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Laminar shear stress induces the expression of aquaporin 1 in endothelial cells involved in wound healing

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ABSTRACT

Laminar shear stress (LSS) due to blood flow contributes to the maintenance of endothelial health by multiple mechanisms including promotion of wound healing. The present study examined the hypothesis that the induction of water channel aquaporin 1 (AQP1) expression by LSS might be functionally associated with endothelial wound healing. When human umbilical vein endothelial cells were exposed to LSS at 12 dyn cm^{-2} for 24 h, significant increases in AQP1 expression were observed at the mRNA and protein levels as compared with static control. In the *in vitro* scratch wound healing assay, LSS treatments before and after wound creation enhanced endothelial wound healing and this effect was significantly attenuated by selective suppression of AQP1 expression using small interfering RNA. Ectopic expression of AQP1 enhanced wound healing in the absence of LSS. This study demonstrated that LSS stimulates the endothelial expression of AQP1 that plays a role in wound healing.

1. Introduction

The interior surfaces of blood vessels are coated with the thin layer of endothelial cells called endothelium which functions as a barrier to the transfer of water, solutes and blood cells out of blood vessels. The endothelium also secretes various factors involved in the regulation of vascular physiology. Therefore, the structural and functional integrities of the endothelium are critical for the prevention of atherosclerosis and cardiovascular diseases [1,2].

Shear stress of flowing blood is known to regulate endothelial physiology and pathophysiology in a magnitude- and flow pattern-dependent manner [3,4]. It has been established that laminar and oscillatory shear stresses experienced by endothelium in straight arterial regions, and in curved or bifurcated regions, respectively, have different effects on the development of atherosclerosis [5,6]. These phenomena may be associated with changes in the rate of nitric oxide (NO) production by nitric oxide synthase 3 (NOS3) in endothelial cells [7,8]. Endothelial barrier function is also affected by shear stress and it has been suggested that low shear stress regions in arteries are more permeable to macromolecules and more susceptible to atherosclerosis [9].

Removal of a small portion of endothelium in blood vessels can stimulate a highly coordinated wound healing process that involves inflammation, cell proliferation, matrix deposition, and remodeling [10]. Of interest, shear stress has been shown to promote endothelial wound healing *in vitro* [11–13], despite inhibitory effects on the cell cycle [14]. The shear stress applied to the luminal surfaces of endothelium can trigger a variety of signal leading to lamellipodial protrusion. The formation of focal adhesions in the flow direction and the disassembly of focal adhesions at the rear induce directional cell migration [15].

Aquaporins are small integral membrane proteins that function as molecular water channels in plasma membranes [16]. AQP1 is abundant in erythrocytes and has been detected in various other cells including epithelial and endothelial cells [17]. The physiological functions of AQP1 have been characterized most extensively in the kidney and these studies have revealed that AQP1 knockout mice have a severe defect in the urine concentrating mechanism [18]. AQP1 gene disruption also leads to the impairment of angiogenesis and endothelial cell migration [17,19]. Thus AQP1 is likely to play a role in endothelial wound healing stimulated by LSS but no previous studies have addressed this issue.

In the present study, we investigated specific issues whether LSS stimulates AQP1 expression and whether AQP1 contributes to endothelial wound healing stimulated by LSS.

Abbreviations: AQP1, aquaporin 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HUVECs, human umbilical vein endothelial cells; LSS, laminar shear stress; NO, nitric oxide; NOS3, nitric oxide synthase 3; RT-PCR, reverse transcriptase-polymerase chain reaction; siRNA, small interfering RNA.

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2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVECs) obtained from Clonetics Cambrex (Rockland, ME) were cultured in EBM-2 medium containing endothelial growth supplements (Clonetics Cambrex), 10% fetal bovine serum (Gibco BRL, Grand Island, NY) and antibiotics (100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 0.25 μg mL^{-1} amphotericin B) on 0.2% gelatin-coated culture dishes at 37 °C and 5% CO_2 [20].

