

Fluid shear stress suppresses ICAM-1-mediated transendothelial migration of leukocytes in coculture model

著者	Naoya Sakamoto, Yosuke Ueki, Masaki Oi, Takuya Kiuchi, Masaaki Sato
journal or publication title	Biochemical and Biophysical Research Communications
volume	502
number	3
page range	403-408
year	2018-05-31
URL	http://hdl.handle.net/10097/00125388

doi: 10.1016/j.bbrc.2018.05.182

Fluid shear stress suppresses ICAM-1-mediated transendothelial migration of leukocytes in coculture model

Naoya Sakamoto¹⁾, Yosuke Ueki²⁾, Masaki Oi²⁾, Takuya Kiuchi²⁾, Masaaki Sato^{2, 3, 4)}

1) Graduate School of Systems Design, Tokyo Metropolitan University,

1-1Minami-Osawa, Hachioji, Tokyo192-0397, Japan

2) Graduate School of Engineering, Tohoku University, Japan

3) Graduate School of Biomedical Engineering, Tohoku University, Japan

4) Frontier Research Institute for Interdisciplinary Sciences, Tohoku University, Japan

Abbreviated title: Effect of Shear Stress on Leukocyte Migration in Coculture Model

Correspondence to:

Naoya Sakamoto, Ph.D.

Department of Mechanical Systems Engineering

Graduate School of Systems Design, Tokyo Metropolitan University

Mailing address: 1-1Minami-Osawa, Hachioji, Tokyo192-0397, Japan

Tel/Fax: +81-42-677-2709

E-mail: sakan@tmu.ac.jp

Abstract

The adhesion and migration of leukocytes to arterial endothelial cells (ECs), one of the indicators of early atherogenesis, is believed to be correlated with the blood flow conditions and interactions between vascular cells including vascular smooth muscle cells (SMCs). In this study, we investigated the effect of fluid shear stress on the transendothelial migration of leukocytes in a coculture model (CM) composed of human umbilical ECs and SMCs, a layer of collagen type I, and a porous membrane. Following exposure to a fluid shear stress of 1.5 Pa for 24 h, human mononuclear leukocytes were seeded on the EC surface and cultured for 1 h. Leukocytes migrating across the EC layer were observed by confocal laser scanning microscopy. The number of migrating leukocytes in the statically cultured CM was significantly larger than that in the static EC monoculture model. The exposure to the shear stress significantly decreased the leukocyte migration induced by the coculture condition. In the static CM, fluorescence staining and Western blotting showed a higher expression of intercellular adhesion molecule-1 (ICAM-1) of ECs. These results indicate that SMC-derived bioactive soluble factors may stimulate the ICAM-1 expression of cocultured ECs, possibly leading to leukocyte migration into the subendothelial space.

Keywords: Coculture system, Atherosclerosis, Endothelial–Smooth muscle cell interaction, Adhesion molecules

Introduction

The effects of fluid shear stress on the functions of vascular endothelial cells have been investigated because the formation of atherosclerosis tends to localize at the bifurcation and curved sites of arteries, which are expected to be predominated by a low shear stress, the flow separation from the vessel wall, and complex flow patterns [1, 2]. The adhesion of blood mononuclear leukocytes to the endothelial cells of arteries is one of the indicators of early atherogenesis [3, 4]. Leukocytes adhere to the endothelium and migrate into the vessel walls mediated by adhesion proteins expressed on the endothelial cell surface such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). Till date, in vitro experiments using endothelial cells in a monoculture have revealed that the expression of adhesion proteins is not only affected by exogenous cytokines such as tumor necrosis factor- α (TNF- α) and interleukine-1 β (IL-1 β) but also by the shear stress acting on endothelial cells [5–7]. Concurrently, previous studies have also reported that smooth muscle cells interact with endothelial cells and have an effect on the properties of leukocyte adhesion to endothelial cells. For example, Rainger and Nash showed that endothelial cells cocultured with smooth muscle cells were sensitized to respond to TNF- α , and they changed the E-selectin expression used for leukocyte capture [8]. Chiu et al. demonstrated that a coculture with smooth muscle cells induced the endothelial cell gene expression of VCAM-1 and ICAM-1, whereas shear stress inhibited the coculture-induced gene expression of these adhesion proteins [9].

