Bone Morphogenic Protein 4 Produced in Endothelial Cells by Oscillatory Shear Stress Stimulates an Inflammatory Response*

Atherosclerosis is now viewed as an inflammatory disease occurring preferentially in arterial regions exposed to disturbed flow conditions, including oscillatory shear stress (OS), in branched arteries. In contrast, the arterial regions exposed to laminar shear (LS) are relatively lesion-free. The mechanisms underlying the opposite effects of OS and LS on the inflammatory and atherogenic processes are not clearly understood. Here, through DNA microarrays, protein expression, and functional studies, we identify bone morphogenetic protein 4 (BMP4) as a mechanosensitive and pro-inflammatory gene product. Exposing endothelial cells to OS increased BMP4 protein expression, whereas LS decreased it. In addition, we found BMP4 expression only in the selective patches of endothelial cells overlying foam cell lesions in human coronary arteries. The same endothelial patches also expressed higher levels of intercellular adhesion molecule-1 (ICAM-1) protein compared with those of non-diseased areas. Functionally, we show that OS and BMP4 induced ICAM-1 expression and monocyte adhesion by a NFκB-dependent mechanism. We suggest that BMP4 is a mechanosensitive, inflammatory factor playing a critical role in early steps of atherogenesis in the lesion-prone areas.

Endothelial cells are constantly exposed to shear stress (a dragging force generated by blood flow), which controls cellular structure and function such as regulation of vascular tone and diameter, vessel wall remodeling, hemostasis, and inflammatory responses (1). The importance of various types of shear stress is highlighted by the focal development of atherosclerosis (2). Atherosclerosis is now known as an inflammatory disease caused by endothelial dysfunction (3, 4). One of the first visible markers of endothelial dysfunction in the lesion-prone areas is up-regulation of inflammatory adhesion molecules such as E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and ICAM-1 (3–6). These endothelial adhesion molecules play essential roles in adhesion and recruitment of monocytes to the subendothelial layer (3, 4).

How do unstable shear conditions such as low and oscillating shear stress (OS) cause inflammation in those lesion-prone areas, whereas LS exerts athero-protective effects? The opposite effects of LS and OS may be determined by differential expression of genes and proteins, ultimately inducing anti- and pro-inflammatory and atherogenic responses. Recently, several studies (7–10) have begun to address the initial question to determine the expression profiles of mechanosensitive genes. However, the functional importance of those genes has not been clearly established.

Here, we report identification of a mechanosensitive gene, BMP4, by DNA microarray analyses and subsequent verification by a variety of additional approaches in both cultured endothelial cells and human coronary arteries. More importantly, we discovered a novel role of BMP4 as an inflammatory cytokine, providing a potential mechanistic link from shear forces to inflammatory responses and atherogenesis.

MATERIALS AND METHODS

Endothelial Cells—Mouse aortic endothelial cells (MAEC) were cultured and used at passages 4–8 as described by us (11). Human aortic endothelial cells (HAEC) purchased from Clonetics were cultured using the EGM-2 bullet kit (Clonetics) and used at passages 4–8.

Shear Stress Studies—Confluent endothelial monolayers grown in 100-mm tissue culture dishes were exposed to an arterial level of unidirectional LS (5 dyn/cm²) in the growth medium by rotating a Teflon cone (0.5° cone angle) as described previously by us (12). To mimic unstable shear conditions in vivo, endothelial cells were exposed to OS with directional changes of flow at 1 Hz cycle (± 5 dyn/cm²) by rotating the cone back and forth using a stepping motor (Servo Motor) and a computer program (DC Motor Company, Atlanta, GA). In some studies, 5 dyn/cm² unidirectional LS was used for comparison to OS (± 5 dyn/cm²).

Preparation of Cell Lysates and Immunoblotting—Following experimental treatments, endothelial cell lysates were prepared and analyzed by Western blot analysis as described by us (13, 14). Briefly, cells were washed in ice-cold phosphate-buffered saline and lysed in 0.1 ml of boiling lysis buffer A (10 mM Tris-HCl, pH 7.8, 1 mM sodium vanadate, and 1% SDS). The lysate was further homogenized by repeated aspiration through a 25-gauge needle. Protein content of each sample was measured by using a Bio-Rad DC assay (15). To detect secreted BMP4 in conditioned media, endothelial monolayers were first washed in serum-free Dulbecco’s modified Eagle’s medium supplemented with minimum non-essential amino acids and pyruvic acid and exposed to OS, LS, or static conditions for 1 day. The conditioned media were then centrifuged at 1,000 × g for 10 min. Aliquots (2 ml) of the supernatant were subjected to Western blot analysis as described by us (13, 14). The secreted BMP4 was detected using a rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with horseradish peroxidase-conjugated secondary antibody. The protein bands were visualized by enhanced chemiluminescence. The bands were quantified using the ImageQuant technology (Molecular Dynamics, Sunnyvale, CA).

