From the Society for Vascular Surgery

Controlled release of small interfering RNA targeting midkine attenuates intimal hyperplasia in vein grafts

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Objective: **Intimal hyperplasia is a major obstacle to patency after vein grafting. Despite of a diverse array of trials to prevent it, a satisfactory therapeutic strategy for clinical use has not been established. However, sufficient inhibition of early stages of intimal hyperplasia may prevent this long-term progressive disease. Midkine (MK) is a heparin-binding growth factor that was originally discovered as the product of a retinoic acid-responsive gene. We previously demonstrated that MK-deficient mice exhibit a striking reduction of neointima formation in a restenosis model, which is reversed on systemic MK administration. In this study, we evaluated a strategy of using small interfering RNA (siRNA) targeting MK as a therapy for vein graft failure.**

Methods: **We first made a highly effective siRNA to rabbit MK. Jugular vein-to-carotid artery interposition vein grafts, which are applied to a low flow condition, were made in Japanese white rabbits. Small interfering RNA mixed with atelocollagen was administrated to the external wall of grafted veins. Cy3-conjugated stabilized siRNA was used to confirm its stability and successful transfer into the vein graft wall. Neointimal hyperplasia was evaluated 4 weeks after the operation. The proliferation index and leukocyte infiltration were determined.**

Results: **MK expression was induced and reached the maximum level 7 days after operation. Fluorescence of Cy3-labeled siRNA could be detected in the graft wall even 7 days after operation. Knockdown of the gradually increasing expression was achieved by perivascular application of siRNA using atelocollagen. The intima-media ratio and the intima thickness at 28 days after grafting were both reduced >90% by this treatment compared with controls. This phenomenon was preceded by significant reductions of inflammatory cell recruitment to the vessel walls and subsequent cell proliferation in MK siRNA-treated grafts.**

Conclusions: **These results suggest that midkine is a candidate molecular target for preventing vein graft failure. Furthermore, for clinical applications of siRNA, a single intraoperative atelocollagen-based nonviral delivery method could be a reliable approach to achieve maximal function of siRNA in vivo. This strategy may be a useful and practical form of gene therapy against human vein graft failure. (J Vasc Surg 2006;44:633-41.)**

Clinical Relevance: **Intimal hyperplasia is a major obstacle to patency after vein grafting. Despite a diverse array of trials to prevent it, a satisfactory therapeutic strategy for clinical use has not been established. However, sufficient inhibition of early stages of intimal hyperplasia may prevent this long-term progressive disease. Here, we report almost complete suppression of intimal hyperplasia. Knockdown of growth factor midkine expression was achieved by the perivascular application of small interfering RNA (siRNA) using atelocollagen. Our data indicate that the combination of siRNA and its controlled release could be a therapeutic strategy for vein graft failure and that midkine is a potent molecular target for the prevention of intimal hyperplasia.**

An autologous vein is the most commonly used conduit for peripheral bypass grafting in the treatment of ischemia resulting from occlusive vascular disease. However, the vein graft patency is limited by progressive intimal hyperplasia and subsequent atherosclerosis, with patencies of 80% for 1 year and 60% for 5 years.[1,2] The patencies can become lower, particularly in cases with poor runoff vessels. Hemodynamic factors such as a low flow velocity and a low shear stress result in intimal thickening.[3-8] Considering the high use of the procedure, approximately 100,000 lower extremity bypasses are performed in the United States each year,[9] a means to solve the stenosis problem has been awaited. In addition, coronary artery bypasses and percutaneous transluminal coronary angioplasty are also problematic at similar frequencies.[10,11]

A diverse array of molecular targets for each step have been postulated, including monocyte chemoattractant protein-1, E2F, c-myc, matrix metalloproteinase, and transforming growth factor β, and many trials for the prevention of stenosis have been performed.[9,12-25] Nevertheless, a satisfactory strategy for clinical use has not been established.[9,12]

Midkine (MK) is a heparin-binding growth factor that was originally discovered as the product of a retinoic acid-responsive gene.[26,27] MK exhibits 50% sequence identity with the pleiotrophin/heparin-binding growth-associated molecule (PTN/HB-GAM).[28-31] The three major biologic

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roles of MK are in the nervous system, cancer, and inflammation.[30] MK supports neuronal cell survival and neurite extension.[32,33] The neuronal cytoprotective effect of MK has been demonstrated in various in vivo models, including retinal degeneration induced by constant light exposure,[34] cerebral infarction,[35] and ischemia-induced neuronal death.[36,37] In human carcinomas, MK expression is often elevated and is associated with a poor prognosis. MK antisense oligodeoxyribonucleotide (ODN) can suppress MK expression and tumor formation.[38] In addition, MK is involved in inflammation, as revealed by studies involving MK-deficient (*Mdk−/−*) mice. Ischemic reperfusion-induced renal damage,[39] cisplatin-induced renal damage,[40] and rheumatoid arthritis[41] are manifested to a lesser extent in *Mdk−/−* mice. MK antisense ODN can suppress ischemic reperfusion-induced renal damage[42] and cisplatin-induced renal damage in *Mdk+/+* mice.[40]

We previously reported that *Mdk−/−* mice exhibit a striking reduction of intimal hyperplasia in an arterial restenosis model.[43] MK expression is transiently increased, the maximum level being reached at day 7, in a rat balloon injury model, in which the intimal hyperplasia is completed at about day 14.[43] Supporting these data, MK antisense oligodeoxyribonucleotide (ODN) suppresses MK induction and consequently intimal hyperplasia in a rabbit balloon denudation model.[44] However, the ODN suppressed intimal hyperplasia by <40% compared with control treatment.[44] We speculated that this modest effect was attributable to MK expression being gradually induced, whereas the ODN was administered only once at the time of operation. Therefore, a means of longstanding, full knockdown of MK appeared to be needed.

