some experiments, ASC suspension was dropped around the femoral artery from the adventitial side after wire injury.

Histochemistry. The femoral arteries were fixed by perfusing them with 4% paraformaldehyde and processed for paraffin embedding. Cross sections (2 μm) were cut, deparaffinized, rehydrated, and stained with hematoxylin and eosin. For immunohistochemistry, the sections were incubated with primary antibodies reactive to PCNA and CD31. The sections were then incubated with biotinylated secondary antibody and finally horseradish peroxidase-labeled streptavidin according to the instructions provided by the manufacturer (DAKO). The sections were counterstained with hematoxylin.

Statistical analysis. Values are means ± SE. Statistical analyses were performed using analysis of variance followed by the Student-Newman-Keuls test. Differences with a P value of <0.05 were considered statistically significant.

RESULTS

Characterization of ASCs. Cell surface markers were first analyzed using flowcytometry analysis (Fig. 1). In contrast to previous reports showing that ASCs isolated from humans expressed CD34 to some extent (9, 15, 16), CD34 expression was negative in ASCs derived from Wistar rats. The expression of VE-cadherin was also negative, suggesting that the contamination of ECs was negligible. The expression of CD29 and CD90 was positive. These cell surface markers were reportedly positive in BMMSCs (14). Therefore, our results suggested that ASCs isolated from Wistar rats resembled mesenchymal stem cells rather than hematopoietic stem cells.

Expression patterns of mRNA and protein in ASCs. We cultured ASCs on fibronectin-coated dishes and examined mRNA expression in ASCs cultured in either EBM or EGM (Fig. 2*A*). The expression of Flk-1 was not significantly induced in ASCs cultured in EGM compared with those cultured in EBM. The expression of mature EC markers such as CD31 and VE-cadherin was significantly suppressed when ASCs were cultured in EGM (data not shown). In contrast, the expression of Flt-1 significantly increased in ASCs cultured in EGM compared with those cultured in EBM. We also examined the mRNA expression of proangiogenic factors and anti-apoptotic factors that ASCs might secrete. The expression of VEGF-A and IGF-1 significantly decreased in ASCs cultured in EGM compared with those cultured in EBM, probably because EGM contains VEGF-A and IGF-1 to induce the differentiation into ECs. The expression of bFGF was not significantly changed between EBM-cultured ASCs and EGM-cultured ASCs. The expression of HGF was significantly suppressed in ASCs cultured in EGM. In contrast, the expression of Ang-1 significantly increased in ASCs cultured in EGM compared with those cultured in EBM. We next examined the expression level of some of these genes at the protein level (Fig. 2*B*). The expression of Flk-1, CD31, or VE-cadherin was not detected in ASCs cultured in EGM until up to 14 days after incubation with EGM. In contrast, the expression of Flt-1 was detected 3 days after incubation with EGM and peaked 7 days after culture in EGM. The ASCs cultured in EGM also secreted a higher amount of Ang-1 into the culture medium than EBM-cultured ASCs. Since Flt-1 is expressed in monocytes/macrophages as well as ECs (20), the expression of CD14 was examined by Western blot analysis and immunostaining, but its expression was not detected in ASCs cultured in EGM (data not shown), suggesting that the contamination of monocytes/macrophages was negligible. Collectively, our data suggested that ASCs do not appear to have the potential to differentiate into mature ECs in vitro, although ASCs express Flt-1. Our results also suggested that ASCs might have the capacity to promote neovessel formation via a stimulation of the recruitment of ECs in situ, because ASCs, especially cultured in EGM, produced a significant amount of Ang-1.

ASCs stimulate migration of HUVECs. We, therefore, examined whether ASCs would stimulate the migration of ECs by chemotaxis assay (Fig. 3). Ang-1 was used as the positive control for the chemotaxis assay. Ang-1 (10 ng/ml) significantly stimulated the migration of HUVECs, and this effect was significantly suppressed when Ang-1 was preincubated with anti-Ang-1 antibody. Culture medium harvested from EGM-cultured ASCs significantly stimulated the migration of HUVECs compared with that harvested from EBM-cultured ASCs. This stimulatory effect was partially but significantly blocked by the preincubation of the culture medium with anti-Ang-1 antibody, suggesting that Ang-1 was, at least partly, responsible for the migration-stimulating effect.

