Inhibition of collagen-induced platelet aggregation by anopheline antiplatelet protein, a saliva protein from a malaria vector mosquito

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During blood feeding, mosquitoes inject saliva containing a mixture of molecules that inactivate or inhibit various components of the hemostatic response to the bite injury as well as the inflammatory reactions produced by the bite, to facilitate the ingestion of blood. However, the molecular functions of the individual saliva components remain largely unknown. Here, we describe anopheline antiplatelet protein (AAPP) isolated from the saliva of Anopheles stephensi, a human malaria vector mosquito. AAPP exhibited a strong and specific inhibitory activity toward collagen-induced platelet aggregation. The inhibitory mechanism involves direct binding of AAPP to collagen, which blocks platelet adhesion to collagen and inhibits the subsequent increase in intracellular Ca2+ concentration ([Ca2+]i). The binding of AAPP to collagen effectively blocked platelet adhesion via glycoprotein VI (GPVI) and integrin αIIbβ3. Cell adhesion assay showed that AAPP inhibited the binding of GPVI to collagen type I and III without direct effect on GPVI. Moreover, intravenously administered recombinant AAPP strongly inhibited collagen-induced platelet aggregation ex vivo in rats. In summary, AAPP is a malaria vector mosquito-derived specific antagonist of receptors that mediate the adhesion of platelets to collagen. Our study may provide important insights for elucidating the effects of mosquito blood feeding against host hemostasis. (Blood. 2008; 111:2007-2014) © 2008 by The American Society of Hematology

Introduction

The saliva of blood-sucking arthropods contains a number of pharmacologically active molecules that counteract the host defenses triggered by blood feeding, such as inhibitors of the clotting cascade, platelet aggregation and vasodilators.1 Mosquitoes search for blood by repeatedly thrusting their mouthparts into the host’s deep network of skin vessels. This probing causes vessel laceration by the mouthparts. After laceration of blood vessels, platelets are generally exposed to subendothelial collagen in the damaged vessel walls, and subsequently adhere to the collagen, become activated, release their granular contents and form aggregates, with the result that the injuries are plugged by platelets.2 Thus, platelet activation by collagen is an initial step in normal hemostasis. However, mosquitoes can successfully enorge on their hosts within a half minute because antihemostatic components of their saliva facilitate location of blood vessels.3 Although mosquito saliva is known to show inhibitory activity toward platelet aggregation in response to adenosine diphosphate (ADP), collagen, and thrombin,4 a limited number of saliva components involved in the inhibition of platelet aggregation have been characterized. Specifically, Aedes aegypti apyrase inhibits ADP-induced platelet aggregation by metabolizing ADP released from injured cells,4 while Anopheles albimanus anopheline inhibits thrombin-mediated blood coagulation.5 However, no inhibitors of collagen-induced platelet aggregation have been identified to date.

In anopheline mosquitoes, saliva and the salivary glands have been extensively studied due to their direct involvement in the transmission of malaria parasites to human hosts.6 Recently, the salivary gland transcriptosome and proteosome (termed the sialome) of anopheline mosquitoes have been reported.8 In a previous study that characterized a salivary gland-specific promoter by using a transgenic Anopheles stephensi,9 we cloned a gene encoding a putative saliva protein that was specifically expressed in the female salivary glands. Sialome searches revealed that the gene product is a homolog of a 30-kDa A aegypti allergen of unknown function12 and well conserved among anopheline mosquitoes as a member of the GE-rich protein family.8,9,10 In the present paper, we demonstrate that this A stephensi homolog protein specifically inhibits collagen-induced platelet aggregation. Therefore, we have renamed it anopheline antiplatelet protein (AAPP). To the best of our knowledge, this is the first report of a mosquito saliva component that is capable of specifically inhibiting collagen-induced platelet aggregation in vitro and ex vivo.

Methods

Preparation of a salivary gland extract

A stephensi mosquitoes (SDA 500 strain) were maintained at Jichi Medical University as described previously.14 The salivary glands were dissected from unfed female A stephensi mosquitoes, transferred to 1.5-mL conical polypropylene tubes containing cooled PBS and disrupted by sonification


S.Y. and T.S. contributed equally to this study.

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on ice. After centrifugation at 15 000g for 5 minutes, the resulting supernatant was assayed for platelet aggregation.