2.2. LSS treatment

HUVECs cultured on an 100 mm-culture dish (BD Biosciences, San Jose, CA) were exposed to steady LSS at 12 dyn cm^{-2} by rotating a Teflon cone (0.5° cone angle) mounted onto a culture dish, as described previously [21,22]. Control cells were kept under static conditions for the same period.

2.3. cDNA microarray analysis

Total cellular RNAs extracted from static and LSS-exposed HUVECs were subjected to cDNA microarray analysis using GeneChip® HG-U133 Plus 2.0 (Affymetrix, Santa Clara, CA) [23]. Complete datasets were deposited in the Gene Expression Omnibus database [Accession Number, GSE13712].

2.4. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Cellular RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA) and the mRNA expressions of the specified gene were analyzed by semi-quantitative RT-PCR, which was conducted as described previously [24]. The sequences of PCR primers used were: AQP1 (GeneBank accession number, NM_198098.2, NM_001185060.1, NM_001185061.1, NM_001185062.1) 5′-ATC ACA CAC AAC TTC AGC AAC CAC-3′ (sense) and 5′-ACA CCC CCA TAA GAG GCT TGA C-3′ (antisense); NOS3 (NM_000603.3) 5′-TGC TGG CAT ACA GGA CTC AG-3′ (sense) and 5′-TAG GTC TTG GGG TTG TCA GG-3′ (antisense); GAPDH (NM_002046.3) 5′-GCC AAA AGG GTC ATC ATC TC-3′ (sense) and 5′-GTA GAG GCA GGG ATG ATG TTC-3′ (antisense). Resulting PCR products were electrophoresed in 1.0% agarose gels with a DNA Ladder Marker (ELPIS-Biotech, Daejeon, Korea). Gels were ethidium bromide-stained and monitored using the Gel Doc system (BioRad, Hercules, CA).

2.5. Western blotting

Cells were lysed in a lysis buffer (20 mM Tris–Cl, 2.5 mM EDTA, 1.0% SDS, pH 7.5) supplemented with 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Roche, Mannheim, Germany) and then subjected to Western blotting as described previously [24]. Rabbit polyclonal AQP1 antibody from Santa Cruz Biotech (Santa Cruz, CA), mouse monoclonal NOS3 antibody from BD Transduction Laboratories (San Diego, CA), and mouse monoclonal β-actin antibody from Sigma–Aldrich (St. Louis, MO) were used as primary antibodies. Goat anti-mouse IgG and anti-rabbit IgG antibodies conjugated to horseradish peroxidase were purchased from Cell Signaling (Danvers, MA) and Santa Cruz Biotech, respectively, and used as secondary antibodies. Immunoreactive bands were detected using a picoEPD Western Reagent kit (ELPIS-Biotech.) and quantified using the NIH Image program.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Cell surface expression of AQP1 was determined by ELISA. Cells were fixed with 4% paraformaldehyde in PBS for 10 min, and blocked with 0.1% BSA in 0.3% Triton X-100/PBS for 1 h at room temperature. Cells were incubated with AQP1 antibody diluted 1:300 overnight at 4 °C followed by 1 h-incubation with a secondary antibody conjugated to horseradish peroxidase diluted 1:1000. Then, cells were washed and incubated with 3,3′,5,5′-tetramethyl benzidine (Sigma–Aldrich) for 3 min. The reaction was stopped with 2 N HCl and the formation of yellow reaction products was determined at 450 nm using a BioRad Model 680 microplate reader reader (BioRad Laboratories, Inc., Hercules, CA).

2.7. Small interfering RNAs (siRNAs)

Human AQP1 siRNA (#1299001, HSS179896) with nucleotide sequences corresponding to the coding region of the human AQP1 gene transcript (NM_109098.2) and a negative control siRNA with scrambled sequences (#12935200) were purchased from Invitrogen (Grand Island, CA). The nucleotide sequences of AQP1 siRNA were as follows: 5′-GCC AUC CUC UCA GGC AUC ACC UCC U-3′ (sense) and 5′-AGG AGG UGA UGC CUG AGA GGA UGG C-3′ (antisense).

