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The Anti-Inflammatory Effects of Heat Shock Protein 72 Involve Inhibition of High-Mobility-Group Box 1 Release and Proinflammatory Function in Macrophages

Daolin Tang,² Rui Kang,²† Weimin Xiao,*‡ Haichao Wang,³§ Stuart K. Calderwood,|| and Xianzhong Xiao³*

High-mobility-group box 1 (HMGB1), a nuclear protein, has recently been identified as an important mediator of local and systemic inflammatory diseases when released into the extracellular milieu. Anti-inflammatory regulation by the stress response is an effective autoprotective mechanism when the host encounters harmful stimuli, but the mechanism of action remains incompletely delineated. In this study, we demonstrate that increases in levels of a major stress-inducible protein, heat shock protein 72 (Hsp72) by gene transfection attenuated LPS- or TNF-α-induced HMGB1 cytoplasmic translocation and release. The mechanisms involved inhibition of the chromosome region maintenance 1 (CRM1)-dependent nuclear export pathway. Overexpression of Hsp72 inhibited CRM1 translocation and interaction between HMGB1 and CRM1 in macrophages post-LPS and TNF-α treatment. In addition, overexpression of Hsp72 strongly inhibited HMGB1-induced cytokine (TNF-α, IL-1β) expression and release, which correlated closely with: 1) inhibition of the MAP kinases (p38, JNK, and ERK); and 2) inhibition of the NF-κB pathway. Taken together, these experiments suggest that the anti-inflammatory activity of Hsp72 is achieved by interfering with both the release and proinflammatory function of HMGB1. Our experimental data provide important insights into the anti-inflammatory mechanisms of heat shock protein protection. The Journal of Immunology, 2007, 179: 1236–1244.

Sepsis is a systemic inflammatory syndrome that can lead to lethal organ damage. Despite advances in antibiotic therapy and intensive care, the overall mortality from severe sepsis exceeds 30% in the United States (1). Initiated by an infection, the pathogenesis of sepsis is attributable, at least in part, to dysregulated systemic inflammatory responses characterized by excessive accumulation of various proinflammatory cytokines (2, 3).

Recent evidence indicates that high-mobility-group box 1 (HMGB1), a ubiquitous nuclear protein, is an important proinflammatory cytokine that mediates the response to infection, injury, and inflammation (4–8). Although residing predominantly in the nucleus of quiescent macrophages/monocytes, HMGB1 can be actively secreted in response to exogenous and endogenous inflammatory stimuli such as endotoxin, TNF-α, IL-1, IFN-γ, and hydrogen peroxide (4, 9–11). In addition, HMGB1 can be passively released by necrotic cells (6). HMGB1 has a chromosome region maintenance 1 (CRM1)-dependent nuclear active export pathway (12). Once released from cells, HMGB1 can bind to cell surface receptors including the receptor for advanced glycation end products, TLR-2, and TLR-4 and mediate cellular responses including chemotactic cell movement and release of proinflammatory cytokines (e.g., TNF-α and IL-1); Refs. 13–17). Anti-HMGB1 Abs or specific antagonists protect mice from lethal endotoxemia or sepsis, even when the first dose are given 24 h after onset of diseases (4, 18), establishing HMGB1 as an important therapeutic target for inflammatory diseases (5, 19).

MAPKs and the NF-κB/IκB pathway play essential roles in inflammation because of the rapidity of activation and potency in activation of transcription of NF-κB (20, 21). The MAPK family mainly includes ERK, p38 kinase, and JNK. Once activated, the MAPKs modulate the functional responses of cells through phosphorylation of transcription factors and activation of other kinases. NF-κB regulates the transcription of many genes involved in immunity, inflammation, and protection from programmed cell death (22).

The heat shock (HS) response (HSR) is a universal stress response activated in all prokaryotes and eukaryotes and is represented at the molecular level by the induction of heat shock protein (HSP) synthesis (23). The Hsp70 family is the best characterized
of these and include the constitutive Hsp73, and stress-inducible Hsp72. The HSPs are involved in protecting the organism from a variety of insults including ischemia/reperfusion and oxidative injury (24, 25). Although the underlying mechanism of this protection involves their chaperone functions (e.g., preventing abnormal protein folding or aggregation; Ref. 26), recent work also shows that HSPs directly interfere with cell death pathways including apoptosis and necrosis (27, 28). Induction of Hsp72 in vitro by heat shock or Hsp72 overexpression can reduce mortality in experimental models of septic shock, endotoxemia, and adult respiratory distress syndrome, and can regulate expression of inflammatory genes, such as TNF-α, IL-1, IL-12, IL-10, and IL-18 (29–36).

