Abstract. Endothelial progenitor cells (EPCs) play an important role in endothelial repair and vascular regeneration. Growth arrest-specific gene 6 (Gas6) is a novel key regulator of the vascular system, which is linked to a number of cardiovascular diseases. However, the effects of Gas6 on EPCs have not been elucidated to date. The present study was designed to determine the biological function of EPCs treated with Gas6 and to elucidate the underlying mechanisms. EPCs were isolated from umbilical cord blood and treated with various concentrations (25, 50, 100 and 200 ng/ml) of Gas6. The proliferation, migration and angiogenesis of the Gas6-treated EPCs were evaluated by MTT assay, Transwell assay and in vitro tube formation assay, respectively. The phosphorylation status of AKT and ERK was evaluated by western blot analysis. The results demonstrated that treatment with Gas6 enhanced the proliferation and migration of the EPCs in a dose-dependent manner. However, Gas6 did not promote the differentiation of EPCs on Matrigel. Gas6 induced the phosphorylation of AKT, but not that of ERK. The enhanced proliferation and migration induced by Gas6 was markedly suppressed by the inhibitor of PI3K but not by that of ERK. These results suggest that Gas6 activates the AKT signaling pathway, which, in turn, promotes the proliferation and migration of EPCs.

Introduction

The enhancement of re-endothelialization is a critical therapeutic option to repair injured blood vessels. Regeneration of the injured endothelium is linked to the proliferation and migration of neighboring endothelial cells (ECs) (1). Mature ECs are a group of cells with low proliferative potential and a limited capacity to substitute the damaged endothelium. Accumulating evidence has indicated that endothelial progenitor cells (EPCs), which can home to sites of tissue injury and differentiate into mature ECs and participate in re-endothelialization after vascular injury, may be an endogenous repair mechanism to maintain the integrity of the endothelial monolayer by replacing denuded areas of the artery (2-4). The discovery of new methods to improve the EPC re-endothelialization process is currently the subject of intensive investigation.

Growth arrest-specific gene 6 (Gas6), is a member of the vitamin K-dependent family of proteins, which includes the procoagulant factors II, VII, IX, and X, and the anticoagulant factors, protein C and S, as well as protein Z (5). Even though Gas6 was discovered as a homolog of protein S more than a decade ago, it plays no role in the generation of fibrin (6). Instead, Gas6 exerts several other functions that belong to the repertoire of growth or survival factors. Firstly, the original observation that Gas6 is upregulated in growth-arrested cells suggests a role in the protection from certain cellular stresses, such as apoptosis (7). Subsequently, a number of studies have demonstrated the ability of Gas6 to promote either cell survival (8) and/or proliferation (9). Additional growth factor-like properties of Gas6 have been reported, including the stimulation of cell migration and cell-cell adhesion (10). The first hint that Gas6 may be important in the vasculature came from the purification of Gas6 from the conditioned medium of vascular smooth muscle cells (VSMCs) that potentiated the growth response of VSMCs treated with angiotensin II (11). It was subsequently discovered that the expression of Gas6 was increased in the injured rat carotid, with a time course paralleling that of neointima formation (12). Further experimental evidence indicated that ECs in culture express and release Gas6, and that it promotes cell survival, possibly through an autocrine pathway (13-16). These findings highlight the importance of Gas6 in vascular function. Gas6 was found to bind to the extracellular regions of three distinct receptor tyrosine kinases, namely Axl, Mertk and Tyro3. A recent study demonstrated that Axl may be a potential angiogenic target (17). Considering that Gas6 has various potential bioactivities, it is of great interest to identify those that have a significant impact on EPCs.

