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Pivotal roles of shear stress in the microenvironmental changes that occur within sentinel lymph nodes

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ABSTRACT

A sentinel lymph node (SLN) is the first lymph node that receives drainage from a primary tumor. According to their physiological and biomechanical characteristics, we hypothesized that SLN contain lymphatic endothelial cells that are constantly loaded with high levels of shear stress, which might contribute to the production of a suitable environment for micrometastasis within them. To test this hypothesis, we investigated the effects of shear stress stimulation on the expression of adhesion molecules on human lymphatic endothelial cells (LEC) isolated from the lymph vessels nearest the SLN of breast cancers, and on the release of ATP from human LEC. Next, we studied whether shear stress-mediated adhesion molecule expression accelerates the attachment of carcinoma cells to human LEC. Finally, in *in vivo* experiments we evaluated whether exogenous ATP facilitates the expression of carcinoma cell-ligated adhesion molecules in rat SLN. In conclusion, shear stress stimulation induces ICAM-1 expression on LEC by activating cell surface F_1/F_O ATP synthase, which might contribute to the creation of a premetastatic environment within SLN.

INTRODUCTION

A sentinel lymph node (SLN) is the first lymph node that receives drainage from a primary tumor. Morton *et al.* (1) initially demonstrated the SLN concept in a feline model and later confirmed it in a clinical study of patients with breast cancer and melanoma. The SLN concept has subsequently become one of the most important topics in surgical oncology (2, 3). SLN are also the most common and earliest site of malignant tumor metastasis.

Many factors such as the humoral, immunological, and morphological heterogeneity of collecting lymph vessels can contribute to deciding which lymph node within the regional lymph network draining a primary tumor becomes the SLN. However, it remains unclear which factors play pivotal roles in deciding which lymph node becomes the SLN. In addition, we consider that SLN are constantly subjected to a high lymph flow rate. We reached this conclusion in our recent study, in which a high lymph flow rate was detected within the SLN of the stomach using contrast-enhanced ultrasonography with Sonazoid in a porcine model (4). Thus, the endothelial cells in the marginal sinuses of SLN and their nearest afferent lymph vessels might be constantly stimulated by the high shear stress generated by the abovementioned high lymph flow rate because collapsible tubes such as the collecting lymph vessels display unchanged diameters in such conditions. Therefore, we have hypothesized that SLN contain lymphatic endothelial cells that are constantly loaded with a high level of shear stress.

Vascular endothelial cells (EC) are constantly exposed to shear stress, the mechanical force generated by the flow of blood or lymph. Upon detecting shear stress, these EC transmit signals to their interiors, where they trigger cell responses including changes in a

variety of cell functions (5, 6). In the case of lymphatic endothelial cells, lymph flow stimulation results in the rapid production and release of endogenous nitric oxide (NO), which contributes to the cyclic GMP-mediated relaxation of lymphatic smooth muscles (7) and inhibitory effects on lymph pump activity (8). We have also demonstrated that in response to shear stress, lymphatic EC release endogenous ATP, which activates endothelial constitutive NO synthase (ecNOS) expression at the mRNA and protein levels through the inositol 1,4,5-triphosphate-mediated release of intracellular Ca^{2+} and the activation of Ca^{2+}-activated K^+ channels in lymphatic EC (9).

Recently, it was shown that primary tumors influence the microenvironments of distant organs during the development of metastasis (10, 11). However, it is unclear whether similar environmental changes occur within SLN in order to encourage carcinoma micrometastases to develop within them. If such environmental changes occur within SLN, the mechanisms responsible for these changes need to be elucidated. Therefore, to address the pathophysiological roles of shear stress stimulation in the development of environmental changes within the SLN, we attempted to investigate (1) the effects of shear stress on the expression of adhesion molecules on cultured human lymphatic EC isolated from the afferent lymph vessels nearest to SLN and (2) on the release of ATP from human lymphatic EC (LEC), and (3) to study whether shear stress-mediated increases in adhesion molecule expression accelerate the attachment of carcinoma cells to cultured human LEC. Finally, in *in vivo* rat experiments we (4) evaluated whether the ATP released from lymphatic endothelial cells in response to shear stress stimulation facilitates the expression of carcinoma cell-ligated adhesion molecules within rat SLN.

MATERIALS AND METHODS

Cell culture

The isolation and culturing of the human LEC were performed according to the techniques described by Kawai et al. (9) using the afferent lymph vessels nearest to the sentinel lymph nodes in breast cancer patients. The human LEC experiments were approved by the ethical committee for human studies of Shinshu University School of Medicine. All subjects were informed of the risks and purpose of the study before their written consent was obtained. The human LEC were maintained in endothelial growth medium (EGM)-2 supplemented with 10% fetal bovine serum (FBS) and used at the fourth to ninth passage.

The human breast adenocarcinoma cell line MDA-MB-231 was purchased from the American Type Culture Collection (Manassas, VA). The carcinoma cells were maintained in Dulbecco's modified Eagle's medium/Nutrient Mixture F12 Ham (DMEM/F12) culture medium supplemented with 10% FBS. The human LEC were incubated under atmospheric conditions of 5% O_2, 5% CO_2, and 90% N_2 at 37°C, whereas the carcinoma cells were incubated under normoxic conditions of 21% O_2, 5% CO_2, and 74% N_2 at 37°C.

Flow loading experiments

A parallel plate-type apparatus was used to apply laminar shear stress to the human LEC using a slightly modified version of Yamamoto's method (6). Briefly, one side of the flow chamber consisted of a 1% gelatin-coated glass plate, on which the cultured human LEC rested, and the other side consisted of a polycarbonate plate. These two surfaces were held 200 μm apart with a Teflon gasket. The chamber contained an entrance and an exit for the fluid, and the entrance was connected to an upper reservoir by a silicone tube. The exit led

to a lower reservoir. The flow of the fluid (37°C, EGM-2) was driven by a peristaltic pump with a flow rate controller. The intensity of the shear stress (τ, dynes/cm^2) acting on the LEC layer was calculated using the formula $\tau = 6\mu Q/a^2b$, where μ is the viscosity of the perfusate (poise), Q is the flow volume (mL/s), and a and b are the cross-sectional dimensions of the flow path (cm).

ATP assay

The ATP concentrations of the human LEC culture media supernatants were determined using a luciferin-luciferase assay based on the Cell Titer-Glo® Luminescent Cell Viability Assay (Promega, WI), as described previously (12). Briefly, in order to develop a calibration curve for ATP measurement, we first established a luciferin-luciferase assay using culture media containing several concentrations of ATP. Next, 100 μL of the flow-stimulated human LEC supernatant were collected in a 96-well plate, into which 100 μL of luciferin-luciferase solution had been added, and light emission was recorded using a luminometer (Dainippon Sumitomo Pharma, Japan). Thus, we calculated the concentrations of ATP in the supernatants of the flow-stimulated human LEC culture media using the calibration curve for ATP measurement.

Immunocytochemistry

Indirect immunohistochemical studies were performed using cultured LEC seeded on glass slides coated with type I collagen, and then the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline solution (PBS) for 20 min at room temperature. Next, the cells were washed three times with PBS and then incubated overnight at 4˚C with primary polyclonal human antisera to E-selectin/CD62E (dilution: 10

μg/mL; R&D Systems, MN), P-selectin/CD62P (dilution: 10 μg/mL; R&D Systems, MN), VCAM-1/CD106 (dilution: 10 μg/mL; R&D Systems, MN), ICAM-1/CD54 (dilution: 10 μg/mL; R&D Systems, MN), and ATP synthase (dilution 1 : 50; Millipore, Billerica, MA). After washing them three times in PBS, the cells were incubated for 1hr at room temperature with 1 : 100 diluted Alexa Fluor 488 donkey anti-mouse IgG secondary antibody (Invitrogen, CA). The nuclei of the cultured cells were counterstained and mounted with ProLong Gold antifade reagent and 4'-6-diamidine-2-phenylindole (DAPI) (Molecular Probes, OR), before being examined with a fluorescent microscope (Leica, Germany) and photographed.

