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ORIGINAL ARTICLE

Vitamin D Improves Cardiac Function After Myocardial Infarction Through Modulation of Resident Cardiac Progenitor Cells

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Background Vitamin D has been implicated in the prevention of heart failure. However the underlying mechanism remains unclear. We hypothesised that these effects may be partially mediated by cardiac stem/progenitor cells (CPCs). Therefore, we examined the effects of 1,25-dihydroxyvitamin D3 (1,25D) on cell cycle activity and differentiation of a previously described CPC population called cardiac colony-forming unit fibroblasts (cCFU-Fs).

Methods cCFU-Fs were isolated from adult male C57Bl/6 mouse hearts using fluorescence-activated cell sorting. The effect of 1,25D on cell proliferation and differentiation were was assessed by colony-forming and fibroblast differentiation assays. Cell cycle was analysed by flow cytometry. Mice with induced myocardial infarction (MI) were treated with 1,25D or vehicle controls and cardiac function assessed by echocardiography.

Results 1,25D dose-dependently increased expression of vitamin D receptor (Vdr) and reduced large colony formation. Addition of 1,25D to cCFU-Fs slowed cell proliferation, promoted cell cycle arrest and decreased expression of pro-fibrotic factors during TGF-β-induced fibroblast differentiation of cCFU-Fs. After MI, 1,25 D-treated mice had less left ventricular wall thinning and significant improvement in left ventricular systolic function compared to vehicle-treated controls. Although, no significant changes in myocardial fibrotic area and cardiomyocyte size were noted, treatment with 1,25D significantly inhibited cardiac interstitial cell proliferation after MI.

Conclusions Vitamin D signalling promotes cardioprotection after myocardial infarction. This may be through modulation of cCFU-F cell cycle. The role of 1,25D and VDR in regulating cardiac stem/progenitor cell function therefore warrants further investigation.

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Introduction

Although the role of vitamin D in maintaining bone homeostasis is well known, its effects on muscle and vasculature are being increasingly appreciated. Pre-clinical studies suggest that vitamin D alters cardiac calcium handling and collagen production after induced myocardial infarction (MI) or left ventricular (LV) pressure overload [1,2]. Furthermore, clinical studies link low levels of vitamin D to coronary artery disease and heart failure (HF) [3–5]. This raises the enticing possibility of a low-cost adjunct to current standard treatment for coronary artery disease and HF, but clinical studies have been inconsistent. More targeted clinical trials, guided by a better understanding of how vitamin D confers favourable cardiac effects, are needed.

We recently described, in murine and human hearts, a population of cardiac-resident mesenchymal progenitors (cardiac colony-forming unit fibroblasts [cCFU-Fs]) [6,7]. These progenitors can differentiate into endothelial, smooth muscle and cardiomyocyte-like cells, as well as into myofibroblasts. Manipulation of this population in the injured heart may result in favourable cardiac functional effects. We hypothesise that attenuating pro-fibrotic factors in cCFU-F fibroblast differentiation can alter cardiac fibrosis, resulting in improvement of cardiac function after MI. We aimed to examine the effects of 1,25-dihydroxyvitamin D_3 (1,25D) supplement on cCFU-Fs and whether vitamin D supplement improves cardiac function after MI.

Material and Methods

Animals

We conducted all experiments using male C57Bl/6 mice (8–12 weeks) and study protocols were approved by the Western Sydney Local Health District Animal Care and Ethics Committee. All experiments were conducted in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes. Mice were housed in a facility with 12-hour light and dark phases, and offered ad libitum food and water intake.

Cell Isolation, Culture and Treatment

cCFU-Fs were isolated from mouse hearts as previously described [6]. Briefly, hearts were isolated, minced and then digested in 0.1% collagenase type II (Worthington) in phosphate buffered saline (PBS) at 37 °C for 30 minutes. Debris was removed with a 40 μm filter. Cells were washed and collected by centrifugation before staining with fluorophore-conjugated antibodies: Sca1, PDGFRα and CD31, as previously described [6]. Cells were sorted for Sca1+ PDGFRα+ CD31− using fluorescence-activated cell sorting (FACS) (BD FACS Arial; Becton and Dickinson).

cCFU-Fs were cultured in Dulbecco's modified Eagle's medium (DMEM) (Lonza) containing 20% fetal bovine serum (FBS, Sigma-Aldrich), L-glutamine (2 mM), penicillin (10 U/mL) and streptomycin (10 μg/mL). Cells were grown in six-well plates at 37 °C in a humidified atmosphere with 5% CO_2. When the cells reached ~80% confluence, they were harvested for experiments. To determine the effective dose of vitamin D, cells were treated with 1 nM, 10 nM or 100 nM concentrations of 25(OH)VitaminD_3 (vitamin D precursor [25D]; Sigma-Aldrich) or 1,25$(OH)_2$Vitamin D (the active form of vitamin D; Sigma-Aldrich) for 14 days and harvested for RNA analysis.

Quantitative Real-Time Polymerase Chain Reaction (qPCR) Analysis

Total RNA was prepared using Trisure reagent (Bioline) according to the manufacturer's protocol. Total RNA abundance was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Reverse transcription was carried out using a first-strand cDNA synthesis kit (Bioline) according to the manufacturer's instructions. mRNA expression of the *Vdr*, *Cyp24a1*, *Cyp27b1*, *Acta*2 and *TnC* genes and the housekeeping gene *Tbp* were measured quantitatively using SensiFAST SYBR No-ROX Mix (Bioline) and gene-specific primers (primer sequences available on request). Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, which normalised against housekeeping gene.

CFU-F Assay

The colony-forming assay was performed as previously described [6]. Briefly, 2000 cells per 35 mm were plated on tissue culture plastic and cultured in DMEM (Lonza) supplemented with 20% FBS. Concentrations of 1 nM, 10 nM or 100 nM of 25D or 1,25D were added daily to the well. After 12 days, the cells were stained with crystal violet to enable visualisation of colony formation.

Flow Cytometry

cCFU-Fs were harvested, washed in PBS and stained with anti-pHH3-488 antibody (Abcam), anti-PDGFRα-APC (BioLegend), anti-CD31-488 (BioLegend) and anti-Sca1-PE (BioLegend) for 30 minutes in the dark. We analysed the fluorescence intensity of the cells using flow cytometry (BD FACS Canto II; BD Biosciences).

Fibroblast Differentiation Assay

To study fibroblast differentiation, cCFU-Fs were seeded in 24-well plates. At 50% confluency, media was supplemented with 10 μg/mL of TGF-β1 (R&D Systems) with or without 1,25D at the doses indicated for 7 or 14 days. Media was changed second daily. The cells were stained with Sirius red, Gomori trichrome or Acta2, or harvested for qPCR or Western blotting.

Western Blot

Total protein was extracted using RIPA buffer (Sigma-Aldrich). Protein concentrations were measured using the BCA assay kit (Thermo Scientific) and10 μg of protein was loaded onto 10% SDS-PAGE gels. After electrophoresis, proteins were transferred to a nitrocellulose membrane

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