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Arterial shear stress augments the differentiation of endothelial progenitor cells adhered to VEGF-bound surfaces

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ABSTRACT

Our ongoing studies show that vascular endothelial cell growth factor (VEGF)-bound surfaces selectively capture endothelial progenitor cells (EPCs) *in vitro* and *in vivo*, and that surface-bound VEGF stimulates intracellular signal transduction pathways over prolonged culture periods, resulting in inductive differentiation of EPCs. In this article, we investigated whether simulated arterial shear stress augments the differentiation of EPCs adhered to a VEGF-bound surface. Human peripheral blood-derived mononuclear cells adhered to a VEGF-bound surface were exposed to 1 day of shear stress (15 dynes/cm^2, corresponding to shear load in arteries). Shear stress suppressed the expression of mRNAs encoding CD34 and CD133, which are markers for EPCs, and augmented the expression of mRNAs encoding CD31 and von Willebrand factor (vWF) as well as vWF protein, which are markers for endothelial cells (ECs). Shear stress enhanced expression of ephrinB2 mRNA, a marker for arterial ECs, but did not significantly change expression of EphB4 mRNA, a marker for venous ECs. Focused protein array analysis showed that mechanotransduction by shear stress activated the p38 and MAPK pathways in EPCs. Thus, arterial shear stress, in concert with surface-bound VEGF, augments the differentiation of EPCs. These results strongly support previous observation of rapid differentiation of EPCs captured on VEGF-bound stents in a porcine model.

1. Introduction

Cardiovascular disease accounts for a high degree of mortality. Once vessels are stenosed or occluded, a life-threatening situation is created. Current therapeutic strategies include implantation of stents or artificial grafts. Clinically significant outcomes have been verified for stent technology, whereas small-diameter artificial diameter grafts suitable for replacement of diseased coronary arteries have not yet been developed for clinical applications [1,2]. Regardless of the type of implant, it is necessary to use materials with strict nonthrombogenic potential.

Natural vessels are lined with a monolayer of endothelial cells (ECs), which is the only cell type expressing nonthrombogenic potential in the body. In animal models, artificial grafts seeded *ex vivo* with ECs harvested from autologous veins have demonstrated nonthrombogenic potential [3–5], similar to that of natural endothelium. Endothelial progenitor cells (EPCs), which are derived from bone marrow and circulate in small numbers in the arterial bloodstream [6], provide an alternative source of ECs. Artificial grafts seeded *ex vivo* with EPCs have also demonstrated high patency in animal models [7–9]. However, despite high initial enthusiasm for these grafting methods, neither approach to *ex vivo* cell-based grafts has been used clinically, largely because these methods involve labor-intensive, time-consuming, and costly procedures.

To eliminate or circumvent the problems inherent in cell seeding technology, such as clonal mass culture followed by full-coverage cell seeding, recent studies have been focused on *in situ* capture of EPCs from the arterial bloodstream. The preceding study was CD34 antibody-bound stent [10], since EPCs are in the CD34 positive fraction of mononuclear cells (MCs) [11]. Although the authors observed rapid cell adhesion upon implantation, it is possible that cell types other than EPCs also adhered. Very recently, Korean group reported that a VE-cadherin-bound stent could capture EPCs that were subsequently capable of endothelialization, resulting in minimal intimal hyperplasia [12]. Our own ongoing study share a common working hypothesis with Korean group: our approach is based on the highly selective capture of EPCs via biospecific interactions between cell surface markers exclusively expressed on EPCs and proteins bound to the substrate. Our *in vitro* studies have shown that vascular endothelial growth factor (VEGF)-bound

Abbreviations: VEGF, vascular endothelial cell growth factor; EPCs, endothelial progenitor cells; ECs, endothelial cells; MCs, mononuclear cells; PEVA, poly(ethylene-co-vinyl alcohol); HFIP, hexafluoroisopropanol; CDI, *N*,*N*′-carbonyldiimidazole.

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surfaces significantly induce EPC adhesion, spreading, differentiation, and endothelialization, as well as stimulate intracellular signal transduction pathways via VEGF receptor-2, even after prolonged period [13–16]. These results indicate that solid-phase VEGF appears to act as strong inductive promoter for capturing and thereafter cellular events.

