Enhancement of in vitro human tubulogenesis by endothelial cell-derived factors: implications for in vivo tubular regeneration after injury

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The kidney has the capacity to regenerate completely after various insults (35). Differentiated tubular cells are thought to dedifferentiate and proliferate after injury. Following a surge of cell proliferation, regenerating cells are considered to acquire an immature phenotype, repopulate the damaged area, and redifferentiate into mature epithelial cells, leading to reconstruction of the functional integrity of nephrons (3). During tubular recovery after injury, hepatocyte growth factor (HGF) is considered to function as a renotrophic factor. Previous reports demonstrated that HGF promotes tubular regeneration after various insults, such as renal ischemia (9), HgCl2 (13, 19), and unilateral nephrectomy (23). HGF secreted by infiltrating macrophages after injury plays roles as a mitogen, motogen, and regenerative factor for renal tubular cells (24). Receptors for HGF are induced to form tubular structures by HGF (20, 21). Using this model, various factors have been discovered to be involved in branching tubulogenesis. Murine inner medullary collecting duct (IMCD)-3 cells (1, 5, 7, 26) or rat primary tubular cells (4) are induced to form tubular structures by HGF (20, 21). Using this model, various factors have been discovered to be involved in branching tubulogenesis. Murine inner medullary collecting duct (IMCD)-3 cells (1, 5, 7, 26) or rat primary tubular cells (4) are utilized for the study of tubulogenesis. These in vitro models, in which HGF has been shown to induce tubular formation, are well defined, practical, and contribute to an understanding of the mechanism of tubulogenesis. However, there have been no in vitro human tubulogenesis models established to date.

Here, we established a novel in vitro tubulogenesis model using human renal proximal tubular epithelial cells (RPTEC). In the presence of HGF, RPTEC cultured in gel form tubular structures morphologically equivalent to renal tubules in vivo. When cocultured with human umbilical vein endothelial cells (HUVEC), HGF-induced tubular formation was significantly enhanced. This could not be reproduced by the addition of VEGF, basic FGF, or PDGF. Protein array revealed that HUVEC produce various matrix metalloproteinases (MMPs). The stimulatory effects of coculture with HUVEC or HUVEC-derived condition medium were almost completely abolished by the addition of the tissue inhibitor of metalloproteinase (TIMP)-1 or TIMP-2. These data suggest that endothelial cell-derived factors including MMPs play a critical role in tubulogenesis and imply a potential role of peritubular capillary endothelium as a source of factor(s) required for tubular recovery after injury.

Materials and Methods

Reagents. Human recombinant proteins including HGF, bone morphogenetic protein (BMP)-7, insulin-like growth factor (IGF)-I, basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF)-BB, and recombinant mouse follistatin 288 were obtained from R&D Systems, (Minneapolis, MN). Recombinant human epidermal growth factor (EGF) was purchased from Promega (Madison, WI). Growth factor-reduced Matrigel was obtained from Becton Dickinson (Mountain View, CA), and atelocollagen was obtained from Koken (Tokyo, Japan).

Cell culture. Primary human RPTEC (Lonza, Walkersville, MD) were maintained in renal epithelial cell basal medium (REBM) supplemented with REGM complex (0.5 µg/ml hydrocortisone, 10 ng/ml...
hEGF, 0.5 μg/ml epinephrine, 6.5 ng/ml triiodothyronine, 10 μg/ml transferrin, 5 μg/ml insulin, 1 μg/ml gentamicin sulfate, and 0.5% FBS). Primary HUVEC (Clonetics, Walkersville, MD) were cultured in endothelial basal medium (HuMedia-EB2, Kurabo, Osaka, Japan) supplemented with HuMedia-EG (1 μg/ml hydrocortisone, 10 ng/ml hEGF, 5 ng/ml hFGF-B, 10 μg/ml heparin, 50 μg/ml gentamicin, 50 ng/ml amphotericin B, and 2% FCS). These cells were cultured under humidified conditions of 95% air-5% CO₂ at 37°C, and the culture medium was changed every 2–3 days.

