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p16\textsuperscript{INK4a} Exerts an Anti-Inflammatory Effect through Accelerated IRAK1 Degradation in Macrophages

Yousuke Murakami,*† Fumitaka Mizoguchi,* Tetsuya Saito, Nobuyuki Miyasaka,*† and Hitoshi Kohsaka*†

Induction of cyclin-dependent kinase (CDK) inhibitor gene p16\textsuperscript{INK4a} into the synovial tissues suppresses rheumatoid arthritis in animal models. In vitro studies have shown that the cell-cycle inhibitor p16\textsuperscript{INK4a} also exerts anti-inflammatory effects on rheumatoid synovial fibroblasts (RSF) in CDK activity-dependent and -independent manners. The present study was conducted to discern how p16\textsuperscript{INK4a} modulates macrophages, which are the major source of inflammatory cytokines in inflamed synovial tissues. We found that p16\textsuperscript{INK4a} suppresses LPS-induced production of IL-6 but not of TNF-\(\alpha\) from macrophages. This inhibition did not depend on CDK4/6 activity and was not observed in RSF. p16\textsuperscript{INK4a} gene transfer accelerated LPS-triggered IL-1R–associated kinase 1 (IRAK1) degradation in macrophages but not in RSF. The degradation inhibited the AP-1 pathway without affecting the NF-\(\kappa\)B pathway. Treatment with a proteosome inhibitor prevented the acceleration of IRAK1 degradation and downregulation of the AP-1 pathway. THP-1 macrophages with forced IRAK1 expression were resistant to the p16\textsuperscript{INK4a}–induced IL-6 suppression. Senescent macrophages with physiological expression of p16\textsuperscript{INK4a} upregulated IL-6 production when p16\textsuperscript{INK4a} was targeted by specific small interfering RNA. These findings indicate that p16\textsuperscript{INK4a} promotes ubiquitin-dependent IRAK1 degradation, impairs AP-1 activation, and suppresses IL-6 production. Thus, p16\textsuperscript{INK4a} senescence gene upregulation inhibits inflammatory cytokine production in macrophages in a different way than in RSF. The Journal of Immunology, 2012, 189: 000–000.

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovial inflammation, hyperplasia, and destruction of the cartilage and bone. In rheumatoid synovial tissues, lymphocytes and macrophages are recruited and activated. These cells, especially activated macrophages, release a large amount of inflammatory cytokines. In response to these cytokines, synovial fibroblasts proliferate vigorously and form villous hyperplastic synovial tissues called pannus. These fibroblasts also secrete inflammatory mediators that further attract inflammatory cells and stimulate growth of the synovial fibroblasts as well as vascular endothelial cells (1). The pannus becomes the source of tissue-degrading proteinases and activators of osteoclasts, leading to destruction of the affected joints (2, 3).

The pannus formation results from proliferation of the synovial fibroblasts driven by the inflammatory processes in RA. This fact led us to explore a new therapeutic approach that directly controls the cell cycle of synovial fibroblasts. If the synovial fibroblasts become refractory to the proliferative stimuli, no pannus should develop (4). It has been known that the master molecules that control cell cycling are cyclin-dependent kinases (CDK) (5). Especially CDK4/6 phosphorylates retinoblastoma gene product (pRb), which liberates active E2F transcription factors for cell-cycle progression. CDK inhibitors (CDKI) are intracellular proteins that inhibit kinase activity of cyclin/CDK complexes. CDKI p16\textsuperscript{INK4a} inhibits CDK4/6 specifically, whereas CDK p21\textsuperscript{CIP1} inhibits a broad spectrum of CDK (5). The intra-articular gene transfer of p16\textsuperscript{INK4a} as well as p21\textsuperscript{CIP1} suppressed RA in animal models (4, 6). It inhibited histological findings characteristic to RA: synovial hyperplasia, mononuclear cell infiltration, and destruction of the bone and cartilage of the joints. Comparable therapeutic effects were observed when the small-molecule (sm)CDKI was administered orally or i.p. (7). A separate series of our experiments disclosed that cell-cycle progression is not the only function of CDK (8). CDK kinase activity also regulates production of inflammatory molecules in a pRb-independent manner. Also, it has been suggested that CDKI can affect expression of inflammatory molecules in a CDK kinase-independent manner.

