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Fibroblast growth factor 21 plays an inhibitory role in vascular calcification in vitro through OPG/RANKL system

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ABSTRACT

Vascular calcification is prevalent and associated with adverse outcome without available therapy. The benefits of fibroblast growth factor (FGF)-21 on metabolism and atherosclerosis make it a promising therapeutic agent for vascular calcification. We investigated the effects of FGF21 on vascular smooth muscle cell (VSMC) calcification by culturing rat VSMCs in a calcifying medium for 9days. FGF21 markedly attenuated mineral deposition and apoptosis at the indicated time points. In the presence of FGF21, the expression levels of osteoblastic protein including bone morphogenic protein-2, alkaline phosphatase(ALP), runt-related transcription factor(RUNX)-2 and nuclear factor-kappa B ligand (RANKL) were down-regulated, whereas the expression of osteoprotegerin (OPG) increased. Knockdown of OPG significantly impaired inhibition of FGF21 on apoptosis and the expression of pro-apoptotic genes including caspase-3 and Bax and osteoblastic −promoting markers including ALP, RUNX-2 and RANKL. Furthermore, FGF21 facilitated the phosphoryl of AKT but suppressed P38, while OPG knockdown attenuated the effects. LY29400 (inhibitor of PI3K) abrogated the activation of PI3K/AKT and SB203580 (inhibitor of P38) abolished the inhibition of FGF21 on P38, while alteration was observed in the expression of RUNX-2. FGF21 inhibited VSMCs calcification via OPG/RANKL system, and through P38 andPI3K/AKT pathways.

1. Introduction

Vascular calcification (VC) is highly prevalent and increases mortality and cardiovascular events [1–3]. VC is an active process relating with down-regulation of inhibitors and up-regulation of inducers under promineralizing conditions [4,5]. Besides, available evidence implicates VC is concerned with passive process without requiring cellular activity [5]. Despite investigations having documented valuable information, no effective intervention is for available [4].

FGF21is a powerful metabolic regulator and activates the complex assembled by FGFR and β-Klotho. FGF21 regulates energy metabolism, protects against cardiac insults and atherosclerosis, and reduces bone mass that is still debated [6,7]. Benefits in metabolic syndrome associated vasculopathy and divergent regulation of bone homeostasis highly suggest FGF21 may participate in the pathogenesis of VC.

Coincidence of VC and osteoporosis, extremely in the elderly and postmenopausal women, suggests connections between them [8,9]. Analyses of gene-targeted mice implied the RANK/RANKL/OPG cytokine triad was the possible link [8–10]. RANKL generates multiple intracellular signals by binding to RANK [11]. It has been well established that hormones and cytokines exert effects on bone remodeling through RANK/RANKL/OPG [11]. OPG acts as the decoy receptor for RANKL and tumour necrosis factor related apoptosis-inducing ligand (TRAIL). Studies showed spatial variation in expression of this triad was observed in vasculature [12]. In normal mice vessels, OPG was constitutively expressed, whereas RANK and RANKL were undetectable. Furthermore, OPG was frequently observed in less calcified atheromatous plaques, while RANKL and

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RANK preferentially in calcification lesion. Panizo et al. showed RANKL was highly expressed in calcified sites of vascular and increased VC [13]. Considering the result of previous studies, it has been proposed that RANK/RANKL/OPG system involves in VC. Our preliminary experiments showed FGF21 prevents VSMCs calcification in vitro. In the present study, we further investigated whether the underlining signaling pathway is mediated by RANK/RANKL/OPG system.

2. Materials and methods

2.1. Cell culture and in vitro calcification

Primary VSMCs were isolated from aortas of Sprague Dawley (SD) rats and identified by immunofluorescence staining for α-smooth muscle actin (Abcam plc, Cat.No. ab5694,S1). All animal procedures were approved by the Institutional Animal Care and Use Committee of Capital Medical University, Beijing, China. To induce calcification, VSMCs were exposed to calcifying medium (CM) for 9 days, which consisted of growth medium supplemented with 4.5 g/L glucose, 10^{-7} mol/L insulin, 50 μg/mL ascorbic acid, 10 mM sodium pyruvate and 10 mM BGP (Sigma,Cat.No.G9422) [14]. RecombinanFGF21(R&D Systems, Cat. No. 2539 -FG) at 50 ng/ml was added to the culture 48 h before the testing time points. 10 μM SU5402(Sigma,Cat.No.SML0443), 1 μM LY294002(CST, Cat.No. 9901S) and 2.5 μM SB203580 (CST, Cat.No.5633S) were inhibitors targeting FGFR1, PI3Kand P38, respectively.