Materials

Fibrillar type I collagen from equine tendon (Horm), for use in platelet aggregation, cell adhesion, and Ca\(^{2+}\) influx assays, was from Nycomed Pharma (Munchen, Germany); nonfibrillar type I collagen from rat tail collagen, for use under static conditions, was from BD Biosciences (San Jose, CA); collagen type III, collagen type IV, ADP, bovine serum albumin (BSA), normal goat serum, platelet-activating factor (PAF) and A23187 were from Sigma-Aldrich (St Louis, MO); epinephrine was from Daiichi Pharmaceuticals (Tokyo, Japan); U-46 119 was from Cayman Chemicals (Ann Arbor, MI); convulxin was from Alexis Biochemicals (San Diego, CA); Thrombin receptor-activating peptide (TRAP) was synthesized by Sawadey Technology (Tokyo, Japan); Collagen-related peptide (CRP) was synthesized by Peptide Institute (Osaka, Japan); Antihuman integrin α\(\text{IIb}\)/β\(\text{IIIa}\) mAb OM-2 has previously been described.15 Anti-(human immunoglobulin G)-conjugated goat anti-mouse IgG (H + L; Vector Laboratories), followed by incubation with a mouse anti-rAAPP immune serum. A Polypeptide band recognized by the serum was detected by fluorography. Anti-(human integrin α\(\text{IIb}\)/β\(\text{IIIa}\)) or electrophoretically transferred to an Immobilon Transfer Membrane (Millipore, Bedford, MA). The baseline was set with PRP and the maximum possible increase in light transmission (platelet aggregation rate: 100%) was set with PPP. The percentage inhibition was calculated based on the maximum aggregation rate of the test samples relative to the appropriate buffer control.

Preparation of human washed platelets

Blood was isolated from an arm vein of healthy human volunteers. The blood was from a 21-G needle into a plastic syringe containing 0.1 vol 3.8% trisodium citrate solution, and centrifuged at 180g for 10 minutes. PRP was removed and mixed with one-fourth volume of ACD buffer (44.8 mM sodium citrate, 20.9 mM citric acid, 74.1 mM glucose, pH 5.0). After the centrifugation, the platelet pellet was resuspended in 0.9% saline and recentrifuged at 500 g for 20 minutes to create a platelet pellet. The final pellet was resuspended in incubation buffer (134 mM NaCl, 12 mM NaHCO\(_3\), 0.34 mM Na\(_2\)HPO\(_4\), 2.9 mM KC1, 1 mM CaCl\(_2\), 0.8 mM MgCl\(_2\), 5 mM Hepes, 5 mM glucose, pH 7.4).

AAPP binding assay

Binding of rAAPP to collagen was measured in 96-well collagen-coated microtiter plates (Nunc, Rochester, NY). After blocking with blocking buffer (PBS containing 0.5% BSA) for 1 hour at room temperature, various concentrations of rAAPP was added and incubated for 1 hour at room temperature. Binding of rAAPP to collagen was detected using the ExpressDetector Nickel-HRP (KPL, Gaithersburg, MD), which can bind to the His-tag at the C-terminal of rAAPP, according to the manufacturer’s protocol. As a control, rTrx was used.

Platelet adhesion assay

Ninety-six–well microtiter plates were coated with 40 µg/mL of collagen for 1 hour at room temperature, followed by blocking with the blocking buffer for 1 hour. Control wells were coated with the blocking buffer only. The wells were rinsed 3 times with PBS. rAAPP was serially diluted, and 50 µL of each dilution was added to the plates and incubated for 30 minutes at room temperature. Next, 50 µL of human washed platelet suspension (6 × 10\(^{5}\) cells/mL) was added to each well and incubated for 45 minutes at room temperature. Nonadherent platelets were removed, and the wells were washed 3 times with PBS. The number of adherent platelets was determined using the Dc protein assay kit (Nippon Bio-Rad, Tokyo, Japan).
**GPVI-expressing Jurkat cells**

The human GPVI cDNA was cloned from a cDNA library of the human placenta (Clontech, Palo Alto, CA) and inserted into a lentivirus vector,21 kindly provided by Dr X. Wu (University of Alabama, Birmingham, AL), and transfected with Jurkat cells. A stable GPVI-expressing Jurkat cell line was established by a method of Kappes et al21 and maintained in RPMI1640 10% FCS medium supplemented with 0.5 μg/mL Puromycin (Sigma-Aldrich). Surface expression of GPVI was confirmed by flow cytometry using antihuman GPVI mAb OM-2. Nontransfected Jurkat cells do not express endogenous GPVI.