The above studies were all carried out with synovial fibroblasts. In rheumatoid synovial tissues, macrophages are the major source of inflammatory cytokines that are critical for the resultant pathology (9–11). The present study was conducted to explore how p16\textsuperscript{INK4a} affects expression of inflammatory cytokines from activated macrophages. We found that p16\textsuperscript{INK4a} suppresses IL-6 production in macrophages. This effect was mediated by accelerated degradation of IL-1R–associated kinase 1 (IRAK1).
Materials and Methods

Reagents

Anti-p-p38 MAPK, anti-p-JNK, anti-p-IκB kinase (IKK)α/β, anti-p-MAPK kinase 4 (MKK4), and anti-IκBα Abs were purchased from Cell Signaling Technology (Danvers, MA). Biotin-labeled anti-TLR4 Ab and PE-labeled streptavidin were purchased from eBioscience (San Diego CA). Biotin-labeled IgG1 was purchased from Beckman Coulter (Tokyo, Japan). Anti-actin Ab and MG132 were purchased from Sigma-Aldrich (St. Louis, MO). Anti-p16INK4a Ab was purchased from Millipore (Billerica, MA). Anti-IRAK1 Ab was kindly provided by Dr. Shizuo Akira [Osaka University, Osaka, Japan (12)]. An smCDK4/6 selective inhibitor, PD0332991, was provided by Pfizer (Boston, MA) (13). IRAK1 wild-type and knockout bone marrow-derived macrophage (BMM) lysates were kindly provided by Dr. James A. Thomas (University of Texas Southwestern Medical Center, Dallas, TX).

Cells

BMM were isolated from 6–8-wk-old DBA1/J mice (Charles River Laboratories, Yokohama, Japan) and cultured as described previously (14). They were cultured in RPMI 1640 medium containing rM-CSF (50 ng/ml) or 10% CMG14-12-conditioned media as a source of M-CSF (15). Human synovial tissues were derived from RA patients undergoing total joint replacement surgery or synovectomy at Shimoshizu National Hospital. Consent forms were completed by the patients prior to the surgery. RA was diagnosed according to the 1988 criteria of the American College of Rheumatology (16). Human synovial cells were prepared as described previously (8). Human acute monocytic leukemia cell line THP-1 was cultured as described elsewhere (17). RAW264.7 cells were cultured as described elsewhere (18). For activation, cells were stimulated with the optimal doses of LPS, which were minimum doses to induce maximum IL-6 production in each cell type.

Western blot analyses and electrophoresis mobility shift assay

Total cell lysate of BMM and rheumatoid synovial fibroblasts (RSF) were subject to Western blot analyses with specific Abs. A primary Ab against mouse actin was used for loading control. Peroxidase-conjugated anti-mouse or rat IgG Abs were used as secondary Abs.

After preparation of nuclear lysates with the Nuclear Extraction kit (Active Motif, Carlsbad, CA), EMSA was performed with the second-generation gel shift assay kit (Roche, Tokyo, Japan). AP-1 and NF-κB consensus sequence was purchased from Promega (Madison, WI).

Proliferation assay

Measurement of [3H]thymidine uptake by RSF and BMM was performed as described elsewhere (8, 14).

Flow cytometry analysis

BMM were stained with biotin-labeled anti-TLR4 mAb or biotin-labeled isotype-matched IgG1 followed by PE-labeled streptavidin. Data were acquired with the FACSCalibur system (BD Biosciences, San Jose, CA) and analyzed by CellQuest (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR).

Preparation of retroviral and adenoviral vectors

The human p16INK4a and IRAK1 genes were cloned into the retroviral expression vectors, pMX-IP and pMX-IN, respectively (pMX-p16INK4a and pMX-IRAK1). They had an internal ribosomal entry site and a resistance gene for pharmacological selection (19). Using pSilencer5.1 (Applied Bio systems, Tokyo, Japan), recombinant retroviral vectors containing murine CDK4- and IRAK1-specific short hairpin (sh)RNA sequences (IRAK1 sense, 5′-GATCCGAGGCCATCCCTCCTCGGTTCAAGAGACGGGGAGGGATGGGCTCTTTTTGGAAA-3′, and IRAK1 antisense, 5′-AGCCTTTCCAAAAAGAGCCCATCCTCCCGTTCTCTGAAAA-3′) and pMX-IP and pMX-IRAK1 were synthesized by gene synthesis (GenScript, Piscataway, NJ).

