Soluble HLA-G Inhibits Cell Cycle Progression in Human Alloreactive T Lymphocytes

Rajia Bahri, Francois Hirsch, Adeline Josse, Nathalie Rouas-Freiss, Nicolas Bidere, Aime Vasquez, Edgardo D. Carosella, Bernard Charpentier and Antoine Durrbach

*J Immunol* 2006; 176:1331-1339; 
doi: 10.4049/jimmunol.176.3.1331
http://www.jimmunol.org/content/176/3/1331

References
This article cites 38 articles, 15 of which you can access for free at:
http://www.jimmunol.org/content/176/3/1331.full#ref-list-1

Why *The JI*? Submit online.
- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Soluble HLA-G Inhibits Cell Cycle Progression in Human Alloreactive T Lymphocytes

Rajia Bahri,* Francois Hirsch,* Adeline Josse,* Nathalie Rouas-Freiss,† Nicolas Bidere,* Aime Vasquez,* Edgardo D. Carosella,‡ Bernard Charpentier,* and Antoine Durrbach**

HLA-G is involved in regulating T cell responses. Various mechanisms have been proposed to explain the inhibition of T cell proliferation. In this context, the possible role of HLA-G in cell cycle regulation remains to be explored. Using stably transfected M8 cells expressing the secreted isoform (HLA-G5) of HLA-G, we investigated the role of HLA-G in inducing apoptosis and in controlling the cell cycle of activated T cells. Soluble HLA-G (HLA-G5) inhibited both CD4 and CD8 T cell proliferation. However, HLA-G5 did not induce T cell apoptosis, as determined by 3,3′-diethoxyacarbocyanine and propidium iodine labeling. It induced accumulation of the retinoblastoma protein, but not its phosphorylated and active form. Treatment of activated T cells with HLA-G5 also reduced the amounts of cyclin D2, E, A, and B by >80%. In contrast, it induced an accumulation of p27kip1, but not p21cip1, in activated T cells. HLA-G does not induce apoptosis of alloreactive T cells, but induces p27kip1 and inhibits cell cycle progression. The Journal of Immunology, 2006, 176: 1331–1339.

Human leukocyte Ag G is a nonclassical MHC class I molecule with limited polymorphism and a restricted tissue distribution: it is only expressed in physiological conditions in medullary thymic epithelial cells (1), cornea (2), and extraembryonic tissues. During pregnancy, HLA-G is expressed on the cytotrophoblast and is believed to inhibit maternal NK cell cytotoxicity, allowing the development of the embryo (3). Four membrane-bound (HLA-G1 to -G4) and three soluble HLA-G (HLA-G5 to -G7) isoforms have been described. All of these isoforms are derived from the alternative splicing of the single primary transcription product (4–6). HLA-G inhibits immune celluar function by interacting at least with the killer Ig-like receptors (KIR) (KIR2DL4) (7) and Ig-like transcripts (ILT) (ILT2) (8, 9). These receptors are mostly expressed on NK cells, but are also expressed intracellularly by most CD4 and CD8 T cells and by a significant fraction at their surface (10), suggesting that HLA-G can regulate the functions of NK, CD4, and CD8 T cells. During human organ transplantation, in which CD4 and CD8 T cells have a more important role than NK cells, HLA-G expression correlates with a better acceptance of the allograft (11–13). In vitro, HLA-G modulates the functions of several immune effectors: it acts on NK cells by inhibiting their cytotoxicity (14–16) and their transendothelial migration properties (17); it also inhibits Ag-specific CD8+ T cell cytolytic function (18, 19). In addition, HLA-G interacts with CD4 T cells, but also dendritic cells, which are involved in the initiation of the CD4 cell activation cascade during the alloimmune response. HLA-G suppresses CD4+ T cell proliferation in response to allogeneic stimulation (20–22) and promotes (Th2-type) responses. HLA-G also inhibits dendritic cell maturation (23, 24), thus increasing allogeneic skin graft survival. These data led to the conclusion that HLA-G is involved in the inhibition of the alloimmune response.