2.8. Plasmid constructs

The full coding sequence of human AQP1 was PCR-amplified from a clone (IMAGE ID 4799283, #MGC-26324, Clone sequence: BC022486.1) obtained from the American Type Culture Collection (Manassas, VA) using following primers: 5′-CCG GAT ATC ATG GCC AGC GAG TTC AAG AAG AAG-3′ and 5′-GCG CTC GAG CTA TTT GGG CTT CAT CTC CAC C-3′ (*EcoRV* and *XhoI* sites were incorporated into sense and antisense primers, respectively (underlined). PCR products were gel-purified using a Power gel extraction kit (TaKaRa Bio Inc., Shiga, Japan), digested with *EcoRV* and *XhoI* (New England Biolabs, Ipswich, MA) and inserted in pcDNA3.1(+) vector (Invitrogen) using the Ligation Mix Kit (TaKaRa Bio Inc.). The plasmid was cloned and propagated in *Escherichia coli* strain DH5α (TaKaRa Bio Inc.) and purified using the Endo-free Maxi-Prep DNA purification kit (Qiagen). The AQP1 coding sequence was verified to be identical to the reference sequence (NM_109098.2). The pcDNA-NOS3 construct encoding bovine NOS3 has been described previously [25].

2.9. Transfection

HUVECs were transfected with siRNAs using lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Cells were treated with a mixture of 25 nM siRNA and 1.25 μL mL^{-1} Lipofectamine RNAiMAX in 5 mL Opti-MEM (Invitrogen) for 4 h. For plasmid transfection, TrueFect™ (United BioSystems Inc., Rockville, MD) was used. Briefly, cells were treated with 1 μg mL^{-1} of plasmid DNA and 3 μL mL^{-1} of TrueFect in Opti-MEM for 4 h.

2.10. Scratch wound healing assay

HUVECs were cultured for 24 h under static conditions and then exposed to LSS or continuously kept static for 18 h. The confluent endothelial cell monolayer was scraped in a straight line with a 200 μL pipette tip to create a wound parallel to the direction of the flow. Wounded monolayers were then washed once with 1 mL of growth medium to remove cell debris, and exposed to LSS or kept static for 5–13 h. Images of cell monolayer around wounds were taken using an Eclipse TS 100 inverted phase micro-

scope (Nikon, Melville, NY). Wound areas in microscopic images were determined using the image J 1.42q program (NIH-http://rsb.info.nih.gov/ij). Data are presented as wound closure (%):

$$\text{Wound closure } (\%) = [(A_0 - A_t)/A_0] \times 100\%$$

where A_0 is the area of the original wound and A_t is the area of wound measured at a specified time after scratching [26].

2.11. Statistical analysis

Data are presented as means ± SEMs. The statistical analysis was conducted using the Sigma Stat 3.1 program. One-way ANOVA was use to determine the significant intergroup differences Duncan's multiple-range test was conducted if intergroup differences had p values of <0.05.

3. Results

Effects of LSS on AQP1 expression at the mRNA and protein levels were first examined. As shown in Fig. 1A, microarray data for the two different oligonucleotide probes against AQP1 (207542_s_at, and 209047_at) indicated that the mRNA level of AQP1 was increased by 11–13 folds due to exposure to LSS at 12 dyn cm^{-2} for 24 h. We also analyzed AQP1 mRNA levels by semi-quantitative RT-PCR using gene-specific primer sets and GAPDH as an internal control, and found that the AQP1 mRNA level of LSS-exposed cells was much higher than that of static control cells (Fig. 1B). No significant differences were observed for GAPDH. AQP1 expression at the protein level was also analyzed by Western blotting using β-actin as a loading control. As shown in Fig. 1C, the AQP1 protein level was much higher in LSS-exposed cells compared to static controls. Expression of AQP1 on the cell surface was also examined by ELISA. The results showed that LSS increased the cell surface expression of AQP1 (Fig. 1D).