The mechanisms of anti-inflammatory regulation by HS or Hsp72 are mostly uncharacterized. However, our previous study showed that HS reduces LPS-induced HMGB1 active release from macrophage cultures (37), and nuclear Hsp72 is a negative regulator of oxidative stress-induced HMGB1 cytoplasmic translocation and release (38). Here we demonstrate that Hsp72 not only inhibits HMGB1 release induced by exogenous bacterial endotoxins or the endogenous proinflammatory cytokines TNF-α but also suppresses the proinflammatory activities of HMGB1.

Materials and Methods

Cell culture and treatment

Murine macrophage-like RAW264.7 cells were obtained from the Shanghai Type Culture Collection and cultured in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FBS, 2 mM glutamine, and antibiotic-antimycotic mix in a humidified incubator with 5% CO2 and 95% air. Recombinant human Hsp72 plasmid vector (pCDNA3.1-Hsp72) was introduced into RAW264.7 cells by liposomal delivery. After transfection, cells were cultured in RPMI 1640 (Invitrogen Life Technologies) supplemented with genetecin G418 (Promega) for further selection of transfected cells. The surviving cells in medium containing G418 were considered to be stable cell lines that contained overexpression of Hsp72 gene, and expression of Hsp72 was confirmed by Western blotting.

At 70% confluence, RAW264.7 cells were removed mechanically and resuspended in serum-free Opti-MEM I medium (Invitrogen Life Technologies). After preincubation for 2 h, RAW264.7 cells were treated with LPS (Escherichia coli 0111:B4; Sigma-Aldrich), recombinant TNF-α protein (PeproTech), or recombinant HMGB1 protein (Ref. 39; provided by Dr. Kevin J. Tracey and Dr. Huan Yang, Laboratory of Biomedical Science, and Center for Immunology and Inflammation, Feinstein Institute for Medical Research, Manhasset, NY) as indicated.

Preparation of cellular extracts

At indicated time points after the treatment, cells were harvested and washed twice with cold PBS; and nuclear and cytoplasmic extracts were prepared according to the method of Schreiber et al. (40).

HS treatment

RAW264.7 cells were sealed in screw-cap flasks containing an atmosphere of 5% CO2 and 95% air. These flasks were then immersed completely in a water bath with a measured temperature of 42.5°C. With this protocol, the medium within the flask reached 42.5°C within 5 min of immersion. After 1 h of immersion, cells were left at 37°C for 12 h and then treated with HMGB1.

Western blotting

Proteins in the whole-cell lysate, subcellular fractions, or concentrated cell culture supernatants were resolved on 10% SDS-PAGE gel, and transferred to a polyvinylidene fluoride membrane. After blocking, the membrane was washed twice with cold PBS; and nuclear and cytoplasmic extracts were added by boiling in 2x SDS sample buffer before SDS-PAGE electrophoresis.

EMSA for NF-κB activation

Nuclear extracts were prepared, and EMSA was performed using biotin-labeled oligonucleotides to measure NF-κB DNA binding activity (Pierce Chemicals). In brief, 10 μg of nuclear extract were incubated with biotin-labeled, double-stranded DNA fragment corresponding to the NF-κB site (sense strand, 5′-AGTGCAGGGAGCTTCCAGGC-3′) at 4°C in the presence or absence of 100× unlabeled NF-κB and subjected to electrophoresis at 110 V for 4 h at 4°C on a 5% polyacrylamide gel. The gels were transferred to a nylon membrane and subjected to cross-link with a UV lamp, and the signals were detected by chemiluminescence. For supershift experiments, NF-κB p65 Ab (Santa Cruz Biotechnology) was incubated with the binding reaction mixture for 30 min at room temperature before addition of the labeled oligonucleotide.

Statistical analysis

Significance of differences between groups was determined by two-tailed Student’s t test or Fisher’s least significance difference test, as indicated. Values of P < 0.05 were considered significant.