Here, we report that the combination of siRNA with MK and its controlled release using atelocollagen led to striking suppression of intimal hyperplasia in a vein graft model. This study provides a strategy for sufficient prevention of intimal hyperplasia.

MATERIALS AND METHODS

SiRNAs. Five siRNAs targeting rabbit MK and one scrambled siRNA with the following sense and antisense sequences were synthesized: #1 (bases 65-83), 5'-CCAAAAAGAAAGACAAGGUdTdT-3' (sense), 5'-ACCUUGUCUUUCUUUUUGGdTdT-3' (antisense); #2 (bases 316-334), 5 CUGAAGAAGGCUCGGUACAdTdT-3' (sense), 5'-UGUACCGAGCCUUCUUCAGdTdT-3' (antisense); #3 (bases 383-401), 5'-AGACCAAAGCCAAGGCCAAdTdT-3' (sense), 5'-UUGGCCUUGGCUUUGGUCUdTdT-3' (antisense); #4 (bases 404-420), 5'-CCAAGAAAGGGAAGGCAAAdTdT-3' (sense), 5'-UUUGCCUUCCCUUUCUUGGdTdT-3' (antisense); #5 (bases 410-428), 5'-AAGGGAAGGCAAAGGACUAdTdT-3' (sense), 5'-UAGUCCUUUGCCUUCCCUUdTdT-3'(antisense); and #2-SCR, 5'-GGCGGCUCAUGAACGAAUAdTdT-3' (sense), 5'-UAUUCGUUCAUGAGCCGCCdTdT-3' (antisense). All the siRNAs were designed by B-Bridge International, Inc, Sunnyvale, Calif, and synthesized by Dharmacon, Inc, Lafaytte, Colo, using 2'27-bis (acetoxyethoxy)-methyl ether (ACE) protection chemistry. We selected siRNA sequences as reported by Elbashir et al.[45] Each freeze-dried siRNA was reconstituted in RNase-free water to prepare a 20 μmol/L stock solution. For the in vivo study, we used stabilized siRNAs, which were modified siRNAs with improved stability and silencing longevity (siSTABLE, Dharmacon). We also used Cy3-conjugated stabilized siRNAs to confirm their stability and successful transfer into vein grafts.

Cell culture conditions and transfection of siRNAs in vitro. RK13 cells (American Type Culture Collection, Manassas, Va) derived from rabbit kidney were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing heat-inactivated fetal bovine serum at 37°C under a humidified atmosphere of 5% carbon dioxide. Cells were plated at a density of 1×10^5 cells/35 mm tissue culture dish 20 hours before transfection, resulting in 30% to 50% confluence on the day of transfection. In a separate tube, 5 μL of each siRNA stock solution (20 μmol/L) was diluted with 95 μL of Opti-MEM (Invitrogen, Carsbad, Calif), and 3 μL of Lipofectamine 2000 (Invitrogen) was diluted with 97 μL Opti-MEM. The diluted siRNAs and diluted Lipofectamine 2000 were then combined, gently mixed, and incubated for 20 minutes at room temperature. The siRNA and Lipofectamine 2000 mixture was added directly to the cells in 0.8 mL of culture medium. The medium was replaced with serum-free DMEM containing heparin (20 μg/mL) 48 hours after transfection. After 24 hours of incubation, the conditioned medium was collected for Western blot analysis.

Vein graft model and in vivo transfection. Japanese white rabbits (2.5 to 3 kg) were anesthetized by intramuscular administration of ketamine (35 mg/kg) and xylazine (10 mg/kg). The no-touch technique was used to dissect out a 25-mm segment of the right external jugular vein through a midline vertical neck incision, and all branches were carefully ligated with 8-0 polypropylene sutures and divided. The animals were systemically heparinized (200 U/kg). The ipsilateral common carotid artery was clamped distally and proximally, and a graft was anastomosed in an end-to-end fashion into the divided artery with interrupted 8-0 polypropylene sutures.

Atelocollagen (Koken Co, Ltd, Tokyo, Japan) was used for transfection in vivo. Atelocollagen is a highly purified type 1 collagen derived from calf dermis by pepsin treatment. Before the operation, siRNAs and atelocollagen complexes were prepared as follows: 300 μL of 3.5% atelocollagen and 300 μL of a stabilized siRNA solution (20 μmol/L) or saline alone were mixed and kept at 4°C before use. Then the mixture was used to coat the external surface of the vein graft. This complex formed a gel after appropriate heat treatment with a hair dryer, which was used for <10 seconds and did not dry or injure the vein grafts. This resulted in incubation of siRNAs around the graft for at least 1 week. Before wound closure, the grafts were subjected to low flow conditions as described previously.[46] The animal experiments in the present study were performed in

