Recombinant Domain V of beta (2)-Glycoprotein I Inhibits OxLDL-induced TF Expression in Macrophages

Yan Chi, Mingying Li, Tiantian Yu, Jianzhong Qin, Jingda Li, Yuhua Chen, Qingping Liu

Abstract

Objective: To investigate if lectin-like oxidised low density lipoprotein receptor is implicated in oxidised low density lipoprotein induced up-regulation of tissue factor and whether recombinant domain V of beta (2)-Glycoprotein I expressed in Pichia pastoris inhibits the binding of oxidised and lectin-like low density lipoprotein.

Methods: The expression of tissue factor and lectin-like oxidised low density lipoprotein receptor was detected using Western blot methods. Small interference ribonucleic acid of lectin-like oxidised low density lipoprotein receptor was used to block lectin-like oxidised low density lipoprotein receptor expression. Flow cytometry was used to test the effect of beta (2)-Glycoprotein I expressed in Pichia pastoris on the binding of oxidised low density lipoprotein with lectin-like oxidised low density lipoprotein receptor by using the lectin-like oxidised low density lipoprotein receptor-expressing 293T cells.

Results: Oxidised low density lipoprotein at 5-10 μg/mL increased tissue factor and lectin-like oxidised low density lipoprotein receptor expression, whereas 20-50 μg/mL oxidised low density lipoprotein attenuated tissue factor expression. Inhibiting lectin-like oxidised low density lipoprotein receptor expression by small interference ribonucleic acid of lectin-like oxidised low density lipoprotein receptor impaired oxidised low density lipoprotein-induced tissue factor over expression in macrophages. Pretreatment with beta (2)-Glycoprotein I expressed in Pichia pastoris led to a strong inhibition of tissue factor and lectin-like oxidised low density lipoprotein receptor expression in a dose-dependent manner in macrophages. Flow cytometry analysis showed that beta (2)-Glycoprotein I expressed in Pichia pastoris attenuated the interaction of oxidised low density lipoprotein with lectin-like oxidised low density lipoprotein receptor in lectin-like oxidised low density lipoprotein receptor-expressing 293T cells.

Conclusions: Lectin-like oxidised low density lipoprotein receptor was implicated in the expression of tissue factor induced by oxidised low density lipoprotein, and beta (2)-Glycoprotein I expressed in Pichia pastoris inhibited oxidised low density lipoprotein-induced tissue factor and lectin-like oxidised low density lipoprotein receptor expression, at least in part, via inhibition of the interaction between oxidised low density lipoprotein and lectin-like oxidised low density lipoprotein receptor.

Keywords: Tissue factor, Oxidised low density lipoprotein, Lectin-like oxidised LDL receptor, Beta (2)-Glycoprotein I. (JPMA 68: 1644; 2018)

Introduction

Atheromatous plaque disruption with superimposed thrombosis is a major cause of acute coronary events such as acute myocardial infarction (AMI) and unstable angina.[1] Tissue factor (TF) is a protein that triggers the coagulation cascade, facilitating thrombosis and atherosclerosis.[2,3] TF has been detected in various cell types within atheromatous plaques, including endothelial cells, vascular smooth muscle cells, monocyte, and, especially, macrophages, which account for about 60% of the total cells in the atheromatous plaque.[1] TF found in the atheromatous lipid-core is thought to be largely derived from the macrophages present in the plaque.[4] Macrophages contribute to thrombogenesis and atherogenesis partially via the mediation of TF.[5]

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Under physiological conditions, macrophages express a low basal TF level. In response to injury or various agonists, TF expression is rapidly increased.[6] Oxidised low density lipoprotein (oxLDL), a factor known to play an important role in atherogenesis by interacting with macrophages, has been shown to induce TF expression in macrophages.[7,8] OxLDL can bind to lectin-like oxidised LDL receptor (LOX-1), one of the scavenger receptors expressed in macrophages, to activate signalling pathways involved in the pathogenesis of atherosclerosis. However, it remains poorly understood whether oxLDL induces TF expression by the mediation of LOX-1.

