Evidence for a role of platelet endothelial cell adhesion molecule-1 in endothelial cell mechanosignal transduction: is it a mechanoresponsive molecule?

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Fluid shear stress (FSS) induces many forms of responses, including phosphorylation of extracellular signal–regulated kinase (ERK) in endothelial cells (ECs). We have earlier reported rapid tyrosine phosphorylation of platelet endothelial cell adhesion molecule-1 (PECAM-1) in ECs exposed to FSS. Osmotic changes also induced similar PECAM-1 and ERK phosphorylation with nearly identical kinetics. Because both FSS and osmotic changes should mechanically perturb the cell membrane, they might activate the same mechanosignaling cascade. When PECAM-1 is tyrosine phosphorylated by FSS or osmotic changes, SHP-2 binds to it. Here we show that ERK phosphorylation by FSS or osmotic changes depends on PECAM-1 tyrosine phos-phorylation, SHP-2 binding to phospho-PECAM-1, and SHP-2 phosphatase activity. In ECs under flow, detectable amounts of SHP-2 and Gab1 translocated from the cytoplasm to the EC junction. When magnetic beads coated with antibodies against the extracellular domain of PECAM-1 were attached to ECs and tugged by magnetic force for 10 min, PECAM-1 associated with the beads was tyrosine phosphorylated. ERK was also phosphorylated in these cells. Binding of the beads by itself or pulling on the cell surface using poly-L–coated beads did not induce phos-phorylation of PECAM-1 and ERK. These results suggest that PECAM-1 is a mechanotransduction molecule.

Introduction

Endothelial cells (ECs)* cover the entire inner surface of the cardiovascular system and consequently are exposed to fluid shear stress (FSS) of flowing blood. It is now established that FSS modulates the physiology, gene expression, and morphology of ECs (Davies, 1995). Also established is the notion that atherosclerotic lesions are likely to develop in areas with turbulent flow and low time averaged mean FSS (for review see Gimbrone, 1999). In vitro studies show that both temporal (Bao et al., 1999) and spatial (Nagel et al., 1999) FSS gradients cause the expression and accumulation in the nucleus of transcriptional factors such as egr-1 and c-fos. Activation of extracellular signal–regulated kinase (ERK) is one of the early FSS responses by ECs (Tseng et al., 1995), and many of such early responses may be caused by a sudden application of flow to cultured ECs (i.e., change in FSS gradient). ERK activation in the turbulent flow area may lead to the increased egr-1 expression and AP-1 activation, which are thought to activate athero-genic genes, such as a tissue factor (Schwachtgen et al., 1998; Houston et al., 1999) and MCP-1 (Shyy et al., 1994). As for the upstream events leading to ERK phosphorylation by FSS, involvement of various signaling molecules, including trimeric G-protein (Gudi et al., 1996; Jo et al., 1997), PKCε (Traub et al., 1997), shc (Chen et al., 1999), integrin (Chen et al., 1999), and VEGF receptor (Chen et al., 1999) has been proposed. How all of these events relate to each other is not known, but it appears that FSS activates more than one signaling cascade.

In spite of extensive studies on the effects of FSS on ECs, the molecular mechanism for mechanotransduction is largely unknown. One hypothesis is that membrane pertur-bation by FSS alters the functional state of some membrane

protein(s) and such a change initiates signaling (Gudi et al., 1998). Another idea is that deformation of the whole cell caused by FSS changes tension on certain cytoskeletal structures, triggering mechanosignaling (Davies, 1995). Because mechanosensing by certain prokaryotic and lower eukaryotic cells is tied to the activity of stretch-activated ion channels (Sukharev et al., 1994; Kanzaki et al., 1999), activation of stretch-sensitive channels by FSS is also a possibility. However, it is not known if the proteins homologous to the mechanosensing channels of other cells are expressed in ECs. Certain receptors may have the sensor function, as the activities of P2Y (Mo et al., 1991) and P2X4 (Yamamoto et al., 2000) are modulated by flow. It is possible that all of these systems are simultaneously activated by FSS.

Platelet EC adhesion molecule-1 (PECAM-1, CD31) is a glycoprotein with six Ig-like loops in the extracellular domain, a transmembrane domain, and a short cytoplasmic piece, and is expressed by ECs, platelets, and leukocytes (Newman et al., 1990). It plays a role in leukocyte extravasation (Muller et al., 1993; Vaporciyan et al., 1993). In ECs forming a monolayer, it is localized to the cell junction, establishes homophilic binding between neighboring ECs, and forms Ca-independent cell–cell adhesion (Albelda et al., 1990; Ayalon et al., 1994). PECAM-1 may be anchored to actin filaments as it binds to β- and γ-catenins (Ilan et al., 2000), although ∼70% of it is Triton extractable (Ayalon et al., 1994). We have shown that PECAM-1 is tyrosine phosphorylated within 1 min in cultured bovine aortic ECs (BAECs) exposed to FSS of 5 dyn/cm² or higher (Harada et al., 1995; Osawa et al., 1997). It is also tyrosine phosphorylated with similar kinetics when ECs are treated with hypo- or hyperosmotic medium, which, like FSS, should cause cell deformation and perturb the plasma membrane (Osawa et al., 1997). Thus, it appears that PECAM-1 is tyrosine phosphorylated when the EC plasma membrane is mechanically perturbed. Tyrosine-phosphorylated PECAM-1 (pY-PECAM-1) binds to SHP-2 (Jackson et al., 1997; Masuda et al., 1997), a protein tyrosine phosphatase involved in the activation of ERK in cells stimulated by growth factors (Milarski and Saltiel, 1994; Noguchi et al., 1994). Thus, we hypothesized that SHP-2 binding to pY-PECAM-1 might be an upstream event in the ERK activation by FSS. Gab1 is also involved in ERK activation by growth factors. It is a multi-site docking protein with a PH domain, SH3 domains, polyproline sequences, and a bisphosphoryl tyrosine–based activation motif (BTAM) that recruits and activates SHP-2 (Cunnick et al., 2001). Here, we used ERK phosphorylation by FSS as a model system for studying mechanosignal transduction in ECs. We show that this FSS response depends on both PECAM-1 tyrosine phosphorylation and the catalytic activity of SHP-2. When ECs were exposed to FSS, SHP-2 and Gab1 translocated from the cytoplasm to the cell junction, and their translocation depended on PECAM-1 expression. We also show that direct application of mechanical force to PECAM-1 on the EC surface causes its tyrosine phosphorylation and ERK phosphorylation in the cell, indicating that PECAM-1 has an ability to respond to mechanical force.

Results

ERK activation by FSS depends on PECAM-1 tyrosine phosphorylation

ERK phosphorylation is a readily detectable EC response to FSS. To ascertain that our experimental system worked properly, we applied 15 dyn/cm² of FSS to confluent BAECs whose ERK activity was then assayed. Flow phosphorylated ERK but not p38, another MAP kinase (Fig. 1). Because hyperosmotic shock (HOS) is known to activate p38, we challenged ECs with HOS. Indeed, p38 was activated, but, more interestingly, ERK was also activated (Fig. 1). The kinetics of this ERK phosphorylation was practically identical to that induced by FSS, both peaking at 5–10 min of stimulation. Our earlier studies showed that both FSS and HOS rapidly induced PECAM-1 tyrosine phosphorylation in ECs with similar kinetics and that in both cases, the PECAM-1 tyrosine phosphorylation reached a plateau within 2 min and was sustained for >30 min (Osawa et al., 1997; Masuda et al., 1998). We suggest that although a set of signaling events activated by FSS should be different from a set mobilized by HOS, certain common signaling events, including PECAM-1 tyrosine phosphorylation and ERK activation, are elicited by both FSS and HOS. Because HOS is more conveniently applied to cells than FSS, many of the initial experiments were done using HOS, and their results were confirmed by FSS experiments.

The mechanism of ERK phosphorylation by FSS and HOS is not fully understood. We wondered if PECAM-1 phosphorylation might be an event upstream of the ERK phosphorylation. To test this, we decreased PECAM-1 expression in BAECs using PECAM-1 antisense S-oligonucleotide (S-oligo). As shown in Fig. 2 a, PECAM-1 expression
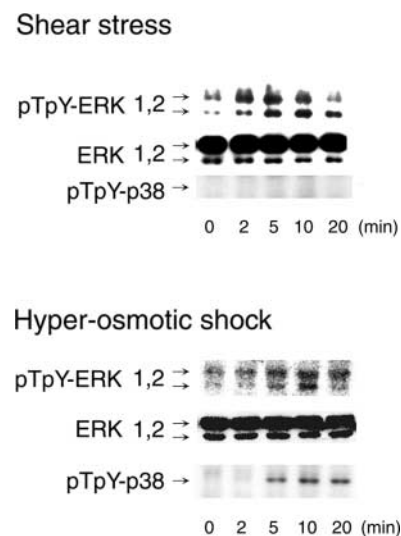