Three-dimensional gel culture. RPTEC were suspended at 5 × 10³ cells/ml in the mixture of Matrigel and atelocollagen 1:1. The cell solution dispensed into Transwell filter inserts (pore size 0.4 μm; Corning, Corning, NY) was incubated at 37°C. After the solution had gelled, REBM supplemented with REGM complex with the indicated agents was added. In some experiments, HUVEC were cocultured under the filter. When cocultured with HUVEC, RPTEC were cultured in endothelial basal medium (HuMedia-EB2) supplemented with HuMedia-EG. Cultures were photographed under phase contrast using a Leica DM IRB (Leica, Wetzlar, Germany).

Ischemia-reperfusion injury. Ischemia-reperfusion injury was induced in male Wistar rats aged 7 wk (Charles River Japan, Tokyo, Japan), as described previously (17). The care and use of animals described in this study conformed to the procedures of the laboratory’s Animal Protocol, as approved by the Ethics Review Committee for Animal Experimentation of Gunma University Graduate School of Medicine.

Immunohistochemistry. Samples were fixed in 4% formaldehyde and embedded in paraffin. Sections (4 μm) were deparaffinized, rehydrated, and microwaved at 500 W three times for 5 min, or autoclaved at 120°C for 15 min in 10 mmol/l citric acid buffer to retrieve antigens. The sections were blocked for 1 h with a protein block (Dako Cytomation, Carpinteria, CA) and then incubated overnight at 4°C with primary antibodies as follows: goat anti-PCNA Ab, rabbit anti-aquaporin-1 (AQP1) Ab, rabbit anti-E-cadherin Ab, rabbit anti-megalin Ab, goat anti-zona occludens-1 (ZO-1) Ab (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Ki67 Ab, mouse anti-vimentin Ab (Lab Vision, Fremont, CA), and rabbit anti-Pax-2 Ab (Covance, Emeryville, CA). After a washing with PBS, sections were incubated with Alexa Fluor 488- or 568-conjugated anti-mouse/rabbit/goat IgG (Molecular Probes, Eugene, OR). Nuclei were stained with 4′-diamidino-2-phenylindole (DAPI). Immunofluorescent images were recorded as described previously (15). For immunohistochemical controls, the primary antibody was replaced with normal mouse/rabbit/goat serum and did not show positive staining, thus confirming specificity.

Light microscopy. Samples were fixed in 4% formaldehyde in PBS, dehydrated, and embedded in paraffin. Sections (4 μm) were cut and stained with hematoxylin and eosin (HE) or periodic acid-Schiff (PAS).

Electron microscopy. Samples fixed with 2% glutaraldehyde were postfixied with 2% osmium tetroxide and embedded in epoxy resin (Quetol812, Nisshin EM, Tokyo, Japan). Ultrathin sections (70 nm) were stained with uranyl acetate and lead acetate and were observed under a transmission electron microscope (JEM100CX-II, JEOL, Tokyo, Japan).

Cell growth. Cell growth was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide assay, as described previously (16).

Quantitative analysis of tubular formation. RPTEC were cultured in gels with the indicated factors. After the indicated periods, 30 colonies were randomly selected and the number of colonies with processes or tubular structures was measured; the results are expressed as the percentage of total colonies. Values are means ± SE (n = 4).

Transwell invasion and migration assay. For the invasion assay, RPTEC were cultured on a mixture of Matrigel and atelocollagen (1:1) on Transwell filters, under which HUVEC were plated and cultured for 10 days. The number of structures invading into gels in each well was counted.

For the migration assay, RPTEC cultured on type I collagen (BD Biosciences, San Jose, CA)-coated Transwell filters (pore size 8 μm; Corning, Corning, NY) were cocultured with HUVEC plated under filters for 6 h at 37°C in endothelial basal medium supplemented with HuMedia-EG. After removal of cells on the filter, samples were fixed in 4% formaldehyde in PBS and stained with hematoxylin. The number of cells migrating through the membrane in each randomly selected field was then counted. Data are means ± SE (n = 3).