To quantitatively evaluate the data obtained in the immunocytochemical studies, in each experiment we examined the cultured cells in more than three culture dishes and then took three photographs of each culture dish. Each image contained 7 to 11 cells; thus, approximately 100 cultured cells were examined.

For non-specific staining, the primary antiserum was replaced with Block-ace (Dainippon Sumitomo Pharma, Japan) as a negative control.

Quantitative RT-PCR

The expression of ICAM-1 mRNA was evaluated using the quantitative reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from the cultured human LEC using the ISOGEN reagent (Nippon Gene, Toyama, Japan), according to the manufacturer's instructions. The concentration of each RNA was calculated by measuring the absorbance of the RNA samples at 260 nm with a spectrophotometer. The extracted RNA was reverse-transcribed with M-MLV reverse transcriptase (Ambion, Austin, TX). For RT-PCR analysis, each superscript first-strand synthesis kit (Invitrogen) was used

with 1.0 μg of total RNA. The following forward and reverse primers for ICAM-1 and cyclophilin A were used for each specific probe: ICAM-1 (Takara, Kyoto, Japan) and cyclophilin A: 5'-TTCGTGCTCTGAGCACTGGAG-3' (forward) and 5'-GGACCCGTATGCTTTAGGATGAAG-3' (reverse). The cDNA was diluted five-fold prior to PCR amplification. Quantitative RT-PCR was performed using a Light Cycler rapid thermal cycler system (Roche Diagnostics, Burgess Hill, UK). The reactions were performed in a 20 μL volume with 0.5 μM primer, Taq DNA polymerase, and the buffer included in the SYBR Premix Ex Taq kit (Takara). The PCR protocol included a 10s denaturation step followed by 45 cycles of denaturation for 5s at 95 °C and annealing for 20s at 60°C. The fluorescent product was detected at the end of the 60°C extension period. Negative controls including the PCR reaction products produced from each primer pair were subjected to melting curve analysis. The data were analyzed with the Light Cycler analysis software. The results are presented as ratios of the expression of ICAM-1 mRNA to that of cyclophilin A.

In vitro human LEC attachment assay

Human LEC were plated on type I collagen-coated 35 mm plates and then incubated in 5% O_2, 5% CO_2, and 90% N_2 at 37 ˚C to form confluent monolayers. The LEC were kept in serum-starved EBM-2 medium supplemented with 3% FBS. Selected plates were treated with 10^{-7} M ATP or the MDA-MB-231 cell culture medium supernatant for 48 hrs. In some experiments, 10^{-6} M suramin was simultaneously added to the plates during 48 hrs treatment with 10^{-7} M ATP or the MDA-MB-231 cell supernatant.

In some experiments, the plates were also treated with 10 μg/mL anti-human ICAM-1

antibody (R&D Systems, MN) for 30 min after 48 hrs treatment with the MDA-MB-231 supernatant or 10^{-7} M ATP. Breast cancer cells that had been stained with PKH26 fluorescent dye (SIGMA, MO) were then plated at 5×10^4 cells per plate and incubated for 30 min at 37 ˚C. Unbound cells were removed by aspiration, and the plates were washed three times with DMEM/F12. Attachment was quantitated by counting the number of attached cells under × 100 magnification using a Leica microscope.

In vivo immunocytochemical experiments

Male Sprague-Dawley rats (6 to 8 weeks old; n = 4; BW 142 ± 5 g; Japan SLC, Hamamatsu, Japan) were used for the present studies. The rats were housed in an environmentally-controlled vivarium and fed a standard pellet diet and water ad libitum. All experimental protocols were approved by the animal ethics committee of Shinshu University School of Medicine in accordance with the Japanese Physiological Society's "Guide for the Care and Use of Laboratory Animals". As *in vivo* model experiments used to evaluate the effects of shear stress on lymphatic endothelial cells within SLN, the rats were administered 1.0 mL PBS (negative control) or 1.0 mL PBS containing 10^{-6} M ATP into the roots of their tails. At 24 hours after the administration of the test chemicals, the rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p., Tokyo Chemical Industry, Tokyo, Japan) and exsanguinated. Their iliac lymph nodes were rapidly isolated and fixed with liquid nitrogen for the immunocytochemical studies. Slices of the tissue samples were then fixed with 100% acetone and permeabilized with 0.1% Triton X-100. Next, the slices were washed three times with PBS and then incubated for 2 hours at room temperature with primary polyclonal rat antiserum to 1 : 20 diluted FITC-labeled ICAM-1/CD54 (R&D Systems,

Minneapolis, MN). After being washed three times in PBS, the tissues slices were mounted with ProLong Gold antifade reagent and 4'-6-diamidine-2-phenylindole (DAPI) (Molecular Probes, OR) to counterstain the cell nuclei in the lymph nodes, examined with a fluorescent microscope (Leica, Wetzler, Germany), and photographed.

In vivo intravital experiments

Male Sprague-Dawley rats (6 to 8 weeks old; n = 4; BW 140 ± 6 g; Japan SLC, Hamamatsu, Japan) were used for these experiments. The rats were housed in an environmentally-controlled vivarium and fed a standard pellet diet and water ad libitum. All experimental protocols were approved by the animal ethics committee of Shinshu University School of Medicine in accordance with the Japanese Physiological Society's "Guide for the Care and Use of Laboratory Animals". In the *in vivo* model experiments, the rats were also subcutaneously administered 1.0 mL PBS (negative control) or 1.0 mL PBS containing 10^{-6} M ATP. At 24 hours after the administration of the test chemicals, the rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p., Tokyo Chemical Industry, Tokyo, Japan). Additional anesthetic was added intramuscularly as needed. A tracheostomy was performed to ensure that the airway remained patent. The right carotid artery was cannulated to allow arterial blood pressure monitoring. The left external carotid vein was also cannulated to allow the continuous intravenous administration of isotonic saline solution (30 μl/min/100 g body wt) throughout the experiments.

After an incision had been made in the abdomen, the iliac lymph nodes and the afferent and efferent lymph vessels were isolated from the surrounding adipose and connective tissue and then placed in a custom-made chamber for intravital video microscopic observation

(Leica, Wetzler, Germany). The equipment did not obstruct the flow of lymph through the vessels. The fluorescent changes in the lymph nodes were evaluated time-dependently after the subcutaneous administration of 0.3 mL FITC-labeled ICAM-1 antibody diluted 1 : 10 with PBS containing 1% bovine serum albumin (BSA) into the roots of the rats' tails.

Drugs

All salts were obtained from Wako (Tokyo, Japan), and ATP, suramin, piceatannol, and acetazolamide were purchased from SIGMA (St Louis, MO). The piceatannol was diluted in ethanol. The concentration of ethanol used did not affect the biological viability of the cultured EC. Drug concentrations are expressed as the final concentration in the culture plate.

Statistical analyses

All results are expressed as the mean ± standard error of the mean (SEM). Statistical significance was analyzed with the Student's t-test for unpaired observations or one-way ANOVA, followed by Duncan's post-hoc test, as appropriate. $p < 0.05$ was considered statistically significant.

RESULTS

Effects of shear stress stimulation strength and duration on the expression of adhesion molecules on human LEC

First, to evaluate the antibody specificity controls used for the immunocytochemical analyses, we stained the adhesion molecules (E-selectin, P-selectin, VCAM-1, and ICAM-1) on cultured human LEC stimulated by 2 hours treatment with 10 ng/mL LPS (positive control). Significantly high expression of E-selectin and ICAM-1, but only weak expression of P-selectin and VCAM-1, was detected on the LEC (Fig. 1A, m~p). In preliminary experiments, we found that stimulation with a shear stress of 1.0 dyn/cm^2 significantly increased the ICAM-1 immunocytochemical and mRNA expression levels of human LEC. In the present experiments, the expression of adhesion molecules on human LEC was measured at different time points up to 24 hours after their stimulation with a shear stress of 1.0 dyn/cm^2. Figure 1A shows representative photomicrographs of the effects of shear stress stimulation for different time periods on the expression of adhesion molecules on human LEC. At 0 hours, little or no expression of E-selectin, P-selectin, VCAM-1, or ICAM-1 was observed on the cultured human LEC (Fig. 1A, a~d). The photomicrographs were merged with nuclear staining images of the same cultured cells. Overnight culturing of the cells in starvation medium containing 3% FBS induced little or no adhesion molecule expression on human LEC (negative control). In contrast, 2 or 24 hours stimulation with a shear stress of 1.0 dyn/cm^2 selectively increased the immunocytochemical expression of ICAM-1 on human LEC (Fig. 1A, h and l). However, little or no expression of E-selectin, P-selectin, or VCAM-1 was observed on the human LEC subjected to 2 or 24 hours stimulation with a shear

stress of 1.0 dyn/cm^2 (Fig. 1A, e~g and i~k). The ICAM-1 expression results are summarized in Fig. 1B. Thus, stimulation with a shear stress of 1.0 dyn/cm^2 for 2 hours or more significantly increased the expression of ICAM-1 on human LEC.

