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Fluid Shear Stress Induces Actin Polymerization in Human Neutrophils

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Abstract We have previously reported that a physiological range of shear stress induces neutrophil homotypic aggregation mediated by lymphocyte function-associated antigen-1 (LFA-1) and intercellular adhesion molecule-3 (ICAM-3) interactions. To further characterize the homotypic aggregation, actin polymerization was investigated in neutrophils stimulated by shear stress in comparison with formyl-methionyl-leucyl-phenylalanine (fMLP). In fMLP-stimulated neutrophils, actin polymerization was localized in the pseudopods, and this reaction was not mediated by a cytosolic level of Ca^{2+}. In contrast to fMLP stimulation, the actin polymerization induced by shear stress in a cone-plate viscometer was localized in cell-cell contact regions, and this polymerization required the increase of intracellular Ca^{2+}. This shear stress–induced actin polymerization was not observed when neutrophils were pretreated with anti-LFA-1 or anti-ICAM-3 antibody. In conclusion, LFA-1 and ICAM-3 interaction mediated by the increase of $[Ca^{2+}]i$ generated the intercellular signal in order to accumulate F-actin in the cell-cell contact regions.

Key words: shear stress, actin polymerization, LFA-1, ICAM-3, homotypic aggregation

Regulated reorganization of the microfilamentous cytoskeleton is essential for normal neutrophil functions, including adhesion, locomotion, chemotaxis, phagocytosis, and secretion [Howard and Meyer, 1984; Wallace et al., 1984; Howard et al., 1994]. The basic structure element of the microfilamentous cytoskeleton in neutrophils is filamentous actin (F-actin), a dynamic polymer composed of 43 kD globular, monometric actin subunits (G-actin). In neutrophils, G-actin is reversibly converted to F-actin through polymerization and depolymerization processes [Carlsson et al., 1979; Fechheimer and Zigmond, 1983; Howard and Oresajo, 1985; Rao, 1985; Yano et al., 1994]. In response to a variety of humoral stimuli, such as a chemotactic peptide formyl-methionyl-leucyl-phenylalanine (fMLP), an accumulation of F-actin in newly formed pseudopods of neutrophils has been observed [Sha'afi et al., 1983; Rao and Varani, 1985; Zimmerman et al., 1988; Keller et al., 1990; Niggli and Keller, 1991; Downey et al., 1992]. The signals generated after fMLP stimulation include a pertussis-toxin-sensitive G-protein step and activation of the phosphotidyl-inositol pathway with subsequent protein kinase C (PKC) activation and Ca^{2+} mobilization [Berridge, 1987]. The role of these two second messenger systems in fMLP-induced actin polymerization responses has been studied extensively but is still controversial. The PKC inhibitor H-7 has no effect on fMLP-induced actin polymerization, while actin polymerization is observed by stimulation with PMA, a direct activator of PKC [Keller et al., 1990]. As fMLP-induced actin polymerization still occurs when intracellular Ca^{2+} is held stable, it is generally accepted that increases in intracellular Ca^{2+} are not necessary to initiate actin polymerization [Sha'afi et al., 1986; Downey et al., 1990]. In contrast to humoral stimuli such as fMLP, the physical stimulation is one of important roles for neutrophil activation. The actin polymerization of neutrophils is observed by the adhesion to a plastic surface that requires the presence of extracellular Ca^{2+} but is not mediated through a pertussis-toxin-sensitive G-protein and thereby differs from polymerization elicited by fMLP [Southwick et al., 1989].

Abbreviations used: BAPTA, 1,2-Bis (o-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid; GP, glycoprotein; ICAM-3, intracellular adhesion molecule-3; LFA-1, lymphocyte function-associated antigen-1; SLe^a, sialyl-Lewis A; SLe^x, sialyl-Lewis X.

