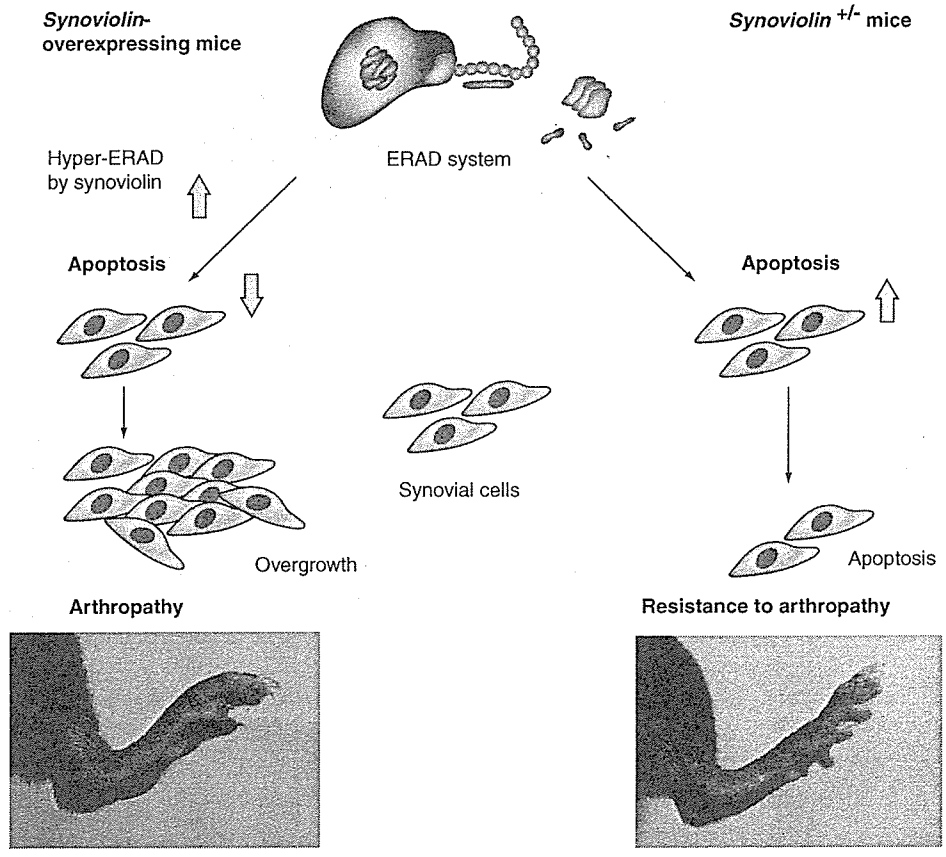
Figure 2. Drug targets for treatment of RA.



Gain-of-function of synoviolin results in the development of spontaneous arthropathy through the anti-apoptotic effects of synoviolin. On the other hand, *syno*$^{+/-}$ mice are resistant to the CIA model. Therefore, synoviolin could be a novel therapeutic target for RA.
CIA: Collagen-induced arthritis; ERAD: Endoplasmic reticulum associated degredation
RA: Rheumatoid arthritis.

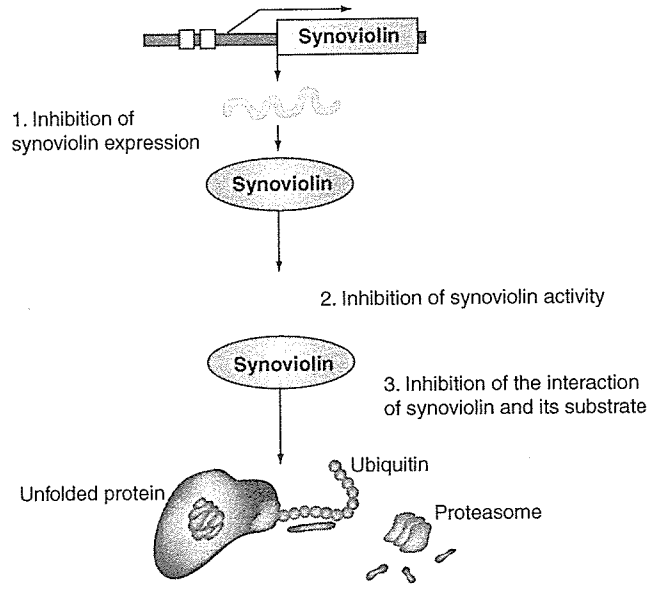
the cells from cell death through ER dysregulation [25–27]. Synoviolin is involved in this ERAD system as an ubiquitin ligase [19,28,29] and takes part in rescuing the cells from cell death. In synovial cells of CIA-*syno*$^{+/-}$, which have impaired the ERAD system owing to the lack of synoviolin, the apoptotic cells are significantly increased [19]. Therefore, in CIA-*syno*$^{+/-}$, synovial cells cannot avoid apoptotic cell death because of lack of synoviolin, thus preventing synovial cell overgrowth. However, in RSCs, hyperactivation of the ERAD system by overexpression of synoviolin could prevent synovial cell apoptotic death, consequently leading to synovial hyperplasia.

Dysfunction of the ERAD system has been implicated in various disorders. For instance, production of expanded polyglutamine causes certain inherited neurodegenerative disorders [30–32]. Furthermore, mutation of the *parkin* gene, a well known ubiquitin ligase protein in the ERAD system, is thought to result in neuronal death of the substantia nigra in patients with autosomal recessive juvenile parkinsonism [33]. The authors first postulated that hyperactivation of the ERAD system could lead to proliferative diseases and then introduced a novel concept, the hyper-ERAD disease (Figure 1).

Possible drug target for the treatment of RA

It was demonstrated that the etiology of RA could be based on hyperactivation of the ERAD system due to overexpression of synoviolin and it was concluded that synoviolin could be considered as

Figure 3. Development of synoviolin-based therapy for rhematoid arthritis.



Possible pathways in which synoviolin could be targeted for the treatment of rheumatoid arthritis.

a novel therapeutic target of RA (Figure 2). In making synoviolin a therapeutic target, three methods can be considered (Figure 3).

First, overexpression of synoviolin in RSCs could lead to the suppression of hyper-ERAD. In order to control the amount of synoviolin, it is important to elucidate the transcriptional regulation of synoviolin. Clarification of the mechanisms of transcriptional regulation of synoviolin should allow the suppression of transcription of synoviolin, thus avoiding a hyper-ERAD state. The authors recently identified a crucial site for *synoviolin* expression in the *synoviolin* proximal promoter, in other words, the Ets binding site, and that the growth-associated binding protein (GABP)-α/β complex is essential for its transcriptional regulation [34]. Thus, it is expected that RA gene therapy will become possible in the future.

Second, synoviolin is an E3 ubiquitin ligase that acts in the ERAD system, which suggests inhibition of its enzymatic activity. Blockade of synoviolin enzymatic activity should be associated with suppression of the hyper-ERAD state. In this regard, human *synoviolin*-overexpressing mice with a lack of enzymatic activity did not show any phenotype, including arthritis [Unpublished Data]. Therefore, there is a need to develop synoviolin inhibitors.

Third, since synoviolin is an E3 ubiquitin ligase, one could intercept synoviolin-substrate interaction. Synoviolin cannot function as an enzyme in the absence of an interaction with the substrate. Moreover, it is conceivable that not only synoviolin but also its substrate are expressed highly or specifically in RSCs. This is supported by the results of studies in *synoviolin*-overexpressing mice only showing arthritis despite the systemic expression. However, a substrate of synoviolin has not yet been identified. Thus, there is a need for further studies to identify a synoviolin substrate. In this regard, the authors are currently conducting such studies using the yeast two-hybrid system [Yamasaki S *et al.*, Zhang L *et al.*, Unpublished Data]. If a specific substrate of synoviolin in RSCs is identified, it is expected that disturbances of the synoviolin–substrate interaction could be used to prevent RA flares. Moreover, it is predicted that this may enhance the development of antibody therapies.

In any case, since CIA was almost completely suppressed in *syno*$^{+/-}$, further studies should be conducted to investigate the impact of approximately 50% inhibition of the amount and/or activity of synoviolin. Moreover, since synovial cell outgrowth is a common event in RA, new drugs designed to block *synoviolin* expression/activity might help find the cure of RA.

The development of a synoviolin-based marker for the diagnosis of RA, together with the development of drugs designed to block synoviolin expression and/or activity could perhaps allow the identification of the disease at an early stage and administration of effective therapy for RA.

Conclusion

Synoviolin is an important causative factor of RA, and our findings could open new avenues of investigation into the pathogenesis of RA.

Future perspective

RA has a negative impact on quality of life. Cytokines released from immune cells cause chronic inflammation and stimulate the proliferation of synovial cells that destroy bone and cartilage of joints. However, nearly 25% of RA patients do not respond to anticytokine or anti-inflammatory therapies. This may be because synovial cells acquire the autonomous proliferation ability that could be controlled by an ERAD-associated E3 ubiquitin ligase synoviolin. Therefore, we believe

that our findings will help design novel therapies for RA and expect development of selective inhibitors of synoviolin in the future.

Moreover, bone and cartilage destruction of joints in RA is one of the most serious terminal symptoms, thus we are considering the possibility of synoviolin involvement in these phenomena. Clarification of this point is our next subject, and we hope our research will help in the establishment of a new therapy.

Acknowledgements

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Executive summary

Background

- Rheumatoid arthritis (RA) affects approximately 1% of the adult population worldwide.
- RA is a disease associated with generalized symptoms related to the whole body especially to painful joints.
- The burden of musculoskeletal diseases on society has been recognized throughout the world and RA is defined as one of the most important diseases in the Bone and Joint Decade, launched by the World Health Organization in 2000 to reduce the social and financial costs of musculoskeletal disorders to society.

Pathology

- RA includes chronic inflammation of systemic joints associated with overgrowth of synovial cells, which eventually causes cartilage and bone destruction in the joints.

Synoviolin

- Cloning of synoviolin from rheumatoid synovial cells ocurred using immunoscreening.
- Synoviolin is an endoplasmic reticulum (ER)-resident membrane protein.
- Synoviolin is an E3 ubiquitin ligase associated with ER-associated degredation (ERAD).
- Synoviolin is expressed ubiquitously, however it is highly expressed in the rheumatoid synovium.

Animal study

- Mice overexpressing *synoviolin* exhibit spontaneous arthropathy and a progressive synovial hyperplasia characteristic of RA patients.
- Reduced expression of *synoviolin* in mice correlated with protection from arthritis. This resistance is not due to an impaired cytokine response or reduced inflammatory cell infiltration, but to an increase in synovial cell apoptosis.

New disease concept

- RA is an ERAD activated disease caused by by overexpressed *synoviolin* in rheumatoid synovial cells.