In recent studies, no apoptosis could be detected at the end of 10 days of MLR in presence of soluble HLA-G5, suggesting that soluble HLA-G inhibits T cell alloproliferation in an apoptosis-independent manner (21, 25). However, one could argue that apoptosis may be detected earlier after the allogenic stimulation and not at the end of MLR. In contrast, a first study demonstrated that soluble HLA-G induced apoptosis of T cells activated by PHA during 5 days (26). In addition, recent work with blast CD8 T cells stimulated for 15 days by PHA has demonstrated that the inhibition of CD8 T cell-dependent cytotoxicity induced by HLA-G involves Fas-dependent apoptosis (19, 27). This mechanism of death is believed to activate Fas and its downstream cascade, leading to the activation of caspase family proteins. Although stimulation of the Fas cascade is a very potent inducer of activated T cell apoptosis, it is not efficient during the early phase of T cell activation and takes place later during the activation of T cells. Consequently, the mechanisms by which HLA-G inhibits T cells proliferation in allogenic situations remain elusive, although this is an important issue: recent reports indicate a possible role for HLA-G in the acceptance of allografts (11–13).

The aim of this study was to investigate the early events of HLA-G-related inhibition of T cell proliferation. We found that soluble HLA-G5 inhibited T cell proliferation and cell cycle progression, but did not induce T cell apoptosis.

Materials and Methods

Cell lines

The HLA class I-positive M8 melanoma cell line was kindly provided by F. Jotereau (Institut National de la Santé et de la Recherche Médicale Unité 211, Nantes, France) and was transfected with a full-length HLA-G5.
HLA-G inhibits cell cycle progression in T cells

Peripheral blood leukocytes were isolated from blood bank leukopheresis packs obtained from healthy volunteers (Etablissement Français du Sang). After Ficol-Isoaque density (d = 1.078) (Eurobio) gradient centrifugation, adherent cells were removed by incubating cells on plastic dishes and passed through nylon wool columns. CD4+ and CD8+ T cells were then negatively separated by immunomagnetic selection using anti-CD8- or anti-CD4-coated magnetic beads (Miltenyi Biotec).

T lymphocytes were stimulated for 3 or 5 days with 0.25 μg/ml OKT3 plus 100 U/ml IL-2 in RPMI 1640 medium with 10% decomplemented FCS. The mean concentration of HLA-G5 used in culture medium to inhibit activated T cells stimulated by OKT3 and IL-2 was 40 μg/ml.

Flow cytometric analyses of mitochondrial transmembrane potential (Δψm)

To evaluate changes in the inner Δψm, cells were stained for 15 min at 37°C with 40 nM potential sensitive fluorescent dye 3,3′-diethyloxacarbocyanine (DiOC6) from Molecular Probes. Cells with complete Δψm loss were obtained by a 10-min incubation with 5 μM carbonyl cyanide m-chlorophenyl hydrazone (CCCP; Sigma-Aldrich). As a positive control, cells were incubated with the Fas agonist Ab Apo1.3 (100 ng/ml) for 16 h in absence or in presence of Boc-D-fmk during the same period of time.

Immunoblot analyses

Cells were washed and lysed in 1% SDS and protease inhibitors (Complete; Boehringer Mannheim). The insoluble material was removed by spinning the cellular extract at 12,000 × g for 2 min. Protein concentrations were determined by the bicinchoninic acid protein assay (Pierce). Aliquots of 10–50 μg of total protein were separated by SDS-PAGE (with 10–15% polyacrylamide) and transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore). The membranes were blocked with 5% nonfat dried milk or with 5% BSA in PBS-0.1% Tween 20 for 1 h at room temperature and incubated with primary Abs for 45 min at room temperature. Secondary Abs conjugated with HRP were used, and they were detected by using the ECL detection system. For phospho-Rb, the membranes were blocked with 5% BSA in PBS-0.1% Tween 20. When necessary, the blots were stripped with a Western blot recycling kit (Euromedex; Chemicon International).

Proliferation assays

Proliferation responses were evaluated by culturing 10⁵ T lymphocytes in the presence of 5 × 10⁶ M8 cells pretreated with mitomycin in 0.150 ml of complete medium, or by directly activating T lymphocytes with OKT3 and IL-2, in 96-well flat-bottom plates.

