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Full paper

Matrix Gla protein negatively regulates calcification of human aortic valve interstitial cells isolated from calcified aortic valves

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ABSTRACT

Calcified aortic valve stenosis (CAS) is a common heart valve disease in elderly people, and is mostly accompanied by ectopic valve calcification. We recently demonstrated that tumor necrosis factor-α (TNF-α) induces calcification of human aortic valve interstitial cells (HAVICs) obtained from CAS patients. In this study, we investigated the role of matrix Gla protein (MGP), a known calcification inhibitor that antagonizes bone morphogenetic protein 2 (BMP2) in TNF-α-induced calcification of HAVICs. HAVICs isolated from aortic valves were cultured, and calcification was significantly induced with 30 ng/mL TNF-α. Gene expression of the calcigenic marker, BMP2, was significantly increased in response to TNF-α, while the gene and protein expression of MGP was strongly decreased. To confirm the role of MGP, MGP-knockdown HAVICs and HAVICs overexpressing MGP were generated. In HAVICs, in which MGP expression was inhibited by small interfering RNA, calcification and BMP2 gene expression were induced following long-term culture for 32 days in the absence of TNF-α. In contrast, HAVICs overexpressing MGP had significantly decreased TNF-α-induced calcification. These results suggest that MGP acts as a negative regulator of HAVIC calcification, and as such, may be helpful in the development of new therapies for ectopic calcification of the aortic valve.

1. Introduction

Aortic valve stenosis (AS) is one of the most common heart valve conditions in elderly people.[1] The main etiologies of AS are rheumatic changes, congenital bicuspid valve, and degenerative calcification.[2] As the country's population has shifted towards older ages, calcific aortic valve stenosis (CAS) has become the most common cause of AS instead of rheumatic changes.[3,4] Aortic valves from patients with CAS are characterized by extensive fibrotic thickening of the valve leaflets and focal ectopic calcification.[5] Aortic valve calcification is irreversible. The most effective treatment is surgical aortic valve replacement; however, this treatment is invasive.[6]

Recently, transcatheter aortic valve replacement (TAVR) has become popular as a less invasive treatment for AS patients.[7] In combination with TAVR, to delay the progression of CAS, development of a medication-based treatment is important. It is necessary to establish the detailed mechanism underlying aortic valve calcification. Although several groups have focused on identifying this mechanism, it has not yet been established.[8–10] CAS is an active process that appears to correlate with several inflammatory factors.[11] Inflammation is a prominent feature of aortic valve calcification, and may develop due to endothelial dysfunction fueled by atherosclerotic risk factors.[12,13] Macrophages and T lymphocytes have been identified in calcified aortic valve lesions.[14,15] These immune cells release cytokines, including tumor necrosis factor-α (TNF-α),[16] transforming growth factor-β_1,[17] and interleukin-1,[18] all of which contribute to extracellular matrix formation, remodeling, and local calcification.

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TNF-α is a pleiotropic cytokine that is mainly secreted by activated macrophages and T lymphocytes in response to many factors including exposure to oxidized low-density lipoprotein,[19] damaged extracellular matrix,[20] or bacterial infection.[21] TNF-α has been detected in the atherosclerotic lesions and calcified aortic valves of both humans and mice, and plays an important role in ectopic calcification of the aortic valve.[16,22] Our recent study reported that TNF-α promotes osteogenic differentiation of human aortic valve interstitial cells (HAVICs) obtained from CAS patients by stimulating the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway. NF-κB activates the gene expression and activity of bone morphogenetic protein 2 (BMP2), which induces the gene expression of SMAD4, Runt-related gene 2 (Runx2), and Distal-less homeodomain 5 (Dlx5) and alkaline phosphatase (ALP) activation.[23] However, inhibition of the signaling pathway upstream of BMP2 only partially inhibits TNF-α-induced calcification of HAVICs. Consequently, we hypothesized that another pathway for inducing HAVIC calcification may exist.

Matrix Gla protein (MGP), a γ-carboxyglutamic acid-rich and vitamin K-dependent protein, is a well-known ectopic calcification inhibitor.[24] Mice born lacking the MGP gene have extensive calcification of the large elastic and muscular arteries and heart valves, and die within 2 months from arterial rupture and heart failure.[25] Recently, Yao et al. reported that MGP inhibits BMP2 and protects against atherosclerosis and vascular calcification.[26] These reports suggest that MGP may be a naturally occurring inhibitor of ectopic cardiovascular calcification. However, there have been no reports on the interaction between TNF-α and MGP.

In this study, we investigated and confirmed the role of MGP in TNF-α-induced calcification of HAVICs. Our results showed that TNF-α substantially downregulated MGP gene expression in HAVICs. Investigating the role of MGP using MGP knockdown HAVICS and HAVICs overexpressing MGP, which were obtained from CAS patients, revealed that MGP is a negative regulator of aortic valve calcification.