Figure 1.   **ERK activation by FSS and HOS.** BAECs were exposed to 15 dyn/cm² of FSS or to culture medium containing 300 mM sucrose (HOS; ∼600 mOsm) for 0–20 min. Phospho-ERK, total ERK, and phospho-p38 were detected by immunoblotting using anti–phospho-ERK (pTpY-ERK1,2), anti-ERK (ERK1,2) and anti–phospho-p38 (pTpY-p38), respectively. The levels of ERK2 phosphorylation at 10 min over the control level were 2.1 ± 0.2 (n = 7) and 1.7 ± 0.1 (n = 6) times by HOS and FSS, respectively. p38 was activated by HOS but not by FSS.
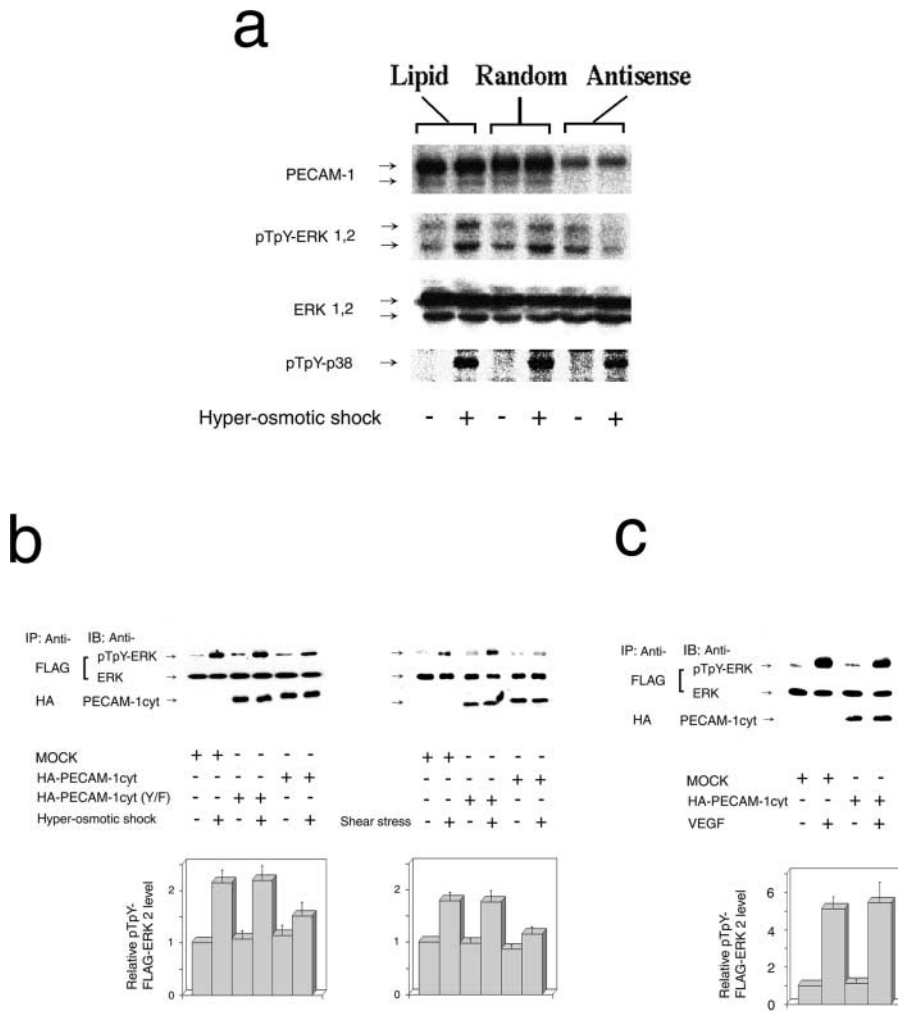
Figure 2. **ERK activation by mechanical stresses depends on the presence and tyrosine phosphorylation of PECAM-1.** (a) BAECs were treated with PECAM-1 antisense S-oligo (Antisense), control scrambled S-oligo (Random), or lipid carrier (Lipid) using the high transfection efficiency condition. Cells were grown to confluency and treated with (+) or without (−) HOS for 10 min. Cell lysates were immunoblotted with anti–PECAM-1 (PECAM-1), anti–phospho-ERK (pTpY-ERK1,2), anti-ERK (ERK1,2), and anti–phospho-p38 (pTpY-p38). PECAM-1 downregulation inhibited ERK, but not p38, phosphorylation by HOS. The data are from one of five sets of similar experiments. (b) FLAG–ERK2 was transiently coexpressed with HA–PECAM-1cyt or HA–PEAM-1cyt (Y/F) in BAECs. Confluent transfectants were treated with (+) or without (−) HOS (left) or FSS (right) for 10 min. Cell lysates were immunoprecipitated (IP) with anti-FLAG (FLAG) or anti-HA (HA) followed by immunoblotting (IB) with anti–phospho-ERK (pTpY-ERK), anti-ERK (ERK), or anti–PECAM-1 (PECAM-1cyt). Note that PECAM-1cyt, but not PECAM-1cyt(Y/F), has a dominant negative effect. (c) BAECs coexpressing FLAG–ERK and PECAM-1cyt were treated with 50 ng/ml of VEGF for 10 min. PECAM-1cyt did not inhibit ERK activation by VEGF. Control cells were transfected with FLAG–ERK and lipid carrier (MOCK) and treated similarly with VEGF. Bar graphs in b and c show relative FLAG–ERK2 phosphorylation levels (mean ± SEM) of 4–12 experiments.

was suppressed to ∼30% of the level of control cells by using the high transfection efficiency protocol (see Materials and methods). ECs treated similarly but with random S-oligo or carrier lipid had the same PECAM-1 expression levels as untreated cells. ERK expression (Fig. 2 a), as well as SHP-2 and Gab1 expression (see Fig. 5 c), was not affected by the antisense treatment. When ECs with reduced PECAM-1 were exposed to FSS, most cells detached from the plate. This reduced cell adhesion was caused by the high transfection efficiency procedure, because ECs treated with random S-oligo or carrier lipid alone could not overcome FSS. HOS, on the other hand, did not cause massive cell detachment. When ECs were treated with HOS and their ERK phosphorylation was assayed, the antisense-treated ECs showed reduced ERK phosphorylation whereas the two control groups exhibited normal ERK activation. Decreased PECAM-1 expression did not affect p38 phosphorylation, suggesting that the observed decrease in ERK phosphorylation was a specific effect of reduced PECAM-1 expression. HOS induced PECAM-1 tyrosine phosphorylation in the control-transfected cells, but pY-PECAM-1 was undetectable in the antisense-treated ECs. This is likely due to greatly reduced PECAM-1 engagement between neighboring cells (discussed more later), as the chance of two cells with sufficient amounts of PECAM-1 being next to each other would be small. These results suggest PECAM-1's involvement in ERK activation by HOS.

Overexpression of the cytoplasmic domain of PECAM-1 (PECAM-1cyt) might interfere with the ERK activation by FSS and HOS. To effectively detect ERK phosphorylation without using the high efficiency condition, we used FLAG-tagged ERK2 (FLAG–ERK2). BAECs were cotransfected with FLAG–ERK2 and either HA epitope–tagged PECAM-1cyt (HA–PECAM-1cyt) or HA–PECAM-1cyt whose Tyr663 and Tyr686 were replaced with Phe (HA-PECAM-1cyt[Y/F]). The monolayer morphology and endogenous PECAM-1 localization (determined by staining cells with antibodies against the external region of PECAM-1) were not affected by overexpressing these constructs, and the overexpressed tagged proteins were present diffusely in the cytoplasm. Transfected cells were exposed to HOS or FSS for 10 min, and tagged proteins were immunoprecipitated from cell lysates and immunoblotted with anti–phospho-ERK, anti-ERK, and anti–PECAM-1 (Fig. 2 b). In mock control ECs exposed to HOS or FSS, phosphorylation of FLAG–ERK2 increased 2.1 ± 0.25 and 1.8 ± 0.17 times over the levels in unstimulated cells, respectively. These increases were comparable to those of endogenous ERK (Fig. 1), indicating that FLAG–ERK2 is a good reporter of endogenous ERK. Expression of HA–PECAM-

1cyt inhibited FLAG–ERK2 phosphorylation by HOS and FSS by ∼70%, but this dominant negative effect was lost when phosphorylatable Tyr663 and Tyr686 were replaced with Phe, showing that these tyrosines play important roles in the ERK activation.

High concentrations of PECAM-1cyt in the cell may non-specifically inhibit ERK phosphorylation. To exclude this possibility, confluent BAECs coexpressing FLAG–ERK2 and HA–PECAM-1cyt or mock-transfected cells were treated with 50 ng/ml VEGF for 10 min. FLAG–ERK2 was phosphorylated to the same level in both transfectants (Fig. 2 c). Thus, PECAM-1cyt inhibits specifically the ERK phosphorylation by HOS or FSS, and the signaling pathway activated by the mechanical stimuli is different from the one activated by a chemical stimulus.

