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An Experimental Model for Studying Molecular Behavior of Platelet-Endothelial Cell Adhesion Molecule-1 during Mechanical Interactions between Monocytes and Vascular Endothelial Cells*

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Abstract

Monocyte accumulation in the arterial intima is a hallmark in early atherosclerosis. Monocyte extravasation involves sequential processes including rolling, adhesion, and transmigration across vascular endothelial cells (ECs), where abundant mechanical interactions between these two cells exist. Platelet-endothelial cell adhesion molecule-1 (PECAM-1) is a junctional protein expressed on both cells, and participates in paracellular transmigration via homophilic binding between these cells, while PECAM-1 binds homophilically between neighboring ECs in the absence of monocytes. During monocyte transmigration, PECAM-1-bearing membrane can be recruited to the transmigration spot from intracellular pools. The mechanism by which PECAM-1 binding between ECs in control state is switched to that between monocytes and ECs during transmigration, and its relationship with recruited PECAM-1 remain unclear. In this study, we built an experimental model *in vitro* for studying molecular behavior of PECAM-1 during monocyte transmigration. A plasmid vector containing PECAM-1 tagged with green (GFP) or red (DsRed) fluorescent protein was constructed, and separately transfected into ECs. The mixture of both transfectants in culture achieved a monolayer that contains an intercellular PECAM-1 boundary between PECAM-1-GFP- and PECAM-1-DsRed-expressing cells which is visualized as yellow in merged image. Using this model together with confocal laser scanning microscope-based system, molecular behavior of PECAM-1 on neighboring ECs during monocyte transmigration was observed in live cells. This model can be essential to directly visualize binding/dissociation state of PECAM-1 between neighboring ECs during monocyte trans-endothelial migration in relation to mechanical monocyte-EC interactions.

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1. Introduction

Accumulation of blood monocytes in the arterial intima is a crucial step during atherogenesis[1,2]. They infiltrate the arterial endothelium to enter the underlying tissue, and participate in plaque development. Extravasation of monocytes involves well-coordinated, sequential series of processes including rolling, firm adhesion, locomotion on, and transmigration across vascular endothelial cells (ECs)[3,4], where abundant mechanical interactions between these two cells are present. Previously we have shown that monocyte adhesion decreases elastic modulus of ECs with a reduction in stress fibers[5] and the amount of focal adhesion kinase[6]. Moreover, gaps between endothelium and underlying substrate immediately increase when monocytes adhere[5]. These data indicate that monocyte adhesion induces micro-mechanical changes in ECs, i.e. softening of ECs and weakening of EC-substrate adhesiveness, which can promote subsequent transmigration.

While molecular mechanisms in rolling and adhesion have been well described, those in transmigration are less understood. Leukocytes have been shown to invade endothelium mainly by paracellular (between ECs), and in part by transcellular (through the body of single EC) routes. Many molecules are involved in the former, including platelet-endothelial cell adhesion molecule-1 (PECAM-1)[7-10], vascular endothelial cadherin (VE-cadherin)[9,11], junctional adhesion molecules (JAMs)[12], and CD99[8]. Recently we have demonstrated that oxidized LDL alters the conformation of endothelial junctions, upregulating PECAM-1 and downregulating VE-cadherin, which leads to augmented monocyte transmigration[13].

PECAM-1 is a transmembrane protein expressed on both monocytes and ECs, and is concentrated at cell-cell borders in ECs where it binds homophilically with each other between neighboring ECs via its extracellular domain. Recent studies[7,14] reported that part of PECAM-1 exists in the intracellular pool called lateral border recycling compartment (LBRC) which is located below the surface of the cell at endothelial borders, and PECAM-1 is constitutively recycled between LBRC and the cell surface. During paracellular transmigration, this recycling PECAM-1-bearing membrane is targeted extensively to the transmigration spot[7], where endothelial PECAM-1 binds homophilically to monocyte PECAM-1[8]. This targeted recruitment is mediated by kinesin-microtubule-based system[14]. However, little is known about precise regulatory mechanism of these complicated trafficking and binding/dissociation processes of PECAM-1. First, it remains unclear whether and how the PECAM-1 binding between neighboring ECs is disrupted during transmigration to serve as a binding partner for monocyte PECAM-1. Second, the role of recruited PECAM-1 from intracellular pools in these processes is unknown.