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Neutrophil adhesion to endothelial cells is an important initial step for inflammatory response. This adhesion is mediated by LFA-1/ICAM-1 interaction, and LFA-1 that belongs to the β_2 integrin family is the numerically and functionally dominant adhesion receptor of neutrophils [Dustin and Springer, 1988; Diamond et al., 1990; Lawrence et al., 1990; Lawrence and Springer, 1991]. The stimulation of this adhesion receptor by antibody against β_2 integrin induces actin polymerization, and the capacity of β_2 integrins to induce phosphatidylinositol trisphosphate formation correlates with their ability to induce actin polymerization [Löfgren et al., 1993].

In contrast to the heterotypic cell adhesion such as neutrophils and endothelial cell interaction, homotypic cell contact plays an important role for immune response [Rothlein and Springer, 1986; Keizer et al., 1988; Springer, 1990]. The relationship between actin polymerization and leukocyte homotypic aggregation has been intensively examined. In B lymphoblastoid cells, the reorganization of the F-actin was observed at the sites of intercellular boundaries in β1 integrin-induced homotypic cell aggregation [Sanchez-Mateos et al., 1993; Ohta et al., 1995].

However, little is known about the relationship between F-actin distribution and neutrophil homotypic aggregation. We have recently reported that neutrophils undergo homotypic aggregation under fluid shear stress [Okuyama et al., 1996b]. This neutrophil homotypic aggregation is mediated by the LFA-1/ICAM-3 pathway and required the increase of intracellular Ca^{2+} concentration through Ca^{2+} influx. We have no clear answer to what happens through neutrophil homotypic aggregation-induced shear stress in vivo. Thereby, we examined the effect of fluid shear stress on actin polymerization of human neutrophils.

MATERIALS AND METHODS

Preparation of Neutrophils

Neutrophils were prepared from 20 ml of citrated venous blood from normal human subjects. Blood was mixed with an equal amount of 3% dextran saline in a plastic syringe and left to sit vertically for 60 min at room temperature. The resultant upper phase was taken and placed gently in the same volume of Ficoll-Paque (sp. gr. 1.077). By centrifugation at 450*g* for 20 min at 4°C, neutrophils were sedimented at the bottom of the tube. After removing both the upper phases and interface, the contaminating erythrocytes were lysed by the addition of 5 ml of ice-cold distilled water for 30 s, followed by 5 ml of ice-cold, 1.8% NaCl solution. After centrifugation at 100*g* for 10 min, the cells were resuspended to make 1×10^7/ml with a modified Tyrode solution buffer (135 mM NaCl, 2.7 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.3 mM NaH_2PO_4, 12 mM $NaHCO_3$, 5.6 mM glucose, pH 7.4). The neutrophils were used within 3 h after preparation [Okuyama et al., 1995].

Activation of Neutrophils

FMLP and 1,2-bis (o-aminophenoxy) ethane-N,N-N′,N′-tetraacetic acid (BAPTA)-AM were stored in dimethyl sulfoxide (DMSO) at −20°C (Sigma Chemical Co., St. Louis, MO). For use, they were thawed and added to 1 ml of cell suspension to achieve the desired final concentration. Neutrophils were pretreated with 100 μM BAPTA-AM for 30 min in modified Tyrode buffer without Ca^{2+}. Then, the cells were washed once with phosphate-buffered saline (135 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, pH 7.4) and resuspended in the modified Tyrode buffer containing 1 mM $CaCl_2$. In some experiments, neutrophils were incubated with 10 ug/ml of anti-LFA-1 (CD11a) antibody 25.3.1 (Immunotech, Westbrook, Maine), anti-ICAM-3 antibody HP2/19 (Immunotech) or nonimmune mouse IgG (Sigma) for 30 min at 37°C in modified Tyrode buffer. A neutrophil suspension (400 μl) was applied to the center of the plate in the cone-shaped shear loading apparatus (Toray Industries Inc., Kanagawa, Japan) and then exposed to a constant shear force of 12 dynes/cm^2 for various times by rotating the cone located 40 μm above the plate. Shear stress (π) was calculated by the formula $\pi = \mu rw/d$, where π is shear stress (dynes/cm^2), μ is viscosity of medium (poise), r is distance (cm) from the center of the dish, w is angle velocity of rotation of the disc (rad/s), and d is the distance (cm) between the plate and disc. At the end of loading shear stress, the cells were subjected to morphological study by inverted phase microscopy [Ikeda et al., 1993; Okuyama et al., 1996b].