Cultures were pulsed with 1 μCi of [³H]thymidine (Amersham Biosciences) on day 3 or 5 and harvested 18 h later. The dry filters were counted in a beta counter with scintillation fluid. Results are given as mean values of triplicate cultures.

Results

Soluble HLA-G inhibits T cell proliferation

To test the ability of HLA-G to inhibit allogenic T cell proliferation, we used stimulatory cells, M8 cells stably transfected with the empty vector (M8-pcDNA) or with the vector encoding the secreted protein HLA-G5 (M8-HLA-G5). The production of HLA-G5 was determined by Western blot analysis on cell lysate (Fig. 1A) and immunofluorescence (Fig. 1B). According to Western blot analysis, HLA-G5 exhibited the expected molecular mass of 37 kDa (14). In addition, HLA-G5 was determined in the supernatant of M8-pcDNA cells or M8-HLA-G5 cells by dot blot. HLA-G5 was only detected in the supernatant of M8-HLA-G5 cell cultures were collected and added to stimulated T cells with OKT3 and IL-2. The supernatants of M8-pcDNA cells inhibited T cell proliferation by >65%, whereas M8-pcDNA cells did not (Fig. 2A).

Then, M8-pcDNA and M8-HLA-G5 cells were treated with mitomycin C to inhibit their proliferation. They were then incubated with allogenic T lymphocytes for 1, 2, 3, or 4 days. M8-HLA-G5 cells inhibited T cell proliferation by >65%, whereas M8-pcDNA cells did not (Fig. 2A). The supernatants of M8-pcDNA and M8-HLA-G5 cell cultures were collected and added to stimulated T cells with OKT3 and IL-2. The supernatant of M8-HLA-G5 (Fig. 2B), but not that of the M8-pcDNA cells inhibited T cell proliferation in a dose-dependent manner, suggesting that the soluble form of HLA-G5 has an effect on alloreactive T cells (Fig. 2C).

A concentration of 40 μg/ml HLA-G5 was used in the subsequent experiments. To rule out any production of HLA-G5 by T lymphocytes, as previously observed, we determined by dot blot the production of HLA-G5 in the supernatant of T cells incubated with...
The concentration of HLA-G5 in the supernatant of M8 cells.

In presence of the supernatant of M8-pcDNA cells (Fig. 2A, B), HLA-G was detected in M8-HLA-G5 cells by indirect immunofluorescent staining using 4H84 Ab. Detection of HLA-G5 in the supernatant of M8-pcDNA (smM8-pcDNA) or in M8-HLA-G5 cells (snM8-HLA-G5). A standard curve of rHLA-G5 was used to quantify by dot blot the concentration of HLA-G5 in the supernatant of M8 cells.

The inhibition of T cell proliferation could be due to the death of T cells in the presence of HLA-G5 or secondary to the inhibition of their expansion. Puppo et al. and Fournel et al. (19, 26) reported that HLA-G5 induced CD8 T cell apoptosis through the activation of the Fas cascade by activating caspase. We determined whether the inhibition of caspase by the pan caspase inhibitor boc-D-fmk restored the proliferation of T cells in presence of M8-HLA-G5 supernatant. After 4 days of culture, T cell proliferation was similarly affected in the presence and in the absence of boc-D-fmk (Fig 4A). In addition, the number of T cells was similar in the presence and in the absence of boc-D-fmk (data not shown) during the early stage of activation. Similar results have been observed when using z-VAD.fmk instead of boc-D-fmk (data not shown). Both observations suggest that no caspase-mediated apoptosis occurs in the presence of HLA-G5. Moreover, using a marker of cell viability, the propidium iodide, we observed similar percentages of dead cells in the presence of HLA-G5 supernatant and in that of a control supernatant (Fig. 4B). Apoptosis involved the proteolytic activation of the effector caspase 3 in lymphocytes. We have determined the time course of the activation of caspase 3 corresponding to the occurrence of p17 and p12 form of caspase 3 detected by Western blotting in T cells incubated or not with HLA-G5. No p17/p12 caspase 3 can be identified in cells incubated with HLA-G5, whereas the Fas agonist Ab is associated with a significant activation of caspase 3. Because apoptosis is a rapid event leading to the inhibition of the $\Delta\psi_m$, we followed the time course of the occurrence of the decrease of the $\Delta\psi_m$ using the DiOC6 marker. As a positive control, T lymphocytes were incubated with the agonist anti-Fas Ab (Apo 1.3). In addition, the $\Delta\psi_m$ is affected by caspase-dependent or independent apoptosis pathway. They exhibited a high percentage of dying cells that are propidium iodide positive or DiOC6 negative (Fig. 4, D and E). In presence of boc-D-fmk, T lymphocytes had an elevated DiOC6-positive staining, and no propidium iodide can be detected, indicating, as previously described, that Boc-D-fmk inhibits caspase-dependent DiOC6 decrease. The $\Delta\psi_m$ of activated T cells (OKT3/IL-2) incubated with the supernatant of M8-HLA-G5 cells or control cells (M8-pcDNA) has also been determined. In cultures, up to 20% of lymphocytes were engaged in apoptosis and had a low $\Delta\psi_m$ (DiOC6$^{low}$), and >80% were alive and had a high $\Delta\psi_m$ (DiOC6$^{high}$) (Fig. 4F). In contrast, cells incubated with the decoupling agent CLCCP had a low $\Delta\psi_m$ (DiOC6$^{low}$). Cultures incubated with the supernatant containing HLA-G5 or with the supernatant of control cells had similar percentages of DiOC6-positive cells (Fig. 4F). These results indicate that HLA-G5 does not induce apoptosis in these conditions.
Soluble HLA-G5 inhibits T cell progression through the cell cycle

