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Interleukin-24 attenuates β-glycerophosphate-induced calcification of vascular smooth muscle cells by inhibiting apoptosis, the expression of calcification and osteoblastic markers, and the Wnt/β-catenin pathway

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ABSTRACT

Vascular calcification is a hallmark of cardiovascular disease. Interleukin-24 (IL-24) has been known to suppress tumor progression in a variety of human cancers. However, the role of IL-24 in the pathophysiology of diseases other than cancer is unclear. We investigated the role of IL-24 in vascular calcification. IL-24 was applied to a β-glycerophosphate (β-GP)-induced rat vascular smooth muscle cell (VSMC) calcification model. In this study, IL-24 significantly inhibited β-GP-induced VSMC calcification, as determined by von Kossa staining and calcium content. The inhibitory effect of IL-24 on VSMC calcification was due to the suppression of β-GP-induced apoptosis and expression of calcification and osteoblastic markers. In addition, IL-24 abrogated β-GP-induced activation of the Wnt/β-catenin pathway, which plays a key role in the pathogenesis of vascular calcification. The specificity of IL-24 for the inhibition of VSMC calcification was confirmed by using a neutralizing antibody to IL-24. Our results suggest that IL-24 inhibits β-GP-induced VSMC calcification by inhibiting apoptosis, the expression of calcification and osteoblastic markers, and the Wnt/ β-catenin pathway. Our study may provide a novel mechanism of action of IL-24 in cardiovascular disease and indicates that IL-24 is a potential therapeutic agent in VSMC calcification.

1. Introduction

Vascular calcification is observed in a number of diseases, including atherosclerosis, diabetes, and end-stage renal disease [1]. It is characterized by decreased arterial wall elasticity and impeded blood flow and can result in heart attacks and stroke [1,2]. Vascular calcification has been recognized as a regulated process similar in many ways to bone mineralization [3]. Vascular cells such as vascular smooth muscle cells (VSMCs) and pericyte-like cells play a significant role in vascular calcification [4]. In VSMCs, high ambient inorganic phosphate levels induce apoptosis, which occurs before the onset of VSMC calcification [5]. In addition, inorganic phosphate induces the expression of calcification and osteoblastic markers, including bone morphogenetic protein-2 (BMP-2), runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP), collagen type I alpha 1 (COL1A1), osteopontin (OPN), matrix metalloproteinase-2 (MMP-2) in VSMCs [6]. Inorganic phosphate also regulates intracellular signaling pathways, such as the Wnt/β-catenin and Axl/growth arrest-specific gene 6 (Gas6) pathways [7,8], and subsequently leads to vascular calcification [6–8].

Interleukin-24 (IL-24), previously known as melanoma differentiation antigen 7 (mda-7), belongs to the IL-10 family of cytokines, which also includes IL-19, IL-20, IL-22, and IL-26 [9]. Binding of IL-24 to either of its two heterodimeric receptor complexes IL-20R1/IL-20R2 and IL-22R/IL-20R2 activates the JAK/STAT pathway [10]. Recent studies have shown that IL-24 exhibits anticancer activity, including cancer-specific induction of apoptosis, cell cycle regulation, and the inhibition of angiogenesis in a variety of cancer cells [11,12]. However, it has been reported that the cancer cell-specific killing activity of IL-24 is independent of the JAK/STAT pathway [13]. Although the functions of IL-24 in various cancers are the subject of active investigation, the role of IL-24 in other diseases has not been studied. Because cancers share elements of molecular pathogenesis with cardiovascular disease [14], we investigated the role of IL-24 in cardiovascular disease, particularly in VSMC calcification. Here, we have shown for the first time that IL-24 attenuates β-glycerophosphate (β-GP)-induced VSMC calcification by

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inhibiting apoptosis, the expression of calcification and osteoblastic markers, and the Wnt/β-catenin pathway.

