Microenvironment of HMGB1 "Clusters": A Potential Drug Target for Cancer and Diabetes

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The IAFOR International Conference on Sustainability, Energy & the Environment – Hawaii 2017
Official Conference Proceedings

Abstract
The quest for potential therapeutic strategies to treat inflammatory diseases represents one of the topical progresses the medical sciences field. High Mobility Group Box 1 (HMGB1) is a late mediator of myriads of pathophysiological diseases such as cancer and diabetes, which makes it a potential target for drug development. Despite its intracellular role in controlling DNA expression and architectural assembly, the HMGB1 which is released by damaged cells interact with cell surface receptors such as Receptor for Advanced Glycation End-products (RAGE) and Toll-like Receptor (TLR), subsequently activating signal cascades which then induces various pro-inflammatory reactions. In connection with its DNA-related biological functions, it appears to be necessary for HMGB1 to form clusters. We found that this self-association is influenced by specific physiochemical factors: ionic strength, pH, metal ions especially zinc, and redox environment. The HMGB1 cluster possibly influences its interaction with the receptors and the concomitant inflammatory responses. It is also expected that future study can also address the possibility of other physiochemical factors such as formaldehyde in changing HMGB1 structure and whether it may induce cluster formation. This may be an important effort to understand the molecular mechanism of the effect of formaldehyde towards HMGB1, since formaldehyde is a carcinogen and largely used as an illegal food preserving agent, especially in eastern world.

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Cell microenvironment and diseases

Microenvironment is a term used to describe a specific place within the habitat of a microorganism where it lives and carries out its biological functions (Madigan & Martinko, 2006). Controlling cell microenvironment is crucial in determining its behavior (Barthes et al., 2014). One of the regulators of the microenvironment is physiochemical conditions which can change rapidly (Madigan & Martinko, 2006). Any disorders or failure to control the microenvironment and/or physiochemical factors may result in genetic instability, irregular pattern of cell necrosis or differentiation, and changes in cellular signals and metabolism. These are the determining aspects of human diseases and disorders (Barthes et al., 2014; Kang et al., 2014). The microenvironment of a single cell also consists of its surrounding cells, as well as proteins. For example, tumor microenvironment is established from interactions between malignant and normal cells, which may account for .50% of the mass of the whole tumor (Balkwill, Capasso, & Hagemann, 2012). Although the non-malignant cells are speculated to promote tumor formation at all stages of carcinogenesis, their exact biological functions are still under detailed investigation (Hanahan & Coussens, 2012).

Examination of several different aspects of the tumor microenvironment, instead of just targeting the malignant cells during cancer treatment, is increasingly considered to be important (Balkwill et al., 2012). In particular, the examination of the molecular bases may offer more insights of the shifts between tumor state and activation of immune system (Balkwill et al., 2012), wound healing and inflammation (Hanahan & Coussens, 2012), which subsequently may lead to the development of new diagnostic biomarkers (Kang et al., 2014). High Mobility Group Box 1 (HMGB1) protein is significantly involved in chronic pathophysiological diseases including cancer, diabetes and Alzheimer's disease, by various mechanisms such as by binding to its receptors (Sims, Rowe, Rietdijk, Herbst, & Coyle, 2010). Thus, understanding the molecular mechanism of HMGB1 may lead to the discovery of potential therapeutic target to block tumor growth and development, as well as for the treatment of other diseases (Kang et al., 2014).

HMGB1: between structure and function

HMGB1, a highly conserved protein, is frequently found in the nucleus of almost all eukaryotic cells (Ito & Maruyama, 2011). Inside the cell, HMGB1 serves as a protein that modifies DNA structure and promotes protein assembly (Scaffidi, Misteli, & Bianchi, 2002). However, extracellular HMGB1 possess different function altogether. HMGB1 is released from the tumor cells and the surrounding cells under hypoxia or other environmental stimuli (Kang et al., 2014). HMGB1 forms a complex with DNA, lipids and pro-inflammatory cytokines, which subsequently binds to the cell surface receptors such as Receptor of Advanced Glycation End products (RAGE) (Sims et al., 2010) and Toll-like Receptor (TLR) (Yang et al., 2010) to induce inflammation. By interacting with HMGB1, TLR9 mediates the release of HMGB1 from nucleus (Kierdorf & Fritz, 2013). TLR4 specifically stimulates the production of reactive oxygen species (ROS), subsequently promotes hypoxia-induced HMGB1 secretion (Leventhal & Schroppel, 2012). The dual functions of HMGB1 is illustrated in Figure 1.