To proliferate, T cells must progress through the cell cycle. Following Ag recognition, the progression of T cells through the cell cycle is regulated by the phosphorylation of the Rb by the cyclin/cyclin-dependent kinase (cdk) complex, leading to the release of E2F that activates various genes. We first used quantitative Western blotting to follow the appearance of phosphorylated Rb in activated lymphocytes in the presence and in the absence of HLA-G5. In control lymphocytes, phosphorylated Rb accumulated between days 0 and 3. The accumulation of phosphorylated Rb was lower in cells incubated with HLA-G5 (Fig. 5A). In contrast, Rb did not accumulate in lymphocytes incubated with control supernatant, whereas it did in lymphocytes incubated with M8-HLA-G5 cells.

Phosphorylation of Rb is due to the sequential action of the cyclin/cdk complex that regulates progression through the cell cycle. Cyclins involved in T cell activation and progression through the cell cycle. Cyclin D2 involved in the G1 phase, cyclin E that regulates the G1-S transition, and cyclin A and B that regulate the S-G2 phase transition were all less abundant in cells incubated with the supernatant containing HLA-G5 than in control cells (Fig. 5C), in which their levels increase following T cell activation. This suggests that HLA-G5 regulates the initial step of progression through the cell cycle.

The regulation of cell cycle progression and the activation of the cyclin/cdk complex are controlled by the inhibitory kinase family (p21Cip1/p27Kip1). In resting lymphocytes, p27Kip1 is high and decreases when T cells become activated, leading to the activation of the cyclin/cdk complex. We followed the expression of p27Kip1 and p21Cip1 in cells incubated with or without supernatant containing HLA-G5. p27Kip1 was increased, whereas p21Cip1 was not significantly affected (data not shown). These data suggest that HLA-G5 impairs the progression of activated T cells from G1 to S phase.

To test whether HLA-G5 is able to interfere with the cell cycle activation in activated T cells, we have tested the modification of phosphorylated Rb and p27Kip1 in fully activated T cells. Four hours after the introduction of HLA-G5 supernatant, a decrease of phosphorylated Rb and an increase of p27Kip1 were observed (Fig. 6A).
Soluble HLA-G5 did not impair T cell phenotype but reduced cytokine production by activated T cells

During allostimulation, T cell expansion is associated with T cell activation, leading to their volume increase, acquisition of phenotypic markers, and the production of large amounts of cytokines. We first observed that the volume of T lymphocytes increased in alloreactive T cells incubated with allogenic M8-pcDNA or M8-HLA-G5, compared with resting lymphocytes, indicating that T cells even in the presence of HLA-G5 become activated. The mean volume was 208 μm³ in resting T cells, 329 μm³ in stimulated T cells, and 327 μm³ in stimulated T cells incubated with HLA-G5 (Fig. 6B). A similar percentage of stimulated T cells incubated with or without HLA-G5 expressed markers such as CD28, CD154 (CD40L), CTLA4, CD95, CD45RA (data not shown), CD45RO, or CD25 (α-chain of the IL-2R) (Fig. 6C). In contrast, the same percentage of CD4⁺CD25⁺ high that are the enriched population of regulatory T cells has been observed in cells incubated or not with HLA-G5.