2. Materials and methods

2.1. Cell culture and treatment

Rat VSMCs were isolated from the aortas of 4-week-old male Sprague–Dawley (SD) rats using the explant method [15] and were cultured in DMEM supplemented with 20% FBS, 100 units/ml of penicillin, and 100 mg/ml of streptomycin at 37 °C with 5% CO_2. VSMCs that had migrated from the explants were collected and maintained in the growth medium. The cells used in our experiments were not more than eight passages old. When confluent, the cells were pretreated with recombinant human interleukin-24 (rhIL-24, 50 ng/ml, R&D Systems) or rhIL-24 plus neutralizing anti-IL-24 (500 ng/ml, R&D Systems) in growth medium for 12 h. The treatment media were then replaced with DMEM containing 10% FBS and 3 mM β-glycerophosphate. The medium was replaced with fresh medium every 2–3 days for 21 days.

2.2. In vitro calcification of VSMCs

VSMC calcification was visualized by von Kossa staining. Each well was fixed with 2% paraformaldehyde and then washed with deionized water three times. Von Kossa staining was performed using the Von Kossa Method for Calcium Kit (Polysciences, Inc., Warrington, PA, USA) according to the manufacturer's instructions. Images were captured with an Olympus BX51 Microscope equipped with an Olympus DP11 digital camera.

2.3. Calcium content measurement

Calcium content was measured using the Calcium Colorimetric Assay Kit (Biovision, Mountain View, CA, USA) according to the manufacturer's instructions.

2.4. Apoptosis assay

Apoptosis was determined using the Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. The samples were analyzed by FACScalibur flow cytometry (BD Biosciences San Diego, CA, USA).

2.5. Real-time PCR

RNA isolation and quantitative real-time PCR were performed as described previously [16]. The primers used for quantitative real-time PCR are listed in Supplementary Table 1.

2.6. Western blot analysis

Preparation of cell homogenates and SDS–polyacrylamide gel electrophoresis were performed as described previously [16]. The membranes were incubated with antibodies to BMP-2, Runx2, ALP, Wnt1, Wnt7a, α-tubulin (Santa Cruz Biotechnologies, CA, USA), p-β-catenin, β-catenin, p-GSK3β, and GSK3β (Cell Signaling Technology, Beverly, MA, USA). The enhanced chemiluminescence (ECL) kit (Millipore, Billerica, MA, USA) and Lumino Image Analyzer, Las-3000 (Fujifilm, Tokyo, Japan) were used for detection.

2.7. Reporter gene assay

The mouse VSMC line MOVAS [17] was transfected in triplicate with the FOPflash or TOPflash TCF/LEF reporter plasmids (Upstate Biotechnology, NY, USA) together with a CMV-lacZ internal transfection control plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After transfection, the cells were pretreated with rhIL-24 (50 ng/ml) or rhIL-24 (50 ng/ml) plus anti-IL-24 (500 ng/ml) for 12 h and were then treated with 3 mM β-GP. After 24 h, the luciferase and β-galactosidase activities in cell lysates were determined luminometrically, and luciferase activity was normalized to β-galactosidase activity.

2.8. Statistical analysis

For the statistical analysis of data, *P* values were analyzed using a two-way ANOVA and post hoc tests. Results were expressed as mean±SEM and were considered statistically significant when *P* values were <0.05.