Correspondence to: Professor Cheng-Yun Liu, Department of Geriatrics, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1277 Jiefang Avenue, Wuhan, Hubei 430022, P.R. China
E-mail: lcyun@medmail.com.cn

Key words: growth arrest-specific gene 6, endothelial progenitor cells, proliferation, migration, PI3K/AKT pathway
Materials and methods

Isolation and culture of late EPCs. Ethical approval was granted by the Institutional Review Board of Tongji Medical College, Hubei, China. Informed consent was obtained from healthy donors prior to the collection of umbilical cord blood. The mononuclear cells were isolated from umbilical cord blood by Ficoll density gradient centrifugation with Histopaque 1077 (Sigma, St. Louis, MO, USA). The isolated cells were resuspended using an EGM-2 BulletKit system (catalog number CC-3202; Lonza) consisting of endothelial basal medium, 5% fetal bovine serum, human epidermal growth factor (hEGF), vascular endothelial growth factor (VEGF), human fibroblast growth factor (hFGF), hFGF-B, insulin-like growth factor (IGF)-1 and ascorbic acid. Mononuclear cells were seeded on fibronectin-coated (Sigma) dishes and maintained in a 5% CO₂ incubator at 37°C. Three days after planting, the non-adherent cells were removed and, thereafter, the medium was changed every 2 days. Cobblestone-like cell colonies were observed after 2 weeks.

Characterization of EPCs. Direct fluorescent staining was used to detect the dual binding of 1, 1-dioctadecyl-3, 3, 3, 3-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (Dil-ac-LDL; molecular probe) and fluorescein isothiocyanate (FITC)-conjugated Ulex europaeus agglutinin lectin (UEA-1; Sigma). The cells were incubated with 2.4 µg/ml Dil-ac-LDL for 4 h in a cell incubator. The cells were then washed and fixed with 4% paraformaldehyde for 10 min and incubated with 10 µg/ml FITC-labeled UEA-1 for 1 h. Subsequently, the cells were washed and incubated with Hoechst 33258. Double-positive cells were observed under a laser confocal microscope (FV500; Olympus, Tokyo, Japan). To assess the expression of surface antigen on the cells, we performed fluorescence-activated cell sorter (FACS) analysis. Five million EPCs per sample were stained for 30 min at 4°C with fluorescein isothiocyanate-conjugated monoclonal mouse anti-human CD34 (BD Pharmingen, San Diego, CA, USA) antibody, phycoerythrin-conjugated monoclonal mouse anti-human VEGFR2 (BD Pharmingen) antibody and mouse anti-human CD3133 antibody conjugated to allophycocyanin (Miltenyi Biotec, Auburn, CA, USA). Data were processed using FlowJo software (version 7.6).

Proliferation assay. The effects of Gas6 on EPC proliferation were determined by MTT assay. A total of 5x10⁴ cells/well were seeded on 96-well culture plates and then deprived of serum for 12 h to achieve cell cycle synchronization. The dose range (25, 50, 100 and 200 ng/ml) of Gas6 (R&D Systems, Minneapolis, MN, USA) was the same as that used in previous studies (18,19). The control groups received a dilution of water used in previous studies (18,19). The control groups received a dilution of water.

Migration assay. The migration ability of the EPCs was evaluated using a Transwell migration assay (Costar, Cambridge, MA, USA) with 6.5-mm-diameter polycarbonate filters (8 µm pore size). Gas6 with various concentrations plus endothelial basal medium-2 and 0.2% FBS were placed in the lower wells. EPCs (4x10⁴ cells/well) were seeded onto the upper chamber supplemented with serum-free endothelial growth medium. After 12 h of incubation in the cell incubator, the upper chamber was removed and wiped clean with a cotton swab; the lower side of the filter was washed with PBS and fixed with 4% paraformaldehyde for 10 min. For quantification, the cell nucleus was stained with crystal violet. Cell migration into the lower chamber and attachment to the lower side of the filter were manually counted in 16 fixed microscopic fields (magnification, x400) by independent investigators blinded to the treatment regimen, randomly. Each test was performed in triplicate, and assays were repeated 3 times with individual EPCs. When investigating the effects of PI3K and ERK inhibitors on the migration ability of the cells, the EPCs were cultured in the absence or presence of 200 ng/ml Gas6 and the indicated concentrations of 20 µmol/l LY294002 or 5 µmol/l PD98059 for 24 h. The following steps were the same as those described above.

In vitro tube-formation assay. In vitro tube-formation assay was performed using Matrigel (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Passage-3 EPCs were incubated with an additional 50, 100 and 200 ng/ml Gas6 and 10 ng/ml VEGF or 200 ng/ml Gas6 plus 10 ng/ml VEGF for 24 h. The Matrigel solution was thawed at 4°C for 30 min to allow gelation, then 1x10⁴ EPCs with the previous treatment were placed on top of the Matrigel. After 12 h of incubation, the mean tube length was calculated in 3 randomly selected fields from each well (x100) using Image-Pro Plus software and was calculated against the value of the control groups. The experiment was repeated 5 times.