The inhibition of the cell cycle progression can be due to the decreased activation of the TCR of T cells. The activation of TCR is associated with the phosphorylation of its ζ-chain. Fig. 6D showed that the phosphorylated ζ-chain observed in activated T cells was not reduced in presence of HLA-G5 supernatant (Fig. 6D).

The cytokine profile of T cells incubated with or without HLA-G5 has also been determined. In T lymphocytes incubated with allogenic M8-pcDNA cells, a high concentration of IL-2, IFN-γ, GM-CSF, and IL-10 (Fig 7), but not IL-4 and IL-5 (data not shown), was produced in a time-dependent manner. In contrast, IL-2, IFN γ, GM-CSF, and IL-10 were produced in small amount in cells incubated with allogenic M8-HLA-G5 cells. However, no IL-4 was produced, suggesting that HLA-G5 impaired the production of Th1 cytokines, but is not able to induce a polarized secretion of Th2 cytokines. Granzyme B and perforin, both produced by CTLs, were studied by Western blotting. The abundance of both proteins was lower in T cells incubated with HLA-G5 than in control cells (Fig. 7).

Discussion

HLA-G is a nonclassical HLA class I-associated molecule expressed during pregnancy by the extravillous cytotrophoblast at the interface between the maternal immune system and the semiallogenic embryo (29). HLA-G is believed to participate in graft acceptance by inhibiting NK cells. HLA-G is also expressed in the...
thymic epithelium and in cardiac, hepatic, and renal allografts in which its expression is associated with a reduction of acute rejection (11, 12). This suggests that HLA-G also regulates T cell functions. HLA-G interacts with CD8 and CD4 T cells via killing inhibitory receptors (KIR) and/or ILT, which are expressed by activated T cells (10, 30, 31). However, the mechanisms of action of HLA-G remain elusive. HLA-G inhibits CD8 T cell proliferation by inducing T cell apoptosis in CD8 blasts stimulated by PHA (19, 26). The induction of apoptosis is mediated by the activation of the Fas pathway, which activates intracellular caspases (32–34). T cell apoptosis through the Fas pathway requires the recruitment of the cytoplasmic TNFR-associated factor and activation of caspase 8, and then activation of the effector cysteine proteases, caspases 3, 6, and 7. In T cells incubated with the HLA-G supernatant, we did not observe any proteolytic cleavage of caspase 3 and then the occurrence of p17/p12 active caspase 3. The activation caspase 3 following the activation of Fas can be prevented by small peptides such as boc-D-fmk. We found that the presence of boc-D-fmk did not relieve the HLA-G5-mediated inhibition of the CD8 T cell proliferation, indicating that soluble HLA-G5 did not induce caspase activation. In addition, we found that CD8 T cell expansion was regulated rapidly in presence of HLA-G5 during the allogenic response: T cell proliferation (both CD4 and the CD8 T cells) was inhibited during the first hours following an allogenic response in MLR or after strong activation of T cells through TCR activation with IL-2 and anti-CD3. This regulation occurred before the establishment of the Fas-Fas ligand pathway in T lymphocytes, which requires a prolonged activation over several days (35). As previously reported, we confirmed that human T lymphocytes are not sensitive to the agonist anti-Fas Ab during the first days of allogenic stimulation or following IL-2/anti-CD3 stimulation (data not shown). Moreover, we used two sensitive markers of apoptosis, namely DiOC6, which indicates the transmembrane potential of mitochondria (ΔΨm), and propidium iodide. There was no evidence of apoptosis in T cells incubated with HLA-G5 during the first day of stimulation. These results indicate that HLA-G5 does not mediate inhibition of T cell proliferation through apoptosis during the early step of activation. However, we cannot exclude the possibility that later during a complete T cell activation, HLA-G can induce T cell apoptosis.

