Original Article
Endothelial progenitor cells transplantation ameliorates atherosclerosis through transfection of vitamin D in ApoE\(^{-/-}\) mice

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Abstract: Objective: Previous researches have reported that endothelial cells status was important to atherosclerosis (AS). In this study, our aim is to figure out effect of the over-expressed vitamin D in endothelial progenitor cells (EPCs) during therapy of AS. Methods: EPCs were separated from bone marrow in wild type (WT) mice. Constructed pAd-VDR and pAd-VDR shRNA adenovirus vectors which were GFP tagged and then transfected in EPCs those were separated from bone marrow in wild type (WT) mice. Finally, the EPCs transfected with pAd-VDR and pAd-VDR shRNA were transplanted into ApoE\(^{-/-}\) mice. Transfection efficiency, cell proliferation, cell apoptosis, cell cycle, cell senescence and migration were measured. Aortas from each group were harvested and examined. Lipid measurements were performed. MMP-2, TIMP2, eNOS, iNOS and NO serum levels were also been detected in each group. Results: We found that the overexpression of vitamin D receptor in endothelial progenitor cells could promote cell proliferation and migration. Furthermore, those EPCs inhibited cell apoptosis and senescence as well. Transplantation of Vitamin D-transfected EPCs in ApoE-deficient mice significantly ameliorates atherosclerosis by decreasing the serum lipid level and probably inhibiting the synthesis of extracellular matrix (ECM), promoting ECM degradation, overexpressing eNOS and NO. Conclusion: In conclusion, our data presented a novel potential strategy for the therapy of atherosclerosis by transplantation of endothelial progenitor cells with the transfection of Vitamin D.

Keywords: Endothelial progenitor cells, Vitamin D receptor, atherosclerosis

Introduction

In the Western world, coronary heart disease has been the major cause of death \([1]\). The coronary heart disease is mainly due to atherosclerosis where the development of thrombosis may cause fatal complication such as acute myocardial infarction and sudden cardiac death \([2]\).

The first manifestation of atherosclerotic disease, endothelial dysfunction, results from accumulating cardiovascular risk factors including hyperlipidemia, hypertension, age, smoking and diabetes. This endothelial dysfunction is already present in healthy, asymptomatic humans and is characterized by an activation of endothelial cells (EC), decreased nitric oxide (NO) availability and constitutional changes at the cell’s outer surface \([3-5]\). Ongoing deterioration of the endothelial monolayer finally leads to endothelial cell death, invasion of inflammatory cells and vascular smooth muscle cell proliferation. An initial functional impairment of the endothelial monolayer with a high chance of reversibility has turned into a structural damage. This damage of the endothelial cell layer is followed by the development of an atherosclerotic lesion unless sufficient repair mechanisms lead to a restoration of the endothelial monolayer.

Recently, a novel mechanism contributing to endothelial dysfunction and cardiovascular risk has been proposed, that is, the impaired endogenous endothelial repair capacity in patients
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exposed to cardiovascular risk factors. Accordingly, patients with 1 or more cardiovascular risk factors have a reduced number of circulating endothelial progenitors, which are also defective in their repair function [6]. Conversely, increasing number of circulating progenitors, induced by cell transfusion or enhanced mobilization, can restore the integrity of the endothelial lining, suppress neointimal formation and increase blood flow to the ischemic sites [7]. Hence, an imbalance between endothelial injury and repair in favor of an increased risk-factors-mediated endothelial damage might be today considered another possible link between the exposure to traditional risk factors and atherosclerotic cardiovascular risk.

The positive relationship between 25(OH)D and baPWV is likely to be mediated by blood pressure has been proved by Kang JY [8]. There also is overwhelming epidemiologic and clinical evidence that links vitamin D deficiency to cardiovascular disease [9], including coronary stenosis, coronary artery calcification, and carotid atherosclerosis [10-12]. Clinical and epidemiologic studies reveal that vitamin D molecules calcitriol and paricalcitol could reduce the expression of TF induced by the proinflammatory cytokine TNF-α in human aortic VSMCs [13]. However, our understanding of vitamin D in vascular disease remains very limited. One Korean group investigated that serum vitamin D levels was negatively associated with carotid atherosclerosis [14]. Recent studies have suggested that vitamin D is able to inhibit foam cell formation by suppressing cholesterol uptake in macrophages from type 2 diabetes patients [15] and to decrease atherosclerosis by regulating T-lymphocyte functions [16]. Previous studies showed that VDR signaling inhibits atherosclerosis in part by blocking activation of the local RAS in macrophages [17].