These previous studies used a coculture system in which endothelial cells and smooth muscle cells were cocultured on the opposite sides of a porous membrane. Because there was no space in their coculture systems to allow leukocytes to migrate, it was difficult to observe the transendothelial migration of leukocytes. Several studies, on the other hand, have cocultured endothelial cells and smooth muscle cells separated by a collagen layer and assessed the migration of leukocytes [10, 11]. Moreover, it was shown that the number of leukocytes that migrated into the collagen layer under the coculture condition increased compared with that under a monoculture condition [10]. However, the coculture systems used by these studies were not appropriate for the flow-exposure experiment, possibly owing to the lack of sufficient mechanical stiffness to endure the fluid shear stress conditions. Hence, the effect of shear stress on leukocyte migration under a coculture condition has not been completely elucidated so far.

We have studied the cellular interaction between endothelial cells and smooth muscle cells using a coculture model that is suitable for the fluid-flow exposure experiment at a physiological level of the shear stress [12–

14]. In this study, we evaluated the adhesion and migration of leukocytes using the cocultured model subjected to a fluid shear stress. We observed that leukocytes migrated to the collagen layer separating the endothelial and smooth muscle cells in the model.

Materials and Methods

Cell culture

Human endothelial cells and smooth muscle cells were isolated from umbilical cords using enzymatic digesting methods, as previously described [12, 14]. They were grown in gelatin-coated culture dishes in Medium 199 (M199) (Invitrogen, USA) containing 20% fetal bovine serum, 1 unit/mL penicillin–streptomycin (Invitrogen), and 100 ng/mL human basic fibroblast growth factor (bFGF, Austral Biological, USA). Cells with 2–8 passages were used for the experiments.

Construction of Coculture model

The endothelial–smooth muscle cells coculture model was constructed as previously described [12, 13]. Smooth muscle cells were cultured in dishes until they attained confluence. Collagen gels were prepared by mixing 5 mg/mL bovine skin collagen type I (Koken, Japan), $10 \times$ M199, and reconstruction buffer (0.05 M NaOH, 0.2 M HEPES, and 0.26 M NaHCO₃). The mixture was poured into a dish, and immediately after, a porous membrane (pore diameter: 5 μ m) was placed on the collagen solution. The mixture was allowed to polymerize for at least 1 h at 37 °C, and endothelial cells were then seeded on the membrane (Fig. 1). The coculture models were cultured for at least 48 h prior to the experiments. A model constructed without smooth muscle cells, termed as the monoculture model, was also used in the experiments (Fig. 1).

Flow-exposure experiment

The flow circuit and flow chamber have been previously described [12, 14]. The cocultured dish was incorporated into a parallel-plate flow chamber and exposed to a steady wall shear stress of 1.5 Pa, which could be assumed to be an average magnitude for the physiological shear stress, for 24 h at 37 °C in 95% air/5% CO₂ atmosphere.

Leukocyte adhesion and migration assay

Mononuclear leukocytes were isolated from human peripheral blood by density gradient centrifugation with Lymphoprep™ (Axis-shield, Norway). To aid visualization, the leukocytes were incubated with a culture medium containing 10 μM of calcein-AM (Molecular Probes, USA) for 90 min, which did not affect the adhesion [15]. The study protocols were officially approved by the Ethics Committee for Clinical Research with Human Subjects of Tohoku University.

After the flow-exposure experiments, the coculture model was incubated statically for 1 h with a medium containing mononuclear leukocytes (2.0×10^5 cells/cm²), allowing the leukocytes to bind to the surface of the endothelial monolayer. The model was then gently washed thrice with phosphate-buffered saline (PBS) to remove the unadhered leukocytes. After the model was fixed with 10% formalin, the cells in the model were stained with 150 nM rhodamine-phalloidin (Molecular Probes, USA). Sequential fluorescent images of the model along the z-axis were captured at an interval of 1 μm using a confocal laser scanning microscope (Olympus, Japan).

Immunofluorescent staining

After exposure to the shear stress, the coculture model was incubated for 1 h at room temperature (RT) with mouse monoclonal antibody directed to ICAM-1 (R&D systems, USA). Subsequently, the model was rinsed twice with PBS and incubated for 45 min at RT with FITC-conjugated anti-mouse IgG. Fluorescent images of ICAM-1 were captured by fluorescent microscopy (Olympus).

