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Shear stress augments the endothelial cell differentiation marker expression in late EPCs by upregulating integrins

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ABSTRACT

Vascular endothelial cell injury has been implicated in the onset of atherosclerosis. A number of previous studies have demonstrated that endothelial progenitor cells (EPCs), in particular late EPCs, play important roles in endothelial maintenance and repair. Recent evidence has revealed shear stress as a key regulator for EPC differentiation. However, the detailed events that contribute to the shear stress-induced EPC differentiation, in particular the mechanisms of mechanotransduction, remain to be identified. The present study was undertaken to further confirm the effects of shear stress on the late EPC differentiation, and to investigate the role of integrins in this procedure. Shear stress was observed to increase the expression of endothelial cell differentiation markers, such as vWF and CD31, in late EPCs isolated from rat bone marrow. Shear stress moreover enhanced the mRNA expression of integrin subunits β_1 and β_3 in a time-dependent manner, and also upregulated specific integrins in late EPCs plated on substrates containing various extracellular matrix (ECM) proteins. In addition, the shear stress-induced vWF and CD31 expression were found to be related to the levels of integrin β_1 and β_3, and were inhibited in late EPCs treated with RGD peptide (Gly-Arg-Gly-Asp-Asn-Pro, GRGDNP) that blocks the binding of integrins to the extracellular matrix. Additionally, this increase was also attenuated by both anti-β_1 integrin and anti-β_3 integrin antibodies. The integrin subunits β_1 and β_3 thus play important roles in regulating the shear stress-induced endothelial cell differentiation marker expression in late EPCs. This may provide novel insights into the mechanisms of mechanotransduction in shear stress-mediated late EPC differentiation.

1. Introduction

Atherosclerosis is a chronic inflammatory disease, associated with endothelial dysfunction in its early stages. During the pathogenesis of arteriosclerosis, endothelial cells on the arterial wall are damaged by various means such as hyperlipidemia, hypertension, diabetes and infections [1]. Reendothelialization after arterial injury, and maintenance of the integrity of vascular endothelium are therefore critical for the prevention and treatment of atherosclerotic vascular diseases. It was traditionally believed that the damaged endothelial cells would be replaced by neighboring endothelial replication. However, this pool is very limited, and proliferation is infrequent and not sufficiently active to be able to satisfy this regeneration process [2]. Evidence accumulated in recent years suggests that endothelial progenitor cells (EPCs) play an essential role in repairing endothelial injury [3,4].

EPCs are heterogeneous and can be classified at least into early and late EPCs. Early EPCs appear within 4–7 days while late EPCs develop after 2–3 weeks in ex vivo culture systems. They share some endothelial phenotype but are identified with different morphology, proliferation rate and survival feature [5]. Up until now, most of the studies performed have mainly focused on early rather than late EPCs. However, late EPCs, which express a variety of endothelial markers and functionally differentiate into mature endothelial cells, seem to also be important in promoting vascular integrity and neovascularization [6].

It is generally accepted that many types of cells, such as osteoblasts, endothelial cells and fibroblasts, respond to mechanical forces in their environment. For example, shear stress, the tangential hemodynamic force per area, has been implicated in altering the structural and functional properties of endothelial cells at the cellular and molecular levels with an effect on the homeostasis of vascular endothelium [7,8]. Several studies have shown that shear stress increases EPC differentiation into a mature endothelial phenotype characterized by for example the expression of platelet endothelial cell adhesion molecule1 (PECAM1/CD31) and von Willebrand factor (vWF) [9–11]. These findings suggest that EPCs, similar to mature endothelial cells, respond to shear stress and transmit signals into the cell. The details, however, and in particular the precise mechanism of mechanotransduction, remain to be identified.