3. Results

3.1. IL-24 prevents β-GP-induced calcification of rat VSMCs

Vascular calcification is considered to be one of major risk factors for cardiovascular diseases [18]. β-GP promotes the calcification of VSMCs in vitro through the deposition of calcium in a manner similar to mineralization by osteoblasts [19]. In this study, rat VSMCs were mainly treated with 50 ng/ml of rhIL-24, which does not fall within the physiological range (1.6–4.8 pg/ml in rat serum in our data) (data not shown). In addition, it has been known that the concentration from 10 to 200 ng/ml of rhIL-24 is commonly used for treatment of cells [20,21]. In our preliminary study, 50 ng/ml of rhIL-24 treatment was most effective in the inhibition of VSMC calcification without cytotoxic effect (data not shown). Therefore, most experiments were performed at the concentration of 50 ng/ml of rhIL-24. To determine the effect of IL-24 on vascular calcification, von Kossa staining was performed in rat VSMCs after treatment with β-GP in the presence or absence of rhIL-24. β-GP-induced calcification of VSMCs compared with the control are shown in Fig. 1A, and rhIL-24 inhibited the β-GP-induced VSMC calcification. The inhibitory effect of rhIL-24 on β-GP-induced VSMC calcification was restored to the control IgG level by treatment with anti-IL-24. To further dissect whether IL-24 is capable of preventing calcium deposition, the calcium contents were measured by the o-cresolphthalein complexone method. Treatment with IL-24 or anti-IL-24 alone in the absence of β-GP did not affect the calcium contents of VSMCs (Fig. 1B). The calcium content was much higher in β-GP-treated VSMCs than in control IgG-treated VSMCs (10.1 ± 1.0 in control vs. 26.3 ± 6.2 in β-GP) (Fig. 1B). The β-GP-induced calcium content was remarkably reduced in a dose-dependent manner by treatment with rhIL-24, and this effect was also prevented by anti-IL-24. These results suggest that IL-24 may be capable of preventing the calcification of VSMCs.

3.2. IL-24 suppresses the β-GP-induced apoptosis of rat VSMCs

Inorganic phosphate is released when β-GP induces the apoptosis of VSMC and consequently leads to VSMC calcification, suggesting that apoptosis is a causative factor of VSMC calcification [22,23]. To corroborate the effect of IL-24 on the prevention of VSMC calcification, VSMC apoptosis was analyzed by flow cytometry using Annexin V/PI staining. When VSMCs were treated with β-GP, the apoptotic rate was increased by 1.85 ± 0.09-fold when compared with untreated control cells (Fig. 2A). The increased rate of apoptosis was significantly suppressed by treatment with rhIL-24 (1.85 ± 0.09 in β-GP vs. 1.33 ± 0.15 in rhIL-24), and the suppres-

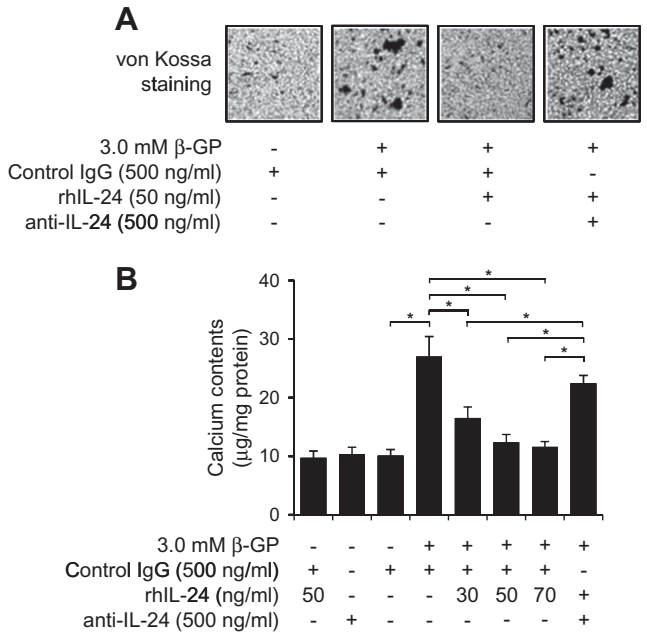

Fig. 1. rhIL-24 prevented β-GP-induced VSMC calcification. Rat VSMCs were treated with 3 mM β-GP in the presence or absence of rhIL-24 and/or anti-IL-24 as indicated in Fig. 1 for 3 weeks with media changes every 3 days. (A) VSMC calcification was determined by von Kossa staining. (B) Calcium content was measured by the *o*-cresolphthalein complexone method and was normalized to the cellular protein concentration. The data shown are representative of the results from three independent experiments, each performed in duplicate. $^{*}P < 0.05$ compared with the individual controls.