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The nuclear import of RNA helicase A is mediated by importin-α3

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Abstract

RNA helicase A (RHA), an ATPase/helicase, regulates the gene expression at various steps including transcriptional activation and RNA processing. RHA is known to shuttle between the nucleus and cytoplasm. We identified the nuclear localization signal (NLS) of RHA and analyzed the nuclear import mechanisms. The NLS of RHA (RHA-NLS) consisting of 19 amino acid residues is highly conserved through species and does not have the consensus classical NLS. In vitro nuclear import assays revealed that the nuclear import of RHA was Ran-dependent and mediated with the classical importin-α/β-dependent pathway. The binding assay indicated that the basic residues in RHA-NLS were used for interaction with importin-α. Furthermore, the nuclear import of RHA-NLS was supported by importin-α1 and preferentially importin-α3. Our results indicate that the nuclear import of RHA is mediated by the importin-α3/importin-β-dependent pathway and suggest that the specificity for importin may regulate the functions of cargo proteins.

Keywords: RNA helicase A; Nuclear localization signal; Importin

RNA helicase A/nuclear DNA helicase II (RHA) was isolated as a human homologue of *Drosophila* maleless (MLE) involved in sex-specific gene dosage compensation of fruit fly [1–3]. It belongs to the DExH family of ATPase/helicase and unwinds both double-stranded DNA (dsDNA) and RNA (dsRNA) [4,5]. RHA contains several functional domains. The amino terminus has two double-stranded RNA-binding domains (dsRBD1 and dsRBD2). The catalytic core domain, containing seven well-conserved motifs and ATP binding site, is located within the central region. The minimal transactivation domain (MTAD) is next to the ATP binding site and the carboxyl terminus contains glycine-rich single-stranded nucleic acid-binding domain (RGG) [6,7].

RHA displays various functions at several stages of gene expression. For example, it is involved in transactivation in an ATP-dependent manner and/or functions as a bridging factor [8]. We showed previously that RHA mediates the recruitment of RNA polymerase II (Pol II) through MTAD to cAMP-responsive element binding protein (CREB)-binding protein (CBP) and enhancement of cAMP-mediated transcriptional activation [6]. RHA also mediates various transactivation, such as BRCA1 [9], the activation function 1 domain of mineralocorticoid receptor (MR) in a ligand-selective manner [10], and nuclear factor κB (NF-κB) [11]. In addition, recent studies showed that RHA directly binds to promoters such as the *cis*-acting transactivation response element (TAR) of HIV-1 [12],

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the *p16INK4a* promoter [13], and multidrug resistance gene (MDR1) via MEF1 [14]. These studies indicated that RHA has important roles in the transcription of several genes.

RHA localizes predominantly in the nucleus. It is also known that RHA is a shuttling protein and regulates the gene expression in the cytoplasm. In the nucleus, RHA plays roles in post-transcriptional regulation. It is included in spliceosome and involved in the processing of transcripts [15]. Furthermore, it promotes the viral gene expression and the export of the constitutive cytoplasmic transport element (CTE) containing viral mRNA. Thus, the translocation of RHA between the nucleus and cytoplasm seems to be regulated in a transcription-dependent manner [16,17]. In the cytoplasm, RHA is reported to relate with translation. These studies indicate that RHA plays a common role in the expression of a wide variety of genes at various steps.

The transport of proteins between the cytoplasm and the nucleus occurs through nuclear pore complexes (NPCs) in the nuclear envelope. These proteins contain specific sequences, the nuclear localization signal (NLS), required for the nuclear import. SV40 large T-antigen (T-NLS) and nucleoplasmin have the classical NLSs and are well analyzed. They contain one or more clusters of basic amino acids, particularly lysine, which are important for their activity. Other types of NLSs have also been identified such as M9 sequence of heterogeneous nuclear ribonucleoprotein (hnRNP) A1, which bears no sequence similarity to classical NLSs [18,19].

The nuclear import of classical NLSs is mediated by a heterodimeric receptor complex composed of importin-α and -β. Importin-α is responsible for binding to the NLS, while importin-β mediates binding of the transport complex to the NPC. Importin-α interacts with importin-β through its N-terminal importin-β-binding (IBB) domain, is rich in basic amino acids, and functions as an adapter molecule [20,21]. In addition to the import receptors, a small GTPase Ran is required for nuclear import pathway. In the cytoplasm, the GTPase-activating protein (RanGAP) hydrolyzes GTP bound with Ran to GDP and Ran-GDP rapidly is imported into the nucleus. The nuclear Ran is created by asymmetric distribution of regulatory factors for Ran. The nuclear exchange factor RCC1 (Ran GEF) promotes the exchange of Ran-GDP to Ran-GTP. Ran-GTP binds to the import receptor at the nuclear side of NPC and causes the dissociation of the NLS-import receptor complex [22–24].

Only one gene coding for importin-β has been identified. In contrast, six isoforms of importin-α have been identified in human cells, whereas there is one importin-α gene in *Saccharomyces cerevisiae*. The importin-α isoforms are classified into three groups based on their sequence homology. The first group contains importin-α1/Rch1. Although importin-α2 has similarity with importin-α1, it is found in *Xenopus laevis* and other vertebrates but not in mammals. The second group has importin-α3/Qip1 and α4/hSRP1γ, and they have 85% sequence identity. The third group consists of importin-α5/NPI-1, α6, and α7, which has 80% homology. Although there are differences in their expression levels, almost all importins are expressed ubiquitously. Previous studies showed that members of the importin-α family have different substrate specificities [25–27].

Previous study reported that the transport of RHA is mediated by the domain of 110 amino acids at its C-terminus (termed NTD for nuclear transport domain), and that the import and export activities of this domain can be separated. It is also suggested that the nuclear import of NTD is importin-α/β-dependent [16]. Moreover, it is reported that methylation of NTD by protein arginine methyltransferase 1 (RPMT1) regulates the import of RHA [28]. However, the involvement of importin-α and -β has neither been demonstrated in binding studies nor in vitro nuclear import assays. For further understanding of the nuclear import of RHA, we identified in the present study the NLS of RHA and characterized its nuclear import mechanisms.

Materials and methods

Transfection. For in vivo expression experiments, HeLa cells were seeded onto 14-mm^2 square coverslips and incubated for 24 h before transfection. Transfections were performed by using FuGENE 6 reagent (Roche Diagnostics) with 0.5 μg of each construct, according to the protocol provided by the manufacturer. Cells were incubated for 24 h after transfection, washed three times with PBS, and fixed with 3.7% formaldehyde in PBS for 15 min at room temperature. After removal of formaldehyde, cells were washed with PBS and water, then the coverslips were mounted and analyzed by TCS4D confocal laser microscopy system (Leica).

Construction of plasmids. The plasmids encoding the enhanced green fluorescent protein fusion protein (pEGFP-RHA) were constructed by inserting the RHA fragments into pEGFP-C2 (Clontech Laboratories). The fragments of RHA1, 2, 3, and 4 regions were obtained from pGEX RHA1, 2, 3, and 4 [8], respectively, and inserted into pEGFP-C2. To create the expression plasmid encoding the EGFP-pyruvate kinase (PK) fusion protein, the fragment corresponding to the PK sequence was generated by PCR. The pyruvate kinase fragment was inserted into pEGFP-C2 and named pEGFP-PK. To generate the deletion mutants, the fragments were generated by PCR and termed RHA 4b, RHA C4, RHA N2, RHA NC2, and RHA NC4, respectively. These fragments were subcloned into pEGFP-PK. For mutational analysis, substitutions of arginine or lysine with alanine were introduced into the RHA4b region by sequential PCR steps. The plasmids encoding GFP-RHA R1160A, K1163A, and R1166A were constructed by inserting the fragments of RHA derived from pEGFP-RHA and the corresponding mutated RHA4b fragments into pEGFP-C2.

For glutathione *S*-transferase pull-down assay, the fragments of deletion mutants and point mutated RHA were inserted into the bacterial expression vector pGEX-5X-1 (Amersham Biosciences). The control plasmids GST-M9-GFP and GST-T-NLS-GFP were described previously [29]. To generate pGEX-5X-1-GFP for import assay, the coding sequence of EGFP was amplified by PCR and inserted into pGEX-5X-1. For GST-RHA-NLS-GFP, a PCR fragment corresponding to RHA-NLS was inserted into pGEX-5X-1-GFP and pGEX-5X-1. To express the biotinated importin proteins as probes, importin-α1 and importin-β fragments were obtained from pGEX-2T-PTAC58 [30] and pGEX-2T-PTAC 97 [31], respectively. Fragments of other importin families were generated by RT-PCR. These fragments were subcloned into PinPoint-Xa-3 (Promega).

Expression and purification of recombinant proteins. GST fusion proteins were expressed in *Escherichia coli* (*E. coli*) strain BL21 (DE3) and purified with glutathione–Sepharose beads (Amersham Biosciences), using the instructions provided by the manufacturer. To prepare import substrates for the in vitro import assays, purified GST proteins were eluted from beads and concentrated. The eluted proteins were purified with Sephadex 75 (Amersham Biosciences). For in vitro import assay,

expression and purification of recombinant importin-αs and importin-β proteins were performed as described previously [32]. The recombinant wild type and G19V Ran were expressed, purified, and charged with GDP and GTP, respectively, as described previously [29]. As probes for the binding assay, importin-αs and -β were expressed and labeled with biotin in *E. coli*. The cells were suspended into lysis buffer (20 mM Hepes, pH 7.3, 500 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1 μg/ml of aprotinin, leupeptin, and pepstatin A). The lysates were centrifuged to remove cell debris and used for probes.

In vitro import assay. Nuclear import assays were performed essentially as described previously [33,34]. Total cytosol from Ehrlich ascites tumor cells was prepared as described previously. For the competition experiments, biotinylated BSA, which was chemically coupled to a synthetic peptide containing the T-NLS (T-BSA) [34] and recombinant IBB [35], was prepared as described previously and added to the reaction mixtures as an unlabeled competitor.

Pull-down assay. The bacterial lysates containing biotin-tagged importin-α and -β were diluted fivefold with Probe dilution buffer (20 mM Hepes, pH 7.3, 1 mM EDTA, 1 mM DTT, 0.0625% Tween 20, 6.25% glycerol, and protease inhibitors). The diluted bacterial lysates were precleaned by incubation with GST bound to glutathione–Sepharose beads in binding buffer (20 mM Hepes, pH 7.3, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.05% Tween 20, 5% glycerol, and protease inhibitors) for 1 h at 4 °C. After centrifugation, supernatants were incubated with 2 μg of GST fusion proteins immobilized on glutathione–Sepharose beads for 4 h at 4 °C. At the end of incubations the beads were washed four times with binding buffer and bound fractions were separated by SDS–PAGE. Recovered importin-αs and -β were detected by using streptavidin conjugated with horseradish peroxidase (HRP) (Amersham Biosciences).