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Being located at the cell surface, integrins are possible candidates for the transduction of hemodynamic forces into biochemical signals. Accordingly, a variety of published data suggest that integrins play important roles in the mechanotransduction [12,13]. Cells, such as for example endothelial cells, must be anchored to their matrix in order to sense and transduce signals in response to shear stress. The attachment sites, termed focal adhesions, are complexes of integrins, cytoskeletal and signal proteins. The mechanosensitive integrins can interact dynamically with extracellular matrix proteins, resulting in the activation of many molecules in focal adhesions and the cytoplasm [14,10]. It may thus be proposed that the shear stress-induced endothelial mark expression in late EPCs is mediated by integrins. To address the validity of this assumption, the effects of shear stress on the expression of endothelial marks in late EPCs were investigated. At the same time, the role of integrins in shear stress-mediated EPC differentiation was evaluated. This study may provide useful information on the understanding of the molecular mechanisms involved in the regulation of shear stress on late EPC differentiation, and thus aid the development of new therapeutic strategies to improve late EPC activity.

2. Materials and methods

2.1. Cell culture and identification

The method has been described before [15] and is detailed in the supplementary data.

2.2. Shear stress experiments

Shear stress was applied to the EPCs using a flow chamber system that has been described previously [16]. The intensity of shear stress was calculated using the following formula: $\tau_w = \frac{6\mu}{h^2 b} Q$, where is the shear stress, the medium viscosity (0.0077 g/cm s), Q the volumetric flow rate (2.05 cm^3/s), h the chamber height (0.03 cm) and b the chamber width (2.5 cm). Shear stress at 12 dyne/cm^2 was used for most experiments. In some experiments, the synthetic peptide GRGDNP (GIBCO-BRL), the neutralizing integrin β3 antibody (GIBCO-BRL), or the neutralizing integrin β1 antibody (clone: MEM-101A; Dianova) was pre-incubated for 30 min before exposure to shear stress.

2.3. RNA isolation and quantitative reverse transcription-polymerase chain reaction

Total cellular RNA was isolated with TRIzol reagent (Invitrogen, USA) and reverse-transcribed to cDNA using the SYBR® PrimeScript® RT-PCR Kit (Takara, Japan) at 37 °Cfor 15 min. Gene expression were evaluated by SYBR® Premix Ex Taq™ (Takara, Japan). Rat vWF was amplified with the sense primer 5′-GCG TGG CAG TGG TAG AGT A-3′ and the anti-sense primer: 5′-GGA GAT AGC GGG TGA AAT A-3′; CD31 with the sense primer 5′-GAC AGC CAA GGC AGA TGC AC-3′ and the anti-sense primer: 5′-ATT GGA TGG CTT GGC CTG AA-3′. GAPDH (sense primer: 5′-GGC ACA GTC AAG GCT GAG AAT-3′, anti-sense primer: 5′-ATG GTG GTG AAG ACG CCA GTA-3′) was used as a housekeeping gene, in order to normalize the expression target gene. The thermal cycling conditions were as follows: 30 s at 95 °C for pre-denaturation, 40 cycles for 15 s at 95 °Cfor denaturation, 1 min at 59 °C for annealing, and 10 s at 72 °C for elongation. At the end of each cycle, the fluorescence emitted by the SYBR Green I was measured. After the completion of the cycling process, samples were immediately subjected to a temperature ramp for melting curve analysis. The relative gene expression was analyzed by the $2^{-\Delta\Delta Ct}$ method.

2.4. In situ hybridization

The gene expression of the late EPC integrin subunits β1 and β3 were determined by in situ hybridization according to the manufacturer's protocol (Boster, China). In short, denaturation was performed by incubating the slides on a 96 °C heating block for 5 min, and hybridization was performed by placing the slides in a humidity chamber at 37 °C overnight. After removing the cover-slips, a stringent wash was performed in 0.5× saline-sodium citrate buffer at 80 °C for 5 min. The endogenous peroxidase activity and unspecific staining were blocked by applying 3% hydrogen peroxide and the CAS-Block™, respectively. An antidigoxygenin antibody was added to the slides for 45 min at RT followed by incubation with a polymerized peroxidase-goat anti-mouse antibody (Dako) for 45 min at RT. A DAB chromogen substrate system was used to generate a sensitive signal. After counterstaining with Mayer's haematoxylin, the expression of the integrins could be viewed with microscope (Olympus, Japan).