Measurement of Intracellular Ca^{2+} Concentration

Neutrophils were incubated with 10 μM 1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxy-9-xan-

thenyl)phenoxy]-2-(2′-amino-5′-methyl-phenoxyl) ethane-N,N,N′,N′-tetra-acetic acid, acetoxymethyl derivative (fluo-3 AM) at 37°C for 60 min in modified Tyrode buffer without Ca^{2+}. The cells were washed once with phosphate-buffered saline and resuspended in modified Tyrode buffer in the presence of 1 mM $CaCl_2$. Fluo-3 AM–loaded neutrophils were preincubated at 37°C with various agents described above, and neutrophils were exposed to 12 dynes/cm^2 of shear force for an appropriate time or stimulated by 10^{-7} M fMLP. The cell suspension was placed into the cuvette immediately, and fluorescence intensity was monitored at the setting of 480 nm (excitation) and 530 nm (emission) by a fluorescence spectrometer (F-4000; HITATI, Tokyo, Japan). The intracellular Ca^{2+} concentration was determined according to the method of Vadenberghe [Vandenbergh and Ceuppens, 1991; Okuyama et al., 1995].

Confocal Fluorescence Microscopy

Resting and activated neutrophils were fixed with 2% paraformaldehyde (diluted in 0.1 M phosphate buffer, pH 7.5) for 15 min. After fixation, neutrophils were washed twice with phosphate-buffered saline. Neutrophils were prepared by permeabilizing neutrophils with Triton X-100 (0.1%) in phosphate-buffered saline for 10 min. After washing, to study the distribution of F-actin in neutrophils, neutrophils were stained with rhodamine phalloidin (0.033 μM) (Molecular Probes, Eugene, OR) for 15 min. Neutrophils rewashed twice with phosphate-buffered saline were examined with a Multi-Probe 2001 confocal laser scanning microscope (Molecular Dynamics Inc., Sunnyvale, CA). Differential interference contrast (DIC) was performed to examine the morphological changes.

F-Actin Content

The F-actin content was measured by rodamine phalloidin–pretreated neutrophil as described above. The cell suspension was placed into the cuvette, and intensity was continuously monitored at the setting of 540 nm (excitation) and 580 nm (emission) by a fluorescence spectrometer (F-4000; HITATI) [Yano et al., 1995].

Statistical Analysis

The statistical significance of the results in F actin content was evaluated by the paired *t*-test.

RESULTS

Effect of Shear Stress on Neutrophil Homotypic Aggregation

Neutrophils were challenged under a constant shear force of 12 dynes/cm^2 for 5 min. We decided on the loading of 12 dynes/cm^2 on neutrophils because in the normal circulation the time-average range of shear stress in arterial vessels is in the order of 12 dynes and shear stress–induced neutrophil homotypic aggregation was maximum under this shear stress. Shear stress–loaded neutrophils underwent a solid homotypic aggregation with large clusters observed by inverted phase microscopy (Fig. 1).

Effect of Shear Stress on F-Actin Distribution of Neutrophils

To study the distribution of F-actin during homotypic aggregation of neutrophils, we performed rodamine phalloidin staining to visualize F-actin. As shown in Figure 2, in neutrophils exposed to a shear stress of 12 dynes/cm^2 a high fluorescence intensity was observed exclusively in the cell-to-cell contact regions. In contrast to shear stress, we found the development of F-actin–rich pseudopods after fMLP treatment.

