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Growth differentiation factor 11 supports migration and sprouting of endothelial progenitor cells

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ABSTRACT

Background: Neovascularization plays an important role in tissue engineering applications. In animal models, it was demonstrated that implantation of endothelial progenitor cells (EPCs) from cord blood led to the formation of a complex functional neovasculature, whereas EPCs isolated from peripheral blood (pbEPCs) showed a limited vasculogenic potential, which may be attributed to age-related dysfunction. Growth differentiation factor 11 (GDF11) was recently identified as a rejuvenation factor, which was able to reverse age-related dysfunction of stem cells. Therefore, we hypothesized that GDF11 may improve the vasculogenesis-related phenotype of pbEPCs.

Materials and methods: pbEPCs were isolated from adult peripheral blood. Transforming growth factor (TGF)-β type-I receptor expression was analyzed by immunostaining. pbEPCs were treated with recombinant GDF11 for various time periods. Thereafter, phosphorylation of Smad2/Smad3, adhesion, proliferation, cell survival, migration, and *in vitro* sprout formation was investigated.

Results: pbEPCs express the TGF-β type-I receptors ALK4 and ALK5, but not ALK7. Treatment of pbEPCs with recombinant GDF11 resulted in activation of the Smad2/Smad3 pathway and in increased migration, which was inhibited by the TGF-β1 superfamily type-I activin receptor-like kinase inhibitor SB431542, demonstrating that the TGF-β receptor-Smad2/Smad3 pathway is involved in GDF11 induced migration. Moreover, *in vitro* sprout formation was increased as well by GDF11 treatment. However, other parameters such as adherence, proliferation, and apoptosis were not affected by GDF11.

Conclusions: This study provides evidence that GDF11 improves vasculogenesis-related growth parameters in pbEPCs and may represent a therapeutic option to ameliorate the angiogenic and vasculogenic properties of pbEPCs.

1. Introduction

Growth differentiation factor 11 (GDF11) is a member of the transforming growth factor (TGF)-β superfamily that regulates diverse cellular processes [1]. Recently, GDF11 was identified as a rejuvenation factor in heterochronic parabiosis experiments. In this surgical procedure, the circulatory system of a young animal is coupled to that of an aged animal. This procedure can rejuvenate a number of organs in the older of the pair [2]. During aging, levels of circulating GDF11 decline. Interestingly, injecting recombinant GDF11 into aged animals recapitulates the effects of heterochronic parabiosis [3]. Recently, it was also shown that GDF11 has rejuvenating effects on the central nervous system and skeletal muscle of mice [4,5] by reversing

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age-related dysfunction of neuronal stem cells and muscle fiber-associated stem cells, respectively.

Endothelial progenitor cells (EPCs) gain much attention in the context of prospective therapeutical applications because these cells can be isolated noninvasively from the peripheral blood (pbEPCs) of patients and display a high proliferative potential *ex vivo* [6]. EPCs, also referred to as outgrowth endothelial cells [7,8], can be isolated from pbEPCs and from cord blood (cbEPCs). We [9] and others [10,11] have shown that EPCs isolated from adult pbEPCs were unable to form blood vessels on implantation, whereas late EPCs isolated from showed a high vasculogenic potential. The reason for this functional difference is still unclear, but it may be assumed that the impaired vasculogenic potential of adult pbEPCs may be attributed to age-related dysfunction [12]. Based on the fact that GDF11 is considered to be a rejuvenation factor, we developed the working hypothesis that GDF11 treatment may rejuvenate pbEPCs thereby improving vasculogenesis-related cell parameters such as adhesion, proliferation, cell survival, migration, and sprout formation.