2.5. Flow cytometry

The expression of CD31, vWF, integrin β1 and β3 were also determined by flow cytometry. Cells were trypsinized and incubated with antibodies (eBioscience, USA) for 1 h. For each antibody, preliminary experiments were performed to determine the antibody concentration necessary to saturate the binding sites. Typically, around 20,000 late EPCs were measured for fluorescent intensity per experiment. In addition, isotype controls were performed for each sample condition, and the mean fluorescent intensity found for the isotype control was subtracted from the mean fluorescent intensity of the antibody-bound cells.

2.6. Statistical analyses

Unless otherwise indicated, results are reported as means ± SD from three independent experiments. Statistical analyses were performed using Student's t test or one-way ANOVA followed by Tukey's test, and $p < 0.05$ was considered statistically significant. All data were analyzed using SPSS software (version 15.0; SPSS, Chicago, IL, USA).

3. Results

3.1. Shear stress increased the expression of vWF and CD31

To further identify the effects of shear stress on the late EPC differentiation, late EPCs were treated with different levels of shear stress (2, 6, 12, and 20 dyne/cm^2) for 12 h, and the gene expression of CD31 and vWF were analyzed by real-time RT-PCR. The results show a dose-dependent increase in the shear-stress induced gene expression of CD31 and vWF in late EPCs above 2 dyne/cm^2, while no effects were observed at or below this level. (Fig. 1A). To confirm the gene data, the protein expression of CD31 and vWF of late EPCs were analyzed by FACS. As for the gene expression, the application of shear stress at 6, 12, and 20 dyne/cm^2 for 24 h led to enhanced CD31 and vWF protein expression, while no effects were observed at the stress level of 2 dyne/cm^2 (Fig. 1B).

3.2. Shear stress induced the expression of integrin β_1 and β_3

To investigate the effects of shear stress on the integrin expression, late EPCs were exposed to shear stress (12 dyne/cm^2) for 3, 6 and 12 h. In Situ Hybridization was used to analyze the gene expression of integrin β_1 and β_3. Shear stress increased the expression of integrin β_1 and β_3 in a time-dependent manner with a max-