Result

NLS of RHA consists of 19 amino acids

It is reported that RHA contains the bidirectional nuclear transport domain in its C terminal region. The region includes 110 amino acids mapped to 1150–1259 [16]. To understand further the nuclear import mechanism of RHA, we identified the minimal sequence necessary for the NLS activity. A series of deletion mutants were constructed and expressed as GFP fusion proteins in HeLa cells (Fig. 1A). The C terminal region of RHA which comprises 1138–1270 amino acids and contains NTD (RHA 4b) was localized in the nucleus, while the mutant which has the region between 1155 and 1172 deleted (RHAΔ4b) was localized in the cytoplasm (Fig. 1B). As indicated in previous studies, this region has nuclear import activity.

To identify the minimal region required for nuclear import of RHA, we generated another series of deletion mutants of RHA 4b. The mutants were termed RHA C2 (aa 1138–1173), N2 (1173–1270), NC2 (1155–1222), and NC4 (1155–1173) (Fig. 1C). To prevent the nuclear entry by passive diffusion, mutants were expressed as a fusion protein with GFP-pyruvate kinase. PK has been used as a reporter protein for NLS identification because it is normally located in the cytoplasm and can localize to the nucleus when attached to a functional NLS. As shown as Fig. 1D, GFP-PK was localized in the cytoplasm. Three mutants containing the NC4 region accumulated in sufficient amounts in the nucleus, while RHA N2 was localized in the cytoplasm. To test the importance of the NC4 region in the context of full-length RHA, NC4 region was deleted and monitored for the effect on GFP-RHA localization. This mutant deleted the NC4 region from RHA full length (RHA ΔNLS) as well as RHA Δ4b showed cytoplasmic localization (Fig. 1B). These results indicate that the region extending from amino acid 1155 to 1173 is required for

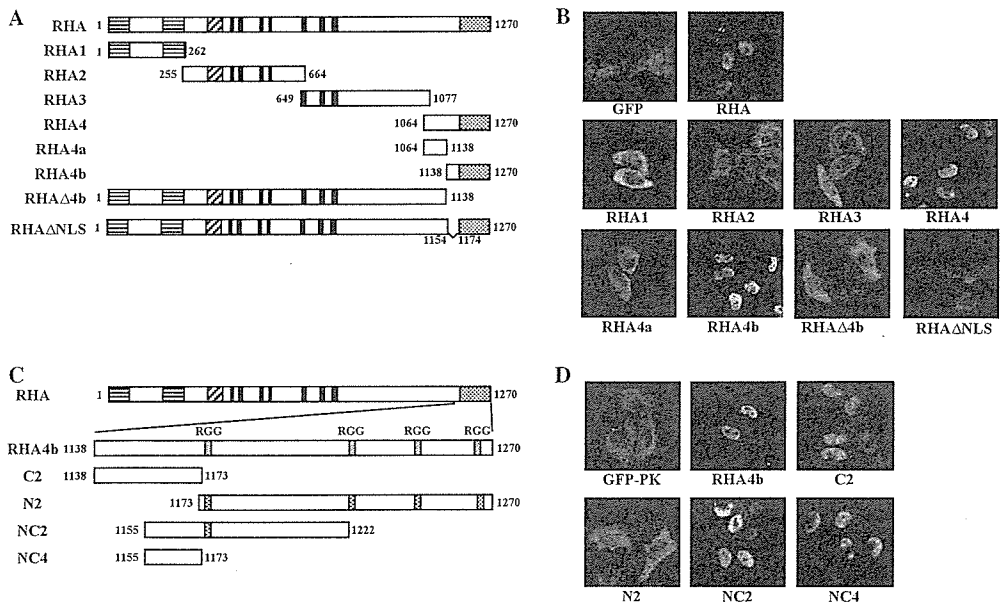

Fig. 1. NLS of RHA (RHA-NLS) consists of 19 amino acid residues. (A,C) Schematic representation of RHA and deletion mutants fused to the C-terminus of GFP. Relative position of individual domains of RHA. Solid boxes indicate the functional domain, the N-terminal double-stranded RNA binding domain (dsRBD), transactivation domain, central core helicase domain (I–VII), and C-terminal RGG box (B,D). Fluorescent microscopy of wild type (wt) and deletion mutants of RHA expressed in HeLa cells as GFP-fusion proteins.

human	1150	YGDGPRPPKMARYDNGSGY
bovin	1147	YGDGPRPPKMARYDNGSGY
mouse	1151	YGDGPRPPKMARYDNGSGY
fruit fly	1165	FSDGGGPPKRGRFETGRFT
SV40		PKKKRKV
nucleoplasmin		KRPAAIKKAGQAKKKKLD
c-Myc		PAAKRVKLD
RanBP3		PPVKRERTS

Fig. 2. RHA-NLS is highly conserved through species. The amino acid sequences of the RHA-NLS region in RHA homologues.

nuclear localization of RHA. The NC4 region consists of 19 amino acids and does not include RGG motifs. It has no significant similarity to other known NLSs such as the classical monopartite NLS, bipartite NLS or M9 of hnRNP A1 (Fig. 2).

Nuclear import of RHA is mediated by importin-α/β complex

It is reported that the nuclear import of RHA competes with NLS peptide, suggesting that the nuclear import of RHA is mediated by importin α/β pathway [16]. To characterize the nuclear import mechanism of RHA-NLS through importins, we performed the nuclear import assays in digitonin-permeabilized HeLa cells. As a transport substrate, we constructed and purified a fusion protein that comprised RHA-NLS between GST and GFP. To compare the nuclear import pathway of RHA-NLS with those of other known NLSs, T-NLS, and M9 fused to GST-GFP were used as controls. The nuclear import of T-NLS is mediated by importin-α/β and that of M9 is mediated by transportin. As shown in Fig. 3A, RHA-NLS, T-NLS, and M9 substrates all failed to effectively enter the nuclei when incubated in buffer alone (panels a, f, and k). The addition of cytosol as a source of the soluble import factors resulted in efficient nuclear entry of all three substrates (panels b, g, and l). Next, to test the involvement of Ran in the nuclear import of RHA-NLS, we used Ran mutant (G19V Ran) which lacks GTPase hydrolysis activity and inhibits several nuclear import pathways mediated by importin-β family. Addition of G19V Ran-GTP markedly inhibited the nuclear accumulation of all three substrates (panels c, h, and m). These results indicate that the nuclear import of RHA-NLS utilizes Ran-dependent pathway and requires soluble factors. To determine the contribution of nuclear import receptors for the import of RHA, two factors known to block importin-α- and -β-mediated nuclear import pathways were used in the import assays. A synthetic T-NLS peptide chemically coupled to bovine serum albumin (T-BSA) is known to bind directly to importin-α and competitively inhibit the nuclear import of classical NLSs. The N-terminus of importin-α containing IBB domain inhibits importin-β-dependent nuclear import by saturating the importin-α binding site of importin-β. Consistent with

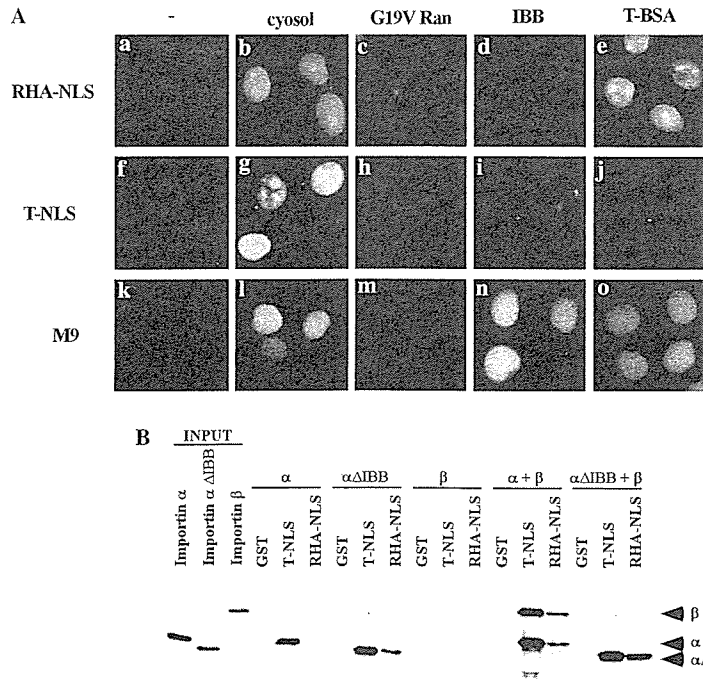

Fig. 3. RHA-NLS is transported into nuclei of digitonin-permeabilized cells. (A) In vitro nuclear import assay. Digitonin-permeabilized HeLa cells were incubated with a reaction mixture containing an ATP regeneration system, each import substrate (GST-T-NLS-GFP, GST-RHA-NLS-GFP, and GSTM9-GFP), and/or indicated effectors, a cytosol (cytosol), cytosol and G19V Ran-GTP (G19V Ran), cytosol and of IBB (IBB), cytosol and of T-BSA (TBSA). (B) Pull-down assay with GST, GST-RHA-NLS or GST-T-NLS-GFP was performed using biotinated importin-α, importin-αΔIBB and importin-β.

previous reports, the nuclear import of T-NLS was markedly inhibited in the presence of excess amount of T-BSA and IBB (panels i and j) while that of M9 was not affected (panels n and o). The nuclear accumulation of RHA-NLS was abrogated by IBB but not by T-BSA (panels e and f). These results suggest that the nuclear import mechanism of RHA-NLS is importin-β-dependent.

To determine whether the RHA-NLS region directly interacts with importin complex, pull down assay was performed. As a probe, GST-NLS expressed in bacterial cells were incubated with biotinated importin-α or -β. As shown as Fig. 3B, RHA-NLS interacted with importin-α/β complex but not with only importin-α alone, whereas T-NLS interacted with importin-α and importin-α/β complex. It is known that the IBB domain has autoinhibitory activity for nuclear import. The IBB domain interacts with its ARM domain which is a binding domain of the NLS of cargo protein. This interaction prevents importin-α to form a complex with cargo proteins and its inhibition is released by interaction of importin-α and -β [36]. To confirm whether the interaction between importin-α and RHA-NLS is inhibited by the autoinhibitory mechanism, importin-α lacking IBB domain (importin-αΔIBB) was used as a probe for pull-down assay. RHA-NLS bound with importin-αΔIBB but not with importin-α. These results suggest that RHA-NLS and importin-α could form a nuclear import complex.