In this study, we investigated the effect of the vitamin D receptor gene transfected endothelial progenitor cells on the therapy of atherosclerotic plaque in ApoE-deficient mice, which was a classic model of atherosclerosis [18].

Materials and methods

Vector construction and transfection

The rat VDR gene was cloned into pYr-adshuttle-4 to obtain pYr-ads-4-VDR. The VDR shRNA (5’-TGCCATTGAGGTGATCATGTT-3’) was cloned into pYr-1.1 to obtain pYr-1.1 VDR shRNA. The pAd-VDR/pAd-VDR shRNA were obtained by the recombination of pYr-ads-4-VDR/pYr-1.1-VDR shRNA and pAd/PL-DES adenovirus vector using LR Clonase II (Invitrogen). Cell transfection was performed using Lipofectamine 2000 (Invitrogen) or calcium phosphate methods.

MTT assay

Cells in 96-well plates were incubated with MTT. The formazan precipitate was dissolved in 200 ml dimethyl sulphoxide and absorbance at 550 nm was measured with a Benchmark microplatereader (Bio-Rad, CA, USA). After adding different concentration of Rapamycin in EPCs, the cellular viability was evaluated by MTT assay. Rapamycin suppressed cellular viability obviously, and appeared atrend-stationary at 100 ng/ml.

Cell apoptosis

Apoptosis was determined by translocation of phosphatidylserine to the cell surface using an Annexin V-FITC apoptosis detection kit (Nanjing KeyGen Biotech. Co. Ltd., China) according to the manufacturer’s protocol. Briefly, after treatment with DPB-5 for 48 h, cells were harvested and washed twice with ice-cold PBS, then evaluated for apoptosis using a FACSC alibur flow cytometer (BD Biosciences) with Annexin V-FITC and PI double staining. Fluorescence was measured with an excitation wavelength of 480 nm through FL-1 filter (530 nm) and FL-2 filter (585 nm).

BrdU

Cellular proliferation was detected by BrdU Elisa kit (BD, USA). Briefly, the classical method is the flow cytometric measurement of DNA content which can simultaneously determine the incorporation of Bromodeoxyuridine (BrdU). The procedure requires that DNA is partially denatured to expose incorporated BrdU to a specific antibody. Denaturation is necessary because antibodies developed so far bind only to BrdU in single-strand DNA. Green fluorescence from the fluorescein-conjugated antibody is a measure of BrdU incorporation. The protocol described here uses high-molarity HCl for the denaturation of DNA. Furthermore, this
method may be utilized either for unfixed or for fixed cells in suspension.

**Cell cycle**

The cells were harvested and washed twice with PBS, then fixed in ice-cold 70% (v/v) ethanol for 24 h at 4°C. Before analysis, cells were washed with PBS, suspended in 1 ml of cold PI solution (50 g/ml PI, 1% (v/v) Triton X-100, 100 g/ml RNase A) and incubated on ice for 30 min in darkness. Cytometric analysis was performed using flow cytometer and Cell Quest software. Fluorescence was measured with an excitation wavelength of 480 nm through FL-2 filter. Apoptotic cells were detected on a PI hist as a Pro G1 peak.

**Cell senescence**

Cell senescence was measured by Senescence β-Galactosidase Staining Kit (Beyotine) followed by the manufacturer’s instructions.

**Transwell**

Cell migration was detected using a transwell chamber (Sigma) followed by the manufacturer’s instructions.

**Atherosclerotic plaque assessment**

Aortas were harvested and stained with Oil Red O as described. Digital micrographs were taken, and total area of atherosclerotic plaque was determined using Image J (NIH) by a blinded observer.

**Lipid measurements**

Serum lipid profiles, including total cholesterol (TC), triglycerides (TG), HDL-C and LDL-C were measured with a 7600-120 Hitachi automatic analyzer (Hitachi, Tokyo, Japan). TC and TG were measured enzymatically (Roche Diagnostics GmbH) while HDL-C and LDL-C were measured using a direct assay method (Sekisui Medical Co., Ltd., Tokyo, Japan).