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FIGURE 1. Effect of p16INK4a expression on IL-6 production in LPS-stimulated macrophages. BMM, THP-1, and RAW264.7 cells were infected with pMX-IP (control) and pMX-p16INK4a (p16INK4a) retroviruses. (A) Cellular proteins were harvested, p16INK4a and actin expression were detected with Western blot analyses. (B) [3H]thymidine was added to culture media of the BMM transductants. Incorporation of [3H]thymidine was assessed after 8 h. (C) The BMM transductants were stimulated with LPS (10 ng/ml). Total RNA were harvested for TNF-α and IL-6, and mRNA was quantified with real-time PCR. The amounts of the cytokine mRNA were normalized to that of GAPDH mRNA and presented as fold change relative to nontreated control cells. The TNF-α levels at 3 h and IL-6 levels at 6 h after stimulation were depicted because they were highest during the observation. (D) The BMM transductants were stimulated with LPS (10 ng/ml) for 24 h. Culture supernatants were collected, and IL-6 and TNF-α levels quantified with ELISA. (E) TLR4 surface expression on BMM transductants was detected by flow cytometry analyses. (F) Differentiated THP-1 macrophages were stimulated with LPS (1 μg/ml) for 24 h for quantification of IL-6 in the culture supernatants. (G) RAW264.7 cells were treated in the same way with LPS (100 ng/ml). Data are representative of three independent experiments and expressed as the mean ± SD of triplicate wells. *p < 0.01.
OGGGAAGCAGTGCGCTCTG-3'; CDK4 sense, 5'-GATCCGGTTGAGTT-
CATGGAAGATCTCTGATCGTATCGTACCGGTGTCTTG-GAA-3', and CDK4 antisense 5'-AGGTTTCTCCAAATAAGGCCGTG-
AGACCATTAAAGATTGACAGAGATGCTCAAGGCACCTAACCAG-3') were
constructed (murine CDK4 short hairpin [shCDK4] and murine IRAK1
short hairpin [shIRAK1]). Short hairpin RNA vector for negative control
(shNC) was purchased from Applied Biosystems. Recombinant retroviruses
were prepared as described previously (20). Cells were infected with the retro-
viruses in the presence of 6 μg/ml polybrene. One day after the infection,
they were exposed to puromycin (6 μg/ml) or neomycin (500 μg/ml).
Replication-defective adenoviruses containing a human p16 gene (AxCA-
p16) and LacZ gene (AxCA-LacZ) were prepared as described previously (8).

Quantification of cytokine and IRAK1 expression
Specific ELISA kits to quantify human IL-6 and TNF-α in the culture supernatants were purchased from R&D Systems (Minneapolis, MN).
Quantitative real-time PCRs for IL-6, TNF-α, IRAK1, and GAPDH were carried out as previously described (21, 22). A p16INK4a gene-specific primer set was purchased from Qiagen (Tokyo, Japan).

Small interfering RNA transfection
To introduce small interfering RNA (siRNA) into BMM, 2 × 10^5 BMM
was incubated with 200 μl 1.2 μM siRNA containing 24 μl FuGENE-
HD transfection reagent (Roche) in Opti-MEM for 16 h. p16INK4a
specific siRNA (si-p16A and B) and control siRNA were purchased from
Qiagen.

Statistic analyses
[3H]Thymidine uptake, IRAK1 and cytokine mRNA measurements, and IL-6
concentrations in the supernatants were compared with the Mann–Whitney
U test.