2. Materials and methods

2.1. Materials

Alpha Modified Eagle's Medium (α-MEM) was obtained from Nacalai Tesque (Kyoto, Japan). Fetal bovine serum (FBS, Biofluids™) and penicillin and streptomycin (Gibco™; Gaithersburg, MD, USA) were obtained from Invitrogen (Carlsbad, CA, USA). TNF-α and other analytical grade reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan). All primers used for quantitative real-time polymerase chain reaction (qPCR) were obtained from Fasmac (Kanagawa, Japan). Power SYBR® Green PCR Master Mix was supplied by TOYOBO (Osaka, Japan). Control small interfering RNA (siRNA) and MGP siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All chemicals used were of the highest purity commercially available. All solutions were freshly made at sufficiently high concentrations that only very small volumes had to be added to the culture medium. Primary mouse antibodies against human MGP and β-actin, and a secondary goat anti-mouse IgG antibody conjugated to Alexa Fluor 680, were purchased from Invitrogen.

2.2. Isolation and culture of HAVICs

Human aortic valves were obtained from patients with calcific aortic valve stenosis (CAS group; mean age 64.3 ± 5.8 years, n = 6) and patients with aortic dissection or aortic regurgitation (Non-CAS group; mean age 66.0 ± 10.0 years, n = 4) who underwent aortic valve replacement at Hirosaki University Hospital (Aomori, Japan). There were no statistically significant differences in clinical features associated with CAS between these two groups (data not shown). All patients gave written informed consent, and the study was approved by the Institutional Review Board of the Hirosaki University Hospital. Human aortic valve specimens were gently cut into 2 ± 1 mm long pieces, and HAVICs were isolated by collagenase digestion as previously described.[23,27] The cells were cultured in α-MEM containing 10% FBS, and the fourth passage of cells was used in all experiments. After the HAVICs reached confluency they were further cultured in the presence or absence of 30 ng/mL TNF-α for 7 or 14 days, with the medium changed every 3–4 days.

2.3. Identification of calcification

Cells were seeded in a 12- or 96-well plate and grown for 3 d until confluent. Then they were further cultured with or without 30 ng/mL TNF-α for 7, 12, 14, 20, or 32 day. The degree of calcification was quantified using Alizarin Red S staining[28] visualized with a digital camera (Nikon, Tokyo, Japan). Alizarin Red S dye released from the extracellular matrix following incubation of the cells in 100 mM aqueous cetyl-pyridinium chloride solution was quantified by spectrophotometry at 550 nm.[29]

2.4. Measurement of gene expression

Total RNA was isolated from cells using a QuickGene RNA cultured cell kit S (Fujifilm, Tokyo, Japan). An aliquot of total RNA was reverse transcribed to cDNA using random primers. For qPCR, the cDNA was amplified (ABI PRISM 7000, Life Technologies, Carlsbad, CA, USA) for 40 PCR cycles (95 °C for 15 s and 60 °C for 1 min) after initial denaturation at 95 °C for 1 min. The reaction volume contained 3 μL of a 1:4 dilution of the first–strand reaction product, 0.6 μL of 10 μM specific forward and reverse primers, 0.4 μL of 50 × ROX reference dye, 5.4 μL pure water, and 10 μL SYBR qPCR, and was adjusted to a final volume of 20 μL. The primers used for MGP, BMP2, osteocalcin, and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) were designed using NCBI Primer BLAST (The National Center for Biotechnology Information, Bethesda, MD, USA) and their sequences are shown in Table 1. Amplification of the housekeeping gene G3PDH served as the normalization standard. The qPCR data are represented as cycle threshold (Ct) levels and were normalized using the individual G3PDH control Ct values. Relative gene expression was calculated using the 2(-delta delta Ct) method.[30]

2.5. Western blot analysis of MGP

Cytoplasmic extracts were obtained by lysing the cells in 20 mM Tris–HCl, pH 7.4, containing 0.05% Triton X-100. After a

Table 1
Primers used for quantitative real-time PCR.

Gene symbol	Genbank Accession no.	Sequences (5′–3′)
BMP2	NM_001200	Forward: cggactgcggtctcctaa Reverse: ggaagcagcaacgctagaag
MGP	NM_000900	Forward: tcacatgaaagcatggaatctta Reverse: acaggcttagagcgttctcg
Osteocalcin	NM_199173	Forward: tgagagccctcacactcctc Reverse: acctttgctggactctgcac
G3PDH	NM_002046	Forward: tgcaccaccaactgcttagc Reverse: ggcatggactgtggtcatgag

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