SHP-2 phosphatase activity and PECAM-1–dependent ERK activation

SHP-2 binds to pY-PECAM-1, and this binding is thought to activate the phosphatase activity of SHP-2, which then initiates certain signaling events (Masuda et al., 1997). To test if the ERK activation by HOS and FSS depends on the enzymatic activity of SHP-2, BAECs were cotransfected with FLAG–ERK2 and HA-tagged SHP-2 in which Cys459 was changed to Ser (HA–SHP-2[C/S]). SHP-2 with this mutation has no phosphatase activity (Noguchi et al., 1994). Monolayers of transfected cultures were treated with HOS or FSS for 10 min (Fig. 3). Whereas cells transfected with the vector alone (MOCK) showed stimulus-dependent FLAG–ERK2 phosphorylation, ECs expressing HA–SHP-2(C/S) did not show the similar FLAG–ERK2 phosphoryla-

Figure 3. **ERK activation by FSS and HOS depends on SHP-2 phosphatase activity.** Confluent BAECs cotransfected with FLAG–ERK2 and HA–SHP-2(C/S) were treated with (+) or without (−) HOS (left) or FSS (right) for 10 min. Cell lysates were immuno-precipitated (IP) with anti-FLAG (FLAG) or anti-HA (HA) followed by immunoblotting (IB) with anti–phospho-ERK (pTpY-ERK), anti-ERK (ERK), or anti–SHP-2 (SHP-2). Relative FLAG–ERK2 phosphorylation levels (bar graphs) are shown as mean ± SEM ($n = 4$). Overexpression of phosphatase-negative SHP-2 inhibited ERK activation. Control cells were transfected with FLAG–ERK2 and lipid carrier (MOCK).

tion. These results suggest that SHP-2 phosphatase activity is important for ERK activation by HOS and FSS.

Translocation of SHP-2 and Gab1 to the cell junction

PECAM-1 is tyrosine phosphorylated in ECs exposed to HOS or FSS, but its localization was not affected by this modification (Fig. 4 a). Because SHP-2 binds to pY-PE-CAM-1, this association may be visualized in live ECs. SHP-2 tagged with GFP was expressed in BAECs. Its localization was diffuse in unstimulated cells, but in cells exposed to HOS, a detectable amount of GFP–SHP-2 moved to the cell–cell adhesion (Fig. 4 b). This accumulation was detectable from ∼3 min of stimulation and reached the plateau intensity at ∼5 min. We also overexpressed GFP–SHP-2(C/S) and found that it accumulated at the cell junction regardless of stimulation (Fig. 4 c). To see if this localization depends on PECAM-1, BAECs were cotransfected with PECAM-1 antisense S-oligo and GFP–SHP-2(C/S). Whereas the control cells cotransfected with carrier lipid and GFP–SHP-2(C/S) showed accumulation of the GFP protein at the cell junction, the antisense-treated cells did not exhibit a similar accumulation (Fig. 4 d). To confirm that GFP–SHP-2(C/S) indeed binds to PECAM-1, BAECs were transfected with either HA–SHP-2(C/S) or HA–SHP-2, cultured until confluent, and treated with or without HOS for 10 min. Cell lysates were immunoprecipitated with anti-HA, followed by immunoblotting with anti–PECAM-1. Consistent with our earlier finding on the interaction between endogenous SHP-2 and PECAM-1 (Masuda et al., 1997), HA–SHP-2 bound to PECAM-1 only when cells were stimulated. On the other hand, HA–SHP-2(C/S) bound to PECAM-1 independent of stimulation (Fig. 4 e). Note that a significantly higher amount of PECAM-1 is associated with SHP-2(C/S) than with wild-type SHP-2.

Immunofluorescence microscopy was used to study if endogenous SHP-2 behaved in a similar manner. Confluent BAECs exposed to either low (0.1 dyn/cm^2) or high (15 dyn/cm^2) FSS for 10 min were fixed and stained with anti–SHP-2. These FSS levels were chosen because the threshold for PECAM-1 tyrosine phosphorylation is ∼3 dyn/cm^2 (Harada et al., 1995). In cells exposed to no or low FSS, SHP-2 showed diffuse localization throughout the cell, making it difficult to identify cell borders. However, under the high FSS condition, the cell–cell border was clearly labeled, indicating that high FSS induced SHP-2 translocation from the cytoplasm to the cell junction (Fig. 5 a). Gab1 is an SHP-2 binding multisite docking protein and a positive regulator of ERK activation (Cunnick et al., 2001). Its localization in ECs exposed to no or low FSS was also diffuse, but in the cells exposed to high FSS, anti-Gab1 staining became detectable at the cell junction (Fig. 5 a). The similar translocation of SHP-2 and Gab1 was induced by HOS. In BAECs whose PECAM-1 expression was reduced by the antisense treatment, the FSS-induced translocation of SHP-2 and Gab1 to the cell junction was suppressed (Fig. 5 b). Confocal optical sections at the midlevel of cells sometimes showed reduced anti–SHP-2 or anti-Gab1 staining in antisense-treated cells. When the same cells were observed under a conventional fluorescence microscope, they showed similar staining intensity, suggesting that the overall expression of
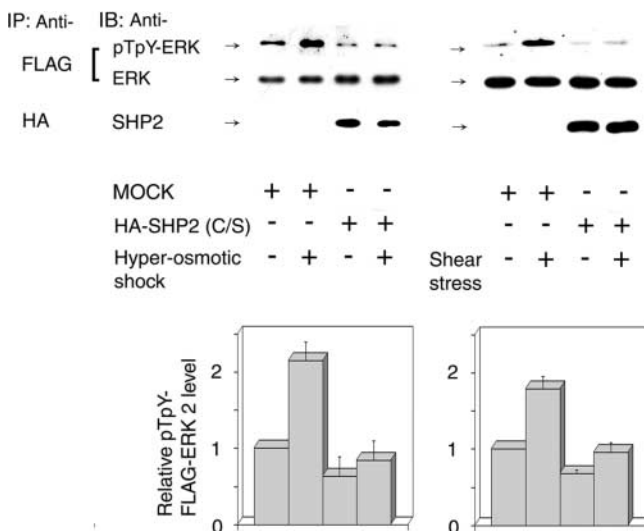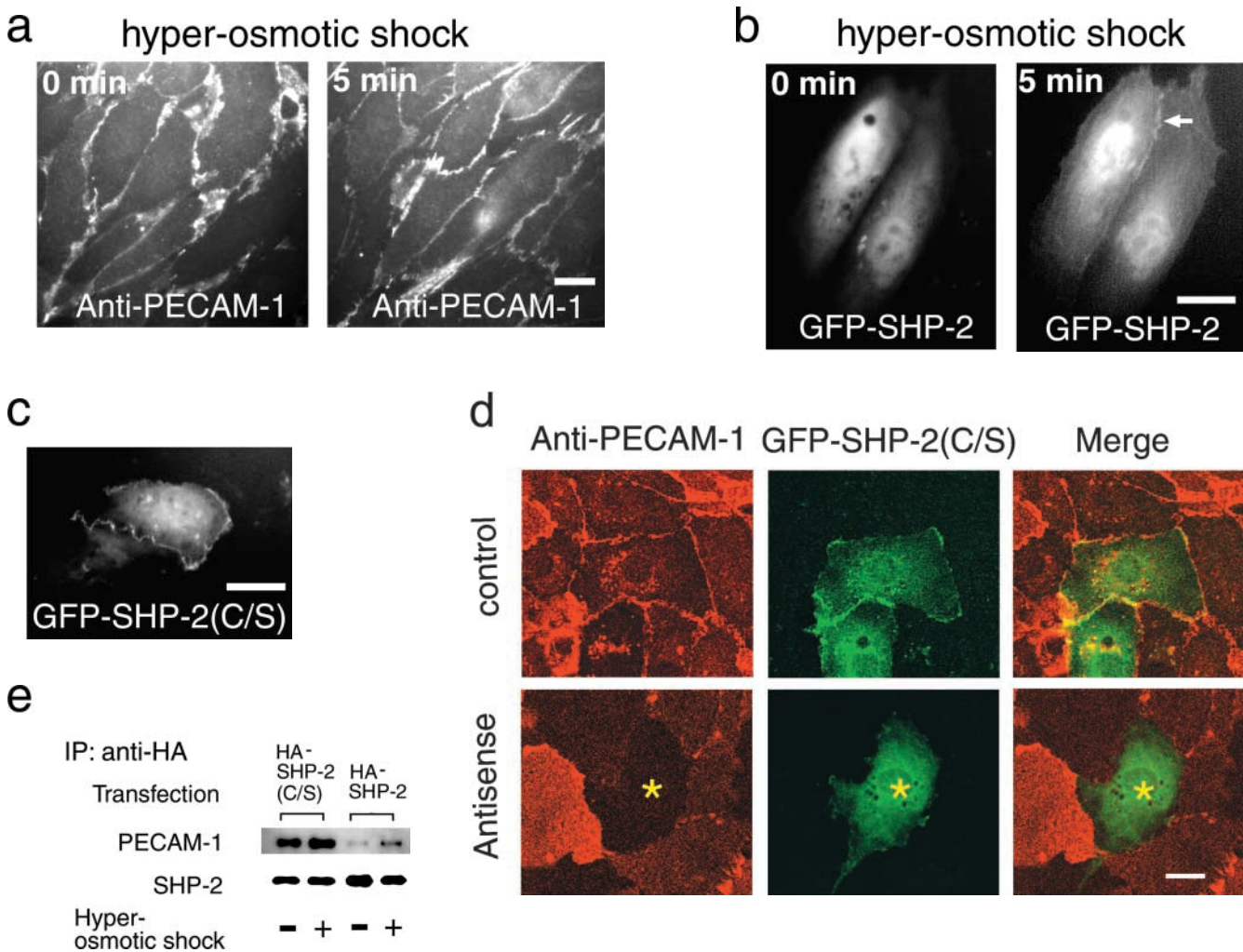
Figure 4. **PECAM-1 dependent translocation of SHP-2 to the cell junction.** (a) BAECs were stained with anti–PECAM-1cyt before (0 min) or after (5 min) HOS treatment. The general pattern of anti–PECAM-1 staining was unaffected by the osmotic treatment. (b and c) GFP–SHP-2 or GFP–SHP-2(C/S) was transiently expressed in confluent BAECs and its localization was observed in living cells. GFP–SHP-2 was diffusely distributed in the cell (b, 0 min), but when the same cells were exposed to HOS for 5 min, a detectable amount of GFP–SHP-2 translocated to the cell border (b, 5 min, arrow). Note the clearer cell outline after HOS treatment. GFP–SHP-2(C/S) was associated with the cell border without HOS stimulation (c). (d) GFP–SHP-2(C/S) was transiently expressed in BAECs with or without PECAM-1 antisense S-oligo using the low transfection efficiency (roughly 10%) condition. Cells were fixed, and the localization of PECAM-1 by immunofluorescence staining and of GFP-SHP-2(C/S) was studied using a confocal laser scanning microscope. In control cells (treated with lipid carrier and GFP–SHP-2[C/S]), GFP–SHP-2(C/S) was colocalized with PECAM-1 at the cell border (control, merge). However, when PECAM-1 expression was downregulated by the antisense (asterisk), GFP–SHP-2(C/S) was no longer associated with the cell border. Bars, 10 μm. (e) HA–SHP-2 and HA–SHP-2(C/S) were transiently expressed in BAECs. Transfectants were treated with (+) or without (−) HOS for 10 min followed by immunoprecipitation using anti-HA and by immunoblotting with anti–SHP-2 and anti–PECAM-1. Whereas SHP-2 was associated with PECAM-1 in an HOS-dependent manner, SHP-2(C/S) was always associated with PECAM-1. The same results were obtained in three independent experiments.