ASCs inhibit neointimal formation via stimulation of endothelial repair in a paracrine fashion. We next examined the function of ASCs in vivo using the wire injury model of the rat femoral artery. When injected in the femoral artery, EBM-cultured ASCs slightly but significantly inhibited neointimal formation compared with wire injury without cell administration. EGM-cultured ASCs potently and more significantly inhibited neointimal formation compared with EBM-cultured ASCs (Fig. 4*A*). In accordance with these results, the number of PCNA-positive cells in the neointima was significantly suppressed in the group that was administered EBM-cultured ASCs compared with the group that received no cells. The number of PCNA-positive cells in the

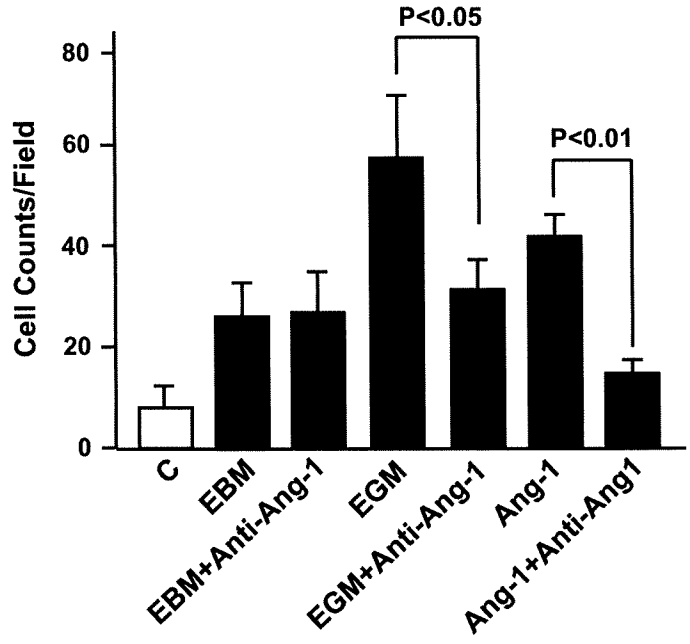

Fig. 3. ASCs produce paracrine factors that stimulate migration of HUVECs. ASCs were cultured in EBM or EGM for 7 days, and the medium was replaced with DMEM-0.2% FBS. The medium was collected after 12 h and used for chemotaxis assay. The conditioned medium was also preincubated with anti-Ang-1 antibody to neutralize Ang-1. Chemotaxis assays were performed as described in MATERIALS AND METHODS (n = 6 experiments). Human Ang-1 (10 ng/ml) was used as the positive control for the chemotaxis analysis.