sive effect of rhIL-24 was abrogated by anti-IL-24 (1.33 ± 0.15 in rhIL-24 vs. 2.23 ± 0.20 in anti-IL-24). Because apoptosis is regulated by the expression ratio of Bax proapoptotic to Bcl-2 antiapoptotic factors and the expression of activated caspase-3 [24], the effect of rhIL-24 on the expression ratio of Bax/Bcl-2 mRNAs was examined in β-GP-treated VSMC using real-time PCR. The mRNA expression ratio of Bax/Bcl-2 was increased 3.1-fold in β-GP-treated VSMC as compared to the untreated control (1.0 ± 0.02 in untreated control vs. 3.1 ± 0.23 in β-GP), and this effect was prevented by rhIL-24 treatment (3.1 ± 0.23 in β-GP vs. 1.3 ± 0.05 in rhIL-24) (Fig. 2B). The reduction of the Bax/Bcl-2 mRNA ratio was reduced 2-fold by anti-IL-24 (1.3 ± 0.05 in rhIL-24 vs. 2.5 ± 0.42 in anti-IL-24). Furthermore, the mRNA expression of caspase-3 was remarkably upregulated in β-GP-treated VSMCs (1.0 ± 0.07 in the untreated control vs. 1.8 ± 0.11 in β-GP), whereas it was downregulated by rhIL-24 treatment (1.8 ± 0.11 in β-GP vs. 1.0 ± 0.09 in rhIL-24) (Fig. 2C). Anti-IL-24 also abrogated the downregulating effects of rhIL-24 on caspase-3 mRNA expression (1.0 ± 0.09 in rhIL-24 vs. 1.4 ± 0.11 in anti-IL-24). These data suggest that IL-24 exerts anti-apoptotic effects on β-GP-induced VSMC apoptosis, at least in part through the regulation of the Bax/Bcl2 ratio and caspase-3 expression.

3.3. IL-24 inhibits the β-GP-induced gene expression of calcification and osteoblast markers

VSMC calcification is associated with an increased expression of calcification and osteoblast markers, including BMP-2, Runx2, ALP, OCN, OPN, MMP-2, and COLIA1 [6,25]. In the present study, the mRNA expression of BMP-2, Runx2, ALP, OPN, MMP-2, and COL1A1 was substantially increased in β-GP-treated calcified VSMCs as compared to the uncalcified control (Fig. 3A). The increase in the mRNA levels of BMP-2, Runx2, ALP, OPN, MMP-2, and COL1A1 were inhibited by rhIL-24, and this effect was neutralized by anti-IL-24 treatment. Moreover, the effects of rhIL-24 on BMP-2, Runx2, and ALP protein expression in β-GP-calcified VSMCs, as determined by western blot analysis, were consistent with the mRNA expression data (Fig. 3B). These data suggest that IL-24 is capable of inhibiting β-GP-induced vascular calcification by suppressing the expression of calcification and osteoblast markers.

3.4. IL-24 inhibits the Wnt/β-catenin pathway in β-GP-induced, calcified VSMCs

The Wnt/β-catenin pathway plays an important role in the pathogenesis and progression of atherosclerosis and cancer [14]. The Wnt/β-catenin pathway causes vascular calcification by stimulating the expression of calcification and osteoblast markers [26]. We found that the protein expression of the Wnt ligands Wnt1 and Wnt7a was upregulated by the treatment of VSMCs with β-GP (Fig. 4A and B). The β-GP-induced upregulation of Wnt1 and Wnt7a proteins was suppressed by rhIL-24, and this effect was neutralized by anti-IL-24. Activation of the Wnt pathway prevents the degradation of cytoplasmic β-catenin due to the inhibition of GSK-3β-mediated phosphorylation of β-catenin, which allows cytoplasmic β-catenin to translocate into the nucleus [23]. In this study, β-GP-induced GSK-3β phosphorylation resulted in reduced β-catenin phosphorylation and consequently increased cytoplasmic β-catenin expression (Fig. 4C). The rhIL-24 inhibited the effects of β-GP on the phosphorylation of GSK-3β and β-catenin and cytoplasmic β-catenin expression, and this effect was neutralized by treatment with anti-IL-24 (Fig. 4D). β-Catenin that is translocated