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were collected and placed on ice with 10 ml of ice-cold acetone to precipitate protein for 30 min. Samples were pelleted by centrifugation (15,000 × g for 10 min) and resuspended in 100 μl of sample buffer for SDS-PAGE (13, 14). Aliquots of cell lysates (20 μg of protein each) were resolved on a 10% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was incubated with a primary antibody overnight at 4 °C and then with a secondary antibody conjugated to alkaline phosphatase (1 h at room temperature), which were detected by a chemiluminescence method (15). The intensities of immunoreactive bands in Western blots were analyzed by using the NIH Image program. The following primary antibodies were used: a monoclonal BMP4 antibody, rabbit ICAM1 antibody, goat VCAM1 antibody, and goat actin antibody (Santa Cruz Biotechnology).

Analysis—DNA microarray analyses were performed with an Affymetrix murine gene chip containing 12,000 genes (U74Av2; Affymetrix) and a Motorola murine genome chip containing 10,000 genes, according to the protocols provided by each manufacturer (16). Affymetrix chips were scanned and analyzed at the DNA core facility at Emory University School of Medicine; studies with Motorola chips were scanned and analyzed at the DNA core facility at the University of California at San Francisco or purchased from R&D Systems. The analyses of these studies showed that LS exposure significantly reduced cell-associated fluorescence intensity of cell lysates by fluorescence spectrophotometry using a plate reader. Both assays showed similar results. Some studies were performed with MAEC pretreated with 5 μg/ml mouse-ICAM1 antibody (Y11; Southern Biotechnology) (22).

RESULTS

Differential Regulation of the BMP4 Gene by LS and OS in Endothelial Cells—To identify the genes that may be responsible for the athero-protective and pro-atherogenic effects of LS and OS, respectively, we performed DNA microarray studies using cultured MAEC. Exposing MAEC to LS, but not OS, for 1 day using the modified “cone-and-plate” device (12) induced a cell shape alignment to the direction of the flow from a typical polygonal “cobblestone shape” found in static cultured cells (Fig. 1). The total RNAs prepared from these cells were used to determine mRNA expression profiles by using Affymetrix and/or Motorola DNA chips according to the manufacturers’ protocols. The analyses of these studies showed that LS exposure signif-

**FIG. 1. Morphology of endothelial cells exposed to LS, OS, or static conditions.** Confluent monolayers of MAEC were exposed to static condition (Static), LS (15 dynes/cm²) or OS (∼5 dynes/cm², 1 Hz cycle) for 24 h using the cone-and-plate apparatus. Following shear exposure, cell morphology was determined by light microscopy. Arrows indicate the direction of imposed shear stress.

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**Quantitative Real-time PCR**—Real-time PCR for BMP4 was carried out as previously described (17). Briefly, 4 μg of total RNA was reverse-transcribed by using random primers and a Superscript-II kit (Invitrogen) to synthesize first-strand cDNA. The cDNA was purified using a microbiospin 30 column (Bio-Rad) in Tris buffer and stored at −20 °C until used. The cDNA was amplified using a LightCycler (Roche Applied Science) RT-PCR machine. The mRNA copy numbers were determined based on standard curves generated with murine BMP4 and 18 S templates. The 18 S primers (50 nM at 61 °C) using endothelial RNA sample yielded a 130-base pair reverse, 5'-TTATACGGTGGAAGCCCTGTTCCCAGTCAG-3'. The pair of primers was verified by a nested PCR using other BMP4 primer pairs (forward, 5'-ATGGACTGTTATTATGCCTTGTTTTCTGTCAACACCATGATTC-3', reverse, 5'-CCACGTTAATGTAATGGCGACGCGAGTTCT-3'; and forward, 5'-GTCAACACCATGATTCTGTAACCCAGACCCTGTA-3', reverse, 5'-TTATACGTTAGACCCGCCCTCCCCGCA-3) and by running DNA gels. Real-time PCR for BMP4 was carried out using the annealing temperature 65 °C and extension time for 7 s in the PCR buffer (20 mm Tris-Cl, pH 8.4, at 25 °C, 4 mm MgCl₂, which was added 250 μg/ml bovine serum albumin, 200 μm deoxynucleotides) containing SYBR green (1:84,000 dilution), 0.05 unit/μl Taq DNA polymerase, and Taq start antibody (1:100 dilution).