ECs are constitutively exposed to shear stress induced by blood flow. Shear stress influences cellular responses, including cell shape, alignment and various other biochemical functions, via mechanotransduction pathways [17]. We wished to investigate whether and how arterial shear stress augments the biochemical function of EPCs captured on VEGF-bound surfaces. In this study, we report the effects of laminar arterial shear stress on human peripheral blood derived-MCs adhered to VEGF-bound surfaces, focusing on differentiation at both mRNA and protein levels.

2. Materials and methods

2.1. VEGF-bound surface

VEGF-bound glass substrates (70 mm × 100 mm) were prepared as follows: The glass plates were sequentially cleaned using 1:1 ethanol:chloroform, H_2SO_4, and 0.2 N NaOH. Cleaned glass plates were coated with 0.5% poly(ethylene-co-vinyl alcohol) [PEVA; vinyl alcohol content: 68 mol% (Polysciences, Warrington, PA, USA)] in hexafluoroisopropanol (HFIP, Wako Pure Chemical, Osaka, Japan) and dried. Then surface hydroxyl groups on the PEVA-coated glass were activated using 50 mg/ml *N*,*N*′-carbonyldiimidazole (CDI, Wako Pure Chemical) in acetonitrile for 1 day, after which the glass plates were immersed in 50 mg/ml VEGF (R&D Systems, Minneapolis, MN, USA) in PBS for 1 day (Fig. 1A).

2.2. Mononuclear cell isolation and culture

Mononuclear cells (MCs) were isolated from 100 ml human peripheral blood by density gradient centrifugation using Histopaque-1077 (Sigma–Aldrich, St. Louis, MO, USA) as described [18]. Cells were then plated on VEGF-bound glass substrates at a density of 4×10^5 cells/cm^2 and cultured in EBM-2 (Lonza, Brussels, Belgium) supplemented with 5% fetal bovine serum, human FGF-2, human EGF, IGF-1, and ascorbic acid at 37 °C in atmosphere containing 5% CO_2. Media were replaced every 3 days. To confirm the endothelial lineage, adhered MCs were incubated with 1, 1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine perchlorate-labeled acetylated LDL (DiI-acLDL; 10 mg/ml; Biomedical Technologies, Stoughton, MA, USA) and FITC-labeled Ulex europaeus agglutinin (FITC-Ulex lectin; 10 mg/ml; Sigma–Aldrich) for 2 h.

2.3. Shear stress experiment

After 6 days in culture, cells were exposed to laminar shear stress for 24 h using a parallel plate-type device (Yasuhisa Koki, Tokyo, Japan), as described previously [19]. The intensity of the wall shear stress (τ dynes/cm^2) on the cell layer was calculated using the formula $\tau = 6\mu Q/a^2 b$, where μ is the viscosity of the perfusate (0.0095 poise), Q is the flow volume (0.583 ml/s), and a and b are the cross-sectional dimensions of the flow path (a: 0.02 cm, b: 5.6 cm). All shear stress experiments were performed at 37 °C in a CO_2 incubator.

2.4. Real-time RT PCR analysis

Total RNA was extracted using an RNeasy Kit (Qiagen, Hilden, Germany), and first-strand cDNAs were synthesized using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo-dT primers. After reverse transcription, gene expression was monitored via real-time PCR using a PRISM 7500 (Applied Biosystems, Foster City, CA, USA). PCR was performed using FastStart Universal SYBR Green Master (Roche Diagnostics, Basel, Schweiz). Primer pairs are shown in Table 1.

2.5. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde in PBS and probed with mouse anti-Ki67 antibody (Immunotech, Marseille, France) or rabbit anti-von Willebrand factor (vWF) antibody (Dako Cytomation, Glostrup, Denmark) in PBS containing 3% BSA. Cells were rinsed with PBS and labeled with AlexaFluor 488-labeled chicken anti-mouse IgG (Invitrogen) or anti-rabbit IgG (Invitrogen). Fluorescence images were obtained using a BioZero microscope system (Keyence, Osaka, Japan).