In this study, we built an *in vitro* experimental model for studying molecular behavior of PECAM-1 during monocyte transmigration to shed light on the role of the molecule in relation to mechanical monocyte-EC interactions. A plasmid vector containing PECAM-1 tagged with green (GFP) or red (DsRed) fluorescent protein was constructed, and separately transfected into human umbilical vein endothelial cells (HUVECs). The mixture of both transfectants in culture achieved a monolayer that contains an intercellular boundary of PECAM-1 between those transfectants, i.e. in two different colors, to directly visualize binding/dissociation state of PECAM-1 between neighboring ECs. Using this model with confocal laser scanning microscope (CLSM), we observed three-dimensional molecular

behavior of PECAM-1 at such boundary during monocyte transmigration in live cells.

2. Methods

All protocols involving human subjects were approved by an institutional review committee, and informed consent was obtained from all participants.

2.1. Cells

HUVECs were purchased from Kurabo (Japan), and cultured in HuMedia-EG2 complete medium (Kurabo) supplemented with or without Primocin (Amaxa, Germany), which protects against microbial contaminants including mycoplasma. For experiments, cells at passage 3 were used. Human $CD14^+$ monocytes were freshly isolated from the peripheral blood of young, healthy volunteers as described[15].

2.2. Construction of GFP- or DsRed-tagged PECAM-1

Total RNA from HUVECs, which was extracted using TRIZOL (Invitrogen, CA), was reverse-transcribed using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, Sweden). Full length PECAM-1 cDNA (2200 bp) was amplified by polymerase chain reaction (PCR) with the following primers: 5'-TCGAC *GGTACC* GACACC ATG CAG CCG AGG TGG GCC CAA-3' (forward primer), and 5'-CCGC *GGTACC* GTCGA AGT TCC ATC AAG GGA GCC TTC CGT-3' (reverse primer). Restriction sites for *KpnI* are italicized. The PCR products were cloned into *KpnI* restriction site in mammalian expression vector pAcGFP1-N1 or pDsRed-Monomer-N1 (Takara Bio, Japan) using DNA ligation kit version 2 (Takara Bio). This procedure produced a construct in which *Aequorea coerulescens*-derived GFP (AcGFP1) or monomeric mutant of the *Discosoma sp.* red fluorescent protein DsRed (DsRed-Monomer), respectively, was fused to the intracellular C-terminus of PECAM-1. Absorption/fluorescence emission maxima for AcGFP1 and DsRed-Monomer is 475/505 and 556/586 nm, respectively. The plasmid from transformed *E. coli*, in which PECAM-1 cDNA was inserted in the desired direction, was selected by the difference in digestion pattern with *EcoRI*. The correct construction of PECAM-1-GFP/DsRed was further confirmed by DNA sequencing (BigDye Terminator v3.1, Applied Biosystems, CA).

2.3. Analysis of PECAM-1-PECAM-1 interaction in neighboring two ECs during monocyte transmigration

HUVECs were separately transfected with PECAM-1-GFP or PECAM-1-DsRed using nucleofection system (Amaxa). In brief, pelleted HUVECs of 3~4 $\times$ 10^5 cells were transfected with either plasmid (1.4~3 μg) in 100 μL nucleofector solution. The mixed cell suspension of both transfectants was seeded onto 35 mm glass-bottomed dish (cover glass ϕ = 12 mm, Asahi glass, Japan) coated with fibronectin. Mixing ratio of PECAM-1-GFP- and PECAM-1-DsRed-transfected cells was determined to be 5-6 : 8, which gave the best result in this system. The cells were cultured for 2 days, and observed using CLSM (TCS-NT, Leica) with a 40 $\times$ 1.25 numerical aperture oil-immersion objective. For experiments with monocytes, transfected HUVECs were pre-stimulated with 5 ng/mL interleukin-1beta (IL-1β) on day 1 after transfection. On day 2, freshly-isolated human monocytes were stained with 2 $\times$ 10^{-7} M CellTracker orange-CMRA (Invitrogen, Absorption/fluorescence emission maxima: 548/576 nm), and approximately 1.5 $\times$ 10^5 cells/dish were added to HUVECs. The molecular behavior of PECAM-1-GFP/DsRed during monocyte transmigration was observed on a microscopic stage equipped with a mini-CO_2 incubator (TK-INCO_2, Tokken, Japan) at 37°C using CLSM at 1-9 min intervals (Fig.1), as described[13,15].