**Cell adhesion assay**

Ninety-six–well microtiter plates were coated with various concentrations of collagen or rAAPP. The wells were blocked with the blocking buffer at room temperature for 1 hour, then rinsed once in PBS. GPVI-expressing Jurkat cells were washed with PBS twice and resuspended in the blocking buffer at 1 × 10^6 cells/mL. One hundred microliters of cell suspension was added to each well and incubated at room temperature for 1 hour. The wells were washed with PBS 3 times to remove nonadherent cells. Adherent cells were quantified by the BCA protein assay kit (Pierce, Rockford, IL).

**GPVI- and αβ1-mediated platelet adhesion assay**

Ninety-six–well microtiter plates were coated with insoluble equine tendon fibrillar type I collagen or soluble rat tail type I (nonfibrillar) collagen maintained in acetate buffer, pH 4.5. Various concentrations of rAAPP were added to the 96-well plates immobilized with the fibrillar type I collagen with Tyroad’s-Hepes buffer (136.7 mM NaCl, 13.8 mM NaHCO3, 0.36 mM Na2HPO4·H2O, 2.6 mM KCl, 1.0 mM MgCl2·6H2O, 5.5 mM glucose, 0.25% BSA, pH 7.4) containing 50 μM EDTA. Similarly, various concentrations of rAAPP were added to the 96-well plates immobilized with the nonfibrillar type I collagen with Tyroad-Heps buffer and incubated for 1 hour. After washing with PBS 3 times, washed platelet suspension (10^5 cells/μL) was allowed to adhere under static conditions to each well for 1 hour. As positive controls, washed platelet suspension was pre-incubated with antihuman GPVI mAb OM-2 (1 μg/mL) or anti-human integrin α2-subunit) mAb 6F1 (5 μg/mL). Adherent platelets were quantified fluorimetrically as described by Nakamura et al.22 Results are representative of 3 independent experiments and expressed as the mean of triplicate reading plus or minus SEM for the indicated concentrations.

**Measurement of intracellular Ca2+ concentration**

Ca2+ concentration ([Ca2+]i) transients were monitored by fura-2 fluorescence. Human PRP was incubated with 5 μM fura-2 AM (Dojindo, Tokyo, Japan) for 60 minutes at 37°C. After 2 washes with the wash buffer, the platelets were resuspended to 3 × 10^6 cells/mL with the incubation buffer. After a 1-minute incubation with various concentrations of rAAPP, the platelets were stimulated with collagen (0.5 μg/mL) and the fura-2 fluorescence was measured at an excitation wavelength of 340/380 nm and emission wavelength of 500 nm using a Hitachi F-2000 fluorescence spectrophotometer.

**Ex vivo platelet aggregation of rats**

Platelet aggregation of rats was investigated as described previously.23 Crl:SD rats (Charles River Japan, Tokyo, Japan) for 60 minutes at 37°C. After 2 washes with the wash buffer, the platelets were resuspended to 3 × 10^6 cells/mL with the incubation buffer. After a 1-minute incubation with various concentrations of rAAPP, the platelets were stimulated with collagen (0.5 μg/mL) and the fura-2 fluorescence was measured at an excitation wavelength of 340/380 nm and emission wavelength of 500 nm using a Hitachi F-2000 fluorescence spectrophotometer. 

**Results**

AAPP is a predominant protein in the female salivary glands

The complete AAPP cDNA has an ORF encoding 269 amino acid residues. The gene product has characteristic features of acidic secreted proteins (pI 3.8) containing 10 unique repeats of a 6-amino acid unit (GEEGGA) or related sequences (Figure S1A, available on the Blood website; see the Supplemental Materials link at the top of the online article). We named this protein anopheline antiplatelet protein (AAPP), according to its biologic function identified in the present study.

