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Localized Activation of m-Calpain in Migrating Human Umbilical Vein Endothelial Cells Stimulated by Shear Stress

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Abstract Using a parallel-plate flow-chamber and confocal laser scanning microscopy (CLSM), we studied the mode of cytoskeletal reorganization in migrating HUVECs stimulated by shear stress. Activation of m-calpain associated with a change in the spatial distribution of cytoplasmic ionized Ca^{2+} concentration ($[Ca^{2+}]_i$) was studied. Shear stress (10 dyne/cm^2) caused migration and decrease in the F-actin content of HUVECs. Migrating individual HUVECs showed the lamellipodium formed in the direction of cell migration, in which $[Ca^{2+}]_i$ elevated to 148 ± 12 nM in a localized fashion. We found the appearance of activated m-calpain in the local area of the migrating HUVECs, which was associated with a decrease in the amounts of pp125FAK and ezrin. The localized rise in $[Ca^{2+}]_i$ might be closely related to morphological change to regulate the direction of cell migration induced by shear stress through localized activation of m-calpain. J. Cell. Biochem. 81:184–192, 2001. © 2001 Wiley-Liss, Inc.

Key words: shear stress; $[Ca^{2+}]_i$; m-calpain; pp125FAK; ezrin

Vascular endothelial cells (ECs) have the ability to sense their hemodynamic environment [Izumo and Malek, 1994]. Fluid shear stress causes various biological responses in ECs including changes in morphology or alignment [Franke et al., 1984; Levesque and Nerem, 1985; Wechezak et al., 1989], and changes in their functions, such as the release of endothelium-derived relaxing factor (EDRF) [Cooke et al., 1990] or prostaglandin I_2 (PGI_2) [Grabowski et al., 1985], low-density lipoprotein uptake [Sprague et al., 1987], and synthesis of tissue plasminogen activator [Diamond et al., 1989], endothelin-1 [Yoshizumi et al., 1989], or nitric oxide synthase [Nishida et al., 1992], and proliferation [Levesque et al., 1989]. Although these alterations in cellular functions induced by shear stress have been studied extensively, little is known about the system responsible for signal transduction from the cell surface to the cell interior. To address this question, several groups including ours focused on the involvement of cytoplasmic ionized Ca^{2+} ($[Ca^{2+}]_i$) as a second messenger and confirmed that the activation of ECs is associated with an increase in $[Ca^{2+}]_i$ [Nollert et al., 1990; Shen et al., 1990; Dull and Davies, 1991; Mo et al., 1991]. They have confirmed an inhomogeneous, but flow direction-related $[Ca^{2+}]_i$ rise in shear stress-activated ECs [Geiger et al., 1992; Yoshikawa et al., 1997]. We reported the appearance of the $[Ca^{2+}]_i$ wave from upstream to the downstraem of the cells in shear stress-stimulated HUVECs [Yoshikawa et al., 1997]. Although a close relationship between localized increases in $[Ca^{2+}]_i$ and changes in cell morphology was suggested [Yoshikawa et al., 1999], it is still unclear how a localized $[Ca^{2+}]_i$ rise regulates cell morphology. It is likely that $[Ca^{2+}]_i$-dependent signal transduction pathway, including calmodulin (CaM)-dependent reactions [Anraku et al., 1991], protein kinase C (PKC)-mediated pathway [Nishizuka, 1986], and calcium-activated neutral protease-(calpain) mediated pathway [Kambayashi and Sakon, 1989], play an important role as an

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effector for elevated $[Ca^{2+}]_i$, but the exact mechanism integral to cytoskeletal reorganization is not fully documented.

Calpains (EC 3, 4, 22, 17) are Ca^{2+}-requiring intracellular cysteine proteases that are uniformly distributed in mammalian and avian cells [Murachi, 1983]. There are at least three major calpain isozymes: m-calpain, which requires millimolar Ca^{2+} for its activation, μ-calpain, which requires 10–100 μM Ca^{2+} [Sakon et al., 1981], and tissue-specific forms of calpain [Sorimachi et al., 1994]. Most cells contain an endogenous inhibitor protein, calpastatin [Murachi et al., 1981], which is specific for the calpains [Shiba et al., 1983]. While their exact physiologic function has not been established, a variety of substrate proteins including enzymes, surface glycoproteins, or cytoskeletal proteins [Phillips and Jakabova, 1977; Tsujinaka et al., 1982; Duri et al., 1986; Kambayashi et al., 1986; Kishimoto et al., 1989; Ariyoshi et al., 1993; Oda et al., 1993; Cooray et al., 1996] has suggested an attractive hypothesis that localized $[Ca^{2+}]_i$ rise might cause localized cytoskeletal reorganization through calpain-mediated limited proteolysis of important cytoskeletal cross-linking proteins, such as ezrin [Shcherbina, 1999] and pp125FAK [Ariyoshi et al., 1998], which might be integral to change in cell morphology or migration. In this study, attempts were made to elucidate the possible roles of calpains in cell migration in shear stress-stimulated HUVECs. We employed a specific antibody against 78 kDa-activated m-calpain, which enabled us in situ capture of calpain activation.