The *in vitro* scratch wound healing assay was conducted under four different conditions which included LSS treatments of cells before and/or after wound scratching (Fig. 2). L/S and L/L groups were pre-exposed to LSS for 18 h, wounded and post-exposed to LSS for 6 h (L/L) or kept static (L/S). S/S and S/L groups were kept static until they were wounded, and post-exposed to LSS (S/L) or kept static (S/S). As shown in Fig. 2A, S/L and L/L groups exhibited higher wound healing efficiencies compared to S/S and L/S groups, indicating post-exposure of wounded cells to LSS enhanced wound healing process. A significant enhancement of wound closure was also observed in L/L cells compared to S/L cells. Thus it was thought that pre-exposure to LSS contributed to wound healing process. However, S/S and L/S groups showed similar wound closure, indicating pre-exposure to LSS alone is not sufficient to enhance wound closure. Overall these results demonstrate that efficient wound healing requires not only pre-exposure to LSS but also post-exposure to LSS. Typical endothelial cell images right after wound creation and 6 h later, under different experimental conditions, are shown in Fig. 2B.

To examine the role of AQP1 in endothelial wound healing stimulated by LSS, we first established experimental conditions to selectively deplete AQP1 using a siRNA approach. Cells were transfected with siRNA targeting the coding region of AQP1 gene transcripts or negative control siRNA containing scrambled sequences, and then exposed or not exposed to LSS at 12 dyn cm^{-2} for 24 h. As shown in Fig. 3A and B, LSS increased the expression of AQP1 and NOS3 at the mRNA and protein levels. Of these, only AQP1 expression was strongly suppressed by AQP1 siRNA transfection. LSS and AQP1 siRNA have no significant effects on GAPDH mRNA or β-actin protein levels. We then examined whether AQP1 participates in the wound healing effects of LSS in endothelial cells. The *in vitro* scratch wound healing assay was conducted under L/S and L/L conditions. As shown in Fig. 3C, AQP1 siRNA-transfected cells exhibited less wound closure than control cells under both experimental conditions.

Additional experiments were undertaken to examine if AQP1 expression alone can enhance wound closure in the absence of LSS. For this purpose, cells were transfected with a plasmid encoding AQP1 or NOS3 or the empty vector (pcDNA). As shown in Fig. 4A, overexpression of AQP1 and NOS3 in the transfected cells were confirmed by Western blotting. The transfected cells were wounded and then wound closure monitored at different time points under static conditions. As shown in Fig. 4B, AQP1-overexpressing cells showed faster wound closure rate compared to control cells (pcDNA) or NOS3-overexpressing cells. These results indicate that AQP1 expression levels are associated with the efficiency of wound closure.

4. Discussion

Atherosclerosis usually occurs in curved or bifurcated regions of vessel where wound healing is poorer due to low shear stress or turbulent flow [5,6]. Endothelial wounding injuries also occur after other vascular pathogenic events such as vein bypass graft failure, ischemia, coarctation, and mechanical trauma [27]. Endothelial cell migration in vessel walls represents an essential phase of the wound healing process after disruption of the intima by such injuries and loss of this function can lead to severe disabilities of endothelium [10,15]. Previous studies have demonstrated that shear stress enhances endothelial cell migration during wound healing