Results
p16INK4a suppressed IL-6 expression in LPS-stimulated macrophages
To study the effect of p16INK4a on macrophages, BMM were infected with
pMX-p16 or control pMX-IP retroviruses. Ectopic p16INK4a
protein expression in the pMX-p16INK4a-infected cells had been
confirmed with Western blots analyses (Fig. 1A). [3H]Thymidine
uptake by the p16INK4a-expressing BMM was almost completely
suppressed in comparison to control virus-treated BMM (Fig. 1B).
Quantitative PCR showed that p16INK4a gene transfer significantly
suppressed IL-6 mRNA expression by BMM stimulated with LPS, whereas TNF-α mRNA expression was not affected
(Fig. 1C). IL-6, but not TNF-α, production at the protein level was
also suppressed significantly in BMM expressing p16INK4a (Fig. 1D).
LPS recognition receptor TLR4 expression was not modified by
p16INK4a overexpression, showing that IL-6 reduction was not due
to the TLR4 downmodulation (Fig. 1E). The murine macrophage
cell line RAW264.7, as well as human THP-1 cells that had
been induced to differentiate to macrophages produce IL-6 in re-
sponses to LPS. This response was also reduced by the p16INK4a
gene transfer (Fig. 1F, 1G). Thus, p16INK4a gene transfer suppressed
IL-6 expression in murine and human macrophages.

CDK4/6 inhibition did not affect IL-6 expression in LPS-stimulated macrophages
Our previous study demonstrated that p16INK4a inhibited matrix
metalloproteinase (MMP)-3 expression in RSF by suppressing
CDK4/6 kinase activity (8). To determine if the inhibitory effect of
p16INK4a on LPS-induced IL-6 production in BMM depends on
CDK4/6 kinase activity, CDK4/6 selective inhibitor (PD0332991)
was added to the BMM culture. The CDK4/6 inhibitor suppressed
[3H]thymidine uptake of BMM in a dose-dependent manner. Max-
imal inhibition was observed at 2 μM PD0332991 (Fig. 2A, which
did not affect viability of BMM (data not shown). The CDK4/6
inhibitor did not modulate LPS-induced IL-6 expression and pro-
bution by BMM, but it completely suppressed BMM proliferation

FIGURE 2. Effects of direct CDK4/6 inhibition on IL-6 production in LPS-stimulated BMM. (A) BMM were treated with or without indicated concentrations of smCDK4/6-selective inhibitor PD0332991 (smCDKI) for 24 h, and [3H]thymidine uptake was assessed for the last 8 h. (B) BMM were treated with or without 2 mM smCDKI for 1 h prior to LPS stimulation (10 ng/ml) for 6 h. IL-6 mRNA in the treated BMM were quantified with real-time PCR. The amounts of the mRNA were normalized to that of GAPDH mRNA and presented as fold change relative to nontreated cells. (C) BMM were pretreated in the same manner and then stimulated with LPS (10 ng/ml) for 24 h. IL-6 in the culture supernatants was measured with ELISA. (D) BMM were transduced with shCDK4 or shNC. CDK4 and CDK6 mRNA in the BMM were quantified with real-time PCR. (E) BMM infected with shCDK4 and shNC were examined for [3H]thymidine uptake during 8 h incubation. (F) BMM infected with shCDK4 and shNC were stimulated with LPS (10 ng/ml) for 24 h. IL-6 in the culture supernatants was measured with ELISA. Data are representative of three inde-
pendent experiments and expressed as the mean ± SD of triplicate wells. *p < 0.01.

p16INK4a expression in BMM promoted IRAK1 degradation
TLR4 signaling is mediated by quite a few signaling molecules. To
explore how p16INK4a inhibits IL-6 production in LPS-stimulated
BMM, we studied the expression of signaling molecules with
Western blot analyses and found that p16INK4a gene transfer af-
fected IRAK1 protein expression (Fig. 3A). Upon ligand binding
to TLR, MyD88 is recruited to the receptor and brings IRAK1 and
IRAK4 to the receptor. This leads to phosphorylation of IRAK1,
which activates kinase activity of IRAK1 and then triggers degra-
dation of IRAK1 itself (23). The IRAK1 protein level in p16INK4a-
expressing BMM was comparable to that in control cells at the
unstimulated status. Upon LPS stimulation, the IRAK1 expression
was significantly downmodulated in p16INK4a-expressing cells as
compared with control cells (Fig. 3A). The specific band was ob-
erved in wild-type BMM but not in IRAK1 knockout cells, dem-
onstrating that binding of anti-IRAK1 Abs was specific (Fig. 3A). p16INK4a expression did not affect IRAK1 mRNA levels, arguing that the decrease of the IRAK1 protein was not due to the decrease in gene transcription (Fig. 3B).