β2-glycoprotein I (β2-GPI) is a 50kDa single-chained polypeptide composed of 326 amino acid residues, composed of 5 homologous repeats known as complement control protein domains. The fifth domain of β2-GPI, called DV, contains a positively-charged amino acid sequence and a C-terminal loop that serves likely as the binding region for phospholipids, including oxLDL.[9,10] Our prior investigations have demonstrated that the recombinant domain V of β2-GPI expressed in Pichia pastoris (P.rbeta2-GPI DV) was able to bind to oxLDL in high affinity.[11,12] Whether this binding would have an effect on the interaction between oxLDL and LOX-1, and accordingly suppressed the expression of TF induced by oxLDL, was unknown.

The current study was planned to investigate whether LOX-1 is implicated in oxLDL-induced up-regulation of TF and whether P.rbeta2-GPI DV inhibits the binding of oxLDL to LOX1, and, accordingly, inhibits TF expression.

Materials and Methods

Chemicals and antibodies were first obtained. These included J774A.1 and 293T cells (ATCC, Manassas, VA, USA); Rabbit polyclonal anti-LOX-1 antibody (ab-60178), anti-TF antibody (ab-85145), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) polyclonal antibody (sc-25778), and normal mouse immunoglobulin G1 (IgG1) (sc-3877) (Abcam, UK, or Santa Cruz, USA); Lipofectamine 2000 (Invitrogen, Carlsbad, USA); and bicinchoninic acid (BCA) protein assay reagent (Pierce Biotechnology, Rochford, IL. USA). Small interference ribonucleic acid (RNA) of LOX-1 (SiRNA-LOX-1) was synthesised (Shanghai GenePharma Co., China), and P.rβ2-GPI DV was produced and purified in our lab.

OxLDL was generated by oxidising LDL (d=1.019-1.063 g/mL) via incubation with 5 μM copper sulfate (CuSO4) for 8 hrs at 37°C[13].

The expression and purification of the β2-GPI DV protein was performed according to literature.[12]

J774A.1 murine macrophages and 293T cells were cultured in Dulbecco's modified eagle medium (DMEM) containing 100 IU/mL penicillin, 0.1 mg/mL streptomycin, 10 mmol/L sodium bicarbonate, 1 mmol/L sodium pyruvate and 10% (v/v) foetal bovine serum (FBS) at 37 in a 5% carbon dioxide (CO_2) atmosphere. Cells were seeded at 2×2105 cells/mL in culture medium and passaged twice per week. For all experiments, J774A.1 cells were starved in FBS-free DMEM for 6 hrs, and then treated with oxLDL for indicated concentration and times. P.rβ2-GPI DV was dissolved in phosphate-buffered saline (PBS). To examine the inhibitory effect of P.rβ2-GPI DV, a mixture of P.rβ2-GPI DV and oxLDL was co-incubated in vitro for 30 min and added to J774A.1 macrophages in DMEM.

Protein (30μg) extracted from cells was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking for 3 hrs with 5% non-fat milk in tris-buffered saline and polysorbate 20 (TBS-T) at room temperature. The membrane was incubated overnight at 4C with primary antibodies and subsequently with a horseradish peroxidase (HRP)-conjugated anti-rabbit or -mouse secondary antibody for another 1 hr at room temperature. Enhanced chemiluminescence (ECL) plus chemiluminescent substrate (Amersham Biosciences) was used to visualise protein bands.

For LOX-1 siRNA transfection, J774A.1 cells were transfected with100 nM LOX-1 siRNA using the lipofectin 2000 transfection reagent (Invitrogen, USA). A scrambled siRNA was used as a negative control. After 6 hrs transfection, the culture medium was replaced with fresh DMEM supplemented with 10% FBS, and cells were cultured for 24 hrs. The efficiency of gene silencing was tested by Western Blot analysis.

For the binding of oxLDL complexed with DiI dye (DiI-oxLDL) to cell-surface LOX-1, human LOX-1 gene was amplified by polymerase chain reaction (PCR) and cloned into expression the vector pIRES2-AcGFP1-Nuc to obtain pIRES2-AcGFP1-LOX-1. Human embryonic kidney 293T cells were transiently transfected with the pIRES2-AcGFP1-LOX-1 plasmid or pIRES2-AcGFP1-Nuc. After 48 hrs post-transfection, cells were harvested and RNA or protein was extracted for testing LOX-1 expression in 293T cells by

reverse transcription PCR (RT-PCR) or Western Blot analysis, respectively. To assess whether LOX-1 was expression on 293T cell surface, the cells was examined by immunofluorescence microscopy with an anti-LOX-1 antibody and an Alexa Fluor 488-conjugated secondary antibody. To evaluate the binding activity of LOX-1 with oxLDL, 293T cells were incubated with 20μg/mL Dil-oxLDL for 1 hr at 4 or 37, washed, and examined by confocal microscopy (Olympus, Japan).