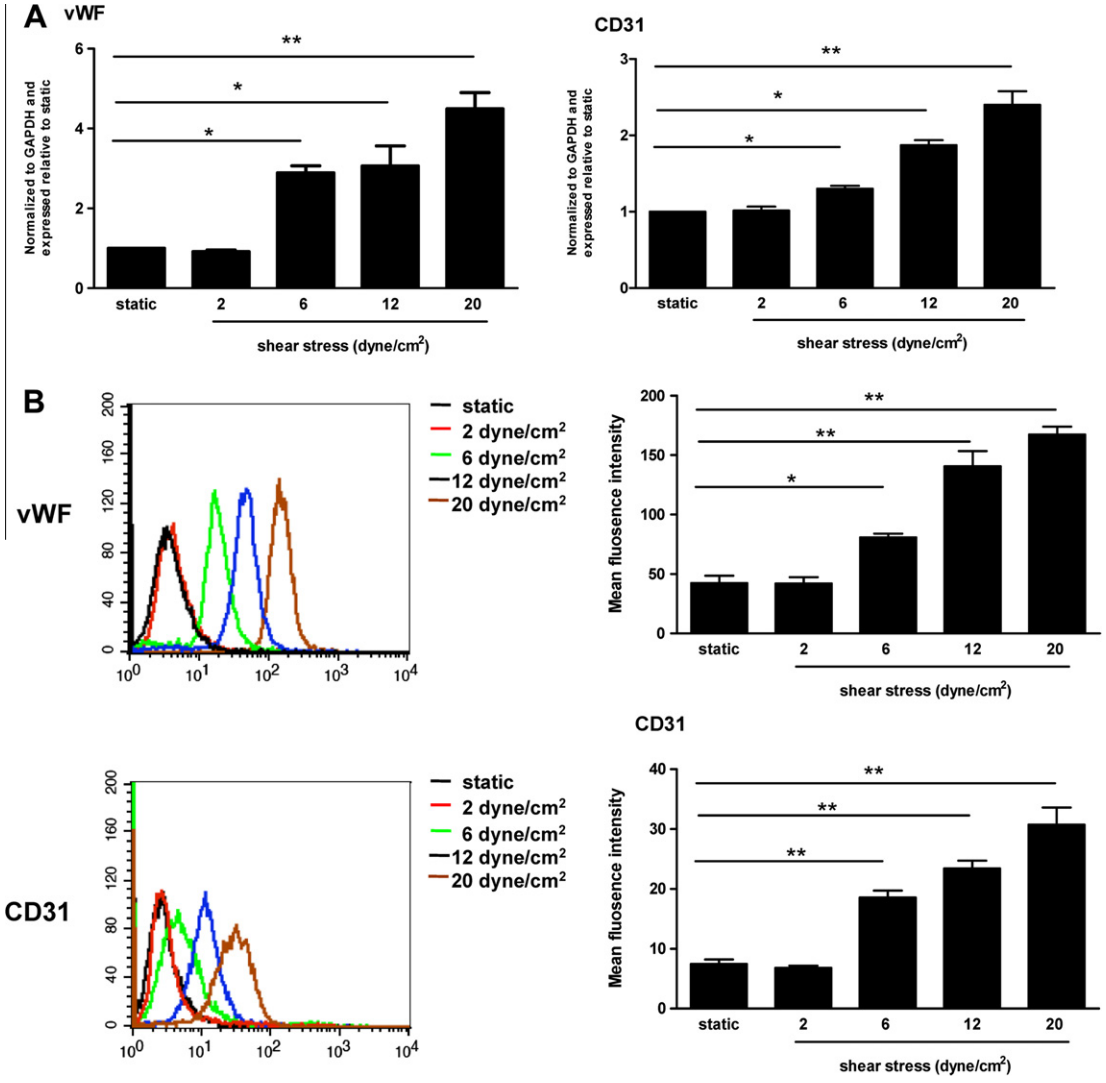

Fig. 1. Shear stress increased the expression of endothelial cell differentiation markers in late EPCs. (A) Late EPCs were seeded onto a glass slide that had been coated with fibronectin (50 μg/cm^2), after which they were either cultured in static condition or exposed to shear stress at varying levels for 12 h. The gene expression of vWF and CD31 were then determined using real time quantitative RT-PCR. The bars, representing the mean ± SD from three separate experiments. (B) Late EPCs were treated with shear stress at varying levels for 24 h, and the protein expression of vWF and CD31 were determined by FACS. Representative FACS profiles of three independent experiments are shown in the left panels. The right panels show the relative fluorescence intensity that is normalized with the mean fluorescence intensity of isotype control. $^{**}p < 0.01$ and $^{*}p < 0.05$.

imum observed at 12 h (Fig. 2A). Moreover, FACS analysis showed that the expression of the integrin subunit β_1 on the late EPC surface was elevated by around a factor 1.74 above the control level after exposure to shear stress for 12 h. In addition, the protein expression of integrin β_3 was also increased in late EPCs treated with shear stress for 12 h (156 ± 12% of static control) (Fig. 2B).

3.3. Effects of various ECM proteins on the shear stress induced-EC mark and integrin expression

To study the effects of various ECM proteins on the shear stress-induced late EPC differentiation, late EPCs were seeded onto culture surfaces coated with fibronectin, collagen I or laminin, followed by exposure to shear stress (12 dyne/cm^2). In comparison to the fibronectin-coated surfaces, laminin and collagen I did not have such a promoting effect on the shear stress-induced CD31 and vWF gene and protein expression (Fig. 3A and B).

As the shear stress-induced signaling and gene expression are possibly mediated by integrins [13], the varying levels of the shear stress-induced EC mark expression among the different ECM proteins can be expected to be related to the different levels of integrin expression. As shown in our FACS analysis, there is no difference of the integrin β_1 and β_3 expression among cultures grown on fibronectin, collagen I and laminin in static conditions. However, the expression of integrin β_1 was increased on all substrata after exposure to shear stress at 12 dyne/cm^2 for 12 h. Among these ECM proteins, fibronectin had the maximal enhancing effect on the shear stress-induced integrin β_1 expression, followed by laminin and finally collagen I. Shear stress also increased the expression of integrin β_3 on fibronectin and laminin, but not on collagen I (Fig. 3C).