rAAPP was produced as a thioredoxin fusion protein by using an E coli expression system. Anti-rAAPP immune serum specifically reacted with a salivary gland protein with an apparent molecular mass of around 37 kDa (Figure 1A lane 2), which corresponds to one of the most abundant proteins found in the
female salivary glands (Figure 1A lane 1). To confirm the tissue-
and sex-specificity of AAPP expression, we performed RT-qPCR
analysis of the aapp mRNA extracted from the salivary glands and
carcasses of male and female adult mosquitoes. Abundant
aapp mRNA was detected in the salivary glands of female mosquitoes,
compared with the very low expression level (<1%) in the male
salivary glands (Figure 1B). No aapp mRNA was detected in the
carcasses of either male or female mosquitoes. These results
demonstrate that abundant AAPP specifically expressed in the
female salivary gland, suggesting that the protein plays important
roles in blood feeding.

To further analyze the location of AAPP, the salivary glands
were immunostained with an anti-rAAPP immune serum. AAPP
was specifically detected in the distal lateral lobes of the
salivary glands but not in the medial lobes or proximal lateral
lobes (Figure 1C panel 2). Trace amounts of AAPP were rarely
detected in the medial lobes (data not shown). These AAPP
expression sites are consistent with our previous study showing
that the aapp promoter can strongly drive the expression of
Discosoma sp red fluorescent protein (DsRed) at the same
regions of the salivary glands in a DsRed transgenic mosquito
(Figure 1C panel 4).11

AAPP inhibits platelet aggregation via a collagen
activation pathway

To investigate the biologic functions of AAPP, we focused on its
effect on platelet aggregation using various platelet aggregation
agonists. AAPP inhibited collagen-induced human platelet aggre-
gation (Figure 2), but had no effects on ADP-, TRAP-, epinephrine-, PAF-, U-46 619- or A23187-induced platelet
aggregation. The IC_{50} of rAAPP for collagen-induced platelet
aggregation was approximately 25 nM (Figure 3A,B). Collagen induces platelet aggregation by interacting with the
immunoglobulin-like receptor GPVI24,25 on the surface of
platelets. GPVI plays the central role in platelet-collagen
interactions by activating different adhesive receptors.26,27
2 specific agonists were used to examine the interaction between
rAAPP and GPVI: the synthetic, triple-helical, collagen-related
peptide (CRP) based on a repeated GPO (glycine-proline-
hydroxyproline) motif,27 and convulxin, a multimeric C-type
lectin-like toxin from the venom of a rattlesnake28,29 These
two molecules activate platelets selectively via GPVI. Figure 3A
shows that rAAPP had no effect on CRP- or convulxin-induced
platelet aggregation at concentrations of up to 1000 nM.
Direct binding of AAPP to collagen interferes with adhesion of platelets to collagen

When rAAPP was incubated with immobilized collagen in a 96-well plate, rAAPP effectively bound to collagen in a dose-dependent manner (Figure 3C). Moreover, the rAAPP binding strongly interfered with adhesion of platelets to the immobilized collagen in a dose-dependent manner (Figure 3D). The IC_{50} of the platelet adhesion blocking was 250 nM, and 1000 nM rAAPP completely prevented detectable platelet adhesion to collagen. Next, we examined the possible involvement of AAPP in interactions with GPVI. Figure 3E shows that GPVI-expressing Jurkat cells bound to collagen type I-coated plate, but not to AAPP-coated plate, indicating that AAPP dose not involved in direct interaction with GPVI. This result is consistent with the data that rAAPP had no effect on 2 GPVI agonists (Figure 3A).

AAPP acts as an antagonist of receptors that mediate adhesion of platelets to collagen