Patient arrays. HUVEC were cultured in HuMedia-EB2 supplemented with HuMedia-EG for 48 h, and HUVEC-derived conditioned media (HUVEC-CM) were collected. Protein extraction was performed as described previously (36). Human Matrix Metalloproteinase Antibody Array I (Raybiotech, Norcross, GA) was used according to the manufacturer’s instructions. Briefly, after blocking, arrayed antibody membranes were incubated with 500 mg of protein for 2 h at room temperature. Membranes were then incubated with biotin-conjugated antibodies overnight at 4°C. After washing, membranes were incubated with HRP antibodies for 2 h at room temperature and were reacted with chemiluminescent substrate. The signal was detected with the Typhoon, Storm, and Image Quant Systems and quantified using Image J (National Institutes of Health).

Statistical analysis. The differences between means were compared by Student’s t-test, with P values of < 0.05 considered significant.

RESULTS

Histological changes in the kidney after renal ischemia. We first examined the histological changes in the kidney after renal ischemia (Fig. 1). Ischemia-reperfusion injury was induced in normal rats, and the kidneys were removed for histological analysis at 24 h after reperfusion. PAS staining confirmed that tubular dilation, cast formation, and tubular necrosis were present in the outer medulla of the ischemic kidneys (Fig. 1E) but not in normal data (not shown) or sham-operated kidneys (Fig. 1A). Most tubular cells positive for proliferating cell nuclear antigen (PCNA), a marker of cell proliferation, were colocalized with aquaporin-1 (AQP1), a marker of the brush border of proximal tubules, in ischemic kidneys (Fig. 1F), but not in sham-operated kidneys (Fig. 1B). Consistent with previous reports (10, 15), expression of Pax-2, a developmental gene indispensable for kidney organogenesis, was observed in tubular cells of the ischemic kidneys (Fig. 1G), but not in sham-operated kidneys (Fig. 1C). Most PCNA-positive cells expressed vimentin, a mesenchymal marker (Fig. 1H). These data suggest that regenerating cells with immature phenotypes were primarily present in proximal tubules of the kidney after renal ischemia.

Induction of tubular structures induced by HGF. Based on the above results, we attempted to establish an in vitro three-dimensional (3D) model that would mimic the in vivo regeneration processes of renal tubules after injury. Human RPTEC were cultured in gels in the presence of several growth factors, and the morphological changes of RPTEC were examined. RPTEC cultured in gels formed tubule-like structures in the presence of HGF (Fig. 2A). There were no tubulogenic effects by other growth factors such as BMP-7, EGF, IGF-I, and follistatin. Quantitative analysis demonstrated that the tubulogenic effects of HGF were dose dependent (Fig. 2B).

Characteristics of tubular structures induced by HGF. We then examined the phenotype of HGF-induced tubular structures. RPTEC formed multiple processes in the presence of HGF after 2 days of culture. Consistent with regenerating
tubular cells after renal ischemia (Fig. 1), these processes expressed the cell proliferation marker Ki67, the mesenchymal marker vimentin, and the developmental gene Pax-2 (Fig. 3A). Quantitative analysis demonstrated that HGF significantly increased the number of vimentin-positive cells and Pax-2-positive cells, but not Ki67-positive cells (Fig. 3B). At 5 days and thereafter, slit-like structures were observed (Fig. 3C). HE-stained sections revealed that these structures had a lumen (Fig. 3D), at the apical site of which AQP1, a marker of the brush border, was detected (Fig. 3E). E-cadherin, megalin, and ZO-1 could not be detected in HGF-induced tubular structures (data not shown). Electron microscopic evaluation confirmed a polarized tubular structure with a microvilli-rich apical surface facing the lumen and smooth basal surface in contact with the gel (Fig. 3F). These findings suggest that HGF-induced tubular structures are morphologically equivalent to renal proximal tubules in vivo.