Results

Hsp72 inhibits LPS- and TNF-α-induced release and translocation of HMGB1 in RAW264.7 macrophages

We have previously shown that nonlethal HS inhibits LPS-induced HMGB1 release (37). To investigate whether this effect is mediated by Hsp72, we stably transfected RAW264.7 cells with Hsp72 encoding expression vector (RAW/Hsp72). Control cells were obtained by transfection of RAW264.7 cells with empty vector (RAW/Neo). Western analysis demonstrated overexpression of Hsp72 in RAW/Hsp72 cells compared with RAW/Neo cells (Fig. 1, A and B). Consistent with previous reports, LPS and TNF-α induced active HMGB1 release in macrophages, which was not...
altered in control-transfected cells (RAW/Neo; Fig. 1, A and B; Refs. 4, 9, and 41). However, overexpression of Hsp72 significantly inhibited LPS- and TNF-α-induced HMGB1 release (Fig. 1). Interestingly, although LPS did not induce Hsp72 expression (36), TNF-α dose dependently induced Hsp72 expression (Fig. 1, A and B; Refs. 42 and 43).

To elucidate the mechanisms underlying Hsp72-mediated suppression of HMGB1 release, we determined the effects of Hsp72 overexpression on LPS- or TNF-induced HMGB1 cytoplasmic translocation. HMGB1 was predominantly localized in the nucleus of unstimulated macrophages (Fig. 1C). However, by 12 h after LPS or TNF stimulation, HMGB1 staining was found in both the nuclear and cytoplasmic compartments of macrophages (Fig. 1C). To confirm HMGB1 cytoplasmic translocation, cytoplasmic fractions were isolated and assayed for relative content for HMGB1 or β-tubulin using specific Abs. The levels of HMGB1 in the cytoplasmic fractions were significantly increased after treatment with LPS or TNF-α (Fig. 1D). However, overexpression Hsp72 significantly inhibited LPS- and TNF-α-induced HMGB1 translocation (Fig. 1, C and D), suggesting that Hsp72 attenuates HMGB1 release partly through interfering with its cytoplasmic translocation.
Hsp72 inhibits LPS- and TNF-α-induced cytoplasmic translocation of CRM1 in RAW264.7 macrophages

CRM1 is a widely expressed importin involved in the maintenance of chromatin structure and export of nuclear proteins (44). Previously, we and others demonstrated that CRM1 was involved in LPS- or H2O2-mediated HMGB1 active export from nucleus to cytoplasm (11, 12). To investigate whether the inhibitory effect of Hsp72 on HMGB1 translocation is involved in the regulation of CRM1, we examined the effects of Hsp72 increases on CRM1 subcellular distribution using immunocytochemical analysis and Western blotting. In quiescent macrophages, CRM1 was primarily located in the nucleus (Fig. 2A). After stimulation with LPS or TNF-α, CRM1 was also found in the cytoplasmic regions (Fig. 2), indicating a potential CRM1 cytoplasmic translocation. However, overexpression of Hsp72 in RAW/Hsp72 substantially attenuates cytoplasm translocation of CRM1 (Fig. 2), indicating that Hsp72 inhibits cytoplasmic translocation of HMGB1 and CRM1.

Hsp72 inhibits the interaction between HMGB1 and CRM1 by competitively binding to HMGB1 in RAW264.7 macrophages after LPS and TNF-α treatment

Our previous study has suggested that HMGB1 may interact with CRM1 in response to oxidative stress (11). After stimulation with LPS or TNF-α, the amounts of CRM1 were increased in the complex immunoprecipitated with HMGB1-specific Abs (Fig. 3A), indicating a potential enhancement of CRM1/HMGB1 interaction. However, overexpression of Hsp72 substantially attenuated this LPS- or TNF-α–induced CRM1/HMGB1 interaction (Fig. 3A). Hsp72 was not co-immunoprecipitated with HMGB1 in resting Hsp72-transfected RAW264.7 cells (Fig. 3, B and C). After stimulation with LPS or TNF-α, there was a significant increase in interaction between Hsp72 and HMGB1 (Fig. 3, B and C). In contrast, we did not observe any interaction between Hsp72 and CRM1 even after LPS and TNF stimulation (data not shown). It remains elusive whether Hsp72-HMGB1 interaction interferes with HMGB1-CRM1 interaction, thereby suppressing CRM1-mediated nuclear export of HMGB1.
Hsp72 inhibits HMGB1-induced release of cytokines in RAW264.7 macrophages