For flow cytometry analysis, 293T cells were harvested after transfection with the plasmid pIRES2-LOX-1 or blank plasmid pIRES2-AcGFP1-Nuc for 48 hrs. The cells were washed in PBS and incubated in the presence of 20 μg/mL Dil-oxLDL or combination of 20 μg/mL Dil-oxLDL and β2-GPI DV at various concentrations for 1 hr at room temperature. After three washes with PBS, the samples were subjected to flow cytometry analysis (Becton, Dickinson FACS Calibur, USA). Approximately 10,000 events were counted for each sample.

Data was presented as means ± standard errors (SE). Each major experiment was performed for at least three times. Statistical comparisons between selected groups were carried out using Student's t-test with statistically significant and extremely significant differences considered at $p<0.05$ and $p<0.01$, respectively.

Result

Low-concentration oxLDL (5-20 μg/mL) increased TF expression by 2.65 folds compared to the control level, while high-concentration oxLDL (50 μg/mL) inhibited TF expression (Figure 1A). As for LOX-1, its expression was increased in the presence of oxLDL at concentrations 2.5-50 μg/mL within 24 hrs (Figure 1B). The expression of LOX-1 reached the peak level with 10μg/mL oxLDL.

Knockdown of the LOX-1 gene reduced the oxLDL-induced upregulation of TF by 69% (Figure 1C), suggesting that oxLDL induced TF expression and this process was partially mediated by LOX-1.

