Journal of Surgical Research **145,** 142–149 (2008)
doi:10.1016/j.jss.2007.03.077

Endothelial Bmp4 Is Induced During Arterial Remodeling: Effects on Smooth Muscle Cell Migration and Proliferation

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Submitted for publication January 30, 2007

Background. **Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-beta superfamily of proteins that have multiple functional roles in mammalian development. A role for BMP4 in adult vascular remodeling has recently been suggested. We evaluated the expression of Bmp4 during neointimal lesion development *in vivo*.**

Materials and methods. **Heterozygous *Bmp4*$^{lacZ/+}$ mice were used to evaluate *in vivo Bmp4* expression after carotid ligation. β-galactosidase (β-gal) activity was evaluated in histological sections 1 to 14 d after carotid ligation and this was compared with control carotid arteries. The effects of recombinant human (rh) BMP4 on smooth muscle cell (SMC) migration and proliferation were evaluated using a rat aortic SMC line. We next assessed the effects of BMP4 signaling by over-expressing a constitutively active BMP receptor (BMPR-IA/Alk-3) using adenovirus-mediated gene transfer. SMC proliferation, migration, and apoptosis were evaluated in adenovirus transfected cells.**

Results. **Ligated carotid arteries expressed endothelium-specific β-gal staining after 1 d. Staining intensity increased at both 3 d and 1 wk after ligation and remained stable at 2 weeks while no β-gal staining was observed in control vessels. Endothelial-specific expression of β-galactosidase was confirmed through positive staining for PECAM-1. When human recombinant BMP4 was added to cultured SMCs, it inhibited migration but did not affect cultured SMC proliferation. SMCs infected with adenovirus encoding for the active BMP receptor Alk-3 demonstrated dose-dependent receptor expression. Alk-3 over-expressing cells showed a dose-dependent decrease in proliferation and migration but no effect on apoptosis.**

Conclusions. **These results demonstrate that endothelial *Bmp4* expression is upregulated after carotid ligation *in vivo*, and furthermore, that activating the BMP signaling cascade results in decreased SMC proliferation and migration. This suggests that BMPs may counterbalance the effect of mitogen up-regulation observed during the development of neointimal hyperplasia.**

Key Words: **bone morphogenetic proteins; BMPs; shear stress; endothelial cell; smooth muscle cell; proliferation; migration; arterial remodeling; mouse.**

INTRODUCTION

The endothelium of actively remodeling arteries is known to secrete factors that control medial smooth muscle cell (SMC) function [1]. The inward remodeling and neointimal development seen in diseased arteries is mediated by numerous factors that may stimulate or inhibit vascular smooth muscle cell migration and proliferation. One family of proteins thought to play an important role of vascular development and remodeling is the transforming growth factor-beta (TGF-β) superfamily of signaling molecules. The bone morphogenetic proteins (BMPs) are the largest subgroup of the TGF-β protein superfamily, and over 20 BMP subfamily members have been identified in human and mouse [2]. Although they were named for their ability to induce ectopic cartilage and bone formation *in vivo* [3], these proteins have subsequently been shown to play key regulatory roles in a diverse set of developmental processes [4]. In embryonic cardiovascular development, BMPs are a key element in the induction of cardiac myogenesis [5]. BMP2 and BMP6 have been

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0022-4804/08 $34.00

detected in adult human atherosclerotic arteries and are implicated in pathologic arterial calcification [6, 7]. BMPs act primarily through binding with two types of transmembrane tyrosine kinase receptors that are classified as Type 1 (Alk-2, -3, and -6) and Type 2 (BMPR-II, ActR-IIA, and ActR-IIB). Activation of BMP receptors initiates phosphorylation of downstream signaling molecules known as receptor-regulated Smads [8]. BMP signaling is tightly controlled by extracellular inhibitory molecules including noggin, and through a series of positive and negative intracellular cofactors including the inhibitory Smads 6 and 7 [9].

BMP4 is one of the regulatory molecules in the TGF-β/BMP family. It functions at multiple stages of mammalian development and adult tissue remodeling including mesoderm induction, cartilage and bone induction, limb growth and differentiation, and fracture repair [10, 11]. Recently, cultured endothelial cells have been shown to express BMP4 in response to both cytokines and oscillatory shear stress [12]. Additionally, flow alterations in PTFE arterial grafts result in increased BMP4 mRNA levels that are accompanied by decreased expression of the extracellular BMP inhibitor noggin [13]. In this study, we evaluated the *in vivo* expression of BMP4 in mice after carotid ligation. We then evaluated the effects of BMP signaling on SMC proliferation, migration, and apoptosis using adenovirus-mediated delivery of a constitutively active BMP receptor. Our results suggest that BMP4 may counterbalance the effects of mitogens and chemoattractants that are activated during neointimal lesion formation.