Western blotting

In the models, the endothelial cells were washed thrice with ice-cold PBS and lysed in ice-cold triton/NP-40 lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate, 1% NP-40, 1% SDS, 100 μM sodium orthovanadate, 1 μM phenyl-methylsulfonyl, and 1% protease inhibitor cocktail). The cell lysates were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membrane, and stained with a primary antibody against ICAM-1 (R&D systems). An alkaline phosphatase (AP)-conjugated secondary antibody (Chemicon International, USA) was detected by an amplified AP immuno-blot kit (Bio-Rad, USA). The band intensity was quantified from scanned membrane images using ImageJ software (National Institutes of Health, USA).

Data Analysis

The results are expressed as means with standard deviations determined from at least three independent experiments. The significance of the differences was determined by the student's *t* test. A *p* value less than 0.05 was considered statically significant.

Results and Discussion

We observed that leukocytes adhered to the endothelial cell monolayer in the coculture models (Fig. 2A), and there were no specific regions on the endothelial cells where leukocytes were prone to adhere. We also observed that leukocytes migrated to the collagen layer in the endothelial cells in the coculture model (Fig. 2B). Based on these fluorescence images, we counted the number of leukocytes both adhered to the endothelial cell monolayer and migrated to the collagen layer in the observed area of the model. We then found that the total number of leukocytes (the sum of the adhered and migrated leukocytes) observed in the models was not affected by the coculture and shear stress conditions (monoculture static, 12.4 ± 5.7 cells/0.1 mm²; monoculture sheared, 13.3 ± 9.3 cells/0.1 mm²; coculture static, 12.5 ± 2.1 cells/0.1 mm²; coculture sheared, 14.0 ± 2.8 cells/0.1 mm²) (Fig. 3A). However, the number of leukocytes that migrated in the static coculture model was approximately ten times higher than that in the monoculture model (monoculture static, 0.65 ± 0.65 cell/0.1 mm²; monoculture sheared, 0.54 ± 0.38 cell/0.1 mm²; coculture static, 7.3 ± 1.5 cells/0.1 mm², $p < 0.001$), and the exposure to shear stress decreased the leukocyte migration (coculture sheared, 2.3 ± 0.39 cells/0.1mm²). More than 40% leukocytes were found to migrate to the collagen layer in the static coculture model, whereas approximately 10% leukocytes migrated to the monoculture and sheared coculture models (Fig. 3B). We also used a model composed of only smooth muscle cells embedded in the collagen layer without the endothelial monolayer (SMC model) (Fig. 1) and found no leukocyte migration in the SMC model (data not shown).

After immunostaining, we observed that a higher fluorescence of ICAM-1 was expressed on the endothelial cells in the static coculture model compared with the other models (Fig. 4A). The result of Western blotting also indicated a higher expression of ICAM-1 of endothelial cells in the co-culture model cultured statically, and the ICAM-1 expression was decreased by exposure to the wall shear stress (Fig. 4B). We performed Western blotting of VCAM-1 but could not detect the expressions (data not shown).

Previous studies have shown that cytokine stimulation such as with TNF- α and IL-1 β increases the adhesion and migration of leukocytes to the endothelial cell monolayer [16, 17]. In contrast, the present study shows

leukocyte adhesion to endothelial cells and migration across the endothelial monolayer cocultured with smooth muscle cells without exogenous cytokine stimulation. We also find that the migration of leukocytes rarely occurs in the endothelial monoculture and SMC models. These results indicate that leukocyte adhesion and migration observed in the coculture model are caused by smooth muscle cell-derived cytokines. Furthermore, cellular interactions with both endothelial cells and smooth muscle cells play important roles in the phenomena of leukocyte migration to arterial walls.

Although there were no significant differences in the total number of leukocytes observed in the monoculture and coculture models both under static and sheared conditions, the number of migrating leukocytes was approximately ten times higher in the static coculture model compared with the other conditions. Moreover, an increase in ICAM-1 expression of endothelial cells was observed in the static coculture model. These results suggest that leukocytes adhered to ICAM-1 of endothelial cells, whose expression was stimulated by the endothelial–smooth muscle cell interactions, tended to migrate under the subendothelial space. Previous studies using endothelial cells in a monoculture showed that VCAM-1 expression was suppressed by the exposure to fluid shear stress. It was therefore, suggested that leukocyte adhesion to endothelial cells via VCAM-1 was involved in atherosclerogenesis [18, 19]. However, it is still controversial that Burns and DePaola showed flow-induced variations in the leukocyte adhesion to endothelial cells, but they could not detect the VCAM-1 expression changes by either immunofluorescence or Western blotting under both static and sheared conditions [20]. Alternatively, it is now recognized, that the ICAM-1 expression also plays an important role in the transendothelial migration of leukocytes [21–23]. The adhesion and migration of leukocytes adhered to ICAM-1 of endothelial cells under the effect of the smooth muscle cells are considered to be crucial for the pathogenesis of atherosclerosis because in the *in vivo* atherosclerotic regions, endothelial cells are believed to have an interaction with the smooth muscle cells migrated under the subendothelial spaces.