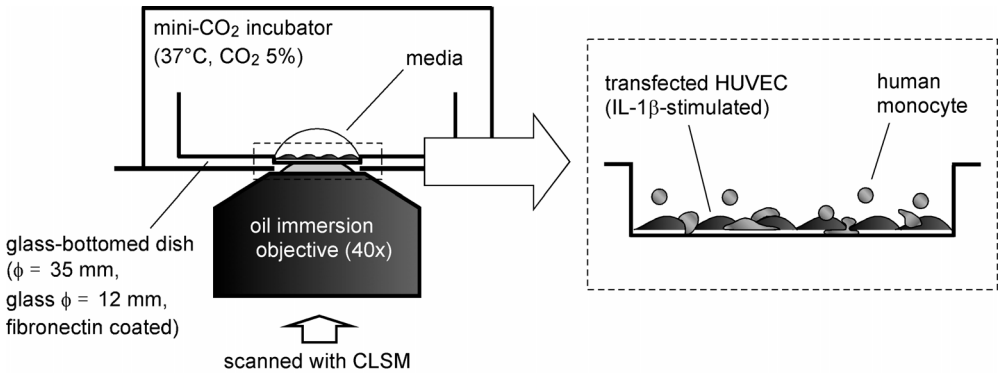

Fig.1. Experimental setup for monocyte trans-endothelial migration. The molecular behavior of PECAM-1-GFP/DsRed during monocyte transmigration was observed in live cells on a microscopic stage equipped with a mini-CO_2 incubator at 37°C using CLSM.

2.4. Western blotting

HUVECs (3~7 × 10^5 cells) were transfected either with PECAM-1-GFP or PECAM-1-DsRed using nucleofection system as mentioned in section 2.3 with minor modifications. The cells were seeded onto 35 mm plastic dish or glass-bottomed dish (cover glass ϕ = 27 mm, Asahi glass) coated with fibronectin, and cultured for 1~2 days. The cells were lysed with M-PER (Pierce, IL). After quantifying total protein by the bicinchoninic acid method, equal amounts of protein were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (GE Healthcare). After blocking the membranes with ECL blocking agent (GE Healthcare), they were incubated with a primary mouse anti-human antibody for PECAM-1 (clone 9G11, R&D systems), followed by a secondary horseradish peroxidase-conjugated anti-mouse IgG (GE Healthcare), and finally visualized by enhanced chemiluminescence (ECLplus, GE Healthcare). Untransfected HUVECs cultured on collagen gel layer were used as a control.

3. Results

3.1. Construction of GFP- or DsRed-tagged PECAM-1

Full length PECAM-1 cDNA (2200 bp) was correctly amplified by PCR (Fig.2(a)). After cloning of the PCR products into the vector, the plasmid in which PECAM-1 cDNA was inserted in the desired direction was selected by the difference in digestion pattern with *EcoRI* (Fig.2(b)). For example in Fig.2(b), four desired plasmids of PECAM-1-DsRed (lanes 1, 2, 4, and 6) were selected, as each had three digested fragments of expected size (6300, 560, and 30 bp). The plasmid with PECAM-1 cDNA inserted in the opposite direction had another set of three expected fragments of 5200, 1700, and 30 bp (lanes 5 and 8). Self-ligation of the vector without inserted cDNA resulted in the original vector size of 4700 bp (lanes 3, 7, 9, and 10). After high-grade purification of the selected plasmids, the correct construction of PECAM-1-DsRed was re-confirmed by the digestion pattern with *EcoRI* (see above), *KpnI* (4700 and 2200 bp), and without digestion (6900 bp) (Fig.2(c)). The correct construction of PECAM-1-GFP was also verified (data not shown). Note that cDNA bands less than 500 bp were not shown in Fig.2(a)-(c).

