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Slow and sustained nitric oxide releasing compounds inhibit multipotent vascular stem cell proliferation and differentiation without causing cell death

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ABSTRACT

Atherosclerosis is the leading cause of cerebral and myocardial infarction. It is believed that neointimal growth common in the later stages of atherosclerosis is a result of vascular smooth muscle cell (SMC) de-differentiation in response to endothelial injury. However, the claims of the SMC de-differentiation theory have not been substantiated by monitoring the fate of mature SMCs in response to such injuries. A recent study suggests that atherosclerosis is a consequence of multipotent vascular stem cell (MVSC) differentiation. Nitric oxide (NO) is a well-known mediator against atherosclerosis, in part because of its inhibitory effect on SMC proliferation. Using three different NO-donors, we have investigated the effects of NO on MVSC proliferation. Results indicate that NO inhibits MVSC proliferation in a concentration dependent manner. A slow and sustained delivery of NO proved to inhibit proliferation without causing cell death. On the other hand, larger, single-burst NO concentrations, inhibits proliferation, with concurrent significant cell death. Furthermore, our results indicate that endogenously produced NO inhibits MVSC differentiation to mesenchymal-like stem cells (MSCs) and subsequently to SMC as well.
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1. Introduction

Atherosclerosis, characterized by narrowing and hardening of the arterial wall, inflammation, and arterial plaque formation [1–3], is present in 90% of patients suffering from cardiovascular related illnesses. Current understanding states that the migration and proliferation of vascular smooth muscle cells (SMC) are key events in the formation and progression of atherosclerotic lesions [4]. For decades, scientific investigations have operated under the hypothesis that mature SMCs de-differentiate into a proliferative phenotype (pSMC) following denudation of the vascular endothelium. pSMCs migrate from the tunica media to the tunica intima, proliferate, and secrete extracellular matrix (ECM) proteins, which serve as the core and preliminary foundation of atherosclerotic plaques [5,6]. However, a recent study has introduced new evidence in support of an alternate theory, which suggests that atherosclerosis is caused by elevated stem cell differentiation [7].

The vascular tunica media was believed to be comprised of only mature SMCs. However Tang et al. [7] identified two medial non-SMC cell types, MVSC and MSC. Lineage-tracing experiments reveal several contradictions to the SMC de-differentiation theory. First, MVSCs can differentiate into MSCs and subsequently to mature SMCs. Second, MVSCs and pSMCs cannot be derived from the de-differentiation of mature SMCs. Third, MVSCs convert from a quiescent to an activated (proliferative and differential) state in response to endothelial denudation or *in vitro* isolation, and contribute toward vascular remodeling and neointimal formation. Finally, MVSCs and MSCs are capable of differentiating into other cell types including chondrocytes and osteoblasts, which would explain the arterial hardening that occurs in patients suffering from atherosclerosis. Taken together these findings introduce a new paradigm: MVSC differentiation, rather than SMC de-differentiation

Abbreviations: SMCs, smooth muscle cells; NO, nitric oxide; iNOS, inducible nitric oxide synthase; MTT, (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide); cDMSO, cellular grade dimethyl sulfoxide; LDH, lactate dehydrogenase; MSC, mesenchymal-like stem cell; MVSC, multipotent vascular stem cell; DETA-NO, diethylenetriamine NONOate; ABH, amino-2-borono-6-hexanoic acid; LPS, lipopolysaccharide; IFN-γ, interferon gamma; bFGF, basic fibroblast growth factor.

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contributes to vascular disease, and establishes a population of pSMCs [7,8]. Staunch proponents of SMC de-differentiation theory maintain its validity [9], and call into questions the methods and findings of Tang et al. [7]. Therefore, additional studies are needed.

The salutary role of nitric oxide (NO) as an inhibitor of oxidative stress [10], inflammation [11], proliferation [12] and platelet aggregation [13], has made it an ideal mediator against atherosclerosis [14]. Specifically, NO has been implicated in the inhibition of SMC proliferation [15,16]. We have recently reported the preparation of a variety of slow and sustained, low molecular weight NO releasing compounds [17–19], which have effectively inhibited SMC proliferation *in vitro* [18,19]. We report herein the inhibitory effect of NO on MVSC proliferation using three different NO-donors: the commercially available, diethylenetriamine NONOate (DETA-NO, **3**′), along with **1**′ and **2**′, two slow and sustained NO donors prepared in our laboratory [18,19]. In addition, our report includes the inhibitory effects of endogenous NO on MVSC differentiation to MSC.

2. Materials and methods

2.1. Chemicals and equipment

Hyclone Dulbecco's Modified Eagle Medium (DMEM, Fisher), Fetal Bovine Serum (FBS, ScienCell), chick embryo extract (CEE, MP Biomedical), N2 (Invitrogen), B27 (Invitrogen), basic fibroblast growth factor (bFGF, Sino Biological Inc.), retinoic acid (Sigma–Aldrich), 2-mercaptoethanol (Sigma–Aldrich), Penicillin/Streptomycin (P/S, ScienCell) were procured. PureLink® RNA Mini Kit, TRIzol®, On-column PureLink® DNase Treatment, and High-Capacity cDNA Reverse Transcription Kit were purchased from Invitrogen. Primers were obtained from Integrated DNA Technology. MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) assay kit and cellular dimethyl sulfoxide (cDMSO) were purchased from ATCC. Lactate dehydrogenase (LDH) cytotoxic assay kit was supplied by BIOO Scientific. Diethylenetriamine NONOate (DETA-NO) and amino-2-borono-hexanoic acid (ABH) were purchased from Cayman Chemical. Lipopolysaccharide (LPS) and interferon-γ (IFN-γ) were obtained from Sino Biomedical Laboratories and Peprotech, respectively. Absorbance was determined using and Molecular Devices micro plate reader and SoftMax Pro software.