2.6. Western blot analysis

Total cellular protein was extracted in lysis buffer containing 50 mM Tris–HCl (pH 7.5), 1% (v/v) Triton X-100, 1% deoxycholic acid, 150 mM NaCl, 2 mM PMSF, 2.5 mg/ml antipain, 5 mg/ml aprotinin, 5 mg/ml pepstatin, and 5 mg/ml leupeptin. The concentrations of protein were determined using a BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Lysates were subjected to SDS–PAGE and subsequent Western blotting. Proteins transferred to polyvinylidene difluoride membranes were probed with rabbit anti-von Willebrand factor antibody (Dako Cytomation) or mouse anti-GAPDH antibody (Cell Signaling Technology, Danvers, MA, USA). The membranes were further incubated with horseradish peroxidase-conjugated anti-mouse IgG antibody or anti-rabbit IgG antibody (Dako Cytomation). The signals were detected using ImmunoStar chemiluminescence reagents (Wako Pure Chemical).

2.7. Focused protein array

Total cellular protein was hybridized to Proteome Profiler Human Phospho-Kinase Arrays (R&D systems) following extraction using the Kit's proprietary lysis buffer. Signals were detected using ImmunoStar chemiluminescence reagents (Wako Pure Chemical). Images were obtained using an LAS-3000 (FujiFilm, Tokyo, Japan), and pixel densities were measured using the Multi-Gauge software (FujiFilm).

2.8. Statistical analysis

All data are shown as mean ± SD. Differences between groups were assessed using Student's *t*-test. Values of $P < 0.05$ were considered significant.

3. Results

3.1. VEGF-bound surfaces induce differentiation of mononuclear cells

We sought to determine whether and how much human mononuclear cells (MCs) adhered to a VEGF-bound surface express the characteristic features of endothelial lineage cells after prolonged culture periods. MCs cultured for 6 days on a VEGF-bound surface were mostly double-positive for acLDL uptake and Ulex-lectin binding, both of which are characteristic of endothelial lineage cells (Fig. 1B), indicating that the adhered cells include EPCs. In addition, we measured mRNA expression of endothelial lineage cell surface markers in MCs adhered to VEGF-bound and FN-bound surfaces using real-time RT PCR. Relative to cells grown on an