**Flow-activated Cytometry Sorting (FACS) Analysis**—Treated cells were dissociated into single-cell suspensions using 0.25% trypsin-EDTA and resuspended in a FACS buffer (Hank’s buffered solution containing 5% fetal bovine serum). Aliquots of cell suspensions were incubated with ICAM1 antibody (R&D Systems) for 20 min on ice, washed twice with FACS buffer, and incubated with secondary antibody (fluorescein-5-isothiocyanate- or phycoerythrin-conjugated; Chemicon) for 20 min on ice in the dark. Then samples were washed again, fixed in 1% paraformaldehyde, and analyzed by FACS (Calibur; Becton-Dickinson) using CellQuest software. The fluorescence intensity of ICAM1 and forward cell scattering of 30,000 cells were measured, and the geometric means calculated from histograms were shown. In some studies, HAEc were transfected with either BMP4 (Dr. Elizabeth J. Robertson, Harvard University) cloned in a bicistronic pAdTrack CMV vector (Dr. Bert Vogelstein, The Johns Hopkins University) or an empty vector, both expressing green fluorescent protein (GFP), using LipofectAMINE 2000. In these experiments, ICAM1 expression was measured in the red phycoerythrin channel, while the green channel was used to monitor GFP expression. GFP expression was determined by FACS analysis and fluorescence microscopy (20–30% transfection efficiency). Because expression of GFP was similar among different treatment groups within the same experiment, we did not need to normalize ICAM1 expression data to the GFP level. Human recombinant nognin was also used in this study (Dr. Arturo Alvarez-Buylla (University of California at San Francisco) or purchased from R&D Systems and used in all experiments at 50 ng/ml (18–20).

**Immunohistochemical Study**—Frozen sections of human coronary arteries obtained from patients undergoing heart transplants were prepared (17) and stained with antibodies specific for BMP4 (1:1,000 dilution, goat antibody, Santa Cruz Biotechnology), ICAM-1 (1:50 dilution, mouse antibody; R&D), or von Willebrand factor (1:100 dilution, mouse antibody; Dako) for 2 h at room temperature, washed, and followed by incubation with secondary antibodies (anti-goat IgG or anti-mouse IgG) conjugated to alkaline phosphatase. Then the slides were washed and developed with a DAKO kit (Dako). Photographs of the slides were taken using a Zeiss microscope. Fifteen different human coronary arteries, containing various stages of atherosclerosis from minimally diseased to fatty streak to advanced atheroma stage, from six different patients were examined.

**Monocyte Adhesion**—Monocyte binding was determined under no-flow conditions using THP-1 monocytes (ATCC) by the method described by Chappel et al. (21). Briefly, THP-1 cells (5 × 10⁶ cells/ml) were labeled with a fluorescent dye 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein-AM (BCECF; Molecular Probes) (1 μg/ml) in serum-free RPMI medium for 45 min at 37 °C. Following exposure to shear stress or BMP treatments in the presence or absence of noggin or vehicle, the endothelial cells were washed in RPMI medium before adding BCECF-loaded THP-1 cells (1:1 ratio). After a 30-min incubation at 37 °C under no-flow conditions, unbound monocytes were removed by washing the endothelial dishes five times with Hank’s phosphate-buffered saline. Bound monocytes were quantified by either counting the cells under a fluorescent microscope or by measuring the fluorescent intensity of cell lysates by fluorescence spectrophotometry using a plate reader. Both assays showed similar results. Some studies were performed with MAEC pretreated with 5 μg/ml mouse-ICAM1 antibody (Y11; Southern Biotechnology) (22).