2.2. Calcification assay

Cells were fixed in 95% ethanol for 15 min and stained with 1% alizarin red solution (Sigma, Cat.No.A5533) for 5 min. Photographs were taken at the microscope. Calcium content was detected using a Calcium Colorimetric Assay Kit (Nanjing Jiancheng Bioengineering Institute).

2.3. Detection of apoptosis

Flow cytometer was performed according to the protocol. Briefly, cells were collected by centrifugation and resuspended in binding buffer containing Annexin V and propidium iodide, followed by being analyzed with flow cytometer (Beckman FC500, USA). The apoptotic index was determined as the percentage of apoptotic cells in a 10.000 cell-cohort.

2.4. Short hairpin RNA (shRNA) transfection

Lentiviral vectors encoding shRNAs were purchased from GENECHEM (Lenti-KD™ Easy I-RNAi-puromycin lentiviral, Shanghai, China). To knockdown OPG, cells were transduced with OPG shRNA carrying lentivirus for 6–8 h and then switched to GM for 72 h.

2.5. Real-time RT-PCR analysis

Total RNA was extracted using TRIzol reagent (Tiangen Biotech, Cat. No.DP 405 -02). Reverse transcription was carried out using PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Cat. No. RR047B). Amplification of cDNA templates were then performed using Real-time PCR through the Ex Taq SYBR Green Supermix (TaKaRa, Cat.No. RR82L R), followed by detection of expression of all genes. Gene expression was normalized to18srRNA. The primer sequences of genes of interest were available in the Supplemental Table 1.

2.6. Western-blot

To extract total protein, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Beyotime Institute of Biotechnology) for30min. Protein concentration was measured using a bicinchoninic acid (BCA) Protein assay kit. Subsequently, sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (SDS-PAGE) were loaded with protein samples, followed by incubated overnight at 4 °C with primary antibodies against BMP2(Abcam plc,Cat. No.ab14933), ALP (Abcam plc,Cat. No.ab95462), OPG(Abcam plc, Cat. No.ab73400), RANKL (Santa, Cat.No.Sc-7628), *p*-AKT (Ser473) (CST, Cat.No.40 60S) and p-P38 (Thr180/Tyr182) (CST, Cat. No.9210S). Afterwards, bands were visualized using gel documentation system (Bio-tanon, Shanghai, China).

2.7. Statistical analyses

Data were presented as mean ± SEM. One-way analysis of variance (ANOVA) was used for the comparison. The Statistical significance was defined as the values of $p < 0.05$.

3. Results

3.1. CM-induced mineralization and apoptosis in VSMCs

As shown in Fig. 1A, cell layer with positive staining for Alizarin Red revealed mineralization occurred as early as day 3 and increased in number through day 9. In contrast, no visible mineralization was found in control (Fig. 1A). Detection of calcium concentration demonstrated increased calcium deposition was observed in CM at the three time points examined (***$P < 0.001$) (Fig. 1B).

Previous studies support apoptosis of VSMCs initiate mineral deposition [15–17]. In the present study, about 4.84% apoptotic cells were detected at day 0 (data not shown). As shown in Fig. 1C, the apoptotic cell count slightly increased with time in control. However, apoptosis in CM increased greatly and even rose to 20% at day 9. The differences reached statistical significance (***$P < 0.001$,respectively) (Fig. 1C). Collectively, findings confirmed VSMCs in CM became calcified but not in control.

3.2. BGP-induced osteoblastic differentiation in VSMCs

Calcified VSMCs express osteogenic phenotypes including ALP and RUNX-2 [17]. In the present study, VSMCs grown in CM exhibited higher expression of ALP and RUNX-2 at 3, 6 and 9 days compared to control. The expression of ALP was substantially increased over the time course of mineralization (***$P < 0.001$) (Fig. 1D). Up-regulation of RUNX-2 induced by BGP was also observed (***$P < 0.001$) (Fig. 1E). Our results show VSMCs grown in CM spontaneously undergo phenotypic transdifferentiation into osteoblast-like cells.

3.3. FGF21 attenuated BGP-induced mineralization and osteoblastic differentiation in vitro

The effects of FGF21 on mineralization and apoptosis were evaluated. Fig. 2A shows FGF21 at 50 ng/ml attenuated mineral deposition in a time-dependent manner (Fig. 2A). Results of calcium concentration were completely concordant with the results of Alizarin Red staining (Fig. 1B). FGF21 at 50 ng/ml greatly reduced BGP-induced calcium deposition at 3, 6 and 9days (**$P < 0.01$, ***$P < 0.001$ and ***$P < 0.001$,respectively). Notably, elevated calcium concentration in VSMCs treated with FGF21is significant

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