical removal, as indeed apoptotic cells do (33). But why the need for chromatin condensation and breakdown (features of apoptosis, as explained in the Introduction) in these cells?

The reason for these complicated apoptotic manoeuvres in the nucleus would be simply to avoid signalling trauma to neighbouring cells if there is none. HMGB1 spillage is a simple way to signal necrosis. Absence of HMGB1 spillage prevents the other cells from reacting to, or even knowing about, an event with no adverse consequences. Thus, as already mentioned in the Introduction, apoptotic cells do not release HMGB1 even after undergoing secondary necrosis and partial autolysis, and these cells also fail to promote inflammation even if not cleared promptly by phagocytic cells. During apoptosis, HMGB1 is bound irreversibly to chromatin, but since HMGB1 is not chemically altered during apoptosis, it must be chromatin itself that gets modified (25).

One of the features of apoptosis is that chromatin gets cleaved to nucleosome-sized fragments. Another, already mentioned modification of chromatin during apoptosis is its condensation. Chromatin in apoptotic nuclei is much more condensed than in mitotic chromosomes, suggesting that nuclear proteins might just get trapped in collapsed chromatin. However, the mobility of most nuclear proteins (including HMGNs, histone H1, and several transcription factors) is not significantly different in the chromatin of living and apoptotic cells: HMGB1 alone gets locked in. Not much more was known about chromatin modification during apoptosis until a single report indicated that apoptotic chromatin is generally underacetylated (34). Subsequently, there has been shown that underacetylation of histone H4 in particular, indeed correlates with HMGB1 locking (24). Also, histone H2B phosphorylation and, as mentioned, chromatin condensation might serve to prevent HMGB1 release (25).

Thus, cells undergoing apoptosis are programmed to withhold the signal that is broadcast by cells that have been damaged or killed by trauma (24). Summarized, dying cells use their own chromatin to signal how they have died. Thus, the nuclear events in apoptosis serve to control the molecular signals that dying cells send out.

### 4.2.3 Damage signal

HMGB1 is known as one of the most important damage associated molecular patterns (DAMPs) or alarmins. Alarmins are endogenous promoters of the immune response to injury or infection. Other major and important DAMPs, except for HMGB1 and DNA, are heat shock proteins and uric acid. Among these DAMPs, HMGB1 is the most studied. Recently, reactive oxygen intermediates,
extracellular-matrix breakdown products, neuromediators and cytokines like the IFNs have also been discovered as damage signals (35).

Several cell types (in particular inflammatory cells, such as activated monocytes, macrophages, dendritic cells and endothelial cells), have the ability, when distressed, to secrete HMGB1 actively, via a dedicated pathway, and thus produce a damage signal without dying (31). For HMGB1, this active translocation occurs in antigen-presenting cells that have been stimulated by Toll-like receptor (TLR) ligands, as well as cytokines. Stimulating factors are: endotoxins, LPS, IL-1, TNF-Alp and IFN-Gamma (36). Microbes (and microbial particles obviously) may also stimulate later active release from antigen-presenting cells (28).

Summarized, HMGB1 is actively secreted by certain inflammatory cells and endothelial cells, and is passively released by necrotic or damaged cells. This way, HMGB1 signals to neighbouring cells that tissue damage has occurred. Figure 6 illustrates that.
19

Figure 6: Release of alarmins into the extracellular space. HMGB1 (and possibly other alarmins or danger-associated molecular patterns), is actively secreted by inflammatory cells and passively released by necrotic cells. By contrast, HMGB1 is irreversibly bound to the chromatin of apoptotic cells (37).

4.2.4 Receptors and pathways

Outside the cell, HMGB1 binds with high affinity to RAGE (the receptor for advanced glycation end products) to act as a potent mediator of inflammation (24). RAGE is a multiligand receptor of the immunoglobulin superfamily, expressed on monocytes and macrophages, but HMGB1 signaling through RAGE also occurs in endothelial cells, neurons and smooth-muscle cells (38).

Cell activation by HMGB1 binding RAGE results in the release of proinflammatory cytokines and chemokines: TNF-Alpha, IL-1Alpha, IL-1Beta, IL-1RA (Interleukin-1 Receptor Antagonist), IL-6, IL-8, and MCP1 (Monocyte Chemotactic Protein-1). It also results in the upregulation of adhesion
molecules: ICAM1 (Intercellular Adhesion Molecule-1) and VCAM1 (Vascular Cell Adhesion Molecule-1), the HMGB1 receptor RAGE, and HMGB1 itself. TNF-Alpha acts locally to amplify responses initiated by HMGB1. Extracellular HMGB1 and RAGE induce both migration and proliferation of vessel-associated stem cells, and thus may play a role in tissue regeneration (38). Thus, upon binding RAGE, HMGB1 alerts leukocytes to extravasate from the blood into the affected tissue, activate inflammation, trigger adaptive immunity and promote the migration and proliferation of cells (including stem cells) to repair the damaged tissue (31).