We found that the AAPP-mediated platelet aggregation inhibition is due to interfere with collagen-platelet interaction by direct binding of AAPP to collagen. Collagen induces platelet aggregation by interacting with GPVI^{24,25} and integrin αβ_{1}^{30,31} on the surface of platelets. GPVI- and integrin αβ_{1}-mediated platelet adhesion are well characterized. Integrin αβ_{1} is known to mediate platelet adhesion to collagen in a Mg^{2+}-dependent manner.^{32} Human platelets adhere to soluble monomeric collagen in a Mg^{2+}-dependent manner, while they adhere to insoluble polymeric collagen in a Mg^{2+}-independent manner.^{33} The studies with anti-integrin αβ_{1} and anti-GPVI antibodies indicate that integrin αβ_{1} mediates the platelet adhesion to soluble monomeric collagen in a Mg^{2+}-dependent manner,^{22,34,35} and that GPVI mediates the platelet adhesion to insoluble fibrillar collagen in a Mg^{2+}-independent manner.^{22} Therefore, to address whether AAPP interferes with platelet adhesion by selectively binding to collagen receptors, we examined the effects of AAPP on platelet adhesion to soluble collagen in the presence of Mg^{2+} (integrin αβ_{1}-mediation) and to insoluble collagen in the absence of Mg^{2+} (GPVI-mediation). Various concentrations of rAAPP were incubated with insoluble fibrillar type I collagen immobilized in a 96-well plate before adding platelet suspension under static conditions in the absence of Mg^{2+}. Figure 4A shows that GPVI-mediated platelet adhesion was abolished by increasing amounts of rAAPP. The IC_{50} was approximately 22 nM, and complete inhibition was reached with 222 nM (10 μg/mL). As a positive control, a mouse mAb specific for human GPVI, OM-2 (1 μg/mL), strongly inhibited the platelet adhesion (> 95%). Next, we examined the ability of rAAPP on the binding of integrin αβ_{1} under static conditions. In the presence of Mg^{2+}, integrin αβ_{1}-mediated platelet adhesion to nonfibrillar type I collagen was reduced by increasing amounts of rAAPP (IC_{50}=222 nM) (Figure 4B). As a positive control, a mouse mAb specific for human integrin α_{2}-subunit, 6F1 (5 μg/mL), strongly inhibited the platelet adhesion (> 95%). Thus, AAPP effectively inhibited both GPVI- and integrin αβ_{1}-mediated platelet adhesion to collagen.

AAPP inhibits GPVI-mediated platelet adhesion to collagen type I, III, but not type IV

Because different types of collagen are among the most thrombogenic component of the vessel wall responsible for the initiation of platelet adhesion, we examined the ability of rAAPP to bind to these collagens. Various concentrations of rAAPP were incubated either with collagen type I, III or IV immobilized in a 96-well plate. The binding of rAAPP to each collagen was evaluated by studying the inhibition of GPVI-expressing Jurkat cells to binding to each collagen. Figure 5 shows that rAAPP inhibited the binding of GPVI-expressing Jurkat cells to collagen types I and III in a dose-dependent manner, but not to type IV, indicating that AAPP competes with GPVI for the binding to collagen types I and III, but not to type IV.
AAPP inhibits the collagen-induced increase in [Ca^{2+}]i

Because an increase in [Ca^{2+}]i is considered to play a pivotal role in platelet aggregation, we investigated the possible involvement of AAPP in the regulation of [Ca^{2+}]i. Addition of collagen to a human platelet suspension in the presence of 1 mM CaCl2 resulted in an increase in [Ca^{2+}]i (Figure 6 control). To determine the effects of AAPP on the collagen-induced increase in [Ca^{2+}]i, platelets were incubated with various concentrations of rAAPP for 1 minute before their activation with collagen. Even at a low concentration (30 nM), rAAPP drastically decreased the collagen-induced change in [Ca^{2+}]i, and 300 nM rAAPP completely abolished the change (Figure 6).

Intravenous administration of AAPP inhibits collagen-induced platelet aggregation ex vivo in rats

rAAPP also exhibited identical inhibition of collagen-induced aggregation of rat platelets in vitro (data not shown). The in vivo effects of rAAPP were tested by giving SD rats a bolus intravenous injection of rAAPP (0.1–1.0 mg/kg) and performing ex vivo aggregation studies on citrated PRP. The ED_{50} for the aggregation inhibition was 0.3 mg/kg, and rAAPP at 1.0 mg/kg prevented all detectable collagen-induced platelet aggregation (Figure 7). ADP-induced aggregation remained unaltered, even as concentrations as high as 1.0 mg/kg. There was no significant change in platelet aggregation after rTrx injection, which was used as a negative control.

Discussion

In the present study, we have identified and characterized an abundant saliva protein, AAPP, from the *A. stephensi* mosquito. AAPP has characteristic features of acidic secreted proteins with a glycine and glutamic acid-rich region, and is specifically expressed in the distal lateral lobes of the adult female salivary glands. This expression pattern is consistent with the finding in our previous study on transgenic mosquitoes that the strong *aapp* promoter specifically drives foreign gene expression in the distal lateral lobes.11 We have demonstrated that AAPP is unique in that it specifically blocks platelet adhesion to collagen and subsequent platelet aggregation.