Intracellular Ca^{2+} Concentration ($[Ca^{2+}]i$) and F-Actin Content

To evaluate the role of $[Ca^{2+}]i$ in shear stress–induced actin polymerization, we examined the $[Ca^{2+}]i$ stimulated by shear stress or fMLP and the effect of BAPTA on F-actin accumulation. As shown in Figure 3 $[Ca^{2+}]i$ increased gradually to 750 nM under loading of a shear stress of 12 dynes/cm^2. In fMLP (10^{-7} M)-stimulated neutrophils, $[Ca^{2+}]i$ increased rapidly to about 1 μM within 1 min, followed by a slow decrease. The intracellular Ca^{2+} chelator BAPTA completely blocked the increase of $[Ca^{2+}]_i$ caused by shear stress or fMLP stimulation. Under these conditions, we examined F-actin content. The cells pretreated with BAPTA did not increase their F-actin content to the extent of that seen in the control cells (data not shown). The content of F-actin increased 1.82-fold within 1 min after fMLP stimulation (Fig. 4). Although the increase pattern of F-actin resembled that of $[Ca^{2+}]i$, fMLP still caused 1.65-fold increases in F-actin content in the absence on any rise of $[Ca^{2+}]i$ in BAPTA-pretreated neutrophils. In contrast to fMLP stimulation, shear stress–induced actin polymerization increased gradually 1.68-

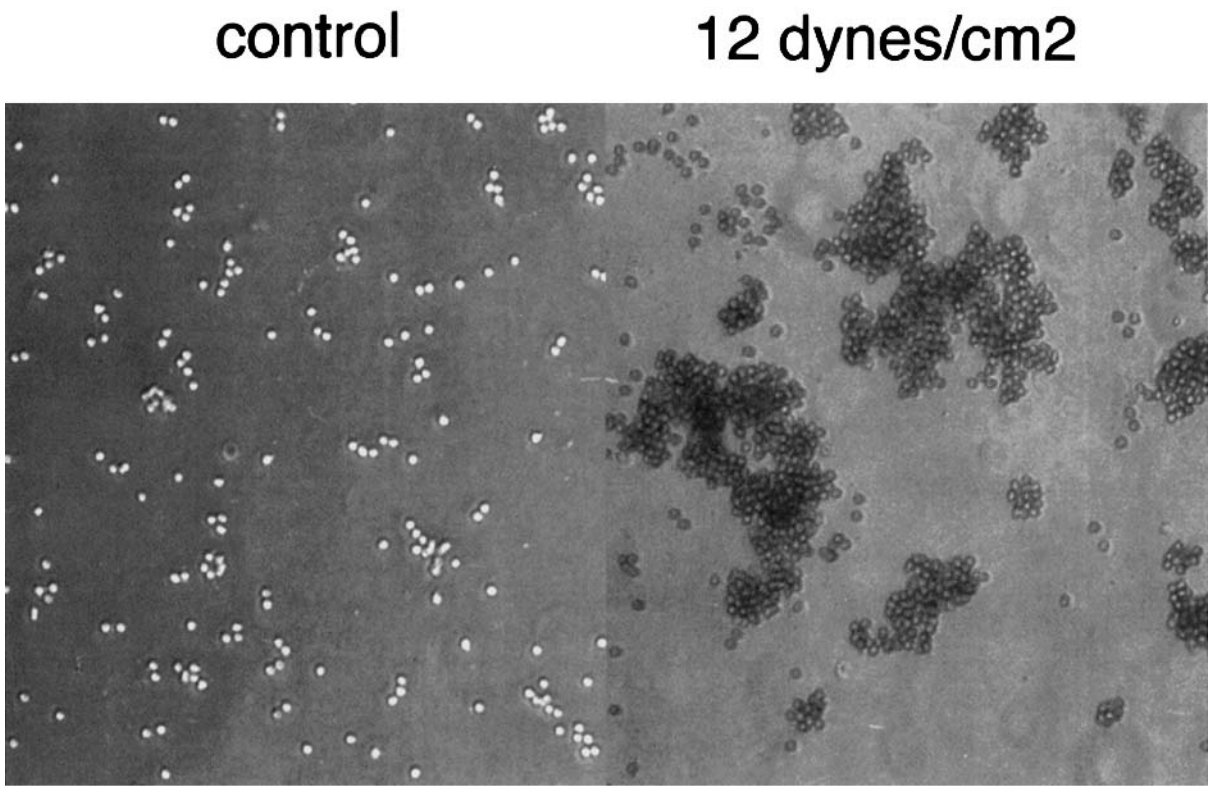

Fig. 1. Neutrophil homotypic aggregation induced by shear stress. Neutrophils (400 μl) in modified Tyrode buffer (1×10^7/ml) were exposed to a constant shear force of 12 dynes/cm^2 for 5 min at room temperature. After exposure, an aliquot was subjected to phase contrast microscopic observation. The figure shows a representative of three different experiments that had essentially the same results.

fold for 5 min during the loading of shear stress (Fig. 5). This shear stress–induced actin polymerization was completely inhibited by BAPTA pretreatment with a significant difference.