2.2. Isolation and culture of multipotent vascular stem cells

The target cells were obtained utilizing a tissue explant method outlined previously [20]. Briefly, rat aortae (kindly supplied by Professor Gary Dunbar) were dissected in a sterile environment. The tunica media was isolated from the accompanying connective tissue and endothelium, sectioned into 2 mm cubes, and placed onto a Cellstart treated 6 well plate, luminal surface down. Once attached, 1 mL of enhanced medium (EM, DMEM with 10% FBS and 1% P/S) was added to each well and plates were incubated. EM was replaced every four days. On day 15, EM was replaced with an equal volume of maintenance medium (MM) (DMEM with 1% FBS, 2% chick embryo extract, 1% N2, 2% B27, 20 ng/mL bFGF, 100 nM retinoic acid, 50 nM 2-mercaptoethanol, and 1% P/S) to prevent MVSC differentiation. Every 2–4 days any detached tissue was removed, and half of the medium was replaced. After 2–3 weeks any remaining explants were removed, and the entire volume of MM was replaced in each well. Cells were incubated until a suitable monolayer formed, at which point they were transferred to a CellBIND treated flask at a density of 8×10^5 cells/mL for culture expansion. Following passage six, cells were transferred to CellBIND treated 96 well plates (2500 cells/well), and incubated for 16–24 h to ensure attachment. The cellular population was synchronized with serum-free MM (100 μL), and incubated at 37 °C for 24–48 h prior to subsequent tests.

To allow for MVSC differentiation to MSC, MVSCs were allowed to grow in EM and harvested after six days. Therefore, MVSCs had a total exposure to EM for 21 days to yield the MSC control group. To activate inducible nitric oxide synthase (iNOS), MVSCs (in EM) on day 15 were treated with LPS (50 μg/mL) and IFN-γ (20 ng/mL) for 6 h. The medium was removed and ABH, a potent arginase inhibitor [18,21] (5 mM) dissolved in EM, was added and the cells were incubated for an additional six days.

2.3. Characterization of isolated vascular cells (qPCR experimental procedure)

Total RNA was isolated using PureLink® RNA Mini Kit, quantified using a Nano-drop ND-1000 spectrophotometer, and reverse transcribed to cDNA utilizing the High capacity cDNA Reverse transcription kit (Applied Biosystems) and random primers (Supl. Mat.). All qPCR reactions were conducted in triplicate using Maxima SYBR Green/Rox qPCR Master Mix (Thermo Scientific). Thermo cycling was performed on an ABI 7500 Real-time PCR System (Applied Biosystems) starting at a hold stage of 95 °C for 10 min, and 40 cycles of denaturation at 95 °C for 15 s, annealing/extension at 60 °C for 60 s followed by disassociation melt curve analysis. Negative controls (no template) were also conducted in triplicate for all target genes.

The C_t for each sample was determined by taking the mean of three technical repeats. The data was normalized to 18S ribosomal RNA, and relative expression was assessed against MSC cells in EM at 3 weeks using the $2^{-\Delta\Delta Ct}$ method [22]. Data are the averages of two biological replicates, repeated three times. Data were analyzed using a Student's *T* test where $p < 0.05$ was considered significant.

2.4. MVSC exposure to secondary amines

Solutions of secondary amines **1** and **2** were prepared in cDMSO at three concentrations (10, 20, and 40 μM and 0.5, 1.0, and 2.0 mM, respectively). DETA (**3**) was dissolved in cellular grade water (cH_2O) to prepare 0.5, 1.0, and 2.0 mM solutions. An aliquot of each amine solution was combined with MM to render a 1% solution (v/v). The serum-free medium was replaced with 100 μL of each sample medium solution and plates were incubated for 48 h, followed by the addition of 100 μL of fresh sample containing medium to each well for a final volume of 200 μL. Plates were again incubated for 48 h, before conducting MTT or LDH analysis for cell viability or cell death assay respectively, according to the manufacturer's instructions and as described elsewhere [18].

2.5. MVSC exposure to NO-donors

Solutions of NO-donors (*N*-nitrosated amines), **1**′, **2**′, and **3**′ were prepared at the same concentrations, and added to synchronize MVSCs in the same manner as their corresponding amines.

3. Results and discussion

3.1. Characterization of proliferation and differentiation of isolated cells

In order to authenticate that the isolated vascular cells were MVSCs, which can proliferate and differentiate to MSCs, we took recourse to quantifying the expression of seven genes. Cells treated in MM from day 15 (MVSC) and those treated in EM for 21 days

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