Western blot analysis. The EPCs pre-treated with 200 ng/ml Gas6 were lysed with RIPA buffer and electrophoresed on 10% SDS-PAGE gels at 100 V for 2 h, and electroblotted onto a PVDF membrane at 275 mA for 12 h. The membrane was incubated with 5% fat-free milk PBS for 2 h at room temperature. The membrane was then incubated with anti-AKT, anti-ERK (1:500; Cell Signaling Technology, Inc.), anti-phospho-AKT and anti-phospho-ERK (1:500; Cell Signaling Technology, Inc.) rabbit monoclonal antibodies followed by the addition of a goat anti-rabbit peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The immunoreactive bands were then visualized with a chemiluminescence reagent (Thermo Fisher Scientific Inc., Rockford, IL, USA) and exposed to X-ray film. The density of each band was quantified using ImageJ software. All the assays were performed in triplicate with individual EPCs.
Statistical analysis. Results were obtained from at least 3 independent experiments and data are presented as the means ± SD. The Student’s t-test was performed for statistical comparisons between 2 groups and ANOVA was used for comparisons between >2 groups. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Characterization of EPCs. Late EPCs appeared after 1-2 weeks as small colonies in cultures of mononuclear cells (MNCs) and developed a cobblestone-like cell morphology over time (Fig. 1A). To confirm the EPC phenotype, attached mononuclear cells were incubated with Dil-ac-LDL and FITC-UEA-1. Cells demonstrating double-positive fluorescence were identified as differentiated EPCs. Almost all adherent cells were shown to endocytose Dil-ac-LDL and bind FITC-UEA-1 (Fig. 1B). Flow cytometric analysis for positive staining with CD34, VEGFR2 and CD133 further confirmed the EPC characteristics (Fig. 1C).

Gas6 stimulates the proliferation of human EPCs. To determine the effects of Gas6 on the growth of human EPCs, we performed a time- and dose-response experiment. Gas6 at concentrations of 50, 100 and 200 ng/ml induced an increase in the proliferation of EPCs of 7.26% (P < 0.05 vs. control), 14.10% (P < 0.001 vs. control) and 18.40% (P < 0.001 vs. control), respectively, after 48 h of culture (Fig. 2). However, treatment with increased concentrations of Gas6 for 24 h did not affect the proliferative ability of the EPCs compared with the controls. The effects on cell proliferation induced by Gas6 occurred in a dose- and time-dependent manner.

Gas6 stimulates EPC migration. The effects of Gas6 on late EPC migration were determined by a Transwell assay (Fig. 3).
A large number of cells treated with Gas6 had migrated to the lower side of the membrane in the Transwell chamber. Treatment with Gas6 increased EPC migration in a dose-dependent manner (control, 15.33±5.50; 25 ng/ml, 36.00±6.55; 50 ng/ml, 53.00±12.00; 100 ng/ml, 103.33±13.57; and 200 ng/ml, 111.66±13.57 cells, respectively), with a significant effect at a dose of 50 ng/ml (P﹤0.05 vs. control); the most significant effect was observed with the highest Gas6 dose used (200 ng/ml; P﹤0.01 vs. control). However, there was no statistically significant difference between the 100 and 200 ng/ml groups (P=0.384).

Gas6 does not promote EPC differentiation on Matrigel. Gas6 promoted the proliferation and migration of EPCs; we thus investigated whether this protein affects the capillary-like structure formation of EPCs. Following stimulation for 12 h, Gas6 at 50, 100 and 200 ng/ml did not ameliorate the capacity of the cells to form capillary-like structures compared with the control. VEGF-A is recognized as a key regulator of tube formation in the process of angiogenesis (35). Further investigation indicated that Gas6 did not alter VEGF-A-dependent tube formation (Fig. 4).