To discern if the same suppression is observed in RSF, p16INK4a or control LacZ were transduced using an adenoviral vector to RSF and IL-6 production was measured after LPS stimulation. Although [3H]thymidine uptake was inhibited in the p16INK4a-infected RSF, IL-6 production was not altered (Supplemental Fig. 1A, 1B). In accordance with this, IRAK1 expression in RSF in p16INK4a-expressing and control RSF was comparable throughout the LPS stimulation (Supplemental Fig. 1C).

**p16INK4a suppressed the AP-1 signaling pathway without suppressing the NF-κB signaling pathway**

Upon LPS stimulation of macrophages, activated IRAK1 triggers phosphorylation of p38 MAPK/JNK and IKKα/β, which leads to activation of the AP-1 and NF-κB transcription factors (24). Western blot analyses illustrated that ectopic p16INK4a expression suppressed phosphorylation of p38 MAPK and JNK but not of IKKα/β in BMM stimulated with LPS (Fig. 4A). Phosphorylation of MKK4, which is upstream of JNK, was suppressed by p16INK4a (Fig. 4A). Downstream AP-1 binding activity was also reduced in p16INK4a-expressing BMM (Fig. 4B). In contrast, IκB degradation, which is downstream of IKKα/β, as well as NF-κB-binding activity were not affected by p16INK4a overexpression (Fig. 4A, 4B).

We assumed that the impairment of the AP-1 pathway in p16INK4a-expressing BMM should be directly due to reduced IRAK1 protein expression. To substantiate this, shIRAK1 was retrovirally transduced in BMM. IRAK1 protein was downregulated in the shIRAK1-transduced cells in comparison with control cells (Fig. 4C). When these cells were stimulated with LPS, AP-1 activation, but not IκB degradation, was significantly suppressed in the shIRAK1-

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**FIGURE 3.** Promotion of IRAK1 degradation by exogenous p16INK4a. BMM transduced with pMX-IP (control) and pMX-p16INK4a (p16INK4a) were stimulated with LPS (10 ng/ml). (A) Cellular proteins were harvested at the indicated time points. IRAK1 and actin expression was detected with Western blot analyses (top panel). The density of the IRAK1 bands was normalized to that of actin and presented as fold change relative to nontreated control cells (bottom panel). Data are representative of three experiments and expressed as the mean ± SD of triplicate wells. (B) IRAK1 mRNA in the BMM was quantified with real-time PCR. The amount of the IRAK1 mRNA was normalized to that of GAPDH mRNA and presented as fold change relative to nontreated control cells. Data are representative of three experiments and expressed as the mean ± SD of triplicate wells.

**FIGURE 4.** Suppression of phosphorylation of p38 MAPK and JNK without affecting the IKKα/β–IκB pathway by p16INK4a. (A) Transduction of BMM with pMX-IP (control) and pMX-p16INK4a (p16INK4a) retroviruses was followed by LPS (10 ng/ml) stimulation. Total cell lysates were collected at the indicated time points and examined for p-p38, p-JNK, p-MKK4, p-IKKα/β, IκBα, and actin expression with Western blot analyses. (B) Nuclear extracts of the control and p16INK4a-expressing BMM stimulated with LPS (10 ng/ml) for 1 h were examined for AP-1 and NF-κB binding activity with EMSA. (C) shIRAK1 or shNC were transduced retrovirally into BMM. Protein levels of IRAK1 and actin in total cell lysates were determined with Western blot analyses. (D) Cells were stimulated with LPS (10 ng/ml). After 1 h, the cells were lysed, and AP-1 binding activity in the nuclear extracts was examined with EMSA. After 30 min stimulation, the protein levels of IκBα in total cell lysates were determined with Western blot analyses. Data are representative of three independent experiments.
Inhibitory effect of endogenous p16INK4a on IL-6 production

To discern if expression of endogenous p16INK4a exerts the same inhibitory effects, BMM were cultured to senescence as described previously (25). No endogenous p16INK4a protein was detectable in log-phase growing BMM, whereas the senescent BMM substantially upregulated the protein and mRNA of p16INK4a (Fig. 6A). These cells were then treated with two different siRNAs against p16INK4a to reduce p16INK4a expression levels (Fig. 6B). IL-6 production from BMM was upregulated by LPS stimulation and inversely proportional to p16INK4a mRNA expression levels (Fig. 6C). Thus, endogenous physiological p16INK4a expression can contribute to the suppression of the LPS-induced IL-6 production.

