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CXCR1 and CXCR2 are novel mechano-sensors mediating laminar shear stress-induced endothelial cell migration

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ABSTRACT

The migration of endothelial cells (ECs) plays critical roles in vascular physiology and pathology. The receptors CXCR1 and CXCR2, known as G protein-coupled receptors which are essential for migratory response of ECs toward the shear stress-dependent CXCL8 (interleukin-8), are potential mechano-sensors for mechanotransduction of the hemodynamic forces. In present study, the mRNA and protein expression of CXCR1 and CXCR2 in EA.hy926 cells was detected by RT-PCR and Western blot analysis under three conditions of laminar shear stress (5.56, 10.02 and 15.27 dyn/cm^2) respectively. Using a scratched-wound assay, the effects of CXCR1 and CXCR2 were assessed by the percentage of wound closure while CXCR1 and CXCR2 were functional blocked by the CXCL8 receptor antibodies. The results showed that the mRNA and protein expression of CXCR1 and CXCR2 was both upregulated by 5.56 dyn/cm^2 laminar shear stress, but was both downregulated by 15.27 dyn/cm^2. The wound closure was inhibited significantly while cells were treated with those antibodies in all the conditions. It was suggested that CXCR1 and CXCR2 are involved in mediating the laminar shear stress-induced EC migration. Taken together, these findings indicated that CXCR1 and CXCR2 are novel mechano-sensors mediating laminar shear stress-induced EC migration. Understanding this expanded mechanism of laminar shear stress-induced cell migration will provide novel molecular targets for therapeutic intervention in cancer and cardiovascular diseases.

1. Introduction

In vascular blood vessels, shear stress is borne primarily by endothelial cells (ECs) [1]. Shear stress has been shown to modulate endothelial cell (EC) migration by Ando et al. [2]. The migration of ECs plays critical roles in vascular physiology and pathology. For example, EC migration is a prominent component of re-endothelization, vascular remodeling and angiogenesis. In post-operative (i.e., balloon angioplasty or bypass surgery) wound progress, the migration of nearby ECs into the injured area is crucial for wound closure and it could prevent the development of restenosis eventually.

Persistent cell migration is a complex process that is physically coordinated both spatially and temporally, including the dynamic processes of cytoskeleton, the regulation of signal transduction, and the adhesion and detachment to neighboring cells or extracellular matrix (ECM) [3–5]. There are many studies focused on both the molecular basis underlying modulation of EC migration by the fluid dynamic forces, and the spatially and temporally cellular signaling transduction [1,6]. However, how the mechanical forces are transduced into ECs is unclear until now. So far as is known, there are mechanical effects induced by fluid shear stress, contributing to cell deformation, spreading, movement, and shape changes [7,8]. The mechanical force can be highly sensitive by "biochemical receptors" that act as mechano-sensors transducing shear stress into cell biological responses [1,6].

CXCR1 and CXCR2 are G protein-coupled receptors. The migration of human umbilical vein endothelial cells (HUVECs) toward CXCL8 is related to both CXCR1 and CXCR2 *in vitro* static conditions [9]. CXCL8 is one of the important chemokines that regulates cell cytoskeleton reassembly and migration [10]. Our previous studies showed that CXCL8 and shear stress were interacted in modulation the functional activities of HUVECs [11], and the mRNA expression of CXCL8 induced by shear stress was force intensity-dependent and time-dependent [12,13]. When HUVECs exposed to low fluid shear stress (2.23 dyn/cm^2) for 1 or 2 h, the expression of CXCL8 mRNA increased about 68 or 52 times as that of HUVECs exposed to high fluid shear stress (19.29 dyn/cm^2). In additional to endothelial cells, CXCR1 and CXCR2 are expressed on melanoma [14], neutralization of CXCR1 and CXCR2 using small molecule antagonists affects cell proliferation and migration [14], and

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downregulation of CXCR1 or CXCR2 by employed a gene knockdown strategy decreasing melanoma cell migration [15]. Therefore, these results suggested that CXCR1 and CXCR2 are potential mechano-sensors mediating the shear stress-induced EC migration.

Specially, different culture conditions *in vitro* have various influences on the expression of CXCR1 and CXCR2 in ECs, although the expression level of CXCR1 and CXCR2 is very low *in vitro* static conditions [9,16–18].

Our investigations in this study focused on the mechanical–biological mechanism of the migratory response of ECs during exposed to laminar shear flow. Firstly, the mRNA expression of CXCR1 and CXCR2 in EA.hy926 cells under three conditions of laminar shear stress (5.56, 10.02 and 15.27 dyn/cm^2) was detected by RT-PCR analysis, and the protein expression of them was also detected by Western blot analysis. Secondly, a scratched-wound assay was used while the receptors CXCR1 and CXCR2 were functional blocked by anti-CXCR1 alone, anti-CXCR2 alone and anti-CXCR1 in combination with anti-CXCR2. Finally, the effects of CXCR1 and CXCR2 were assessed by the index of the percentage of the wound closure. Our results showed that the mRNA and protein expression of CXCR1 and CXCR2 was both upregulated by 5.56 dyn/cm^2 laminar shear stress, but was both downregulated by 15.27 dyn/cm^2. The wound closure was inhibited significantly when CXCR1 and/or CXCR2 were blocked by those CXCL8 antibodies. Our results confirmed that the receptors CXCR1 and CXCR2 are involved in mediating the laminar shear stress-induced EC migration. Overall, these findings suggested that CXCR1 and CXCR2 played mechano-sensitive roles in modulation of laminar shear stress-induced EC migration.

2. Materials and methods

2.1. Cell culture

The EA.hy926 cells (Hematology Research Institute of Jiangsu Province, China) are a hybridoma between HUVECs and the epithelioma A549 and retain most of the features of HUVECs, including the expression of endothelial adhesion molecules and human factor VIII–related Ag [19]. EA.hy926 cells were cultured at 37 °C with 5% CO_2 in RPMI1640 medium (pH 7.2–7.4, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., China), 20 mmol/L HEPES (Sigma, USA), 2‰ $NaHCO_3$ and 2% HAT (Sigma, USA). Cells of passage 3 or 4 were then seeded onto glass slides that were pre-coated with 50 μg·ml^{-1} Fibronectin (Roche, Germany) for 45 min at 37 °C. In most experiments, the cells were used 1–2 days after attaining confluence, although a few experiments were performed 3–5 days postconfluence. The postconfluence time had no influence on the results.

2.2. Shear stress experiments

EA.hy926 cells were exposed to steady laminar shear stress using a parallel flow chamber as previously described elsewhere [20,21]. Briefly, the flow chamber was connected to a recirculation flow loop via Masterflex PharMed tube (Cole-Parmer Instrument, Vernon Hills, IL). Flow in the loop was provided by a peristaltic pump (Cole-Parmer, USA) drawing fluid (serum-free RPMI1640 medium) from a main reservoir, which subsequently passed into two buffer reservoirs to dampened pulsatility before entering the flow chamber. Flow exiting from the chamber was recirculated back into the main reservoir. Medium was equilibrated with 5% CO_2 /95% air, and the temperature was maintained at 37 °C by placing the main and buffer reservoirs in a temperature-controlled water bath. Laminar shear stress was controlled by changing the medium flow rate. Three levels of shear stress were applied in this study: a relatively "low" shear stress of 5.56 dyn/cm^2 that typifies shear stress levels within disturbed flow regions, a relatively "high" shear stress of 15.27 dyn/cm^2 that is typical for undisturbed flow zones within large arteries, and a relatively "mid" shear stress of 10.02 dyn/cm^2 reflecting within the physiological range in human major arteries. Static controls were also concurrently performed.