Regarding the mechanism by which AAPP specifically inhibits collagen-induced platelet aggregation, AAPP directly binds to collagen I and III, and interferes with the interaction between collagen and its receptor GPVI on platelets, thereby inhibiting an increase in [Ca^{2+}]i, which is an important second messenger in the platelet activation cascade. The current view of collagen-induced platelet activation is that collagen initially interacts with GPVI and integrin α2β1, and platelet activation signals are then conveyed by GPVI (reviewed in Nieswandt and Watson36). However, we excluded the possibility of direct interactions between AAPP and these molecules by the following pieces of evidence: (1) the 2 GPVI receptor agonists, CRP and convulxin did not compete with AAPP for platelet aggregation; (2) GPVI-expressing Jurkat cells did not bind to AAPP; and (3) the sequence motifs of the specific α2β1 ligand, GFGER-GPP,7 within the collagen triple-helical region and the RGD fibrinogen receptor antagonist were not contained in the AAPP sequence. Our recent investigations have shown that, although the GE-rich region located in the N-terminal half of AAPP shows 65% similarity with the triple-helix region of the α1 chain of human collagen IV (Figure S1B), this region is involved in the binding neither to GPVI (Figure 5E) nor collagen (S.Y. and T.S., unpublished data, July 2005). Further studies are currently in progress to identify AAPP receptor on collagen by using truncated rAAPPs.

To develop new therapeutic agents for the treatment of cardiovascular and ischemic disorders, a number of new exogenous factors have recently been identified from animal sources, and the search is still on for novel factors that interfere with platelet aggregation and blood coagulation (reviewed in Kini38). Although some factors effectively affect platelet function and aggregation in vitro, most of them fail to retain their biochemical and pharmacologic characteristics in vivo. To date, limited numbers of drugs have been developed or are in the process of being developed from animal sources, for example, desmoteplase (a plasminogen activator) from vampire bat saliva,39-41 calin (a plasminogen activator) from *Hirudo medicinalis* leech saliva42,43; and hirudin (an anticoagulant) from *H medicinalis* leech saliva.44,45 Our finding that intravenously administered rAAPP strongly inhibited collagen-mediated platelet aggregation ex vivo in rats may provide a potential use of AAPP for developing new drugs that maintain the flow of blood.

Malaria transmitted by anopheline mosquitoes is the worst health problem in the world, and kills 1 to 2 million people every year. Our recent studies on malaria survey have shown that approximately 90% of people living in the Solomon Islands, where malaria is hyperendemic, have high levels of antibodies against AAPP and the antibody levels are positively correlated with the antibody levels against malaria antigens (data not shown). From the
aspect of malaria epidemiology, this information may provide important insights regarding bite exposure or monitoring the inroads of anopheline mosquitoes, thereby leading to successful prediction of the emergence of malaria. In addition, it is also of great interest to address whether high levels of anti-AAPP antibodies affect mosquito biting behavior and/or malaria infection. These studies on mosquito-malaria biology are now in progress in our laboratory.

In conclusion, we have demonstrated for the first time that AAPP is a malaria vector mosquito–derived specific antagonist of receptors that mediate the adhesion of platelets to collagen. AAPP shows no similarity to any other protein in the GenBank database, indicating that it is a novel antiplatelet aggregation protein that acts via an alternative pathway. The female salivary gland–specific AAPP expression pattern and its high levels of expression suggest that AAPP may play a critical role in facilitating location of blood vessels and preventing host hemostasis during feeding. In fact, more than 50% of antiplatelet aggregation activity in the salivary glands is brought about by AAPP (S.Y. and T.S., unpublished data, June 2005). Importantly, AAPP is an attractive molecule for developing a novel antithrombosis drug. Further studies are currently underway to understand the molecular details of the AAPP-collagen interaction and evaluate the pharmacologic effectiveness in vivo.

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Authorship

Contribution: S.Y., T.S., M.N., L.T., B.S., J.K., H.W., E.L., and H.M. designed and performed the research; S.Y. and T.S. interpreted and analyzed the data; and S.Y. and T.S. wrote the paper.

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