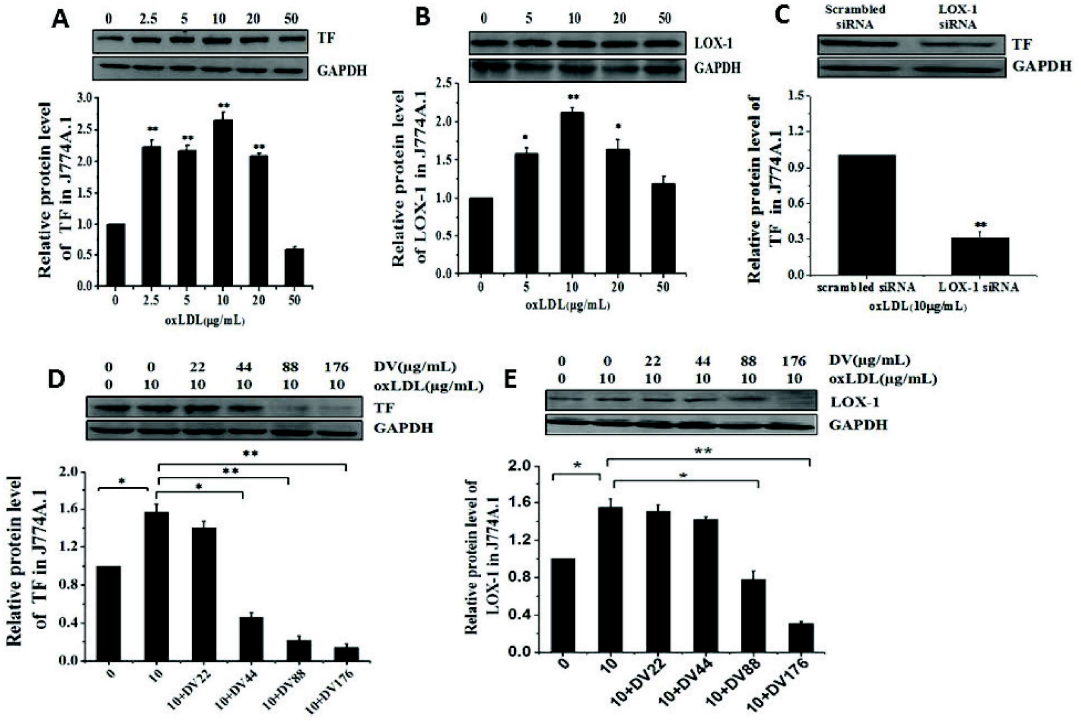

Figure-1: oxLDL up-regulated TF expression mediated by LOX-1 and P.rβ2-GPI DV inhibited the expression of TF and LOX-1 induced by oxLDL. To test the effect of oxLDL on TF (A) and LOX-1 (B) expression. J774A.1 macrophages were incubated with oxLDL at different concentrations (0-50 μg/mL) for 24 hrs. Cell lysates were analyzed by immunoblot analysis with an anti-TF antibody and anti-LOX-1 antibody respectively. GAPDH was used as an internal control. To investigate whether LOX-1 was involved in oxLDL-induced TF upregulation, the LOX-1 gene was downregulated by using the siRNA approach. The scrambled-siRNA or LOX-1-siRNA was added to J774A.1 macrophages cells. After 24 hrs post transfection, cell lysates were tested by immunoblot analysis with an anti-TF antibody (C). To test whether P.rβ2-GPI DV suppresses TF (D) and LOX-1 (E) expression, oxLDL (10μg/mL) and different concentrations of P.rβ2-GPI DV (0,22,44,88,176μg/mL) was co-incubated in vitro for 30 min, then added to J774A.1 macrophages. Cell lysates were analyzed by immunoblot analysis with an anti-TF antibody and anti-LOX-1 antibody respectively.

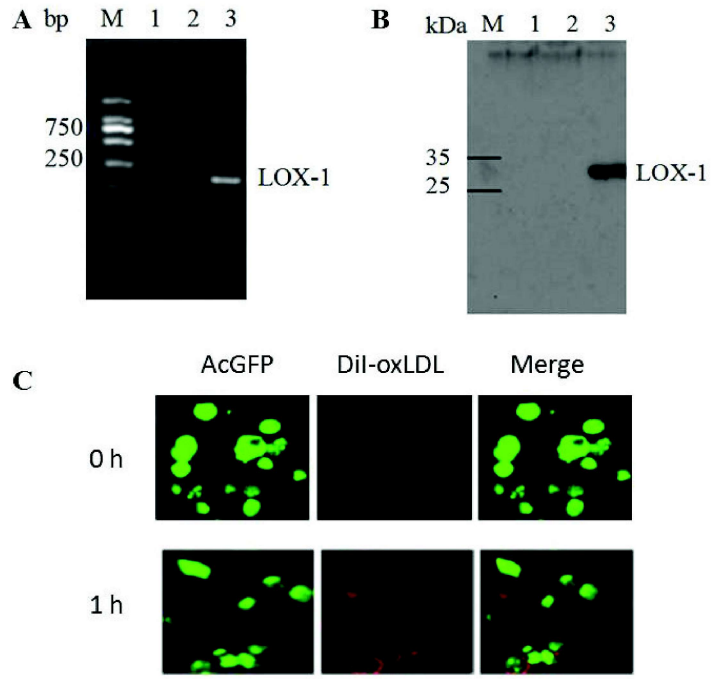

Figure-2: The expression and activity of LOX-1 on 293T cells. 293T cells were transiently transfected with pIRES2-AcGFP1-LOX-1 plasmid or pIRES2-AcGFP1-Nuc. After 48 hrs post transfection, RT-PCR, Western blot and confocal were performed respectively. (A) RT-PCR analysis of LOX-1. Lane 1: untransfected cells; Lane 2: transfected with empty vector; Lane 3: transfected with pIRES2-LOX-1 plasmid; M:DL 2000 marker; (B) Western blot analysis of LOX-1. Lane 1: untransfected cells; Lane 2: transfected with empty vector; Lane 3: transfected with pIRES2-LOX-1 plasmid; M:DL 2000 marker; (C) After 48 hrs posttransfection, 293T cells was incubated with 20 μg/mL DiI-oxLDL for a further 1 h at 37ºC. After being washed, the cells were photographed under a confocal microscope.