3.4. Role of integrins in the shear stress-induced EC mark expression

Having demonstrated that the EC marks upregulated by shear stress were related with the expression of integrin β_3 and β_1, we further went onto determine whether those integrins mediate the fibronectin-enhanced and shear stress-induced EC mark expression in late EPCs. Incubation of late EPCs with RGD peptides (GRGDNP; 0.5 mmol/L), anti-integrin β_3 (10 μg/ml) or anti-integrin

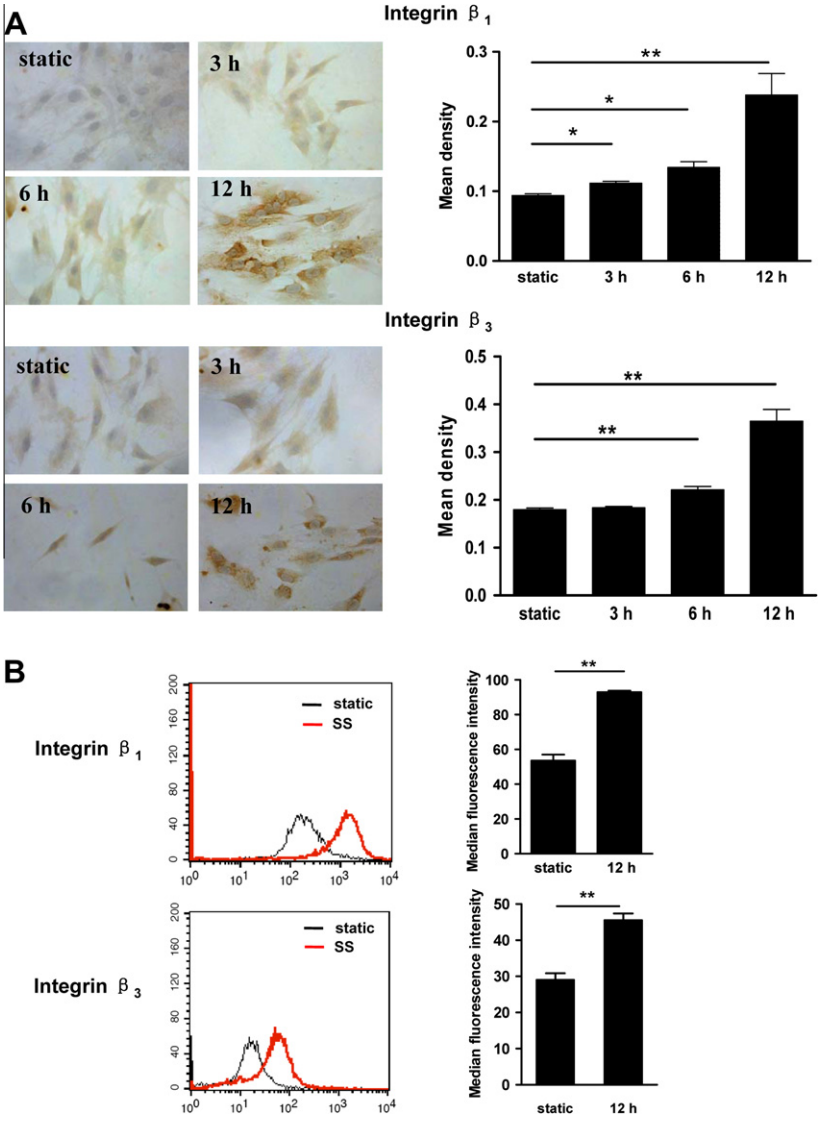

Fig. 2. Shear stress upregulated the expression of integrin β1 and β3. (A) Late EPCs were seeded onto a glass slide that had been coated with fibronectin (5 μg/cm^2), after which they were exposed to 12 dyne/cm^2 shear stress for different exposure times. The gene expression of integrin β1 and β3 were then determined by situ hytohybridization. Representative profiles of three independent experiments are shown in the left panels. The right panels show the mean density that is analyzed by Image-Pro Plus. (B) Late EPCs were treated with 12 dyne/cm^2 shear stress for 12 h. The expression of integrin β_1 and β_3 on the surfaces of late EPCs were determined by FACS. Representative FACS profiles of three independent experiments are shown in the left panels. The right panels show the relative fluorescence intensity that is normalized with the mean fluorescence intensity of isotype control. $^{**}p < 0.01$ and $^{*}p < 0.05$.