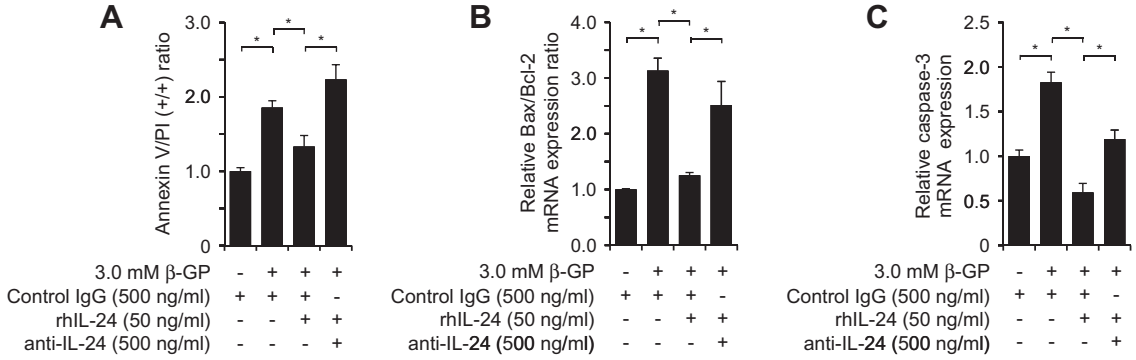

Fig. 2. rhIL-24 suppressed β-GP-induced VSMC apoptosis. Rat VSMCs were treated with 3 mM β-GP in the presence or absence of rhIL-24 (50 ng/ml) and/or anti-IL-24 (500 ng/ml) for 3 days. (A) The relative ratio of the apoptotic cells (Annexin V positive/PI positive) was determined by flow cytometric analysis. The relative mRNA expression ratio of Bax to Bcl2. (B) The mRNA expression level of caspase-3, and (C) were determined by real-time PCR. Representative results from three independent experiments are shown. $^{*}P < 0.05$ compared with the individual controls.