**NFkB Assay**—NFkB activity was determined by using a NFkB reporter construct, NFkB-SEAP vector (1 μg; Clontech) expressing a secreted form of placent al alkaline phosphatase driven by 4-b sequences in tandem. This construct was co-transfected with 0.5 μg of either pAdTrack BMP4 or empty vector control using LipofectAMINE 2000. Six hours post-transfection, conditioned media were centrifuged and heat treated at 65 °C (to inactivate endogenous alkaline phosphatase) for 30 min, followed by chemiluminescence alkaline phosphatase assay according to the manufacturer’s instructions.

**Differential Regulation of the BMP4 Gene by LS and OS in Endothelial Cells**—To identify the genes that may be responsible for the athero-protective and pro-atherogenic effects of LS and OS, respectively, we performed DNA microarray studies using cultured MAEC. Exposing MAEC to LS, but not OS, for 1 day using the modified “cone-and-plate” device (12) induced a cell shape alignment to the direction of the flow from a typical polygonal “cobblestone shape” found in static cultured cells (Fig. 1). The total RNAs prepared from these cells were used to determine mRNA expression profiles by using Affymetrix and/or Motorola DNA chips according to the manufacturers’ protocols. The analyses of these studies showed that LS exposure signif-
FIG. 2. Differential regulation of BMP4 mRNA and protein expression by LS and OS. MAEC were exposed to static condition (St), LS, or OS for 24 h (A–C), except for a time-course study (C, left panel). A. DNA microarray assay. Total RNA prepared from each dish exposed to LS, OS, or static condition was used in DNA microarray analysis using Affymetrix murine chips (n = 3 each for St and LS) or Motorola murine chips (n = 3 each for St, LS, and OS). Bar graph is the mean % of BMP4 ± S.E. using all data sets compared with that of static control values (*, n = 6, p < 0.05). B. Quantitative real-time PCR assay. Total RNAs obtained from above as well as known amounts of murine BMP4 standards were analyzed by real-time PCR. The BMP4 mRNA copy numbers were normalized against the 18S mRNA copy numbers. Bar graph shows the BMP4 mRNA levels expressed as % of static control values (mean ± S.E., n = 6 for St and LS and n = 3 for OS) (*, p < 0.001). C, Western blot (IB). Cell lysates obtained from cells exposed to LS, OS, or St were analyzed by Western blot with a BMP4 antibody or an actin antibody (used as a loading control). The band intensities were quantified and expressed as % of static controls as shown in the bar graphs. Left panel, LS significantly decreased BMP4 protein expression (*, p < 0.05, n = 3–6, except for 20-h group where n = 2); Middle panel, OS significantly increased BMP4 expression (*p < 0.05, n = 6); Right panel, MAEC were exposed to 15 dyn/cm^2 of LS (LS 15 dynes), 5 dynes/cm^2 of LS, or ±5 dynes/cm^2 of OS. After the shear, equal volumes of medium were precipitated, and Western blot was performed with a BMP4 antibody. (* and **, p < 0.05, n = 3). Cell lysates (left and middle panels) and conditioned media (right panel) obtained from cells exposed to LS or OS were analyzed by Western blot with a BMP4 antibody or an actin antibody (used as a loading control). The band intensities were quantified and expressed as % of static controls (mean ± S.E.).

BMP4 protein expression was determined by immuno blot studies. BMP4 protein is synthesized as an inactive precursor (48–55 kDa) that is proteolytically cleaved by proprotein convertases, and the active ~23-kDa protein is secreted (23, 24). In endothelial cell lysates, the BMP4 precursor was detected as a 54-kDa protein, and the mature form (p23) was detected in the conditioned media collected from static or shear-exposed cells (Fig. 2C). Exposure of cells to LS significantly down-regulated expression of BMP4 precursor in a time-dependent manner (Fig. 2C). After 16–24 h of LS exposure, BMP4 precursor expression was virtually undetectable (Fig. 2C, left panel, p < 0.05). In contrast, exposure of MAEC to OS significantly increased BMP4 precursor protein level by 2-fold above control (Fig. 2C, middle panel, p < 0.05). Consistent with the cell lysate result, the conditioned media of MAEC exposed to LS (15 dyn/cm^2) showed a barely detectable amount of secreted form of BMP4 (p23) (Fig 2C, right panel). In contrast, OS exposure did not significantly change the p23 BMP4 level in the conditioned medium (Fig. 2C, right panel, p < 0.05). Because the cells were exposed to LS (15 dyn/cm^2) and OS (±5 dyn/cm^2), we next determined whether it was the shear magnitude difference that accounted for our results observed so far. To address this question, we compared the effects of LS and OS using the same magnitudes (5 dyn/cm^2 LS versus ±5 dyn/cm^2 OS). As shown as Fig. 2C, right panel, at the same shear magnitude, OS-exposed cells had more than 3-fold BMP4 protein that of LS. However, the higher LS magnitude (15 dyn/cm^2) showed a much lower amount of BMP4 than that of lower LS (5 dyn/cm^2). These results show that LS exposure inhibits BMP4 expression in a force-dependent manner, whereas OS maintains high BMP4 expression.