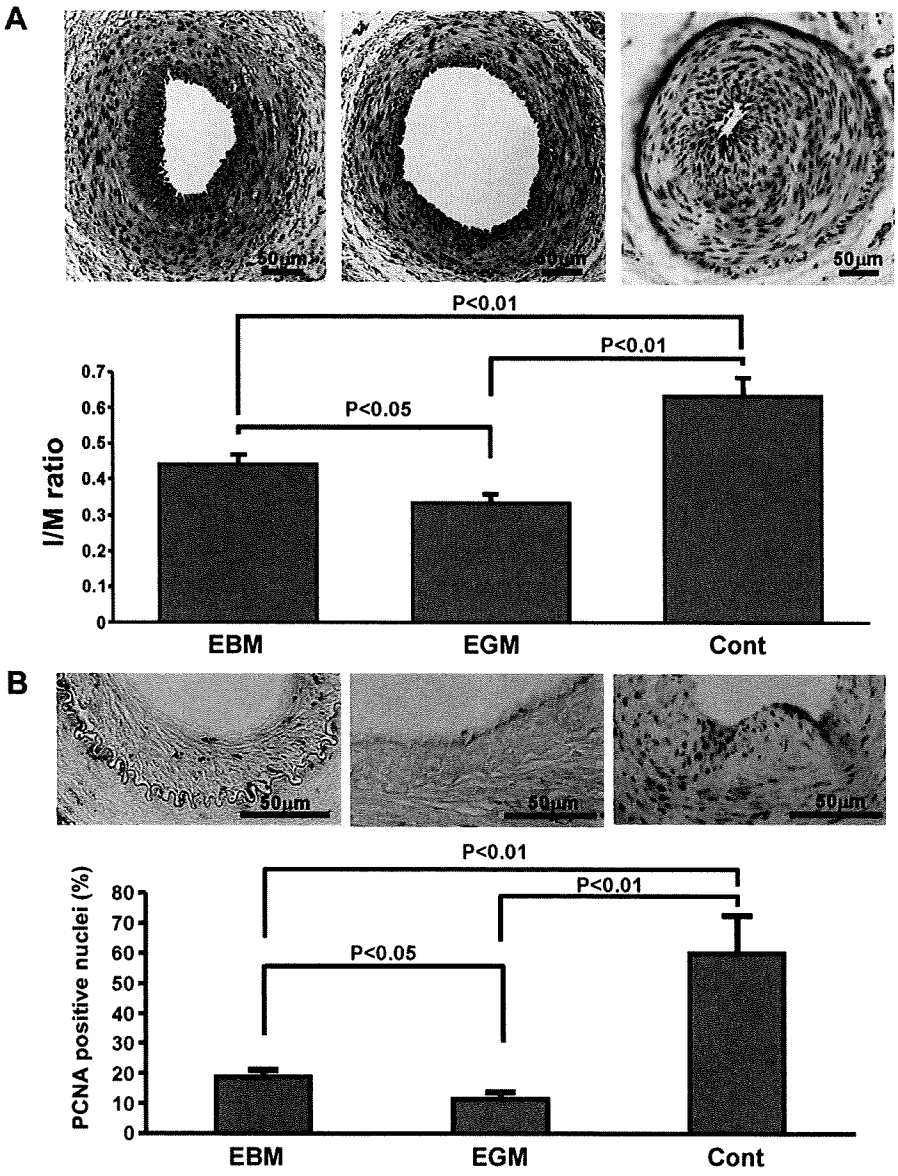

Fig. 4. ASC administration inhibits neointimal formation. *A*: ASCs cultured in EGM or EBM for 7 days (10^6 cells) were injected into the femoral artery immediately after wire injury, and the femoral arteries were harvested 14 days after the injury for histological analysis. Wire injured femoral artery without cell injection was also harvested and analyzed as the positive control (Cont). The ratio of the intimal to medial area (I/M) was compared between the groups (n = 8 experiments). *B*: proliferating cell nuclear antigen (PCNA)-positive cells in the neointima significantly decrease by administration of ASCs. Experiments were performed as described in *A*. The number of PCNA-positive cells was compared between the groups (n = 6 experiments).

neointima was more significantly reduced in the group that was administered EGM-cultured ASCs compared with the group that was administered EBM-cultured ASCs (Fig. 4*B*). Because ASCs cultured in EGM potently inhibited neointimal formation, we studied the mechanism whereby these cells inhibited neointimal formation. We examined whether EGM-cultured ASCs were engrafted into the endothelial layer and contributed to the repair of the endothelial layer after the wire injury. EGM-cultured ASCs were infected with AdGFP before injection into the artery. One day after injection, EGM-cultured ASCs were detected in the endothelial layer. However, EGM-cultured ASCs were barely detected in the endothelial layer 3 and 14 days after injection (Fig. 5*A*), suggesting that EGM-cultured ASCs inhibited neointimal formation without integrating into the endothelial layer. To confirm the specificity of the green fluorescence detected in the endothelial layer, we also injected ASCs without AdGFP infection and examined autofluorescence of the femoral artery (Fig. 5*B*). Green fluorescence was not detected in the endothelial layer in this case, suggesting that the green fluorescence detected in the endothelial layer was derived from AdGFP-infected ASCs that stayed in the endothelial layer. We, therefore, hypothesized that EGM-cultured ASCs potently inhibited neointimal formation by secreting paracrine factors that stimulate the repair of the endothelial layer, because we found that these cells produce a significant amount of Ang-1. To examine this possibility, we next administered EBM- and EGM-cultured ASCs from the adventitial side of the femoral artery after wire injury (Fig. 5*C*). Interestingly, EGM-cultured ASCs more significantly inhibited neointimal formation compared with EBM-cultured ASCs even when these cells were administered from the adventitial side. To further examine the role of paracrine factors secreted by ASCs, we originally tried to knock down endogenous Ang-1 production using small interfering RNA technology. However, the transfection efficiency of small interfering RNA by lipofection into rat ASCs was <10%, making it very difficult to examine the effect of gene knockdown in ASCs. We instead used rat VSMCs that also produce Ang-1 and NRK-52E cells that barely produce Ang-1. Rat VSMCs produced ~50% of Ang-1 mRNA com-