2.3. RT-PCR detection

The mRNA expression of CXCR1 and CXCR2 in shear stress stimulated EA.hy926 cells was semi-quantitatively detected by reverse transcription–polymerase chain reaction (RT-PCR) after various exposure time. Total RNA was isolated from the cells using the TRIzol reagent (Invitrogen, USA), and then RT-PCR was carried out by using the TaKaRa One Step RNA PCR Kit (AMV) (TaKaRa, Dalian, China) according to the manufacturer's instructions. To obtain the relative quantitative values for gene expression, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. Sequences of the primers used for RT-PCR were designed by Dalian Biotechnology (China) as follows:

Gene name: CXCR1.
Sense primer: 5′CCAGTCCAGTTTGCTATGAGGT3′.
Anti-sense primer: 5′TGTAGGAGGTAACACGATGACG3′.
Gene name: CXCR2.

Sense primer: 5′TACTCTTTGCCCTGACCTTGC3′.
Anti-sense primer: 5′GGGTGAATCCGTAGCAGAACA3′.

Gene name: GAPDH.

Sense primer: 5′AGAAGGCTGGGGCTCATTTG3′.
Anti-sense primer: 5′AGGGGCCATCCACAGTCTTC3′.

The PCR products were visualized by electrophoresis on a 1% agarose gel in 1 × Tris–acetate-EDTA (TAE) buffer after staining with 0.5 μg·ml^{-1} ethidium bromide. Images were analyzed by Image Pro Plus 6.0 (Media Cybernetics, USA).

2.4. Western blot analysis

EA.hy926 cells were treated with different shear stress magnitudes at various exposure time, and then proteins were extracted according to protein extraction kit (PMSF methods, Beyotime Biotech, China). Protein concentration was measured using a bicinchoninic acid protein assay (BCA method, BioDev-Tech. Co., Ltd., China). Protein samples (80 μg/lane) were separated on precast SDS–PAGE (10% resolving gel with 5% stacking) and semi-dry transferred to the polyvinylidene fluoride membranes (PVDF, Millipore, USA). Membranes were blocked for 2 h at room temperature with blocking buffer containing 5% non-fat dry milk powder in Tris buffered saline containing 0.1% Tween-20 (TBS-T), and probed with 1:500 (or 1:200) diluted primary antibodies (Santa Cruz Biotechnology, USA) specific for the target protein. Membranes were incubated at 4 °C overnight, followed by incubation for 1 h at room temperature with a 1:5000 diluted HRP-linked secondary antibodies goat antimouse IgG (Zhongshan Goldenbridge Biotechnology Co., Ltd., China). Immunoreactive proteins were visualized using the enhanced chemiluminescence (ECL, Pierce, USA) following the manufacturer's instructions. Images were captured by Omega 16ic™ AFP-Imaging System (ULTRA·LUM, INC., USA), and analyzed by Image Pro Plus 6.0 (Media Cybernetics, USA).

2.5. Scratch wound migration assay

Cell migration was measured using a monolayer scratch injury assay, as described previously [22,23]. Briefly, the EA.hy926 cells

were seeded on glass chamber slides and cultured until confluence. Then, a uniform straight scratch was performed in the cell monolayer by using a plastic Cell Scrapper (Corning, USA). Monolayers were washed gently, marked (for reference) and photographed using an inverted microscope (Nikon Eclipse TE2000-Y system, Japan). Cells treating without or with 50 ng · ml^{-1} anti-CXCR1 or/and anti-CXCR2 (Santa Cruz Biotechnology, USA), and laminar shear flows were applied in the direction that is orthogonal to the wound. Images of the wounds under both static and flow conditions were acquired at 1, 2, 4 and 8 h. Shear controls (cells were treated without antibodies) were also concurrently performed.

2.6. Assessment of wound closure

EA.hy926 cell migration during the process of wound closure was analyzed using the image analysis software (Image Pro Plus 6.0, Media Cybernetics, USA). The acquired image was converted from pixels to micrometers with the use of a calibration image, then the area could be tabulated while an outline of the wound was traced [24,25]. The levels of wound closure could be assessed by the ratio of closure area to initial wound as follows:

$$R_n = \frac{(A_0 - A_n)}{A_0} \times 100\%$$

where R_n represents the percentage of wound closure; A_n represents the residual area of wound at the metering point n (h) and A_0 represents the area of initial wound (Fig. 1).

2.7. Statistical analysis

Data were presented as means ± SD at least 10 images obtained from three different experiments, unless otherwise indicated. Statistical analysis was performed by one-way ANOVA test using SPSS 17.0 software package. Differences in means were considered significant if $P < 0.05$.

3. Results and analysis

3.1. The mRNA expression of CXCR1 and CXCR2 in EA.hy926 cells is upregulated by 5.56 dyn/cm^2, but is downregulated by 10.02 and 15.27 dyn/cm^2

The mRNA expression of CXCR1 and CXCR2 was assessed by RT-PCR (Fig. 2). Under low shear stress of 5.56 dyn/cm^2, a trend toward increasing of the mRNA expression of CXCR1 and CXCR2 with exposure time was observed (Fig. 2B). Specifically, the mRNA expression of CXCR1 was significantly increased at 25 min ($P < 0.05$), peaked at 2 h (63.87 ± 9.45% at 2 h vs. 45.27 ± 0.97% under static conditions, $P < 0.01$), subsequently, slight descent occurred at 4 h (60.02 ± 1.23% at 4 h vs. 45.27 ± 0.97% under static conditions, $P < 0.01$). The mRNA expression of CXCR2 was significantly increased at 30 min ($P < 0.05$), and peaked at 2 h (37.68 ± 2.7% at 2 h vs. 21.4 ± 1.12% under static conditions, $P < 0.01$), respectively.

Under 10.02 and 15.27 dyn/cm^2, the mRNA expression of CXCR1 had an apparent descending trend with exposure time (Fig. 2C and 2D). It was significantly decreased after 25 min ($P < 0.05$). Similarly, under 15.27 dyn/cm^2, the mRNA expression of CXCR2 also had a declinate trend with exposure time (Fig. 2D). It was significantly decreased at 20 min later ($P < 0.05$). However, the mRNA expression of CXCR2 under 10.02 dyn/cm^2 was relatively stable (Fig. 2C).

Interestingly, the mRNA expression of CXCR1 and CXCR2 appeared a fast increase in 30 min $\sim$ 1 h (about 9.79% and 6.12%, respectively) under 5.56 dyn/cm^2, a rapid decrease in 30 min $\sim$ 1 h (about 6.52% and 2.01%, respectively) under 10.02 dyn/cm^2 and also a rapid decrease in 25 $\sim$ 30 min (about 8.16% and 3.70%, respectively) under 15.27 dyn/cm^2. These results suggested that a rapid onset occurred at different exposure time while cells respond to different shear stress.