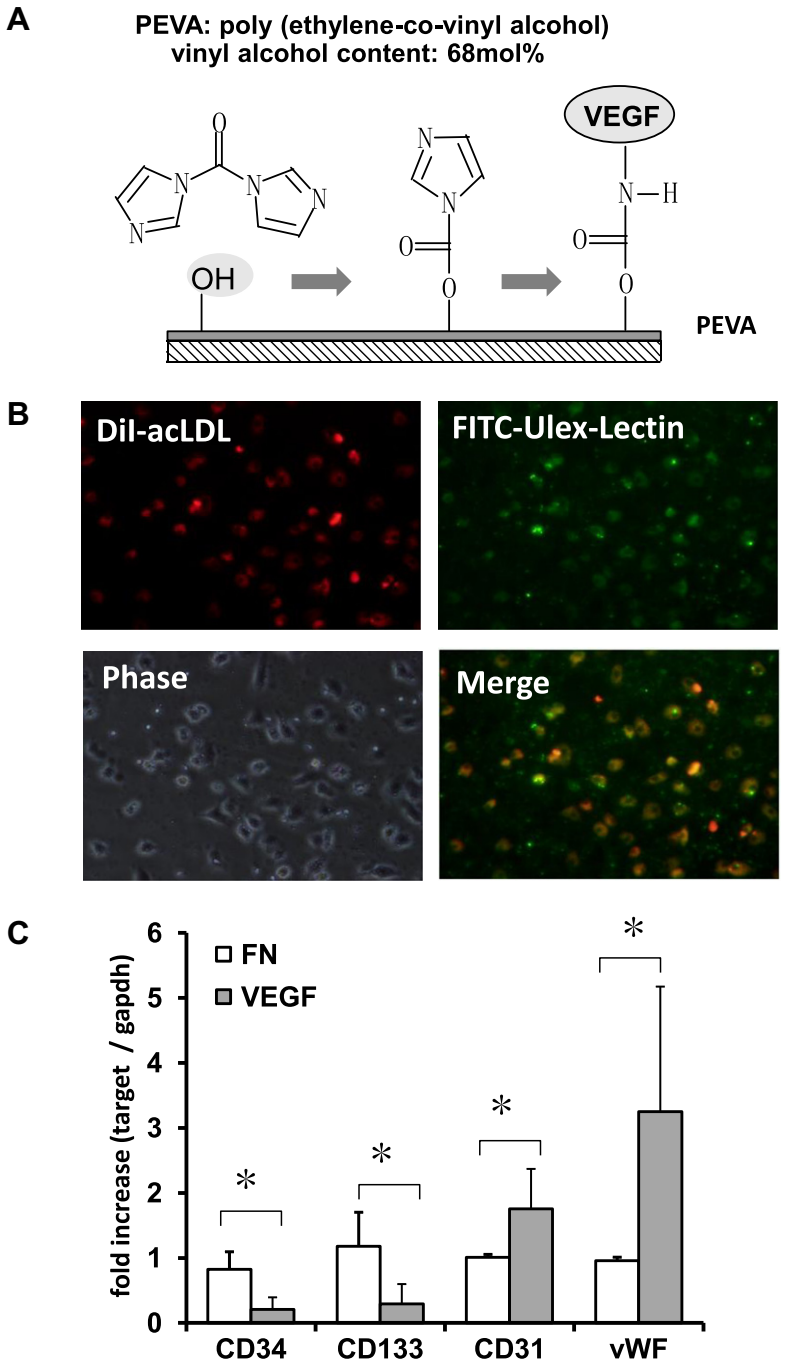

Fig. 1. Mononuclear cells adhered to a VEGF-bound surface. (A) VEGF-bound glass substrate was prepared by thin-layer coating of PEVA, followed by activation of surface hydroxyl groups. Finally VEGF was covalently bound to the surface. (B) DiI-acLDL uptake (red) and FITC-Ulex lectin binding (green) were observed in MCs cultured for 6 days. (C) The mRNA expression of endothelial lineage markers was determined using real-time RT PCR. (Culture period: 7 days) Data are shown as means ± SD of four samples. $^{*}P < 0.05$.

Table 1
Oligonucleotide primers used for gene expression analysis by real-time RT-PCR.

Gene	Primer sequence, 5′–3′
GAPDH	GTCGGAGTCAACGGATTTGG/GCAACAATATCCACTTTACCAGAGTTAA
CD34	AACCCTATACATCATCTTCTCCTATCCT/CACTTCTCTGATGCCTGAACATTT
CD133	GCACTCTATACCAAAGCGTCAAGA/TCCTAGTTACTCTCTCCAACAATCCA
CD31	GGGATCTTTCTTAGTGGATTTAATGG/TGCCGAGGAAGGCTAAAGC
vWF	CCTCAAAGGCGGTGGTCAT/AGCGATCTCCAATTCCAATAGG
ephrinB2	GAAGGGACTCCGTGTGGAAGT/AGGTAGAAATTTGGAGTTCGAGGAA
EphB4	GTGGGAGGTGATGTCATTTGG/GCAGCCGGTAGTCCTGTTCA

FN-bound surface, MCs cultured on a VEGF-bound surface exhibited markedly lower expression of CD34 and CD133 mRNAs, which are markers for progenitor cells, and markedly higher expression of CD31 and vWF mRNAs, which are markers for ECs (Fig. 1C). These data indicate that a VEGF-bound surface induces differentiation of EPCs to ECs more efficiently than an FN-bound surface.

3.2. Arterial shear stress (I): suppression of proliferation potential

We next examined the effect of shear stress on the proliferation of EPCs adhered to a VEGF-bound surface. MCs cultured for 6 days on a VEGF-bound surface were subjected to laminar arterial shear stress (15 dynes/cm^2) in a parallel plate-type device for 24 h (Fig. 2A). Immunocytochemical analysis using anti-Ki67 antibody (proliferating cellular nuclei) and DAPI (all cellular nuclei) showed that the ratio of Ki67-positive nuclei to DAPI-positive nuclei under shear stress was significantly decreased compared to static condition (Fig. 2B). Thus, arterial shear stress suppresses the proliferative activity of EPCs adhered to a VEGF-bound surface.