3.2. The expression of the fusion proteins in HUVECs

We confirmed the protein expression of PECAM-1-DsRed (Fig.2(d)) or PECAM-1-GFP (data not shown) in HUVECs transfected with each plasmid by western blotting, which showed a band of the expected size of each fusion protein (160-kDa), in addition to a 130-kDa band of endogenous PECAM-1 (Fig.2(d)). Only the latter was expressed in untransfected control cells.

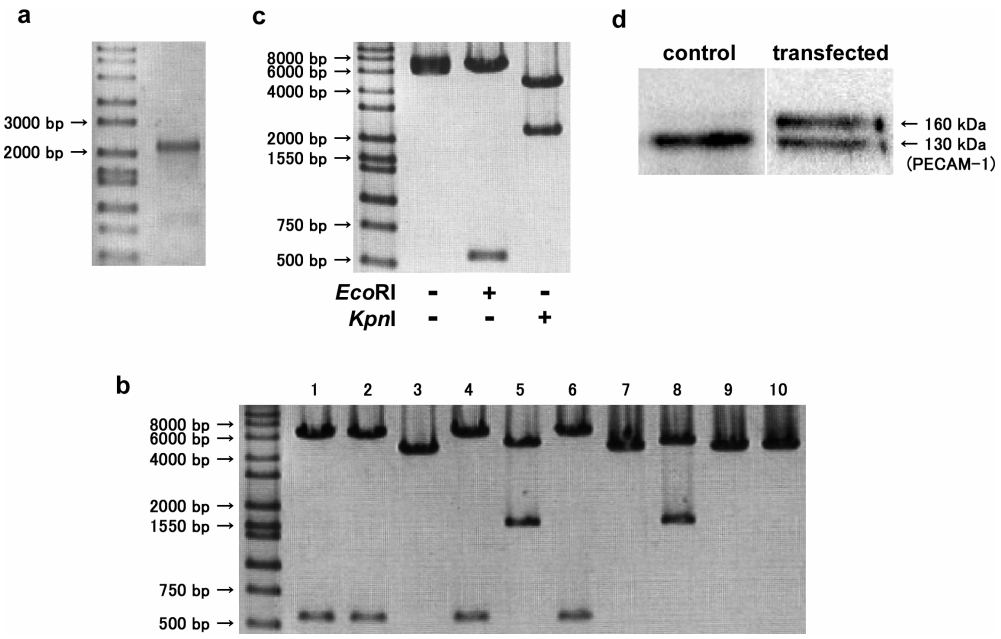

Fig.2. Construction of GFP- or DsRed-tagged PECAM-1. (a) Full length PECAM-1 cDNA (2200 bp) was correctly amplified by PCR. (b) The plasmid in which PECAM-1 cDNA was inserted in the desired direction was selected by the difference in digestion pattern with *EcoRI*. (c) After high-grade purification, the correct construction of PECAM-1-DsRed was re-confirmed by the digestion pattern with *EcoRI*, *KpnI*, and without digestion. Note that cDNA bands less than 500 bp were not shown in (a)-(c). (d) HUVECs transfected with PECAM-1-DsRed were confirmed to express both the fusion protein (160-kDa) and endogenous PECAM-1 (130-kDa), assessed by western blotting. In (a)-(c), assessed by agarose gel electrophoresis.

3.3. Analysis of PECAM-1-PECAM-1 interaction in neighboring two ECs during monocyte transmigration

First, we confirmed that separate transfection of PECAM-1-GFP (Fig.3(a)) or PECAM-1-DsRed (Fig.3(b)) alone resulted in the expression of each fusion protein, and its localization mainly at intercellular borders, which is the similar distribution as endogenous PECAM-1. Next, mixture of cell suspension from both transfectants in culture achieved a monolayer that contains an intercellular PECAM-1 boundary between PECAM-1-GFP/DsRed-expressing cells which is visualized as yellow in merged image (Fig.3(c), arrows). These boundaries appear to result from homophilic interaction of PECAM-1 between PECAM-1-GFP-expressing and PECAM-1-DsRed-expressing cells.