HMGB1 was claimed to be significantly expressed in certain primary tumors, for example melanoma and colon, prostate, pancreatic, and breast cancers (Musumeci, Roviello, & Montesarchio, 2014). The interaction of HMGB1 and RAGE is considered to be a crucial factor for the tumor cells growth, motility and invasive migration by inducing tumor survival (Luan et al., 2010). Other than cancer, patients of Diabetes type 2 were reported to have high levels of serum HMGB1 which subsequently activate TLR2 and progress the pro-inflammatory state (Dasu et al., 2010). HMGB1 is also found to be upregulated in other diseases involving cell necrosis, such as Alzheimer's disease (Musumeci et al., 2014).

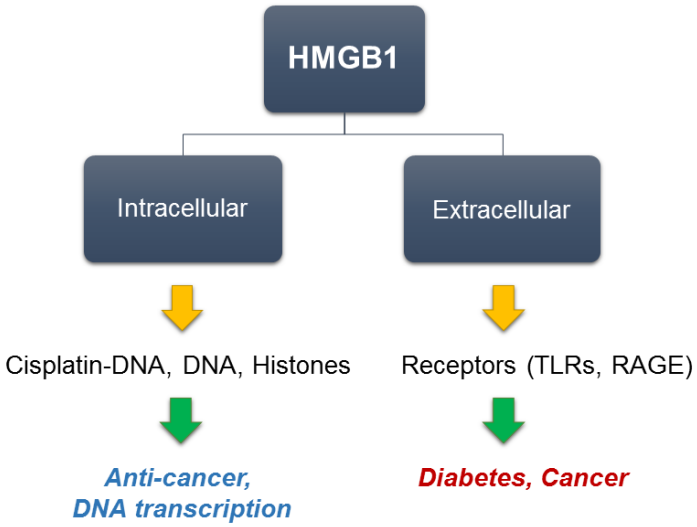

Figure 1. Intracellular and Extracellular Functions of HMGB1

Structurally, HMGB1 consists of two box-like regions, termed Box A and Box B, as described in Figure 2. Both boxes are important in DNA recognition. However, they have different functions. Box A binds distorted DNA, while Box B assists in bending DNA (Stott, Watson, Howe, Grossmann, & Thomas, 2010). Closely related in function with the boxes is the acidic C-terminus tail (Figure 2). The tail has a highly negative charge because it consists of only glutamic acid and aspartic acid residues. The acidic tail assists several DNA-related functions such as stimulation of DNA transcription (Watson, Stott, & Thomas, 2007). The tail has been shown to establish an extensive contact with the DNA-binding faces of both HMG boxes, which then triggers the collapse of HMGB1 structure and prevents the boxes from binding DNA (Stott et al., 2010).

HMGB1 is sensitive towards reduction and oxidation (redox) environment due to its three cysteine residues, namely Cys23, Cys45, and Cys106 (Janko et al., 2014), as described in Figure 2. Previous studies demonstrated that the presence of disulfide bond and/or free cysteine residues, or in other words: the different redox states of HMGB1, play a very crucial role towards HMGB1 localization (D. Tang et al., 2010), the change of its activities including the control of its pro-inflammatory activity (Janko et al., 2014), and even determining the interaction partner of HMGB1 (Yang et al., 2012). A disulfide bond between Cys23 and Cys45 of HMGB1 is rapidly formed in their oxidized state. The glutathione and thioredoxin systems in the cellular environment take turn in alternating the redox states of HMGB1, which varies in different cell compartments (Sahu, Debnath, Takayama, & Iwahara, 2008). On the

other hand, the Cys106 residue is in its free, reduced state and was frequently showed to aid HMGB1 in binding to its receptors (Yang, Antoine, Andersson, & Tracey, 2013). Consequently, the reduction or oxidation of the cysteine residues determines the specific physiological impact of the interaction between HMGB1 and each of its receptors. For example, the oxidation of all cysteine residues, or Cys106 alone, or substitution of any of the cysteine residues will prevent TLR4-dependent signaling (Yang et al., 2013), regulate the autophagic flux and translocation of HMGB1 itself (D. Tang et al., 2010). Through the interaction with RAGE, the reduced HMGB1 was reported to specifically mediate autophagy (D. Tang, Billiar, & Lotze, 2012). Recent study demonstrated that oxidation of the free Cys106 hinders the pro-inflammatory activity of HMGB1 and promotes immune tolerance (Janko et al., 2014).