3.3. Arterial shear stress (II): acceleration of differentiation

We investigated the effect of shear stress on the differentiation of EPCs adhered to a VEGF-bound surface by measuring mRNA and protein levels, as follows: measuring of mRNA expression of endothelial differentiation markers, performed using real-time RT PCR, demonstrated that shear stress significantly downregulated mRNA expression of CD34 and CD133, whereas it significantly upregulated mRNA expression of CD31 and vWF (Fig. 3A). Immunocytochemical analysis of vWF showed that weak vWF immunoreactivity was observed in cells under static conditions, whereas a stronger vWF signal was observed in elongated cells under shear stress (Fig. 3B). Consistent with this, Western blot analysis revealed that the amount of vWF protein increased under shear stress relative to static conditions (Fig. 3C). These data indicate that laminar arterial shear stress facilitates the differentiation of EPCs adhered to a VEGF-bound surface. Moreover, shear stress upregulated mRNA expression of ephrinB2, a marker for arterial ECs, but did not change the mRNA expression of EphB4, a marker for venous ECs (Fig. 3D). This data indicates that EPCs adhered to a VEGF-bound surface respond to the arterial shear stress, resulting in appropriate (i.e., arterial) differentiation.

3.4. Arterial shear stress (III): activation of intracellular signaling

To determine whether shear stress activates intracellular signals in EPCs adhered to a VEGF-bound surface, we measured protein phospholyration levels using focused protein array. MCs cultured for 6 days on a VEGF-bound surface were subjected to arterial shear stress for 30 min, and protein extracts were visualized on focused protein arrays. On the arrays, we observed several spots that were altered under shear stress relative to static conditions (Fig. 4A). Phosphorylation of p38 (T108/182), ERK1/2(T202/204, T185/Y187), JNK (T183/Y185, T221/Y223), MEK1/2 (S218/S222, S222/S226), and MSK1/2 (S376/360), all of which are involved in cell growth and differentiation, was significantly upregulated under shear stress. We observed no significant change in phosphorylation of Akt (S473) and Akt (T308), both of which are