Basic residues in RHA-NLS are important for nuclear import and interaction with importin complex

Although the amino acid sequence of RHA-NLS is unique to RHA homologues, they have basic amino acids as known in the classical NLSs. Previous studies indicated that certain basic amino acids in RHA-NTD are important for the nuclear import of RHA. Mutations were introduced into full-length RHA and the mutants (RHA R1160A, K1163A, and R1166A) were expressed as GFP fusion proteins in HeLa cells (Fig. 4A). The mutant R1160A was localized in the nucleus, whereas K1163A was localized in the cytoplasm. The mutant R1166A was localized in both the nucleus and cytoplasm, although the extent of nuclear accumulation was significantly lower (Fig. 4B). As described in previous studies [16], it is consistent that the two basic residues (K1163 and R1166) are important for the nuclear import activity of RHA-NLS and that K1163 is particularly essential. To test whether the basic amino acids are required for the formation of complex with importin-α and -β, we performed in vitro binding assays using each mutant of RHA-NLS fused to GST. As described previously, the wild type RHA-NLS bound to importin-α and -β. The mutant R1160A interacted with the importin complex similar to the wild type, whereas the mutant R1166A bound weakly and K1163A bound at a level similar to that of GST alone (Fig. 4C). These results emphasize the importance of the basic residues for complex formation of importin-α and β.

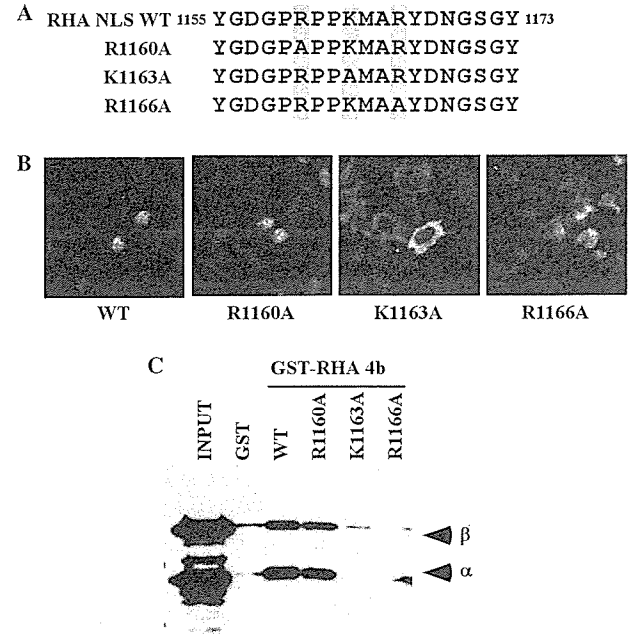

Fig. 4. Alanine substitution of basic residues in RHA-NLS reduces its nuclear migration. (A) Amino acid sequence of RHA-NLS. The mutated three basic residues are indicated by the shaded box. (B) The contribution of basic residues for nuclear import. Full-length RHA and mutant RHAs (R1160A, K1163A, and R1166A) were expressed as GFP-fusion in HeLa cells. (C) Pull-down assay with mutant RHA. RHA 4b with each substitution fused to GST was incubated with biotinated importin-α1 and β.

Importin-α1 and α3 mediate the nuclear import of RHA

Six importin-αs have been identified in human cells, and they are classified into three subfamilies, termed importin-α1, α3, α4, α5, α6, and α7 [27]. We examined the types of importin-α isoforms involved in the nuclear import mechanism of RHA. First, to determine whether RHA-NLS interacts with importin-α1, α3, α4, α5, and α7, pull-down assays were performed using biotinated importin-αs and -β expressed in *E. coli* as probes. Importin-α1, α3, and α4 bound efficiently to RHA-NLS, whereas importin-α5 bound weakly and importin-α7 did not bind (Fig. 5A). The basic residues in RHA-NLS which were important for interaction with importin-α1 had roles for interaction with importin-α3 (data not shown). Next to confirm the specificity of the interaction of RHA-NLS with importin-αs, we carried out in vitro import assays with recombinant transport factors. RHA-NLS and T-NLS substrates were combined with recombinant Ran, importin-β, an ATP-regenerating system, and/or purified importin-αs. As shown in Fig. 5B, incubation without any importin-α failed to induce efficient nuclear import of both substrates. All importin-α isoforms stimulated the nuclear import of T-NLS. For RHA-NLS, importin-α3 showed the best stimulation of the nuclear import and importin-α1 displayed a somewhat weaker effect. Interestingly, importin-α4, α5 or

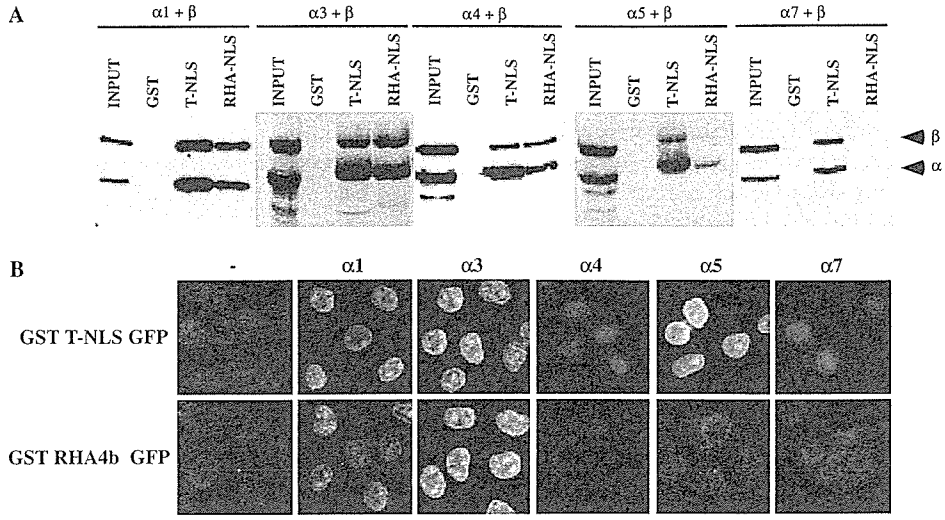

Fig. 5. Qip1 preferentially binds to RHA-NLS. (A) Pull-down assays of recombinant importin isoforms. RHA-NLS fused to GST was incubated with each importin-α, importin-β. (B) In vitro import assay with importin-α isoforms in digitonin-permeabilized HeLa cells.

α7 did not induce nuclear accumulation of RHA-NLS substrate. This was not due to inappropriate preparation of recombinant importin proteins based on the finding that T-NLS was efficiently imported by the addition of these proteins. These results indicate that the nuclear import of RHA is preferentially mediated by importin-α3.

Discussion

In this study, we identified the minimal region of the RHA that is important for its nuclear localization. The NLS of RHA is located at amino acid residue 1155–1173 and consists of 19 amino acids (Fig. 2). RHA formed a nuclear import complex through this region with importin-α and the translocation was mediated by importin-α/β and small GTP-Ran. In this process, importin-α3 was preferentially used for the nuclear import of RHA among six importin-α subfamily members. The 19 amino acid stretch has a few basic residues and the particular residues are important for the interaction with importin complex and the nuclear import of RHA.

It is reported that the NTD comprising 110 amino acids from 1150 to 1250 is essential for the nuclear import and the export of RHA. With regard to nuclear import, it has been indicated that the N terminal half of NTD containing 71 amino acids between 1150 and 1220 is more important, and that the region can transfer into the nucleus [16,28]. The 19 residues identified in the present study are part of the 71 amino acids of NTD and the minimal region for the nuclear import. These results are consistent with previous reports. Furthermore, it is reported that the NTD of RHA is methylated by PRMT1 and the methylation is necessary for the nuclear import mediated by this 110 amino acid stretch [28]. Some proteins, such as heterogeneous nuclear ribonucleoproteins A2 (hnRNP A2) and RNA-binding protein Sam68, are known to have PRMT1-methylated the arginine residues in the RGG domain, and such methylation regulates the translocation [16,37]. However, the relationship between methylation and nuclear import is still not clear. It is thought that the methylation could affect protein interaction. The RHA-NLS region is located next to the RGG domain and does not contain any RGG motifs, although NTD has four RGG motifs. These results suggest that NTD could be divided into two functional regions, the N-terminal for the formation of the import complex and C-terminal for regulation. Methylation in the C-terminal half of NTD may induce conformational change or dissociation from inhibitory factors. These changes may enable the RHA-NLS in the N terminal half of NTD to recruit with importin-α.

The nuclear import of cargo proteins with these classical NLSs utilizes various members of the importin-β families [23]. With regard to RHA-NLS, it has been suggested that the nuclear import is mediated by importin-α/β pathway. Although RHA-NLS directly interacted with importin-α, T-NLS peptide did not compete with RHA-NLS in the nuclear import assay (Fig. 3). While the exact reason for this phenomenon is not clear, several mechanisms could be postulated. One is differences of affinities between NLS and importin-α isoforms. It is reported that importin-α1 and α5 have binding activities for a wide variety of NLS sequences, while importin-α3 exhibits specificity for certain NLSs [38]. Furthermore, it is known that the expression patterns of importin-α subfamilies are tissue-specific and are changed in the differential stages. The binding specificity can be affected by abundance of nuclear import receptors. It is reported that the U1A splicesome protein has two import mechanisms. U1A is translocated through importin-α/β-dependent and cytosol independent pathways. It is suggested that the latter pathway might be negatively regulated under ordinary condition [39,40]. RHA also interacts with various molecules

and has functions in cells. These reports allow us to speculate that nuclear localization mechanisms and the efficiency of the interaction between importin-α and cargo proteins are regulated in the cell type- or signal-dependent manners. These differences in binding specificity may prevent T-NLS from competing with RHA-NLS. The second mechanisms may be related to difference in nuclear import pathway using importin-α/β. NLS interacts with the ARM domains in importin-α. It is known that two T-NLS peptides can bind with each pocket in the ARM domain in vitro binding assay [41]. It is possible that T-NLS and RHA-NLS interact with the different pockets in importin-α. These issues can be addressed by probing the structure of the nuclear import complex.

In the nuclear import process, the two types of classical NLS sequences have been well-analyzed. These sequences are classified into two major groups depending on the numbers of their charged clusters. One is a monopartite sequence with a single consensus motif of basic residues like SV40 T-antigen. The other is a bipartite sequence with two clusters of basic residues with a spacer region like nucleoplasmin. The NLS of RHA which consists of 19 amino acid residues has some basic residues, but it is not consistent with either monopartite or bipartite NLS, basic-basic-X-basic [42]. Comparisons of the amino acid alignment of the NLS sequences show that the RHA-NLS has similarity to that of Ran Binding Protein 3 (RanBP3). Mutational analysis of the NLS of RanBP3 indicated that substitution of the first amino acid in the basic cluster (lysine at amino acid 52) leads to a small nuclear localization and that loss of the double basic residues completely blocked the translocation [43]. In the case of RHA, it has three basic residues and a lysine residue at amino acid 1163 corresponding to lysine at 52 of RanBP3 which is the most important. The next residue of K52 that has only slight effect in RanBP3 is not a basic residue (methionine) in RHA (Fig. 2). RanBP3 NLS also has similarity with c-Myc NLS [43,44]. These results suggest that they could form the same group of NLS.