these proteins was unchanged. To confirm this, we transfected BAECs with PECAM-1 antisense under the high efficiency transfection condition and performed immunoblotting with anti–SHP-2 and anti-Gab1 (Fig. 5 c). Reduced PECAM-1 expression did not decrease the total cellular contents of SHP-2 and Gab1.

Cell–cell adhesion is needed for PECAM-1–dependent mechanosignaling

PECAM-1 is localized at the EC junction of confluent cultures, but is diffusely present in the plasma membrane in sparse ECs. Presumably, PECAM-1 is not engaged in solitary ECs. We investigated if PECAM-1 engagement is important for its tyrosine phosphorylation by FSS or HOS. To obtain sparse cultures, ~70% confluent BAECs were subcultured at a split ratio of 1:32 for 12 h and then serum starved using DF (1:1 mixture of DME and F12 media with 10 mM Hepes) medium with 0.8% FBS for 18 h. Under these conditions, no cell colonies were noted, and cells appeared fibroblastic in shape, occasionally forming small cell–cell contacts. When these ECs were exposed to FSS, no detectable increase in pY-PECAM-1 occurred. By HOS, which elicits a higher PECAM-1 response, we observed a small increase, ~20% of the increase seen in confluent cultures (Fig. 6 a). This low level of increase may come from cells that established cell–cell contacts. ERK activation in
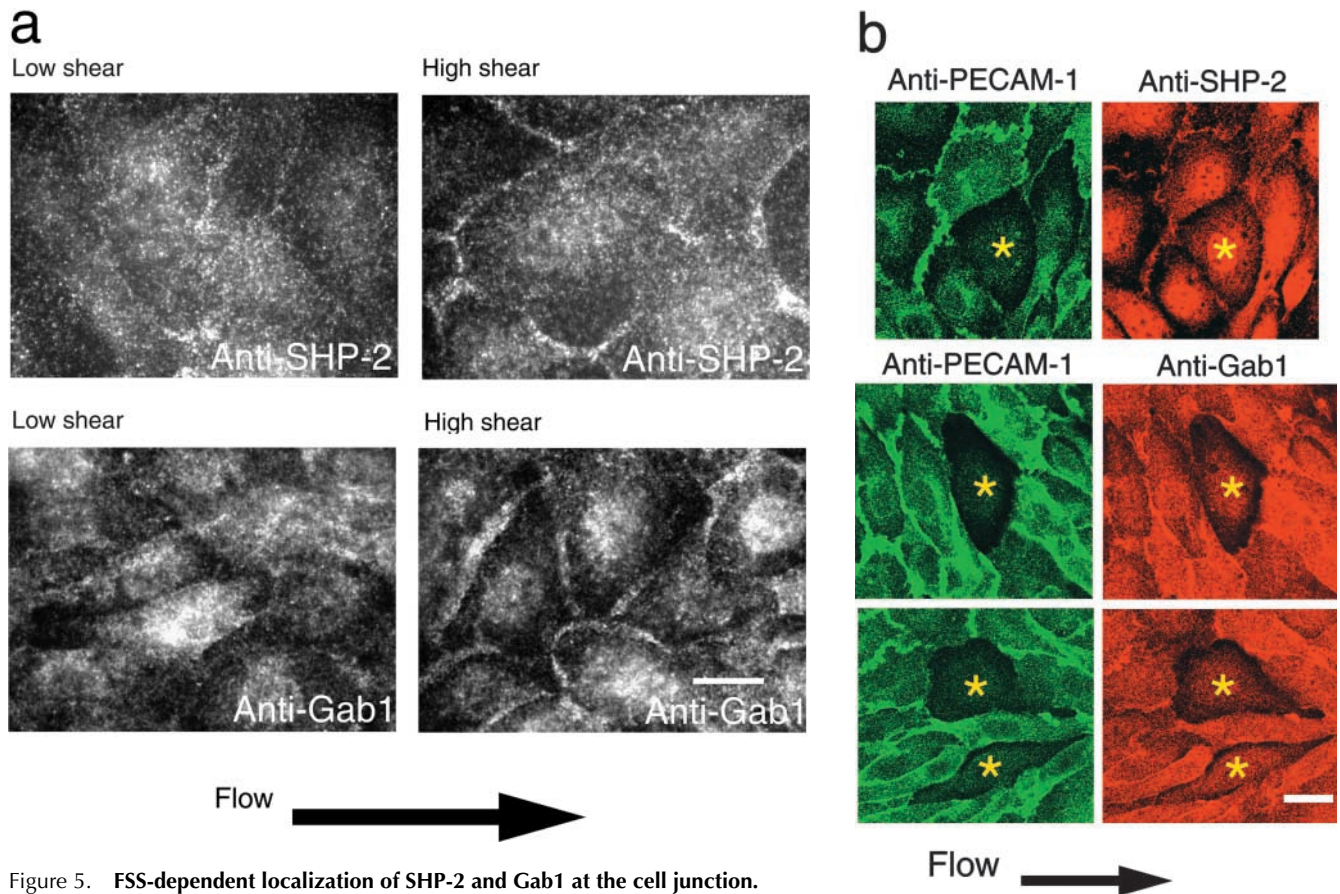
Figure 5. FSS-dependent localization of SHP-2 and Gab1 at the cell junction. (a) Immunofluorescence localization of endogenous SHP-2 and Gab1 in confluent BAECs exposed to 0.1 dyn/cm² (low shear) or 15 dyn/cm² (high shear) for 5 min. Both SHP-2 and Gab1 were distributed diffusely in ECs under low shear stress, but they translocated to the cell border in cells exposed to high shear stress. (b) BAECs were treated with PECAM-1 antisense S-oligo (low transfection condition) and allowed to form a monolayer. They were exposed to 15 dyn/cm² of FSS for 5 min, fixed, stained doubly with anti–PECAM-1 and anti–SHP-2, or with anti–PECAM-1 and anti-Gab1, and observed using a confocal microscope. Although both SHP-2 and Gab1 accumulated at the cell junction in ECs expressing PECAM-1, they did not accumulate at the cell junction in cells with reduced PECAM-1 expression (asterisks). For the experiments shown in a and b, ECs were exposed to flow immediately after adding 1 mM Na3VO4 to the medium. Bars, 10 μm. (c) BAECs were treated with PECAM-1 antisense S-oligo (Antisense), scrambled sequence S-oligo (Random), or lipid carrier (Lipid) using the high transfection efficiency condition as in Fig. 2. Cell lysates were immunoblotted using anti–PECAM-1cyt, anti-Gab1, and anti–SHP-2. Although the antisense treatment greatly reduced PECAM-1 expression, Gab1 and SHP-2 expression levels were not affected. The same results were obtained in three separate experiments.

these sparse cultures was not induced by HOS. It appears, therefore, that PECAM-1 engagement is an important condition for its tyrosine phosphorylation and ERK activation by FSS and HOS.