Overall, these results demonstrated that the mRNA expression of CXCR1 and CXCR2 in EA.hy926 cells was shear stress-dependent. In addition, in all these shear stress conditions, the mRNA expression of CXCR1 was much higher than that of CXCR2 ($P < 0.01$).

3.2. The protein expression of CXCR1 and CXCR2 in EA.hy926 cells is also shear stress-dependent

The protein expression of CXCR1 and CXCR2 was measured by Western blot (Fig. 3). Under low shear stress of 5.56 dyn/cm^2, the protein expression of CXCR1 and CXCR2 was increased in 1 $\sim$ 4 h ($P < 0.05$ at 1 h, $P < 0.01$ at 2 and 4 h), peaked at 4 h and maintained relatively stable later. For instance, under low shear stress for 4 h, the protein expression of CXCR1 and CXCR2 was respectively increased to about 2.3 and 4.3 times than that under static conditions.

Under mid shear stress of 10.02 dyn/cm^2, the protein expression of CXCR1 and CXCR2 was increased slightly. Specifically, the significant increase was observed in the protein expression of CXCR1 at 2 h ($P < 0.05$) while not in that of CXCR2.

Under high shear stress of 15.27 dyn/cm^2, the protein expression of CXCR1 and CXCR2 was decreased with exposure time. Both

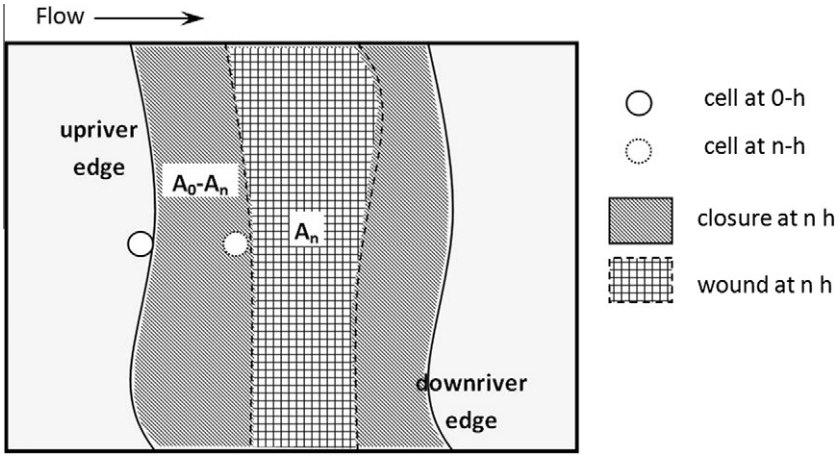

Fig. 1. Definition of quantitatively assess the wound closure by the percentage of wound closure. The percentage of wound closure was computed by $R_n = \frac{(A_0 - A_n)}{A_0} \times 100\%$; where R_n represents the percentage of wound closure; A_n represents the residual area of wound at the metering point n (h) and A_0 represents the area of initial wound.

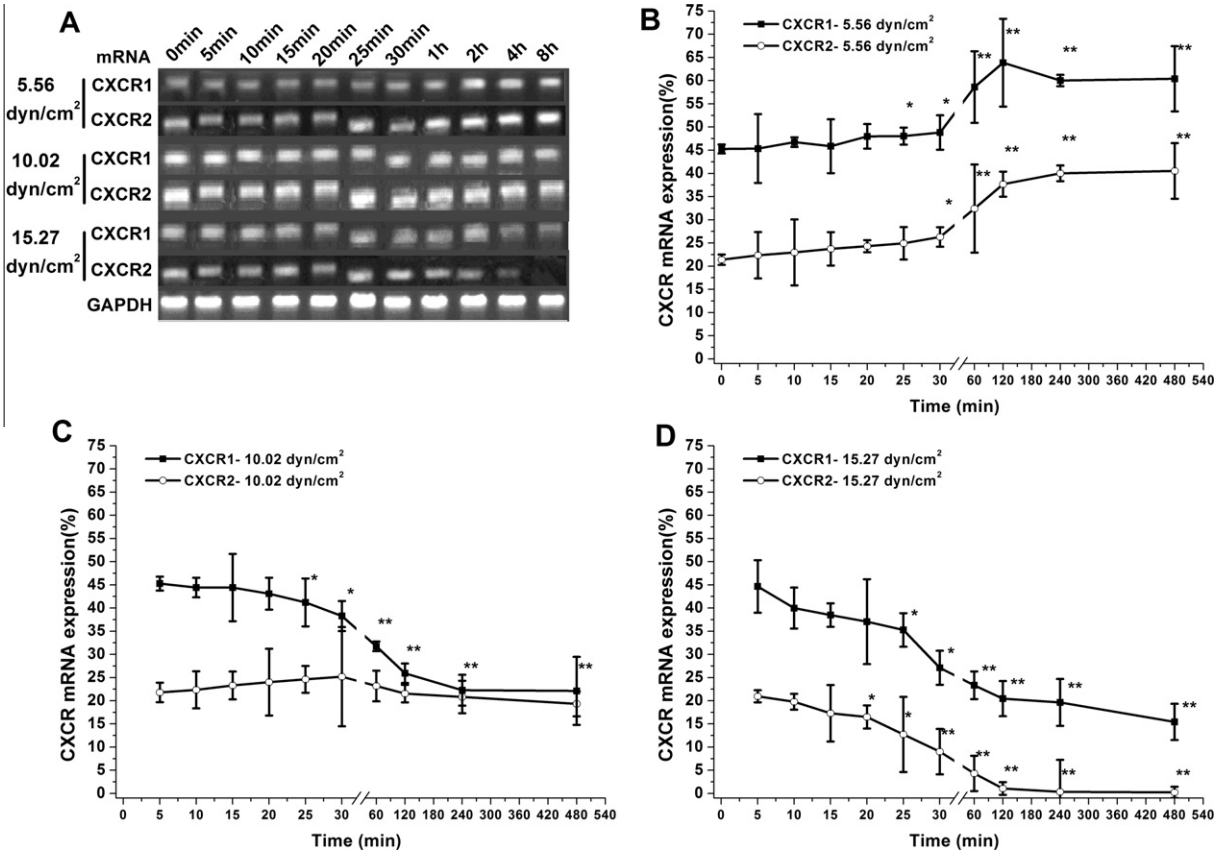

Fig. 2. The mRNA expression of CXCR1 and CXCR2 in EA.hy926 cells under laminar shear flow. The mRNA expression of CXCR1 and CXCR2 at various exposure time was semi-quantitatively detected by reverse transcription–polymerase chain reaction (RT-PCR). In order to obtain the relative quantitative values for gene expression, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. (A) The PCR products were visualized by electrophoresis on a 1% agarose gel in 1 × Tris–acetate-EDTA (TAE) buffer after staining with 0.5 $\mu g \cdot ml^{-1}$ ethidium bromide. (B) ~ (D) Images were analyzed by Image Pro Plus 6.0 (Media Cybernetics, USA). All data presented as the fold-change over control in GAPDH. The CXCR1 mRNA and CXCR2 mRNA were both increased under low shear stress (5.56 dyn/cm^2), and decreased under high shear stress (15.27 dyn/cm^2). Bars indicated mean ± SD, $^{*}P < 0.05$ vs. static control (the CXCR1 mRNA and the CXCR2 mRNA, respectively), $^{**}P < 0.01$ vs. static control.

of them were significantly decreased at 2 h ($P < 0.05$). Specially, the expression of the CXCR2 protein declined rapidly in 2 ~ 4 h ($P < 0.01$ at 4 h later).