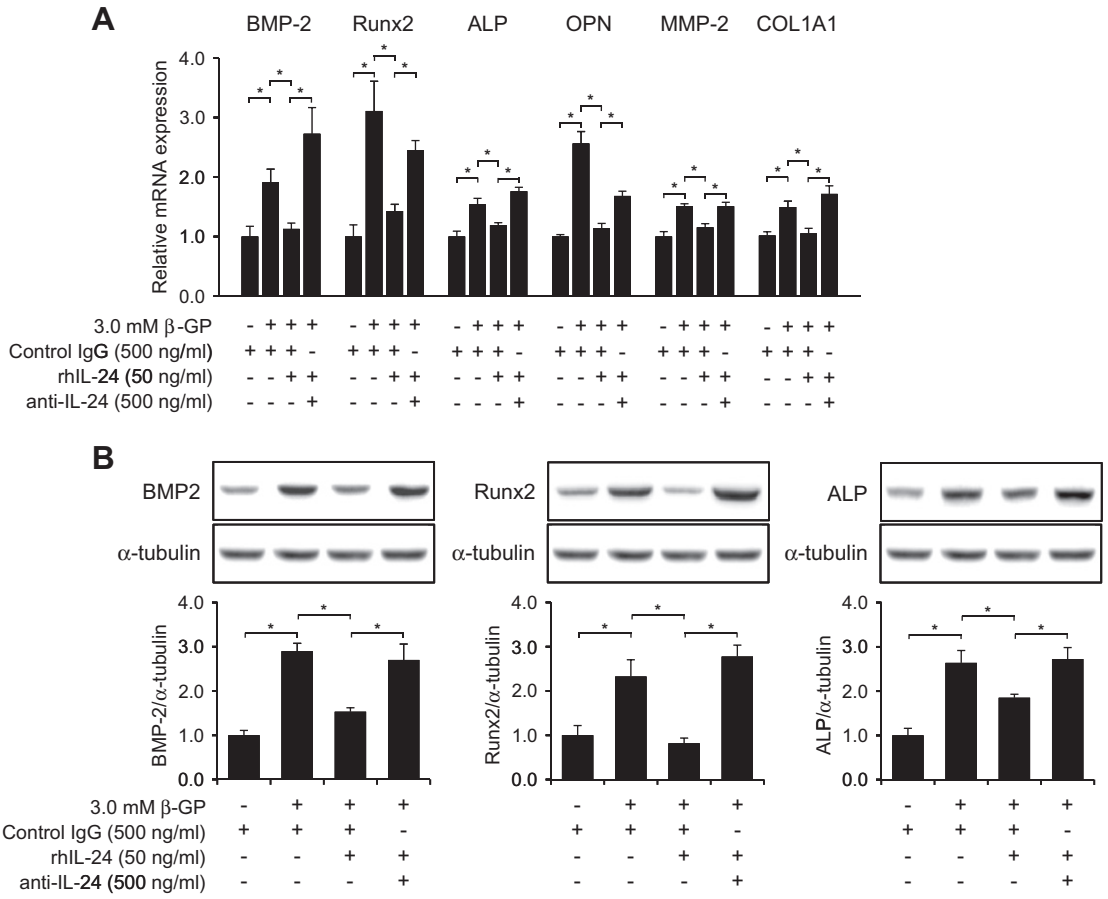

Fig. 3. rhIL-24 inhibited β-GP-induced expression of calcification and osteoblastic markers. Rat VSMCs were treated with 3 mM β-GP in the presence or absence of rhIL-24 (50 ng/ml) and/or anti-IL-24 (500 ng/ml) for 3 weeks with media changes every 3 days. (A) The relative mRNA expression levels of BMP-2, Runx2, ALP, OPN, MMP-2, and COL1A1 were determined by real-time PCR as described in Materials and Methods. (B) Protein expression of BMP-2, Runx2, and ALP was determined by western blot analysis using specific antibodies as indicated in Fig. 3B (upper panel). The intensity of individual protein bands obtained from western blot was quantified by densitometry. α-tubulin was used as an internal loading control (bottom panel). Representative results from three independent experiments are shown. $^{*}P < 0.05$ compared with the individual controls.

to the nucleus interacts with T-cell factor (TCF)/lymphoid enhancer-binding factor (LEF), which activates the transcription of downstream target genes [23]. We assessed the effect of IL-24 on the β-GP-induced transcriptional activity of TCF/LEF by using two luciferase reporter plasmids, TOPflash and FOPflash, in the mouse VSMC line MOVAS, which is used as an in vitro cell line model of vascular calcification [17]. The activity of TOPflash was increased in β-GP-treated VSMCs as compared to the control (Fig. 4E). The rhIL-24 markedly decreased the activity of TOPflash with no significant effect on the activity of FOPflash, and this effect was neutralized by anti-IL-24. These data suggest that IL-24 reduces VSMC calcification by inhibiting the Wnt/β-catenin pathway.

4. Discussion

Many researchers have investigated the molecular mechanism of the cancer cell-specific killing activity of IL-24 in various cancer cells. However, the role of IL-24 in diseases other than cancer has been studied less often. We found that the IL-24 mRNA expression level was lower in the hearts of spontaneously hypertensive rats than in those of Wistar-Kyoto rats, and the addition of IL-24 to VSMCs reduced the expression of genes associated with cardiovascular disease that were elevated by treatment with reactive oxygen species (unpublished data). For these reasons, we examined the role of IL-24 in the pathophysiology of cardiovascular disease, particularly in vascular calcification. Vascular calcification is often detected in patients with osteoporosis, atherosclerosis, and chronic kidney disease and is highly correlated with cardiovascular morbidity and mortality [1]. There are several factors, including high phosphate levels, bone morphogenetic proteins (BMPs), oxidative stress, and vitamin D, that are related to the induction of vascular calcification [5].

In this study, we investigated the role of IL-24 in a calcification model using β-GP as a phosphate source in rat VSMCs. In von Kossa staining and calcium content analysis, we found increased VSMC calcification in β-GP-treated cells as compared to untreated cells. These results were consistent with the finding that β-GP promotes VSMC calcification [19]. Moreover, we found that IL-24 treatment attenuates the β-GP-induced VSMC calcification, and we confirmed the specificity of this effect with a neutralizing antibody to IL-24, suggesting that IL-24 may suppress β-GP-induced VSMC calcification by inhibiting calcium deposition.