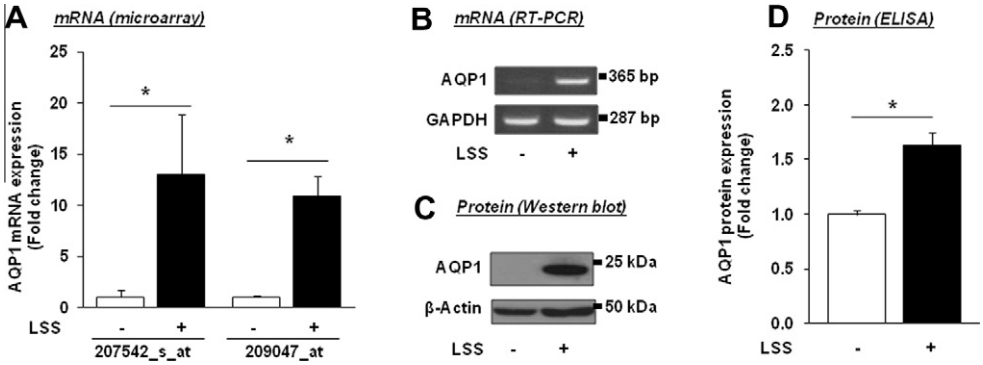

Fig. 1. The induction of AQP1 expression by LSS in endothelial cells. Cells were exposed to LSS at 12 dyn cm^2 or maintained under static conditions for 24 h. The cDNA microarray data were analyzed for two different probes against AQP1 (A). AQP1 mRNA levels were also analyzed by semi-quantitative RT-PCR *versus* GAPDH (B). AQP1 protein was analyzed by Western blotting using β-actin as a loading control (C). Expression of AQP1 on the cell surface was determined by ELISA (D). Data are expressed as means ± SEMs ($n = 3$), $^*p < 0.05$.