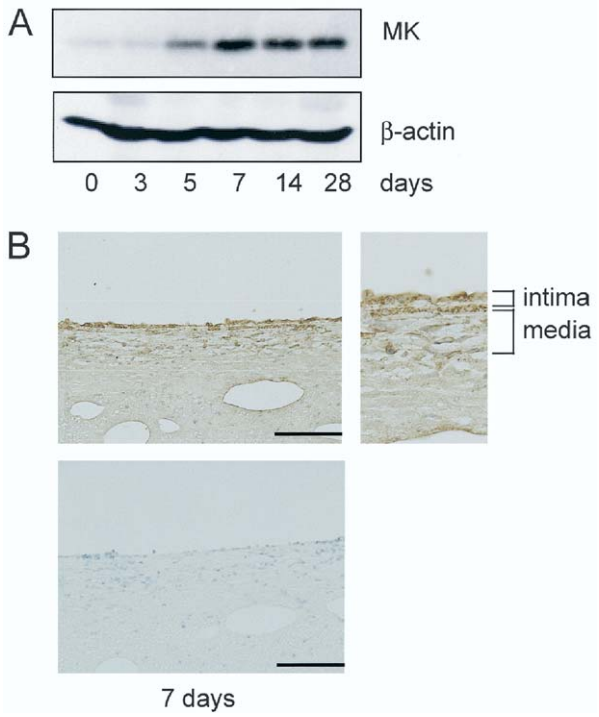

Fig 1. Increased midkine *(MK)* expression in a rabbit vein graft. **A,** The time course of MK expression in the rabbit vein graft is shown. Protein extracts of grafted veins at the indicated times after operation were subjected to Western blot analysis for MK and β-actin. **B,** Immunostaining for MK 7 days after operation is shown (upper left and right). A magnified photo of the upper left panel is shown at upper right. The bottom panel shows a control staining without the first antibody of an adjacent section. Bars, 100 μm.

compliance with the guidelines of the Institute for Laboratory Animal Research, Nagoya University Graduate School of Medicine.

Western Blot analysis. A whole graft explanted on the indicated day after operation was snap-frozen, ground into powder, and then lysed in 1 mL of 10 mM Tris-HCl (pH 7.4) containing 150 mmol/L sodium chloride, 1% Triton X-100, and 1 mmol/L phenylmethylsulfonyl fluoride. The supernatant of the lysate obtained on centrifugation was subjected to Western blot analysis as described previously.[47]

Fluorescence microscopy. Cy3-conjugated stabilized siRNAs were used to confirm their stability and successful transfer into the vein graft wall. Grafts coated with atelocollagen with or without the siRNAs were harvested 1 week after operation and immediately embedded in OCT compound and frozen in liquid nitrogen. A cryostat was used to cut 4-μm-thick sections, which were then observed (MRC-1024, Bio-Rad Laboratories).

Histopathology, immunostaining, and morphometry. Vein grafts were fixed at 100 mm Hg for 20 minutes and immersed overnight in 4% paraformaldehyde, and then four evenly spaced segments (4 to 5 mm apart) of the middle portion of each graft were embedded in paraffin. Serial sections were stained with hematoxylin and eosin or elastic Van Gieson stain, and were also stained for the detection of proliferating cell nuclear antigen (PCNA, 1:100; Ki-67, 1:75; Dako, Glostrup, Denmark). Frozen sections made after 7 days were used for the detection of leukocytes (CD45, 1:100; Pharmingen, San Jose, Calif). Controls for the immunostaining included incubation with class-matched and species-matched immunoglobulins (Ig) and omission of primary antibodies. For MK immunostaining, we used chicken antihuman MK (IgY), which recognizes rabbit MK, as the primary antibody and chicken IgY as the control. Intimal hyperplasia was quantitated by using imaging software (ImageJ, National Institutes of Health, Bethesda, Md).

Intimal and medial cross-sectional areas were measured, and the neointimal thickness was measured at eight randomly selected points for each section. The proliferation index was determined by dividing the number of positively stained nuclei by the total number of nuclei in the intima of each section. The leukocyte count was determined as the total number of $CD45^+$ cells in the graft wall of each section. The intima-media ratio, intimal thickness, proliferation index, and cell counts were determined from these data, and the averages for each vessel were calculated. Two observers controlled the obtained values for randomly selected multiple samples.

Statistical analysis. Stat View 5.0 (SAS Institute, Cary, NC) software was used for statistical calculations. Values are expressed as means ± SEM, and compared with the double-placebo-treated control group by means of analysis of variance involving the Bonferroni post hoc analysis. Comparisons between two groups were performed with the unpaired t or Mann-Whitney U test. $P < .05$ was considered statistically significant.

RESULTS

MK expression in a rabbit vein graft. A controlled release of MK knockdown reagents applied from outside into the vein wall was possible because the vein wall is thinner and looser than that of the artery. In contrast to the limited use of the mouse model because of high-level techniques, rabbit vein graft models are commonly used and we already had a complimentary DNA sequence of rabbit MK. We therefore decided to use a rabbit vein graft model in the present study.

A healthy ungrafted vein expresses tiny amounts of MK. The interposition of a vein into an arterial vessel, however, led to a significant increase in MK expression, the maximum level being reached 7 days after operation. It then decreased but persisted >4 weeks (Fig 1). At 7 days after operation, strong MK expression was noted in intimal and endothelial cells and moderate expression in medial cells (Fig 1).