MATERIALS AND METHODS

Materials

Fluo-3 AM and rhodamine phalloidin was purchased from Do-jin-do Labo (Kumamoto, Japan). Human umbilical vein endothelial cells (HUVECs) were a kind gift from Dr. Suzuki (Department of Biochemistry, Osaka University Medical School, Osaka, Japan). Specific antibodies recognizing pre- and post-autolytic m-calpain [Kikuchi and Imajoh-Ohmi, 1995] were a kind gift from Dr. Shinobu Ohmi (Institute of Medical Science, Tokyo University, Minato-ku, Tokyo, Japan). Monoclonal antibodies against ezrin and pp125FAK were purchased from Transduction Laboratories (CA). FITC-conjugated goat anti-rabbit IgG (second antibody) was purchased from Cappel (OR). A parallel-plate shear stress chamber was designed and developed for a CLSM (confocal laser scanning microscopy, Zeiss, Germany) unit as described previously [Yoshikawa et al., 1997]. Other reagents were of the highest analytical grade available.

Loading of Fluo-3 AM

HUVECs were prepared and passaged as described previously [Ikeda et al., 1996]. For shear stress experiments, cells were grown on glass coverslips (24×24 mm) in Dulbecco's modified Eagle's medium (DMEM, Gibco NY) with 10% fetal calf serum (FCS). Sub-confluent cells were rinsed twice with modified HEPES Tyrode's buffer (129.0 mM NaCl, 10.0 mM HEPES, 8.9 mM $NaHCO_3$, 5.6 mM dextrose, 0.8 mM $MgCl_2$, 0.8 mM KH_2PO_4; pH 7.4) and incubated for 45 min at 37°C in the same buffer containing 10 μM fluo-3 AM and 0.02% Cramphore.

Shear Stress Experimental Setup and $[Ca^{2+}]_i$ Imaging

Fluo-3-loaded HUVECs were subjected to steady, laminar flow of modified HEPES Tyrode's buffer containing 1 mM Ca^{2+} in a parallel plate flow chamber designed for CLSM, which enabled simultaneous observation of both $[Ca^{2+}]_i$ and differential interference contrast (DIC) images. The conditions for image acquisition and $[Ca^{2+}]_i$ calibration were described previously [Ikeda et al., 1996]. Briefly, CLSM recorded the fluorescence from an intracellular thin plane focused by two pinholes (focal plane) to generate $[Ca^{2+}]_i$ images. The depth of the focal plane (focal depth) of 0.6 μm used in our assay conditions was small enough to prevent artifacts due to cell thickness. Furthermore, as the influence from the uneven dye distribution was negligible as reported previously [Ikeda et al., 1996], single acquisition of fluo-3 images provided precise $[Ca^{2+}]_i$ images. The artifacts due to bleaching of the dye was also minimal in our assay conditions by employing the strong and photo-resistant $[Ca^{2+}]_i$ indicator, fluo-3, as described elsewhere [Ikeda et al., 1996].

Immunofluorescence Microscopic Examination of HUVECs for Pre- and Post-Autolytic m-Calpain

Resting or shear stress-stimulated HUVECs were fixed with 2% paraformaldehyde/PBS at

room temperature for 15 min, permeated with 0.2% Triton X-100/PBS, washed three times with 1% BSA/PBS, and were incubated with PBS containing 0.033 μM rhodamine phaloidine. After twice washing with 1% BSA/PBS, cells were exposed for 2 h to antibodies in 1% BSA/PBS. The cells were washed three times with 1% BSA/PBS to remove excess primary antibody, followed by incubation for 2 h with second antibodies diluted 1:100 in 1% BSA/PBS. After washing cells three times with 1% BSA/PBS, the cells were examined by CLSM. The focal plain clearly showing cell edge was employed for image acquisition.

RESULTS

Shear Stress-Induced Migration of HUVECs

In order to examine the change in cytoskeleton in migrating HUVECs stimulated by shear stress, we first observed the change in cell morphology and F-actin fibers. Before initiation of shear stress, HUVECs showed polygonal appearance with circumferential F-actin fiber alignment. As shown in Figure 1, 2 h after the initiation of shear stress at 10 dyne/cm^2, HUVECs changed their morphology to a more irregular shape and started to migrate in a random direction, which was associated with a decrease in F-actin content. The relative amounts of F-actin estimated by fluorescence intensity from rhodamine phaloidine decreased by 56% in migrating HUVECs. Twelve hours later, HUVECs almost completed the change in cell alignment to show a spindle shape in the direction of flow, where the F-actin content recovered to the initial level with well-aligned F-actin fiber in the direction of flow.

Spatial Distribution of $[Ca^{2+}]_i$ in Migrating HUVECs Stimulated by Shear Stress

As shown in Figure 2, migrating HUVECs stimulated by shear stress showed lamellipodium in the direction of migration, which was associated with localized rise in $[Ca^{2+}]_i$. In migrating HUVECs, the mean value of $[Ca^{2+}]_i$ was increased from 96 ± 6 nM (before stimulation, data not shown) to 122 ± 8 nM, whereas