Figure 1C indicates the effects of shear stress strength on the expression of ICAM-1 on human LEC. Two hours stimulation with a shear stress of 0.5 or 1.0 dyn/cm^2 significantly increased the immunocytochemical expression of ICAM-1 on human LEC in a strength-dependent manner.

In addition, the ICAM-1 mRNA expression of the human LEC was also significantly increased in response to 2 hours stimulation with a shear stress of 0.5 or 1.0 dyn/cm^2 (Fig. 1D).

Effects of piceatannol or suramin on the shear stress stimulation-mediated increase in ICAM-1 expression

To address the molecular mechanisms responsible for the shear stress stimulation-mediated increases in ICAM-1 protein and mRNA expression, we investigated the effects of the cell surface F_1/F_O ATP synthase inhibitor piceatannol (6, 13) and the non-selective purinergic P2X/P2Y inhibitor suramin (12) on the shear stress stimulation (1.0 dyn/cm^2, 2 hours)-mediated increases in ICAM-1 protein and mRNA expression in human LEC. Figures 2A 1-3 show representative photomicrographs of shear stress stimulation-induced ICAM-1 immunocytochemical expression on human LEC in the absence or presence of 20 μM piceatannol or 10^{-6} M suramin. Two hours stimulation with a shear stress of 1.0 dyn/cm^2 significantly increased the expression of ICAM-1 on human LEC (Fig. 2A 1). Pretreatment with 20 μM piceatannol or 10^{-6} M suramin produced a significant

reduction in the shear stress stimulation-mediated increase in the expression of ICAM-1 on human LEC (Fig. 2A 2 and 3). These results are shown in Fig. 2B 1-3. Pretreatment with piceatannol or suramin also caused a significant reduction in the shear stress-mediated increase in ICAM-1 mRNA expression in human LEC (Fig. 2C 2 and 3). These results are demonstrated in Fig. 2C 1-3.

Exogenous ATP induces ICAM-1 expression at the protein and mRNA levels in human LEC

To evaluate the hypothesis that shear stress stimulation induces ATP secretion from human LEC by activating F_1/F_O ATP synthase, which might contribute to the shear stress stimulation-mediated increases in ICAM-1 protein and mRNA expression in human LEC, we first examined the effects of exogenous ATP on the protein and mRNA expression of ICAM-1 in human LEC in the absence or presence of suramin. As shown in Figure 2A 4, 2 hours treatment with 10^{-6} M exogenous ATP produced a significant increase in ICAM-1 expression on human LEC. The increase was similar to that produced by shear stress stimulation (1.0 dyn/cm^2, 2 hours) (Fig. 2A 1). The exogenous ATP-mediated increase in ICAM-1 expression on human LEC was significantly reduced by simultaneous treatment with 10^{-6} M suramin (Fig. 2A 5). These results are shown in Figures 2B 4 and 5.

The exogenous ATP also produced a significant increase in ICAM-1 mRNA expression in human LEC. The ATP-induced increase in ICAM-1 mRNA expression was significantly reduced by treatment with 10^{-6} M suramin. These results are shown in Figure 2C.

Effects of piceatannol on shear stress stimulation-mediated ATP release from human LEC

We next measured the concentrations of ATP in the culture supernatants of shear stress stimulation-loaded human LEC. Figure 3A shows the results of these experiments. Two

hours stimulation with a shear stress of 0.5 or 1.0 dyn/cm^2 produced a significant increase in the concentration of ATP. The shear stress stimulation-induced release of ATP from the human LEC was significantly reduced by pretreatment with 20 μM piceatannol (Fig. 3A).

To confirm the existence of cell surface F_1/F_O ATP synthase on human LEC, we next immunocytochemically investigated the expression of F_1/F_O ATP synthase on non-permeabilized cultured cells. Figure 3B shows representative photomicrographs of cell surface F_1/F_O ATP synthase molecules on the surfaces of non-permeabilized human LEC that had been loaded without or with shear stress stimulation (1.0 dyn/cm^2, 2 hours). We confirmed that F_1/F_O ATP synthase was present on the surfaces of human LEC. However, shear stress stimulation had little or no effect on F_1/F_O ATP synthase protein expression on the surfaces of human LEC. Figure 3C shows these results.

Attachment assay involving 2 hours stimulation with shear stress or exogenous ATP in the absence or presence of suramin

As shown in Fig. 4A 1 (negative control) and Fig. 4A 2, 2 hours shear stress stimulation (1.0 dyn/cm^2) caused a significant increase in the attachment of carcinoma cells to human LEC. The increased attachment of carcinoma cells to human LEC was significantly reduced by simultaneous treatment with 10^{-6} M suramin (Fig. 4A 3). These results are shown in Figures 4B 1-3.

Similar to shear stress stimulation, two hours stimulation with 10^{-6} M exogenous ATP produced a significant facilitation of the attachment of carcinoma cells to human LEC (Fig. 4A 5). In addition, simultaneous treatment with 10^{-6} M suramin caused a significant reduction in the exogenous ATP-mediated increase in the attachment of carcinoma cells to

human LEC (Fig. 4A 6). These results are shown in Fig. 4B 5 and 6.

Attachment assay involving 2 hours stimulation with shear stress or exogenous ATP in the absence or presence of anti-ICAM-1 antibody

Next, we examined whether shear stress stimulation- or exogenous ATP-mediated facilitation of the attachment of carcinoma cells to human LEC could be blocked by treatment with anti-ICAM-1 antibody. As shown in Figures 4A 2 and 4, 2 hours shear stress (1.0 dyn/cm^2) stimulation produced a significant increase in the attachment of carcinoma cells to human LEC, which was also significantly reduced by treatment with anti-ICAM-1 antibody. These experimental results are shown in Figures 4B 2 and 4. Similar to the effects of shear stress stimulation, 2 hours stimulation with 10^{-6} M exogenous ATP also caused a significant increase in the attachment of carcinoma cells to human LEC (Fig. 4A 5 and 7). The increased attachment of carcinoma cells to human LEC was significantly reduced by additional treatment with anti-ICAM-1 antibody (Fig. 4B 5 and 7).

ATP-mediated ICAM-1 expression in *in vivo* rat iliac lymph nodes

To evaluate whether shear stress stimulation could produce ICAM-1 expression in *in vivo* lymph nodes by inducing the release of ATP via the activation of cell surface F_1/F_O ATP synthase on LEC, we investigated the effects of subcutaneously-administered exogenous ATP (10^{-6} M) on ICAM-1 expression within the regional lymph nodes (~SLN) in *in vivo* experiments. As shown in Figure 5A, the subcutaneous administration of 1.0 mL 10^{-6} M exogenous ATP into the rats' tail roots at 24 hours before the experiments produced a marked increase in ICAM-1 expression in the rats' iliac lymph nodes. However, the administration of 1.0 mL PBS (negative control) induced little or no ICAM-1 expression in these lymph

nodes (Fig. 5A, left panel). These results are shown in Fig. 5B (n = 10 images).

