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Rosuvastatin elicits KDR-dependent vasculogenic response of human placental stem cells through PI3K/AKT pathway

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ABSTRACT

The growth and plasticity of engrafted human mesenchymal stem cells is regulated by external stimuli. Rosuvastatin (RSV) promotes myocardial neovascularization and limits myocardial remodeling in patients with chronic heart failure (CHF). While these non-lipid benefits may in part depend on the activation of stem cells, experimental evidence that RSV directly elicits vasculogenic differentiation of human mesenchymal stem cells is still lacking.

We assessed whether RSV may drive a gene program of vascular commitment and the secretion of trophic mediators with antiapoptotic, angiogenic and antifibrotic activities in human mesenchymal stem cells from full-term placentas (FMhMSCs).

With real-time RT-PCR, immunofluorescence, chemiluminescence, Western blot analysis, and *in vitro* vasculogenesis assays, we show that RSV enhanced expression of vascular endothelial growth factor (VEGF), kinase insert domain receptor (KDR), encoding a major VEGF receptor, hepatocyte growth factor (HGF), and platelet-derived growth factor-BB (PDGF-BB) in a time- and dose-dependent manner. GATA-4 and Nkx-2.5 transcription was not affected. RSV enhanced capillary-like formation *in vitro*, but capillary-embedded FMhMSCs lacked endothelial marker expression, suggesting a role of pericyte-like elements in tube formation. In HUVEC/FMhMSC cocultures, RSV increases PDGFRβ expression in FMhMSCs, and enhanced capillary density and organizational efficiency, promoting a long-lasting survival of tubular networks. RSV also activated PI3K–Akt pathway; the vasculogenic effects of the statin were abrogated following PI3K inhibition by LY294002.

In conclusion, RSV-induced increase in capillary formation was dependent on VEGF and KDR. RSV promotes the activation of paracrine signals for vascular commitment of FMhMSCs through PI3K–Akt pathway. This observation may pave the way to the use of RSV as a pharmacological enhancer of stem cell potential for cardiovascular cell therapy.

1. Introduction

Statins have been shown to elicit a host of non-cholesterol-lowering responses, including reduced systemic inflammation [1], enhanced angiogenesis [2], and bone marrow cell (BMC) mobilization [3]. Rosuvastatin (RSV), a relatively new molecule, prevented progressive left ventricular remodeling and promoted neoangiogenesis in failing heart of patients [4,5] and animal models [6]. This outcome was partially ascribed to normalization of cardiac cytokine expression, including TNF-α, and increase in circulating BMCs [6] and endothelial progenitor cells [7], an event that

Abbreviations: RSV, rosuvastatin; CHF, chronic heart failure; FMhMSCs, human mesenchymal stem cells from fetal membranes of term placenta; VEGF, vascular endothelial growth factor; KDR, kinase insert domain receptor; PDGF-BB, platelet-derived growth factor-BB; vWF, von Willebrand factor.

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may potentially account for myocardial neovascularization and rescue.

Whether RSV may directly commit human adult stem cells toward a cardiovascular fate and/or enhance the paracrine secretion of trophic mediators with angiogenic, antiapoptotic and antifibrotic properties remains to be established. Addressing this issue may be of particular biomedical relevance. In fact, only poor or negligible myocardial rescue has been obtained so far in randomized clinical trials with simple stem cell transplantation in failing heart [8]. Conversely, Strauer et al. [9] demonstrated that cell therapy improves ventricular performance and prognosis in patients with chronic heart failure when stem cells were administered in addition to standard therapeutic regimes. It is conceivable that pharmacological agents modulate the magnitude of stem cell potential. *Ex vivo* preconditioning with synthetic molecules harboring differentiating and paracrine "logics" remarkably enhanced the cardiovascular commitment in both mouse embryonic [10] and human stem cells [11,12], improving their rescuing properties *in vivo* in animal models of heart failure [11,13,14]. These findings prompt further studies to assess whether drugs already available for conventional cardiovascular therapy (*i.e.* RSV) may also be used for stem cell preconditioning to enhance the potential for a cell therapy.

Here, we investigated whether exposure to RSV of human mesenchymal stem cells from fetal membranes of term placenta (FMhMSCs) may activate the transcription of vascular lineage-promoting genes, and whether RSV may drive the secretion of growth factors enhancing vasculogenesis *in vitro*.

2. Methods

2.1. Rosuvastatin preparation

Rosuvastatin (RSV) Ca^{2+} – salt was kindly provided by AstraZeneca and it was dissolved in DMSO at the final concentration of 50 mM.

2.2. Cell isolation

Term placenta obtained from caesarian sections were rapidly rinsed in PBS containing penicillin and streptomycin and used immediately. Pieces from fetal membranes were minced and digested for 10 min in DMEM with 0.25% trypsin–EDTA, 10 U/ml DNaseI and 0.1% collagenase. Tissues were pipetted vigorously up and down avoiding foam for 5 min; larger pieces of tissue were allowed to settle under gravity for 5 min. Each supernatant was transferred to a fresh tube, neutralized with FBS, then spun at $1000 \times g$ for 10 min. Each pellet was resuspended in 5 ml of DMEM containing 20% FBS, 10 U/ml penicillin and 100 μg/ml streptomycin. FMhMSCs were seeded into culture flasks and grown at 37 °C in 5% CO_2. Non-adherent cells were removed after 1 week and medium (with 10% FBS) was changed subsequently every 4 days.