Promotion of HGF-induced tubular structures by coculture with HUVEC. To investigate whether tubular formation is influenced by endothelial cells, RPTEC in gels on Transwell filters were cocultured with HUVEC plated under the filter (Fig. 4A). Tubular structures were induced in the presence of HGF (Fig. 4B). When cocultured with HUVEC, tubule formation was induced in the presence of HGF, but not in the absence of HGF (Fig. 4B). Quantitatively, the number of tubular structures was significantly increased when cocultured...
Fig. 3. Characteristics of tubular structures induced by HGF. A: phenotype of tubular process induced by HGF. RPTEC were cultured in gels in the presence of HGF (20 ng/ml) for 2 days. Expression of Ki67, vimentin, and Pax-2 in HGF-induced tubular processes was examined by immunostaining. Bars = 5 μm. B: quantification of marker-positive cells. Values are means ± SE (n = 3). *P < 0.05. NS, not significant vs. HGF (−). C: presence of slit-like lumen (arrowheads) in HGF-induced tubular structures after 7 days of culture. Bars = 20 μm. D: hematoxylin and eosin (HE)-stained sections of HGF-induced tubular structures. Bars = 5 μm. E: localization of AQP1 at the apical side of the lumen in HGF-induced tubular structures. AQP1 (green), DAPI (blue). Bars = 5 μm. F: electron microscopic analysis of tubular structure induced by HGF. Bars = 1 μm.
with HUVEC compared with that without HUVEC (Fig. 4C). We further investigated the effects of HUVEC-CM on HGF-induced tubular formation. Consistent with the above results, HGF-induced tubular formation was significantly enhanced in the presence of HUVEC-CM (Fig. 4D), thus suggesting the presence of HUVEC-derived soluble factors that can stimulate HGF-induced tubular formation.

Effects of various growth factors on HGF-induced tubular formation. To identify the factor(s) responsible for the stimulatory effects of HUVEC on HGF-induced tubule formation, we tested the effects of known endothelial cell-derived growth factors such as bFGF, PDGF, and VEGF on tubule formation induced by HGF. No additional tubulogenic activity by these factors was observed (Fig. 5A). HGF-induced tubular formation was not enhanced, even in the presence of a mixture of these factors (Fig. 5A). We also examined the effects of high concentrations of HGF on the morphology of RPTEC cultured in gels. As shown in Fig. 5B, stimulatory effects of HUVEC on HGF-induced tubular formation were observed even in the presence of high concentration of HGF, thus suggesting that endothelial cell-derived factors other than HGF, bFGF, VEGF, and PDGF promoted HGF-induced tubular formation.

Promotion of cell proliferation in RPTEC by coculture with HUVEC. We next examined the effects of coculture with HUVEC on cell growth in RPTEC on monolayer culture by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Fig. 6). RPTEC were cultured on Transwell filters, under which HUVEC were plated. Cell proliferation was not induced by HGF, but a significant increase in cell proliferation was observed under coculture with HUVEC in either the presence or absence of HGF.

Effects of coculture with HUVEC on cell invasion and migration in RPTEC. We also examined the effects of coculture with HUVEC on cell invasion and migration in RPTEC. RPTEC were cultured on gels with Transwell filters, under which HUVEC were plated (Fig. 7A). Cell invasion was observed in the presence of HGF either with or without HUVEC after 7 days of culture (Fig. 7B), but not with HUVEC alone (data not shown). HE-stained serial sections revealed the presence of structures invading the gels after 7 days of culture (Fig. 7C). Quantitatively, the number of invading structures increased significantly under coculture with HUVEC plus HGF compared with that with HGF alone (Fig. 7D).

We then examined the effects of coculture with HUVEC on cell migration in RPTEC. RPTEC grown on type I collagen-coated Transwell filters were cocultured with HUVEC plated under the filters. Cell migration was not induced in the presence of HGF alone. In contrast, there was a significant increase in the number of migrating cells under coculture with HUVEC in either the presence or absence of HGF (Fig. 7E).
Involvement of multiple MMPs in stimulatory effects of coculture with HUVEC. Tubular structures are considered to be formed via cell proliferation, migration, differentiation, and degradation of ECM. The above results suggest that HUVEC produce factor(s) that can degrade ECM components. We thus examined the presence of MMPs and TIMPs in HUVEC-CM by protein array. As shown in Fig. 8, HUVEC-CM contained a variety of MMPs. Several TIMPs were also produced by HUVEC. To further examine whether HUVEC-derived MMPs play a role in tubular formation, the effects of recombinant TIMP-1 and TIMP-2 were tested. To exclude the direct influence of TIMPs on RPTEC, HUVEC were preincubated in the presence of TIMPs for 24 h, and then Transwell filter inserts containing RPTEC in gels were incubated with HUVEC, followed by the addition of HGF. It is also possible that the addition of TIMPs affects HUVEC condition directly. To exclude this possibility, HUVEC-CM were preincubated with TIMPs for 24 h, followed by coculture with RPTEC in the presence of HGF. Both TIMP-1 and TIMP-2 almost completely abolished the stimulatory effect of coculture with HUVEC (Fig. 8C, top) or HUVEC-CM (Fig. 8C, bottom). These results suggest that various MMPs produced by HUVEC accelerate HGF-induced tubular formation via the degradation of ECM components.