Figure 7: Schematic illustration of potential pathways for HMGB1 release leading to inflammatory responses. HMGB1 can be extracellularly released by passive secretion from any necrotic cell or by active secretion from activated macrophages/monocytes (39).

Although the receptor for advanced glycation end products (RAGE) has been shown to interact with HMGB1, other HMGB1 receptors are known to exist but have not been well characterized. There have been done experiments in which the role of RAGE, Toll-like receptors, as well as associated kinases, in HMGB1-induced cellular activation, have been explored.

Actually, HMGB1 by itself has only little proinflammatory activity but it binds to mediators of inflammation such as LPS, DNA or IL-1Beta and induces signaling pathways leading to NF-κB
activation, thereby potentiating inflammatory responses. Although the signaling pathways elicited by HMGB1 are not fully defined, there is evidence that the triggering occurs via several receptors including RAGE, as well as TLR2, TLR3, TLR4, TLR7 and TLR9 (30, 40-41).

HMGB1 binds RAGE to regulate migratory responses, but the use of ultrapure recombinant HMGB1 has demonstrated that it does not bind TLR4. However, HMGB1 which is released upon LPS-induced TLR4 activation, binds LPS even if present in very small amounts and carries it to TLR4. Therefore it perpetuates NF-KappaB (Nuclear Factor-KappaB) activation and inflammation. This is an example of an important mechanism by which HMGB1 performs its activities and has been shown to play a role in sepsis (also see Discussion). A similar mechanism was reported for DNA, which is released into the systemic circulation after traumatic shock or injury, and presented to TLR9 by HMGB1 (41). Thus, HMGB1 is not exactly an endogenous ligand for TLRs but rather an amplifier.

Figure 8: Signaling pathway in sepsis (URL2, 70).

Generally, upon activation of inflammatory cells through binding of IL-1, TNF-Alpha, IFN-Gamma, LPS or HMGB1 itself to their own receptors (IL-1R, TNFR1, TLR4, IFN-GammaR and RAGE respectively), the NF-kB and MAPK (Mitogen-Activated Protein Kinase) pathways are activated. Phosphorylated MAPKs and NF-kB migrate to the nucleus, where directly or via adaptor proteins they
activate HATs (Histone Acetylases) or inhibit deacetylases (21). This in turn promotes acetylation of HMGB1. Exported acetyl-HMGB1 cannot return to the nucleus. Also, the release of proinflammatory mediators is promoted.

Some inflammatory cells are equipped with secretory lysosomes, that can be secreted upon appropriate stimulation and that can accumulate HMGB1, presumably through specific transporters embedded in the lysosomal membrane. A signal can promote the fusion of the secretory lysosomes, carrying HMGB1, with the plasma membrane. Thereby, their cargo, HMGB1, is secreted to the extracellular space (38).

As already mentioned, alarmins (and complement as well) have been shown in experimental studies to play an important role as endogenous triggers of trauma-associated inflammation. Among the alarmins, HMGB1 appears to trigger this posttraumatic sterile inflammation, again, via receptors, such as TLR4 and RAGE (40, 42-44). Figure 9 demonstrates this posttraumatic pathway in the endothelial cell.
Figure 9: Schematic diagram: relation between the release of HMGB1, complement activation and induction of an inflammatory response in the vascular endothelium early after trauma (45).

For completeness there may also be mentioned that HMGB1, when secreted into the extracellular milieu, can mediate downstream neurite outgrowth, smooth muscle cell chemotaxis, fibrinolysis, cell migration (and tumour metastasis, see *Discussion*) (36).
4.3 Dendritic cells – damage signals

Dendritic cells deserve to get their own section in this chapter. There has been done a lot of research on the relationship and interaction between dendritic cells and HMGB1.

4.3.1 Introduction

Dendritic cells are key components of innate and adaptive immune responses. They are important antigen-presenting cells, specialized in antigen presentation to T cells. Indeed, the activation of dendritic cells, necessary for the initiation of primary and secondary immune responses, can be induced by endogenous danger signals - released by tissues undergoing stress, damage or abnormal death - and by exogenous danger signals elaborated by pathogens (35).

4.3.2 The pathway

It has been shown that human dendritic cells actively release their own HMGB1 into the extracellular milieu upon activation. This secreted HMGB1 is necessary for the up-regulation of CD80, CD83, and CD86 surface markers of human dendritic cells and for IL-12 production. The HMGB1 secreted by dendritic cells is also required for the clonal expansion, survival, and functional polarization of naive T cells. There has been demonstrated that RAGE is required for the effect of HMGB1 on dendritic cells. In dendritic cells too, HMGB1/RAGE interaction results in downstream activation of MAPKs and NF-kB (46).