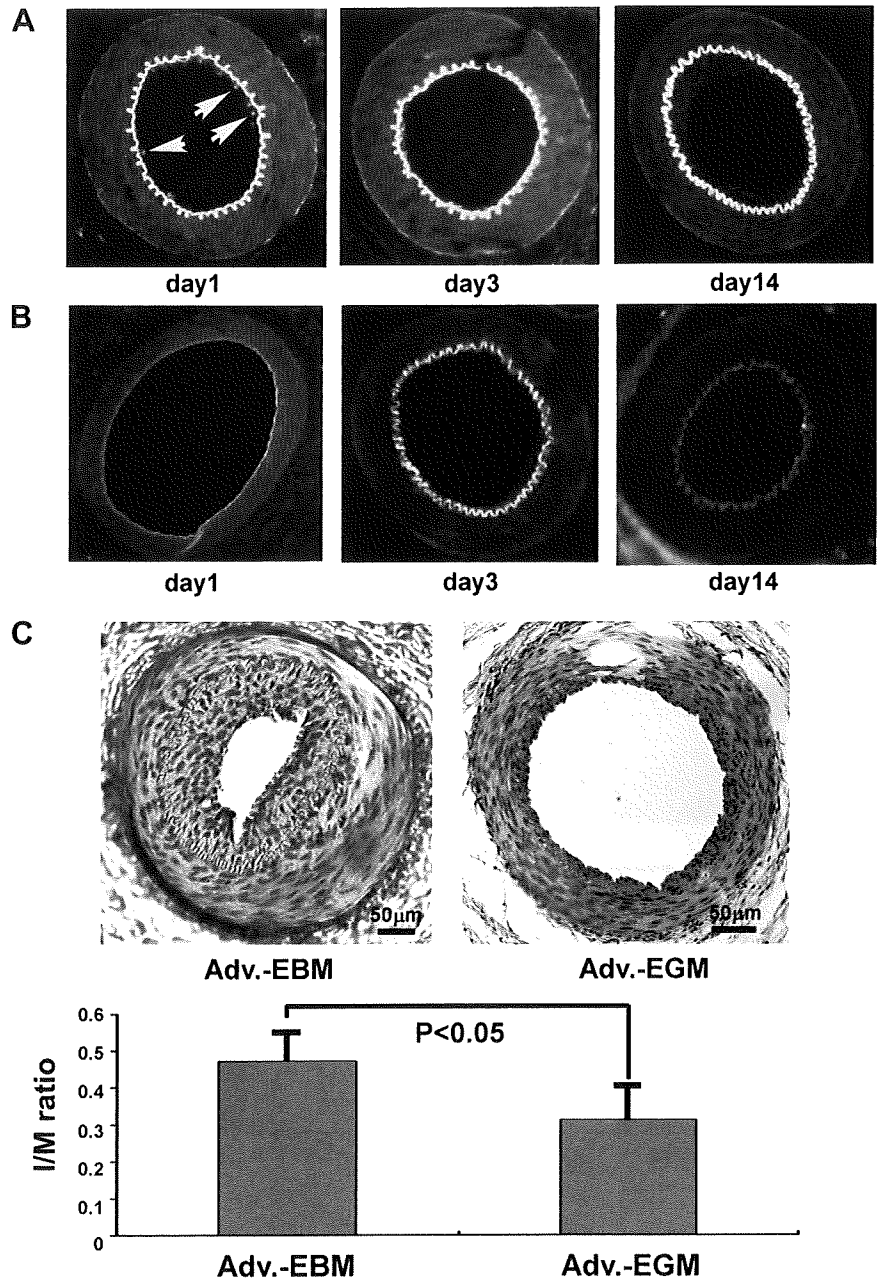

Fig. 5. *A*: time course of ASCs that remain in the endothelial layer. EGM-cultured ASCs were infected with adenovirus expressing green fluorescent protein (AdGFP) (40 multiplicity of infection) before injection into the femoral artery. Fluorescence of GFP was examined at the time points indicated. *B*: analysis of autofluorescence of the femoral artery. EGM-cultured ASCs were injected in the femoral artery without AdGFP infection, and autofluorescence of the femoral artery was examined at the time points indicated. *C*: ASCs administered from the adventitial side also significantly inhibit neointimal formation. ASCs cultured in EGM or EBM for 7 days (10^6 cells) were injected from the adventitial side (Adv.) of the femoral artery immediately after the wire injury. Femoral arteries were harvested 14 days after wire injury, and I/M ratio was compared between the groups (n = 6 experiments).