Some authors have reported that VSMCs undergo apoptosis in high phosphate-induced calcification [19,24], and the correlation between VSMC calcification and apoptosis was supported by the finding that both calcification and apoptosis were suppressed by a caspase inhibitor [22]. To confirm the inhibitory effect of IL-24 on calcification, the effect of IL-24 on the apoptosis of β-GP-treated VSMCs was examined. We found that β-GP augmented VSMC apoptosis and that IL-24 inhibited the β-GP-induced apoptosis by decreasing the Bax/Bcl-2 expression ratio, suggesting that the inhibitory effect of IL-24 on VSMC calcification is mediated, at least

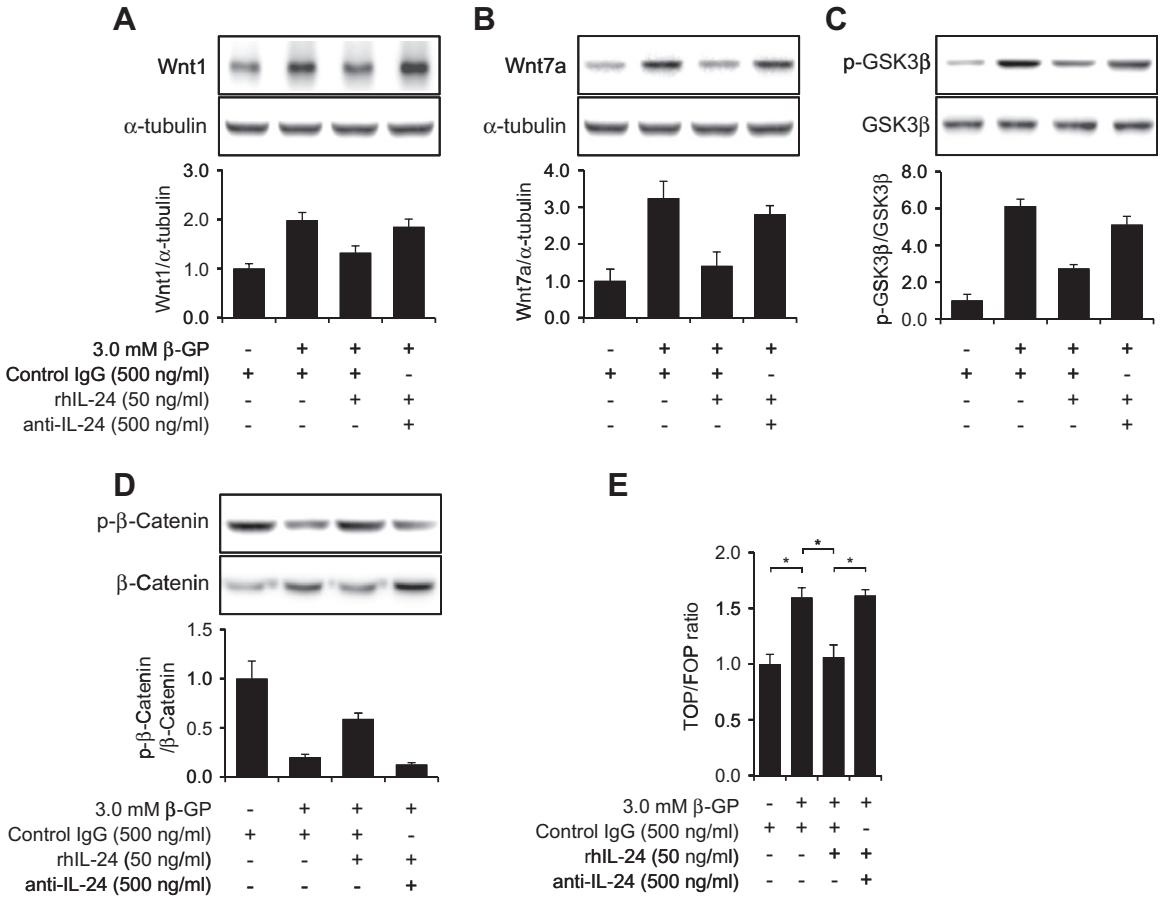

Fig. 4. rhIL-24 attenuated the β-GP-induced activation of the Wnt/β-catenin pathway. Rat VSMCs were treated with 3 mM β-GP in the presence or absence of rhIL-24 (50 ng/ml) and/or anti-IL-24 (500 ng/ml) for 3 weeks with media changes every 3 days. (A)–(D) Western blot analysis of Wnt1, Wnt7a, phosphorylated GSK3β (GSK3β), and phosphorylated β-catenin (β-catenin) was performed in rat VSMCs (upper panel). The intensity of individual protein bands obtained from western blot was quantified by densitometry (bottom panel). (E) MOVAS cells were transfected with TOPflash or FOPflash TCF/LEF reporter constructs together with a β-galactosidase construct for the normalization of transfection efficiency, and were then treated as indicated in Fig. 4E. The data are representative of four independent experiments. $^{*}P < 0.05$ compared with the individual controls.

in part, through its antiapoptotic activity. Interestingly, these effects of IL-24 were similar to those of statins, which are hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, on VSMC calcification [27,28].

Several studies have reported finding calcification and osteoblastic markers, including BMP-2, Runx2, ALP, OPN, MMP-2, and COL1A1, in calcified vessels and valves [29,30]. BMP-2, a member of the transforming growth factor-β superfamily [31], is expressed and secreted from various cell types, including vascular smooth muscle cells, endothelial cells, and osteoblasts [32–34], and it promotes the calcification of adjacent cells in a paracrine manner [35]. BMP-2 induces vascular calcification and accelerates phosphate uptake [36], and its downstream effects are achieved by upregulating Runx2 and ALP expression [5,23,26]. Runx2, a