Effect of Anti-LFA-1 and Anti-ICAM-3 Antibodies on Shear Stress-Induced Actin Polymerization

To study the relationship between actin polymerization and cell aggregation, we examined the effect of anti-LFA-1 or ICAM-3 antibodies. These antibodies concomitantly inhibited the aggregation induced by shear stress. F-actin accumulation was not observed during antibody incubation alone (data not shown). The cells pretreated with these antibodies underwent shear stress, and a slight increase of F-actin was observed (Fig. 6). There were significant differences between the amount of F-actin content pretreatment with these antibodies and control. This inhibitory effect on F-actin accumulation was compatible with the degree of inhibitory effect on the aggregation.

DISCUSSION

We have previously reported that human neutrophils undergo homotypic aggregation under fluid shear stress in the physiological range. This aggregation was mediated by the LFA-1/ICAM-3 pathway that was activated by the increase of $[Ca^{2+}]i$ through a Ni^{2+}-sensitive Ca^{2+} channel. The physiological implication and the intracellular events after aggregation are not clear, so we examined actin polymerization as the intracellular event after aggregation. The present study demonstrates for the first time that shear stress induced actin polymerization. Neutrophils were observed in homotypic aggregation induced by shear stress (Fig. 1), and under this condition we found that regions of cell-cell contact displayed actin polymerization (Fig. 2). These reactions are not a specific, mechanical induced break in cell membrane because shear stress–induced neutrophil aggregation and actin polymerization are reversible (data not shown). The mechanisms of shear stress–