Our results indicated that the RHA-NLS uses importin-α1 and α3, especially α3 for the nuclear import (Fig. 5). importin-α consists of six isoforms classified into three subgroups. Most cargo proteins, such as SV40 T-antigen, hnRNP K, PCAF [45], and mCRY2 [46], are imported into the nucleus with some efficiency for all importin-α members. On the other hand, some substrate proteins have the specificities for each importin-α isoform. For example, STAT1 [47] and thioredoxin-binding protein 2 (TBP-2) [48] are imported via interaction with importin-α1 or α5. Importin-α3 with which RHA interacts preferentially is also used by other proteins. It was originally isolated as an interactant with DNA helicase Q1/RecQL [49]. STAT3 [50] and RanBP3 [43] have the monopartite of NLS sequences and RCC1 [17,45,51] and mCRY2 [46] have the bipartite NLSs. Their factors are imported into the nucleus using importin-α3. The NLSs of RHA and RanBP3 specifically use importin-α3 for the nuclear import, whereas that of c-Myc does not. The consensus motifs for each importin-α isoforms are unknown. Structural analysis of the cargo proteins could clarify these regulation mechanisms.

Recent studies suggested the involvement of importin-α isoforms in cell-specific functions. Most importins are expressed in several types of tissues except importin-α6 which is limited to the testes [17,25]. The expression levels of importin-α are regulated in cell-specific or signal-dependent manners [52]. For example, importin-α3 is strongly detected in the testes, ovaries and small intestine. Downregulation of importins with small interfering RNAs (siRNAs) revealed that importin-α3 could control cell proliferation and apoptosis [45]. In HL60 cells, the expression levels of each isoform of importin-α are affected during the differentiation stage [52]. Genetic analysis of *Drosophila melanogaster* and *Caenorhabditis elegans* also showed that the germ cells use specific importin isoforms and the individual importins cannot rescue the functions of others [27,53]. For example, *Drosophila* homologue of importin-α3 (Dα3) has a more important role in development, and Dα1 and Dα2 partially rescue the Dα3 [27,54]. However, the relationship between their expression patterns and the selectivity of their cargo proteins for remains to be clarified. RHA is expressed ubiquitously and regulates CREB-dependent transcription [6,8]. The CREB family includes CREB, CREM, and ATF-1 and they are expressed ubiquitously. It is known that CREM has specific functions in the testis. There are tissue-specific splicing isoforms CREM-τ and CREM interacts with testis-specific coactivator [55–57]. These reports suggest that RHA plays important roles as a coactivator of the CREB–CBP complex in the testis and this might be the reason why importin-α3 is used by RHA. We speculate that these may be the underlying mechanisms through which importins regulate the tissue-specific functions of ubiquitously expressed factors. In this study, RHA also bound with importin-α4 as well as importin-α3, which are classified under the same subfamily. Unexpectedly, RHA was not mediated the nuclear localization with importin-α4. It is indicated that some proteins, such as RCC1 and RanBP3 which interact with importin-α3 and α4, are translocated into nuclear by importin-α3 but not by importin-α4 in cells. It is unclear why there are such differences between importin-α3 and α4. They might need the other components for forming the stable import complexes or be competed with factors in cytosol.

In addition to transcription, RHA is involved in the splicing [15] and the export of viral RNA and shuttles between the nuclear and the cytoplasm [17]. It is suggested that RHA does not only export the mRNA but also has functions in the cytoplasm. RHA colocalizes with cytosolic Staufen in the dendrites of differentiated neuroblasts and may regulate the translation [58]. In a preliminary study, we observed that RHA could interact with a ribosomal protein (unpublished data). These findings suggest that the ratio of translocation may regulate the functions

of RHA in cells. RHA uses importin-α1 and α3. The expression level of importin-α3 with which RHA preferentially interacts is stable and are regulated such as housekeeping genes. In contrast, importin-α1 is the most widely expressed in tissues and its levels is regulated by various signals [52]. RHA is predominantly localized in the nucleus and has very important roles in transcription. These might be the reasons for the use of these two importin isoforms (importin-α1 and α3) by RHA-NLS rather than the testis-specific importin-α6. These mechanisms of the nuclear import could allow effective regulation of RHA functions.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2005.11.161.

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ORIGINAL ARTICLE

High-mobility group box 1 protein promotes development of microvascular thrombosis in rats

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Summary. *Background:* Sepsis is a life-threatening disorder resulting from systemic inflammatory and coagulatory responses to infection. High-mobility group box 1 protein (HMGB1), an abundant intranuclear protein, was recently identified as a potent lethal mediator of sepsis. However, the precise mechanisms by which HMGB1 exerts its lethal effects in sepsis have yet to be confirmed. We recently reported that plasma HMGB1 levels correlated with disseminated intravascular coagulation (DIC) score, indicating that HMGB1 might play an important role in the pathogenesis of DIC. *Objectives:* To investigate the mechanisms responsible for the lethal effects of HMGB1, and more specifically, to explore the effects of HMGB1 on the coagulation system. *Methods:* Rats were exposed to thrombin with or without HMGB1, and a survival analysis, pathologic analyses and blood tests were conducted. The effects of HMGB1 on the coagulation cascade, anticoagulant pathways and surface expression of procoagulant or anticoagulant molecules were examined *in vitro*. *Results:* Compared to thrombin alone, combined administration of thrombin and HMGB1 resulted in excessive fibrin deposition in glomeruli, prolonged plasma clotting times, and increased mortality. *In vitro*, HMGB1 did not affect clotting times, but inhibited the anticoagulant protein C pathway mediated by the thrombin–thrombomodulin complex, and stimulated tissue factor expression on monocytes. *Conclusions:* These findings demonstrate the procoagulant role of HMGB1 *in vivo* and *in vitro*. During sepsis, massive accumulation of HMGB1 in the systemic circulation would promote the development of DIC.

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Introduction

Sepsis is a complex clinical syndrome resulting from systemic inflammatory and coagulatory responses to infection [1,2]. Hyperactivation of the inflammatory system is the most important feature of sepsis, and has been the most common target of therapeutic strategies. So far, diverse therapies directed against proinflammatory mediators have revealed dramatic effects in animal models of sepsis. However, in humans, most of these strategies have not improved survival of septic patients [2]. Coagulopathy is another important feature of sepsis, and 30–50% of patients show the most severe clinical form, called disseminated intravascular coagulation (DIC) [3]. Intravascular fibrin deposition leads to obstruction of the microvascular bed, resulting in development of multiple organ dysfunction syndrome (MODS), including renal insufficiency and acute respiratory distress syndrome [4]. As DIC and MODS are associated with poor outcomes, anticoagulant therapy during sepsis seems to be a valuable option. Activated protein C (APC), an anticoagulant with a broad spectrum of anti-inflammatory effects, led to improved survival in patients with severe sepsis [5]. However, because of bleeding complications, the use of APC therapy needs to be carefully considered in relation to risks and benefits [6]. Thus, it is highly desirable to identify novel targets for the treatment of sepsis.

High-mobility group box 1 protein (HMGB1) is one promising therapeutic target for the treatment of sepsis. Blockade of HMGB1, even at later time points after onset of infection, was shown to rescue mice from lethal sepsis [7,8]. HMGB1 has pleiotropic effects both inside and outside the cell. In the cell nucleus, HMGB1 bends DNA, and promotes protein assembly on DNA targets [9]. When released from necrotic or activated cells, extracellular HMGB1 triggers inflammation, immune responses, and tissue regeneration

[10–13]. HMGB1 also acts as a lethal mediator in conditions such as sepsis, where serum HMGB1 levels are significantly increased [7,8]. However, the precise mechanisms by which HMGB1 exerts its lethal effects in sepsis have yet to be confirmed.

HMGB1 may induce lethality through multiple mechanisms. Proinflammatory activity of HMGB1 is one possible mechanism [14,15]. Procoagulant effects may be another mechanism. We recently reported that plasma HMGB1 levels correlated with DIC score and sepsis-related organ failure assessment score [16], indicating that HMGB1 might play a role in the pathogenesis of DIC and MODS. To confirm this idea, we explored the effects of HMGB1 in a thrombin-induced DIC rat model. We found that HMGB1 promoted development of microvascular thrombosis, and increased the rate of mortality.

Materials and methods

In vivo *DIC model*

Experiments involving animals were approved by the Institutional Animal Care and Use Committee of Shin Nippon Biomedical Laboratories, Kagoshima, Japan, and were conducted according to the National Institute of Health guidelines. Male Sprague–Dawley rats, each weighing 190–230 g, were used for all experiments. Before use, animals were randomly divided into groups, as indicated in Fig. 1A, were fed with regular chow, and had free access to drinking water for more than 1 week. Bovine thrombin (Mochida Pharmaceutical, Tokyo, Japan) was administered in one of the tail veins by continuous infusion at a rate of 1250 U kg^{-1} h^{-1} for 4 h. This infusion rate, which induced organ dysfunction in a reversible manner but did not affect survival, was determined in a preliminary experiment. One hour after treatment initiation, 0.4 mg kg^{-1} or 2 mg kg^{-1} HMGB1, prepared from calf and porcine thymus (a gift from Shino-Test Corporation, Sagamihara, Japan) [17], was administered as a bolus injection. The theoretical maximum HMGB1 concentration in plasma was 53 μg mL^{-1} when 2 mg kg^{-1} HMGB1 was administered as a bolus injection; however, the actual plasma HMGB1 concentrations at 5 h after administration were as low as 1.06 ± 0.68 ng mL^{-1}. It is likely that plasma HMGB1 levels of these rats are comparable with those of DIC or septic patients [7,16,17]. As a control, an equal volume of physiologic saline was administered instead of thrombin and/or HMGB1. Survival was monitored for up to 1 week (n = 10 per group). Blood tests and pathologic analyses were performed 6 h after treatment initiation (n = 5 per group).