Previous studies have reported the effects of shear stress on ICAM-1 expression of monocultured endothelial cells, but the findings were not completely in agreement; the exposure of the shear stress increased or did not significantly affect the endothelial expression of ICAM-1 [7, 18, 24]. In the present study, we found that ICAM-1 expression of endothelial cells under the static condition was significantly increased by the coculture with smooth muscle cells compared with the monoculture condition. Furthermore, ICAM-1 expression was suppressed by the shear stress. The regulation of the adhesion protein expressions seems to be

related to a complex mechanism, but our results also emphasize the importance of the coculture conditions for endothelial biology and the investigation of the association between endothelial cells and leukocyte adhesion and migration.

The existence of smooth muscle cells may have an effect on the leukocyte migration mediated by soluble bioactive factors secreted by smooth muscle cells. However, leukocyte migration was not observed in the SMC model composed of only smooth muscle cells embedded in the collagen layer without endothelial cells. This indicates that the migration of leukocytes to the collagen layer induced by the coculture condition requires adhesion to endothelial cells. Leukocytes need to undergo a large self-deformation for the migration when they pass through the narrow gaps between the layers of endothelial cells. Moreover, our models consist of a porous membrane with 5- μm -diameter pores, which are smaller than the diameters of the leukocytes. Stiffness of endothelial cells has been reported to be crucial for the transmigration of leukocytes [25, 26]. Similarly, although the detailed mechanisms underlying the modifications in the cell deformability are unclear, adherence to endothelial cells may increase the deformability of leukocytes, facilitating their transmigration through the porous membrane. In addition to ICAM-1, platelet endothelial cell adhesion molecule-1 (PECAM-1), known to mediate the cell–cell adhesion of endothelial cells, are suggested to stimulate the transendothelial migration of leukocytes [27–29]. The adhesion of leukocytes to these molecules expressed on endothelial cells may be necessary for their migration to the subendothelial spaces.

The results of the total number of leukocytes observed in both the monoculture and coculture models were not different for the static and sheared conditions. Hence, this study indicates that the exposure of the models to a shear stress decreased only the migration and did not affect the adhesion of leukocytes. One of the possible mechanisms underlying this decrease is explained by the variations in the production of chemoattractants from endothelial cells and/or smooth muscle cells under a shear stress condition. Chiu et al. showed that the gene expression of growth related oncogene- α (GRO- α) and monocyte chemotactic protein-1 (MCP-1) of endothelial cells was increased by a coculture with smooth muscle cells and eliminated by exposure to a shear stress [9, 30]. Furthermore, a physiological level of the shear stress is thought to maintain endothelial tight junctions, which are known to act as barriers in the leukocyte migration through para-endothelial cell sites. To clarify the mechanisms by which shear stress decreased the coculture-induced leukocyte migration, we need to further investigate the roles of these factors.

In summary, using the endothelial–smooth muscle coculture model that we have developed, the present

study showed that a coculture with smooth muscle cells stimulates the transendothelial migration of leukocytes and ICAM-1 expression of endothelial cells, even though the total number of leukocytes observed in the models were not affected by the coculture condition. We also found that the exposure to shear stress significantly suppressed the coculture-induced leukocyte migration and ICAM-1 expression. Our findings indicate the investigation with coculture systems suitable for leukocyte migration is necessary for further understanding the role of shear stress condition in the pathophysiology of atherosclerosis.

Acknowledgements

The authors would like to thank Drs. Makoto Takahashi and Ikuo Takahashi for kindly providing human umbilical cords with the informed consents of the donors. The present study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan (Nos. 20001007, 15K01304, 16K15837, and 17H02077).