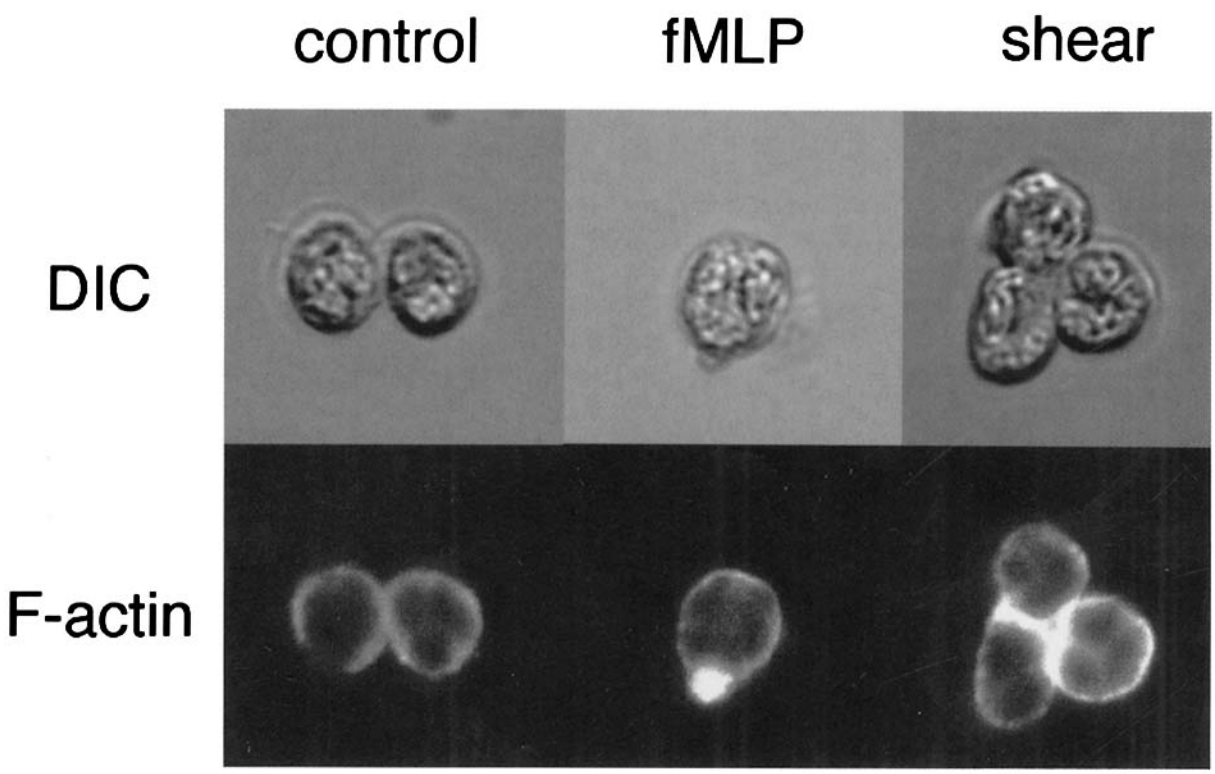

Fig. 2. Effect of shear stress or fMLP on F-actin distribution of neutrophils. Neutrophils were fixed before (control) and after the exposure of shear stress (12 dynes/cm^2 for 5 min) or 10^{-7} M fMLP stimulation (1 min after addition). The cells were stained with rhodamine phalloidin and prepared for confocal laser scanning microscopy as described. The figure shows a representative of three different experiments that had essentially the same results.

induced neutrophil actin polymerization were speculated. There is a possibility that actin polymerization is triggered by shear stress directly as an aggregation-independent reaction or an intracellular signal originated from cell-cell contact regions.

Mechanical stimuli such as shear stress or stretch force can influence functions of various cells including leukocytes, platelets, and endothelial cells. Recently, the relationship between mechanoreceptor and intracellular events has been studied in various cells [Ando et al., 1988; Shirinsky et al., 1989; Biagi and Enyeart, 1990]. It is likely that there are specialized mechanoreceptors in these cells that can convert mechanical stimuli into intracellular second messenger. It is generally accepted that the role of intracellular Ca^{2+} is important for intracellular second messenger [Naruse and Sokabe, 1993]. In platelets, Ikeda et al. [1993] reported that transmembrane calcium influx associated with von Willebrand factor binding to GPIb in the initiation of shear induced platelet aggregation. Naruse and Sokabe [1993] reported that stretching cellular membranes increased intracellular Ca^{2+} concentration in human umbilical endothelial cells. This increase rose from Ca^{2+} entry through stretch-activated channels [Naruse and Sokabe, 1993]. In contrast to stretch force, the increase of intracellular Ca^{2+} induced by shear stress was observed in endothelial cells. This increase was caused by Ca^{2+} mobilization from intracellular Ca^{2+} storage sites and did not seem to require extracellular Ca^{2+} [Ando et al., 1988]. So we first examined the role of $[Ca^{2+}]i$ on shear stress–induced actin polymerization in comparison with the response of fMLP. We previously reported that the increase of $[Ca^{2+}]i$ was observed by loading of shear stress. BAPTA, which is an intracellular Ca^{2+} chelator, completely inhibited the increase of $[Ca^{2+}]i$ induced by shear stress and fMLP stimulation (Fig. 3). BAPTA-pretreated neutrophils did not exhibit the actin polymerization induced by shear stress (Fig. 5). These data suggested that actin polymerization induced by shear stress required the increase of $[Ca^{2+}]i$, but what step during actin polymerization required the elevation of $[Ca^{2+}]i$ was not determined because BAPTA inhibited both the