**Western blot**

Total protein lysates were obtained by lysing the cells with RIPA buffer supplemented with phosphatase inhibitor cocktail (Roche, Canada). The protein content was then measured by using the Bradford assay (Sigma, USA). Then, 100 µg of each sample were subjected to a 10% SDS-Page. The proteins were then transferred to a PVDF membrane (Roche, Laval, Canada) using the Pierce Fast Semi-Dry Blotter (Pierce, USA). Afterwards, the membrane was blocked with 0.5% skimmed milk over night. The following day, the membranes were washed in TBST for 3 times and incubated with the designated antibodies, anti-VDR (ab109234, Abcam, USA), anti-GAPDH (ab181602, Abcam, USA). Afterwards, the membranes were incubated in the appropriate secondary antibodies conjugated with HRP. The western blots were then developed under chemiluminescence condition (SuperSignal West Pico, Pierce, USA) using the ChemiDoc XRS machine (Bio-rad, USA). The bands were then analyzed using the Quantity One 1D Analysis software (Bio-rad, USA).

**Reactive oxygen species (ROS) concentration assay**

ROS concentration was measured using Oxi Select ROS assay kit (Cell Biolabs, SanDiego, CA). The assay was performed as prescribed in the manufacturer’s instructions.

**ELISA**

MMP2, TIMP2, eNOS, iNOS and NO determination of sera in mice was quantified by ELISA (Cell Biolabs, USA). The assay was performed as prescribed in the manufacturer’s instructions; and determination was performed using a WellScan MK3 spectrophotometer (Labsystems Dragon, Helsinki, Finland) at 450 nm.

**Measurements of nitrate-nitrite**

Nitrate-nitrite was estimated by using “Ca yman’s Assay Kit” (Cayman, Itemnumber-78-0001, Ann Arbor, MI). This method involves a two-step process. Briefly, the retinas were homogenized and centrifuged at 10,000 g for 10 minutes. The supernatant was collected to measure nitrates-nitrites. The reaction was started by adding 80 µL of retina samples, 10 µL of enzyme cofactor, and 10 µL nitrate reductase in a 96-well plate. After 1 hour, 50 µL Greiss reagents were added and allowed to incubate for 30 minutes. The changes in absorbance were measured at 550 nm. The amount of nitrate-nitrite was expressed as mM of nitrate-nitrite per milligram protein.

**Statistical analysis**

Data were analyzed by two-tailed unpaired Student’s t test. P value of 0.05 was considered indicative of statistical significance.
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Results

The construction of adenovirus vectors

To investigate the role of VDR in endothelial restoration, we cloned rat VDR gene to obtain pAd-VDR adenovirus vector. In addition, The VDR shRNA (5'-TGCCATTGAGGTGATCATGTT-3') was cloned into pAd-VDR shRNA adenovirus vector. After the construction of adenovirus vectors, the adenovirus, including rAd-EGFP, rAd-VDR and rAd-VDR shRNA, were transfected into NIH/3T3 cells. Figure 1 showed that green fluorescence by fluorescence microscope and VDR expression by western blot. These results suggest that both VDR overexpression and VDR knockdown adenovirus could efficiently infect rat cells.

Purification and identification of endothelial progenitor cells (EPCs)

As cardiovascular risk factors promote atherogenesis by damaging the endothelium, endothelial status has been mainly assessed by focusing the attention on the quantification of the endothelium capacity to modulate arterial vasomotion [19]. Thus, we purified the endothelial progenitor cells from Wistar rats for transplantation. Figure 2 showed the marker of EPCs: CD34 and CD133 were positive green, and VEGFR was positive red. These data indicated that we have successfully obtained EPCs.

VDR promotes the cell growth of EPCs

To ascertain if VDR could increase cell viability in endothelial progenitor cells (EPCs), we measured the cell viability using rapamycin as control. Figure 3A showed that the cell viability of EPCs decreased gradually with the increasing concentration of rapamycin and the stable decrease occurred after 100 ng/ml. VDR encouraged the cell growth of EPCs by enhancing cell...
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viability (Figure 3B), promoting cell proliferation (Figure 3C), inhibiting cell apoptosis (Figure 3D), extending the S phase in the cell cycle (Figure 3E). In addition, Figure 4A and 4B showed that VDR overexpression in EPCs inhibited the expression of pro-apoptosis protein-Caspase 3. Interestingly, in the β-Galactosidase Staining analysis, we found that VDR overexpression inhibited EPCs senescence (Figure 4C). Finally, we detected the migration of EPCs after transfection and adding rapamycin, as Figure 4D showed that VDR promotes the migration of EPCs. All these data indicated that VDR confers a cell growth advantage for EPCs.