Similar to above results of the mRNA expression of CXCR1 and CXCR2, these results suggested that the protein expression of CXCR1 and CXCR2 in EA.hy926 cells was also shear stress-dependent, which was upregulated by 5.56 dyn/cm^2, but was downregulated by 15.27 dyn/cm^2.

3.3. Laminar shear stress enhances the asymmetric EC migration

Fig. 4 showed the percentages of the wound closure when EA.hy926 cells were exposed to different magnitudes of laminar shear stress for various time. It was clear that shear stress caused significantly faster wound closure than the static control ($P < 0.05$). Specifically, compared with the static control, the percentages of wound closure induced by 5.56, 10.02 and 15.27 dyn/cm^2 was respectively increased about 2, 2.4 and 3.3 times at 1 h, about 1.6, 1.9 and 2.5 times at 2 h, about 1.9, 2.4 and 3.1 times at 4 h, and about 2, 2.4 and 3 times at 8 h. Therefore, it can be concluded that laminar shear stress enhances the migration of endothelial cells.

In particular, under static conditions, cells from both edges of the wound migrated equally to cover the wounded area (Fig. 4A). However, under 5.56 dyn/cm^2 shear stress, the covered area on each side of the wound was different. More specifically, cells in the upriver of the wound migrated farther than that in the downriver (Fig. 4A). It could be found distinctly at 8 h. The same phenomenon also occurred in the shear conditions of 10.02 and 15.27 dyn/cm^2. It was indicated that the laminar shear flow has a pushing force to cells in upriver and a retarding force to cells in downriver, which is responsible for the asymmetric migration of ECs, i.e., the pushing force can enhance the wound closure while the retarding force appears inhibiting effects. In other words, laminar shear flow provides a traction force for asymmetric cell migration along the direction of flow.

3.4. Laminar shear stress-enhanced EC migration via CXCR1 and CXCR2

EA.hy926 cells were treated with 50 $ng \cdot ml^{-1}$ anti-CXCR1 and/or anti-CXCR2. Then, the shear stress induced wound closure was obtained as shown in Figs 5–7.

Under 5.56 dyn/cm^2 shear stress (Fig. 5), the levels of wound closure were inhibited while cells were treated with anti-CXCR1 alone (about 43% at 1 h, 45% at 2 h, 42% at 4 h and 42% at 8 h, respectively. $P < 0.05$ vs. 5.56 dyn/cm^2 control), with anti-CXCR2 alone (about 65% at 2 h, 62% at 4 h and 62% at 8 h, respectively. $P < 0.05$ vs. 5.56 dyn/cm^2 control), and with anti-CXCR1 in combination with anti-CXCR2 (about 34% at 1 h, 35% at 2 h, 31% at 4 h and 29% at 8 h, respectively. $P < 0.05$ vs. 5.56 dyn/cm^2 control).

Under 10.02 dyn/cm^2 shear stress (Fig. 6), the levels of wound closure were also inhibited while cells were treated with anti-CXCR1 alone (about 43% at 2 h, 39% at 4 h and 40% at 8 h,

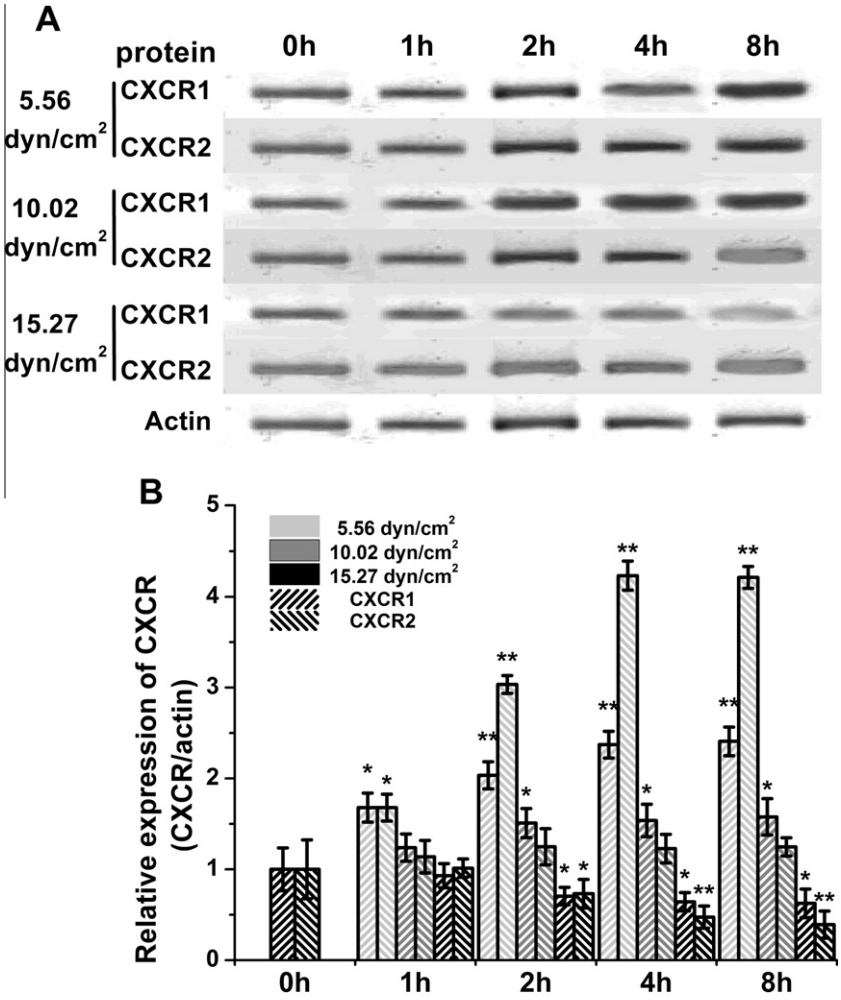

Fig. 3. The protein expression of CXCR1 and CXCR2 in EA.hy926 cells under laminar shear flow. The protein expression of CXCR1 and CXCR2 was measured by Western blot. (A) EA.hy926 cells were treated with different mechanical stimuli for various time and then proteins were extracted. Immunoreactive proteins were visualized using the enhanced chemiluminescence (ECL, Pierce, USA). Images were captured by using Omega 16ic™ AFP-Imaging System (ULTRA·LUM, INC., USA), and analyzed by Image Pro Plus 6.0 (Media Cybernetics, USA). (B) Relative quantification of the CXCR1 and CXCR2 proteins in EA.hy926 cells. Western blot analysis showed that CXCR1 and CXCR2 were both increased under 5.56 dyn/cm^2, but were both decreased under 15.27 dyn/cm^2. Bars indicated mean ± SD, $^{*}P < 0.05$ vs. static control (the CXCR1 protein and the CXCR2 protein, respectively), $^{**}P < 0.01$ vs. static control.

respectively. $P < 0.05$ vs. 10.02 dyn/cm^2 control), with anti-CXCR2 alone (about 57% at 2 h, 51% at 4 h and 49% at 8 h, respectively. $P < 0.05$ vs. 10.02 dyn/cm^2 control), and with anti-CXCR1 in combination with anti-CXCR2 (about 35% at 1 h, 30% at 2 h, 24% at 4 h and 37% at 8 h, respectively. $P < 0.05$ vs. 10.02 dyn/cm^2 control).