Effects of siRNAs on MK expression by RK13 cells. SiRNA degrades RNA and thus inhibits expression of a target molecule at the RNA level. The overall inhibition

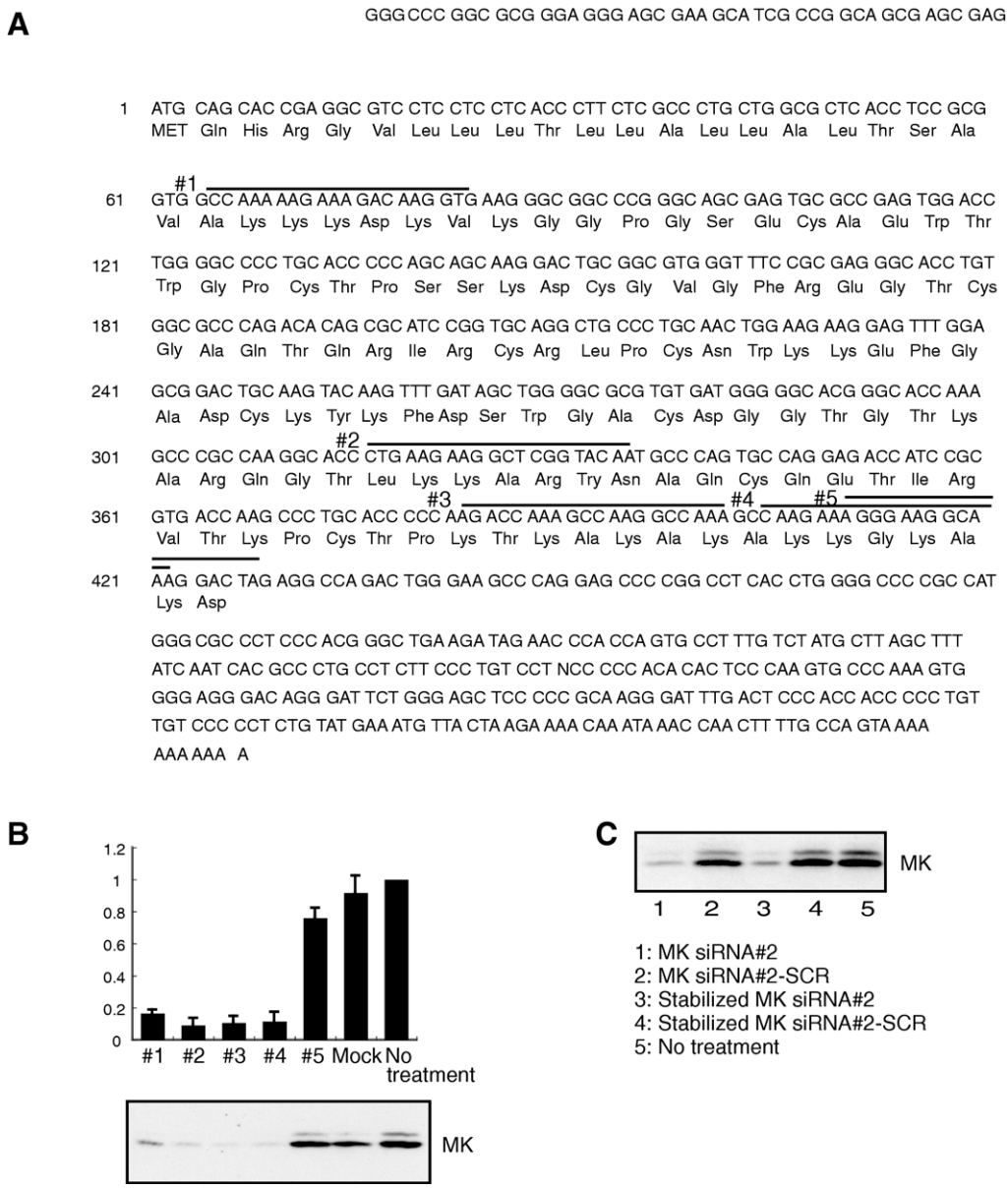

Fig 2. Effects of small interfering RNAs *(siRNAs)* targeting rabbit midkine *(MK)* on its expression in cultured cells. **A,** The full-length rabbit MK complimentary DNA sequence and design of siRNAs targeting rabbit MK are shown. The five siRNAs examined in this study are indicated by bars. **B,** RK13 cells were transfected with MK siRNAs or Lipofectamine 2000 alone (mock), or not treated (n = 3 dishes). A representative Western blot for MK is shown. The relative densitometric intensities of Western blot bands of MK were calculated and are presented as the average ± SEM in the graph (n = 3). **C,** Stabilized siRNAs retained inhibitory effects equal to those of the unmodified siRNAs. A Western blot for MK is shown.

effect of siRNA is stronger than that of ODN. Clinical application of siRNA has become realistic with current techniques; therefore, we used siRNA in this study. We designed five rabbit MK siRNAs (Fig 2). MK siRNA #2 most strongly suppressed the secretion of MK by rabbit kidney cell line RK13 (Fig 2). Densitometric analysis of a Western blot revealed that it suppressed MK production to 9% of that in control cultures (Fig 2). Scrambled siRNA (MK siRNA #2-SCR) showed no effect (Fig 2). We also confirmed that the stabilized siRNAs retained inhibitory effects equal to those of the unmodified siRNAs (Fig 2). Hereafter, we used the stabilized siRNAs for all animal experiments.