pared with ASCs cultured in EBM, and NRK-52E cells produced <1% of Ang-1 mRNA compared with ASCs cultured in EBM, as assessed by real-time PCR (data not shown). When rat VSMCs were administered from the adventitial side, they slightly but significantly inhibited neointimal formation compared with the NRK-52E cells administration (Fig. 6). These results also suggested that Ang-1 produced by ASCs might be, at least in part, implicated in the suppression of neointimal formation. We finally examined whether ASC administration from the adventitial side would stimulate the repair of the endothelial layer (Fig. 7). We examined the ratio of the endothelial layer positively stained with CD31. Endothelial repair was significantly enhanced by the administration of EGM-cultured ASCs compared with administration of EBM-cultured ASCs.

DISCUSSION

In this study, we isolated ASCs from Wistar rats and examined their characteristics in vitro and in vivo. ASCs obtained from Wistar rats expressed CD29 and CD90 but not CD34, suggesting that the ASCs we used resembled BMMSCs rather than hematopoietic stem cells. Although several studies demonstrated that human ASCs contain a large population of cells that express CD34 and that these CD34-positive cells differentiate into endothelial-like cells in vitro and in vivo (9, 15), ASCs do not always express CD34 in mice (6, 11). Although we do not know the reason for this discrepancy, cell surface markers of ASCs may differ among species.

ASCs used in this study expressed Flt-1 when they were cultured in EGM. However, ASCs did not express Flk-1 or