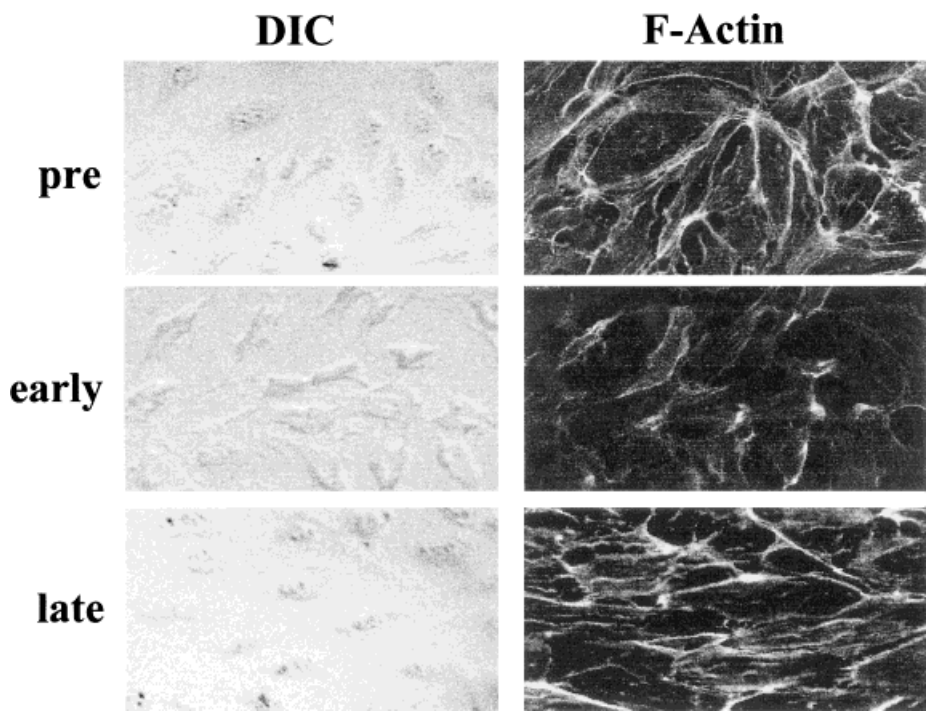

Fig. 1. Change in F-actin in migrating HUVECs stimulated by shear stress. For each flow experiment, fluo-3-loaded HUVECs were gently transferred to the chamber, which was then equilibrated for several minutes with modified HEPES Tyrode's buffer. After acquisition of pre-flow images, steady laminar flow was initiated through the flow pass (0.2×1.0 mm) to generate shear stress of 10 dyne/cm^2. HUVECs were fixed by 2% PFA before, 2 or 12 h after the initiation of shear stress, followed by staining with rhodamine-phalloidine as described in Materials and Methods. Results shown are from one representative of 4 different experiments. Shear stress was calculated using the equation $\gamma = 6\mu Q/a^2 b$: γ; shear stress, μ; viscosity, Q; flow rate, a; height of flow pass, b; width of flow pass.

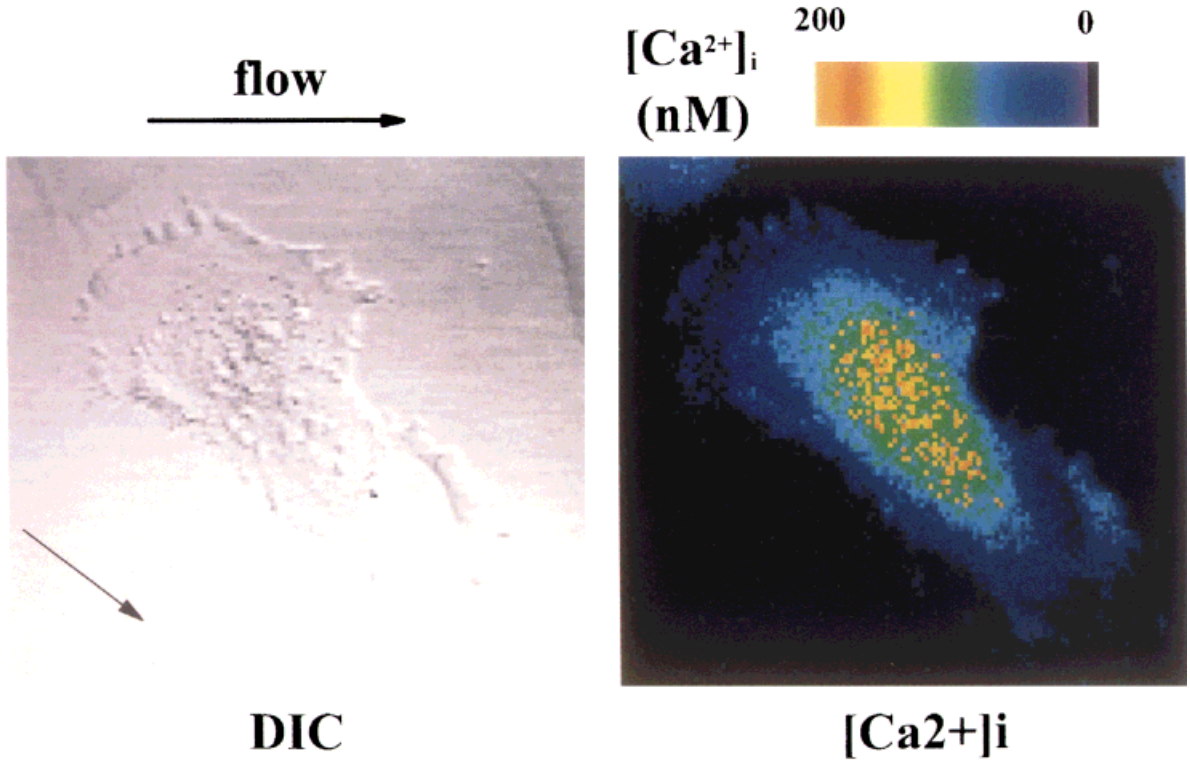

Fig. 2. Shear-stress induced migration of individual HUVECs. In the presence of 1 mM extracellular Ca^{2+}, fluo-3-loaded HUVECs were subjected to shear stress of 10 dyne/cm^2. Typical images of $[Ca^{2+}]_i$ and DIC 2 h after the initiation of shear stress were shown. The arrow in the DIC image means the direction of cell migration. Results shown are from one representative of four different experiments.

the mean value of localized $[Ca^{2+}]_i$ inside lamellipodium was 148 ± 12 nM. These results suggested the possible role of localized $[Ca^{2+}]_i$ rise in the change of cell morphology. In order to clarify the mechanisms of $[Ca^{2+}]_i$ dependent cytoskeletal reorganization, we next studied the activation mode of m-calpain in migrating HUVECs.