Conflicts of Interest

N. Sakamoto, Y. Ueki, M. Oi, T Kiuchi, and M. Sato declare that they have no conflicts of interest.

References

- [1] R.M. Nerem, *Atherosclerosis and the Role of Wall Shear Stress*, Springer, New York, NY, 1995.
- [2] A.M. Malek, S.L. Alper, S. Izumo, Hemodynamic shear stress and its role in atherosclerosis, *JAMA* 282 (1999) 2035-2042.
- [3] R. Ross, The pathogenesis of atherosclerosis: a perspective for the 1990s, *Nature* 362 (1993) 801-809.
- [4] R. Ross, Atherosclerosis--an inflammatory disease, *N. Engl. J. Med.* 340 (1999) 115-126.
- [5] A. Ohtsuka, J. Ando, R. Korenaga, A. Kamiya, N. Toyama-Sorimachi, M. Miyasaka, The effect of flow on the expression of vascular adhesion molecule-1 by cultured mouse endothelial cells, *Biochem. Biophys. Res. Commun.* 193 (1993) 303-310.
- [6] J. Ando, A. Ohtsuka, Y. Katayama, R. Korenaga, C. Ishikawa, A. Kamiya, Intracellular calcium response to directly applied mechanical shearing force in cultured vascular endothelial cells, *Biorheology* 31 (1994) 57-68.
- [7] T. Nagel, N. Resnick, W.J. Atkinson, C.F. Dewey, Jr., M.A. Gimbrone, Jr., Shear stress selectively upregulates intercellular adhesion molecule-1 expression in cultured human vascular endothelial cells, *J. Clin. Invest.* 94 (1994) 885-891.
- [8] G.E. Rainger, P. Stone, C.M. Morland, G.B. Nash, A novel system for investigating the ability of smooth muscle cells and fibroblasts to regulate adhesion of flowing leukocytes to endothelial cells, *J. Immunol. Methods* 255 (2001) 73-82.
- [9] J.J. Chiu, L.J. Chen, C.N. Chen, P.L. Lee, C.I. Lee, A model for studying the effect of shear stress on interactions between vascular endothelial cells and smooth muscle cells, *J. Biomech.* 37 (2004) 531-539.
- [10] M. Navab, G.P. Hough, L.W. Stevenson, D.C. Drinkwater, H. Laks, A.M. Fogelman, Monocyte migration into the subendothelial space of a coculture of adult human aortic endothelial and smooth muscle cells, *J. Clin. Invest.* 82 (1988) 1853-1863.
- [11] M. Takaku, Y. Wada, K. Jinnouchi, M. Takeya, K. Takahashi, H. Usuda, M. Naito, H. Kurihara, Y. Yazaki, Y. Kumazawa, Y. Okimoto, M. Umetani, N. Noguchi, E. Niki, T. Hamakubo, T. Kodama, An in vitro coculture model of transmigrant monocytes and foam cell formation, *Arterioscler. Thromb. Vasc. Biol.* 19 (1999) 2330-2339.
- [12] N. Sakamoto, T. Ohashi, M. Sato, Effect of fluid shear stress on migration of vascular smooth muscle cells in cocultured model, *Ann. Biomed. Eng.* 34 (2006) 408-415.