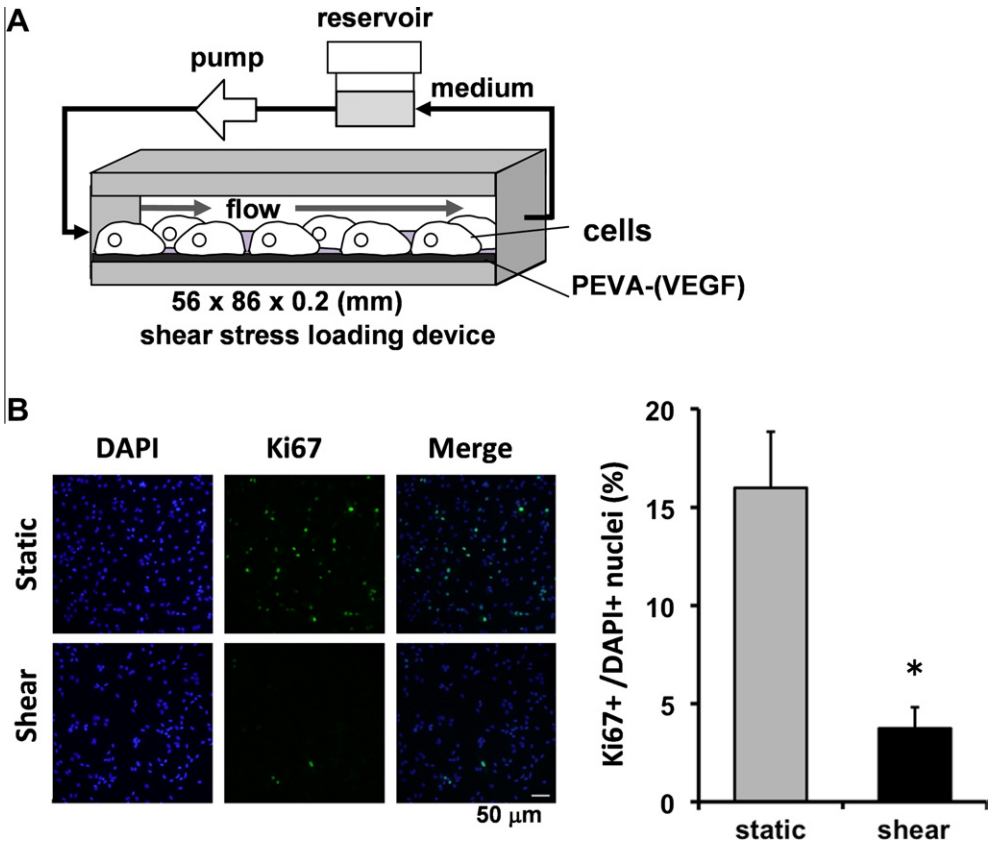

Fig. 2. The effect of arterial shear stress on the proliferation of MCs adhered to a VEGF-bound surface. (A) Schematic of the parallel plate-type device. (B) MCs cultured for 6 days on a VEGF-bound surface were subjected to arterial shear stress (15 dynes/cm^2) for 24 h. Immunostaining with anti-Ki67 antibody (Green: nuclei of proliferating cells) and DAPI (blue: all nuclei). (C) The ratios of Ki67 positive nuclei to DAPI positive nuclei, for shear-stressed and non-shear-stressed cells. Data are shown as means ± SD of four samples. *$P < 0.05$.