Distribution of Pre- or Post-Autolytic m-Calpain, Ezrin, and pp125FAK

As shown in Figures 3 and 4, before initiating shear stress, pre-autoproteolytic m-calpain distributed through the cytoplasm in the nodular pattern and no post-autoproteolytic m-calpain was detected. As shown in Figures 3 and 4, after the initiation of shear stress, pre-autoproteolytic m-calpain was found mainly in the edge of migrating HUVECs, where post-autoproteolytic m-calpain co-localized in the tube or granular appearance, suggesting the localized activation of m-calpain through translocation from cytoplasm to cell edge. Artifacts due to cross-reactivity of the antibodies were negligible because each antibody employed was reported to recognize solely 80 or 78 kDa m-calpain [Kikuchi and Imajoh-Ohmi 1995]. Therefore we next studied the change in endogenous calpain substrate, ezrin, and pp125FAK, which has been well known to regulate cell morphology [Cooray et al., 1996; Ariyoshi et al., 1998; Shcherbina et al., 1999]. As shown in Figures 5 and 6, the content of pp125FAK and ezrin decreased in migrating HUVECs, suggesting the possible roles of m-calpain in regulating cytoskeletal structure in a small compartment of the cells through the proteolysis of cytoskeleton-related proteins, which might be integral to lamellipodium formation.

DISCUSSION

The development of a $[Ca^{2+}]_i$ imaging system applicable in living cells has allowed the spatial analysis of $[Ca^{2+}]_i$, which has proposed the attractive hypothesis that localized $[Ca^{2+}]_i$ gradients might control cell motility or morphology in a localized fashion [Tayler et al.,

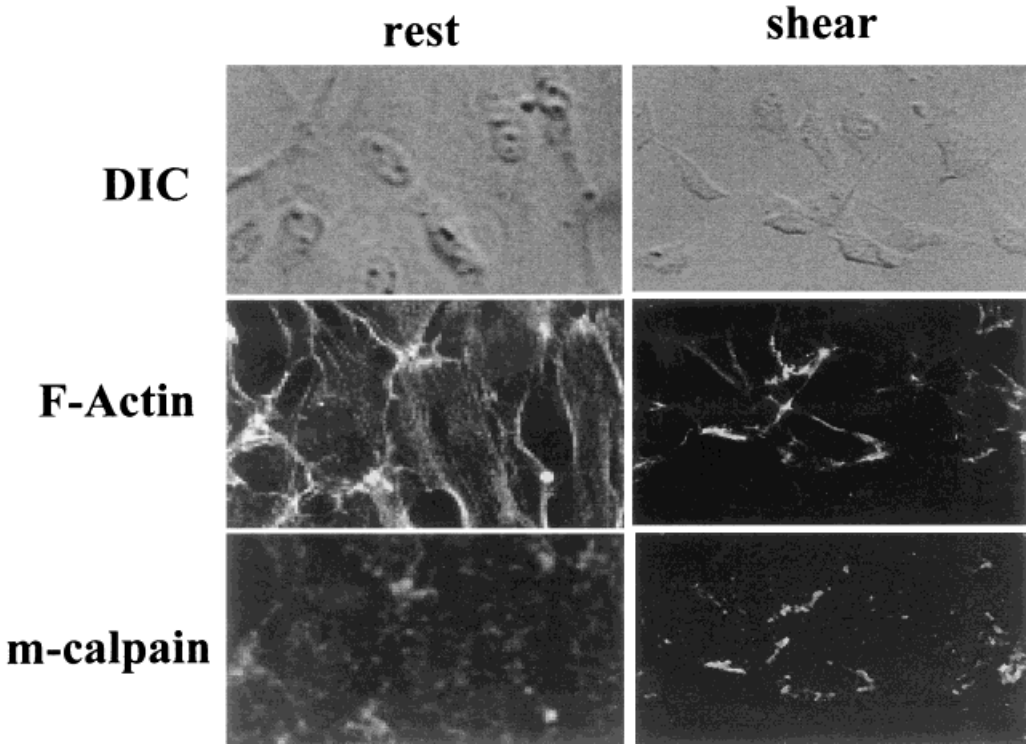

Fig. 3. Distribution of F-actin and pre-autoproteolytic m-calpain in HUVECs. HUVECs were fixed by 2% PFA before and 2 h after the initiation of shear stress. Faction or pre-autoproteolytic m-calpain was stained as described in Materials and Methods. The results presented were from one representative of five different experiments.