Similar to the *in vivo* rat SLN immunocytochemical experiments, the subcutaneous administration of 1.0 mL FITC-labeled anti-ICAM-1 antibody into the roots of the rats' tails demonstrated that the iliac lymph nodes that had been perfused with 1.0 mL PBS containing 10^{-6} M exogenous ATP through their afferent lymph vessels for 24 hours displayed significantly increased ICAM-1 expression (Fig. 5C, right panel) around 10 min after its administration. However, in the rat lymph nodes that were perfused with PBS alone (negative control) for 24 hours, little or no anti-ICAM-1 antibody fluorescence was observed within the iliac lymph nodes (Fig. 5C, left panel). These results are shown in Fig. 5D (n = 10 images).

DISCUSSION

Shear stress stimulation contributes to the production of a suitable environment for micrometastasis within SLN

Sentinel lymph nodes (SLN) are the most common and earliest site of malignant tumor metastasis. The clinical success of sentinel node navigation surgery (14) suggests that SLN are an effective mechanical barrier against migrating cancer cells. SLN also contain marginal endothelial cells, which might be constantly loaded with high shear stress. It is known that primary tumors influence the microenvironments of distant organs during the development of metastasis (10, 11). However, it is unclear which molecules in premetastatic SLN loaded with high shear stress produce a suitable environment for micrometastasis within the node. Thus, we examined the hypothesis that the high shear stress generated by increased lymph flow through the SLN and its afferent lymph vessels contributes to the development of a premetastatic environment that is suitable for carcinoma micrometastases within the node.

The major findings of this study are summarized as follows. Two hours stimulation of cultured human LEC with a shear stress 0.5 or 1.0 dyn/cm^2 caused a significant increase in ICAM-1 expression at the protein and mRNA levels in human LEC. The shear stress stimulation strength used in the present study has been physiologically detected *in vivo* in the lymphatic endothelial cells of collecting lymph vessels (7). Stimulation with the same level of shear stress resulted in the release of a significant amount of ATP through the activation of cell surface F_1/F_O ATP synthase on human LEC. In agreement with these findings, F_1/F_O ATP synthase was immunocytochemically confirmed to be present on the surfaces of human

LEC. In addition, the administration of the selective cell surface F_1/F_O ATP synthase inhibitor piceatannol (20 μM) caused a significant reduction in the shear stress stimulation-induced increases in ICAM-1 protein and mRNA expression in human LEC. These findings were caused by a piceatannol-mediated decrease in shear stress stimulation-induced ATP release from the cells. In addition, shear stress stimulation caused a significant increase in the attachment of carcinoma cells to human LEC. The exogenous addition of ATP also produced a similar response in the carcinoma cell and human LEC attachment assay. Pretreatment with anti-ICAM-1 antibody or the non-selective purinergic P2X/P2Y inhibitor suramin caused a significant reduction in the shear stress stimulation- and exogenous ATP-mediated increases in the attachment of carcinoma cells to human LEC. In agreement with the findings obtained in the present molecular biological studies of human LEC, the expression of ICAM-1 within rat SLN that had been pretreated with exogenous ATP was confirmed both immunocytochemically and using intravital fluorescent-microscopy. In conclusion, shear stress stimulation generated by the high rate of lymph flow through SLN and their afferent lymph vessels produced a significant increase in ICAM-1 expression on the surfaces of lymphatic endothelial cells via the activation of cell surface F_1/F_O ATP synthase, which might contribute, in part, to the creation of a premetastatic environment and the subsequent production of carcinoma micrometastases within SLN. This conclusion is shown schematically in Figure 6. To confirm this conclusion, it will be necessary to evaluate the effects of shear stress stimulation on ICAM-1 expression within the SLN in an *in vivo* animal model study.

Crucial roles of ICAM-1 in micrometastasis

ICAM-1 expression by tumor cells has been reported to be a major contributor to the facilitation of metastatic progression (12, 15). Recently, we also observed strong ICAM-1 expression in human breast cancer SLN tissue that had been subjected to carcinoma cell micrometastasis, but weak or no ICAM-1 expression in SLN tissue that had not been subjected to metastasis (16). On the other hand, studies of leukocyte-endothelial cell adhesion in tumor microvessels have demonstrated diminished adhesive interactions under both basal and cytokine-stimulated conditions (17). It has been suggested that the proposed downregulation of endothelial ICAM-1 expression facilitates tumor progression by allowing tumor cells to avoid immunosurveillance by circulating lymphocytes. However, there have been several other immunohistochemical studies of the tumor vasculature in which the enhanced expression of endothelial ICAM-1, which resembles an inflammatory phenotype, was detected in breast cancer (18). Thus, the adhesion molecule expression profile of human LEC remains unclear.

In the present experiment, shear stress stimulation induced ATP release by activating cell surface F_1/F_O ATP synthase, which resulted in the overexpression of ICAM-1 on human LEC, and hence, facilitated the ICAM-1-mediated attachment of carcinoma cells to human LEC in the afferent lymph vessels of SLN from breast cancer patients. However, the molecular mechanisms of the shear stress stimulation-mediated activation of cell surface F_1/F_O ATP synthase on human LEC need to be clarified in future. Thus, except for that obtained in the present study, no information exists regarding the effects of shear stress stimulation on human LEC located near and/or within SLN, particularly with regard to their expression of adhesion molecules, their interactions with carcinoma cells, and their role in the development of a

premetastatic microenvironment that encourages carcinoma micrometastasis. Therefore, this study might be the first to suggest that shear stress stimulation plays crucial roles in the establishment of a pre-metastatic environment within SLN.

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COMPETING INTERESTS STATEMENT

The authors declare that no competing financial interests exist.

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FIGURE LEGENDS

Figure 1

A: Representative photomicrographs of the effects of starvation culture medium containing 3% fetal bovine serum (FBS) during 24 hours culture (h) (a~d), shear stress stimulation (1.0 dyn/cm^2) for different lengths of time (2 h or 24 h) (e~l), or 2 h treatment with 10 ng/mL LPS (m~p) on the expression of adhesion molecules; i.e., E-selectin, P-selectin, VCAM-1, and ICAM-1, on cultured human lymphatic endothelial cells (LEC). The photomicrographs have been merged with nuclear staining images of the same cells. Each marker represents 50 μm.

B: The density measurements of each photomicrograph, which were obtained using Scion Image analysis. The ordinate denotes the normalized value of each density measurement shown as the mean density/pixel (n = 5). The abscissa shows the length of the shear stress stimulation period. ** $p < 0.01$, significantly different from the no shear stress stimulation-mediated response.

C: The effects of shear stress strength (0.5 or 1.0 dyn/cm^2) on ICAM-1 expression on human LEC. The ordinate represents the same item as in Figure 1B. The abscissa shows the strength of the shear stress stimulus. ** $p < 0.01$, significantly different from the no shear stress stimulation-mediated response.

D: The effects of shear stress strength on ICAM-1 mRNA expression in human LEC, as evaluated by RT-PCR. The ordinate shows the ratio of ICAM-1 signals to cyclophilin A signals (n = 4). The abscissa shows the strength of the shear stress stimulus. ** $p < 0.01$, significantly different from the no shear stress stimulation-mediated response.

Figure 2

A: 1-3, Representative photomicrographs of the effects of shear stress stimulation (1.0 dyn/cm^2, 2 hours) on ICAM-1 expression on human LEC in the absence or presence of 20 μM piceatannol (a selective inhibitor of cell surface F_1/F_O ATP synthase) or 10^{-6} M suramin (a non-selective inhibitor of purinergic P2X/P2Y receptors).

A: 4-5, Representative photomicrographs of the effects of exogenous ATP (10^{-6} M) on ICAM-1 expression on human LEC in the absence or presence of 10^{-6} M suramin.

The photomicrographs have been merged with nuclear staining images of the same cultured cells. Each marker represents 50 μm.

B: The density measurements of each photomicrograph, which were obtained using Scion Image analysis. The ordinate represents the same item as in Figure 1B. Each number on the abscissa corresponds to the item with the same number in A. * $p < 0.05$, significantly different from the shear stress stimulation (1.0 dyn/cm^2, 2 hours)-mediated (positive control) response (column 1). ** $p < 0.01$, significantly different from the exogenous ATP (10^{-6} M, positive control)-mediated response (column 4).