4.3.3 Plasmacytoid dendritic cells

Plasmacytoid dendritic cells are specialized dendritic cells that produce high amounts of type I interferons in response to microbes. It been shown that HMGB1 leaves the nucleus of maturing plasmacytoid dendritic cells following TLR9 activation, and that these cells express on the plasma membrane the receptor for HMGB1, RAGE. Maturation and type I IFN secretion of plasmacytoid dendritic cells is hindered when the HMGB1/RAGE pathway is disrupted. These results reveal HMGB1 and RAGE as the first known autocrine loop modulating the maturation of plasmacytoid dendritic cells, and suggest that antagonists of HMGB1/RAGE might even have therapeutic potential for the treatment of systemic human diseases (47).
4.3.4 Dendritic cell maturation

There is the strong hypothesis that HMGB1 is an immunostimulatory signal that induces dendritic cell maturation. There has been demonstrated that HMGB1, via its B box domain, induces phenotypic maturation of dendritic cells, as evidenced by increased CD83, CD54, CD80, CD40, CD58, and MHC class II expression and decreased CD206 expression. The B box also causes increased secretion of the proinflammatory cytokines IL-12, IL-6, IL-1alpha, IL-8, TNF-Alpha, and RANTES (48).

4.3.5 Summary

HMGB1 released by necrotic cells may be a signal of tissue or cellular injury that, when sensed by dendritic cells, is used by dendritic cells to sustain their own maturation and induces and/or enhances an immune reaction. Dendritic cells can also secrete HMGB1, and such secretion promotes proliferation and Th1 polarization of interacting T cells.
4.4 DNA repair after DNA damage

4.4.1 DNA repair and chromatin modification

There is the strong hypothesis that HMGB1 modulates the repair of DNA damage in mammalian cells. HMGB1 binds cooperatively with nucleotide excision repair (NER) damage recognition proteins to DNA lesions. It also bends the damaged DNA, but the biological consequence of this interaction is not clearly understood.

There has been demonstrated that mammalian cells lacking HMGB1 are hypersensitive to DNA damage induced by UVA irradiation or UVC radiation, showing less survival and increased mutagenesis. In addition, NER efficiency is significantly decreased in the absence of HMGB1 as assessed by the repair and removal of UVC lesions from genomic DNA. A study (27) further explored the role of HMGB1 in chromatin remodeling upon DNA damage. Immunoblotting demonstrated that, in contrast to HMGB1 proficient cells, cells lacking HMGB1 show no histone acetylation upon DNA damage. Additionally, purified HMGB1 protein enhances chromatin formation in an in vitro chromatin assembly system. These results reveal a role for HMGB1 in the error-free repair of DNA lesions. Its absence leads to increased mutagenesis, decreased cell survival, and altered chromatin reorganization after DNA damage (27).
Figure 10: Model of potential effects of HMGB1 on DNA repair and chromatin remodeling. After DNA damage, HMGB1 binds to the DNA lesion (*), causing a greater distortion in the DNA. It then recruits NER proteins XPA, RPA, and XPC-RAD23B (R23B), as well as chromatin-remodeling factors to remove the nearby nucleosome (N) and facilitate accessibility of the damaged DNA to the repair apparatus (27).

4.4.2 Adverse side effects

Because strategies targeting HMGB1 are in development for treatment of sepsis and rheumatoid arthritis, these findings draw attention to potential adverse side effects of anti-HMGB1 therapy in patients with inflammatory diseases (27).
4.5 A curiosity: Thrombomodulin

Thrombomodulin will be discussed as an example of a protein, that performs one of its activities using HMGB1, or actually inhibiting it.

Thrombomodulin is actually a multi-domain proteoglycan found primarily on the endothelium. It can bind thrombin, the terminal enzyme of the blood clotting cascade. This binding blocks the ability of thrombin to clot fibrinogen and activate cells through protease-activated receptors, and it markedly augments the ability to activate protein C, a natural anticoagulant with antiapoptotic and anti-inflammatory activity. Thus, thrombomodulin is an anticoagulant cofactor that promotes thrombin-mediated formation of activated protein C (43).

There has been demonstrated that the N-terminal lectin-like domain (D1) of thrombomodulin has unique antiinflammatory properties. Thrombomodulin, via D1, binds HMGB1, thereby preventing cell signaling via the receptor RAGE and possibly other receptors. These findings highlight a new mechanism, i.e., sequestration of mediators, through which thrombomodulin, suppresses inflammation, distinctly from its anticoagulant cofactor activity. Thus, it prevents the interaction of these mediators with cell surface receptors on effector cells in the vasculature (49).