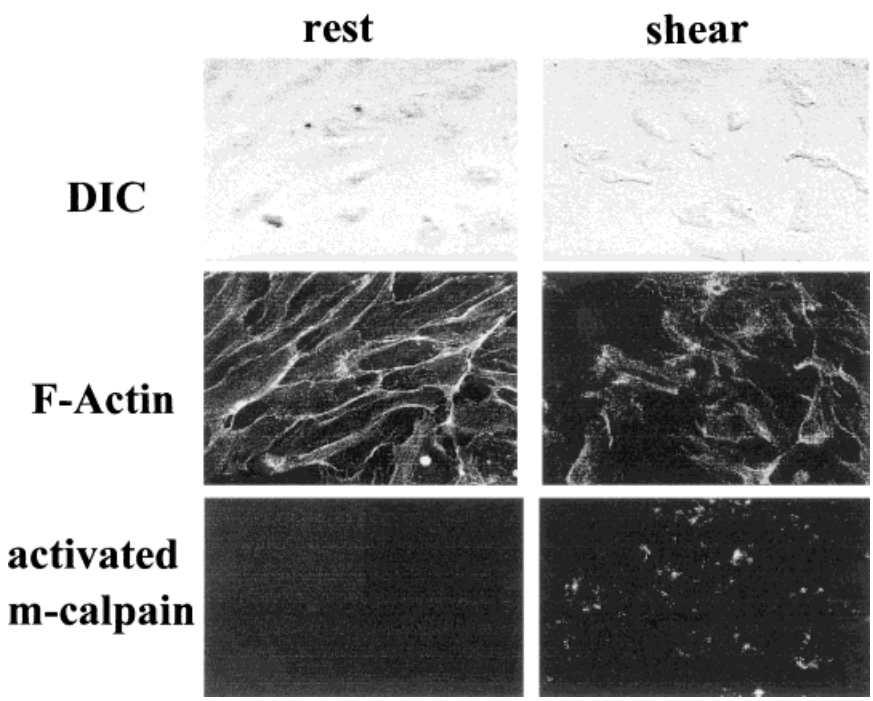

Fig. 4. Distribution of F-actin and post-autoproteolytic m-calpain in HUVECs. HUVECs were fixed by 2% PFA before and 2 h after the initiation of shear stress. F-actin or post-autoproteolytic m-calpain was stained as described in Materials and Methods. The results presented were from one representative of five different experiments.

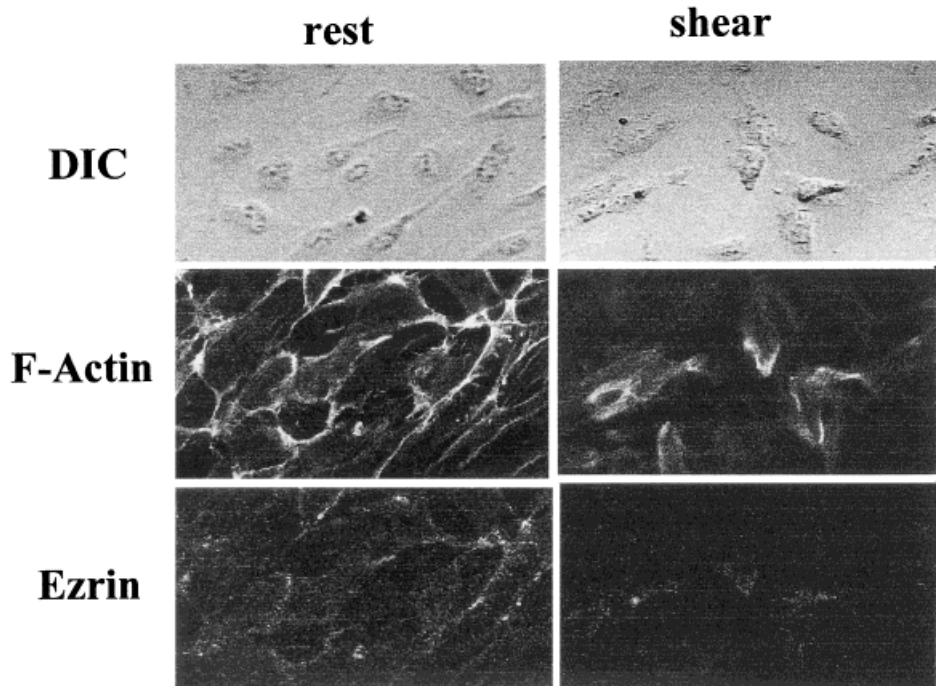

Fig. 5. Distribution of F-actin and ezrin in HUVECs. HUVECs were fixed by 2% PFA before and 2 h after the initiation of shear-stress. F-actin or ezrin was stained as described in Materials and Methods. The results presented were from one representative of five different experiments.