β_1 (50 μg/ml) abrogated the shear stress–induced EC mark expression (Fig. 4A and B). In contrast, the control peptides (GRGESP; 0.5 mmol/L), and the control antibody (IgG), produced no visible effects (Data not shown).

4. Discussion

Shear stress plays a significant role in vascular biology. When considering its effects on stem or progenitor cell functions, an important issue is its capacity to induce cell differentiation, especially into vascular cells. Murine ESC-EC exhibits under static conditions limited localization of vascular endothelial cadherin (VE-cadherin) at cell–cell junctions and poor low density lipoprotein (LDL) uptake, but exposed to flow, has shown increased angiogenic and vasculogenic potentials [17]. Furthermore, shear stress promotes an endothelial cell fate in a murine mesenchymal progenitor cell line C3H/10T1/2: following exposure for up to 12 h at 15 dyne/cm^2, cells have been reported to upregulate EC markers including CD31 (PECAM-1), VE-adherin and von Willebrand Factor, and form capillary-like tube structures in a Matrigel assay [18]. Using early EPCs isolated from human peripheral blood, Suzuki et al. have demonstrated that shear stress suppresses the expression of mRNAs encoding CD34 and CD133, which are markers for EPCs, and augments the expression of mRNAs encoding CD31 and von Willebrand factor (vWF) as well as vWF protein, which are markers for endothelial cells (ECs) [19]. Because under normal development late EPCs are more likely to be subjected to shear stress, one might expect that the latter naturally plays a more significant role in the later stages of endothelial differentiation. The present study has demonstrated that exposure to shear stress induces late EPC differentiation. Moreover, it was observed that shear stress of 6, 12, and 20 dyne/cm^2 significantly increased the expression of endothelial cell differentiation markers such as vWF and CD31 in late EPCs, while no effect was seen at the stress level of 2 dyne/cm^2. These findings indicate that the shear stress-induced EPC differentiation is correlated with the intensity of shear stress. If this holds true also in vivo, an explanation could be offered as to why atheroscle-