Similar to those results under shear stress of 5.56 and 10.02 dyn/cm^2, under 15.27 dyn/cm^2 shear stress (Fig. 7), the levels of wound closure were inhibited while cells were treated with anti-CXCR1 alone (about 50% at 1 h, 47% at 2 h, 46% at 4 h and 48% at 8 h, respectively. $P < 0.05$ vs. 15.27 dyn/cm^2 control), with anti-CXCR2 alone (about 77% at 1 h, 35% at 2 h, 53% at 4 h and 60% at 8 h, respectively. $P < 0.05$ vs. 15.27 dyn/cm^2 control), and with anti-CXCR1 in combination with anti-CXCR2 (about 46% at 1 h, 41% at 2 h, 43% at 4 h and 45% at 8 h, respectively. $P < 0.05$ vs. 15.27 dyn/cm^2 control).

As mentioned above, different percentages of wound closures were obtained under different conditions. It was implied that the mechanism of CXCR1 and CXCR2 mediating cell migration may be different in various shear conditions. However, the wound closure which is depended on shear stress is inhibited by the antibodies at all these magnitudes. These results suggested that laminar shear stress-enhanced EC migration via CXCR1 and CXCR2.

4. Discussion

Our investigation demonstrates CXCR1 and CXCR2 are novel mechano-sensors mediating laminar shear stress-induced EC migration. The vascular endothelial cell monolayer is a metabolically active monolayer which is constantly exposed to both biochemical and biomechanical stimuli in the forms of cyclic stretch, hydrostatic pressure and shear stress [1]. In vascular remodeling, fluid shear stress is a primary stimulus. Blood flow in the straight parts of the aorta is a relatively simple laminar flow with a magnitude of shear stress in the range of 10–40 dyn/cm^2. The flow will become disturbed and unsteady at the atherosclerosis-prone regions of the arterial tree (at curvatures and bifurcations), and there may occur a secondary flow with low velocities [26]. Exposing ECs to low shear stress would lead to an inflammatory and hence proatherogenic cellular phenotype, whereas subjecting cells to high shear stress results in a phenotype that is largely anti-inflammatory and therefore atheroprotective [6,27]. Shear stress dose-dependently enhances migration of HUVECs, with a maximum at 45 dyn/cm^2 laminar shear stress [28]. In present study, three levels of shear stress are adopted: a relatively "low" shear stress of 5.56 dyn/cm^2 that typifies shear stress levels

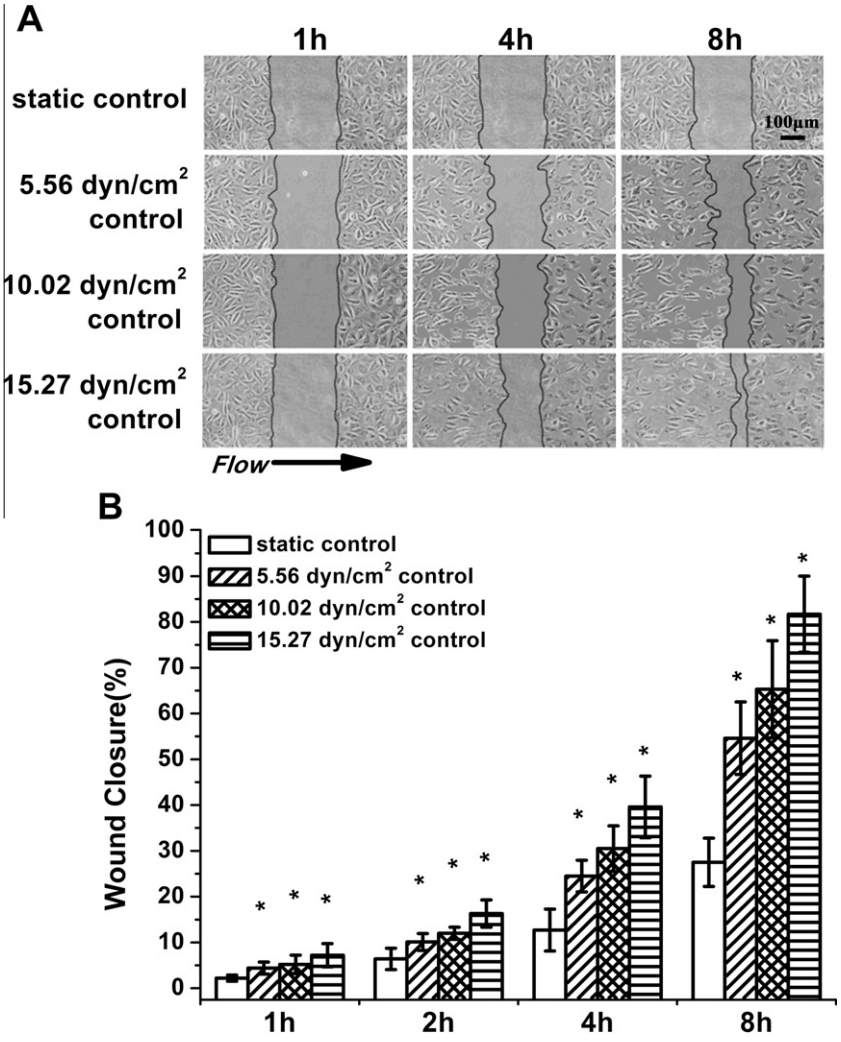

Fig. 4. Effect of laminar shear stress on wound closure and EA.hy926 cell migration. (A) Laminar shear stress enhanced the wound closure progression. EA.hy926 cells were respectively exposed to three different levels of laminar shear stress 5.56, 10.02 and 15.27 dyn/cm^2 for 1, 4 and 8 h. (B) The percentage of wound closure under different laminar shear stress for various time. Shear stress caused significantly faster wound closure than the static control. The wound closure was increased with the shear stress and time. More specifically, cells in the upriver of the wound migrated farther than that in the downriver. It could be found distinctly at 8 h. It was suggested that laminar shear flow provides a traction force for cell migration along the direction of flow. Overall, laminar shear stress enhances the asymmetric EC migration. Bars indicated mean ± SD. Control: cells were treated without CXCL8 receptor antibodies in various conditions. $^*P < 0.05$ vs. static control.

within disturbed flow regions, a relatively "high" shear stress of 15.27 dyn/cm^2 that is typical for undisturbed flow zones within large arteries, and a relatively "mid" shear stress of 10.02 dyn/cm^2 reflecting within the physiological range in human major arteries. Shear stress at all these magnitudes has been shown to activate EC signal transduction and induced the expression of several genes *in vitro* [29].