Stability and successful transfer of siRNAs. We used a vein graft model with poor runoff because this model mimics the clinical situation in which vein grafting is per-

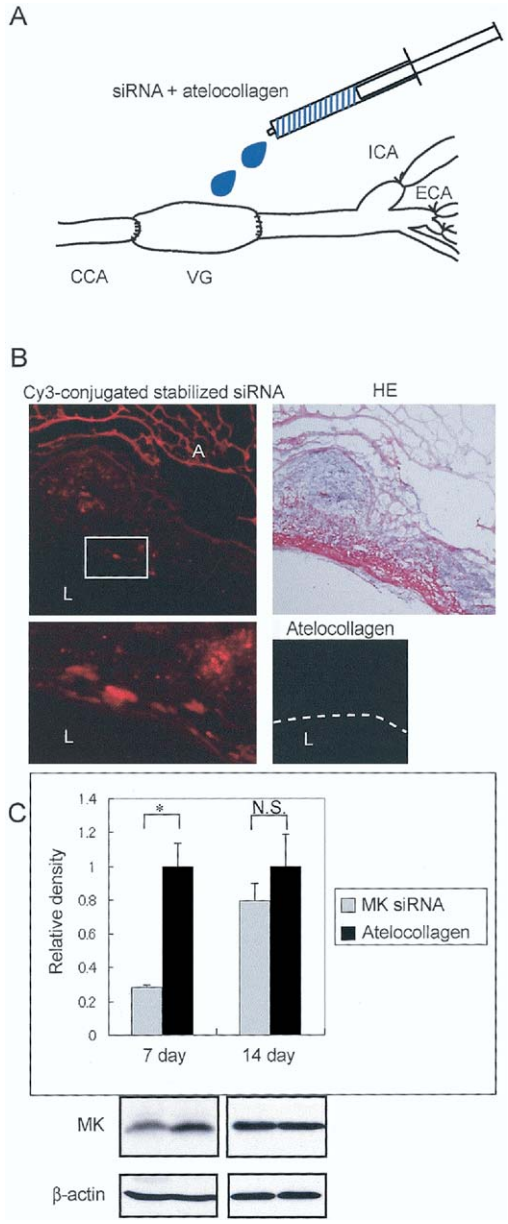

Fig 3. Controlled release of small interfering RNA *(siRNA)* into the grafted vein wall. **A,** A poor run-off vein graft model with distal branch ligation is depicted. The most inferior branch of the external carotid artery serves as the only outflow for the low-flow graft. *CCA,* common carotid artery; *ECA,* external carotid artery; *ICA,* internal carotid artery; *VG,* vein graft. **B,** Successful transfer in vivo was determined by fluorescent photomicrography of atelocollagen with a Cy3-conjugated scrambled siRNA-treated graft 7 days after operation. Low *(upper left)* and high *(lower left)* magnifications are shown. The upper right panel shows an adjacent section stained with hematoxylin and eosin. Note the absence of the Cy3 signal *(red color)* in the graft treated with atelocollagen without siRNA *(lower right)*. The *dotted line* indicates the internal edge of the vessel. *A,* atelocollagen; *L,* indicates lumen. **C,** The effect of midkine *(MK)* siRNA on MK expression at 7 days after operation was assessed by Western blot. Relative densitometric densities are shown in the graph (n=3).

formed for ischemic extremities with poor runoff (Fig 3). After external jugular vein-carotid artery interposition, three of four distal vessel branches were ligated, resulting in an approximately 90% reduction in flow, from 30 to 3 mL/min, as measured by an ultrasonic flowmeter. The consequent low blood flow in the grafted vein facilitates intimal hyperplasia.[29]

To examine the localization and stability of a siRNA in the vein graft wall, a Cy3-conjugated stabilized siRNA was mixed with atelocollagen and then perivascularly applied to the grafted vein. To achieve uniformity of treatment and precise dosing, the concentration of atelocollagen rather than the absolute amount of atelocollagen may be important. We tested 0.5%, 0.75%, 1.0%, and 1.75% (final concentration) of atelocollagen. Atelocollagen at 0.5% to 1.0% was barely solidified and thus not useful for our model. Atelocollagen at 1.75% was easily solidified and uniformly wrapped the vein graft. We also tested a hard sheet of atelocollagen. Although this sheet absorbed siRNA, it did not release siRNA when set around the vein graft of rabbits; therefore, we used atelocollagen at the final concentration of 1.75%.

As atelocollagen becomes solidified at normal body temperature, Cy3-conjugated stabilized siRNA mixed with atelocollagen remained at the application site after operation. Fig 3 shows that transfection involving atelocollagen led to a good infiltration and stability of siRNA in the graft wall for at least 7 days after operation. A graft with the vehicle alone showed no fluorescence signal (Fig 3, bottom on right), indicating that the pictures in Fig 3 do not show autofluorescence.

Treatment of vein grafts with MK siRNAs. We perivascularly applied MK siRNA mixed with atelocollagen to a vein graft at operation. MK siRNA suppressed MK expression to approximately 28% of the scrambled siRNA level, as estimated 7 days after operation (Fig 3). However, the suppression was not observed at 14 days after operation (Fig 3).