C: The effects of shear stress stimulation (1.0 dyn/cm^2, 2 hours) in the absence or presence of 20 μM piceatannol or 10^{-6} M suramin, and exogenous ATP (10^{-6} M) in the absence or presence of 10^{-6} M suramin, on ICAM-1 mRNA expression in human LEC, as evaluated by RT-PCR. Each number on the abscissa represents the same item as in A. The ordinate represents the same item as in Figure 1D. * $p < 0.05$, significantly different from the shear stress stimulation (1.0 dyn/cm^2, 2 hours)-mediated response (column 1). ** $p < 0.01$, significantly different from the exogenous ATP (10^{-6} M)-mediated response (column 4).

Figure 3

A: The effects of shear stress stimulation (0.5 or 1.0 dyn/cm^2, 2 hours) on the concentration of ATP in the human LEC culture supernatant in the absence or presence of 20 μM piceatannol. The ordinate indicates the concentration of ATP in the culture supernatant, which was measured in a luciferin-luciferase assay. The abscissa denotes shear stress strength. * $p < 0.05$, ** $p < 0.01$, significantly different from the no shear stress stimulation-mediated response (negative control) or the 1.0 dyn/cm^2 shear stress stimulation-mediated response (positive control).

B: Representative photomicrographs of the effects of shear stress stimulation (1.0 dyn/cm^2, 2 hours) on the cell surface F_1/F_O ATP synthase expression of non-permeabilized cultured human LEC. The photomicrographs have been merged with nuclear staining images of the same cells. Each marker represents 50 μm.

C: The density measurements of the photomicrographs shown in Figure 2E, which were obtained using Scion Image analysis. The ordinate denotes the normalized value of each density measurement shown as the mean density/pixel (n = 5). NS, no significant difference between the columns.

Figure 4

A: Representative dark-field photomicrographs of the *in vitro* attachment assay assessing the effects of 2 hours shear stress stimulation (Figure 4A 2; 1.0 dyn/cm^2, positive control) or 2 hours treatment with exogenous ATP (Figure 4A 5; 10^{-6} M, positive control) in the absence or presence of 10^{-6} M suramin (Figures 4A 3 and 6, respectively) or anti-ICAM-1 antibody (Figures 4A 4 and 7, respectively).

B: Attachment assay data, which were normalized to the number of adherent carcinoma cells

per field (× 100). ** $p < 0.01$, significantly different from data obtained during shear stress stimulation (A2 and B2; positive control) or after the addition of exogenous ATP (A5 and B5; positive control). ** $p < 0.01$, significantly different from the negative control (A1 and B1; stimulation with starvation culture medium alone).

Figure 5

A: Representative photomicrographs of the effects of the subcutaneous administration of 1.0 mL of exogenous ATP (10^{-6} M; right panel) or PBS (left panel) on ICAM-1 expression within rat iliac lymph nodes in *in vivo* experiments. The lymph nodes were isolated and fixed with liquid nitrogen at 24 hours after the administration of ATP. The photomicrographs have been merged with nuclear staining images of the same cells. Each marker represents 200 μm.

B: Density measurements of the photomicrographs shown in Figure 5A, which were obtained using Scion Image analysis. The ordinate represents the same item as in Figure 3C (n = 10 images). ** $p < 0.01$, the columns are significantly different.

C: Representative photographs the effects of the subcutaneous administration (1.0 mL) of FITC-labeled anti-ICAM-1 antibody on the appearance of ICAM-1-ligated fluorescence within rat iliac lymph nodes in *in vivo* experiments. The lymph nodes had been perfused with 1.0 mL PBS containing no (negative control; left panel) or 10^{-6} M exogenous ATP for 24 hours before the intravital microscopic observation. The upper and lower panels show optical and fluorescent photomicrographs, respectively. Each marker represents 1 mm. The black and white arrows indicate murine iliac lymph vessels.

D: Summary of the density measurements of the photomicrographs shown in Figure 5C, which were obtained using Scion Image analysis. The ordinate represents the same item as

in Figure 3C (n = 10 images). ** $p < 0.01$, the columns are significantly different.

Figure 6

Schematic diagram outlining the conclusion of the present study.