Effects of Gas6 on the phosphorylation of AKT. To elucidate the molecular mechanisms underlying the effects of Gas6 on EPCs, the phosphorylation status of the MAP kinases and AKT, which are implicated in EPC proliferation and function, was examined by western blot analysis (20,21). As shown in Fig. 5A, Gas6 did not cause any significant change in the phospho-ERK/ERK level over 1-h period, indicating that Gas6 had no effect on ERK activation. To confirm that Gas6 promotes EPC viability and motility through the AKT path-
ways, we treated the EPCs with LY294002 (PI3K inhibitor) alone or in combination with Gas6. LY294002 inhibited the phosphorylation of AKT; when used in combination with Gas6, Gas6 partly reversed the inhibition of phospho-AKT induced by LY294002 (Fig. 5C). These results indicate that Gas6 promotes EPC proliferation and migration, most likely through the AKT signaling pathway.

Effects of PI3K or ERK inhibitor on Gas6-induced EPC proliferation. To further confirm the roles of AKT in the Gas6-induced effects on EPCs, we determined the effects of AKT inhibition on EPC proliferation. As shown in Fig. 6, the PI3K inhibitor, LY294002, at dose 20 µmol/l did not alter the viability of the control EPCs, but markedly attenuated the effects of Gas6 on EPC proliferation. However, PD98059, an ERK inhibitor, at a concentration of 5 µmol/l, exhibited a similar effect between the absence and presence of Gas6-induced EPC proliferation vs. their own control. The decrease in EPC proliferation in both the Gas6 group and the control group treated with PD98059 was due to ERK inhibition. These results suggest that AKT, but not ERK, is involved in the Gas6-induced EPC proliferation.

Effects of PI3K or ERK inhibitor on Gas6-induced EPC migration. The possible roles of AKT in the Gas6-induced augmentation of the EPC migration were also assessed. The results revealed that the Gas6-induced increase in EPC migration was substantially attenuated by LY294002 (a PI3K inhibitor) (Fig. 7). By contrast, PD98059 (an ERK inhibitor) did not seem to have any significant effect on the migratory activity of these cells, suggesting that ERK is not involved in the stimulatory effects of Gas6 on EPC migration.

Discussion
To the best of our knowledge, the present study is the first to describe a novel effect by which Gas6 is engaged in the biological function of EPCs. The major findings of this study were...
ZUO et al: ROLE OF GAS6 IN HUMAN EPCs

Role of Gas6 in Human EPCs

Gas6 significantly stimulated EPC proliferation and migration in vitro; it upregulated phospho-AKT but not phospho-ERK expression; it did not promote EPC differentiation on Matrigel; and the positive effects on proliferation and migration were abrogated in the presence of the PI3K-specific inhibitor, LY294002. In recent years, it has become apparent that circulating EPCs are crucial to maintaining cardiovascular homeostasis and vascular integrity compared with mature ECs. It has been found that EPCs contribute up to 25% of ECs in newly formed vessels (22,23). Cell therapy using EPCs in ischemic heart disease has been evaluated and proven to be safe and effective in a number of pre-clinical studies (24,25). Accordingly, it is important to investigate the endogenous or exogenous factors that affect these cells. In the present study, we investigated whether the treatment of EPCs with Gas6 can improve the proliferation, migration and tube formation of EPCs.

Gas6 is widely expressed and has been found in the lungs, heart, kidneys, intestine, ECs, bone marrow, VSMCs and monocytes and, at a very low level, in the liver. With the use of an ELISA-based method, Gas6 was detected in human plasma. It regulates homotypic and heterotypic adhesion (10), promotes proliferation (26,27), survival (15,28,29) and motility (30,31) and amplifies the activity of extracellular stimuli (11,32). In addition to the general effects of Gas6 mentioned above, we focused on its role in the cardiovascular system. Our previous study demonstrated that, in patients with acute coronary syndrome, Gas6 plasma levels at admission reflect the presence of common cardiovascular risk factors and can independently predict cardiovascular events (33). These data indicate that Gas6 may play an additional role in the vascular system. Therefore, on an experimental ground, we investigated the possible roles of Gas6 in the biological function of EPCs.