HMGB1 can be actively secreted by activated monocytes or macrophages and passively released by damaged and necrotic cells, and it mediates an inflammatory response (5). HMGB1 migration to organs/tissue sites induces various inflammatory cytokines including TNF-α, IL-1β, IL-1RA, IL-6, IL-8, MIP-1α, MIP-1β, and thus contributes to the inflammatory cascade (45, 46). To evaluate the potential role of HSR and Hsp72 on the proinflammatory function of HMGB1, RAW264.7 cells were heat shocked at 42.5°C for 1 h and then incubated at 37°C for 12 h. Nonlethal HS inhibited HMGB1-induced release of cytokines TNF-α and IL-1β (Fig. 4, A and B). In addition, HMGB1-induced release of TNF-α and IL-1β was also significantly decreased in Hsp72-overexpressing RAW/Hsp72 cells compared with RAW/Neo control cells (Fig. 4, C and D). These experiments suggest that the effects of Hsp72 on HMGB1 include at least two aspects: 1) inhibition of HMGB1 release; and 2) suppression of the extracellular proinflammatory function of HMGB1.

Hsp72 inhibits HMGB1-induced activation of MAP kinases in RAW264.7 macrophages

Activation of MAPK signaling cascades, including the ERK, p38, and JNK pathways, is an important upstream step in HMGB1-induced expression and release of cytokines such as TNF-α and IL-1β in macrophages, neutrophils, and endothelial cells (14, 46, 47). We investigated whether the HSR and Hsp72 increase inhibit HMGB1-induced cytokine release partly through interfering with MAPK pathways. We cultured cells either recovering from HS or overexpressing Hsp72 with HMGB1 and examined the extent of phosphorylation of p38, JNK1/2, and ERK1/2 MAPK. HMGB1 time dependently induced phosphorylation of p38, JNK, and ERK/MAPK in macrophages (Fig. 5A). However, increased expression of Hsp72 significantly inhibited HMGB1-induced phosphorylation of each kinase (Fig. 5B).

Hsp72 inhibits HMGB1-induced IkBα degradation, NF-κB p65 nuclear translocation, and NF-κB DNA binding activity in RAW264.7 macrophages

In addition to MAPK activation, the NF-κB signal transduction pathways also involved in HMGB1-induced cellular activation, and NF-κB-dependent transcriptional activity is important for cytokine expression (14, 15). In quiescent cells, NF-κB factors p50 and p65 are sequestered as inactive trimers in the cytosol through interaction with IκBα, the most important member of the inhibitors
in the absence or presence of HMGB1 (1 μg/ml) for 1 h and recovery for 12 h at 37°C) or transfected RAW264.7 cells (RAW/Neo and RAW/Hsp72; B) were incubated with HMGB1 (1 μg/ml) for 30 or 60 min, and phosphorylated p38 (P-p38), JNK1/2 (P-JNK1/2), or ERK1/2 (P-ERK1/2) protein levels in the whole-cell lysate were detected by Western blotting. GAPDH was used as a loading control. All blots are representative of three experiments with similar results.

FIGURE 5. Effects of Hsp72 overexpression on HMGB1-induced MAPK phosphorylation in macrophage cultures. Cell pretreatment with HS (42.5°C, 1 h and recovery for 12 h at 37°C; A) or transfected RAW264.7 cells (RAW/Neo and RAW/Hsp72; B) were incubated with HMGB1 (1 μg/ml) for 30 or 60 min, and phosphorylated p38 (P-p38), JNK1/2 (P-JNK1/2), or ERK1/2 (P-ERK1/2) protein levels in the whole-cell lysate were detected by Western blotting. GAPDH was used as a loading control. All blots are representative of three experiments with similar results.