The lectin-like domain can also dampen cell activation signals by dampening the MAP kinase and NF-κB pathways, presumably through interaction with an as yet unidentified receptor. In addition, The thrombin-thrombomodulin complex also increases activation of TAFI, an enzyme that inactivates vasoactive peptides like complement C5a. Each of these diverse functions is mediated by different domains of thrombomodulin (43).

*Figure 11* summarizes all these properties of thrombomodulin.
Figure 11: Thrombomodulin has anticoagulant and anti-inflammatory properties. It can also dampen cell activation signals (43).
5 Discussion

If all these results are collected and interpreted, it is determined that HMGB1 clearly plays an extremely important role in the immune system.

All the HMGB1-mediated proinflammatory responses, we discussed, have multiple effects at various sites. In the Brain, HMGB1-mediated proinflammatory responses are manifested by fever, anorexia and pain sensation. In the lungs, these facilitate neutrophil infiltration, endothelia activation, and may also culminate in inflammation, oedema and injury. In the intestine, HMGB1 signaling results in loss of epithelia barrier function and bactericidal effects. Administration of HMGB1 via intracerebroventricular, intratracheal, intraperitoneal and intraarticular routes induces marked inflammatory responses in an organ-specific manner, and activates various innate immune cells (50).

Because of its powerful activities, it can be concluded that HMGB1, when unregulated, may crucially contribute to immune-related pathology. For instance, cell death is critical to normal homeostasis, although this process, when increased aberrantly, can lead to the production of proinflammatory mediators promoting autoimmunity. Also, severe infections causing sepsis, or tissue damage caused by trauma, ischemia, or hemorrhage, may result in life-threatening out-of-control HMGB1 responses.

<table>
<thead>
<tr>
<th>Early response</th>
<th>Later response</th>
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<td><strong>Stimuli</strong></td>
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<td><strong>HMGB1 sources</strong></td>
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<tr>
<td>Passive HMGB1 release from necrotic cells</td>
<td>Active release of acetylated HMGB1 by macrophages, monocytes and dendritic cells</td>
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<td>Normal HMGB1 response</td>
<td>Excessive HMGB1 response</td>
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<tr>
<td>ADJUVANT</td>
<td>Extreme Release of Inflammatory Mediators</td>
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<td>Dendritic cell activation</td>
<td>Extreme Barrier permeability</td>
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<td>Th 1 Polarization</td>
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<td>Chemotactic</td>
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<td>REPAIR</td>
<td><em>HMGB1 A box peptide, anti-HMGB1 antibodies, Thrombomodulin can antagonize this process.</em></td>
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<td>Proliferation</td>
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Table 2: Cellular injury resulting in necrosis leads to passive HMGB1 release. Microbes, microbial particles, or proinflammatory cytokines may stimulate later active release from antigen-presenting cells. HMGB1 assists in tissue repair. When unregulated it may contribute to pathological hyperinflammatory responses.

Indeed, HMGB1 is involved in several disorders, including rheumatoid arthritis, systemic lupus erythematosus, several types of cancer, Borna disease, stroke, atherosclerosis, cystic fibrosis airway disease,… In this chapter some of them will be discussed.
5.1 Sepsis

5.1.1 Sepsis and septic shock?

Severe sepsis, a lethal syndrome after infection or injury, is the third leading cause of mortality in the United States. The pathogenesis of severe sepsis is characterized by organ damage and accumulation of apoptotic lymphocytes in the spleen, thymus, and other organs (51).

Septic shock, as a result of infection and sepsis, is a serious medical condition caused by decreased tissue perfusion and oxygen delivery, though the microbe may be systemic or localized to a particular site. It can cause multiple organ failure and death.

In rough order of increasing severity there is: septicemia, sepsis, severe sepsis or sepsis syndrome, septic shock, refractory septic shock, multiple organ dysfunction syndrome, and death.

5.1.2 A delayed mediator of sepsis

The pathological sequelae of sepsis are characterized by a systemic inflammatory response, but experimental therapeutics that target specific early inflammatory mediators (tumour necrosis factor (TNF) and IL-1Beta) have not proven efficacious in the clinic (52).

Models of experimental sepsis in mice have shown a strong association between extracellular HMGB1 and lethality. Also, recent findings confirm that high mobility group box-1 protein is persistently elevated in the serum of human patients with severe sepsis, up to 1 week after hospitalization (53-54).

Thus, there is evidence that HMGB1, when released extracellularly, acts as a persistent, downstream mediator of sepsis and exhibits significantly delayed inflammatory kinetics relative to TNF and IL-1Beta. It is therefore a promising candidate for therapeutic intervention.