To externally trap and anchor PECAM-1 of sparsely cultured ECs, we plated cells on culture dishes coated with rabbit antibodies against the external domain of PECAM-1 (anti–PECAM-1ext). Some cells were transfected with FLAG–ERK2. As controls, cells were plated in a similar manner onto dishes coated with preimmune IgG or poly-D-lysine (poly-L). Cells were allowed to spread for 1 h and challenged with HOS for 10 min. Whole cell lysates were probed for pY-PECAM-1 and phospho-ERK by immunoblotting. ECs on anti–PECAM-1ext and stimulated by HOS contained a significantly higher level of pY-PECAM-1 (3.5 times over unstimulated cells) and phospho-FLAG–ERK2, but those plated

on poly-L contained only low levels of phosphorylated PE-CAM-1 and FLAG–ERK with or without HOS (Fig. 6 b). Activation of endogenous ERK was also detected only in ECs plated on anti–PECAM-1ext and treated with HOS. Cells attached to the IgG-coated surface were unable to spread, but when challenged by HOS, they gave the same results as those on poly-L. By immunofluorescence microscopy, we checked to see if PECAM-1 was specifically trapped by anti–PECAM-1 ext (rabbit). Intense mat-like anti–PECAM-1 (chick) staining was observed at the base of ECs. Similar staining was not observed by antivinculin (mouse) or anti–pan-cadherin (mouse). No focal adhesions were detected by antivinculin staining. Cells on the poly-L surface did not show the mat-like anti–PECAM-1 staining. These results support the idea that PECAM-1 engagement is necessary for its tyrosine phosphorylation by mechanical stresses.
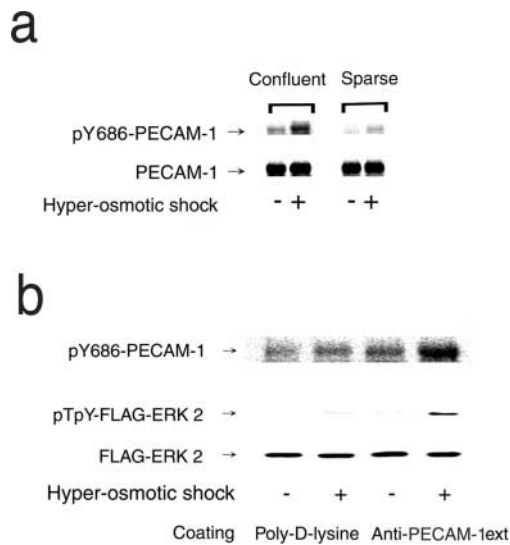
Figure 6. **PECAM-1 engagement is necessary for its tyrosine phosphorylation and ERK activation.** (a) Confluent or sparsely cultured cells were treated with (+) or without (−) HOS for 10 min and PECAM-1 tyrosine phosphorylation was assayed as in Fig. 2 a. Although similar amounts of PECAM-1 were loaded, a significant increase in pY-PECAM-1 was noted in confluent cultures treated with HOS. A low level of pY-PECAM-1 increase in HOS-treated sparse cultures was presumably due to occasional contacts made in these cultures. The same results were obtained in four separate experiments. (b) BAECs transiently expressing FLAG–ERK2 were plated sparsely on a surface coated with anti–PECAM-1ext or poly-L and cultured for 1 h using serum-free DF medium. Cells were then treated with (+) or without (−) HOS for 10 min, and aliquots of total cell lysates were immunoblotted for phospho-PECAM-1 (pY686-PECAM-1). Remaining lysates were mixed with anti-FLAG, and immunoprecipitates were immunoblotted with anti–phospho-ERK and reprobed with anti-ERK. FLAG–ERK2 phosphorylation by HOS was observed in cells plated on the anti–PECAM-1–coated surface. The same results were obtained in three separate experiments.

Is PECAM-1 a mechanotransducer molecule?

FSS and HOS are expected to mechanically perturb the plasma membrane and the structures associated with it. In ECs forming a monolayer, PECAM-1 is engaged and may not be able to move freely with the deforming plasma membrane. Because forces act on the parts of the cell that resist deformation, it is possible that FSS acts on PECAM-1, which then initiates mechanosignal transduction. As magnetic beads were used to exert force onto cells (Glogauer et al., 1997), we employed a similar approach to pull on PECAM-1 on the cell surface. Magnetic beads were coated with anti–PECAM-1ext, applied onto sparsely plated BAECs, and allowed to attach to the cell surface for up to 30 min. Confocal immunofluorescence microscopy revealed that under these conditions, PECAM-1, but not vinculin and cadherin, accumulated under the beads in a time-dependent manner (Fig. 7 a). Magnetic beads coated with preimmune IgG could hardly attach to cells, but those adhering to cells failed to accumulate PECAM-1. Poly-L–coated beads attached to cells to a similar extent as the antibody-coated beads, but were unable to accumulate PECAM-1.

BAECs were plated sparsely on poly-L–coated dishes and allowed to spread for 1 h as described in the Materials and methods. Magnetic beads coated with anti–PECAM-1ext

were added to the culture, and after 20 min, a strong magnet was placed over the cells for 10 min (Fig. 7 b). Cells were lysed, magnetic beads collected, and the materials bound to the beads were immunoblotted with anti-pY686. The tyrosine phosphorylation level of PECAM-1 associated with beads increased only when a magnetic field was applied (Fig. 7 c). PECAM-1 in the cell lysate not bound to beads contained a trace level of pY-PECAM-1, regardless of magnetic field application. These results suggest that PECAM-1 tyrosine phosphorylation occurs when mechanical force is applied directly to it. To examine if ERK was phosphorylated under these conditions, cells expressing FLAG–ERK2 were used to do the same experiment, followed by immunoprecipitation with anti-FLAG. FLAG–ERK2 was activated only when beads were pulled by a magnet (Fig. 7 d).

When the beads on cells are pulled by a magnet, not only PECAM-1 but also the whole cell surface and any associated cytoplasmic structures may be mechanically perturbed. Such general deformation of the cell (surface) may cause the observed PECAM-1 phosphorylation and ERK activation. To test this, we coated magnetic beads with various antibodies against the external domains of EC surface proteins, including anti–P-selectin (Zymed Laboratories and Santa Cruz Biotechnology, Inc.), anti–LDL receptor (Progen and Oncogene Research Products), anti-CR62E (Ancell), and anti–integrin αV (clone LM142; Chemicon). When these beads were applied to sparse EC cultures, only a small number of beads adhered to cells (<1 bead per cell), and extensive biochemical analyses could not be performed. We also coated culture dishes with these antibodies (as in Fig. 6 b) to do HOS experiments, but ECs failed to spread on such surfaces. Beads coated with poly-L attached to BAECs to a similar extent as those coated with anti–PECAM-1ext, but when the magnetic field was applied, neither PECAM-1 nor ERK was phosphorylated (Fig. 7, c and d). These results suggest that phosphorylation of PECAM-1 and ERK is achieved when mechanical force acts on PECAM-1 molecules on the cell surface.

Discussion

We demonstrated the presence in ECs of a novel PECAM-1–dependent signaling pathway activated by FSS and HOS, both of which are thought to exert mechanical perturbation to the plasma membrane. ERK phosphorylation, a known early flow response of cultured ECs (Davies, 1995; Tseng et al., 1995) was found to be a downstream event of this signaling. The initial step of this signaling appeared to be tyrosine phosphorylation of PECAM-1, and we presented evidence suggesting that PECAM-1 might be a mechanoresponsive molecule. The single most important experimental evidence was to elicit this signaling response by applying tugging force directly to PECAM-1 on the cell surface. In such experiments, only the fraction of PECAM-1 that was bound to anti–PECAM-1ext–coated beads and pulled by magnetic force was tyrosine phosphorylated, and ERK was also phosphorylated under these conditions. When PECAM-1 expression was downregulated, the level of ERK phosphorylation by HOS was substantially reduced, suggesting that the PECAM-1–dependent signaling cascade played a significant