Fig. 1

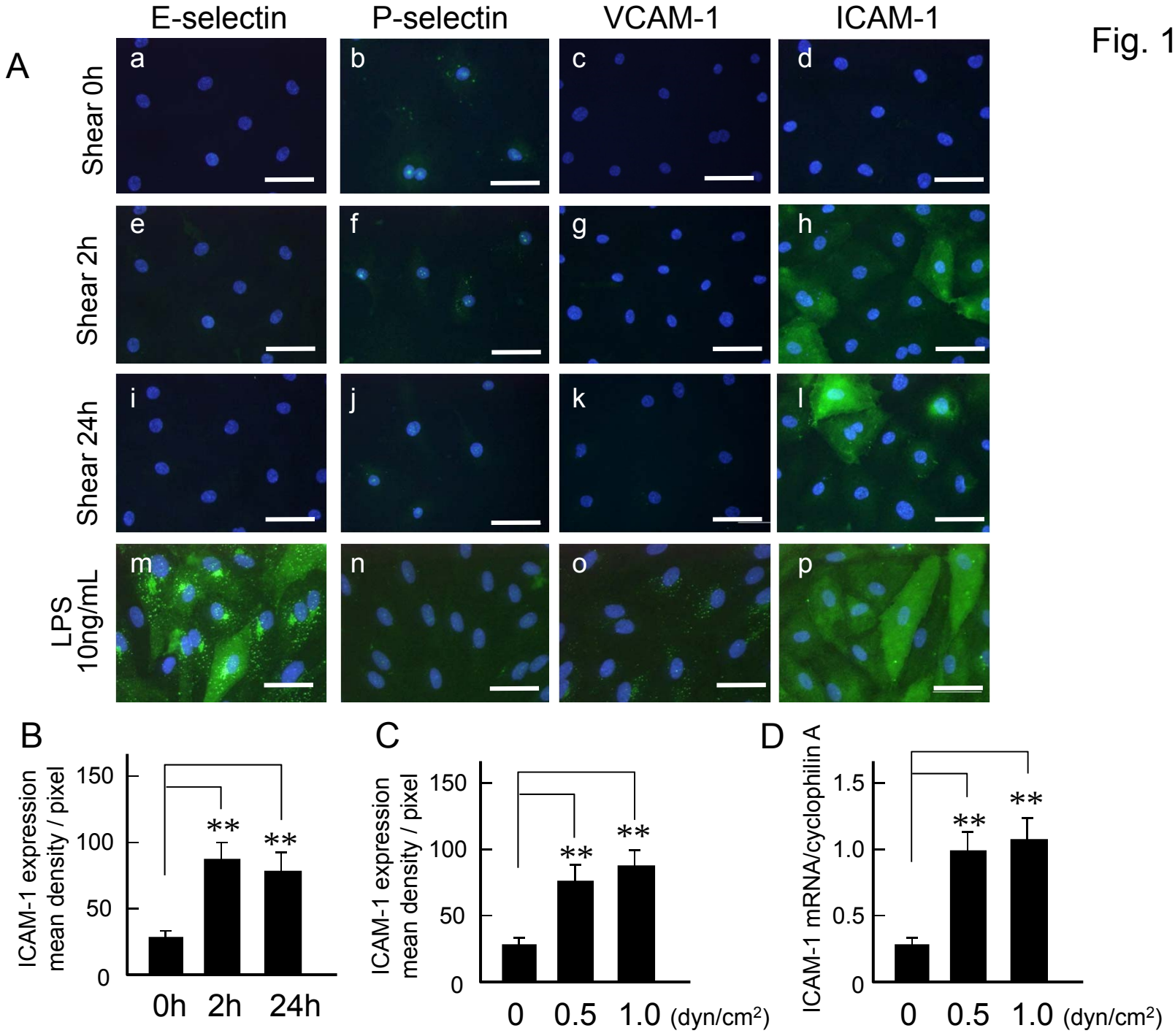
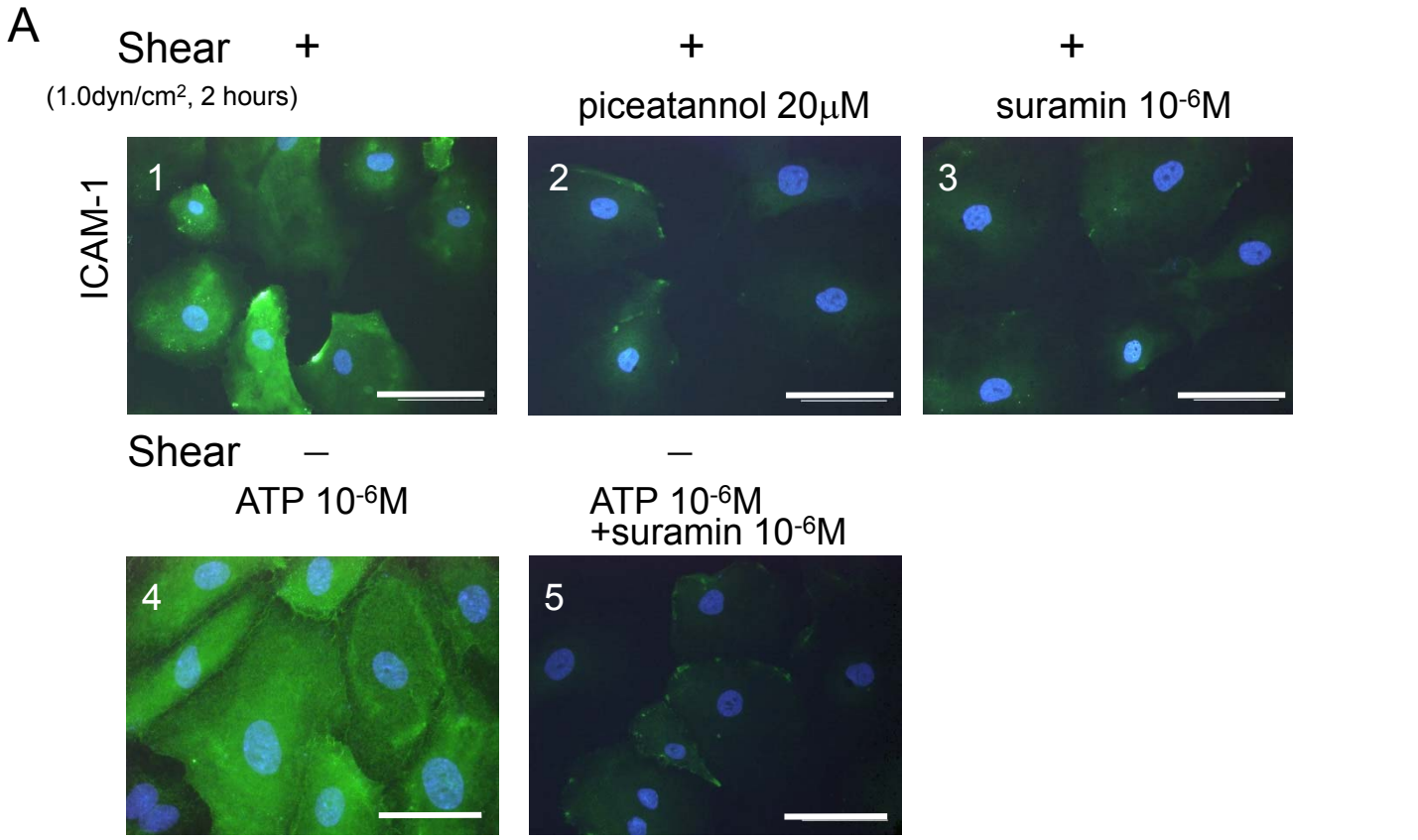
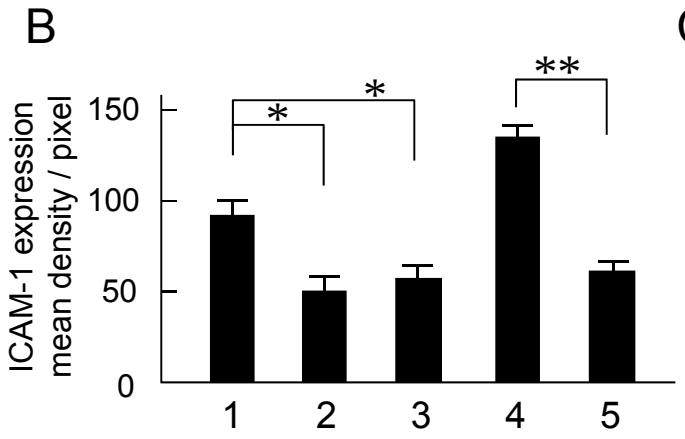

A
E-selectin
P-selectin
VCAM-1
ICAM-1
Shear 0h
Shear 2h
Shear 24h
LPS 10ng/mL
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
B
ICAM-1 expression mean density / pixel
150
100
50
0
**
**
0h
2h
24h
C
ICAM-1 expression mean density / pixel
150
100
50
0
**
**
0
0.5
1.0
(dyn/cm2)
D
ICAM-1 mRNA/cyclophilin A
1.5
1.0
0.5
0
**
**
0
0.5
1.0
(dyn/cm2)

Fig. 2


A
Shear +
(1.0dyn/cm², 2 hours)
+
piceatannol 20μM
+
suramin 10-6M
ICAM-1
1
2
3
Shear –
–
ATP 10-6M
ATP 10-6M
+suramin 10-6M
4
5