Firstly, we demonstrated the biological function of EPCs treated with Gas6. We concluded that Gas6 promotes EPC proliferation and migration. These results are in accordance with those of a previous study by Holland et al, who found that Gas6 silencing reduces EC haptotaxis towards vitronectin (34). Conversely, Gallicchio et al found that Gas6 stimulates Axl and inhibits the ligand-dependent activation of VEGF receptor 2 (VEGFR2) and the consequent activation of an angiogenic program in vascular ECs (35). In a recent study, Ruan et al found that Gas6 did not significantly alter the VEGF-A-dependent activation of VEGFR-2 (17). It is very interesting that the two groups produced different results. We did not investigate VEGF-A-triggered signaling with Gas6; however, we found that Gas6 did not interfere with VEGF-A-induced tube formation in EPCs.

It has been previously demonstrated that Axl knockdown in ECs impairs tube formation (36). Further research has demonstrated that Axl regulates tube formation by the modulation of signaling through the angiopoietin/Tie2 and Dickkopf pathways. In addition, Axl is essential for the VEGF-A-dependent activation of PI3K/AKT (17). Yet, we, as well as others have observed that Gas6 does not promote tube formation (35). Axl is an angiogenic receptor tyrosine kinase that can be engaged by multiple stimuli, including Gas6, VEGF, lactate or hypoxia (37). Thus, we consider that the angiogenic role of Axl may be independent of Gas6 administration. More complex mechanisms may be involved in the bioactivities of Gas6 in EPCs. Angiogenesis is a complex process, requiring the coordinated action of a variety of growth factors in ECs. A
recent study demonstrated that, although Gas6 and Ang1 alone did not promote tube formation in ECs, the combination of Gas6 and Ang1 did (37). We thus consider the possibility that Gas6 alone may not promote tube formation, but it may do so when combined with other factors.

We extended our investigation with the aim of determining the mechanisms associated with the effects of Gas6 on EPCs. Previous studies have indicated that Gas6 plays an important role in some cell types through its regulation of the AKT and ERK signaling pathways following the initial effects on the cellular survival and proliferation (9, 38, 39). Additionally, the AKT and ERK signaling pathways are well-documented signaling pathways involved in EPC biology (40, 41). To explore whether these same kinases play a role in the Gas6-induced proliferation and migration of EPCs, their phosphorylated status following Gas6 treatment was assessed. Our data demonstrated that Gas6 induced the transient activation of AKT but not the ERK kinases.

To further demonstrate the role of AKT and ERK in the Gas6-induced proliferation and migration of EPCs, we performed additional experiments in which AKT and ERK activation was blocked by the pharmacological agents, LY294002 and PD98059, respectively. Our data demonstrated that at concentrations that did not affect the growth of the control EPCs, LY294002 markedly attenuated the cell proliferation and migration induced by Gas6. The decrease in EPC proliferation in both the GAS6 and control groups treated with PD98059 was due to ERK inhibition and not the blockade of GAS6-mediated ERK activation. Taken together, these findings, indicate that Gas6 promotes the EPC proliferation and migration through the PI3K/AKT pathway. It should be noted that the activation of PI3K/AKT is not sufficient to drive angiogenesis, while PI3K/AKT is required in regulating cell angiogenesis (41). The finding that Gas6 did not engage certain downstream signaling effectors, such as ERK, may account for the inability of Gas6 to promote angiogenic responses.

The Gas6-induced growth and differentiation of hematopoietic cells, particularly the erythroid progenitor cells, has been well documented (42, 43). The effects of Gas6 on EPCs have not been demonstrated, although EPCs and hematopoietic cells are derived from the same ancestor, i.e., the hemangioblast (44). It appears that Gas6 regulates EPC growth and differentiation through mechanisms that are distinct from those observed in erythroid progenitor cells. In conclusion, the present study demonstrates that Gas6 promotes EPC proliferation and migration through the PI3K/AKT signaling pathway. This poses an interesting question on the manipulation of EPCs with Gas6 to enhance the therapeutic effects of cell therapy in regenerating the endothelium. However, the results presented in this study are preliminary; therefore, further investigation is required in order for our data to be used as a basis for the development of a therapeutic strategy for re-endothelialization. Moreover, further studies are required in order to explore the effects of Gas6 on animals.

Acknowledgements

This study was supported by a grant from the National Natural Science Foundation of China, no. 81370468.

References