P.rβ2-GPI DV significantly inhibited the expresison of TF (Figure 1D) and LOX-1 (Figure 1E) in a dose-dependent manner. When the concentration of P.rβ2-GPI DV was increased to176 μg/mL, TF and LOX-1 expression was reduced by 90% and 80%, respectively. LOX-1 gene was successfully expressed in 293T cells (Figure 2A--B). Confocal microscopy demonstrated LOX-1 expression on the 293T cell membrane and LOX-1 was capable of binding to oxLDL (Figure 2C).

P.rβ2-GPI DV attenuated the binding of oxLDL to LOX-1 in a dose-dependent manner (Figure 3). When the concentration of P.rβ2-GPI DV was 88 μg/mL, the binding of Lox-1 to oxLDL was reduced from 17.7% to 7.3% ((6) vs (3)).

Discussion

As TF in atheromatous plaques originates primarily from macrophages, we investigated the effect of oxLDL on TF expression in macrophages. It was observed that oxLDL elicited a dose-dependent effect on TF expression in macrophages. Low-concentration oxLDL increased TF expression, whereas higher oxLDL concentrations reduced the expression of TF, indicating that oxLDL has dual effects on the expression of TF. The results of the

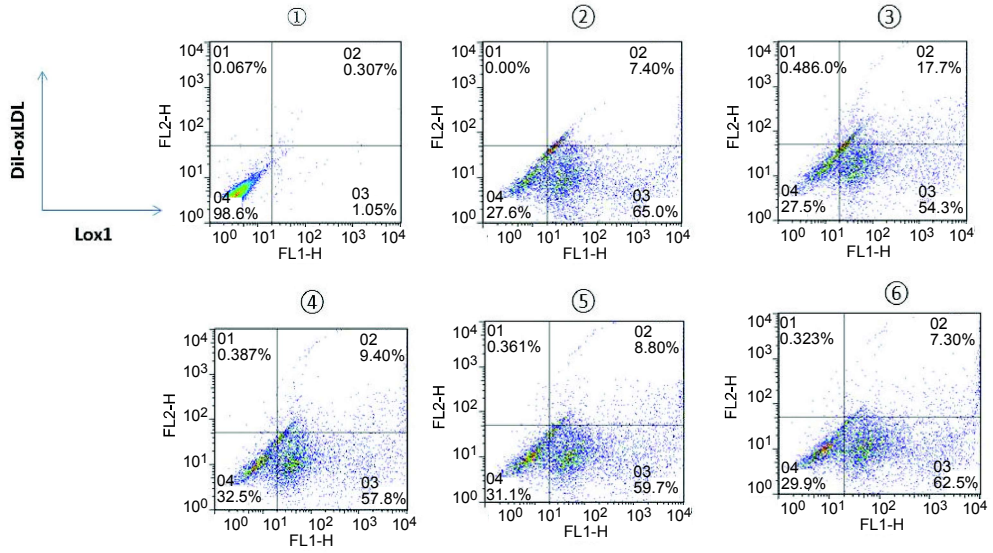

Figure-3: P.rβ2-GPI DV inhibited the binding of oxLDL with LOX-1 on 293T cells. After transfected with plasmid pIRES2-LOX-1 or blank vector plasmid pIRES2-AcGFP1-Nuc for 48 hrs, 293T cells were harvested and washed with PBS. Then 20 μg/mL DiI-oxLDL or the combination mixture of 20 μg/mL DiI-oxLDL and different concentrations P.rβ2-GPI DV were added to the cells for further 1 h at room temperrature. After three washes with PBS, samples were subjected to Flow-Cytometry analysis. Ten thousand events were counted for each sample. (1) transfected with empty vector; (2)-(6) transfected with pIRES2-LOX-1 plasmid; (3)-(6): Cells were treated with different concentration of β2-GPI DV protein (0, 22, 44, 88 μg/mL) in the presence of 20 μg/mL DiI-oxLDL for 1 h.

dual effect of oxLDL on the expression of TF were reported by Meisel et al[7] as well. The main reason for it may be for the complex components of oxLDL. Radhika et al. reported that the protein component of oxLDL activates nuclear factor (NF)-κB in a lysosome-independent way, whereas the lipid component, a lysosomal degradation product generated after oxLDL endocytosis, suppresses NF-κB activation in a lysosome-dependent manner.[16] As NF-κB is one of the transcription factors that regulate TF gene expression,[14] oxLDL performs dual effects on TF expression. This may partially explain the molecular mechanism of the dual effects of oxLDL on TF expression. However, the exact mechanism remains to be tested.