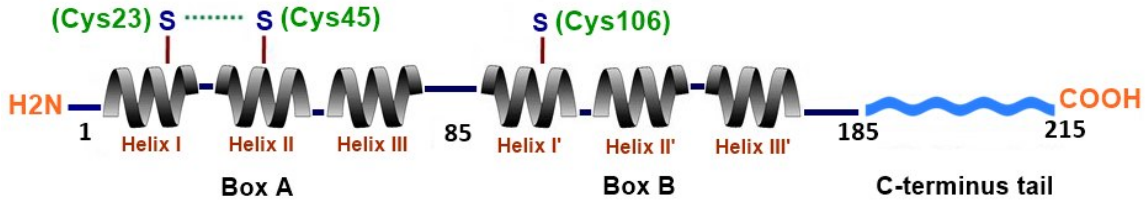

Figure 2. Illustration of HMGB1 Structure.

Structural change of HMGB1 depends on certain physiochemical microenvironment

Proteins represent one of the main components of cell microenvironment, and are paramount for cellular functions. It is known that differentiated cells have myriads of protein expression profile, which is specific to the type of the cells (Barthes et al., 2014). This is important in determining the level of protein expression which subsequently regulates the cell functions and, in cases such as mutation, may causes many types of diseases (Barthes et al., 2014). To carry out their functions, proteins must undergo chemical modification. Such alterations of proteins are crucial in regulating cellular signals and the functions of the protein itself (Liu, Chan, & Chan, 2016). The chemical modifications on protein conformation heavily affect the interactions between proteins. One of the crucial structural changes is the capability of each protein to form oligomer (Ali & Imperiali, 2005), or clusters, in which the protein interacts with itself. It has been suggested that oligomers comprises roughly one third of the cellular proteins. Oligomers also implied to be favored due to its higher activity because it increases the number of active sites, and it has more resistance to degradation (Ali & Imperiali, 2005).

Proteins commonly interact with themselves before binding to their specific partner. This activity is largely influenced by the physiochemical factors of its microenvironment, and promotes a large variety of cellular functions or even diseases. In the disease states, the composition of microenvironment may change, and under that condition protein-protein interactions takes place (Xie et al., 2008). For an instance, two RAGE molecules, or dimer, binds a tetramer of S100B and enhances neurite extension and neuron survival (Ostendorp et al., 2007). A physiochemical factor, Ca^{2+}, is important for the interaction of S100B tetramer with RAGE, where the tetramer exposes a protein-protein interaction site upon Ca^{2+} binding (Ostendorp et al., 2007). RAGE interacts with AGE, which promotes the complications of diabetes, in

the presence of D-(--)-Fructose. The expression of D-(--)-Fructose is increasing in some tissues of diabetic patients and it is about 8-fold more reactive than glucose (Xie et al., 2008).