BMP4 Expression in the Selective Patches of Endothelial Cells over Foam Cell Lesions in Human Coronary Arteries—Next, using the human coronary arteries we determined whether BMP4 protein is expressed in endothelial cells of human atherosclerotic lesions. The coronary arteries exhibiting a
FIG. 3. Selective expression of BMP4 in endothelial cells over foam cell lesions in human coronary arteries. Human coronary arteries were stained with antibodies specific to BMP4 (A and D), von Willebrand factor (B and E), ICAM-1 (F), and non-immune mouse IgG (NI-IgG) (C). Panels A–C are serial sections obtained from minimally diseased (normal) arterial samples, whereas panels D–F show foam cell lesions (marked as * in panel F based on CD68 staining; data not shown). M, medial smooth muscles. Note strong stainings for BMP4 and ICAM-1 in overlapping patches (D and F, arrowheads) in the serial sections. B and E, endothelial cells are marked with arrowheads.

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spectrum of atherosclerotic lesion complexity were obtained from patients undergoing heart transplants and examined by immunohistochemical staining (17). BMP4 protein expression was not apparent in the intimal endothelial cells in relatively normal, “minimally diseased” human coronary arteries (Fig. 3A) or in advanced lesions (data not shown). As shown in Fig. 3D, one exception was found in the endothelial cells (arrowed) overlying foam cell lesions that were stained strongly against the BMP4 antibody. As shown in Fig. 3C, isotype-matched nonspecific mouse IgG used as a negative control further supported the specificity of BMP4 staining. In contrast, the medial smooth muscle cells and macrophages (Fig. 3, A and D) were most intensely stained against a monoclonal BMP4 antibody (smooth muscle cells and macrophages identified by α-actin and CD-68 staining, respectively; data not shown). To verify the identity of endothelial cells, the serial sections were stained with a von Willebrand factor antibody (endothelial marker, Fig. 3, B and E), demonstrating the location of BMP4 staining in select areas of endothelium. Furthermore, immunostaining with an ICAM1 staining showed that the expression of this pro-inflammatory adhesion molecule was selectively increased in the similar endothelial areas expressing BMP4 (Fig. 3, D and F, arrowheads). On the other hand, we failed to detect VCAM-1 in the adjacent serial sections (data not shown). This result is consistent with the finding reported by Endress et al. (7).

BMP4 Produced in Endothelial Cells by OS Stimulates Monocyte Adhesion.—The selective expression of BMP4 protein in endothelial cells above foam cell lesions (an early form of atherosclerotic lesions) prompted a speculation that BMP4 may be involved in the inflammatory responses observed in lesion-prone areas (3, 4). To begin to test this hypothesis, MAEC were treated with increasing amounts of BMP4 for 24 h and then monocyte adhesion to endothelium was determined. As a positive control, some cells were treated with a well known inflammatory cytokine, TNFα (100 units/ml). BMP4 stimulated monocyte binding in a concentration-dependent manner with a maximum activation of 4–7-fold over control (Fig. 4A, p < 0.05). As low as 0.1 ng/ml BMP4 induced a statistically significant increase, whereas 50 ng/ml BMP4 induced a maximum effect. A similar effect of BMP4 on monocyte adhesion was also observed by transfecting MAEC or HAEC with a vector expressing mouse BMP4 (data not shown).

OS has been shown to induce monocyte adhesion both in vivo and in cultured endothelial cells by increasing surface expression of adhesion molecules (21). Therefore, we used a BMP4 inhibitor, noggin (18, 20), to examine whether OS induces monocyte adhesion in endothelial cells in a BMP4-dependent manner. Exposure of endothelial cells to OS for 24 h significantly increased monocyte adhesion (Fig. 4B, p < 0.05). Treatment of MAEC with noggin (50 ng/ml) inhibited OS-induced monocyte adhesion (Fig. 4B).