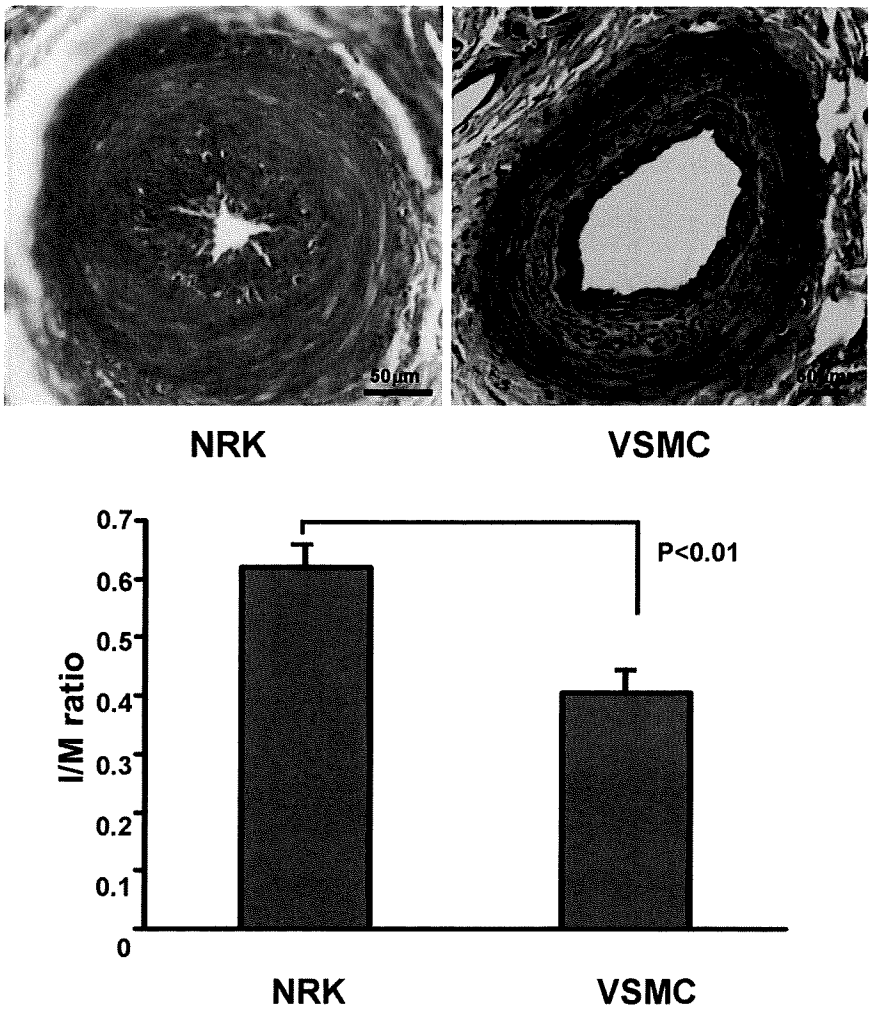

Fig. 6. Effects of administration of rat vascular smooth muscle cells (VSMCs) and NRK-52E cells (NRK) on neointimal formation. These cells were administered from the adventitial side of the femoral artery immediately after wire injury. Femoral arteries were harvested 14 days after wire injury, and I/M ratio was compared between the groups (n = 6 experiments).

mature EC markers such as VE-cadherin and CD31. Thus ASCs used in this study did not appear to have the capacity to differentiate into mature ECs. ASCs may resemble bone marrow-derived cells that express Flt-1 and are recruited to sites of ischemia (7, 12). Although several studies showed that human ASCs had the potential to differentiate toward ECs in vitro by demonstrating the expression of endothelial markers such as CD31, the efficiency varies so much, probably because the methodology whereby they induced differentiation of ASCs into ECs differs from study to study (3, 8, 9, 15). One study demonstrated that mouse ASCs could differentiate into ECs in vitro by examining the expression of CD31 and VE-cadherin, but its efficiency seemed to be very low (11). Recently, Boquest et al. (2) examined the methylation profiles of EC-specific gene promoters such as CD31 and VE-cadherin and showed that the promoters of CD31 and VE-cadherin were hypermethylated in ASCs and that these promoters seemed to have a relatively small potential to be activated in ASCs. Moreover, whether ASCs can differentiate into ECs in vivo remains debatable. Several studies demonstrated that ASCs were integrated into capillaries in hindlimb ischemia models and improved blood flow via the stimulation of angiogenesis (9, 15). However, ASCs could reportedly stimulate angiogenesis and restore blood flow in hindlimb ischemia models without being engrafted into capillaries, probably because of their paracrine effects (11). Therefore, the efficiency of ASCs to differentiate into ECs in vitro and in vivo appears to differ, depending on the cell culture conditions, animal model used, and animal species. Future studies will be required to elucidate an appropriate strategy to efficiently induce differentiation of ASCs into mature ECs.

We, therefore, examined paracrine factors that ASCs produce. ASCs reportedly produce a variety of proangiogenic and antiapoptotic factors such as VEGF, IGF, HGF, and bFGF. Although the production of these factors did not increase when ASCs were cultured in EGM, the production of Ang-1 was significantly increased. Furthermore, ASCs appeared to secrete functionally active Ang-1, as assessed by chemotaxis assay using HUVECs. These results suggested that ASCs potentially promote repair of the endothelial layer via stimulation of migration of ECs in situ.

To test this hypothesis, we administered ASCs in a wire injury model of rat femoral artery. EGM-treated ASCs significantly inhibited neointimal formation without being engrafted into the endothelial layer. EGM-treated ASCs also significantly suppressed neointimal formation even when they were administered from the adventitial side. Endothelial repair occurred more rapidly in rats administered EGM-cultured ASCs compared with those administered EBM-cultured ASCs. The rapid endothelial repair was accompanied by less cell proliferation in