transcription factor, controls the expression of calcification and osteoblastic markers, including ALP, OPN, MMP-2, and COL1A1 [6,24]. ALP uses β-GP as a substrate to release inorganic phosphate and raise the local concentration of phosphorus, which promotes apoptosis and VSMC calcification [23]. We examined whether the expression of these markers was increased in β-GP-treated VSMCs and was regulated by IL-24. We found that β-GP increased the expression of these molecules in VSMCs and that this effect was specifically reduced by IL-24 treatment, suggesting that IL-24 may suppress the expression of downstream molecules by inhibiting BMP-2 expression.

The Wnt/β-catenin pathway is a common signaling pathway in the pathogenesis of cardiovascular disease and cancer [14]. In vascular calcification, the Wnt/β-catenin pathway provokes the expression of calcification and osteoblastic markers through the upregulation of Runx2 transcription [26]. IL-24 induces the apoptosis of pancreatic cancer cells through the downregulation of the Wnt/β-catenin pathway [12]. Therefore, we examined whether the Wnt/β-catenin pathway is regulated by IL-24 in β-GP-induced, calcified VSMCs. Our results showed that β-GP increases the expression of Wnt1 and Wnt7a, favors the accumulation of cytosolic β-catenin by downregulating the phosphorylation activity of GSK3β, and stimulates TCF/LEF transcriptional activity. Furthermore, IL-24 inhibited the activation of the Wnt/β-catenin pathway by β-GP, indicating that the inhibitory effect of IL-24 on VSMC calcification correlates with the inactivation of the Wnt/β-catenin pathway. Although we did not explore the influence of the JAK/STAT pathway on the inhibition of β-GP-induced VSMC calcification by IL-24, the specificity of the effect of IL-24 on the Wnt/β-catenin pathway was confirmed with a neutralizing antibody to IL-24.

In summary, we have shown for the first time that IL-24 inhibits β-GP-induced VSMC calcification and that this inhibition correlated with the suppression of apoptosis, the expression of calcification and osteoblast markers through the downregulation of BMP-2 expression, and the Wnt/β-catenin pathway. The present study

may help elucidate the role of IL-24 in the pathophysiology of vascular calcification.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.145.

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