*Figure 12* puts the contributions and pathways of HMGB1 in sepsis in more detail.
Proposed contributions of HMGB1 to sepsis. HMGB1 is produced by macrophages later in sepsis, in response to inflammatory stimuli such as LPS. HMGB1 may then cause activation of phagocytic cells, resulting in production of proinflammatory mediators (purple) and chemokines (light green). HMGB1 binds to RAGE on endothelial cells and evokes intracellular signaling through kinases (JNK, ERK1/2 and p38MAPK), leading to nuclear translocation of transcription factors (NF-κB and SP-1). In response, endothelial cells express RAGE, adhesion molecules (VCAM-1 and ICAM-1), TNF, chemokines, PAI-1 and tissue plasminogen activator (tPA). HMGB1 may thereby contribute to regulation of fibrinolysis. In enterocytes, HMGB1 increases the activity of intrinsic nitric oxide synthase (iNOS), leading to augmented NO production and permeability, with the result of enhanced bacterial translocation through the gut barrier. Ethyl pyruvate can inhibit HMGB1 production by macrophages, probably by inhibition of intracellular signaling through p38MAPK and NF-κB (55).

5.1.3 Treatment of sepsis

Reducing levels of the protein by anti-HMGB1 treatment may be one way to moderate uncontrolled inflammation seen in sepsis. Yang et al. (52) demonstrated that, in mouse or rat models of sepsis or hemorrhage, inhibiting HMGB1 has been effective in increasing survival. These animals treated with HMGB1 inhibitors were protected against the development of organ injury (as evidenced by improved levels of serum creatinine and blood urea nitrogen). Surprisingly, one such inhibitor is a portion of the...
HMGB1 molecule itself. Of two DNA interaction sites, box B can substitute for the cytokine-like properties of the whole molecule, while box A can inhibit inflammation (52). As explained in the Results, recently, the vascular thrombin binding protein thrombomodulin has been shown to bind and sequester HMGB1, offering protection from its ill effects and at least partially explaining the anti-inflammatory effects of thrombomodulin (49). Thus, therapeutic strategies based on one or more of these inhibitors are attractive, especially considering that the HMGB1 levels peak later than 24 hours after the initiation of sepsis, potentially allowing time for treatment to occur. Of course, further investigations still have to be done.

5.1.4 Acute inflammatory lung injury

HMGB1, as a late mediator of lethality in sepsis, can induce acute inflammatory lung injury. The critical role of alpha-chemokine receptors in the HMGB1-induced inflammatory injury has been identified (56). It has been shown that alpha-chemokine receptor inhibition increases survival in sepsis, in a clinically relevant time frame. Intratracheal instillation of recombinant HMGB1 induces a neutrophilic leukocytosis, preceded by alveolar accumulation of the alpha-chemokine macrophage inflammatory protein-2 and accompanied by injury and increased inflammatory potential within the air spaces. The role of alpha-chemokine receptors in the injury has been investigated, by instilling recombinant HMGB1 directly into the lungs and administering a subcutaneous alpha-chemokine receptor inhibitor, Antileukinate. Alpha-Chemokine receptor blockade reduces HMGB1-induced inflammatory injury within the bronchoalveolar lavage fluid, indicating that HMGB1-induced inflammation and injury are alpha-chemokine mediated. Because HMGB1 can mediate late septic lethality, Antileukinat has been administered to septic mice and increased survival was observed, even when the inhibitor treatment was initiated 24 h after the induction of sepsis. These data demonstrate that alpha-chemokine receptor inhibition can reduce HMGB1-induced lung injury and lethality in established sepsis and may provide a novel treatment in this terrible disease (56).
5.2 Reumatic disease

As shown in studies on patients as well as animal models, HMGB1 can play an important role in the pathogenesis of rheumatic disease, including rheumatoid arthritis, systemic lupus erythematosus, and polymyositis among others. New approaches to therapy for these diseases may involve strategies to inhibit HMGB1 release from cells, its interaction with receptors, and downstream signaling (57).

5.2.1 Rheumatoid arthritis

TNF-Alpha and HMGB1 are two potent proinflammatory cytokines implicated as important mediators of arthritis (58). Increased concentrations of these cytokines have been detected in the synovial fluid of patients with rheumatoid arthritis. In animal models of RA, HMGB1 appears to be crucially involved in the pathogenesis of arthritis since neutralization of HMGB1 significantly ameliorates the disease (59).

HMGB1 is actively released from immune cells in response to TNF-Alpha. Once released, HMGB1 in turn induces production of several proinflammatory cytokines by macrophages. It is suggested that HMGB1-triggered arthritis is probably mediated through IL-1 activation (58). Further research still has to be done.