Wounding of EA.hy926 cells confluent monolayer provokes cell migration in the direction of the wound under both static and flow conditions. Our results show that the wound closure of the EA.hy926 cells monolayer on each side covers equally to the wounded area under static conditions, but appears different migrating behaviors under flow conditions. For instance, cells in the upriver of the wound migrate farther than that in the downriver. It indicates that the flow has a pushing force to the cells in upriver edge and a retarding force to the cells in downriver edge. In other words, laminar shear flow produces a traction force which contributes to the cell migration. It is also documented by Hsu and Gojova et al. that there exists an asymmetry of the speed profiles between the upriver and the downriver sides in the wounded bovine aortic endothelial cells (BAECs) monolayer [21,30]. Recent studies have shown that shear stress-induced asymmetric shape and migration direction of ECs are mediated through Rho and Rac GTPases [31]. Shiu et al. [32] reported that shear stress enhances the migration speed of ECs by modulating the biophysical forces of traction through the biochemical activation of Rho-associated protein kinase Rhop160ROCK. Therefore, shear stress may enhance the asymmetric migration may through the traction and the downstream signaling activation of Rho and Rac GTPases.

Albuquerque et al. [24] reported that cell area increases with time even in static monolayer; shear stress significantly enhances cell spreading, and shear stress (3 dyn/cm^2) also stimulates the largest increase of cell area in both HUVECs and HCAECs. In present study, we assume that both cell spreading and cell area are invariable under the same shear stress and at the same exposure time. Hence, the different percentages of wound closure under the same conditions are due to cell migration.

All ELR + CXC chemokines mediating angiogenesis highlights the importance of identifying a common receptor that mediates their biological function in promoting angiogenesis [33]. The candidate CXC chemokine receptors are CXCR1 and/or CXCR2.

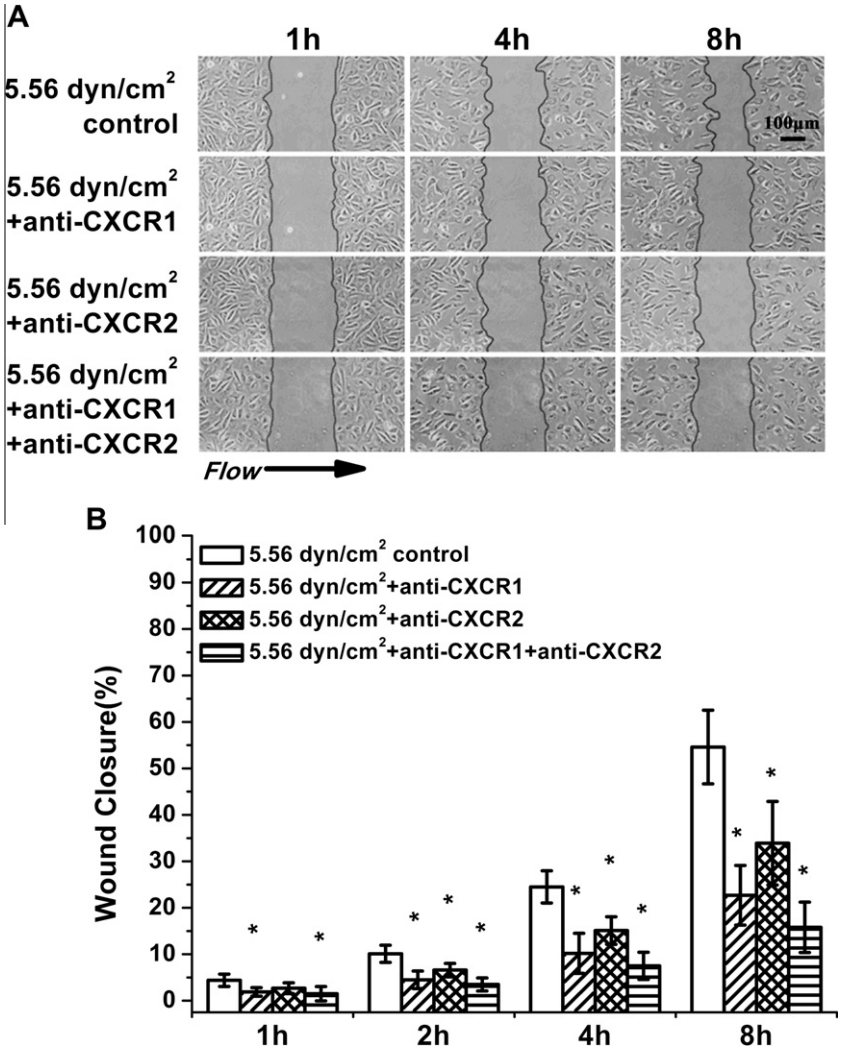

Fig. 5. 5.56 dyn/cm^2 shear stress-enhanced wound closure was inhibited by the CXCL8 receptor antibodies. (A) Effect of the CXCL8 receptor antibodies on the wound closure progression induced by 5.56 dyn/cm^2 laminar shear stress. (B) The percentages of wound closure under 5.56 dyn/cm^2 for various time while cells were respectively treated with anti-CXCR1 alone, anti-CXCR2 alone and anti-CXCR1 in combination with anti-CXCR2. The levels of wound closure were inhibited while cells were treated with anti-CXCR1 alone (about 43% at 1 h, 45% at 2 h, 42% at 4 h and 42% at 8 h, respectively. $P < 0.05$ vs. 5.56 dyn/cm^2 control), with anti-CXCR2 alone (about 65% at 2 h, 62% at 4 h and 62% at 8 h, respectively. $P < 0.05$ vs. 5.56 dyn/cm^2 control), and with anti-CXCR1 in combination with anti-CXCR2 (about 34% at 1 h, 35% at 2 h, 31% at 4 h and 29% at 8 h, respectively. $P < 0.05$ vs. 5.56 dyn/cm^2 control). Bars indicated mean ± SD. Control: cells were treated without CXCL8 receptor antibodies under 5.56 dyn/cm^2. * $P < 0.05$ vs. 5.56 dyn/cm^2 control.

The CXCR1 receptor is highly specific for CXCL8, whereas, the CXCR2 receptor responds to multiple CXC-chemokines including neutrophil-activating peptide-2 (NAP-2), growth-related oncogens (GROα, β, γ), and granulocyte chemotactic protein-2 [17,34]. Although both receptors internalize via arrestin/dynamin-dependent mechanisms, CXCR2 internalizes more rapidly (∼95% CXCR2 vs. ∼10% CXCR1 in the first 5–10 min) and recovers more slowly (∼35% CXCR2 vs. 100% of CXCR1 after 90 min) at the leukocyte cell surface than CXCR1 [35,36]. Generally speaking, expression of the two receptors and their affinity to CXCL8 are different with cell types. For example, migration is primarily mediated by CXCR1 in leukocyte [37], and by CXCR2 in NIH3T3 cells [38]. It is well known that the CXCL8 receptors CXCR1 and CXCR2 exist on HUVECs *in vitro* [9,16,17]; the expression of CXC chemokine receptors may be affected by culture conditions and various stimuli [39]. Although HUVECs express low levels of CXCR1 and CXCR2 in static conditions [9,16,17], both of the mRNA and protein expression of CXCR1 and CXCR2 in EA.hy926 cells is upregulated by 5.56 dyn/cm^2 laminar shear stress, but is downregulated by 15.27 dyn/cm^2. Our results are also confirmed that the mRNA expression of CXCR1 and CXCR2 in HUVECs had the same change trend with that in EA.hy926 cells (supplement 1).