B
ICAM-1 expression
mean density / pixel
150
100
50
0
*
*
**
1
2
3
4
5

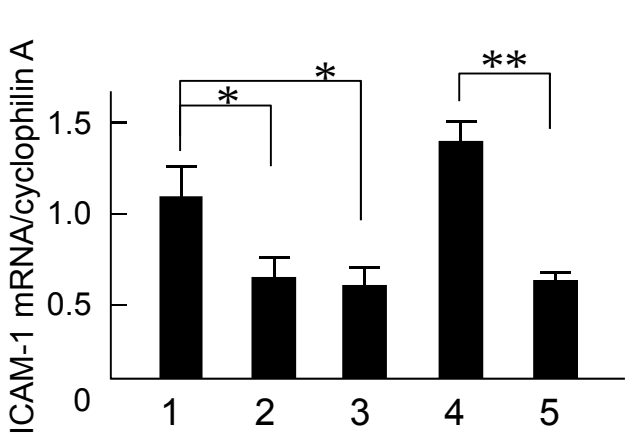

C
ICAM-1 mRNA/cyclophilin A
1.5
1.0
0.5
0
*
*
**
1
2
3
4
5

Fig. 3

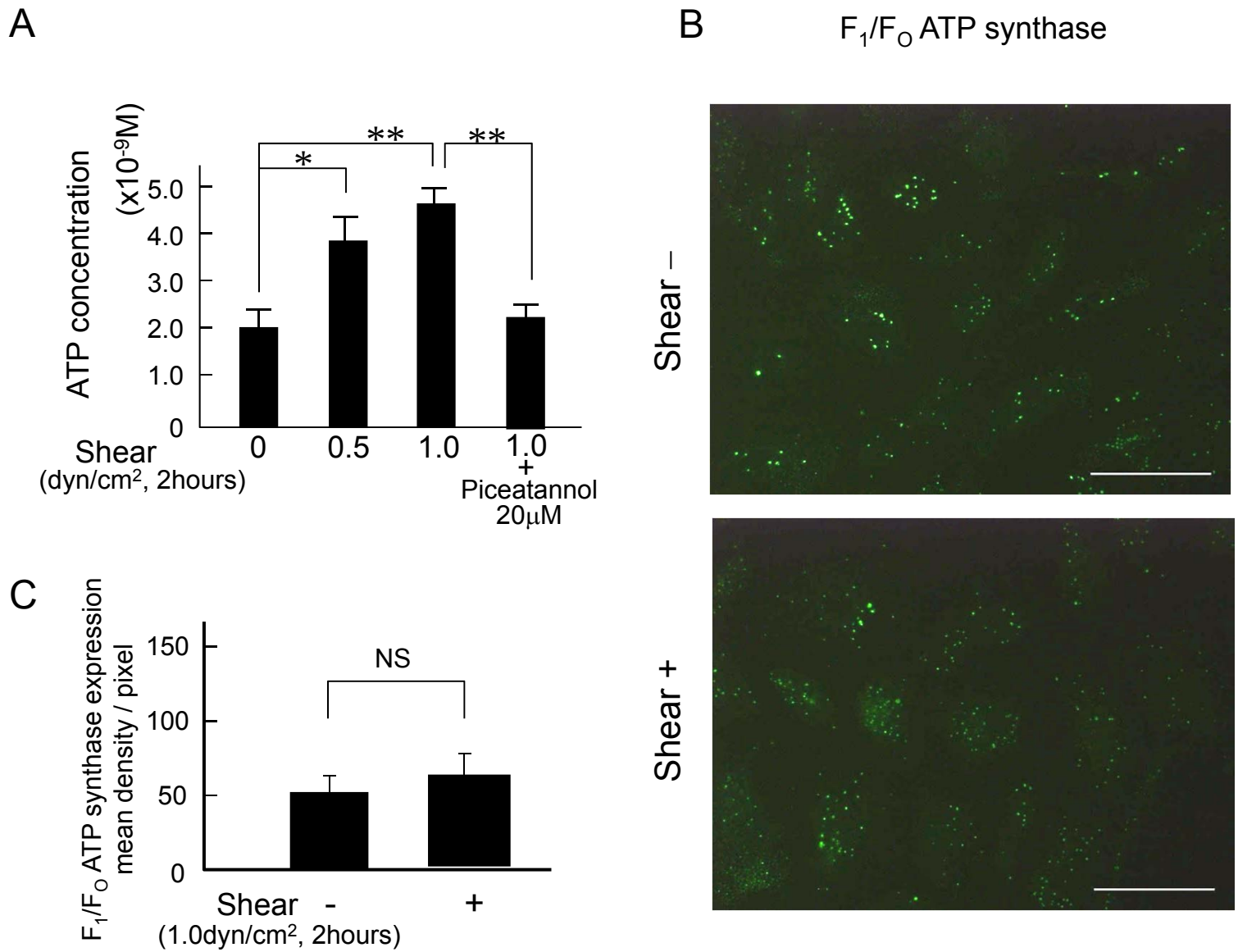
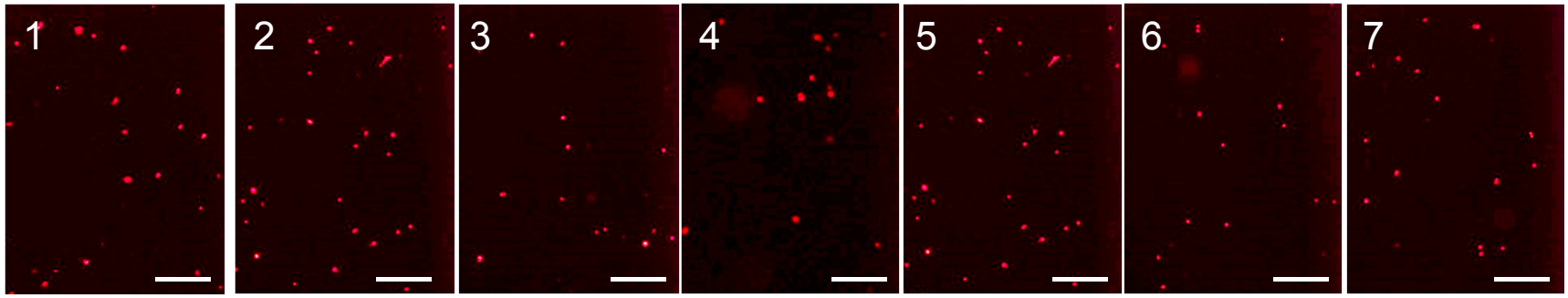

A
ATP concentration
(x10-9M)
5.0
4.0
3.0
2.0
1.0
0
*
**
**
Shear
(dyn/cm2, 2hours)
0
0.5
1.0
1.0
+
Piceatannol
20μM
B
F1/FO ATP synthase
Shear –
Shear +
C
F1/FO ATP synthase expression
mean density / pixel
150
100
50
0
NS
Shear
-
+
(1.0dyn/cm2, 2hours)

Fig. 4

A


1
2
3
4
5
6
7

B

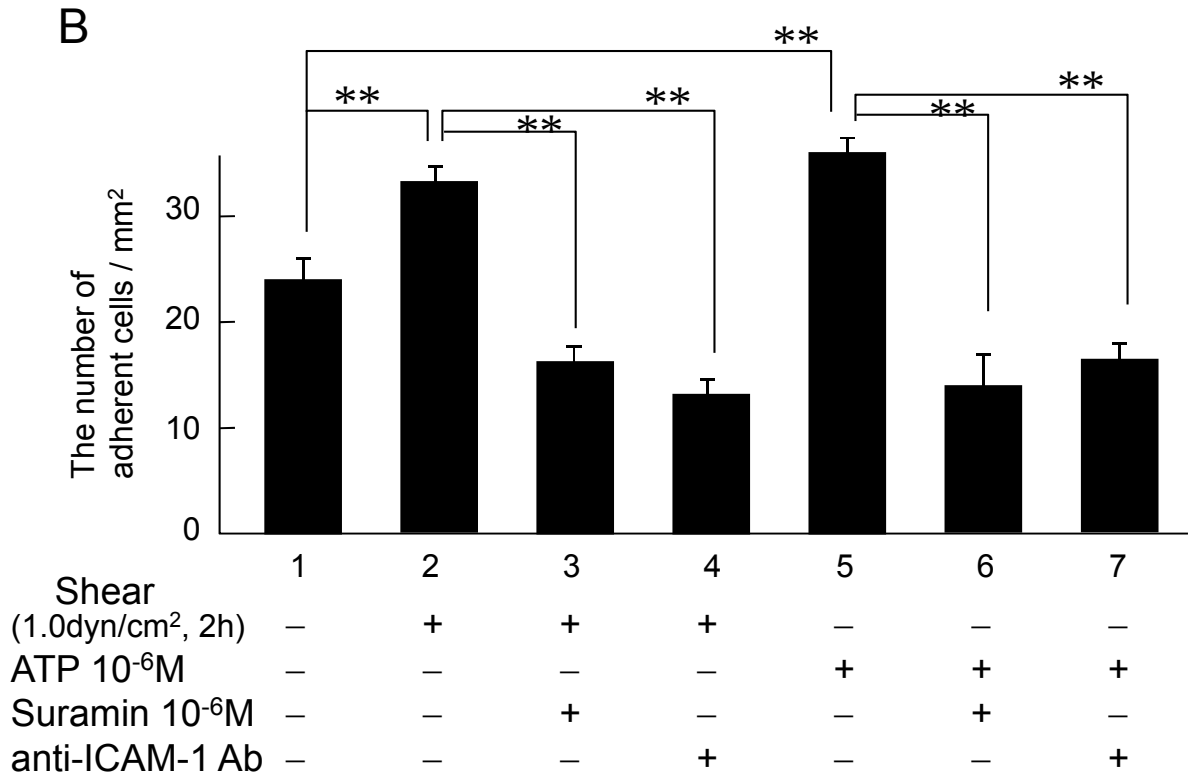
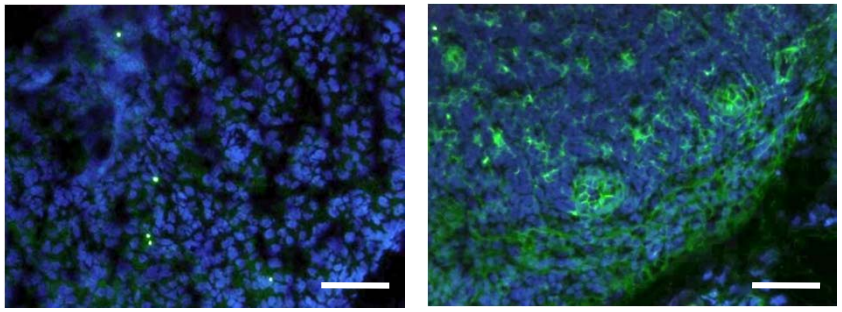