- [13] N. Sakamoto, M. Oi, T. Ohashi, M. Sato, Nitric oxide production and smooth muscle cell phenotype of endothelial cell-smooth muscle cell coculture model, *J. Biomech. Sci. Eng.* 1 (2006) 224-233.
- [14] N. Sakamoto, T. Kiuchi, M. Sato, Development of an endothelial-smooth muscle cell coculture model using phenotype-controlled smooth muscle cells, *Ann. Biomed. Eng.* 39 (2011) 2750-2758.
- [15] K.B. Abbitt, G.E. Rainger, G.B. Nash, Effects of fluorescent dyes on selectin and integrin-mediated stages of adhesion and migration of flowing leukocytes, *J. Immunol. Methods.* 239 (2000) 109-119.
- [16] S. Sheikh, G.E. Rainger, Z. Gale, M. Rahman, G.B. Nash, Exposure to fluid shear stress modulates the ability of endothelial cells to recruit neutrophils in response to tumor necrosis factor-alpha: a basis for local variations in vascular sensitivity to inflammation, *Blood* 102 (2003) 2828-2834.
- [17] R. Oberoi, J. Schuett, H. Schuett, A.K. Koch, M. Luchtefeld, K. Grote, B. Schieffer, Targeting tumor necrosis factor-alpha with adalimumab: effects on endothelial activation and monocyte adhesion, *PLoS One* 11 (2016) e0160145.
- [18] J. Ando, H. Tsuboi, R. Korenaga, Y. Takada, N. Toyama-Sorimachi, M. Miyasaka, A. Kamiya, Shear stress inhibits adhesion of cultured mouse endothelial cells to lymphocytes by downregulating VCAM-1 expression, *Am. J. Physiol.* 267 (1994) C679-687.
- [19] R. Korenaga, J. Ando, K. Kosaki, M. Isshiki, Y. Takada, A. Kamiya, Negative transcriptional regulation of the VCAM-1 gene by fluid shear stress in murine endothelial cells, *Am. J. Physiol.* 273 (1997) C1506-1515.
- [20] M.P. Burns, N. DePaola, Flow-conditioned HUVECs support clustered leukocyte adhesion by coexpressing ICAM-1 and E-selectin, *Am. J Physiol.* 288 (2005) H194-204.
- [21] J. Millan, L. Hewlett, M. Glyn, D. Toomre, P. Clark, A.J. Ridley, Lymphocyte transcellular migration occurs through recruitment of endothelial ICAM-1 to caveola- and F-actin-rich domains, *Nat. Cell. Biol.* 8 (2006) 113-123.
- [22] B. Engelhardt, H. Wolburg, Mini-review: Transendothelial migration of leukocytes: through the front door or around the side of the house?, *Eur. J. Immunol.* 34 (2004) 2955-2963.
- [23] A. Schaefer, P.L. Hordijk, Cell-stiffness-induced mechanosignaling - a key driver of leukocyte transendothelial migration, *J. Cell. Sci.* 128 (2015) 2221-2230.
- [24] R. Sampath, G.L. Kukielka, C.W. Smith, S.G. Eskin, L.V. McIntire, Shear stress-mediated changes in the expression of leukocyte adhesion receptors on human umbilical vein endothelial cells in vitro, *Ann. Biomed. Eng.* 23 (1995) 247-256.

- [25] P.L. Hordijk, Recent insights into endothelial control of leukocyte extravasation, *Cell. Mol. Life Sci.* 73 (2016) 1591-1608.
- [26] K.H. Song, J. Lee, H. Park, H.M. Kim, J. Park, K.W. Kwon, J. Doh, Roles of endothelial A-type lamins in migration of T cells on and under endothelial layers, *Sci. Rep.* 6 (2016) 23412.
- [27] W.A. Muller, Transendothelial migration: unifying principles from the endothelial perspective, *Immunol. Rev.* 273 (2016) 61-75.
- [28] W.A. Muller, S.A. Weigl, X. Deng, D.M. Phillips, PECAM-1 is required for transendothelial migration of leukocytes, *J. Exp. Med.* 178 (1993) 449-460.
- [29] D.E. Conway, M.A. Schwartz, Mechanotransduction of shear stress occurs through changes in VE-cadherin and PECAM-1 tension: implications for cell migration, *Cell Adh. Migr.* 9 (2015) 335-339.
- [30] J.J. Chiu, L.J. Chen, S.F. Chang, P.L. Lee, C.I. Lee, M.C. Tsai, D.Y. Lee, H.P. Hsieh, S. Usami, S. Chien, Shear stress inhibits smooth muscle cell-induced inflammatory gene expression in endothelial cells: role of NF-kappaB, *Arterioscler. Thromb. Vasc. Biol.* 25 (2005) 963-969.

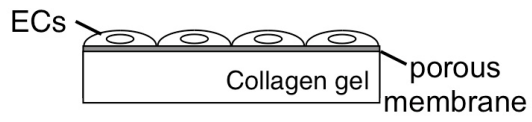
Figure legends

Fig. 1 Schematic of the experimental models used. The coculture model is composed of endothelial cells (ECs) cultured on a porous membrane and smooth muscle cells (SMCs), and a collagen layer. The monoculture model and SMC model do not include SMCs and ECs, respectively.

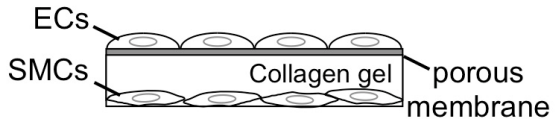
Fig. 2 Typical fluorescence image of leukocytes and the coculture model. (A) Leukocytes (green, arrows) adhered to the surface of the endothelial cells (red) in the coculture model cultured statically. (B) Three-dimensional image of the coculture model reconstructed from the sequential images obtained via confocal microscopy. Leukocytes migrated to the collagen layer (green, arrow heads) were observed between endothelial cells (ECs) and smooth muscle cells (SMCs). The porous membrane was invisible in the fluorescence images.