VDR-transfected EPCs are efficient therapy for atherosclerosis

To test the effect of therapy of EPCs transplantation for atherosclerosis in APOE⁻/⁻ rats, several indicators of atherosclerosis have been detected. As showed in Figure 5A, the concentrations of TC, TG, LDL, lipoprotein in VDR overexpression group are lower than siRNA group.
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and control. Furthermore, the concentrations of HDL, Apo A1, Apo B in overexpression group are higher than siRNA group and control (Figure 5B), which indicated VDR-transfected EPCs transplantation in APOE-/- rats ameliorates atherosclerosis significantly. Figure 6 shows that the expression of TIMP2, eNOS, iNOS of overexpression group are higher than siRNA group and control group. MMP2 decreased significantly after knockdown of VDR.

Discussion

Vitamin D deficiency is a major pandemic, with estimates 1 billion individuals affected worldwide [20]. Present studies have reported that vitamin D deficiency could increase risk of cardiovascular diseases [21], how vitamin D exerted vascular protective effects was yet to be understood fully. One of the most acceptable mechanisms is attributed to its inhibition of endothelial cell dysfunction by suppressing inflammation and oxidative stress [22, 23]. Vitamin D has critical effects in endothelial cells, such as inducing nitric oxide (NO) production [24], protecting against oxidative stress and inhibiting endothelial apoptosis [25, 26] via diverse pathways. With the emerging function of endothelial cells for initiation and progression of atherosclerotic process, vitamin D may contribute for restoring atherosclerosis. Vitamin D receptor (VDR), a member of the nuclear receptor superfamily, mediates the action of the biologically active form of vitamin D, 1a,25-dihydroxyvitamin D3 (1,25(OH)2D3). Activation of VDR either by 1,25(OH)2D3 or its analogue could exhibit atheroprotective effects in several animal experiments [25, 27]. After the VDR binding with vitamin D, it has conformational change and activates target genes transcription. Its biological effects were mediated by mechanisms of genomic and non-genomic mechanisms [28]. Therefore, it seems

Figure 4. VDR promotes cell growth of EPCs. A. The expression of Caspsase 3, which represented the volume of apoptosis, was measured by WB. B. After analyzing the image of WB, the contrast was showed in the diagram clearly. C. Cellular ageing was detected by Senescence β-Galactosidase Staining Kit. D. Cellular ability of migration was detected by Trans-well chamber. (Normal Level Group, N/a; High Level Group, H/b; Low Level Group, L/c; Normal Level Group + Rapamycin, N+R/d; High Level Group + Rapamycin, H+R/e; Low Level Group + Rapamycin, L+R/f).
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Figure 5. The Lipid measurements were performed after EPCs with VDR overexpression or VDR siRNA were implanted in ApoE⁻/⁻ mouse. A. 4 weeks later. B. 8 weeks later. (Total Cholesterol, TC; triglyceride, TG; High density lipoprotein cholesterol, HDL; Low density lipoprotein cholesterol). Two-tailed t test was used for all comparisons between two groups. *P<0.05, **P<0.01.
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that we could overexpress VDR for the uptake of more vitamin D.

In recent years, vitamin D and lipid metabolism and cardiovascular disease relationship is compelling. Our previous study found that VDR knockout mice homozygous for the presence of blood pressure increased significantly with the phenomenon of cardiac hypertrophy and aortic blood pressure levels and the impact of endothelial nitric oxide synthase, endothelin-1 gene expression and atrial natriuretic peptide levels, indicating vitamin D, VDR expression and cardiovascular function and vasoactive substances were significantly related [29]. Under physiological conditions, the integrity of the endothelial monolayer is maintained by replication of adjacent cells; however, in conditions of increased endothelial injury, regeneration of the injured endothelium may be assisted by endothelial progenitor cells (EPCs) homing into the artery wall [30].

In this study we successfully overexpressed VDR in EPCs and transplanted these cells into ApoE−/− mice. In previous study researchers have proved that Vitamin D receptor signaling are related with inhibiting atherosclerosis. Meanwhile EPCs have the potential restoring and renewal ability like stem cells. Different from the previous study, it is the first time that we combine both of these two advantages together for the investigation of atherosclerosis in vivo and this strategy efficiently ameliorates atherosclerosis, indicating it maybe a new method for the therapy of atherosclerosis.

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Disclosure of conflict of interest

None.

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