Figure 7. **PECAM-1 tyrosine phosphorylation and ERK activation by direct application of mechanical force to PECAM-1.** (a) Magnetic beads coated with rabbit anti–PECAM-1ext were allowed to attach to sparsely cultured BAECs for 10–30 min. Cells were fixed and stained with chicken anti–PECAM-1. Confocal micrographs show time-dependent PECAM-1 accumulation under the beads. To see if molecular aggregates similar to the focal adhesion or the adherens junction might be formed under the beads, cells were stained with antivinculin or anti–pan-cadherin. No accumulation of vinculin and cadherin was detected with the beads in 20 min. Beads with autofluorescence were used for anti–pan-cadherin staining. Anti–pan-cadherin staining (left) and a merged image (right) of the antibody (staining green) and autofluorescent beads (red) are shown. Note that anti–pan-cadherin stains the EC junction but not with the beads. Bar, 10 μm. (b) A schematic representation of the experiment. BAECs plated sparsely on a poly-L–coated surface were cultured for 1 h in serum-free DF medium. Magnetic beads coated with anti–PECAM-1ext or poly-L were allowed to adhere to the cell surface for 20 min and a strong magnet was put over the cell for 10 min. In real experiments, beads covered practically the entire apical surface of cells. (c) Cells were lysed and beads collected. Proteins bound to beads were eluted with SDS sample buffer (Bound fraction). The remaining cell lysates were mixed with an appropriate amount of SDS sample buffer (Unbound fraction). Both samples were immunoblotted by anti-686pY and reprobed with anti–PECAM-1. PECAM-1 bound to the beads was tyrosine phosphorylated in a magnetic field–dependent manner. For detail, see text. (d) BAECs were transiently transfected with FLAG–ERK2 and subjected to the same experiment as in c. From cell lysates, FLAG–ERK was immunoprecipitated by anti-FLAG and immunoblotted with anti–phospho-ERK and reprobed with anti-ERK. ERK activation was observed in cells whose PECAM-1 was tugged by the antibody-coated beads. The experiments illustrated in c and d were repeated five to eight times with the same results.
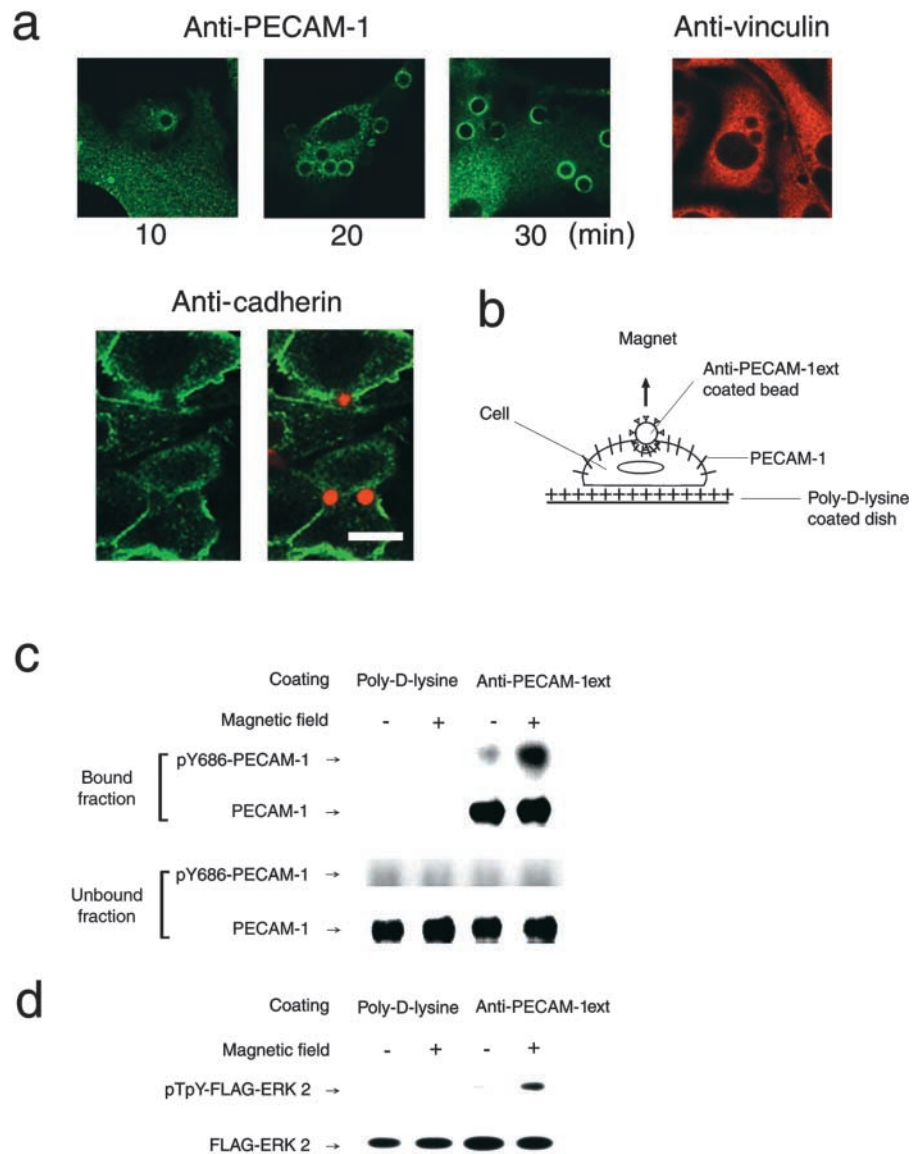
role in the activation of ERK. Other results reported in this paper are consistent with the idea that PECAM-1 is a mechanotransducer and activates a signaling cascade in ECs, leading to ERK activation. Besides ERK activation, there are many other forms of EC responses to FSS. It is generally thought that more than one mechanosignaling pathway is involved in initiating various flow responses, and accordingly, several mechanotransduction schemes have been suggested. We propose that PECAM-1 is a new member of such mechanotransduction molecules in ECs. At present, it is not known how many of the known flow responses are regulated by PECAM-1 tyrosine phosphorylation. Currently, we are investigating what other FSS-induced events might depend on PECAM-1.

Using live BAECs expressing GFP-tagged SHP-2, we showed rapid, mechanical stimulus–dependent translocation of this signaling molecule to the intercellular junction.

The similar translocation of endogenous SHP-2 as well as Gab1 was demonstrated by immunofluorescence microscopy. In ECs with reduced PECAM-1 expression, neither SHP-2 nor Gab1 translocated to the cell junction even though the cells were exposed to FSS or HOS. Thus, the translocation of these molecules to the cell junction depends on the expression of PECAM-1 and its tyrosine phosphorylation, which is induced by mechanical stresses. Several questions remain unanswered regarding the nature of the interaction among PECAM-1, SHP-2, and Gab1 at the cell junction. We have shown direct binding between pY-PECAM-1 and SHP-2 (Masuda et al., 1997), and this is achieved presumably by a BTAM present in PECAM-1. Direct binding between Gab1 and SHP-2 is also reported and is mediated by a BTAM of Gab1 (Cunnick et al., 2000, 2001). These investigators have shown that in cells stimulated by EGF, Gab1 binding activates the phosphatase activ-

ity of SHP-2, which is required for ERK activation. Similar molecular events could occur in ECs stimulated by FSS or HOS. However, in either case in which cells are treated by chemical (EGF) or mechanical (FSS and HOS) stimuli, the precise way in which the membrane protein (EGF receptor or PECAM-1), SHP-2, and Gab1 interact is not known. Because immunoprecipitation experiments did not show direct binding between PECAM-1 and Gab1 (unpublished data), the observed Gab1 accumulation at the cell junction may be due to its interaction with SHP-2. This may create competition between PECAM-1 and Gab1 for binding to SHP-2, because both use BTAM to bind to SHP-2, and such competition might be unfavorable for the observed accumulation of SHP-2 and Gab1 at the cell junction. It is possible that increased concentrations of SHP-2 in this region may work as a sink for Gab1, but it is also possible that some unknown molecules are involved in the observed Gab1 accumulation because it has several protein binding motifs. Attempts to identify other PECAM-1 and Gab1 binding proteins at the cell junction are ongoing. Another question is that for Gab1 to bind to SHP-2, two of Gab1's tyrosine residues (Tyr627 and Tyr659) must be phosphorylated (Cunnick et al., 2001). If Gab1 does bind to SHP-2 in ECs exposed to FSS, how is Gab1 tyrosine phosphorylated? It is quite possible that the answer to this question holds a key to understanding FSS mechanosignaling.

SHP-2(C/S) has no phosphatase activity, but other sites, such as BTAM, are intact (Noguchi et al., 1994). When this mutant was expressed in ECs, it was associated with the cell junction regardless of FSS or HOS. This result provides certain insights into the PECAM-1–dependent signaling. Although its physiological function is not understood, a small fraction of PECAM-1 is always tyrosine phosphorylated in ECs (Masuda et al., 1997; Osawa et al., 1997). Thus, via their BTAM, both SHP-2 and SHP-2(C/S) should bind to pY-PECAM-1 present in unstimulated ECs. By its phosphatase activity, however, SHP-2 would dephosphorylate PECAM-1, causing it to detach from PECAM-1, and no appreciable build-up of SHP-2 would occur at the EC junction. On the other hand, SHP-2(C/S) with no phosphatase activity should bind to PECAM-1 but would not dephosphorylate it. Consequently, the mutant SHP-2 would remain bound, causing its gradual accumulation at the cell junction. In another signaling system in which SHP-2 is involved, similar effects have been observed (Oh et al., 1999).

There is an interesting corollary to the proposed on–off interaction between PECAM-1 and SHP-2. When PECAM-1 is tyrosine phosphorylated by FSS, SHP-2 binds to it. The bound SHP-2 would then dephosphorylate PECAM-1. We suggest that signaling is initiated at some point during these processes. If ECs are still under FSS, PECAM-1 would be rephosphorylated, followed by binding of SHP-2 and dephosphorylation by the bound SHP-2, resulting in another wave of signaling. This cycling allows the system to continuously respond to FSS. We showed earlier that the expression of proteins containing phosphotyrosine is augmented in the area of aorta where FSS is experimentally increased (Kano et al., 2000). One interpretation of this result is that ECs, although they have been under increased FSS for 1 wk, maintain increased levels of signaling. The pro-

tein(s) responsible for the observed increase in phosphotyrosine is not fully identified, but a part of it could be due to pY-PECAM-1. These data support the idea that ECs continuously monitor FSS levels, and the proposed cyclic interaction between PECAM-1 and SHP-2 appears to be a good feature for a system that works continuously.