These boundaries can be essential to analyze dynamic binding/dissociation state of PECAM-1 between two neighboring ECs during monocyte transmigration in live cells. A typical example of such PECAM-1 imaging was shown in Fig.4(a) and supplemental movie I. At time 0 min (before monocyte addition), intact boundaries of PECAM-1 were seen. Monocyte M1 was on the apical surface of endothelium with relatively round-shaped morphology at 8 min after monocyte addition, and started to invade endothelium at the boundary between PECAM-1-GFP/DsRed-expressing cells which is visualized as yellow at 11 min. M1 finished invasion at 22 min and migrated underneath the monolayer thereafter, with dynamic shape change resulting in more flattened morphology (see arrows on xy, xz, and yz images for three-dimensional visualization). Monocyte M2 transmigrated at another boundary (also visualized as yellow) at 8-14 min, and finished transmigration at 14 min. In these observations, it was difficult to distinguish fluorescent signals from PECAM-1-GFP-expressing and PECAM-1-DsRed-expressing cells during monocyte transmigration, since monocytes were labeled with orange-fluorescent dye (CellTracker orange-CMRA) that overlaps with DsRed. This is simply due to an

instrumental limitation, and can be improved in the near future (see *Discussion* for details). In Fig.4(b), closing of PECAM-1 gap at such boundary, which seemed to have occurred after monocyte transmigration, was captured. A PECAM-1 gap, that has been formed apparently by transmigration of monocyte M1 at tri-cellular corner, was clearly seen at 45 min (arrow). Restoration of this gap was observed at 46-50 min (arrows), resulting in the intact boundary at 50 min. Note that in Fig.3(c), a small portion of GFP signals leaked into red channel as expected, but PECAM-1-GFP-expressing and PECAM-1-DsRed-expressing cells were correctly distinguished as in merged image, which is an essential issue in this model. Also note that the images in Fig.3 and 4 were not obtained and manipulated under identical settings, thus fluorescent intensity can not be compared between the images.

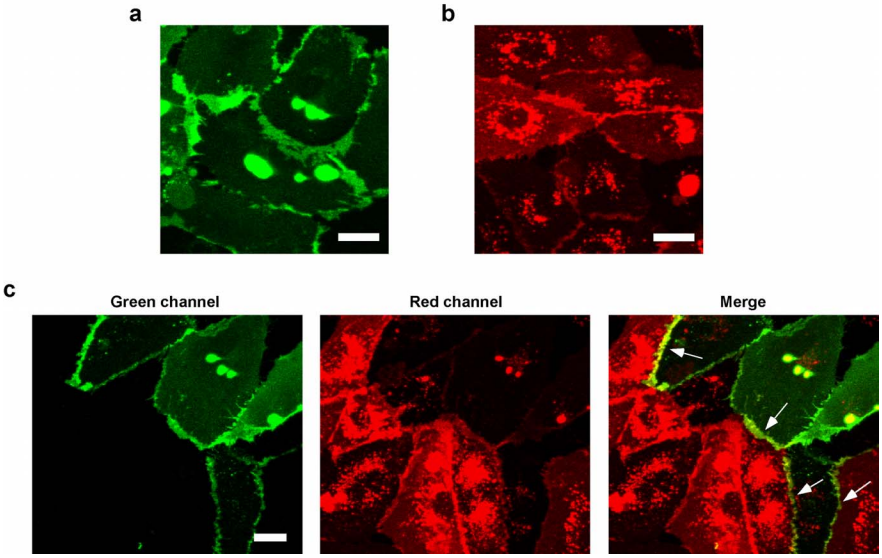

Fig.3. Visualization of PECAM-1-PECAM-1 interaction at intercellular boundary between PECAM-1-GFP/-DsRed-expressing cells. Separate transfection of PECAM-1-GFP (a) or PECAM-1-DsRed (b) alone resulted in the expression of each fusion protein, and its localization mainly at intercellular borders. (c) Mixture of both transfectants in culture achieved a monolayer that contains an intercellular PECAM-1 boundary between PECAM-1-GFP/DsRed-expressing cells which is visualized as yellow in merged image (arrows). In each panel, z-projected xy-images are shown. Bars, 20 μm.