Different flow patterns and magnitudes of shear stress can exert different effects on a number of endothelial cells signaling and gene and protein expressions [40–42]. *In vitro* experiments using parallel-plate flow chamber with cultured endothelial cells have shown that the CXCL8 mRNA expression of HUVECs at 2.23 dyn/cm^2 increased about 68 or 52 times as that at 19. 29 dyn/cm^2 [13]. Sustained exposure to low levels of shear (1.8 dyn/cm^2) or a brief exposure (<1 h) to 10 dyn/cm^2 caused a sustained stimulation of endothelin-1 (ET-1) release, but exposure to 6–25 dyn/cm^2 for ⩾6 h dramatically inhibited ET-1 release [43]. According to Malek et al. [44], the shear stress of arterial level induces endothelial quiescence and an atheroprotective gene expression profile, while the low shear stress, which is prevalent at atherosclerosis-prone sites, stimulates an atherogenic phenotype. Our report here is consistent with Malek's opinion. At the arterial low shear stress area, high expression of CXCR1 and CXCR2 on locally sheared endothelial cells mediates cell migration, may be one of the artherogenic mechanisms.

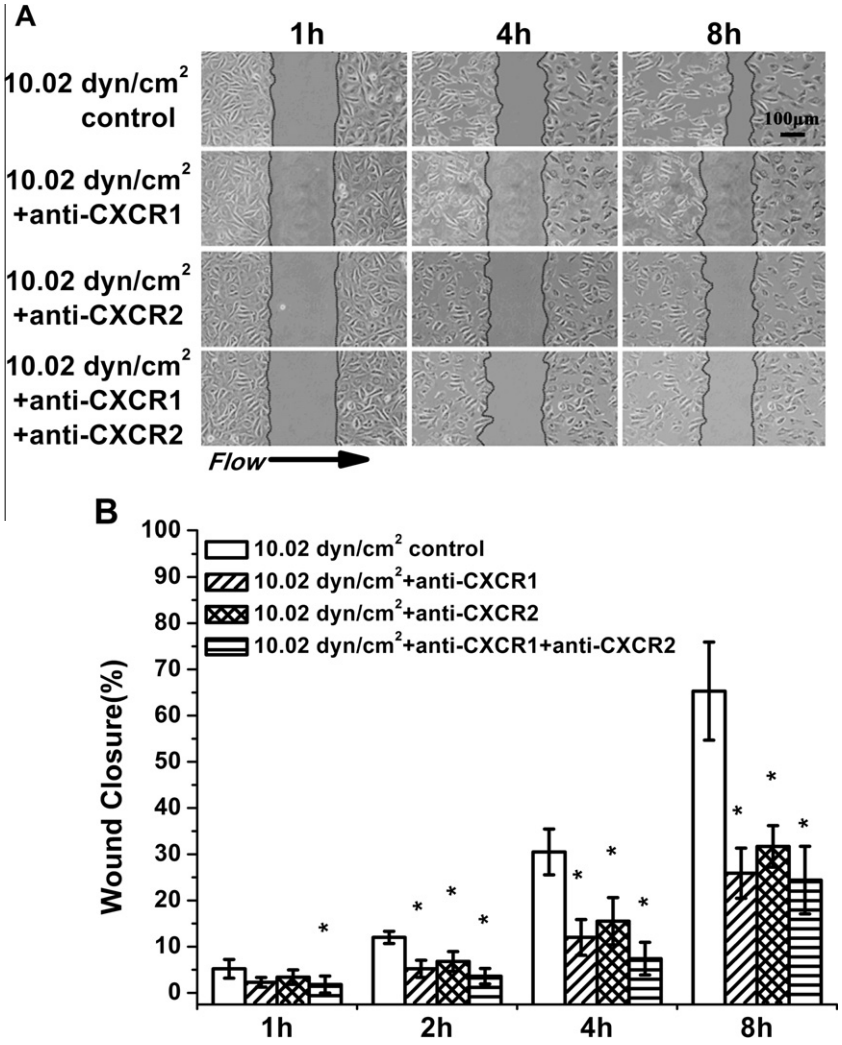

Fig. 6. 10.02 dyn/cm^2 shear stress-enhanced wound closure was inhibited by the CXCL8 receptor antibodies. (A) Effect of the CXCL8 receptor antibodies on the wound closure progression induced by 10.02 dyn/cm^2 laminar shear stress. (B) The percentages of wound closure under 10.02 dyn/cm^2 for various time while cells were respectively treated with anti-CXCR1 alone, anti-CXCR2 alone and anti-CXCR1 in combination with anti-CXCR2. The levels of wound closure were also inhibited while cells were treated with anti-CXCR1 alone (about 43% at 2 h, 39% at 4 h and 40% at 8 h, respectively. $P < 0.05$ vs. 10.02 dyn/cm^2 control), with anti-CXCR2 alone (about 57% at 2 h, 51% at 4 h and 49% at 8 h, respectively. $P < 0.05$ vs. 10.02 dyn/cm^2 control), and with anti-CXCR1 in combination with anti-CXCR2 (about 35% at 1 h, 30% at 2 h, 24% at 4 h and 37% at 8 h, respectively. $P < 0.05$ vs. 10.02 dyn/cm^2 control). Bars indicated mean ± SD. Control: cells were treated without CXCL8 receptor antibodies under 10.02 dyn/cm^2. $^*P < 0.05$ vs. 10.02 dyn/cm^2 control.

Furthermore, fluid shear stress can modulate functions of endothelial cells in blood vessels by activating mechano-sensors, signaling pathways, and gene and protein expressions [45]. In order to study the effect of CXCR1 and CXCR2 in EC migration, we have obtained optimum CXCL8 antibodies concentration under static conditions. The static cultured controls are performed on EA.hy926 cells at various concentrations of anti-CXCR1 or/and anti-CXCR2, while the concentration of CXCL8 receptor antibodies is above 50 ng·ml^{-1}, the wound closure is not significantly changed (data not shown). Therefore, the CXCL8 receptor antibodies with a concentration of 50 ng·ml^{-1} is chosen for the blockade of the receptors CXCR1 and/or CXCR2.

As expected, the migratory response of EA.hy926 cells induced by shear stress can be significantly suppressed by anti-CXCR1 alone, anti-CXCR2 alone or anti-CXCR1 in combination with anti-CXCR2, respectively.

Our results have demonstrated that the receptors CXCR1 and CXCR2 participate in the EC sensing of mechanical stimulations, and the shear stress-induced EC migration can be inhibited while antibodies against CXCR1 and CXCR2. It was suggested that CXCR1 and CXCR2 played important roles in mediating the endothelial cell migration induced by shear stress at all these magnitudes, although there maybe exist other receptors mediating shear stress-induced endothelial cell migration. Therefore, antibodies against CXCR1 and CXCR2 can inhibit EC migration that induced by shear stress at all these magnitudes.