In contrast, exposure of MAEC to LS for 24 h inhibited monocyte adhesion by ~50% of static control level (Fig. 4C, p < 0.05) as expected (26). Because LS exposure significantly inhibited BMP4 expression in endothelial cells (Fig. 2), we next examined whether the inhibitory effect of LS on monocyte adhesion could be reversed by BMP4 addition. For this study, we exposed MAEC in the presence of BMP4 during shear or static control for 24 h, followed by monocyte adhesion assay. The inhibitory effect of LS on monocyte adhesion was lost when MAEC were sheared in medium supplemented with BMP4 (Fig. 4C, p < 0.05). Taken together, these results suggest that BMP4 produced from endothelial cells by OS exposure leads to monocyte adhesion.

BMP4 Stimulates Monocyte Adhesion by Inducing ICAM-1 Expression in an NFκB-dependent Manner.—Next, we examined the mechanism by which BMP4 increases monocyte adhesion to endothelial cells. Adhesion of monocytes to endothelial cells is mediated by sequential coordinated molecular interactions between the integrins expressed on monocyte surface and several adhesion molecules expressed on the endothelial surface, including ICAM-1, VCAM-1, and E-selectin (3). Moreover, it has been shown previously that expression of ICAM-1, VCAM-1, and E-selectin on endothelial cell surface is increased in atherosclerosis-prone areas (3). Therefore, we first determined whether the endothelial expression of ICAM-1, VCAM-1, and E-selectin was modified in response to OS by FACS analysis.

Exposure of HAEC to OS (1 day) increased ICAM-1 expression by 2.8-fold above control (Fig. 5A, p < 0.05). For compar-
ICAM-1 expression in all mock-transfected effects, ICAM-1 expressions in all mock-transfected with each transfection component, LipofectAMINE alone, empty vector alone, BMP4 vector alone, or not. Demonstrate the specific effect of BMP4 on ICAM-1 induction. HAEC were transfected with BMP4 vector or an empty control using MAEC treated with TNFα (100 units/ml for 2 h). MAEC were exposed to OS or static condition in the presence or absence of noggin or vehicle (Veh), and monocyte adhesion was determined. Bar graph represents the numbers of bound monocytes expressed as % of static control (mean ± S.E., n = 4) (*, p < 0.05). C, MAEC were exposed to LS in the presence of recombinant BMP4 or vehicle control, followed by monocyte binding assay. Data are expressed as in panel B (mean ± S.E., n = 4) (*, p < 0.05).

Unlike ICAM-1, unlike ICAM-1, however, neither OS nor BMP4 had any effect on the surface expressions of VCAM-1 and E-selectin in endothelial cells, whereas TNFα strongly increased both (Fig. 5, C and D). Next, we examined the effect of LS on endothelial expression of ICAM-1, VCAM-1, and E-selectin. Unlike OS, LS significantly decreased VCAM-1 expression without any significant effect on ICAM-1 (Fig. 5E) or E-selectin (data not shown). Unlike ICAM-1, the BMP4 effect on VCAM-1 was marginal at best and not significant (p > 0.05, n = 3). These results suggest that LS can inhibit monocyte adhesion directly by down-regulating VCAM-1 expression.

To further investigate the role of BMP4 in ICAM-1 induction, HAEC were transfected with BMP4 vector or an empty control vector by a LipofectAMINE-mediated method. BMP4 stimulated ICAM-1 expression 3–4-fold above that of vector control cells (n = 3, p < 0.001) (Fig. 5F). As further controls, HAEC were mock-transfected with each transfection component, LipofectAMINE alone, empty vector alone, BMP4 vector alone, or not transfected at all. ICAM-1 expressions in all mock-transfected cells were similar to untransfected cells (Fig. 5F). These results demonstrate the specific effect of BMP4 on ICAM-1 induction.

Transfection with BMP4 in HAEC clearly increased expression of both BMP4 precursor (p54 found in cell lysate) and the secreted form (p23 found in conditioned medium) in comparison to controls vector as demonstrated by Western blot (Fig. 5G). This is the first report, as far as we are aware, showing that BMP4 acts as an inflammatory factor by stimulating a specific adhesion molecule, ICAM-1.