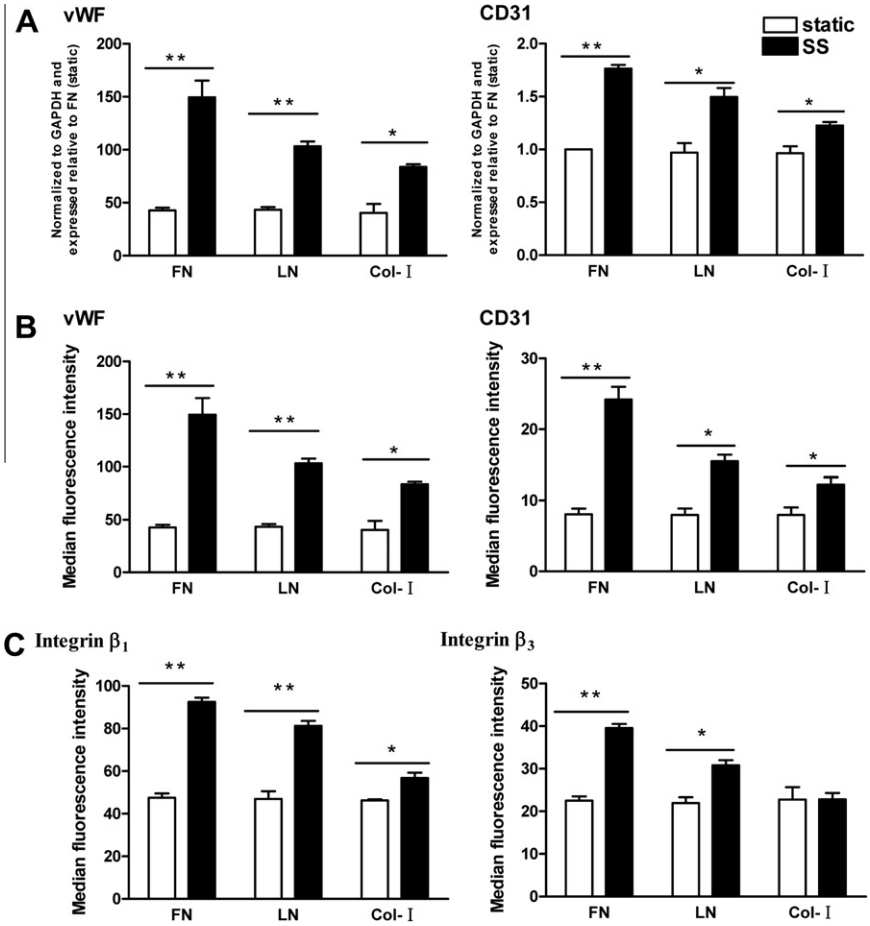

Fig. 3. Effects of different ECM proteins on the shear stress induced-EC mark and integrin expression. (A) Late EPCs were seeded onto culture surfaces coated with fibronectin (5 μg/cm^2), collagen I (5 μg/cm^2) or laminin (5 μg/cm^2), followed by exposure to shear stress (12 dyne/cm^2) for 12 h. The gene expression of vWF and CD31 were determined by real time RT-PCR. The results represent the mean ± SD from three independent experiments. (B) Late EPCs were seeded onto culture surfaces coated with different ECM proteins, followed by exposure to shear stress (12 dyne/cm^2) for 24 h. The protein expression of vWF and CD31 were determined by FACS. The results represent the mean ± SD from three independent experiments. (C) Late EPCs were seeded onto culture surfaces coated with different ECM proteins, followed by exposure to shear stress (12 dyne/cm^2) for 12 h. The expression of integrin β_1 and β_3 on the on the late EPC surfaces were determined by FACS. The results represent the mean ± SD from three independent experiments. $^{**}p < 0.01$ and $^{*}p < 0.05$.

rotic lesions occur predominantly in regions of the vasculature exposed to low shear stress [20].

Due to their specific location at the cell surface, integrins have been recognized as possible candidates for the transduction of hemodynamic forces into biochemical signals [13]. Integrins are moreover responsible for cellular tissue architecture, and they also function as signal transducers regulating survival, proliferation, differentiation and migratory signaling pathways. For example, the upregulation of integrin β_1 is known to induce the expression of endothelial markers, flk-1(VEGFR2/KDR) and VE-cadherin, and may serve as part of the differentiation cue in tumor-associated monocytes [21]. The results in the present study show that shear stress of 12 dyne/cm^2 upregulates the integrin β_1 and β_3 expression in a time-dependent manner. This most likely facilitates the binding of the late EPCs to the matrix, and thus induces the activation of integrins. Therefore, upregulation of the integrin subunits β_1 and β_3 might well explain the augmentation of the shear stress-induced late EPC differentiation. This hypothesis is further strengthened by our finding that late EPCs pretreated with GRGDNP, which blocks the binding of integrins to the ECM, prevented the shear stress-induced endothelial cell differentiation marker gene expression. Moreover, the inhibition was also observed using either anti-integrin β_1 or anti-integrin β_3; however, when β_3 is blocked, the reduction in the CD31 and vWF expression was less than the decrease caused by the blocking of β_1 instead. Thus, integrin β_1 may play a dominant role in the shear stress-induced late EPC differentiation, with a contribution also present from integrin β_3.