Extracellularly, PECAM-1 of an EC binds to PECAM-1 of neighboring ECs primarily in a homophilic manner (Albelda et al., 1990). Although a large fraction of PECAM-1 is extractable by Triton, ~30% is associated with the cytoskeleton (Ayalon et al., 1994). Indeed, PECAM-1 binds to β- and γ-catenins (Kusano et al., 1998; Ilan et al., 2000), suggesting its interaction with actin filaments. Being anchored both extracellularly and intracellularly, PECAM-1 may not be able to move freely as FSS or HOS deforms the cell surface. Thus, when the cell surface is mechanically stressed, the force of stress may act on PECAM-1, which then is tyrosine phosphorylated. This hypothesis is supported by our study using antibody-coated magnetic beads. Our hypothesis predicts that PECAM-1 signaling would be inhibited if PECAM-1's extracellular or intracellular anchoring was disrupted. PECAM-1 of sparsely cultured BAECs would not be engaged, and indeed, PECAM-1 phosphorylation was severely inhibited in such cells. However, sparse ECs responded to HOS as if they were in a confluent monolayer if cells were cultured on the anti–PECAM-1–coated surface. Under these conditions, PECAM-1 would be externally anchored, enabling it to signal. Internal anchoring of PECAM-1 may be modulated by treating cells with drugs that affect actin filaments. Our preliminary results indicate that ECs treated with cytochalasin D or phalloidin caused a significant decrease or increase in pY-PECAM-1 levels, respectively (Masuda et al., 1998). These results are consistent with our hypothesis.

Our pharmacological studies indicate that PECAM-1 tyrosine phosphorylation does not depend on activities of various ion channels, PKC, or the Gq-coupled receptors and is not achieved by stimulating ECs with Ca^{2+} ionophores, NO analogs, or growth factors (Harada et al., 1995; Masuda et al., 1998). To date, no known signaling event has been found to precede PECAM-1 tyrosine phosphorylation in ECs, suggesting that PECAM-1 may initiate a mechanosignaling process. PECAM-1 is localized at interendothelial adhesion, a site proposed for mechanosignal transduction in ECs (Davis, 1993; Kano et al., 2000, Fujiwara et al., 2001). Fig. 8 is our working model for PECAM-1–initiated mechanosignal transduction. We propose that mechanical force acts on PECAM-1 at the cell–cell adhesion and causes phosphorylation of PECAM-1 at two tyrosine residues (Y663 and Y686). Because PECAM-1 is not an autophosphorylating protein, involvement of some kinase(s) is postulated. However, the precise mechanism of PECAM-1 tyrosine phosphorylation is not known at this time. One possible scenario might be that mechanical force causes conformational change in PECAM-1. Without mechanical force acting on it, PECAM-1 might be in a "closed" conformation in which the tyrosine residues would not be available to kinase(s). However, when mechanical force acts on the molecule, PECAM-1's conformation might become "open" so that Tyr663 and Tyr686 would now be available

Figure 8. **A model for mechanosignal transduction by PECAM-1.** The model depicts the following scenario. When an EC is under no mechanical stress, PECAM-1 is in a closed state in which Tyr663 and Tyr686 are unavailable to kinase. When mechanical force acts on PECAM-1, it is converted to an open state in which the tyrosines can be phosphorylated by kinase. SHP-2 and Gab1 are recruited to the cell junction when PECAM-1 is phosphorylated. This activates a signaling cascade leading to ERK phosphorylation. For detail, see text.
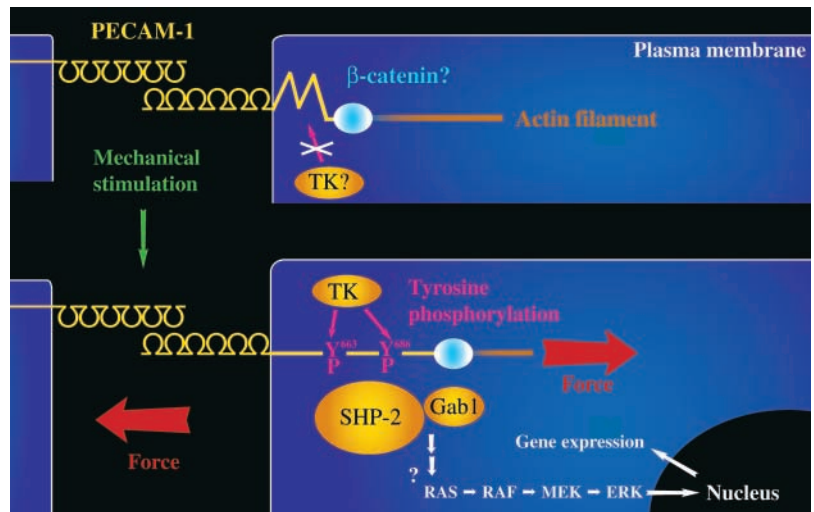
to the kinase. Whether or not such a mechanical stress–induced conformational change occurs in PECAM-1 needs to be investigated. When PECAM-1 is tyrosine phosphorylated, SHP-2 binds to it. This activates SHP-2's enzymatic activity, which then activates, together with Gab1, an ERK signaling pathway.

It is still possible that this signaling is not specific for PECAM-1. Force applied to one of many membrane proteins tethered to the cytoskeleton may activate this or a similar signaling cascade. We attempted to test this possibility by using antibodies against other cell surface proteins, but were unsuccessful for technical reasons. Experiments were possible with poly-L–coated beads, but they provide only a partial answer to the issue. Poly-L beads bind to any negatively charged surface molecules. Although they bind avidly, they are likely adherent to a variety of molecules including phospholipids and random surface molecules, none of which may be clustered under the beads in high enough concentrations to generate a specific signal. Although we have provided reasonable evidence implicating PECAM-1 as a mechanoresponsive molecule, we have not proven that it is the actual molecule that converts the mechanical force of shear stress into the chemical signal of protein tyrosine phosphorylation.

Several important questions remain to be investigated. First, what is the mechanism of PECAM-1 tyrosine phosphorylation? More specifically, what is the PECAM-1 kinase? Although Src family kinases are possible candidates, as they phosphorylate PECAM-1 in vitro (Osawa et al., 1997) and in cells overexpressing it (Cao et al., 1998; Lu et al., 1997), Src family kinase inhibitors (herbimycin A, erbstatin, and PP1) had no effect on PECAM-1 tyrosine phosphorylation by HOS (unpublished data). The expression of PECAM-1 kinase may be limited to ECs because PECAM-1 transfected into non-ECs, including ECV304, COS7, HEK293, and L cells, failed to be phosphorylated when these cells were treated with HOS, although they formed tight monolayers just like ECs, expressing exogenous PECAM-1 at their cell junction (unpublished data). Work is now in progress to identify PECAM-1 tyrosine kinase.

Another set of questions is concerned with the molecular link between PECAM-1 and the actin cytoskeleton. β-Catenin may play a role in this as it can bind to PECAM-1.

However, our preliminary results indicated that the affinity between the two proteins was not strong (Kusano et al., 1998). The actin cytoskeleton that could anchor PECAM-1 includes the cortical actin filament network, the actin bundle associated with the adherens junction, and the apical stress fiber system. One of these actin structures could play the primary role, but it is also possible that all or a combination of them may be necessary. Based on their three-dimensional distribution, we have proposed that the apical stress fibers that run between the apical (where FSS exerts its force) and lateral (where the PECAM-1–dependent signaling is likely to take place) regions of cells may play a role in this signaling (Kano et al., 2000). Morphological as well as biochemical characterization of the link between PECAM-1 and actin filaments is needed.

A third question is the physiological significance of PECAM-1 mechanosignal transduction. Preliminary results indicate that PECAM-1 in ECs is tyrosine phosphorylated in vivo (unpublished data). When a short coarctation was made in guinea pig aorta for 1 wk and the blood vessel was stained with antiphosphotyrosine, increased staining was noted at the cell–cell border (Kano et al., 2000). At present, it is not known how these observations relate to the physiology and pathology of blood vessels. PECAM-1–null mice exhibit no apparent developmental abnormalities, although some minor defects are noted in adult mice (Duncan et al., 1999; Mahooti et al., 2000; Thompson et al., 2001). However, this does not necessarily mean that PECAM-1 plays only a minor role in vivo. In fact, it plays a critical role in leukocyte extravasation, and more recent studies indicate its involvement in signaling (Ji et al., 2002). The primary sequence of its cytoplasmic domain is highly conserved among mammalian species, suggesting that PECAM-1 function is of advantage for survival. ECs might have a backup system for PECAM-1's function, and this is done by expressing other proteins with the same function. The presence of functionally redundant molecules would make knockout mice appear near normal. EC responses to FSS are not usually life or death types of reactions, even though FSS affects a wide variety of EC functions. Many are transient and their long-term effects are difficult to assess. It appears that the FSS effects on ECs that have significant implications for an organism are not acute

types and thus must be determined using a long time span. The life span of a mouse may not be long enough for chronic effects of PECAM-1 deletion to become obvious. Therefore, whether or not there are detrimental effects of the PECAM-1–null condition in man is still an open question.

Materials and methods

Cells and mechanical stimulation
BAECs were cultured as described previously (Osawa et al., 1997). To obtain sparse cultures, 40% confluent BAECs in 10-cm dishes were subcultured at a split ratio of 1:16 for 1 d. ECs were washed three times with PBS containing 0.2 mM EGTA and incubated for 30 min at room temperature. The PBS was replaced with serum-free DF medium, and by gentle pipetting, a suspension of single cells was obtained. These cells were plated sparsely. To prevent the formation of focal adhesions, ECs were plated on poly-L–coated dishes in serum-free medium. Absence of focal adhesions was confirmed by antivinculin staining. Methods for stimulating cells by FSS and HOS and for immunoblotting have been previously described (Harada et al., 1995; Osawa et al., 1997). An ECL (Amersham Biosciences) or I125 streptavidin detection system (Amersham Biosciences) was used for immunoblotting and internal controls were used for quantification.