Blood tests and pathologic analyses

Six hours after treatment initiation, blood was collected from ether-anesthetized rats, and was anticoagulated with either sodium citrate or EDTA. Anticoagulated blood with sodium

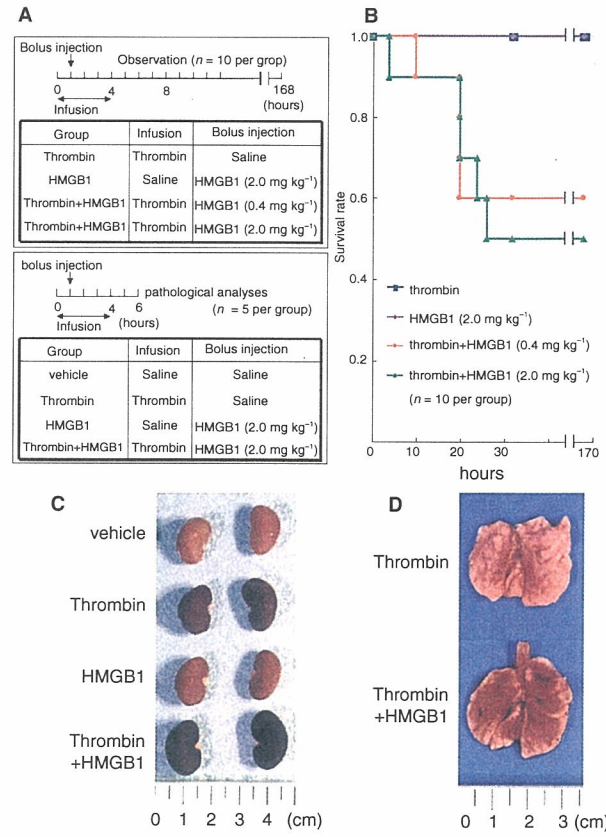

Fig. 1. High-mobility group box 1 protein (HMGB1) exacerbates renal and lung injuries and increases mortality in a thrombin-induced disseminated intravascular coagulation (DIC) model. (A) Experimental designs for survival analysis (upper panel) and pathologic analyses (lower panel) in rats. Thrombin and HMGB1 were administered i.v. by continuous infusion and by a bolus injection, respectively. As control, an equal volume of saline was administered instead of thrombin and/or HMGB1. Survival was monitored for up to 1 week. Pathologic analyses were performed 6 h after treatment initiation. (B) Survival curves of rats treated with thrombin, HMGB1 (2.0 mg kg^{-1}), thrombin plus HMGB1 (0.4 mg kg^{-1}), and thrombin plus HMGB1 (2.0 mg kg^{-1}). n = 10 per group. (C) Representative appearances of kidneys in each group. (D) Representative appearances of lungs in the thrombin-treated group and the thrombin plus HMGB1-treated group.

citrate (3.8% w/v) was centrifuged immediately for 15 min at 1710 × g, and the plasma supernatant was separated. Prothrombin time (PT), activated partial thromboplastin time (APTT) and fibrinogen were measured by standard assays in an automatic coagulometer (CA-5000; Sysmex Corporation, Kobe, Japan). Complete blood counts were performed with EDTA-anticoagulated blood, using an automated counting device (ADVIA120; Bayer Diagnostics, Dublin, Ireland).

After collection of blood samples, histopathologic analyses were performed. Firstly, organ appearance was examined macroscopically. Then, sections of formaldehyde-fixed and paraffin-embedded organs were examined microscopically. The sections were stained with either hematoxylin and eosin or phosphotungstic acid hematoxylin. Fibrin deposition was semiquantitated and given a score of 0–4 as follows: 0, no

fibrin deposition; 1, up to 25% of glomerular cross-section positive for fibrin deposition; 2, 25–50%; 3, 50–75%; and 4, more than 75%. Each group contained five rats, and 40 glomeruli per rat were evaluated in a blinded fashion.

Measurement of inflammatory cytokines

Interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) concentrations in rat plasma, collected 6 h after treatment initiation, were determined using ELISA kits for rat IL-6 and rat TNF-α respectively, as recommended by the manufacturer (BIOSOURCE, Camarillo, CA, USA).

Effects of HMGB1 on clotting time and on thrombomodulin (TM) function

For the *in vitro* clotting assay, we pooled plasma taken from five healthy volunteers, who had given their informed consent. Thrombin time (TT) and PT were measured by standard assays using an automatic coagulometer (KC1 Delta; Trinity Biotech, Bray, Ireland).

The protein C-activating cofactor activity of TM was evaluated by the modified method of Suzuki *et al.* [18], in the presence or absence of HMGB1. Briefly, recombinant human soluble TM, TM-derived peptide P-D1, or TM-derived peptide P-D_{2+3} (final concentration 0.2 nM), prepared as described previously [18,19], was incubated with HMGB1 (final concentration, 0, 1, 10 or 100 nM) at 37 °C for 30 min in a mixture of 50 mM Tris–HCl, 2 mM $CaCl_2$ and 0.1 M NaCl (pH 8.0), containing 0.1% bovine serum albumin. Then, thrombin (final concentration, 1 U mL^{-1}) and protein C (final concentration, 20 nM; donated from Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) were added, and the mixture was incubated at 37 °C for 60 min. Activation of protein C was terminated by adding antithrombin (Sigma-Aldrich, St Louis, MO, USA) and heparin (Mochida Pharmaceutical, Tokyo, Japan). The amount of APC formed during the reaction was then determined with a second assay using a chromogenic substrate (300 μg mL^{-1} S-2238; Chromogenix, Milan, Italy) in a mixture of 20 mM Tris–HCl and 0.1% NaCl (pH 7.4), containing 0.1% bovine serum albumin. Thrombin-mediated cleavage of S-2238 was blocked by antithrombin and heparin in all experiments, and residual thrombin-mediated cleavage of S-2238 was defined as 0 U in this assay. As a positive control, the activity of the ready-made APC (donated from Chemo-Sero-Therapeutic Research Institute) was measured in the presence or absence of HMGB1 (100 nM). The experiment was performed in triplicate. The endothelium-mediated protein C activation assay was performed as above, except that 0.05×10^6 human umbilical vein endothelial cells (HUVECs; Cambrex, Walkersville, MD, USA) per well in a 48-well plate were used instead of recombinant human soluble TM. Kinetic analyses were performed by measuring the activation of protein C in the presence of varying concentrations of protein C (0.1–1 μM), and fixed concentrations of TM (0.2 nM) and thrombin (0.5 U mL^{-1}), with or without HMGB1 (1 μM). The amount of APC generation was measured as above.

Thrombin-activatable fibrinolysis inhibitor (TAFI) activation was determined using a plasma-based chromogenic assay (Pefakit TAFI; Pentapharm, Basel, Switzerland). The experiment was performed in triplicate.

In vitro *assays of tissue factor and TM expression*

Peripheral blood mononuclear cells (PBMCs) from healthy volunteers (as mentioned above) were isolated using Mono-Poly Resolving Medium as recommended by the manufacturer (Dainippon Pharmaceutical, Suita, Japan). PBMCs were stimulated with HMGB1 (100 nM), heat-inactivated HMGB1 (100 nM), or vehicle in RPMI-1640 medium with 1% fetal bovine serum (FBS) and 5 μg mL^{-1} polymyxin B sulfate. The polymyxin B dose was that needed to neutralize 100 pg mL^{-1} lipopolysaccharide (LPS) from *Escherichia coli* 055:B5. Purified HMGB1 contained < 100 pg mL^{-1} LPS as assessed by a kinetic–turbidimetric assay. Following 6 h of incubation, PBMCs were harvested by gentle scraping, and were incubated for 15 min with either fluorescein isothiocyanate (FITC)-conjugated non-immune mouse IgG or an FITC-conjugated mouse monoclonal antibody against human tissue factor (American Diagnostica Inc., Stanford, CT, USA). Then, PBMCs were fixed in OptiLyse C (Beckman Coulter, Tokyo, Japan) for 10 min. Cell fluorescence was measured using an Epics XL flow cytometer equipped with the SYSTEM II software (Beckman Coulter). Monocytes were gated by forward-scatter and side-scatter properties, and were confirmed by expression of CD45 and CD14 antigens in separate experiments. Cells were defined as positive using gate settings, which excluded 99% of cells treated with FITC-conjugated non-immune immunoglobulins.

Human pulmonary artery endothelial HPAE-26 cells (American Type Culture Collection, Manassas, VA, USA) were grown and maintained in F-12K nutrient mixture medium (Gibco BRL, New York, NY, USA) supplemented with 10% FBS, 0.03 mg mL^{-1} endothelial cell growth supplement, and 10 U mL^{-1} heparin. HPAE-26 cells were stimulated with 100 nM HMGB1 or vehicle in F-12K nutrient mixture medium with 10% FBS and 5 μg mL^{-1} polymyxin B sulfate for 16 h. Then, HPAE-26 cells were harvested by gentle scraping, and were incubated for 15 min with either non-immune rabbit IgG or a rabbit antibody against human TM [20]. FITC-conjugated goat anti-(rabbit IgG) (ICN Biomedical, Aurora, OH, USA) was then added. Following 15 min of incubation, HPAE-26 cells were fixed in OptiLyse C. Cell fluorescence was measured using an Epics XL flow cytometer.

Statistical analyses

Data were presented as means ± SD. Statistical analyses were performed using analysis of variance (ANOVA) followed by the protective least significant difference Fisher's test. A probability of < 0.05 was considered significant.

Results

HMGB1 increases thrombin-induced mortality

Using the thrombin-induced DIC rat model, we investigated the effects of HMGB1 on the coagulation system. Six-week-old male rats were randomly divided into groups, as indicated in Fig. 1A. In this model, the thrombin-treated rats all developed reddish urine and dyspnea. However, these signs were reversible, and all rats in the thrombin-alone group survived (Fig. 1B). In contrast, combined administration of thrombin and HMGB1 caused severe reddish urine and dyspnea, and half of the rats were dead within 2 days. No apparent changes were observed in the HMGB1-alone group during the 1-week observation period. These findings suggested that HMGB1 did not act as a lethal mediator by itself, but increased thrombin-induced mortality.

To investigate the potential cause of death, we performed pathologic analyses. In the vehicle group and the HMGB1-alone group, no gross abnormalities were detected in organ morphology, including kidneys and lungs (Fig. 1C, and data not shown). In the thrombin-alone group, kidneys were enlarged and dark in color, and lungs were discolored with brown spots (Fig. 1C,D). These findings were much more severe in the thrombin plus HMGB1 group. Taken together, these observations indicated that HMGB1 increased susceptibility to acute renal and lung injuries in the thrombin-induced DIC rat model.

HMGB1 accelerates glomerular fibrin deposition, renal tubular degeneration and alveolar hemorrhage in a thrombin-induced DIC model

To further explore the effects of HMGB1 on thrombin-induced organ failure, we analyzed histologic changes in each group. As shown in Fig. 2A,B, administration of thrombin resulted in a small amount of fibrin deposition in renal glomeruli and degeneration in renal tubules. Compared to thrombin-treated rats, thrombin plus HMGB1-treated rats had significantly increased fibrin deposition in the glomeruli (fibrin deposition scores 1.2 ± 1.6 and 3.4 ± 1.3, respectively, $P < 0.05$). Administration of HMGB1 alone elicited no changes in glomeruli and tubules (fibrin deposition scores 0). Thus, HMGB1 promoted the development of microvascular thrombosis in the kidneys of thrombin-induced DIC rats.