FIGURE 6. Effects of Hsp72 overexpression on HMGB1-induced the IκBα degradation (by 15 min; Fig. 6, A and B), preceded p65 (Fig. 6, C and D) and p50 (data not shown) translocation into the nucleus (by 30 min) in RAW/Neo cells. However, increased intracellular Hsp72 (induced either by nonlethal HS or Hsp72 transfection) significantly inhibited IκBα degradation, and NF-κB p65 nuclear translocation (Fig. 6). These experiments therefore show that after stimulation with HMGB1, p65, the key activator of NF-κB-regulated transcription, becomes available to κB-regulated genes in the nucleus and that such nuclear localization is effectively inhibited when Hsp72 is expressed to high levels.

We further observed the effect of Hsp72 on HMGB1-induced NF-κB DNA binding activity. Indeed, HMGB1 stimulation of RAW/Neo cells led to a strong increase in NF-κB DNA-binding activity assayed by EMSA (Fig. 6, E, lane 3 and F, lane 4). The DNA-binding activities were specific as indicated by competition with cold κB probe (Fig. 6, E and F, lane 6), and the presence of p65 in the NF-κB-DNA complexes was confirmed by the substantial supershift in the presence of p65-specific Abs (Fig. 6, E and F, lane 7). HMGB1-inducible DNA-binding activity of NF-κB was markedly inhibited in the cells expressing abundant Hsp72 (Fig. 6, E and F, lane 5).

NF-κB regulates the expression of proinflammatory cytokine such as TNF-α and IL-1β (21). To determine whether inhibition of NF-κB activity influences the TNF-α and IL-1β expression, we determined cellular TNF-α and IL-1β level in RAW/Neo or RAW/Hsp72 cells at 1 h after HMGB1 stimulation. Expression of TNF-α and IL-1β was increased in RAW/Neo cells after HMGB1 treatment. In contrast, TNF-α and IL-1β expression was not significantly increased in RAW/Hsp72 cells post-HMGB1 stimulation (Fig. 6G), implicating inhibition of NF-κB activity by Hsp72 in decreasing cellular levels of HMGB1-induced proinflammatory cytokines such as TNF-α and IL-1β.

Discussion

Anti-inflammatory regulation by the stress response is an effective autoprotective mechanism when the host encounters harmful stimuli. The protective roles of stress-inducible HSPs (such as Hsp72) in lethal sepsis and infection are well known, but the mechanism of action remain incompletely delineated. Here we demonstrated that anti-inflammatory effect of Hsp72 is involved in inhibition of HMGB1 release and its proinflammatory signaling function in macrophages.
FIGURE 7. Conceptual relationships between Hsp72 and HMGB1 release and proinflammatory function in macrophages cultures. Hsp72 is an important intracellular protein to inhibition of HMGB1 release and proinflammatory function in macrophages. On the one hand, Hsp72 attenuated LPS or TNF-α induced HMGB1 release partly through inhibiting the CRM1-dependent nuclear export pathway of HMGB1. On the other hand, Hsp72 inhibited HMGB1-induced cytokine (e.g., TNF-α and IL-1β) expression and release potentially via inhibiting MAPKs and NF-κB activation.

HMG1, a nuclear protein widely studied as a transcription factor and growth factor, has recently been identified as a proinflammatory cytokine of lethal systemic inflammation (e.g., endotoxemia and sepsis), arthritis, and local inflammation (e.g., hepatic injury after ischemia/reperfusion and LPS-induced acute lung injury; Refs. 5, 48, and 49). In vitro, HMGB1 displays delayed kinetics of secretion (18–24 h after stimulation), compared with the early mediators of endotoxemia, TNF-α and IL-1, which are secreted within minutes of LPS stimulation (4). HMGB1 has been proven to be a successful therapeutic target in experimental models of diverse infectious and inflammatory diseases, and these findings have renewed the clinical interest of specific cytokine inhibitors and methods (41, 50–52).

Previously, we found that HSR could inhibit the release and translocation of HMGB1 induced by LPS (37), which suggested that HSPs may be involved in this process. Here we demonstrate that overexpression of Hsp72 by gene transfection significantly inhibits HMGB1 release in macrophages exposed to exogenous bacterial products (endotoxin) or endogenous proinflammatory cytokines (TNF-α) induced active release and cytoplasmic translocation of HMGB1 in macrophages. The mechanisms by which HMGB1 is exported from the nucleus to the cytoplasm are beginning to be unraveled. When actively released after stimulation, HMGB1 is heavily acetylated and reaches the cytoplasm through nuclear export sequence-containing proteins in the cytoplasm before their nuclear import (55), but the effects of Hsp72 on the function of CRM1 was previously unknown. Here, we demonstrate that elevated levels of Hsp72, such as are seen in the HSR also substantially attenuate the interaction between CRM1 and HMGB1 potentially by competitively binding to HMGB1. This suggests that Hsp72 may modulate HMGB1 nucleocytoplasmic transport systems by regulating the nuclear export of receptor protein CRM1.