5.2.2 Systemic lupus erythematosus

Furthermore, in the serum and plasma of patients with systemic lupus erythematosus, there have been detected substantial amounts of HMGB1 and increased concentrations of DNA-containing immune complexes, which may contribute to the disease process (41, 59).
Autoantibodies against double-stranded DNA (dsDNA) and nucleosomes represent the essence of systemic lupus erythematosus. However, the mechanisms involved in breaking the immunological tolerance against these poorly immunogenic nuclear components are not fully understood. Impaired phagocytosis of apoptotic cells with release of nuclear antigens may contribute to the immune pathogenesis. As mentioned earlier, HMGB1 is tightly attached to the chromatin of apoptotic cells. It has been shown that late apoptotic cells in vitro can release their chromatin, while HMGB1 still remains bound to these nucleosomes. This way the release of HMGB1 can occur during apoptosis as well (60).
HMGB1-nucleosome complexes form apoptotic cells have indeed been detected in plasma from SLE
patients. HMGB1-nucleosome complexes induce anti-dsDNA and antihistone IgG responses in a TLR2-dependent manner. Nucleosomes from living cells do not (61).

Stimulation of TLR9 by DNA is very important in the activation of plasmacytoid dendritic cells and B cells. Tian et al. (41) have shown that HMGB1-containing nucleosomes stimulate cytokine production through a TLR9–MyD88 pathway involving the multivalent receptor RAGE. Moreover, binding of HMGB1 to class A CpG oligodeoxynucleotides considerably augments cytokine production by means of TLR9 and RAGE. These data demonstrate a mechanism by which HMGB1 and RAGE activate plasmacytoid dendritic cells and B cells in response to DNA, leading to interferon (IFN) production.

The role of HMGB1 is very important in this, since there is evidence that HMGB1 (and HMGB2 and HMGB3) function as universal sentinels for nucleic acids, binding to all/most immunogenic nucleic acids. Yanai et al. (30) show a hierarchy in the nucleic-acid-mediated activation of immune responses: the selective activation of nucleic-acid-sensing receptors is contingent on the more promiscuous sensing of nucleic acids by HMGBs. (The absence of HMGBs severely impairs type-I interferon and inflammatory cytokine induction by DNA or RNA, suppresses the activation of NF-kB and impairs the activation of TLRs.) These findings may have implications for understanding the evolution of the innate immune system and for the treatment of immunological disorders (30).

In conclusion, HMGB1-nucleosome complexes activate antigen presenting cells and, thereby, may contribute to the pathogenesis of SLE via breaking the immunological tolerance against nucleosomes/dsDNA.

The mechanism of stimulation of TLR9 by immune complexes (by means of RAGE), eventually leading to IFN production, is illustrated in figure 13.
Figure 13: Stimulation of interferon (IFN) production by immune complexes in systemic lupus erythematosus (SLE). The figure illustrates a model by which immune complexes in SLE can stimulate IFN production by plasmacytoid dendritic cells. In this model, DNA and HMGB1 are released from dead and dying cells and bind together in immune complexes with anti-DNA. These complexes trigger RAGE and Fc receptor and, in addition, deliver DNA into the cell where it can interact with TLR9. Together, activation of downstream pathways leads to IFN production (62).
5.3 Cancer

The expression of HMGB1 has been described in many types of cancers. HMGB1 is often overexpressed in human cancer, although a direct role for this gene in transformation has not been established. However, it has been shown that HMGB1 can function as an oncogene, contributing to the pathogenesis of several cancers (63) and can also play a role in the progression of certain carcinomas, being an independent prognostic indicator (64). In malignant cells, RAGE activation by HMGB1 leads to MAPK activation and is associated with enhanced tumour growth, metastases, and release of MMPs (Matrix Metalloproteinases) (65).

Two examples, in which HMGB1 plays a role, will be given: nasopharyngeal carcinoma and leukemia.

5.3.1 Nasopharyngeal carcinoma

The role of HMGB1 in nasopharyngeal carcinoma, a carcinoma with poor clinical outcome, has been studied. There has been shown that patients with higher levels of HMGB1 expression have poorer overall survival and disease-free survival, whereas patients with lower levels of HMGB1 expression have better survival. Thus, there has been demonstrated that HMGB1 expression may be an independent prognostic indicator for patient survival. Disruption of endogenous HMGB1 using small interfering RNAs, suppresses the invasive ability of the nasopharyngeal carcinoma cell.

In conclusion, HMGB1 overexpression has a role in the progression of nasopharyngeal carcinoma and hence its poor clinical outcome (64).

5.3.2 Leukemia

There have been done experiments with transgenic mice with HMGB1, targeted to lymphoid cells. All mice developed aggressive lymphoma in a few months. It has also been demonstrated that HMGB1 mRNA and protein are increased in human acute lymphocytic leukemia samples. It is concluded that HMGB1 functions as an oncogene and contributes to the pathogenesis of leukemia (63).