We also analyzed histologic changes in lungs. Administration of thrombin, HMGB1 or thrombin plus HMGB1 increased alveolar wall thickness, interstitial edema, and cell infiltration (Fig. 2C). In addition, administration of thrombin plus HMGB1 caused alveolar hemorrhage (Figs 1D and 2C). In this study, we could not detect fibrin deposition in the lungs of any rats. No other organs, including liver, spleen, and brain, had apparent abnormalities in each group (data not shown). These results suggested that HMGB1 acted as a lethal mediator in the thrombin-induced DIC model, at least in part through acceleration of microvascular thrombosis and subsequent renal and respiratory failure.

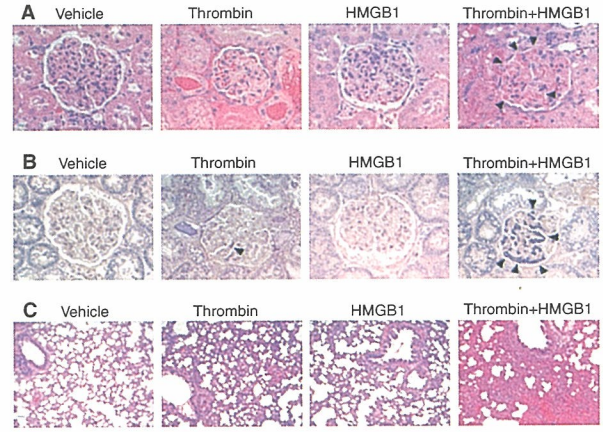

Fig. 2. High-mobility group box 1 protein (HMGB1) accelerates glomerular fibrin deposition, renal tubular degeneration and alveolar hemorrhage in a thrombin-induced disseminated intravascular coagulation model. (A) Hematoxylin and eosin (H&E) staining of kidney tissue sections in vehicle-treated, thrombin-treated, HMGB1-treated and thrombin plus HMGB1-treated rats 6 h after treatment initiation. Arrowheads indicate fibrin deposition. Scale bar: 10 μm. (B) Phosphotungstic acid hematoxylin staining of kidney tissue sections. Arrowheads indicate fibrin fibers stained in dark blue. Scale bar: 10 μm. (C) H&E staining of lung tissue sections. Scale bar: 50 μm.

Thrombin and HMGB1 act synergistically to promote coagulation in vivo

The observation that HMGB1 accelerated fibrin deposition in the thrombin-induced DIC model suggested that HMGB1 might affect hemostatic profiles. As shown in Fig. 3A, the PT and APTT of thrombin-treated rats were prolonged 1.3-fold, compared to those of vehicle-treated rats. HMGB1 treatment did not affect PT or APTT. Interestingly, the PT and APTT of thrombin plus HMGB1-treated rats were prolonged more than 2-fold compared to those of vehicle-treated rats, and more than 1.5-fold compared to those of thrombin-treated rats ($P < 0.001$ and $P < 0.001$, respectively). These results suggested that HMGB1 promoted thrombin-induced consumption of coagulation factors. Thrombin plus HMGB1-treated rats showed significantly lower fibrinogen and platelet concentrations than vehicle-treated or HMGB1-treated rats. They tended to show lower fibrinogen and platelet levels than thrombin-treated rats, although these differences were not significant. Taking into consideration that fibrinogen levels of HMGB1-treated rats were significantly higher than those of vehicle-treated rats ($P < 0.05$), consumption of fibrinogen in thrombin plus HMGB1-treated rats might be canceled out by the upregulation of fibrinogen induced by HMGB1.

Fibrin deposition in blood vessels might cause red blood cell fragmentation and hemolysis. We tested whether these phenomena also occurred in our experimental model. Red blood cell fragmentation and hemolysis were observed in thrombin-treated rats, and to an even greater extent in thrombin plus HMGB1-treated rats (data not shown). It is conceivable that hemolysis may further exacerbate vasculopathy [21]. Thus, not

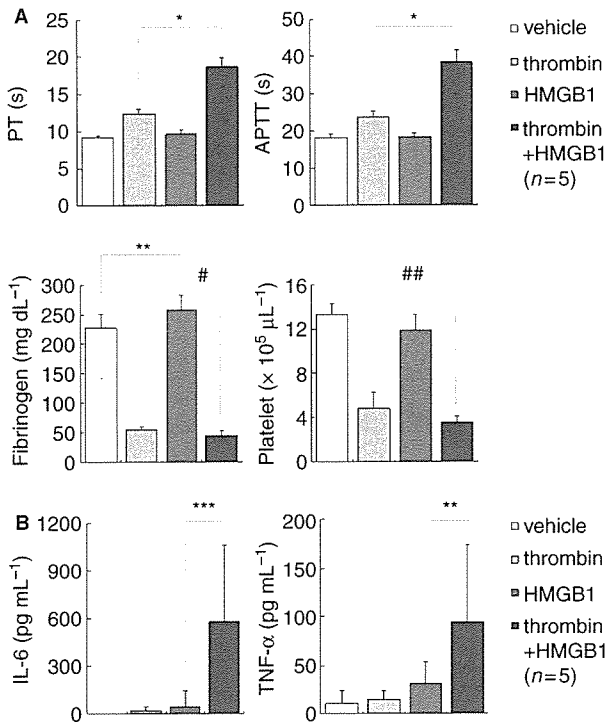

Fig. 3. Thrombin and high-mobility group box 1 protein (HMGB1) act synergistically to promote coagulation and inflammation *in vivo*. (A) Hemostatic profiles (prothrombin time, activated partial thromboplastin time, fibrinogen concentration, and platelet count) of rats treated with vehicle, thrombin, HMGB1 and thrombin plus HMGB1 6 h after treatment initiation. $n = 5$ per group. (B) Plasma levels of interleukin-6 (left panel) and tumor necrosis factor-α (right panel) 6 h after treatment initiation in each group. $n = 5$ per group. $^*P < 0.001$; $^{**}P < 0.05$; $^{***}P < 0.01$; $P = 0.42$; $^{\#\#}P = 0.12$.

only the histologic findings but also the coagulation parameters supported the concept that HMGB1 exacerbated DIC *in vivo*.

Thrombin and HMGB1 act synergistically to promote inflammation in vivo

Thrombin, a coagulation protease, can evoke an inflammatory response through protease-activated receptors [22]. HMGB1 can evoke an inflammatory response through the receptor for advanced glycation end-products and possibly through Toll-like receptors 2 and 4 [14,15]. In our *in vitro* experiments, thrombin and HMGB1 were capable of stimulating proinflammatory cytokine production in murine macrophage-like RAW 264.7 cells (data not shown). In our experiment with rats, thrombin or HMGB1 also induced proinflammatory cytokines such as IL-6 and TNF-α (Fig. 3B). Interestingly, rats stimulated by both thrombin and HMGB1 exhibited more than tenfold higher levels of IL-6 than rats stimulated by thrombin or HMGB1 alone, which exhibited a rather mild inflammatory response ($P = 0.002$ and $P = 0.004$, respectively). Taken together, thrombin and HMGB1 acted synergistically to promote coagulation and inflammation, leading to multiple organ failure (Figs 2 and 3).

HMGB1 stimulates tissue factor expression on monocytes

To identify the mechanisms whereby HMGB1 promotes development of microvascular thrombosis, we examined the effects of HMGB1 on the coagulation system *in vitro*. Neither TT nor PT was affected by HMGB1 *in vitro* (TT 14.8 ± 0.3 s without HMGB1, compared to 14.6 ± 0.2 s with 100 nM HMGB1, and PT 13.2 ± 0.1 s without HMGB1, compared to 13.2 ± 0.1 s with 100 nM HMGB1).

The prothrombotic effects of HMGB1 in rats might be associated with receptor-mediated cellular responses, such as upregulation of procoagulant molecules or downregulation of anticoagulant molecules. We examined the effects of HMGB1 on cell surface expression of tissue factor and TM by flow cytometry (Fig. 4). Although TM expression on endothelial cells did not change, tissue factor expression on monocytes was increased by HMGB1 stimulation. Contaminating LPS was not responsible for the stimulatory effect of the HMGB1 preparation, because: (i) heat-treated HMGB1 did not induce tissue factor expression at all; and (ii) contaminating LPS was < 100 pg mL^{-1}, and this concentration of LPS did not induce tissue factor expression under our experimental conditions with 5 μg mL^{-1} polymyxin B (data not shown). Thus, HMGB1-induced tissue factor expression on monocytes might be one mechanism responsible for the prothrombotic effects.

HMGB1 inhibits protein C activation in vitro

The prothrombotic effects of HMGB1 in rats might be due to inhibitory effects on the anticoagulant system. We first examined the effects of HMGB1 on antithrombin, and found that HMGB1 did not affect antithrombin binding to thrombin (data not shown). We recently reported that TM bound HMGB1, thereby suppressing induction of proinflammatory events [23]. This raised the question of whether HMGB1, in

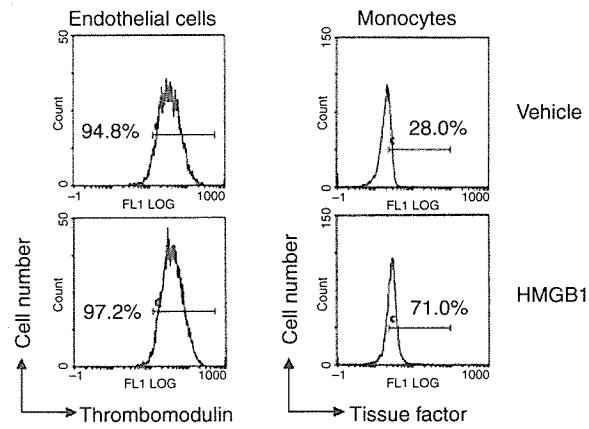

Fig. 4. High-mobility group box 1 protein (HMGB1) stimulates tissue factor expression *in vitro*. Representative data for thrombomodulin expression on HPAE-26 cells (left panel) and tissue factor expression on peripheral blood mononuclear cells (right panel) in the absence (upper panel) or presence (lower panel) of HMGB1 stimulation. Percentages indicate fractions of antigen-positive cells.

turn, would suppress TM function. As TM acts as a cofactor for thrombin-mediated activation of protein C and TAFI [24], we examined the effects of HMGB1 on the cofactor activity of TM. As shown in Fig. 5A, HMGB1 dose-dependently inhibited activation of protein C mediated by the thrombin–TM complex. In kinetic analyses, HMGB1 exhibited no change in the V_{max} and a 2.1-fold augmentation in the K_m for protein C (Fig. 5B). HMGB1 had little effect on the activity of ready-made APC (data not shown), indicating that HMGB1 inhibited conversion of protein C to APC. Also, HMGB1 had no effect on protein C activation mediated by snake venom protein (data not shown), indicating that HMGB1 specifically inhibited protein C activation mediated by the thrombin–TM complex. In addition, HMGB1 had no effect on TAFI activation mediated by the thrombin–TM complex (1 ± 0.06 activity without HMGB1 compared to 0.99 ± 0.08 activity with 100 nM HMGB1), indicating that HMGB1 specifically inhibited TM cofactor activity for protein C activation.