HMGB1 was assumed to form cluster to mediate the structural modification of DNA, when in 1978 it was discovered as tetramers in the rat liver cytosol (Duguet & de Recondo, 1978). The cluster of HMGB1 appears as beads, containing about 20 HMGB1 monomers, which entangled with SV40 DNA (Bonne, Duguet, & de Recondo, 1980). Interestingly, the topic of HMGB1 oligomers is not well developed since those discoveries. The subsequent different studies raised contradictive results as to whether HMGB1 self-associates or not. Based on the existing evidences and the molecular structure of HMGB1 itself, we were interested to conduct research on the self-association of HMGB1 and the possible influences from its environment. Closer examination of the dipolar nature of HMGB1 caused by the polycationic N-terminal part and the polyanionic acidic tail (Fages, Nolo, Huttunen, Eskelinen, & Rauvala, 2000), results in a speculation that HMGB1 may be sensitive towards the modulation of ionic strength in its immediate environment. In fact, we found that increasing ionic strength reduced the strength of HMGB1 tetramer, and the tetramer was more affected by changes of ionic strength than HMGB1 dimer (Anggayasti, Mancera, Bottomley, & Helmerhorst, 2016). Besides ionic strength, pH is one of the physiochemical factors which affect HMGB1 self-association due to its highly acidic C-terminus tail (Figure 2). The rate of HMGB1 self-association gradually increased when the pH value decreased from 7.4. The highest rate of self-association was shown at pH 4.8, however, there was no signal at pH 4.0 (Anggayasti et al., 2016). The effect of lowering the pH value on the extent of HMGB1 cluster formation may have connection with disease states since, for an instance, it is known that during the case of severe diabetes, pH value of blood plasma gets lower than 7.4, which is referred to as acidosis (Nelson, 2008). We also found that the inclusion of low dosage Zn^{2+} promotes HMGB1 tetramer formation (Anggayasti et al., 2016). It possibly ties up with the function of HMGB1 in DNA transcription and subsequently cell proliferation, since Zn^{2+} is associated with those biological functions and is indeed commonly associated with DNA-binding proteins (MacDonald, 2000). Last but not least, the results in our study showed that in a more oxidized condition, which mimics extracellular environment, HMGB1 predominantly exists as tetramer, whereas in a more reduced condition, such as in intracellular environment, more dimer species were detected (Anggayasti et al., 2016). It corresponds to the discussion in the previous section that HMGB1 quickly responds towards the change of its redox environment, which varies in different cell compartments. These findings is summarized in illustration in Figure 3, where we concluded that different cell compartments results in different physiochemical conditions, which promotes the formation of different size of HMGB1 oligomers.

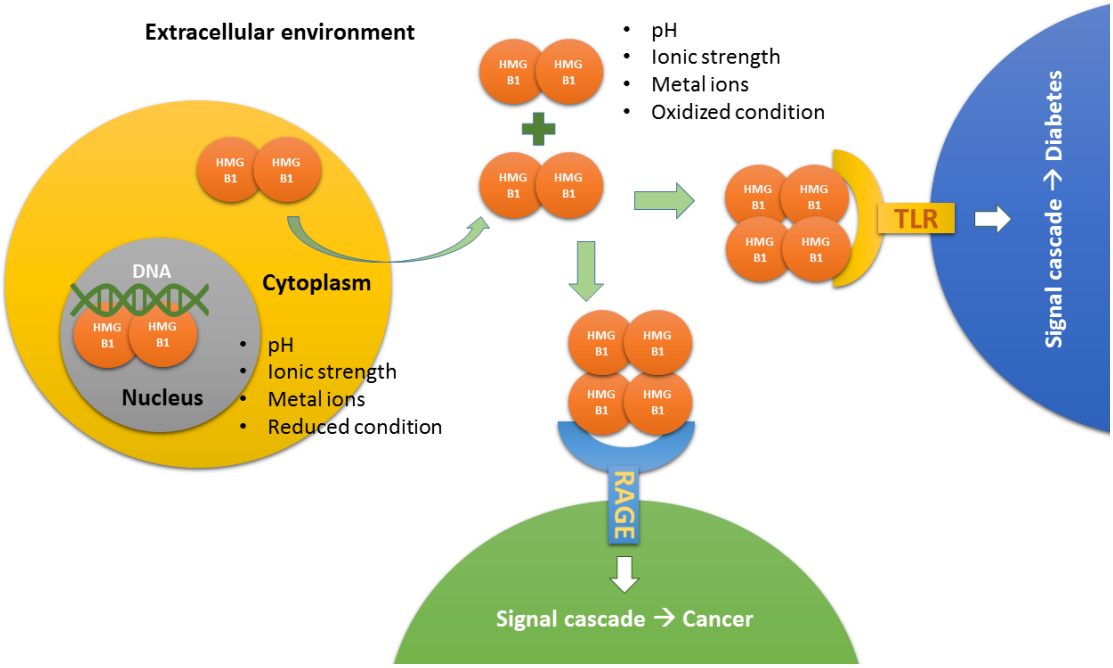

Figure 3. The Projections of HMGB1 Cluster Size, Inside and Outside Cell.