Next, we determined whether OS and BMP4 regulate ICAM-1 induction by an NFκB-dependent mechanism (28–30). HAEC were exposed to OS, BMP4, or static conditions in the presence or absence of a NFκB translocation inhibitor SN50 (31) or the inactive peptide SN50M or the proteosome inhibitor MG132 (32). Either SN50 or MG132 completely prevented ICAM-1 expression induced by BMP4, whereas SN50M did not inhibit ICAM-1 induction induced by BMP4 (Fig. 5F). These results strongly suggest that NFκB plays a critical role in ICAM-1 induction by BMP4.

To demonstrate further whether BMP4 directly stimulates NFκB activity, we examined the effect of BMP4 on NFκB activation using an NFκB reporter construct. In this study, NFκB-SEAP construct expressing a secreted form of placental alkaline phosphatase driven by 4 kb sequences in tandem was co-transfected with either BMP4 vector or empty vector control by the LipofectAMINE method used above (Fig. 5F). As shown in Fig. 5H, expression of BMP4 stimulated NFκB activity by more than 3-fold above controls (n = 3, *, p < 0.05).

Finally, by using a blocking antibody we examined whether OS-induced monocyte binding was ICAM-1-dependent. As shown in Fig. 6, monocyte adhesion induced by OS was prevented by treating MAEC with an ICAM1 blocking antibody. These results further demonstrate that chronic exposure of endothelial cells to OS induces monocyte adhesion in an ICAM-1-dependent manner.
DISCUSSION

Here, we identified BMP4 as a mechanosensitive and pro-inflammatory protein. Whereas the atheroprotective LS inhibited expression of BMP4 protein, the pro-atherogenic OS stimulated it. Through functional studies, we discovered a novel function of BMP4 as an inflammatory factor produced in endothelial cells by OS. We found that OS stimulates production of BMP4, which in turn induces ICAM-1 expression on endothelial surface in a NFκB-dependent manner, eventually leading to monocyte recruitment.

In the current study, we presented most OS and LS results in comparison to static control conditions. However, it seems more appropriate to compare OS effects to LS conditions rather than to static conditions. Currently, most investigators in this field, including us, use static cultured cells as a physiologically "normal" control. This is necessary because of technical constraints of culturing cells under continuous shear conditions. The majority of arterial endothelial cells in vivo, however, are continuously exposed to LS and aligned to the direction of the flow. In contrast, endothelial cells in the hemodynamically defined lesion-prone areas are exposed to unstable and low wall shear conditions. Therefore, endothelial cells cultured under "static"
conditions may represent low or no-shear conditions and may not represent true “control” conditions. Many in vivo studies examine the differences between the normal regions exposed to LS in straight arteries and the lesion-prone areas exposed to disturbed and low shear conditions in branched or curved arteries.

If we compare the OS effects on expression of BMP4 and ICAM-1 as well as monocyte adhesion to those of LS instead of static conditions, the effects of the pro-atherogenic force become more pronounced than what we have shown under “Results.” For example, endothelial cells exposed to LS express BMP4 mRNA and protein at almost undetectable levels. Exposure to OS, however, dramatically up-regulated expression of BMP4 mRNA and protein (Fig. 2). This is consistent with our human coronary artery data showing that BMP4 expression is undetectable in normal (minimally diseased) arteries, whereas it is strongly expressed in endothelial patches overlaying foam cell lesions. In typical studies, OS increases monocyte binding 8–10-fold above that of LS (data not shown).

BMPs are members of the transforming growth factor-β superfamily and play important roles in bone formation, embryonic development, and differentiation (23, 24). Although BMP4 protein has been found previously in calcified atherosclerotic plaques (25), its expression and functional importance in endothelial cells have not been determined. There are two types of signaling receptors specific for BMPs: BMPR-I and BMPR-II. It appears they are both required for signaling (20). Three BMP type I receptors, BMPR-IA (also known as ALK3, Activin-Like Kinase-3), BMPR-IB (ALK6), and ALK2 and one BMP type II receptor have been identified (33). Although somewhat variable depending upon species and vascular bed origins, endothelial cells from mouse arteries as well as cultured murine and bovine aortic endothelial cells have been shown to express both type I (ALK2, 3, and 6) and type II BMPRs (34). Unlike their well-known effects in bone formation and embryonic development, the functional importance of BMPRs in vascular wall is not clear. One notable exception is the link in vascular smooth muscle cells as demonstrated by the loss-of-function mutations of the type II BMPR in familial primary pulmonary hypertension and sporadic primary pulmonary hypertension (35). In endothelial cells, transfection with constitutively active mutants of ALK2, ALK3, and ALK6 has been shown to stimulate expression of id gene and angiogenic responses (34).