2. Materials and methods

2.1. Cell culture

Human pbEPCs were isolated and expanded as previously described [13]. In brief, peripheral blood mononuclear cells were isolated from 50 mL of peripheral blood from human adult volunteers by density gradient centrifugation with biocoll separation solution (Biochrom, Berlin, Germany). Cells were plated on 25-cm^2 culture flasks coated with rat type-I collagen (50 μg/mL) in endothelial growth medium-2 (Lonza, Cologne, Germany), supplemented with SingleQuots (human endothelial growth factor, vascular endothelial growth factor [VEGF], human fibroblast growth factor-B, R3-IGF-1, ascorbic acid, heparin, gentamicin and/or amphotericin-B) and 10% fetal calf serum (FCS), and cultivated at 37°C, 5% CO_2 in a humidified atmosphere. After 4 d in culture, nonadherent cells were removed by washing with phosphate-buffered saline (PBS), new media were applied, and the culture was maintained for another 4–5 wk. Media were changed every 3 d. After reaching about 80% confluence, cells were trypsinized and seeded into 75-cm^2 culture flasks for expansion. EPCs from passage 2–5 were used for experiments. To confirm the endothelial phenotype, EPCs were analyzed for cell surface expression of endothelial markers. EPCs were positive for CD31, von Willebrand factor, vascular endothelial growth factor receptor-2, vascular endothelial-cadherin, CD105, and CD146, whereas the hematopoietic cell surface markers CD45 and CD14 were not expressed. Moreover, EPCs showed a cobblestone morphology and were able to incorporate Dil-labeled acetylated low-density lipoprotein. Usage of the cells was approved by the ethics committee of the University of Freiburg.

2.2. Immunostaining

Cells were fixed with ice-cold methanol and incubated for 20 min in 0.3% H_2O_2 to block endogenous peroxidase activity and subsequently for 30 min with blocking solution (5% goat serum; Sigma, Deisenhofen, Germany), followed by incubation with the primary antibodies mouse monoclonal anti-ALK4, mouse monoclonal anti-ALK5, and mouse monoclonal anti-ALK7 (Abcam, Cambridge, United Kingdom) in 1% goat serum for 2 h at room temperature. In negative controls, incubation with the primary antibodies was omitted. After washing with PBS, the corresponding secondary antibody (ready-to-use horseradish peroxidase–conjugated goat anti-mouse; DAKO, Hamburg, Germany) was applied and incubated for another 1 h at room temperature. Thereafter, cells were washed three times with PBS and then exposed to 3,3′-diaminobenzidine (DAB) chromogen substrate (ready-to-use, DAKO) for approximately 10 min. Thereafter, cells were washed twice in H_2O bidest and weakly counterstained with hematoxylin. Cells were photographed using an inverted microscope at 100-fold magnification.

2.3. Smad phosphorylation assay

Cells were seeded in 6-well plates in endothelial cell growth medium (ECGM) (PromoCell, Heidelberg, Germany), 10% FCS, and incubated overnight. Then, medium was changed to ECGM without FCS. Cells were stimulated with GDF11 (40 ng/mL) for 30 min, and cell lysates were prepared. Phosphorylation of Smad2 and Smad3 was measured using the PathScan Phospho-Smad2/Smad3 sandwich enzyme-linked immunosorbent assay (Cell Signaling Technology, Boston, MA) according to manufacturers' instructions. Shown are mean values and standard deviations.

2.4. Cell proliferation assay

EPCs (2×10^4 cells per well) were seeded in 6-well plates in triplicate in ECGM supplemented with 10% FCS in the presence or absence of 40-ng/mL GDF11 for the indicated days. Medium was changed after 3 d. Cell counting was performed by CASY1 (Innovatis, Reutlingen, Germany). Shown are mean values and standard deviations.

2.5. Cell adhesion assay

EPCs were grown in ECGM, 10% FCS overnight, either in the presence or absence of GDF11 (40 ng/mL). Thereafter, cells were trypsinized and seeded in 48-well plates (4×10^4 cells per well) in triplicate in ECGM supplemented with 10% FCS in the presence or absence of GDF11. After 1 h, nonattached cells were removed by washing three times with PBS and attached cells were trypsinized and counted. Shown are mean values and standard deviations.

2.6. Cell death detection assay

EPCs (5×10^4 cells per well) were seeded in 12-well plates in triplicate for 4 h in ECGM, 10% FCS. The medium was then changed to ECGM, 10% FCS, or ECGM without FCS. Cells were grown in the presence or absence of GDF11 (40 ng/mL) or VEGF-A (50 ng/mL) for 48 h. Thereafter, cells were harvested in incubation buffer, and cell death detection sandwich enzyme-linked immunosorbent assay (Roche, Mannheim, Germany) was performed according to manufacturers' instructions.