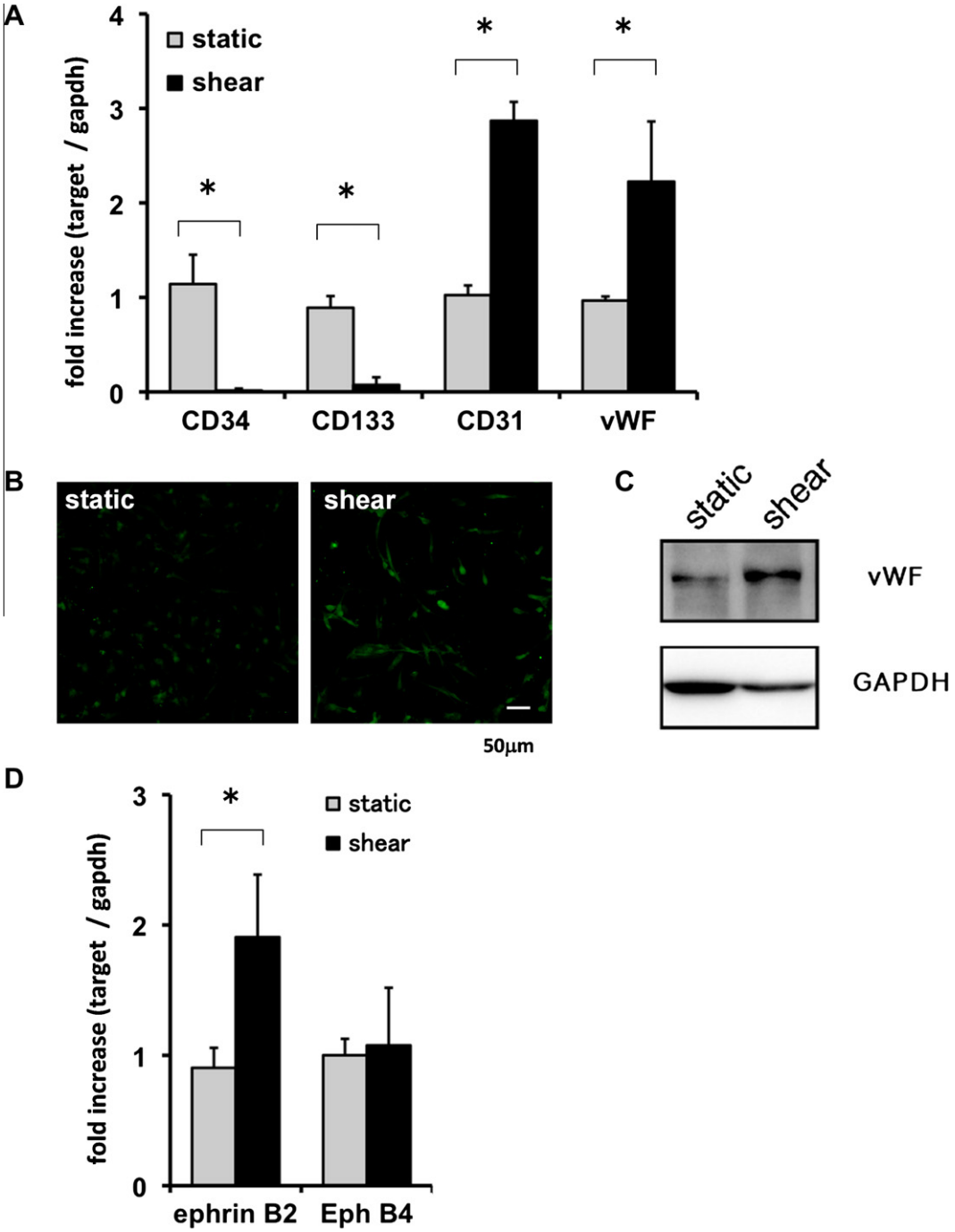

Fig. 3. The effect of arterial shear stress on differentiation of MCs adhered to a VEGF-bound surface for 6 days, followed by 1 day of exposure of arterial shear stress (15 dynes/cm^2). (A) mRNA expression of endothelial differentiation markers under static and shear stress conditions. (B) Immunostaining using anti-vWF antibody (Green). (C) Western blot analysis with anti-vWF antibody and anti-GAPDH antibody. (D) mRNA expression of arterial (ephrinB2) and venous (EphB4) markers. Data are shown as means ± SD of four samples. $^*P < 0.05$.

involved in cell survival and growth. Phosphorylation of S70S6K (T229) and S70S6K (T421/S424), both of which are involved in cell growth, was significantly downregulated (Fig. 4B). Thus, exposure to shear stress even for a short time (30 min) activates intracellular signals especially MAPK pathways in EPCs adhered to a VEGF-bound surface.

4. Discussion

If EPCs circulating in arteries could be effectively and selectively captured on blood-exposed surfaces of implanted vascular devices such as stents and artificial grafts, and subsequently induced rapid differentiation, such surfaces should exhibit nonthrombogenic potential equivalent to native endothelium, a quality long sought by developers of cardiovascular devices. Two challenges must be overcome in order to realize *in situ* capture-based endothelialization technology: effective and selective EPC capture, and rapid differentiation with attainment of the desired cellular characteristics. We have demonstrated that solid-phase VEGF acts as an adhesion site similar to FN, and is furthermore able to induce differentiation, as evidenced by markedly enhanced expression of VEGFR1 and VEGFR2 [13–15]. In clinical applications, more rapid differentiation