The adhesion of stem cells to the ECM is an essential step during differentiation, allowing cells to attach onto a substrate, and to acquire proliferative and survival signals from the underlying matrix. Concerning EPCs, it has earlier been revealed that these cells show higher adhesion and endothelial differentiation when plated on fibronectin [22]. Recently, the role played by integrins in EPC differentiation has been addressed since they involve in VEGF-induced differentiation of EPCs into ECs [23]. The enhanced integrin expression induced by shear stress most likely facilitates binding of the late EPCs to the matrix, promoting EPC differentiation. This conclusion is supported by our findings: the shear stress-induced expression of integrin β_1 was found to be higher on fibronectin-coated surfaces than on surfaces coated with laminin or collagen I. Shear stress also increased the expression of integrin β_3 on fibronectin and laminin, but not on collagen I. In line with this finding, fibronectin exhibited the maximal stimulatory effect on the shear stress-induced EPC differentiation, followed by laminin, and then collagen I.

In summary, this study demonstrates that shear stress upregulates the expression of integrin β_1 and β_3, resulting in late EPC differentiation into endothelial cells. This can hopefully lead to a

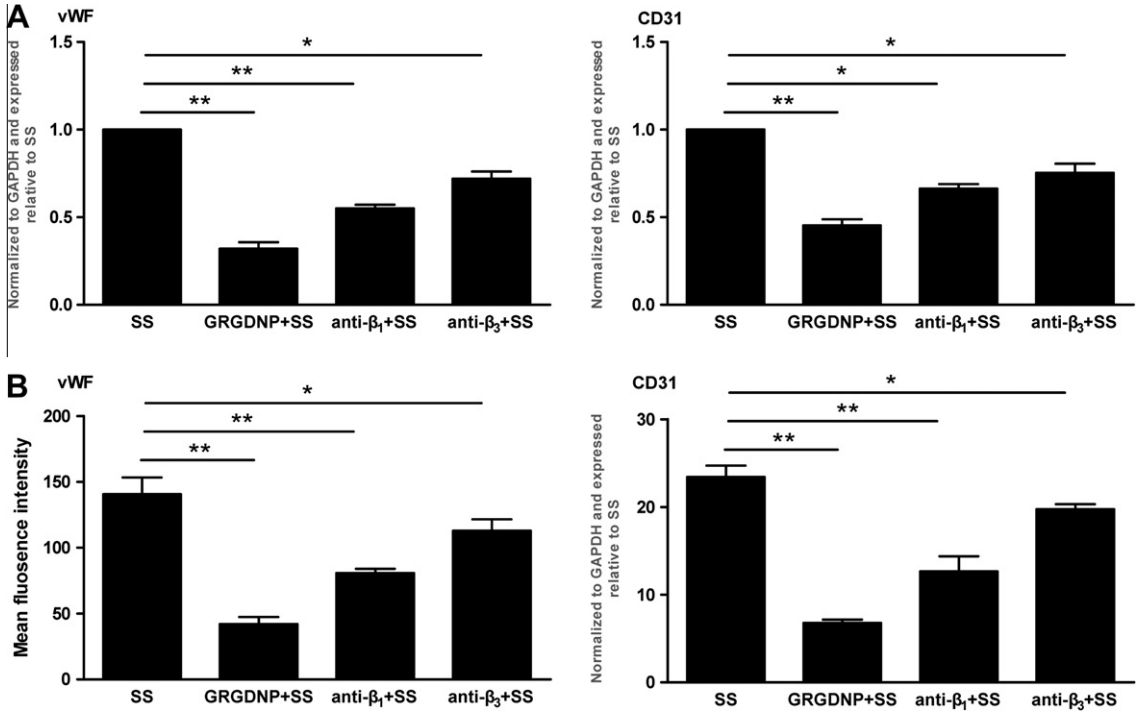

Fig. 4. Role of integrins in the shear stress-induced EC mark expression. Late EPCs were seeded onto culture surfaces coated with fibronectin. Before being exposed to shear stress, late EPCs were pretreated with mouse RGD peptides (GRGDNP; 0.5 mmol/L), anti-integrin β_3 (10 μg/ml) or anti-integrin β_1 (50 μg/ml) for 30 min. The gene (A) and protein (B) expression of vWF and CD31 were determined by real time RT-PCR and FACS, respectively. The results represent the mean ± SD from three independent experiments. $^{**}p < 0.01$ and $^{*}p < 0.05$.

better understanding of the mechanisms of mechanotransduction by which shear stress regulates the EPC differentiation.

5. Competing interests

None.

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.2012.07.115.

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