Antibodies
Rabbit anti–PECAM-1ext and anti–PECAM-1 have been previously described (Osawa et al., 1997). Antibodies against the PECAM-1 cytoplasmic domain were made by immunizing chickens with the GST-tagged recombinant cytoplasmic tail of bovine PECAM-1. IgY was purified using an Eggstruct kit (Promega), and anti–PECAM-1 was affinity purified using the original antigen. Anti-pY686 was made by immunizing rabbits with a synthetic polypeptide corresponding to bovine PECAM-1 (682TETVpYSEIRKADC692) conjugated to keyhole limpet hemocyanin. The antibodies were affinity purified by the original antigen, followed by absorption with the cytoplasmic domain of PECAM-1 (recombinant), the same 12-mer containing unphosphorylated tyrosine, and a 12-mer of scrambled sequence containing pY. In ELISA assays, the lowest dilutions where anti-pY686 gave positive signals were 72,000, 900, and 300 times against the original antigen, the unphosphorylated 12-mer, and the scrambled 12-mer containing pY, respectively. Anti-pY686 was diluted 15,000 times for immunoblotting. Anti-ERK (UBI), anti-SHP-2 (Santa Cruz Biotechnology, Inc.), anti–phospho-ERK (Promega), anti–phospho-p38 (New England Biolabs, Inc.), anti-Gab1 (UBI), agarose-conjugated anti-FLAG(M2) (Sigma-Aldrich), and agarose-conjugated anti-HA (BabCO) were purchased.

Plasmid constructs and transfection
Two tyrosine phosphorylation sites (Y663, Y686) in bovine PECAM-1 were mutagenized to phenylalanine (Stratagene). The cytoplasmic domain and the same domain with Y663F and Y686F mutations were generated by PCR and cloned into pBluescript KS(+) containing an HA epitope with a linker (MYPYDVPDYAG). The HA–PECAM-1 cytoplasmic domain (HA–PECAM-1cyt) and the HA–PECAM-1 cytoplasmic domain with the Y/F mutations (HA-PECAM-1cyt[Y/F]) were subcloned into a mammalian expression vector pcDNA3.1(+) (Invitrogen). A plasmid DNA encoding a phosphatase-inactive mutant SHP-2 (SHP-2[C459S]) was a gift from T. Matozaki and M. Kasuga (Kobe University, Kobe, Japan) (Noguchi et al., 1994) and was subcloned into pBluescript KS(+) containing an HA epitope with a linker (GSMYPYDVPDYAGA). HA-tagged SHP-2(C459S) (HA–SHP-2[C/S]) was subcloned into pcDNA3.1(−). GFP–SHP-2 and GFP–SHP-2(C/S) were made using pcDNA3.1/CT-GFP–TOPO (Invitrogen) according to the manufacturer's instructions. All constructs were sequenced before use. FLAG-tagged ERK2 in pEF-BOS (pEF-BOS-FLAG–ERK2) was provided by M. Ogata and T. Hamaoka (Osaka University School of Medicine, Osaka, Japan) (Ogata et al., 1999). pEF-BOS-FLAG–ERK2 (0.2 μg) was cotransfected with 0.8 μg of pcDNA3.1 (mock), pcDNA3.1-HA–PECAM-1cyt, pcDNA3.1-HA–PECAM-1cyt(Y/F), or pcDNA3.1-HA–SHP-2(C/S) into 70% confluent BAECs in a 6-cm dish using the LipofectAMINE PLUS reagent (Life Technologies) according to the manufacturer's instructions. Transfection efficiencies were 10–20%. Cells were used 40 h after transfection.

Antisense S-oligo treatment
Subconfluent BAECs in 35-mm dishes were washed twice with serum-free OPTI medium (Life Technologies). 12 μl of TransIT-LT1 (PanVera) was mixed with 100 μl of serum-free OPTI medium. After 20 min, 6 μM anti-

sense S-oligo (nucleotide no. 305–322; 5′-GCGGCAGGATCTGCATGT-3′) were added and incubated for 30 min at room temperature. ECs in 1 ml of serum-free OPTI medium were treated with this mixture (final S-oligo concentration 600 nM) for 4 h and then with DF medium containing 10% FBS for 24 h. The cells were washed with DF medium without FBS and treated again with the same OPTI–TransIT-LT1 mixture containing 3 μM antisense S-oligo (final concentration 300 nM) for 4 h. 2.5% FBS and 6 μM antisense S-oligo (without vehicle) were added to the medium, and cells were cultured for 36 h. Prior to use, confluent cells were further treated with DF medium containing 0.8% FBS and 6 μM antisense S-oligo (without vehicle) for 18 h. Under these (high transfection efficiency) conditions, ~90% of cells were transfected and the PECAM-1 expression of the culture was decreased by ~70% (Fig. 2 and Fig. 5 c). ECs used for microscope studies were washed and placed in 1 ml of serum-free OPTI medium as above. They were then treated with antisense S-oligo (final concentration 600 nM) in the OPTI–TransIT-LTI mixture for 4 h. The medium was changed to DF medium containing 10% FBS, and ECs were cultured for 12 h, transferred to DF medium containing 0.8% serum, and cultured for 12 h. Under these low transfection efficiency conditions, PECAM-1 downregulation occurred in 10–20% of ECs.

To cotransfect GFP–SHP-2 and PECAM-1 antisense S-oligo, BAECs were grown in 6-cm dishes until they were 70% confluent and washed twice with serum-free OPTI medium. 2 μg of GFP–SHP-2 or GFP–SHP-2(C/S) were transfected using the LipofectAMINE PLUS reagent according to the manufacturer's instructions. A lipid–DNA complex was applied to the culture medium and 30 min later, a mixture of OPTI–TransIT-LT1 and antisense S-oligo (final concentration 600 nM) was added. Cells were cultured for 4 h, washed with DF medium with 10% FBS, and cultured in the same medium for 30 h.

Direct force application to PECAM-1
Magnetic beads conjugated with sheep anti–rabbit IgG (Dynabeads M-280 sheep anti–rabbit IgG, $\Phi = 2.8$ μm; Dynal) were treated with 30 μg/ml of anti–PECAM-1ext or preimmune IgG for 1 h, blocked with 1 mg/ml heat-denatured BSA (RIA grade; Sigma-Aldrich) for 4 h, and washed three times with PBS. Roughly 2×10^7 beads were added onto a sparse culture of BAECs in a 6-cm dish for 10–30 min and washed. Practically the entire apical surface of cells was covered with beads.

A cylindrical neodium magnet (6 [Φ] $\times$ 2 [H] cm; central magnetic flux density, 3,000 gauss; Niroku) was placed on the cover of the culture dish for 10 min. Under a microscope, we observed upward displacement of beads when the magnet was placed. The magnetic force was estimated to be ~20 pN/bead by using the method employed by Glogauer et al. (1997). Cells were washed twice with ice-cold Dulbecco's PBS containing 1 mM Na$_3$VO$_4$, and lysis buffer (50 mM Tris-HCl, 100 mM NaCl, 20 mM NaF, 2 mM EDTA, 2 mM EGTA, 1 mM Na$_3$VO$_4$, 1 mM PMSF, 10 μg aprotinin, 10 μg leupeptin, and 0.4% Triton X-100) was added. Cells were harvested by scraping and quickly homogenized. Beads were collected using a magnetic particle concentrator (Dynal). PECAM-1 on the beads was eluted with SDS sample buffer. Cell lysates from which beads had been removed were also mixed with SDS sample buffer. pY-PECAM-1 was detected by immunoblotting. Tosylactivated magnetic beads (Dynabeads M-280 tosylactivated, $\Phi = 2.8$ μm; Dynal) were used according to the manufacturer's instructions to make poly-L–coated beads.

To observe PECAM-1 accumulation under beads, a small number of coated beads were added to BAEC cultures. After 30 min, cells were fixed and permeabilized as described below and then stained with chicken anti–PECAM-1, pan-cadherin mAb (Sigma-Aldrich), or vinculin mAb (Sigma-Aldrich), followed by fluorescein-conjugated IgY (Zymed Laboratories) or rhodamine- or fluorescein-labeled anti–mouse IgG (Cappel).

PECAM-1 engagement by antibodies
The surface of 35-mm bacterial culture dishes were treated with 30 μg/ml of anti–PECAM-1ext or control preimmune IgG for 12 h at 4°C, or 1 mg/ml of poly-L for 4 h at room temperature. All dishes were treated with 1 mg/ml of heat-denatured BSA for 4 h at room temperature and washed three times with PBS. Suspensions of single cells were prepared as described above, and cells were plated sparsely onto these dishes.

Immunofluorescence microscopy
Cells were fixed with 2% formaldehyde for 5 min and then with 2% formaldehyde containing 0.2% Triton X-100 for 10 min at room temperature. They were blocked with 50% Block Ace (Dainihon) and treated with appropriately diluted primary antibodies for 30 min at room temperature. Secondary antibodies labeled with Alexa®546 (Molecular Probes) were

used. Images were observed using a confocal laser scanning microscope (Fluoview; Olympus) with a LUMPlanFl60x lens or a fluorescence microscope (Axiophot; Carl Zeiss MicroImaging, Inc.) equipped with a CCD camera and an Acroplan Water 60xW lens.

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