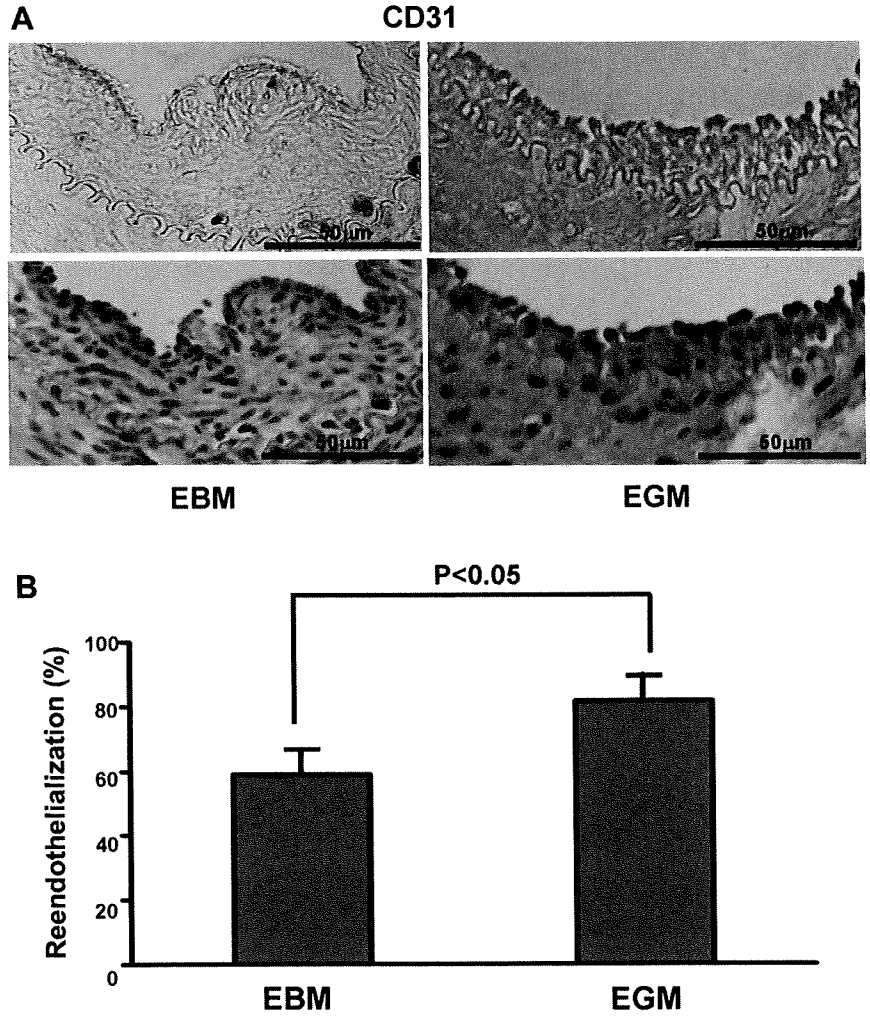

Fig. 7. Administration of EGM-cultured ASCs promotes endothelial repair. *A*: EBM- or EGM-cultured ASCs were administered from the adventitial side after the wire injury. Femoral arteries were harvested 14 days after the injury, and CD31 was stained to detect repaired endothelial layer (*top*). The specimens were next counterstained with hematoxylin to show the nuclei of the endothelial cells (*bottom*). *B*: ratio of the CD31-positive reendothelialized area (n = 6 experiments).

the neointima. Furthermore, the administration of VSMCs that also produce Ang-1 slightly but significantly suppressed neointimal formation, whereas the administration of NRK-52E cells that barely produce Ang-1 did not. These results suggested that ASCs inhibited neointimal formation in a paracrine fashion via the stimulation of endothelial repair. These results also suggested that Ang-1 was, at least in part, implicated in the inhibitory effect of ASCs on neointimal formation. It has been reported that EPCs, when injected in the carotid artery, were engrafted in the endothelial layer in a balloon injury model. However, it remains unclear how long the injected EPCs can survive and proliferate in the endothelial layer. EPCs were not detected in the endothelial layer 30 days after the administration in that study (5). Thus, although EPCs seem to be more effectively integrated in the endothelial layer than ASCs, EPCs may also stimulate endothelial repair via a stimulation of EC migration in situ, because EPCs also reportedly produce paracrine factors such as VEGF (23). These possibilities should be addressed in the future.

In summary, although rat ASCs do not differentiate into mature ECs, they produce paracrine factors such as Ang-1, especially when they were cultured in EGM. These factors seem to stimulate the migration of ECs in situ and the repair of the endothelial layer in vivo. Although the capacity of ASCs to differentiate into mature ECs may be low, ASCs will be useful for cell-based therapy to treat cardiovascular diseases such as hindlimb ischemia, acute myocardial infarction, and the prevention of restenosis after angioplasty via their capacity to produce paracrine factors that stimulate angiogenesis and endothelial repair.

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DISCLOSURES

There exist no conflicts of interest.

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