Fig. 3 Effect of shear stress on the adhesion and migration of leukocytes in the monoculture and coculture models. (A) Number of total and migrated leukocytes observed in the models under static and sheared conditions. (B) Ratio of the migrated cells to the total number of leukocytes. *: $p < 0.05$ vs. static monocultured, †: $p < 0.05$ vs. sheared cocultured.

Fig. 4: Effect of shear stress on ICAM-1 expression of endothelial cells in the monoculture and co-culture models. (A) Typical fluorescence images of ICAM-1 of endothelial cells in the models under static and sheared conditions. (B) A representative result of Western blotting of ICAM-1 detected in whole lysates of endothelial cells. The results shown are representative of three independent experiments.



Monoculture model



Coculture model



SMC model

Fig. 1 Sakamoto N et al.

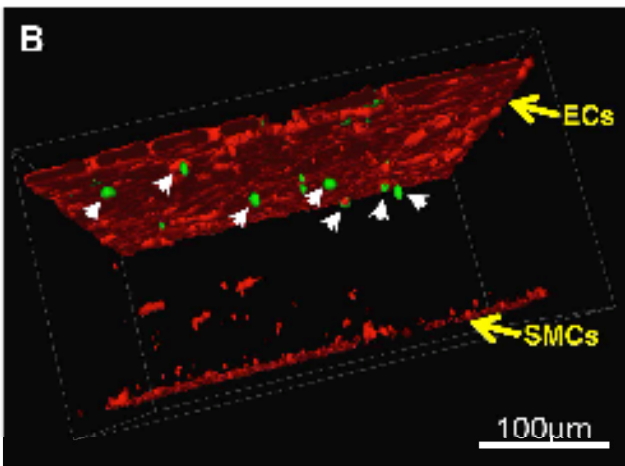
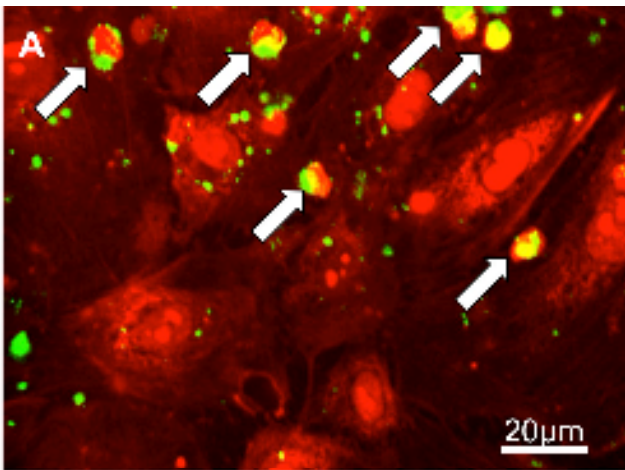


Fig. 2 Sakamoto N et al.