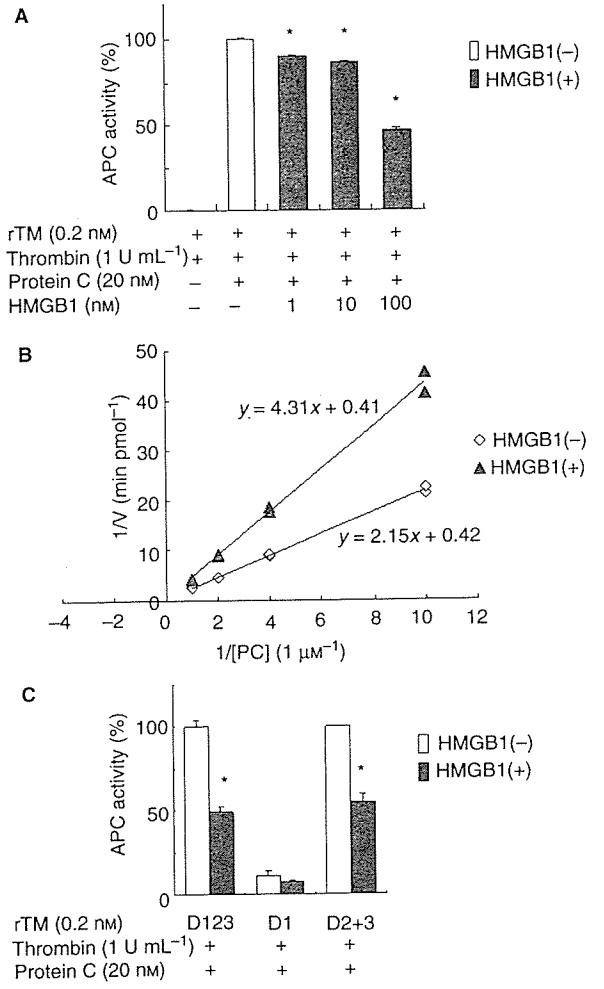

Fig. 5. High-mobility group box 1 protein (HMGB1) inhibits protein C activation *in vitro*. (A) Using a chromogenic substrate S-2238, we examined activated protein C (APC) activity in the absence (white bar) or presence (gray bar) of HMGB1. APC activity was expressed as percentages of that without HMGB1 (white bar). The experiment was performed in triplicate. $*P < 0.001$ compared to APC activity without HMGB1 (white bar). rTM, recombinant thrombomodulin. (B) Lineweaver–Burk plots for thrombin–thrombomodulin-mediated protein C activation in the absence or presence of HMGB1. Enzyme velocity (V = molecules of protein C activated min^{-1}) was calculated, and $1/[PC]$ vs. $1/V$ was then plotted. PC, protein C. (C) APC activity was measured in the absence (white bar) or presence (gray bar) of 100 nM HMGB1 with various TM-derived peptides: full-length TM (D123), P-D1, and P-D_{2+3}. $*P < 0.001$ compared to APC activity without HMGB1 (white bar).

Previous studies have demonstrated that D1 (lectin-like domain) of TM has anti-inflammatory properties and is essential for HMGB1 binding [23,25], and D2 (endothelial growth factor-like domain) of TM is essential for thrombin binding and protein C activation [19]. To examine whether binding of HMGB1 to TM was involved in the inhibitory effects on protein C activation, we used a D1 deletion mutant of TM (P-D_{2+3}) for the protein C activation assay. As shown in Fig. 5C, HMGB1 inhibited protein C activation mediated by P-D_{2+3} to the same extent as that mediated by full-length TM (D123). These results indicated that binding of HMGB1 to TM was not involved in the inhibitory effects on protein C activation. Considering that the concentrations of HMGB1 (100 nM) were much higher than those of TM (0.2 nM) in the assays, unbound HMGB1 might be responsible for these effects. Finally, we examined the effects of HMGB1 on cell surface protein C activation, and found that HMGB1 significantly inhibited protein C activation mediated by HUVECs (1 ± 0.01 APC activity without HMGB1, compared to 0.79 ± 0.03 APC activity with 100 nM HMGB1, $P < 0.001$). Thus, HMGB1 inhibited protein C activation mediated by the thrombin–TM complex *in vitro*.

Discussion

Recent studies have identified HMGB1 as a lethal mediator of sepsis, as well as a promising therapeutic target for sepsis [2,7,8]. Defining the roles of HMGB1 during experimental sepsis in greater detail is now important for understanding the pathogenesis of sepsis and designing novel therapeutic strategies. Here, we have shown that HMGB1 promotes the development of microvascular thrombosis, and increases mortality in a thrombin-induced DIC model. In combination with 1250 U kg^{-1} h^{-1} thrombin, HMGB1 administration at 0, 0.4, 2 and 5 mg kg^{-1} to rats resulted in survival rates of 100%, 60%, 50%, and 50%, respectively (Fig. 1B and data not shown). In contrast, when the dose of thrombin was increased to 2000 U kg^{-1} h^{-1}, the survival rate decreased to 20%. Although these results indicate that HMGB1 is indeed a lethal mediator in the DIC model, they also indicate that 5 mg kg^{-1} HMGB1 may be redundant, and that the lethal activity of HMGB1 may be dependent on other factors such as thrombin.

Thrombin, the principal procoagulant enzyme générated at sites of injury, is converted to an anticoagulant enzyme at distant sites through its interaction with the endothelial cell protein TM, and subsequent generation of APC [20,26]. During sepsis, this anticoagulant mechanism is compromised

[27]. In part, this is due to proinflammatory mediators, such as TNF-α, that reduce TM expression on endothelial cells [28]. In our *in vitro* experiments, HMGB1 reduced the activity of thrombin–TM complexes. The percentage inhibition of APC generation in the presence of 1, 10 and 100 nM HMGB1 was 10%, 14%, and 54%, respectively. As the plasma or serum levels of HMGB1 in DIC or septic patients have been reported to range from 0 to 10 nM [7,16,17], the percentage inhibition of APC generation in such patients may be about 10%. Considering that 40% inhibition of APC generation or heterozygous deletion of TM was previously reported to cause thrombosis in animal models [29,30], HMGB1 in septic patients at a concentration of about 1 nM may not be sufficient to cause thrombosis through inhibition of APC generation alone. It is therefore possible that other mechanisms, such as increased tissue factor expression, are also important in the aggravation of DIC by HMGB1. The molecular balance between HMGB1 and TM may also be important. On the one hand, TM sequesters HMGB1, while on the other hand, HMGB1 inhibits the activity of thrombin–TM complexes. We examined the effects of HMGB1 with 0.2 nM TM, as higher concentrations of TM might sequester 1 nM HMGB1 completely. In our *in vitro* experiments, the percentage inhibition of APC generation decreased when the TM dose was increased, possibly because the TM-unbound fraction of HMGB1 was decreased (data not shown). In our *in vivo* experiments, fibrin deposition was mainly detected in renal glomeruli. Considering that glomerular capillary loops express less TM [31], the relatively high concentration of HMGB1 might promote the development of thrombosis in glomeruli. In addition, it is conceivable that the preceding administration of thrombin might decrease the levels of TM expressed on endothelial cells [32].

Previous studies and our present results suggest possible mechanisms whereby HMGB1 exerts its lethal effects under septic conditions. HMGB1 promotes inflammatory responses by acting on monocytes, endothelial cells, and other types of cell [10,11,23,33,34]. Therefore, massive accumulation of HMGB1 in the systemic circulation would lead to systemic inflammatory response syndrome, an important feature of sepsis. In addition, our results suggest that HMGB1 in the systemic circulation promotes the development of DIC. These dysregulated inflammatory and coagulatory responses may be related to the lethal activity of HMGB1 in sepsis. We recently reported that TM interacts with HMGB1, and protects mice against lethal endotoxemia [23]. TM also interacts with thrombin, resulting in inhibition of the enzyme's procoagulant activity [26]. Binding of HMGB1 and thrombin by TM provides a mechanism for damping the amplification of inflammatory and coagulatory responses. Under septic conditions, HMGB1 and thrombin present in the circulation would propagate inflammatory and coagulatory responses to remote organs [1,34]. However, once an adjacent portion of the vessel wall with intact endothelial cells is encountered, TM-bearing cells can sequester HMGB1 and thrombin, thereby preventing them from reaching remote organs. Replacement with recombinant TM will offer therapeutic value in sepsis, as the expression of endothelial TM is impaired [27].

Some open questions remain. For example, it is important to define the exact mechanisms involved in the aggravation of DIC by HMGB1 *in vivo*, including the involvement of the protein C pathway. It is also important to elucidate the amount of HMGB1 that binds to TM *in vivo*. In our experimental model, i.v. administered HMGB1 at a dose of 2 mg kg^{-1} (theoretical plasma HMGB1 level of 53 μg mL^{-1}) was rapidly cleared from the circulation, and the plasma HMGB1 levels at 5 h after administration were as low as 1.06 ± 0.68 ng mL^{-1}. Binding of HMGB1 to TM might be one mechanism responsible for the rapid clearance of HMGB1 from the circulation. Such phenomena can be seen in clinical settings, suggesting that HMGB1 levels in plasma are lower than those at local injured sites. Another question is whether HMGB1 acts as a lethal mediator by itself. None of 10 rats died after i.v. HMGB1 administration at 5 mg kg^{-1} in our study. Three of five mice died after intraperitoneal administration of 0.5 mg of HMGB1/mouse in a previous study [7]. In contrast, administration of 0.4 mg kg^{-1} of HMGB1 in our study or 0.05 mg HMGB1/mouse in reference 7 could be lethal in DIC or septic conditions, in which other mediators, such as thrombin or LPS, exist. These findings indicate that HMGB1 may be a promoter, rather than an initiator, of DIC or sepsis.

At injured sites, hemostasis, immune responses and subsequent tissue regeneration are necessary. Recent observations have suggested that HMGB1, which is released by necrotic and inflammatory cells at sites of injury, plays important roles in local immune responses and tissue regeneration [12,13]. In addition, our present results suggest that HMGB1 may play a role in hemostasis. All these findings suggest that HMGB1 is a type of general organizer in postinjury wound healing.

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Disclosure of Conflict of Interests

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