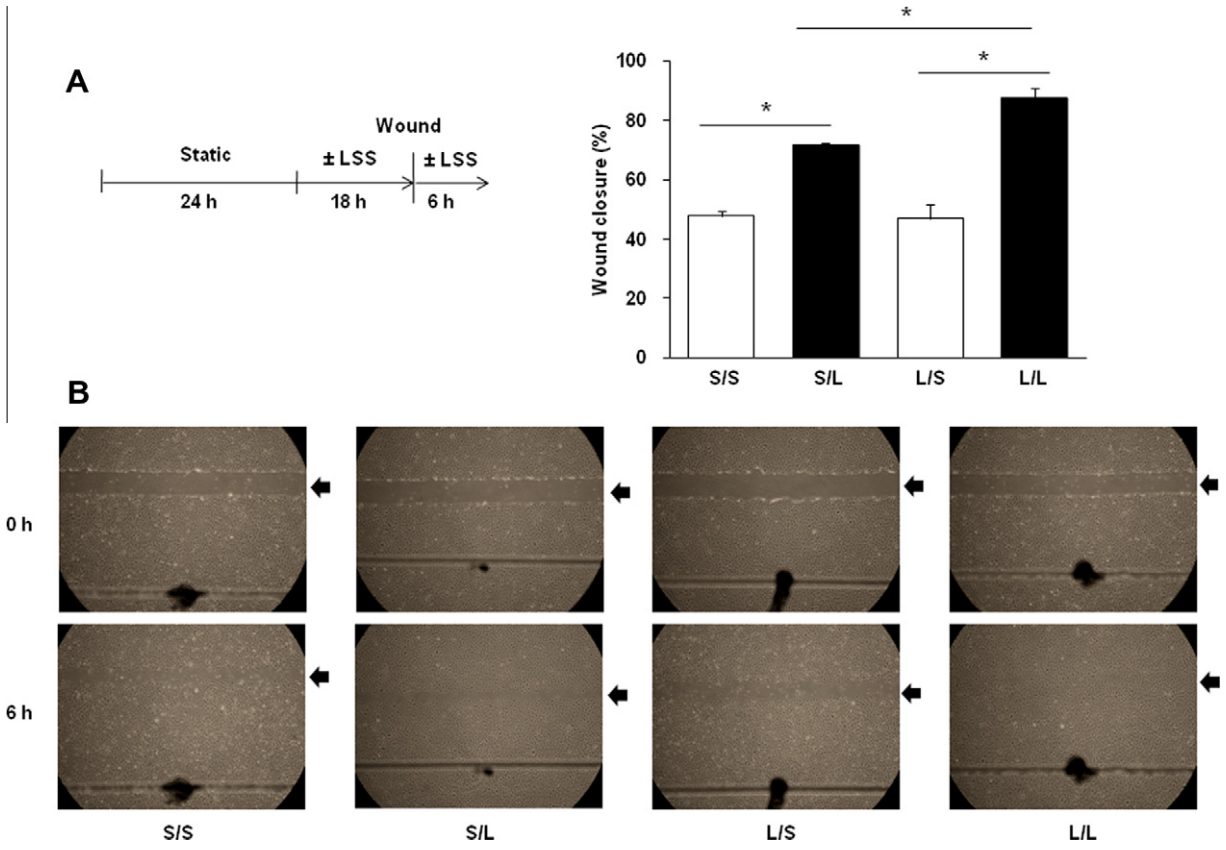

Fig. 2. The enhancement of endothelial wound healing by LSS. The *in vitro* scratch wound healing assay was conducted under four different conditions (A). L/S and L/L groups were pre-exposed to LSS at 12 dyn cm^2 for 18 h, wounded and post-exposed to LSS for 6 h (L/L) or kept static (L/S). S/S and S/L groups were kept static, wounded, and post-exposed to LSS (S/L) or kept static (S/S). Data are presented as wound closure percentage values (A). Representative microscopic cell images are shown (B). Wounded areas are indicated with arrows. Data are expressed as means ± SEMs ($n = 3$), $^*p < 0.05$.

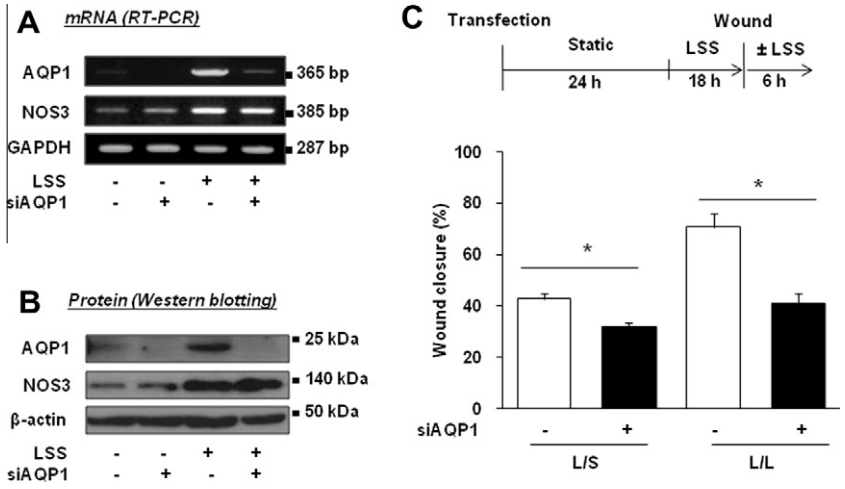

Fig. 3. The suppression of AQP1 expression inhibited endothelial wound closure stimulated by LSS. Cells transfected with AQP1 siRNA or control siRNA were exposed or not exposed to LSS at 12 dyn cm^2 for 24 h. Expressions of AQP1, NOS3, GAPDH and/or β-actin at the mRNA and protein levels were analyzed by RT-PCR (A) and Western blotting (B), respectively. Transfected cells were exposed LSS for 18 h prior to wounding and then the wounded monolayers were exposed (L/L) or not exposed (L/S) to LSS for 6 h (C). Results are wound closure percentage values (means ± SEMs; $n = 3$), $^*p < 0.05$.

in vitro [11–13] and *in vivo* [28,29], and that LSS-induced endothelial cell migration is a complicated process involving mechanosensing, cytoskeleton remodeling, cell-extracellular matrix adhesions, and cell–cell adhesions [12,15].