Once HMGB1 is released into the extracellular space, as a proinflammatory cytokine, it can cause multiple organ failure and contribute to the pathogenesis of diverse disorders including sepsis, cardiovascular shock, rheumatoid arthritis, diabetes, and cancer (19). We further observed the effects of Hsp72 on the proinflammatory function of HMGB1 in vitro. As shown previously, HMGB1 induced the release of TNF-α and IL-1β in macrophages (45). However, overexpression Hsp72 (induced by either nonlethal HS or Hsp72 transfection) inhibited cytokine production. This indicates that Hsp72 is an effective intracellular regulator of HMGB1-induced proinflammatory cytokine release.

We further investigated the possible mechanism of Hsp72 anti-inflammatory function from HMGB1 insult in macrophages. To date, three receptors are reported to mediate the extracellular function of HMGB1. HMGB1 binding to receptor for advanced glycation end products leads to activation of NF-κB and ERK1/2, p38 and SAPK/JNK kinases (56, 57). HMGB1 also binds to TLR-2 and TLR-4, both of which lead to a MyD88-dependent activation of NF-κB (15, 58). This suggests that MAPKs and NF-κB pathways are important signal pathways involved in the proinflammatory function of HMGB1. Thus, we investigated the potential inhibitory effects of Hsp72 on HMGB1-induced activation of MAPKs and NF-κB pathway.

Previous studies have revealed widely divergent effects of Hsp72 expression on MAPK activation in response to various
stimuli. For example, we have found that Hsp72 inhibited LPS-induced production of inflammatory cytokines in a MAPK-independent manner (36); whereas others have reported that Hsp72 can prevent cell death via suppression of JNK (59, 60). Here we found that Hsp72 significantly inhibits HMGB1-induced phosphorylation of p38, JNK, and ERK MAPKs, supporting a potential role for Hsp72 as a negative regulator of HMGB1 signaling (Fig. 7). Transcription factors of the NF-κB family play vital roles in mediating cellular responses to stress, and inflammation (22). In most cells, p50 and p65 form a complex in the cytoplasm with the inhibitor protein IκBα. IκBα inactivates the p50/p65 heterodimer by masking NF-κB nuclear localization sequences (22). Activation of signaling pathways by extracellular signals, such as HMGB1, leads to proteolysis of IκBα, allowing active p50/p65 heterodimer to translocate into the nucleus, and activates κB containing genes such as TNF-α and IL-1β (Fig. 7) (15). Previous studies have shown that elevated Hsp70 expression is associated with inactivation of NF-κB in apoptotic cells (61), or innate immune cells (36). Here, we demonstrated that overexpression of Hsp72 inhibits HMGB1-induced IκBα degradation, and NF-κB (p65) nuclear translocation, and activation (e.g., binding to NF-κB elements, Fig. 7). Consequently, elevation of Hsp72 expression was associated with a blunted inflammatory response to extracellular HMGB1 stimulation, as manifested by a significant decrease in HMGB1-induced production of proinflammatory cytokines (e.g., TNF-α and IL-1β).

In summary, we demonstrated here that intracellular Hsp72 effec-
tively inhibits HMGB1 release and proinflammatory function in macrophages (Fig. 7). On the one hand, Hsp72 attenuated LPS- or cytokine induced HMGB1 release through mechanisms potentially dependent on CRM1 nuclear export pathway. On the other hand, Hsp72 inhibited various signaling molecules (e.g., the MAPKs and NF-κB) of the HMGB1-mediated cytokine production pathway. Our experimental data provide important insights into the anti-inflammatory mechanisms of HSP protection and may lead to the development of novel strategy for treatment of infectious and inflammatory disorders. In light of the important role of extracellular Hsp72 in the regulation of immunity (62), it is important to explore the potential association between Hsp72 and HMGB1 in extracellular space in future studies.

Disclosures
The authors have no financial conflict of interest.

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