**
**
**
**
**
**
The number of adherent cells / mm2
0
10
20
30
1
2
3
4
5
6
7
Shear (1.0dyn/cm2, 2h) – + + + – – –
ATP 10-6M – – – – + + +
Suramin 10-6M – – + – – + –
anti-ICAM-1 Ab – – – + – – +

Fig. 5

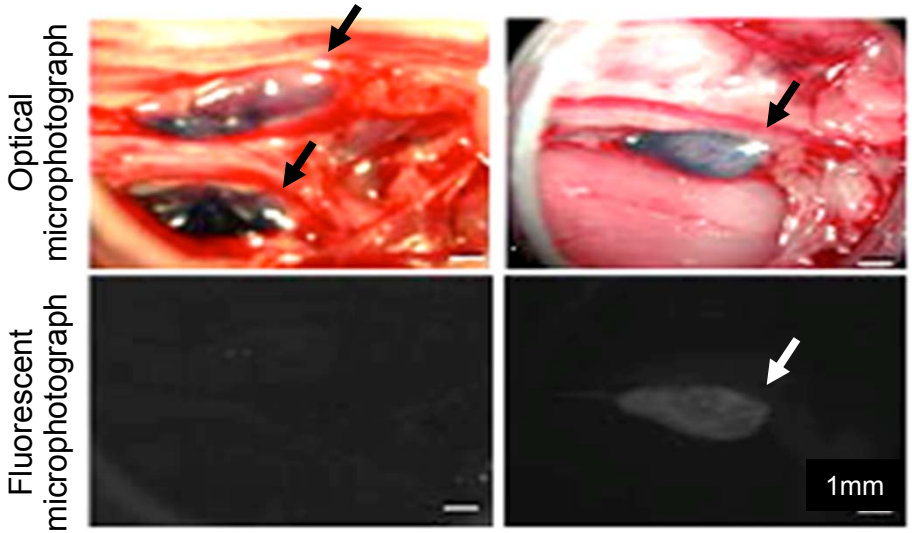
A



Administration of PBS (negative control)

Administration of ATP 10^{-6}M

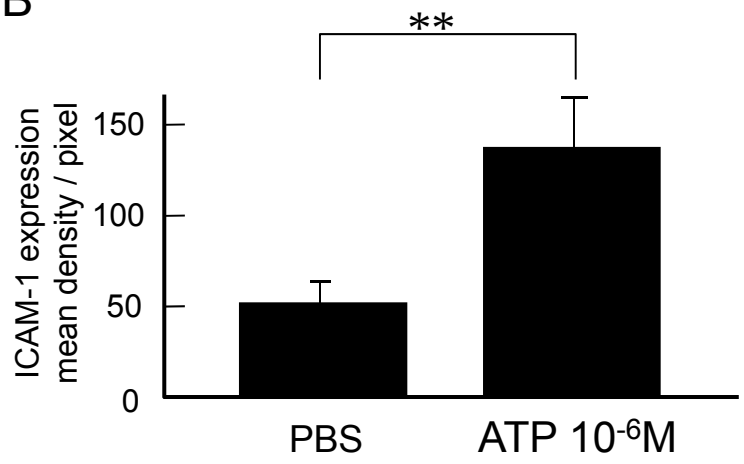
C



Administration of PBS (negative control)

Administration of ATP 10^{-6}M

B



D

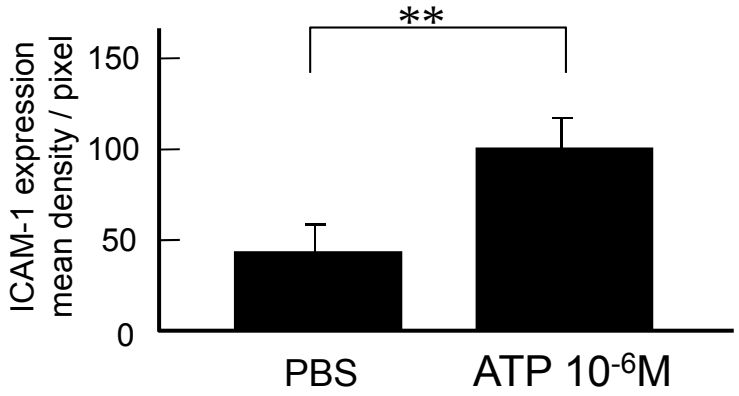
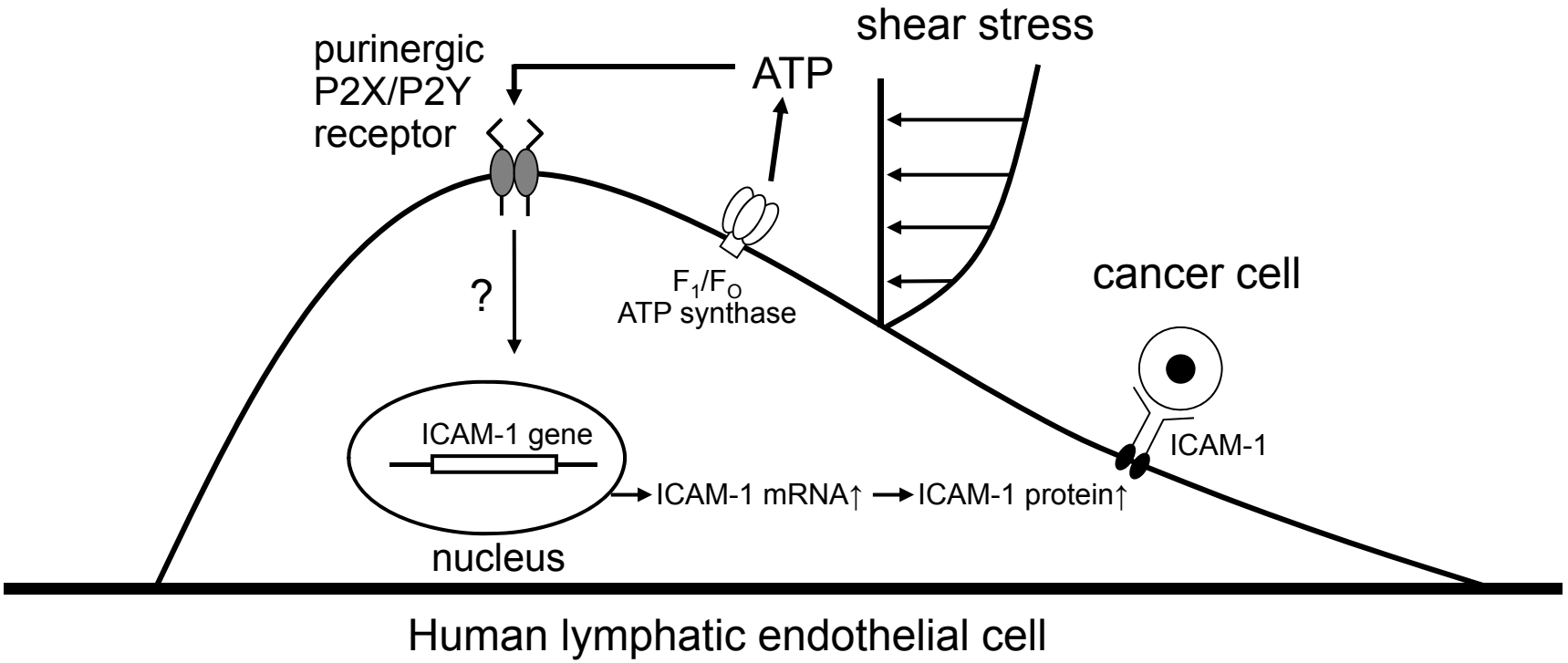


Fig. 6


purinergic
P2X/P2Y
receptor
ATP
shear stress
?
F_1/F_O
ATP synthase
cancer cell
ICAM-1 gene
ICAM-1
ICAM-1 mRNA↑
ICAM-1 protein↑
nucleus
Human lymphatic endothelial cell