The present study newly demonstrates that AQP1 is involved in endothelial wound healing by LSS. The aquaporins have been shown to play a critical role in the migrations of various cell types [30,31]. In addition, aquaporins including AQP1 have been known to be expressed in endothelial cells [17,32]. Therefore, when AQP1 expression levels were observed to be increased by LSS (Fig. 1), it was supposed that LSS-induced AQP1 expression might be associated with the enhancement of endothelial cell migration

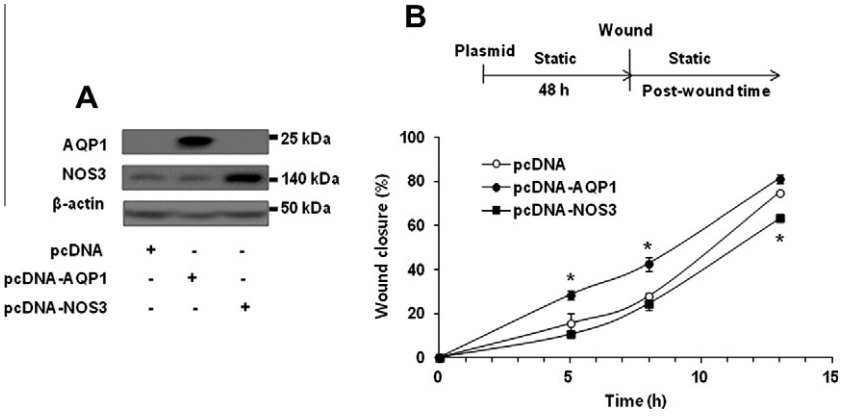

Fig. 4. The ectopic expression of AQP1 enhanced endothelial wound closure in the absence of LSS. Cells were transfected with a plasmid construct encoding AQP1, NOS3, or an empty pcDNA3.1(+) vector. Cell lysates were subjected to Western blotting to determine AQP1, NOS3, and β-actin protein levels (A). Monolayers of transfected cells were wounded and kept static for up to 13 h to monitor wound closure (B). Results are wound closure percentage values (means ± SEMs; $n = 3$), $^{*}p < 0.05$ *versus* vector control.

by LSS. Supporting this supposition, knock down of AQP1 expression by siRNA inhibited wound closure under LSS-exposed conditions (Fig. 3). Furthermore, overexpression of exogenous AQP1 enhanced wound closure under static conditions (Fig. 4). Thus, LSS is considered to enhance endothelial wound closure through a mechanism involving the induction of AQP1 expression.

Although we did not investigate how LSS induces AQP1, evidence indicates that Kruppel-like factor 2 (KLF2), which is known to coordinate transcriptional program in endothelial cells affected by LSS, may be involved [33,34]. Human AQP1 promoter contains nine CACCC regulatory elements which are known to be a consensus motif for KLF2 [35], and AQP1 mRNA has been reported to be up-regulated in cells overexpressing KLF2 [36].

Cell migration begins with the transient formation of membrane protrusions (lamellipodia) at the leading edge of migrating cells. Lamellipodial protrusions require actin reorganization and ion uptake followed by water influx through the cell membrane. It has been postulated that the polarization of aquaporins to the leading edge of migrating cells facilitates water entry and increases hydrostatic pressure causing membrane protrusion [17]. Further studies are needed to address issues whether LSS affects AQP1 polarization in addition to its effect inducing its expression.

Evidence suggests that NO may play a key role in normal wound healing *via* processes as disparate as angiogenesis, proliferation, and matrix deposition [10]. However, we could not observe any enhancement of wound closure rate in NOS3-overexpressing cells (Fig. 4). Previous studies have demonstrated that cell migration rather than proliferation is the major mechanism of wound healing within the first 1–2 days after wounding [11,15]. Thus, NOS3 is not considered to regulate cell migration during initial wound closure process.

In conclusion, the present study shows that AQP1 is a key mediator of the endothelial wound repair stimulated by LSS. This notion is supported by the robust induction of endothelial AQP1 expression by LSS and the essential requirement of AQP1 for LSS-stimulated wound closure. In addition, AQP1 overexpression was found to increase wound closure. Thus, the beneficial effects of LSS on endothelial health are attributable, at least in part, to the enhanced expression of AQP1 which promotes endothelial cell migration and wound repair.

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