4. Discussion

In this study, we used the fusion proteins, i.e. PECAM-1-GFP and PECAM-1-DsRed, that were expressed in HUVECs to analyze molecular behavior of the proteins in relation to monocyte trans-endothelial migration. We linked GFP or DsRed to the intracellular C-terminus of PECAM-1 to maintain its extracellular domain to be free to interact with other PECAM-1 molecules on neighboring ECs or on monocytes during transmigration. It has been shown that the binding of PECAM-1 cytoplasmic domain to Src homology 2 (SH2) domain containing tyrosine phosphatase-2 (SHP-2), the principal interactor of PECAM-1, was unaffected by cytoplasmic GFP tag[16], suggesting that intracellular binding properties of PECAM-1 as a signaling molecule were not affected by this strategy. We confirmed the correct construction of PECAM-1-GFP/DsRed (Fig.2(a)-(c) and data not shown) and the expression of both of the fusion proteins with proper molecular weight (Fig.2(d) and data not shown). Both of the fusion proteins localized mainly at intercellular borders (Fig.3(a) and (b)) as in endogenous PECAM-1, indicating that intracellular transport of GFP/DsRed-tagged PECAM-1 is substantially normal. Taken together, this experimental model is suitable for studying molecular behavior of PECAM-1 during

monocyte transmigration in live cells.