5.3.3 RAGE and carcinogenesis

RAGE is expressed in cell types implicated in tumour formation, including tumour cells, endothelial cells, myeloid cells, MDSCs (myeloid derived suppressor cells), and lymphocytes. Signalling pathways downstream of RAGE that are activated by the accumulation of its ligands (AGE (advanced
glycation end products), HMGB1, S100 proteins) regulate cellular interactions during neoplastic transformation and malignant progression: A pro-tumourigenic microenvironment is established by the secretion of proinflammatory cytokines such as TNF-Alpha, IL-1, and IL-6, and the production of RAGE ligands. RAGE and RAGE ligands activate endothelial and myeloid cells resulting in the recruitment and accumulation of further myeloid cells, including MDSCs. MDSCs inhibit T and natural killer cells leading to T cell tolerance and impaired anti-tumour immunity. RAGE ligands and subsequent signalling also fuel tumour cell proliferation and survival by autocrine and paracrine feedback loops (66).
This mechanism is explained in figure 14.

Figure 14: RAGE functions in inflammation-associated carcinogenesis (66).
5.4 Borna disease

Borna disease virus (BDV) is a noncytolytic, neurotropic RNA virus that has a broad host range in warm-blooded animals, probably including humans. Borna disease is a usually fatal, acute nonsuppurative encephalitis. BDV is now gaining much of the research attention, because the disturbances seen in animals resemble those of neuropsychiatric disorders in humans. These observations raise the possibility that BDV infection may be associated with certain human disorders.

It has been demonstrated that a 24-kDa phosphoprotein (P) of BDV directly binds to HMGB1, and inhibits its function in cultured neural cells in the brain (67). This observation suggests that expression of BDV P may cause deleterious effects in cellular functions by interference with HMGB1. Indeed, Zhang et al. (68) demonstrated that P directly binds to the A-box domain on HMGB1, which is also responsible for interaction with a tumour suppression factor, p53. Binding between HMGB1 and p53 enhances p53-mediated transcriptional activity. Zhang et al. (68) have revealed that p53 and P competitively interfere with the binding of each protein to HMGB1 in a p53-deficient cell line (NCI-H1299). In addition, it has been shown that P is able to significantly decrease p53-mediated transcriptional activation of the cyclin G promoter. Also, the activation of p21(waf1) expression is repressed (68). Moreover, the expression of RAGE is not significantly activated in BDV-infected cells during the process of extension, suggesting that the secretion of HMGB1 from the cell surface is inhibited by the binding of P (67).

Summarized, BDV P (phosphoprotein) can influence the transcriptional activity of p53 by interference of HMGB1 in the nucleus of infected cells, as shown in figure 15. Thus, BDV infection may cause direct damage in the developing brain by inhibiting the function of HMGB1.
Figure 15: Blocking of HMGB1 function by its interaction with BDV P (phosphoprotein).
HMGB1 is secreted in the extracellular milieu and interacts with its receptor RAGE. Based on
the interaction, RAGE induces the signals necessary, not only for neurite outgrowth but for the
cellular survival. In addition, a part of the HMGB1 is located in the nuclei and involved in the
transactivation. BDV P can interact with HMGB1 and could block these functions of HMGB1
(URL3, 71).
5.5 Conclusion and summary

Figure 16 gives an extensive summary of the most important HMGB1 pathways in the immune system.

Figure 16: Summary of important HMGB1 pathways (URL4, 72).
Using figure 16, hereafter, the most important pathways, functions of HMGB1 and other aspects, addressed in this thesis, will be refreshed and summarized. Also, conclusions will be made.

The ability of a cell to respond specifically to various external and internal signals plays an essential role in regulating gene expression, differentiation, and cell death. Most often, the cellular responses to various stimuli involve transduction of signals received by specific receptors to defined targets that elicit specific responses in distinct cellular compartments. HMGB1, a member of the high-mobility group protein superfamily, has the ability to signal to many cellular targets in different cellular compartments and to affect several distinct and apparently unrelated biological pathways. HMGB1, also called Amphoterin, is a highly conserved component of eukaryotic nuclei and is rather known as a DNA binding protein involved in assembly of nucleoprotein complexes, maintenance of nucleosome structure and regulation of gene transcription (38). A series of recent discoveries have revealed the cytokine activity of HMGB1, that when secreted into the extracellular milieu, mediates downstream neurite outgrowth, smooth muscle cell chemotaxis, inflammatory responses and tumour metastasis (36).

Human HMGB1 has 215 residues in its primary amino acid sequence. Structurally, it has a tripartite structure composed of three domains: two homologous DNA-binding motifs termed A and B boxes, each made up of approximately 80 amino acids, and a negatively charged 30 amino acid-long, highly acidic COOH-terminus (22). Like many other nuclear proteins in living cells, HMGB1 molecules are in constant, rapid motion. The high intranuclear mobility of HMGB1 leads to frequent collisions with the chromatin and facilitates interactions with other nuclear proteins. HMGB1 binds to DNA in a sequence-independent manner and modifies DNA structure to facilitate transcription, replication, and repair. The ability of the protein to affect many types of nuclear activities reflects its mode of binding to DNA and its ability to interact with a diverse set of proteins. The architectural changes induced in the DNA promote the assembly of multiprotein complexes at the distorted site (14-15).