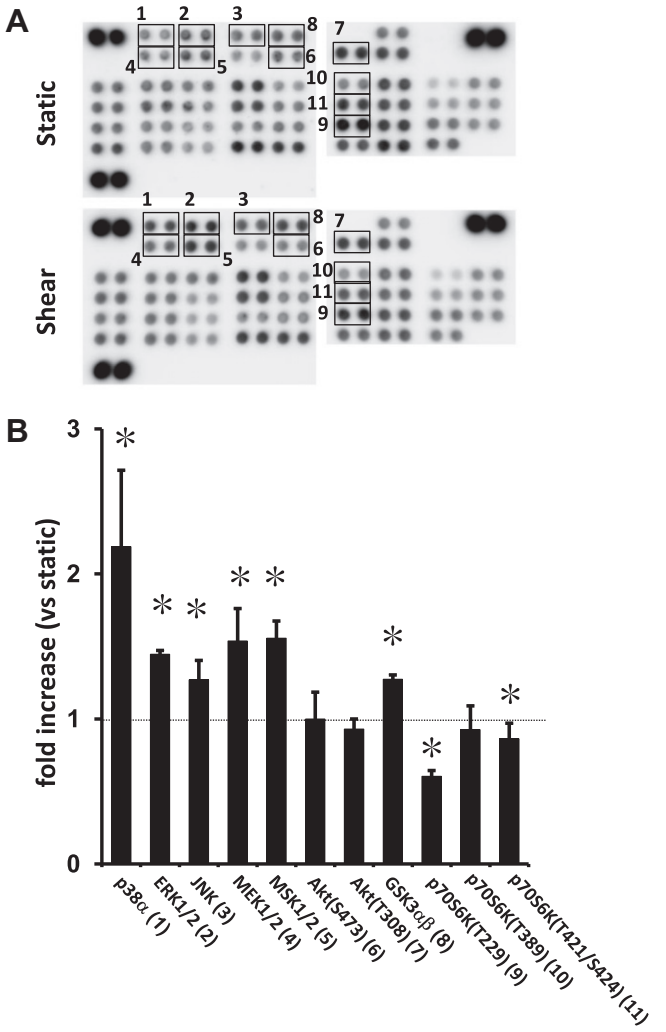

Fig. 4. The effect of arterial shear stress on intracellular signal transduction. MCs cultured for 6 days on a VEGF-bound surface were subjected to arterial shear stress (15 dynes/cm^2) for 30 min. (A) Representative pictures of phospho-kinase protein arrays hybridized with cell lysates. (B) Quantified pixel densities of spots. Data are shown as means ± SD of three samples. $^*P < 0.05$.

from EPCs to ECs is greatly desirable, since blood-exposed surface is subject to competition between (1) EPCs capture, colony formation, and full endothelialization and (2) thrombus formation triggered by the body's own defense mechanisms.

In this study, we investigated whether arterial shear stress augments or attenuates the differentiation of EPCs adhered to VEGF-bound surfaces. We observed significant effects of arterial shear stress on cell surface markers. Compared to static conditions, arterial shear stress markedly suppressed mRNA expression of progenitor markers (CD34 and CD133). Higher mRNA expression of EC markers (CD31 and vWF) and higher expression of vWF protein were observed in EPCs adhered to a VEGF-bound surface in simulated arterial flow (Fig. 3A and B). In addition, arterial shear stress promoted the mRNA expression of an arterial endothelial marker (ephrinB2) but did not change expression of a venous endothelial marker (EphB4) (Fig. 3C). These findings are in accordance with previous studies that observed that, in EPCs adhered to an FN-coated surface, shear stress promotes mRNA and protein expression of VEGFR2 and VE-cadherin, which are cell surface markers characteristic of ECs [20]; these studies also observed characteristic mRNA expression profiles of arterial and venous markers [18]. Furthermore, the data reported in this study demonstrate that EPCs adhered to a VEGF-bound surface exhibit more efficient differentiation, as reflected by expression of cell surface markers, than cells on an FN-bound surface under static conditions (Fig. 1B and C).

Focused protein array analysis showed that shear stress activates intracellular signaling pathways in EPCs, including the p38 and ERK1/2 pathways, but does not activate the Akt pathway (Fig. 4), at least at the time point studied (30 min). In this study, we did not conduct experiments using inhibitors for these pathways aiming at direct relation between the activation of the MAPK pathways and differentiation of EPCs. Previous studies demonstrate that the p38 pathway is involved in shear stress-induced angiogenesis *in vivo* [21] and ERK1/2 pathway regulates EC-specific gene expression (CD31, vWF, VE-cadherin, VEGFR2, etc.) in multipotent adult progenitor cells [22]. Therefore, it is suggested that loading of shear stress on EPCs adhered to a VEGF-bound surface augment differentiation via the MAPK pathways.

Shear stress also markedly suppresses the proliferative activity of EPCs adhered to a VEGF-bound surface (Fig. 2). A previous study of the effect of laminar flow on EPCs adhered to an FN-coated surface showed that, at an early period of culture under static conditions followed by exposure to 1 day of arterial shear stress, differentiation activity increased, but no appreciable proliferative activity was observed [20]. On the other hand, following prolonged static culture, 1 day of shear stress loading induced little appreciable differentiation activity, but proliferative activity was increased [20]. Taken their data together with ours, we conclude that differentiation precedes proliferation. Once differentiation reaches a certain extent of maturity, proliferative activity may be augmented.

This study may have clinical relevance. The characteristic feature of double or biphasic stimuli responsive system is that arterial shear stress augments the differentiation of EPCs, which are constitutively stimulated by solid-phase VEGF [16]. In fact, in our ongoing study of VEGF-bound stent implantation in a porcine model, we have observed rapid EPC capture and subsequent complete endothelialization [23]. Therefore, it is strongly suggested that solid-phase VEGF and continuous shear stress from arterial blood flow cooperate to induce endothelialization of implanted devices.

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