DISCUSSION

In the present study, we established an in vitro tubulogenesis model using human proximal tubular cells. HGF, proven to be renotropic, induced tubular structures in RPTEC cultured in gels (Fig. 2). Similar to regenerating tubular cells after injury in vivo (Fig. 1), HGF-induced multiple processes acquired an immature phenotype and expressed a developmental gene, Pax-2, at 2 days after HGF treatment. Treatment of HGF significantly increased the number of vimentin-positive and Pax-2-positive cells (Fig. 3). HGF is a potent inducer of cell proliferation in various types of epithelial cells. However, mitogenic effect of HGF on RPTEC could not be observed in this model, probably due to that culture media containing supplements that can promote cell growth in RPTEC, for example, EGF or FBS. To support this notion, when cultured in gels, >80% of RPTEC were positive for Ki67 even in the absence of HGF. A proliferative stimulus might not be enough to induce tubular formation. At the later stages of tubular formation, these structures showed an AQP1-positive polarized lumen with microvilli (Fig. 3). Given that E-cadherin, megalin, and ZO-1 could not be detected in HGF-induced tubular structures (data not shown), in vitro tubular structures might be less differentiated compared with renal tubules in vivo. At any rate, these results indicate a similar mechanism between in vitro tubulogenesis and in vivo tubular regeneration after injury. For the identification of additional renotropic factors, tubular cell proliferation assays are widely used. However, this assay represents only one component of the processes of tubular recovery after injury. On monolayer culture, in contrast to the in vivo state, growth factors added to the culture medium interact primarily with the apical surface of tubular cells. In this context, the in vitro 3D tubulogenesis model established here, where the basolateral surface of tubular structures is exposed to the factors released by endothelial cells, would more closely resemble the in vivo situation. This model,
therefore, is appropriate for screening regulators or chemicals involved in tubulogenesis as well as tubular regeneration after injury.

In various organs, tissue regeneration after injury is considered to be modulated by paracrine factors released by neighboring cell types. Given that the kidney is a vascular organ, it is quite possible that tubular recovery after injury will be regulated by peritubular capillaries surrounding renal tubules. However, it is difficult to investigate the direct interaction between tubules and peritubular capillaries during tubular regeneration after injury in vivo. We found that HGF-induced tubular structures were significantly enhanced by HUVEC-derived factors in this model (Fig. 4), thus suggesting that endothelial cell-derived factor(s) are involved in tubulogenesis, as well as in the recovery process of renal tubules after injury.

Previous reports have demonstrated the involvement of several endothelial cell-derived factors in cell growth and differentiation in renal tubular epithelial cells. VEGF induces a proliferative and an antiapoptotic response in tubular cells and acts as a survival factor for these cells (11, 34). The expression of VEGF by endothelial cells is stimulated in response to hypoxia to preserve the capillary blood supply (14) and to promote tubular cell survival and recovery (28). PDGF-B plays an important role in the regeneration of tubular cells from acute ischemic injury (24). bFGF, which is produced by capillary endothelial cells (22, 29), participates in the recovery process after renal ischemia (32, 33). However, these factors could not reproduce the stimulatory effect of coculture with HUVEC (Fig. 5). The activity produced by HUVEC may thus represent either a novel molecule(s) or an unknown function(s) of a known molecule(s). Further study is necessary to clarify this issue. Coculture with HUVEC stimulated RPTEC proliferation (Fig. 6) but could not induce tubular formation in the absence of HGF. In contrast, HGF induced tubular formation in gels (Fig. 2), but did not stimulate cell proliferation in monolayer culture (Fig. 6). These data suggest that the growth-promoting activity is separable from tubule-inducing activity.