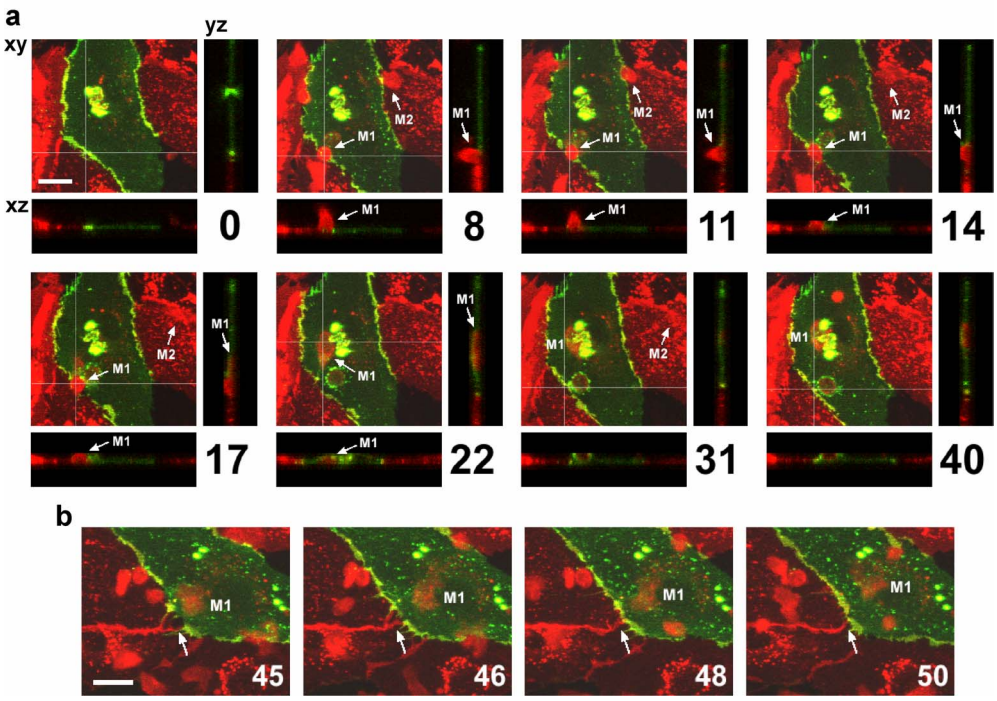

Fig.4. Analysis of PECAM-1-PECAM-1 interaction in neighboring two ECs during monocyte (red) transmigration. (a) Monocyte M1 invaded endothelium at the boundary between PECAM-1-GFP/DsRed-expressing cells which is visualized as yellow. Monocyte M2 transmigrated at another boundary (also visualized as yellow). (b) Restoring of a PECAM-1 gap, which was apparently formed by transmigration of monocyte M1 at tri-cellular corner, was visualized. Bars, 20 μm. The time after monocyte addition in minutes is shown at the lower right corner. In (a), z-projected xy-images are shown along with xz and yz cross-sectional images cut with the designated planes that are indicated as thin lines on xy-images, while in (b), only z-projected xy-images are shown. A movie clip which was integrated from sequential images in (a) is available as a supplemental movie I. Note that in (a), apical portion of monocytes can not be seen in xz and yz image at 14 and 17 min, since z-scanning range was kept minimum to avoid unnecessary laser irradiation.

Using this model, we have depicted such molecular behavior of PECAM-1. A mixture of PECAM-1-GFP- and PECAM-1-DsRed-transfected cells in culture achieved a monolayer that contains an intercellular PECAM-1 boundary between PECAM-1-GFP/DsRed-expressing cells which is visualized as yellow in merged image. These boundaries appear to result from homophilic PECAM-1 binding between those two ECs. Dynamic protein-protein interaction of PECAM-1 between those two ECs during monocyte transmigration was observed in live cells (Fig.4(a)), including closing of PECAM-1 gaps at such boundary which apparently occurred after transmigration (Fig.4(b)). In these observations, as mentioned in *Results*, labeling of monocytes with orange-fluorescent dye impeded the distinct recognition of PECAM-1-GFP and PECAM-1-DsRed at their boundary. This problem can be solved in the near future by use of phase contrast optics or a dye that has fluorescence emission at longer wavelength such as CellTrace Far Red DDAO-SE (Invitrogen), to address the question how PECAM-1 binding between ECs in control state is switched to that between monocytes and ECs during transmigration. Moreover, transfection of monocytes with PECAM-1 cDNA fused to different fluorescent protein like AmCyan1, ZsYellow1, or HcRed1 (all from Takara Bio) can make it an ideal model to simultaneously analyze complex molecular interactions of

PECAM-1 among three cells, i.e. neighboring two ECs and a transmigrating monocyte. Note that IL-1β pre-treatment is a prerequisite for analyzing leukocyte trans-endothelial migration. Total PECAM-1 expression in ECs was suppressed to about 60% by IL-1β treatment (data not shown). Therefore our data of monocyte transmigration (Fig.4) were considered to be obtained at a low basal level of PECAM-1 reduced by IL-1β.

From the mechanical point of view, a number of previous work showed that PECAM-1 is implicated in fluid shear stress-induced mechanotransduction pathways in ECs[17-19], which is an important factor in the localized development of atherosclerotic plaques. It has been shown that PECAM-1 (which directly transmits mechanical force), VE-cadherin (which functions as an adaptor), and vascular endothelial growth factor receptor-2 (VEGFR2; which activates phosphatidylinositol-3-OH kinase) comprise a mechanosensory complex[17]. Intracellular signaling pathway involving PECAM-1, SHP-2, and tyrosine kinase with immunoglobulin and epidermal growth factor homology domain-2 (Tie-2) is important in response to flow[18]. Applying pulling force directly on PECAM-1 on endothelial cell surface using magnetic beads coated with antibodies against the external domain of PECAM-1 induces the same signaling cascade as induced by shear stress[19]. It can be an interesting experiment with our models to study molecular behavior of PECAM-1 in live ECs exposed to flow containing monocytes, in which PECAM-1-mediated transmission of mechanical force exerted by shear stress or monocyte-EC interaction can be analyzed.

In summary, we have built an *in vitro* experimental model for studying three-dimensional molecular behavior of PECAM-1 during monocyte trans-endothelial migration in live cells, with the use of technique in molecular biology including mixture of two different transfectants, to shed light on the role of the molecule in relation to mechanical monocyte-EC interactions. This model can be used to investigate the complicated trafficking and binding/dissociation processes of PECAM-1 during monocyte transmigration, and extensively applicable for other cell-cell interaction events such as tumor invasion, leukocyte infiltration in inflammation, and migration of endothelial progenitor cells in angiogenesis.

Acknowledgments

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