Despite increasing evidence that LOX-1, a cell-surface receptor for oxLDL, is implicated in thrombogenesis and atherogenesis, its involvement in the regulation of TF expression in macrophages remains unclear. In the present study, the expression of LOX-1 was knocked down by LOX-1 siRNA to test the effect of oxLDL on TF expression. Our data showed that inhibiting LOX-1 expression impaired oxLDL-induced TF expression in macrophages by 69%, suggesting that oxLDL induced TF expression partially via the mediation by LOX-1. These results are consistent with the report by Kuge et al., showing that LOX-1 expression is positively correlated with TF expression ($r=0.53$, $p<0.0001$) [18].

It was reported that β2-GPI binds to oxLDL to form stable and non-dissociable complexes.(19,20) It has been thought that the binding of β2-GPI with oxLDL minimises oxLDL-induced inflammatory activities, while promoting oxLDL clearance from the circulation.[21] Hasunuma et al.[22] have reported that β2-GPI can directly bind to ox-LDL to inhibit the uptake of ox-LDL by macrophages. Lin et al.[23] have reported that β2-GPI reduces cellular accumulation of cholesterol by suppressing cholesterol influx, suggesting that β2-GPI may play an important role in the prevention of atherosclerosis. It hypothesised that the binding of beta 2-GPI DV with oxLDL might block the interaction of oxLDL with LOX-1, thereby suppressing the expression of TF and LOX-1. To confirm this hypothesis, we first tested the effect of P.rβ2-GPI DV on oxLDL-mediated expression of TF and LOX-1 in macrophages. As expected, P.rβ2-GPI DV suppressed the expression of TF and LOX-1 by 90% and 80%, respectively. Second, we evaluated the role of P.rβ2-GPI DV in inhibiting the interaction between oxLDL and LOX-1. In addition to LOX-1, other scavenger receptors (SR), including CD36 and SR-A1, also mediate the binding with oxLDL. In order to avoid interference in the binding assay, LOX-1-expressing 293T cells were established and used. Flow cytometry analysis showed that P.rβ2-GPI DV attenuated the interaction of oxLDL with LOX-1 in LOX-1-expressing 293T cells, suggesting that binding of β2-GPI DV with oxLDL inhibited the interaction of oxLDL with LOX-1 and thereby suppressed the expression of TF and LOX-1.

In our experimental conditions, the expression of TF and LOX-1 was inhibited to a higher extent compared to the inhibition of oxLDL and LOX-1 binding by P.rβ2-GPI DV, implying that other mechanisms by P.rβ2-GPI DV inhibit TF and LOX-1 expression induced by oxLDL should be considered and clarified.

The current study demonstrates that LOX-1 played a role in regulating oxLDL-induced expression of TF and P.rβ2-GPI DV inhibited oxLDL-induced TF and LOX-1 expression, at least in part, via inhibiting the interaction between oxLDL and LOX-1. It has been shown that LOX-1 levels were observed to be up-regulated in human carotid-plaque macrophages. Most of the oxLDL effects are mediated by its interaction with LOX-1.[24] Their interactions in macrophages activate several genes involved in the pathogenesis of atherosclerosis. The oxLDL/LOX-1 axis represents one of the key pathogenic tools in the induction of atherosclerosis and, therefore, represents a promising target for anti-atherosclerotic therapy. Therefore, in order to further confirm the protection role of P.rβ2-GPI DV and develop it as a potent drug in the treatment of atherosclerosis targeting oxLDL/LOX-1 axis, the effect of P.rβ2-GPI DV on the expression of other genes and the activity of signal pathways induced by oxLDL and LOX-1 should be tested in vitro and in vivo assay. Next, the pharmacokinetics of P.rβ2-GPI DV deserves to be evaluated in the near future.

Conclusion

LOX-1 was implicated in the expression of TF induced by oxLDL and P.rβ2-GPI DV inhibits oxLDL-induced TF and LOX-1 expression, at least in part, via inhibition of the interaction between oxLDL and LOX-1.

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Conflict of Interest: None.
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