As far as we are aware, BMP4 has not been shown to induce inflammatory responses previously, especially in endothelial cells. Our finding that BMP4 stimulates monocyte adhesion by increasing surface expression of ICAM-1 in endothelial cells seems to be the most interesting and novel aspect of the current study.

Atherosclerosis is a focal and inflammatory disease preferentially occurring at the lesion-prone areas exposed to unstable and low shear stress (1–4). The branched, bifurcated, and curved arteries such as the lesser curvature of the ascending aorta, the outer wall across the apex of the carotid sinus, and the left descending coronary arteries are the preferential sites of atherosclerotic development (1–4). Endothelial cells in the lesion prone areas become dysfunctional and have been shown to express adhesion molecules including ICAM-1, VCAM-1, and E-selectin (3). Circulating blood monocytes and lymphocytes then bind to these adhesion molecules, migrate beneath endothelium, engulf lipids, and transform into macrophage foam cells, eventually becoming the site of advanced atherosclerotic plaques (3, 4).

Unexpectedly, the current study showed that OS and BMP4 selectively regulate expression of ICAM-1 without significantly affecting VCAM-1 and E-selectin. These seemingly conflicting results on ICAM-1 and VCAM-1 have been reported in mouse atherosclerosis models as well. Cybulsky et al. (5) showed that VCAM-1, but not ICAM-1, expression was up-regulated by a high fat diet in low density lipoprotein receptor−/− mice. In contrast, Nakashima et al. (36) reported that only ICAM-1, but not VCAM-1, expression was up-regulated in a disturbed flow-dependent manner in the lesion-prone areas such as the aortic sinus, whereas VCAM-1 expression was robustly increased by high fat diet feeding in ApoE−/− mouse. Evidence from other mouse atherosclerosis models lacking expression of ICAM-1 or VCAM-1 has shown the importance of both adhesion molecules (5, 27). In addition, this concept is consistent with data from Nagel et al. (37), who used cultured endothelial cells to show that ICAM-1 expression, but not VCAM-1 and E-selectin, was up-regulated by shear stress. Moreover, Endres et al. (6) reported that the early atherosclerotic lesions found in the outer wall of human carotid artery bifurcations showed increased expression of ICAM-1, but not VCAM-1 and E-selectin. However, expressions of VCAM-1 and E-Selectin did increase in advanced atherosclerotic plaques (7). These findings are con-
Our findings on the selective effect of OS on ICAM-1 expression are consistent with previous reports in cultured endothelial cells using a similar cone-and-plate shear system (37). However, using a parallel plate shear device and human umbilical endothelial cells, OS has been shown to stimulate VCAM-1 and E-selectin in addition to ICAM-1 (21). These discrepancies may be due in part to subtle differences in flow profiles generated in the two different shear devices. Most importantly, however, BMP4 increased expression of ICAM-1, but not VCAM-1 and E-selectin, demonstrating its selective effect on ICAM-1.

VCAM-1 expression and monocyte adhesion are kept very low in most healthy arteries by unknown mechanisms (7, 36). In our current study, we found that chronic exposure of endothelial cells to LS (a physiological condition expected for disturbed shear stress, including OS, which induces endothelial cells to LS (a physiological condition expected for theroic conditions).

Based on the current data and literature, we propose that BMP4 plays a critical role as a mechanosensitive and pro-inflammatory cytokine mediating the opposite effects of OS and LS. ICAM-1 seems to be more responsive to high cholesterol levels than VCAM-1, whereas VCAM-1 seem to be regulated mainly by oscillatory shear stress, which induces ICAM-1 expression in the lesion-prone areas of atherosclerosis. ICAM-1 expression in the lesion-prone areas of atherosclerosis is consistent with previous reports in cultured endothelial cells (38). Therefore, although OS selectively up-regulates ICAM-1 expression, it may be more responsive to high cholesterol levels.