Human umbilical vascular endothelial cells (HUVECs) were isolated in our laboratory with the method described by Davis [15] from human umbilical cord obtained during elective caesarian sections. The cells collected were grown in EGM-2 medium (Lonza) at 37 °C in 5% CO_2.

2.3. Flow cytometry analysis (FACS) and cellular phenotyping

To assess the mesenchymal origin of fibroblast-like cells isolated from fetal membranes we performed FACS analysis. Cells were harvested by treatment with 0.08% trypsin–EDTA and incubated with 1 μg/10^6 cells conjugated antibodies for 40 min at 4 °C in the dark. The antibodies used were: anti-CD105-Alexa Fluor 488 (Chemicon), anti-CD73-PE (BD), anti-CD29-PE-Cy5 (BD), anti-CD90-R-PE (BD), anti-CD166-R-PE (BD), anti-CD14-APC (BD), anti-CD34-FITC (BD), anti-CD44-FITC (BD), and anti-CD45-Per-CP (BD). After washing, cells were analyzed on a flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA, USA) by collecting 10,000 events and the data analyzed using the Cell Quest Software (Becton Dickinson).

Adipogenic (Chemicon), osteogenic (Chemicon) and chondrogenic (Lonza) differentiations were performed to test the multipotency of FMhMSCs, following the manufacturer's instructions.

For HUVEC characterization, anti-CD31 (Santa Cruz) was used as primary antibody and fluorescent conjugated goat anti-mouse (MP Biomedicals) as secondary antibody. Cells were analyzed on a flow cytometer.

2.4. Cell treatment and viability

Drug sensitivity was estimated by the MTT method, essentially as described by the manufacturer's instructions (SIGMA). FMhMSCs (7500 cells/cm^2) were seeded into 24-well plates and then treated with different concentrations of RSV (0.1–75 μM) for 1, 2, 3 and 6 days. Untreated cells and DMSO treated cells were used as control. Data were collected by reading at 570 nm with a multi-well plate reader (Dynex Technology).

Cell viability was determined by the trypan blue dye exclusion test. The cells were treated with RSV (0.1–75 μM). After 1, 2, 3 and 6 days, both attached and floating cells were harvested and they were counted by using a hemocytometer.

To assess whether RSV, in range between 0.1 and 10 μM, below the toxic concentration, may coax cells into a "cardiovascular decision", we performed comparative analyses of the expression of targeted cardiogenic and angiogenic genes and immunocytochemical assessment of lineage-restricted markers, as detailed below.

2.5. Gene expression

Real-time RT-PCR was used to assess the time- and dose-dependent effect of RSV on the gene expression of vascular endothelial growth factor (VEGF), KDR, hepatocyte growth factor (HGF), GATA-4, Nkx-2.5 and Akt. Primer sequences were: human GAPDH reverse: 5′ TGTGGTCATGAGTCCTTCCA 3′, forward: 5′ CAGCCTCAAGATCATCAGCA 3′; human VEGF reverse: 5′ ACACAGGATGGCTTGAAGATG 3′, forward: 5′ AGAAGGAGGAGGGCAGAATC 3′; human HGF reverse: 5′ ACTCCAGGGCTGACATTTGAT 3′, forward: 5′ ATTTGGCCATGAATTTGACCT 3′; human KDR reverse: 5′ GAGCTCTGGCTACTGGTGATG 3′, forward: 5′ CTGCAAATTTGGAAACCTGTC 3′; human GATA-4 reverse: 5′ TAGCCTTGTGGGGAGAGCTT 3′, forward: 5′ TGGCCTGTCATCTCACTACG 3′; human Nkx-2.5 reverse: 5′ GCGCACAGCTCTTTCTTTTC 3′, forward: 5′ CAAGTGTGCGTCTGCCTTT 3′; human Akt: reverse: 5′ CTTAATGTGCCCGTCCTTGT 3′, forward: 5′ TCTATGGCGCTGAGATTGTG 3′.

Data were normalized using GAPDH as an index of cDNA content after reverse transcription. Amplification included initial denaturation at 95 °C for 10 min, 50 cycles of denaturation at 95 °C for 10 s, annealing at 59–63 °C for 6–10 s, and extension at 72 °C for 10 s, performed at a temperature transition rate of 20 °C/s. Fluorescence was measured at the end of each extension step. Specificity of the product was determined by a melting curve analysis, conducted after completion of the cycling process with the aid of a temperature ramp (from 55 to 95 °C at 0.1 °C/s) and continuous fluorescence monitoring.

Samples were run in duplicate, and the average threshold cycle (C_t) value was used for calculations. Relative quantification of mRNA expression was calculated with the comparative C_t method using the "Delta–delta method" for comparing relative expression results between treatments in Real-time RT-PCR [16].