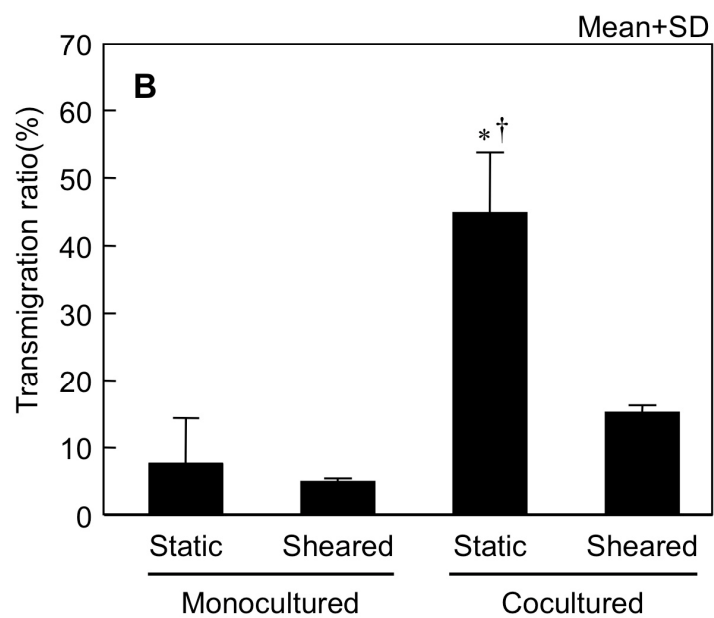
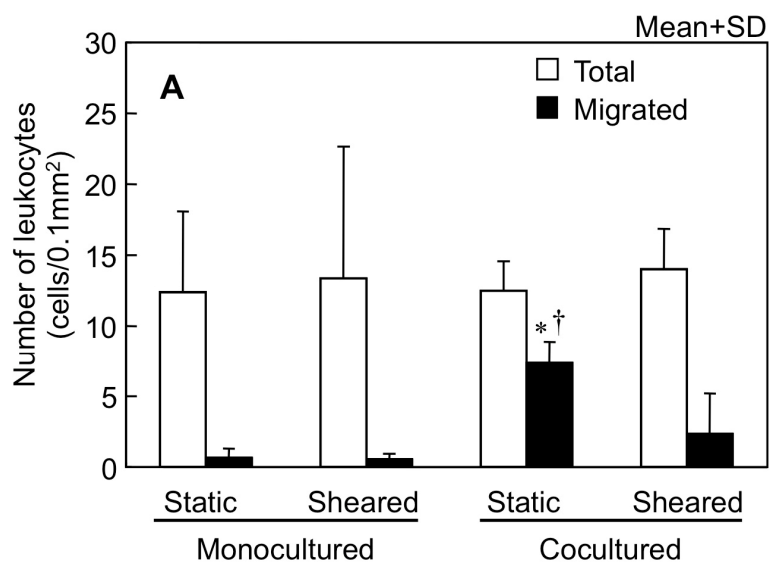


Fig. 3 Sakamoto N et al.

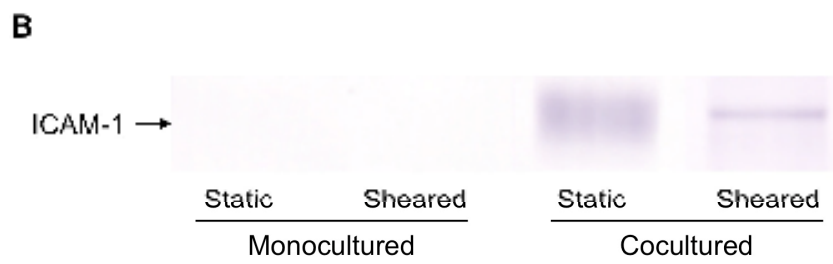
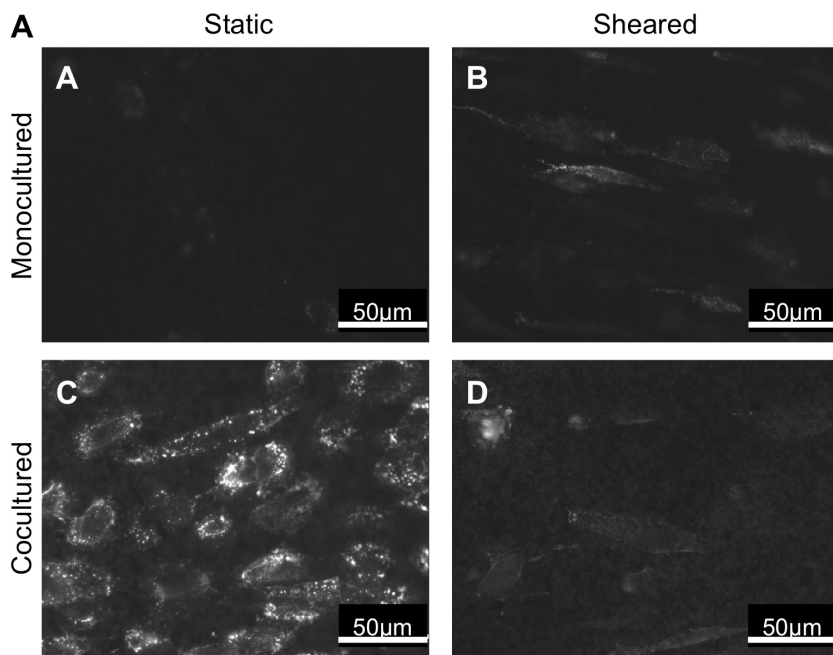


Fig. 4 Sakamoto N et al.