Application of the findings on eastern-world food problems

Formaldehyde is naturally produced in cells, thus it is commonly found in food products. Shiitake mushrooms contain formaldehyde between 40 to 380 mg/kg (X. Tang et al., 2009). Various fresh marine products have natural formaldehyde concentration of 2.177±1.414 mg/kg (Wang, Lee, & Ho, 2007). In fact, human biochemical functions continuously produce residual endogenous formaldehyde. However, low level of formaldehyde detoxification function in mouse model could turn the endogenous formaldehyde to be highly toxic (Ortega-Atienza, Rubis, McCarthy, & Zhitkovich, 2016). WHO International Agency for Research on Cancer officially classify formaldehyde as a Class I carcinogen (Fenech, Nersesyan, & Knasmueller, 2016). Various studies have shown that formaldehyde is significantly connected to cancer, neurotoxicity, and other pathophysiologic effects (Ortega-Atienza et al., 2016).

Formaldehyde becomes a threat since it is used illegally in eastern world to extend the shelf-life of food. Interestingly, the inappropriate use of formaldehyde was recorded as far back as the early 1900 at Indianapolis, United States of America, where the chemical was added to preserve milk given to the infants in an orphan asylum, causing death of three infants (Correspondent, 1900). Currently, it is still common to discover that marine products in markets across China were illegally treated with synthetic formaldehyde preservatives, with extreme formaldehyde concentration range between 300 to 4250 mg/kg (X. Tang et al., 2009). In Indonesia, despite the widespread occurrence of formaldehyde-laced food products across the country in late 2005 (Tamindael, 2011), such case reemerged again in 2011. Formaldehyde was found together, although not in combination with, an antiseptic solution known as borax and a coloring agent rhodamine B (Tamindael, 2011). In 2014, the similar scandal was reported in a large scale within a noodle factory (Correspondent, 2014). Although government authorities has made warnings and officially passed the Food

Law in 2012 (Correspondent, 2014), it seems that there is a little guarantee that formaldehyde abuse will not happen again. Other than the documented cases in China and Indonesia, illegal application of formaldehyde still happens throughout Asian region, such as Vietnam, Thailand, and even Bangladesh (Correspondent, 2009).

In molecular level, recent studies have proposed that formaldehyde is closely related to protein damage. Formaldehyde is suspected to trigger leukocyte genotoxicity, which is assumed to cover several symptoms. In general, the suggested symptoms involved DNA and/or protein damage, which subsequently disrupts nuclear division during mitosis. DNA-protein crosslinks and inflammation-induced ROS formation contribute to the aforementioned damages (Fenech et al., 2016). Other than that, it was found that formaldehyde could promote aggregation of proteins when it is applied to cells, regardless of the cell cycle phases. Interestingly, formaldehyde contributes to the damage of both nuclear and cytoplasmic proteins (Ortega-Atienza et al., 2016). A recent study proposed that formaldehyde can condense with free cysteine in the extracellular fluid of *Escherichia coli*, especially when the cysteine is exposed to oxidative stress. This study also reported that formaldehyde also interacts with cysteine within polypeptide or protein, subsequently results to structural change (Liu et al., 2016).

Natural physiochemical factors in cell microenvironment are the determining factor in modification of protein structure and function, however, the presence of external chemicals are also proven to modify protein architectural assembly. Therefore, in connection with our research findings on the effect of redox states of the cysteine residues of HMGB1, it may be of interest to further investigate whether formaldehyde would interact with HMGB1. Considering its carcinogen property and its ability to induce protein aggregation, which are also related closely to the characteristics of HMGB1, formaldehyde may potentially interact with the cysteine residues of HMGB1, and even promote its self-association. If this research is carried on in the future, it is expected that the results would contribute to enhance the understanding of molecular mechanism of cancer in regards to HMGB1, and may provide an insight on the effect of adding formaldehyde on food which is still quite commonly happen in Asia.

Acknowledgements

The research on the physiochemical factors which contributes to HMGB1 self-association, as published in Biochimica et Biophyisica Acta: Proteins and Proteomics (Anggayasti et al., 2016) was done at the School of Biomedical Sciences, Curtin Health Innovation Research Institute, Curtin University, GPO Box U1987, Perth WA 6845, Australia, by the author under the supervision of Prof. Erik Helmerhorst, Prof. Ricardo L. Mancera, and Dr. Steve Bottomley.

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