The significant effect of MK siRNA on MK expression in a grafted vein prompted us to evaluate its potential for prevention of intimal hyperplasia. Treatment of grafts with atelocollagen containing a MK siRNA led to significant reductions in the intima-media ratio and intimal thickness at 4 weeks (0.21 ± 0.04 μm and 17 ± 3 μm, respectively; $n = 5$; $P < .05$), compared with grafts that were untreated (3.08 ± 0.29 μm and 204 ± 31 μm), treated with atelocollagen (3.16 ± 0.67 μm and 162 ± 44 μm), and treated with atelocollagen containing scramble siRNA (2.96 ± 0.30 μm and 170 ± 11 μm) (Fig 4).

Effects of MK siRNAs on cell proliferation and leukocyte infiltration. Control vein grafts, which were treated with scrambled siRNA, showed highly frequent cell proliferation in the intimal lesion and less but significant proliferation in the media and adventitia at 14 days after operation (Fig 5, *A* and *B*). Vein grafts treated with MK siRNAs showed a significant decrease in the number of proliferating cells in the neointima of the vessel compared with grafts treated with scrambled siRNA (10.4% ± 1.8% vs

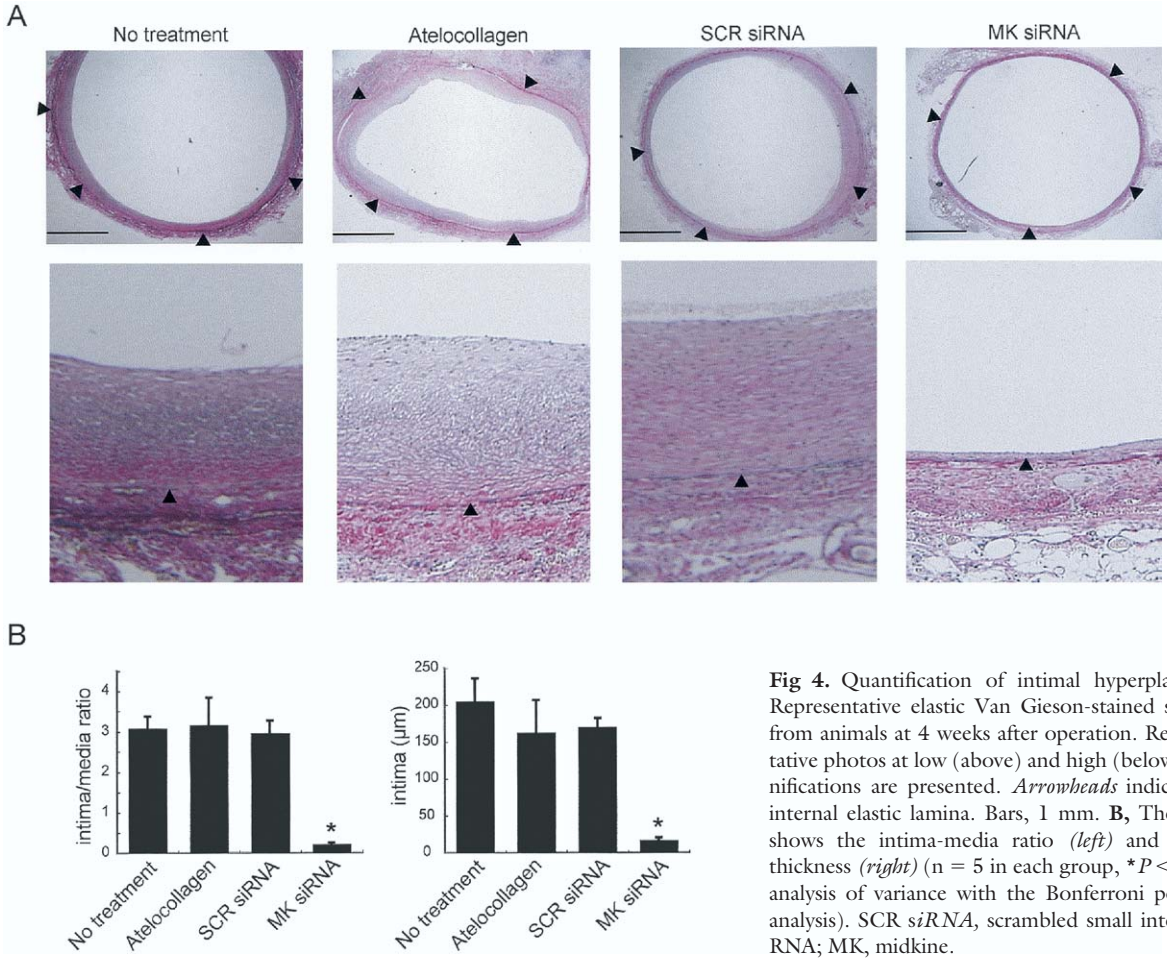

Fig 4. Quantification of intimal hyperplasia. **A,** Representative elastic Van Gieson-stained sections from animals at 4 weeks after operation. Representative photos at low (above) and high (below) magnifications are presented. *Arrowheads* indicate the internal elastic lamina. Bars, 1 mm. **B,** The graph shows the intima-media ratio *(left)* and intimal thickness *(right)* (n = 5 in each group, $*P < .05$ by analysis of variance with the Bonferroni post hoc analysis). SCR s*iRNA,* scrambled small interfering RNA; MK, midkine.