Regeneration processes in the kidney after injury involve not only cell proliferation, migration, and differentiation but also the remodeling of ECM. MMPs, which are ECM-degrading enzymes, are considered associated with episodes of cell invasion during tubulogenesis (25). It has been reported that laminin, entactin, and fibronectin facilitate the formation of tubular structures, while type IV collagen, heparin sulfate proteoglycan, and vitronectin cause marked inhibition of HGF-induced branching morphogenesis. These data suggest the presence of facilitatory ECM and inhibitory ECM on tubulogenesis (27). In the present study, cell invasion and migration in RPTEC were induced by coculture with HUVEC (Fig. 7), suggesting the presence of ECM-degrading enzymes released by HUVEC. The culture gels used in this model consist of laminin, type IV collagen, and type I collagen. Given that type
IV collagen acts as inhibitory ECM on tubulogenesis (27), gelatinases such as MMP-2 and MMP-9, both of which degrade type IV collagen, might be involved in the stimulatory action of coculture with HUVEC. To support this idea, protein array showed that HUVEC-CM contained both MMP-2 and MMP-9 (Fig. 8, A and B). In addition, TIMP-1 as well as TIMP-2 cancelled the stimulatory effects of HUVEC or HUVEC-CM on tubulogenesis (Fig. 8 C). Protein array analysis revealed that HUVEC-CM contained not only MMPs but also TIMPs. Given that recombinant TIMP-1 or TIMP-2 on HGF-induced tubule formation in RPTEC cocultured with HUVEC (top) or with HUVEC-CM (bottom). RPTEC were cultured in gelf in the presence of HGF with HUVEC or HUVEC-CM pretreated with the indicated dose of TIMP-1 or TIMP-2 for 24 h. The number of tubular structures was measured and is expressed as the percentage of the number under coculture with HUVEC or HUVEC-CM in the presence of HGF plus TIMP-1 or TIMP-2 vs. that in the presence of HGF alone. Values are means ± SE (n = 3). **P < 0.01, *P < 0.05 vs. 0 μg/ml.

IV collagen acts as inhibitory ECM on tubulogenesis (27), gelatinases such as MMP-2 and MMP-9, both of which degrade type IV collagen, might be involved in the stimulatory action of coculture with HUVEC. To support this idea, protein array showed that HUVEC-CM contained both MMP-2 and MMP-9 (Fig. 8, A and B). In addition, TIMP-1 as well as TIMP-2 cancelled the stimulatory effects of HUVEC or HUVEC-CM on tubulogenesis (Fig. 8 C). Protein array analysis revealed that HUVEC-CM contained not only MMPs but also TIMPs. Given that recombinant TIMPs have inhibitory effects on HUVEC action, it is possible that HUVEC-derived MMPs are still active even in the presence of TIMPs in this experiment. The local balance between MMPs and TIMPs might be one of the determinants for tubular formation.

It has been reported that significant accumulation of type IV collagen occurs in an ischemic injury model (8). MMP-2 and MMP-9 are reportedly upregulated in the tubulointerstitial space (2) or in the endothelial compartment of the kidney after renal ischemia (6). In the present study, other MMPs were clearly involved in this process, because of the overlapping activity and specificity of MMPs. To identify the MMPs responsible for HUVEC action, we tested the effect of recombinant MMP-1, MMP-2, and MMP-9. However, none of these MMPs could mimic the effect of coculture with HUVEC (Miya M, Maeshima A, unpublished observations). It is possible that other MMPs or synergistic effect of multiple MMPs will reproduce HUVEC action. Further study will be needed to clarify this issue. Nonetheless, it is possible that endothelial cell-derived gelatinases synergistically act as potent enhancers of tubulogenesis, as well as of tubular recovery after injury. It is thought that the potential for tubular recovery after injury declines in the elderly, and it has been reported that the peritubular capillary area surrounding renal tubules decreases with aging (12, 31). Protein array revealed that the expression levels of MMPs in HUVEC were negatively associated with their passage number (unpublished observations). Collectively, it is possible that the capacity for tubular repair is impaired in aged kidneys due to the shortage of endothelial-derived factors such as MMPs. Peritubular capillaries may thus be essential resource-supplying factor(s) indispensable for tubular regeneration after injury, and a target for enhancing tubular repair after various insults, particularly in elderly populations.
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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