Overall, cell migration depends on whether CXCR1 and CXCR2 are blocked, on whether flow is applied, and on what magnitudes of shear stress is exerted on cells.

In particular, under 15.27 dyn/cm^2 shear stress, the expression of CXCR1 and CXCR2 in EA.hy926 cells is downregulated. But the wound closure, which can be blocked by functional CXCL8 antibodies, is greatly enhanced. Furthermore, the immunofluorescence assay was used to determine CXCR1 and CXCR2 expression on the surface of EA.hy926 cells. Our results showed that both CXCR1 and CXCR2 were expression on the surface of ECs. The expression of them enhanced by 5.56 dyn/cm^2 shear stress, but inhibited by 15.27 dyn/cm^2 shear stress, especially exposure for 4 h. It was suggested that the distribution of CXCR1 and CXCR2 on the surface of ECs was shear stress-dependent (supplement 2). Our results

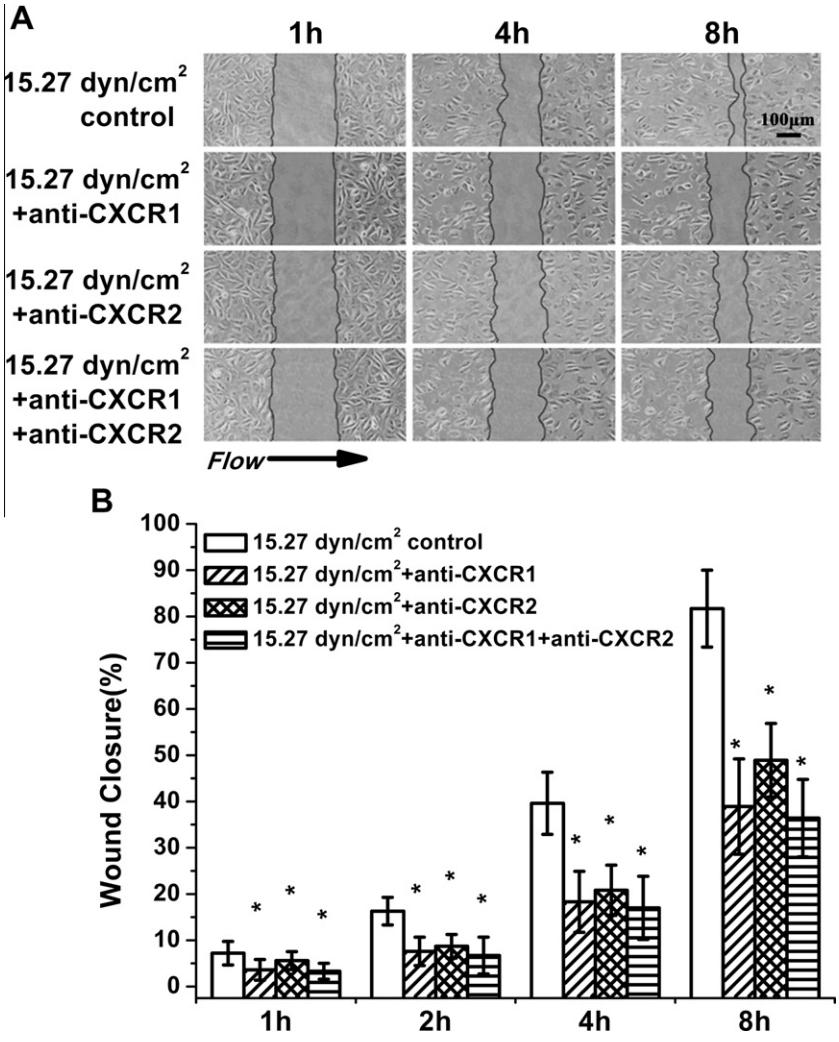

Fig. 7. 15.27 dyn/cm^2 shear stress-enhanced wound closure was inhibited by the CXCL8 receptor antibodies. (A) Effect of the CXCL8 receptor antibodies on the wound closure progression induced by 15.27 dyn/cm^2 laminar shear stress. (B) The percentages of wound closure under 15.27 dyn/cm^2 for various time while cells were respectively treated with anti-CXCR1 alone, anti-CXCR2 alone and anti-CXCR1 in combination with anti-CXCR2. The levels of wound closure were inhibited while cells were treated with anti-CXCR1 alone (about 50% at 1 h, 47% at 2 h, 46% at 4 h and 48% at 8 h, respectively. $P < 0.05$ vs. 15.27 dyn/cm^2 control), with anti-CXCR2 alone (about 77% at 1 h, 35% at 2 h, 53% at 4 h and 60% at 8 h, respectively. $P < 0.05$ vs. 15.27 dyn/cm^2 control), and with anti-CXCR1 in combination with anti-CXCR2 (about 46% at 1 h, 41% at 2 h, 43% at 4 h and 45% at 8 h, respectively. $P < 0.05$ vs. 15.27 dyn/cm^2 control). Bars indicated mean ± SD. Control: cells were treated without CXCL8 receptor antibodies under 15.27 dyn/cm^2. * $P < 0.05$ vs. 15.27 dyn/cm^2 control.

indicated that CXCR1 and CXCR2 are mechano-sensors mediating laminar shear stress-induced EC migration. The traction force maybe has pure classical physics effects on cell migration that is the subject of Newton's third law of motion.

With respect to cell motility, reorganization of the actin cytoskeleton is the primary mechanism [46]. Singh et al. showed that knock-down of CXR1 and CXCR2 affects CXCL-8-induced actin reorganization and phosphorylation of ERK1/2 in melanoma cells, and then decreased the melanoma cell migration [47]. Schraufstatter et al. showed that lung microvascular endothelial cells express CXCR1 and CXCR2 and CXCL-8 activates Rho and Rac signaling pathways through these receptors [17]. Interestingly, CXCL-8 initially activates Rho and actin stress fiber formation in endothelial cells due to activation of the CXCR1; at later time points, Rac is activated in a CXCR2-dependent fashion, leading to cell retraction and gap formation between neighboring cells [17]. Our next work will further validate whether there are differences between CXCR1 and CXCR2 in mechanical signal transduction.

It has become increasingly clear that the CXCL8 receptors CXCR1 and CXCR2 exhibit critical effects in cell migration, which contributes to angiogenesis relevant to cancer. Additionally, it is known that CXCL8 signaling activates members of the Rho GTPase family via the receptors CXCR1/2; then activates a number of non-receptor tyrosine kinases (e.g., Src family kinases and FAK) that regulate the architecture of the cell cytoskeleton and its interaction with the surrounding extracellular environment [10]. Perhaps traction and the CXCL8 receptors CXCR1 and CXCR2 have a common downstream signaling pathway such as the activation of Rho and Rac GTPases. These novel molecular targets can be discovered to facilitate the development of new therapeutic strategies for prevention and treatment of cardiovascular diseases and cancer.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cyto.2010.09.007.

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