The initial inhibition of the T cell proliferation by HLA-G5 is due to the arrest of the cell cycle progression of T lymphocytes. The entry of resting T cells into the cell cycle following TCR activation is regulated by the protein p27kip1. Large amounts of protein p27kip1 are present in resting T cells (36). Following T cell activation and stimulation, the progression of T cell through the cell cycle correlates with the decrease in the amount of p27kip1.
and with the activation of Rb protein, which becomes phosphorylated. p27kip1 knockout mice have more CD4 memory cells than controls, and transgenic mice overexpressing p27kip1 have less T cell proliferation and a lower immune response than controls (37). These observations confirm the importance of p27kip1 in regulating the immune response and T cell proliferation. We found that there was more p27kip1 in T cells activated in presence of HLA-G5 than in control cells. Moreover, the amount of phospho-Rb protein was significantly lower. This is associated with lower expression of the early cyclins D2 and E, and also the expression of cyclins A and B, which are expressed later during the cell cycle. In addition, we observed that HLA-G5 incubated with fully activated T cells induced a rapid decrease of phosphorylated Rb and an increase of p27kip1. All of these results suggest that HLA-G5 regulates the balance between inhibitory molecules and cyclins in T cells, and inhibits the progression of T cells from the G1 to G2-M phase. However, HLA-G5 could regulate the inhibitory protein p27kip1, but does not affect the inhibitory molecules p21cip (data not shown). Although HLA-G could inhibit T cell proliferation in absence of peptides, HLA-E associated with HLA-derived peptides is also able to inhibit T cell proliferation. By an unknown mechanism, some HLA-derived peptides have been demonstrated to inhibit cell cycle progression even when stimulated with OKT3 and IL-2 (38). This is associated with the inhibition of cdk2 and accumulation of p27kip, as we observed. Mechanism(s) that led to the cell cycle arrest in presence of HLA-G5 is not yet known. One hypothesis would be that HLA-G5 might down-regulate TCR activation through the activation of phosphatase following its interaction with KIR. However, we did not observe any decrease of z-chain phosphorylation in presence of HLA-G5 in T lymphocytes stimulated by OKT3. In addition, the concentration of SHP-1 was not modified in presence of HLA-G5 (data not shown), suggesting that the effect of HLA-G5 is not reduced to decrease of TCR signaling. However, we cannot exclude that this pathway may reduce the downstream cascade following the TCR activation, but other mechanisms have to be identified.

During T cell activation, the expansion of cells is associated with differentiation leading to a polarized secretion of cytokines. Treatment of T cells with HLA-G5 decreased the production of the cytokines IL-2, IFN-γ, GM-CSF, and IL-10. This is in accordance with previous studies that have shown that HLA-G has a critical initial impact during the initiation of an allogenic reaction: initiation requires the secretion of numerous cytokines to stimulate the different cells involved in the acute phase of allogenic reaction. HLA-G may participate in the regulation of the allogenic response and may be a key regulator. Clinical studies indicate that HLA-G is associated with a decrease in the number of acute and chronic allogenic rejection events (11–13). However, we did not observe any increase of IL-4 or IL-5 concentrations upon HLA-G5 treatment, suggesting that HLA-G does not induce a Th2 polarization. Possibly, HLA-G is not able to generate regulatory T cells and has to be permanently expressed to control the allogenic reaction. Indeed, there was no difference in the T cell phenotype between those incubated with HLA-G5 and controls (data not shown), and
the removal of HLA-G from T cell culture was associated with T cell proliferation, demonstrating that the inhibitory effect of HLA-G was reversible. However, we observed a dramatic decrease of CD8 T cell proliferation, although a significant fraction, but not all of them expressed IILT2 receptors. It has been reported that most T lymphocytes expressed intracellularly IILT2 and that a significant fraction of activated T cells expressed a functional IILT2 at their cell surface. This suggests that several direct or indirect mechanisms could be involved in the HLA-G5-dependent control of T cell proliferation.

In conclusion, our findings indicate that HLA-G, in its soluble form, is a potent effector controlling the allogeneic response by inhibiting the cell cycle progression of allogeneic T cells and by limiting the production of Th1 cytokines.

Disclosures

The authors have no financial conflict of interest.

References