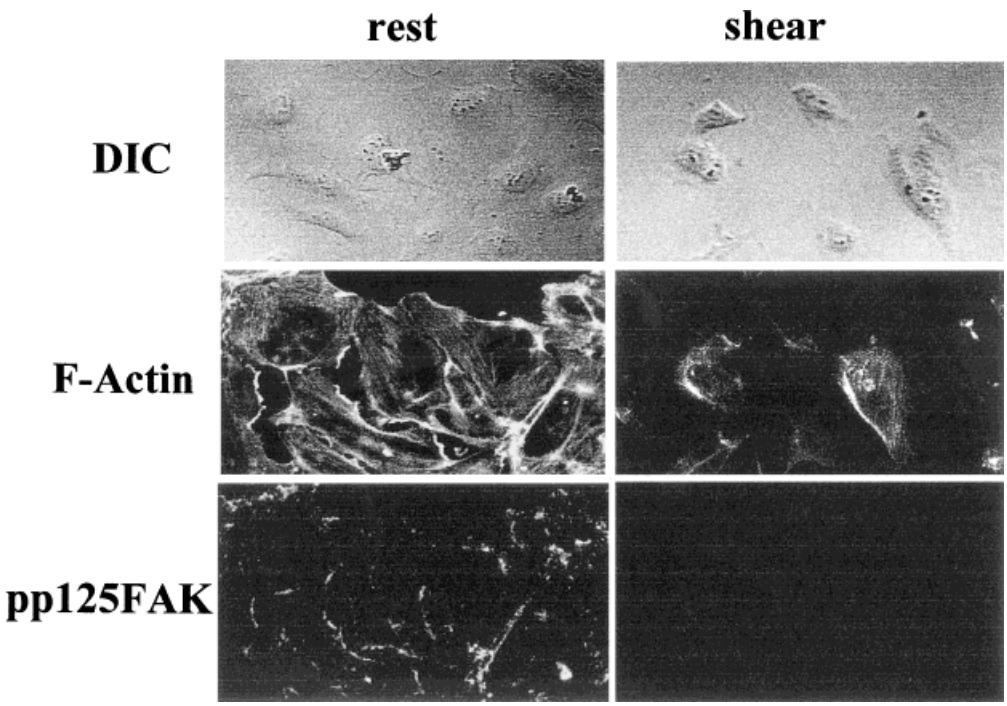

Fig. 6. Distribution of F-actin and pp125FAK in HUVECs. HUVECs were fixed by 2% PFA before and 2 h after the initiation of shear stress. F-actin or pp125FAK was stained as described in Materials and Methods. The results presented were from one representative of five different experiments.

1980; Jacob et al., 1988; Berridge and Irvine, 1989; Ariyoshi and Salzman, 1996]. In ECs, it has been demonstrated that flow shear stress causes change in cell shape from polygonal to fusiform, which is associated with the clear alignment of the cell major axis in the direction of flow [Remuzzi et al., 1984]. By employing fluo-3 in conjunction with CLSM, we developed a unique system which allowed us to carry out the long-term, continuous observation of $[Ca^{2+}]_i$ gradients and morphological changes in HUVECs stimulated by shear stress. Using this system, we successfully demonstrated a localized $[Ca^{2+}]_i$ gradient located inside lamellipodium, protrusion of the cytoplasm, in the direction of cell migration in the previous report [Yoshikawa et al., 1999], suggesting close association of $[Ca^{2+}]_i$ gradients and localized change in cell morphology, such as lamellipodium formation; however it is still obscure how an elevated $[Ca^{2+}]_i$ signal is translated to cause cytoskeletal reorganization. Therefore, we investigated the signal transduction system following $[Ca^{2+}]_i$ rise in migrating HUVECs stimulated by shear stress. It has been well known that $[Ca^{2+}]_i$ activates calmodulin (CaM)-dependent enzymes through the activation of CaM and also directly activate protein kinase C (PKC) and Ca^{2+}-activated neutral proteases, calpains [Nishizuka, 1986; Kambayashi and Sakon, 1989; Anraku et al., 1991]. The major events in cytoskeletal reorganization such as actin polymerization are also reported to be $[Ca^{2+}]_i$-dependent [Stossel, 1988]. In this study, we focused on the possible function of m-calpain in these processes, since HUVECs are rich in m-calpain as well as its endogenous inhibitor, calpastatin [Fujitani et al., 1997]. Calpains have been reported to be activated in a localized fashion through translocation inside cells in various other cell types [Ariyoshi et al., 1993]. Furthermore, calpains are also believed to cleave several cytoskeleton-related proteins and enzymes, such as actin-binding proteins [Phillips and Jakabova, 1977], ezrin [Shcherbina et al., 1999], and pp125FAK [Ariyoshi et al., 1998], which play pivotal roles in cytoskeletal reorganization and cell morphology. Through this knowledge on the role of $[Ca^{2+}]_i$ transients and calpains, one can easily hypothesize that calpain, calcium-activated neutral protease, may posseses an important role as an effector of $[Ca^{2+}]_i$ rise in localized cytoskeletal reorganization and this hypothesis has been strengthened by the findings that a calpain inhibitor affected change in cell morphology in other cell types [Basse et al., 1994]. In order to confirm this hypothesis, we employed an antibody specific for pre- or post-autoproteolytic m-calpain, which enabled in situ capture of the activation of m-calpain. Our observations clearly demonstrated the activation of m-calpain through the translocation of proenzymes from the cytosol to the peripheral region of the cells. In other cell types, several reports suggested the activation of calpains on the membrane after the translocation with lower Ca^{2+} concentration attainable in the physiological conditions [Ariyoshi et al., 1993]. Although the influence of membrane phospholipids [Arthur and Crawford, 1996], calpain activators [Shiba et al., 1992], or substrate [Ariyoshi et al., 1992] has been suggested, the exact mechanism is still unclear. As we reported here, m-calpain was activated in the peripheral region of the cells where localized $[Ca^{2+}]_i$ rise and lamellipodium formation were observed in migrating HUVECs, which was associated with the decrease of pp125FAK and ezrin, suggesting the possible role of locally activated calpain in cell motility. However, we failed to detect the localized decrease in pp125FAK or ezrin. This might be the first report describing the physiological role of localized $[Ca^{2+}]_i$ rise. These findings may be the clue to understanding the nature of the localized $[Ca^{2+}]_i$ rise regulating cytoskeletal reorganization integral to change in cell morphology and cell migration.