Discussion

p16INK4a expression in macrophages suppressed LPS-induced production of IL-6 but not of TNF-α in a CDK4/6-independent manner. This was not observed in synovial fibroblasts. Molecular analyses disclosed that it was due to the acceleration of proteosome-mediated IRAK1 degradation and following suppression of the AP-1 signaling pathway. Thus, p16INK4a gene transfer or its induction in synovial cells inhibits production of a part of macrophage-derived cytokines in addition to proliferation of synovial cells.

Recently, it was shown that TLR triggering is relevant in rheumatoid inflammation. Endogenous ligands for TLR-2 and TLR-4 are found in RA joints. These include gp96, fibrinogen, Hsp60, Hsp70, hyaluronic acid, myeloid-related protein 8/14, and high mobility group box chromosomal protein 1 (26). Indeed, TLR inhibition by a dominant-negative form of the Toll/IL-1R domain containing adaptor protein molecules suppressed the spontaneous production of proinflammatory cytokines and MMPs from RSF (27). LPS was used as a TLR stimulator in this study.

p16INK4a gene transfer suppressed IL-6 production via the promoted IRAK1 reduction, because IRAK1 coexpression prevented the IL-6 reduction. Indeed, the upstream molecule of IRAK1, IKKα/β, was phosphorylated equally in p16INK4a-expressing cells and control cells. These findings also indicated that neither downstream nor upstream molecule of IRAK1 was affected by p16INK4a.

Studies of IRAK1-deficient cells showed that IRAK1 activation is an essential step in the TLR signaling pathways that leads to NF-kB and AP-1 activation and subsequent production of proinflammatory cytokines (23, 28, 29). IRAK1 activation causes degra-
Inhibitory effect of endogenous p16\(^{INK4a}\) on IL-6 production. (A) BMM cultured for 6 d were still in a logarithmic growth phase (6 d), whereas those cultured for 17 d reached replicative senescence (17 d). Endogenous p16\(^{INK4a}\) and actin were detected with Western blotting analyses. p16\(^{INK4a}\) mRNA expression in these cells was quantified with real-time PCR. The amounts of the p16\(^{INK4a}\) mRNA were normalized to that of GAPDH mRNA and presented as fold change relative to BMM cultured for 6 d. (B) BMM were transfected with siRNA against p16\(^{INK4a}\) (si-p16A and B) and siNC. p16\(^{INK4a}\) mRNA expression in the treated BMM was measured with real-time PCR. The amounts of the p16\(^{INK4a}\) mRNA were presented as fold change relative to siNC-treated cells. (C) These cells were stimulated with LPS (100 ng/ml) for 24 h. IL-6 in the culture supernatants was measured with ELISA. Data are representative of two independent experiments and expressed as the mean ± SD of triplicate wells. *p < 0.01.

**FIGURE 6.** Inhibitory effect of endogenous p16\(^{INK4a}\) on IL-6 production. (A) BMM cultured for 6 d were still in a logarithmic growth phase (6 d), whereas those cultured for 17 d reached replicative senescence (17 d). Endogenous p16\(^{INK4a}\) and actin were detected with Western blotting analyses. p16\(^{INK4a}\) mRNA expression in these cells was quantified with real-time PCR. The amounts of the p16\(^{INK4a}\) mRNA were normalized to that of GAPDH mRNA and presented as fold change relative to BMM cultured for 6 d. (B) BMM were transfected with siRNA against p16\(^{INK4a}\) (si-p16A and B) and siNC. p16\(^{INK4a}\) mRNA expression in the treated BMM was measured with real-time PCR. The amounts of the p16\(^{INK4a}\) mRNA were presented as fold change relative to siNC-treated cells. (C) These cells were stimulated with LPS (100 ng/ml) for 24 h. IL-6 in the culture supernatants was measured with ELISA. Data are representative of two independent experiments and expressed as the mean ± SD of triplicate wells. *p < 0.01.
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Disclosures

The authors have no financial conflicts of interest.

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