35.0% ± 3.8% for PCNA, $P < .01$; 5.4% ± 0.5% vs 22.6% ± 1.2% for Ki-67, $P < .001$) (Fig 5). In contrast, an apparent difference in cell proliferation between grafts treated with MK siRNA and those treated with scrambled siRNA-treated was not observed at 7 days after operation (data not shown). Apoptosis in grafted veins was also examined but was barely detectable, even in specimens in which MK siRNA was administered (data not shown).

To identify an earlier event affected by MK siRNA treatment, we examined the inflammatory response. Immunostaining for the CD45 antigen at 7 days after operation revealed extensive leukocyte infiltration in the control graft walls (Fig 6). MK siRNA treatment significantly reduced the leukocyte count from15.0 ± 2.7 per microscopic field (×200) in controls to 2.2± 0.5 (n = 5, $P < .05$).

DISCUSSION

Many trials that have been performed, including genetic interventions to prevent vein graft failure, have taken advantage of the fact that veins to be grafted can be manipulated ex vivo. Antisense or decoy ODNs have been transfected into a graft vein wall via liposomes or high pressure, or both.[12,17,19,21,48,49] However, a conceivable limitation of this strategy is that a drug is administered only once, and a major part of the applied drug does not remain at the application site for very long. The second strategy is ex vivo administration of adenovirus expressing antisense DNAs.[15,16,24,25] An alternative strategy is to wrap a grafted vein with a Pluronic gel containing antisense ODNs or aspirin.[13,14,22,23]

Considering the modest suppression of intimal hyperplasia on a one-shot application of the MK antisense ODN at the initial stage,[44] we attempted in this study to develop a method to achieve long-standing sufficient suppression of MK expression. We used atelocollagen to deliver a siRNA. Atelocollagen is a liquid at <4°C, becomes solid at 37°C, and has been used as a delivery system for expression plasmids, antisense ODNs and siRNAs.[38,50,51] The advantage of the use of atelocollagen is that it becomes stabilized and slowly releases nucleic acid reagents.[51] It is of note that the present combination of MK siRNA and atelocollagen led to almost complete suppression of intimal hyperplasia and the best results among those reported so far.

The present method has two excellent advantages. First, the complicated transfection procedures reported in previous studies are not needed. Second, the siRNA is

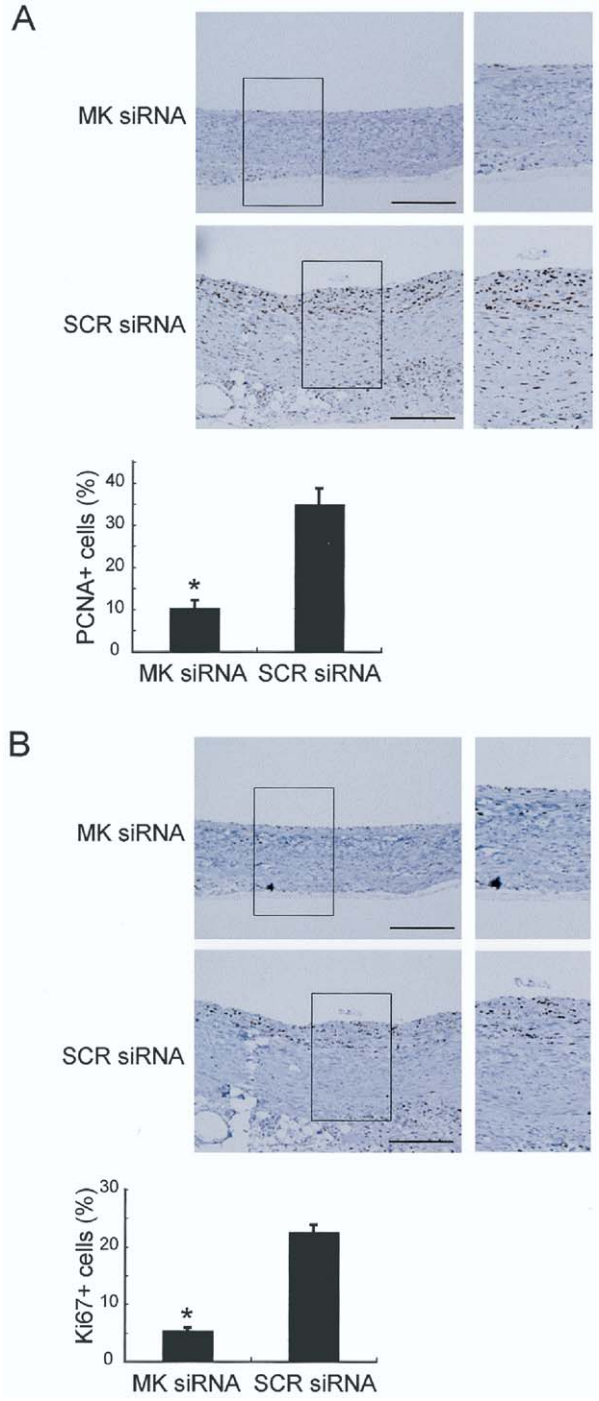

Fig 5. Cell proliferation in a grafted vein 14 days after operation. **A,** Proliferating cell nuclear antigen *(PCNA)*-immunostained sections of grafts treated with midkine *(MK)* small interfering RNA *(SCR siRNA)* (upper panel) and scrambled siRNA *(SCR siRNA)* (lower panel). High magnification views are shown on the right. The bar graph shows the percentage of PCNA-positive cells in the intima (n = 5, $*P < .01$). **B,** Ki-67-immunostained sections of grafts treated with MK siRNA (upper panel) and scrambled siRNA (lower panel).High magnification views are shown on the right. The bar graph shows the percentages of Ki-67-positive cells in the intima (n=5, $*P < 0.001$). Bars, 200 μm.