MATERIALS AND METHODS

Animals

Bmp4^{lacZneo} heterozygous mice were generated and maintained as previously described [14]. The *neo*^{r} cassette was subsequently removed by crossing α-*actin-Cre* transgenic mice with *Bmp4*^{lacZneo} heterozygous mice, and *Bmp4*^{lacZ} heterozygous mice were backcrossed with outbred ICR mice to maintain the line.

Carotid Ligation

A total of 30 male *Bmp4*$^{lacZ/+}$ mice weighing 20 to 25 grams underwent unilateral left carotid ligation as previously described and under protocols approved by the Vanderbilt Animal Care and Use Committee [15]. General anesthesia was administered using ketamine 50-mg/kg and xylazine 10-mg/kg administered by intramuscular injection. Using sterile technique, a midline cervical incision was made and the left carotid bifurcation was exposed. The distal common carotid artery was ligated using 6-0 silk suture. The incision was then closed and animals were allowed to recover in a warm and dry environment. At time 0 (control) and at 1, 3, 7, and 14 d after carotid ligation, animals were euthanized by ketamine and xylazine overdose. Perfusion fixation was then carried out via cardiac puncture using fresh 4% paraformaldehyde in PBS. The right and left carotid arteries were dissected and harvested with care to avoid intimal damage. All protocols complied with the *Guide for the Care and Use of Laboratory Animals* (Institute of Animal Laboratory Resources, Commission on Life Sciences, National Research Council, Washington: National Academy Press, 1996).

Identification of β-Gal Expression in Carotid Specimens

After harvesting, carotid specimens were further fixed for 1 h in 4% paraformaldehyde and stained using X-gal solution as previously described [16, 17]. Gross specimen photography was performed using a Zeiss Axiophot microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY). Carotid vessels with a granular blue staining pattern were counted as positive, while specimens with only mild, nongranular, diffuse blue staining or no staining were counted as negative for β-gal expression. Histological examination was conducted using paraffin-embedded 5 μm sections that were counter-stained with eosin for evaluation of X-gal staining.

Immunohistochemistry

After noting the intimal localization of β-gal positive cells, an additional three mice underwent carotid ligation and harvest at the 3 d post-ligation time point. Carotids were then fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, and cut into 5-μm sections. Immunostaining was then performed using an antibody to the endothelial cell specific molecule PECAM-1 (BD PharMingen, San Diego, CA) and a secondary antibody containing horseradish peroxidase (HRP). HRP positive cells were then identified by colorimetric methods with a Vectastain kit, (Vector Laboratories, Burlingame, CA).

Adenoviral Transfection of SMCs

Embryonic rat aortic SMC (A-10 cells; ATCC, Manassas, VA) were grown to 80% confluence then made quiescent by incubation in medium (DMEM) containing 0.5% fetal bovine serum (FBS) for 24 h. Cells were then exposed to various multiplicities of infection of a replication defective adenovirus encoding for Alk-3, a constitutively active form of the BMP receptor type 1A (BMPR-IA), tagged with hemagglutinin (HA) to allow for confirmation of protein expression (generously provided by M. Fujii, T. Imamura, and K. Miyazono), or Ad.LacZ as a control [18]. High-titered stocks of recombinant viruses were grown in 293 cells and purified. Infection of cells using recombinant adenoviruses was performed at a multiplicity of infection up to 500 plaque forming units (pfu)/cell. After 24 more hours, cells were detached using trypsin, counted, and used for migration or proliferation studies as described below.

Confirmation of transgene expression was confirmed by immunofluorescence staining and Western blotting. Briefly, 24 h after transfection with Alk-3 or control adenovirus, cells in chamber slides were washed, fixed with ethanol, and incubated with rabbit anti-HA-antibody (Novus Biologicals, Inc., Littleton, CO) after appropriate blocking steps. Cells were then visualized by fluorescence microscopy after incubating in secondary FITC-labeled antibody and counterstaining with Hoechst nuclear stain. Transgene expression was also evaluated by Western blotting. Using mouse anti-HA.11 antibody (Covance Research Products, Inc., Berkeley, CA), Western blots were performed on cell lysates of adenovirus-infected and control cells.

SMC Proliferation

Rat A-10 cells were grown in 24-well culture plates to 60% confluence and then made quiescent by incubation in medium containing 0.1% FBS. For experiments using recombinant human BMP4 (rhBMP4) (R&D Systems, Minneapolis, MN), cells were then incubated in DMEM containing 10% serum and rhBMP4 at 0, 50, or 200 μg/mL. ^{3}H-thymidine incorporation was measured 24 h later. For adenovirus transfection experiments, cells were transfected with Alk-3 adenovirus or control adenovirus at various concentrations. After 24 h, cells were placed back into growth medium containing 10% FBS. Cells were then incubated with 1 μCi/mL for 24 h and