REFERENCES

Anraku Y, Ohya Y, Iida H. 1991. Cell cycle control by calcium and calmodulin in *Saccharomyces cerevisiae*. Biochim Biophys Acta 1093:169–177.

Ariyoshi H, Shiba E, Sakon M, Kambayashi J, Kawasaki T, Kang J, Kawashima S, Mori T. 1992. Membrane binding and autolytic activation of calpain-I in human platelets. Biochem Int 27:335–341.

Ariyoshi H, Shiba E, Sakon M, Kambayashi J, Yoshida K, Kawashima S, Mori T. 1993. Translocation of human platelet calpain-I. Biochem Mol Biol Int 30:63–72.

Ariyoshi H, Shiba E, Kambayashi J, Sakon M, Kawasaki T, Yoshida K, Mori T. 1993. Stimulation of human platelet Ca^{2+}-ATPase and Ca^{2+} restoration by calpain. Cell Calcium 14:455–463.

Ariyoshi H, Salzman EW. 1996. Association of localized Ca^{2+} gradients with redistribution of glycoprotein GP-IIIa and F-actin in activated human blood platelets. Arterioscler Thromb Vasc Biol 16:230–235.

Ariyoshi H, Okahara K, Sakon M, Kambayashi J, Kawashima S, Kawasaki T, Monden M. 1998. Possible involvement of m-calpain in vascular smooth muscle cell proliferation. Artherioscler Thromb Vasc Biol 18:493–498.

Arthur JS, Crawford C. 1996. Investigation of the interaction of m-calpain with phospholipids. Biochim Biophys Acta 1293:201–206.

Basse F, Gaffet P, Bienvenue A. 1994. Correlation between inhibition of cytoskeleton proteolysis and anti-vesiculation effect of calpeptin during A23187-induced activation of human platelets: are vesicles shed by filopod fragmentation? Biochim Biophys Acta 1190:217–224.

Berridge MJ, Irvine RF. 1989. Inositol phosphates and cell signaling. Nature 341:197–205.

Cooke JP, Stamler JS, Andon N, Davies PR, Loscalzo J. 1990. Flow stimulates endothelial cells to release a nitrovasodilator that is potentiated by reduced thiol. Am J Pysiol 28:H804–H812.

Cooray P, Yuan Y, Schoenwaelder SM, Mitchell CA, Salem HH, Jackson SP. 1996. Focal adhesion kinase ($pp125^{FAK}$) cleavage and regulation by calpain. Biochem J 318:41–47.

Diamond SL, Eskin SG, McIntire LV. 1989. Fluid flow stimulates tissue plasminogen activator secretion by cultured human endothelial cells. Science 243:177–185.

Dull RO, Davies PF. 1991. Flow modulation of agonist (ATP)-response (Ca^{2+}) coupling in vascular endothelial cells. Am J Physiol 261:H149–H154.

Duri RN, Zhou F, Bradford H, Hu CJ, Colman RF. 1986. Thrombin induced platelet aggregation involves on indirect proteolytic cleavage of aggregin by calpain. Arch Biochem Biophys 271:346–358.

Franke R, Grafe M, Schnittler H, Seiifge D, Mittermayer C, Drenckhahn D. 1984. Induction of human vascular endothelial stress fibers by fluid shear stress. Nature 307:648–649.

Fujitani K, Kambayashi J, Sakon M, Ohmi SI, Kawashima S, Yukawa M, Yano Y, Miyoshi H, Ikeda M, Shinoki N, Monden M. 1997. Identification of μ-, m-calpains and calpastatin and capture of μ-calpain activation in endothelial cells. J Cell Biochem 66:197–209.

Geiger RV, Berk BC, Alexander RW, Nerem RM. 1992. Flow-induced calcium transients in single endothelial cells; spatial and temporal analysis. Am J Physiol 262:C1411–C1417.

Grabowski EF, Jaffe EA, Weksler BB. 1985. Prostacyclin production by cultured endothelial cell monolayer exposed to step increases in shear stress. J Lab Clin Med 105:36–43.

Ikeda M, Ariyoshi H, Kambayashi J, Fujitani K, Shinoki N, Sakon M, Kawasaki T, Monden M. 1996. Separate analysis of nuclear and cytosolic Ca^{2+} concentrations in human umbilical vein endothelial cells. J Cell Biochem 63:23–36.

Izumo S, Malek AM. 1994. Molecular aspects of signal transduction of shear stress in the endothelial cell. J Hypertension 12:989–999.

Jacob R, Merritt JE, Hallam TJ, Rink TJ. 1988. Repetitive spikes in cytoplasmic calcium evoked by histamine in human endothelial cells. Nature 335:40–45.

Kambayashi J, Kajiwara Y, Sakon M, Ohshiro T, Mori T. 1986. Possible participation of calpain in myosin light chain phosphorylation of human platelets. Biochem Int 13:571–578.

Kambayashi J, Sakon M. 1989. Calcium-dependent proteases and their inhibitors in human platelets. Methods in Enzymol 169:442–455.

Kikuchi H, Imajoh-Ohmi S. 1995. Antibodies specific for proteolyzed forms of protein kinase C. Biochem Biophys Acta 1269:253–259.