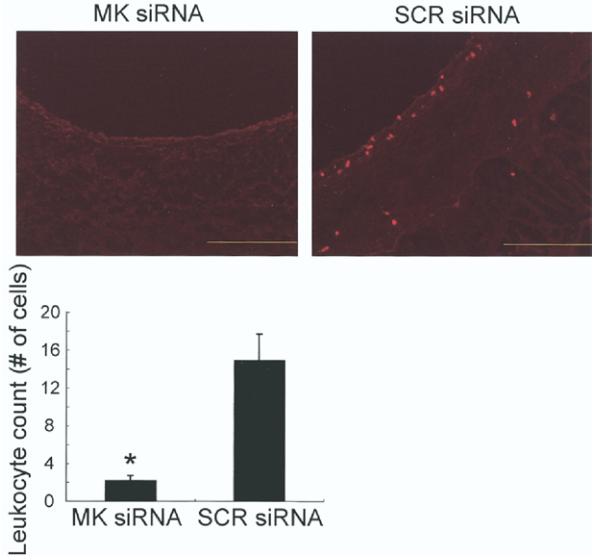

Fig 6. Leukocyte infiltration into a grafted vein 7 days after operation. CD45-immunostained sections of grafts treated with midkine *(MK)* short interfering RNA (siRNA) (left) and scrambled siRNA (SCR siRNA) (right) are shown. The bar graph shows the numbers of $CD45^+$ cells in the graft walls (n = 5, $*P < 0.05$). Bars, 100 μm.

retained at the application site, allowing long-standing suppression of the target gene. Drug delivery systems involving liposomes or high pressure may only allow drugs to remain for a few hours to a day at the application site. Thus, such delivery systems may not provide a sufficient suppression effect of gene expression.

We observed that MK expression was suppressed for at least 7 days in the present study. Although our method did not allow sufficient suppression of MK expression at 14 days after operation, taking into account that neointima formation becomes visible at 7 days and we observed striking suppression of it at 28 days, the early stage suppression of MK expression is probably enough to reduce neointima formation. We speculate that MK expressed in the early stages initiates a chain reaction involving several molecules inducing inflammation in the vessel wall, and that this chain reaction is consequently critical in neointima formation. In addition, although MK does not enhance proliferation of vascular smooth muscle cells, it does enhance migration of macrophages and vascular smooth muscle cells.[43] Together with unknown factor(s) secreted from vascular smooth muscle cells, MK promotes vascular smooth muscle cell migration.[43] This activity may contribute to intimal thickening. Although the molecular cascade after MK remains to be elucidated, our study has provided evidence that the suppression of molecular events at the early stages is a good strategy for the prevention of vascular stenosis.

We have obtained the following evidence:

1. MK is specifically induced in the intima during intimal hyperplasia (present study).[43]

2. MK-deficient mice exhibit decreased intimal hyperplasia and exogenously administered MK restores it[43]
3. Suppression of MK expression leads to suppression of intimal hyperplasia (present study).[44] Overall, MK is a potent molecular target for the prevention of vascular restenosis.

MK was discovered as the product of a retinoic acid-responsive gene and is implicated in inflammation.[26,27,30] Evidence supporting the inflammation-promoting activity of MK has accumulated through the use several systems, such as a neutrophil migration assay, a reperfusion-induced renal injury model, a cisplatin-induced renal injury model, and a rheumatoid arthritis model.[39-41,52] We previously showed that neutrophil and macrophage infiltration into vascular walls is strikingly suppressed in MK-deficient mice in an arterial restenosis model compared with wild-type mice.[26] The present study demonstrated a close association of MK knockdown and suppression of inflammatory cell recruitment and thus strengthens the importance of MK in the inflammation in this disease.

This study used a stabilized siRNA that was developed to have extremely high stability in the blood and thus is resistant to many nucleases. Because atelocollagen stabilizes siRNA,[38] it will be important in terms of the feasibility of clinical application to determine whether a siRNA with a natural backbone is also effective combined with atelocollagen.

CONCLUSION

We demonstrated in this study that MK could be a potent molecular target for the prevention of vein graft failure. The combination of siRNA and its controlled release by means of atelocollagen or other polymers can be used not only for perivascular application on vein grafting but also for siRNA-eluting stents for a broader spectrum of vascular restenosis including that after percutaneous transluminal coronary angioplasty.[11]

AUTHOR CONTRIBUTION

Conception and design: HB, YT, TM, KIK, KEK
Analysis and interpretation: HB, YT, KIK, KEK
Data collection: HB
Writing the article: HB, KEK
Critical revision of the article: HB, YT, KIK, KEK
Final approval of the article: HB, YT, TM, KIK, KEK
Statistical analysis: HB
Obtained funding: TM, KIK, KEK
Overall responsibility: HB, YT, TM, KIK, KEK

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