HMGB1 is actively secreted by macrophages, monocytes, dendritic cells, endothelial cells and others after stimulation with endotoxins, LPS, IL-1, TNF-Alpha and IFN-Gamma (36), and can also be passively released by necrotic cells (31).

Upon activation of inflammatory cells through binding of IL-1, TNF-Alpha, IFN-Gamma, LPS or HMGB1 itself to their own receptors (IL-1R, TNFR1, TLR4, IFN-GammaR and RAGE respectively), the NF-kB and MAPK (Mitogen-Activated Protein Kinase) pathways are activated. Phosphorylated MAPKs and NF-kB migrate to the nucleus, where directly or via adaptor proteins they activate HATs (Histone Acetylases) or inhibit Deacetylases (21). This in turn promotes acetylation of HMGB1. Exported acetyl-HMGB1 cannot return to the nucleus.
Some inflammatory cells are equipped with secretory lysosomes, that can be secreted upon appropriate stimulation and that can accumulate HMGB1, presumably through specific transporters embedded in the lysosomal membrane. A signal can promote the fusion of the secretory lysosomes, carrying HMGB1, with the plasma membrane. Thereby, their cargo, HMGB1, is secreted to the extracellular space. Necrotic cells release HMGB1 by simple diffusion, and thereby trigger inflammation. In contrast, apoptotic cells retain HMGB1 bound to chromatin remnants, even after their eventual lysis (38).

Receptor signal transduction of HMGB1 occurs through the receptor RAGE (Receptor for Advanced Glycation End-products), a multiligand receptor of the immunoglobulin superfamily, expressed on monocytes, macrophages, dendritic cells, endothelial cells, neurons, smooth-muscle cells,... Cell activation by HMGB1 results in the release of proinflammatory cytokines and chemokines: TNF-Alpha, IL-1Alpha, IL-1Beta, IL-1RA (Interleukin-1 Receptor Antagonist), IL-6, IL-8, and MCP1 (Monocyte Chemotactic Protein-1). It also induces upregulation of adhesion molecules: ICAM1 (Intercellular Adhesion Molecule-1) and VCAM1 (Vascular Cell Adhesion Molecule-1), RAGE, and HMGB1 itself (38). TNF-Alpha acts locally to amplify responses initiated by HMGB1. HMGB1-mediated proinflammatory responses have multiple effects at various sites. In the Brain, HMGB1-mediated proinflammatory responses are manifested by fever, anorexia and pain sensation. In the lungs, these facilitate neutrophil infiltration, endothelia activation, and may also culminate in inflammation, oedema and injury. In the intestine, HMGB1 signaling results in loss of epithelia barrier function and bactericidal effects. HMGB1 signaling also plays a significant role in arthritis and inflammation of bone joints. Administration of HMGB1 via intracerebroventricular, intratracheal, intraperitoneal and intraarticular routes induces marked inflammatory responses in an organ-specific manner, and activates various innate immune cells (50).

In neural tissue and malignant cells, RAGE activation by HMGB1 leads to MAPK activation and is associated with enhanced tumour growth, metastases, and release of MMPs (Matrix Metalloproteinases) (65). Extracellular HMGB1 and RAGE also induce both migration and proliferation of vessel-associated stem cells, and thus may play a role in muscle tissue regeneration (38).

Release of HMGB1 from necrotic cells triggers the release of various cytokines, thereby propagating inflammation. The massive release of HMGB1 from the nuclei leads to extensive cell death and septic shock (50). High serum HMGB1 levels in patients with sepsis are associated with increased mortality (36). The discovery of HMGB1 as a potent cytokine mediator of endotoxemia and sepsis and the widespread expression of RAGE on endothelium has initiated a new field of investigation for the development of therapeutics in the treatment of sepsis (52). Intratracheal administration of HMGB1
produces acute lung injury as manifested by neutrophil accumulation. Administration of anti-HMGB1 antibodies, as well as thrombomodulin and HMGB1 A box peptide, inhibit systemic inflammation, even in established cases, because HMGB1 activity is elevated at significantly later time points than TNF or IL-1.

HMGB1 plays a pivotal role in the pathogenesis of chronic arthritis and it may mediate strong, direct bactericidal effects. As already mentioned, the interaction between HMGB1 and RAGE has also been found to be important in tumour formation. Targeting the HMGB1 ligand or its receptor represents an important potential application in cancer therapeutics (65).

Thus, HMGB1 and its counter-receptor, RAGE, represent suitable targets for investigation (for the development of therapeutics), integrating many aspects of modern biology, particularly that associated with sepsis and chronic diseases involving inflammation, dysregulated cell death and cancer.
6 References


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