Kishimoto A, Mikawa K, Hashimoto K, Yasuda I, Tanaka S, Tominaga M, Kuroda T, Nishizuka Y. 1989. Limited proteolysis of protein kinase C by calcium-activated neutral protease (calpain). J Biol Chem 264:4088–4092.

Levesque MJ, Nerem RM. 1985. The elongation and orientation of cultured endothelial cells in response to shear stress. J Biomech Eng 107:341–347.

Levesque MJ, Sprague JEA, Schwartz CJ, Nerem R. 1989. The influence of shear stress on cultured vascular endothelial cells: the stress response of an anchorage-dependent mammalian cell. Biotechnol Prog 5:1–8.

Mo M, Eskin SG, Schilling WP. 1991. Flow-induced changes in Ca^{2+} signaling of vascular endothelial cells: effect of shear stress and ATP. Am J Physiol. 260:H1698–H17607.

Murachi T, Tanaka K, Hatanaka M, Murakami T. 1981. Intracellular Ca^{2+}-dependent protease (calpain) and its high-molecular-weight endogenous inhibitor (calpastatin). Adv Enzyme Regul 19:407–424.

Murachi T. 1983. Calpain and calpastatin. Trends Biochem Sci 8:167–169.

Nishida K, Harrison G, Navas JP, Fisher AA, Docjery SP, Uematsu M. 1992. Molecular cloning and characterization of constitutive bovine aortic endothelial cell nitric oxide synthase. J Clin Invest 90:2092–2096.

Nishizuka Y. 1986. Studies and perspectives of protein kinase C. Science 233:305–312.

Nollert MU, Eskin SG, McIntire LV. 1990. Shear stress increases inositol trisphosphate levels in human endothelial cells. Biochem Biophys Res Commun 170:281–287.

Oda A, Drucker BJ, Ariyoshi H, Smith M, Salzman EW. 1993. pp60src is an endogenous substrate for calpain in human blood platelets. J Biol Chem 268:12603–12608.

Phillips R, Jakabova M. 1977. Ca^{2+}-dependent protease in human platelets-specific cleavage of platelet polypeptides in the presence of added Ca^{2+}. J Biol Chem 252:5602–5605.

Remuzzi A, Dewey CF Jr, Davies PF, Gimbrone MA Jr. 1984. Orientation of endothelial cells in shear fluids in vitro. Biorheology 21:617–630.

Sakon M, Kambayashi J, Ohno H, Kosaki G. 1981. Two-forms of Ca^{2+}-activated neutral protease in platelets. Thromb Res 24:207–214.

Shcherbina A, Bretscher A, Kenny DM, Remold-O'Donnell E. 1999. Moesin, the major ERM protein of lymphocytes and platelets, differs from ezrin in its sensitivity to calpain. FEBS Lett 443:31–36.

Shen J, Luscinskas FW, Connoly A, Dewey CF, Gimbrone MA. 1990. Fluid shear stress modulates intracellular ionized calcium in vascular endothelial monolayers. Biomech 2:196–196.

Shiba E, Tsujinaka T, Kambayashi J, Kosaki G. 1983. Purification and characterization of Ca^{2+}-activated neutral protease inhibitor from human platelet. Thromb Res 32:207–214.

Shiba E, Ariyoshi H, Yano Y, Kawasaki T, Sakon M, Kambayashi J, Mori T. 1992. Purification and characterization of a calpain activator from human platelets. Biochem Biophys Res Commun 182:461–465.

Sorimachi H, Saido T, Suzuki K. 1994. New era of calpain research. Discovery of tissue-specific calpains. FEBES Lett 343:1–5.

Sprague EA, Steinbach BL, Nerem RM, Schwartz CJ. 1987. Influence of a laminar steady-state fluid-imposed wall shear stress on the binding, internalization, and degradation of low-density lipoproteins by cultured arterial endothelium. Circulation 76:648–656.

Stossel TP. 1988. From signal to pseudopod: how cells control cytoplasmic actin assembly. J Biol Chem 264:18261–18264.

Tayler DL, Blinks JR, Reynolds GT. 1980. Contractile basis of amoeboid movement: aequorin luminescence during amoeboid movement, endocytosis, and capping. J Cell Biol 86:599–607.

Tsujinaka T, Sakon M, Kambayashi J, Kosaki G. 1982. Cleavage of cytoskeletal proteins by two forms of Ca^{2+}-activated neutral proteases in human platelets. Thromb Res 28:149–156.

Wechezak AR, Wight TN, Viggers RF, Sauvage LR. 1989. Endothelial adherence under shear stress is dependent upon microfilament reorganization. J Cell Physiol 139: 136–146.

Yoshikawa N, Ariyoshi H, Ikeda M, Sakon M, Kawasaki T, Monden M. 1997. Shear stress causes polarized change in cytoplasmic calcium concentration in human umbilical vein endothelial cells (HUVECs). Cell Calcium 22:189–194.

Yoshikawa N, Ariyoshi H, Aono Y, Sakon M, Kawasaki T, Monden M. 1999. Gradients in cytoplasmic calcium concentration ($[Ca^{2+}]_i$) in migrating human umbilical vein endothelial cells (HUVECs) stimulated by shear stress. Life Sciences 65:2643–2651.

Yoshizumi M, Kurihara H, Sugiyama T, Takaku F, Yanagisawa M, Masaki T. 1989. Hemodynamic shear stress stimulates endothelin production by cultured endothelial cells. Biochem Biophys Res Commun 161: 859–864.