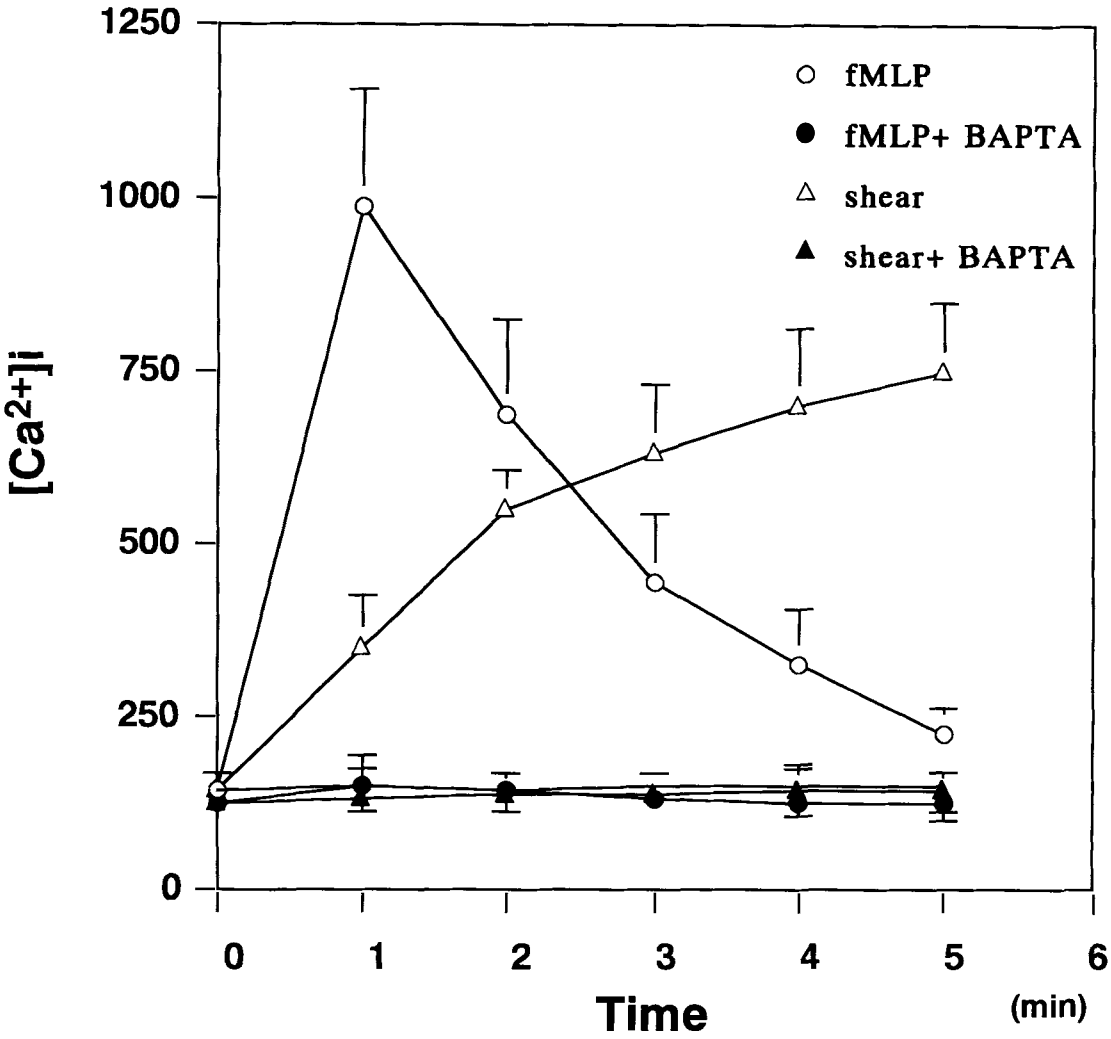

Fig. 3. Intracellular Ca^{2+} concentration induced by shear stress or fMLP and effect of BAPTA. Fluo-3–loaded neutrophils were suspended in modified Tyrode buffer in the presence of 1 mM $CaCl_2$. Also, neutrophils were incubated with 100 μM BAPTA-AM in modified Tyrode buffer without Ca^{2+} for 30 min. Then the cells were washed once with phosphate-buffered saline and resuspended in the modified Tyrode buffer in the presence of 1 mM $CaCl_2$. Incubated neutrophils were exposed to 12 dynes/cm^2 of shear force or stimulated by 10^{-7} M fMLP (no symbol showed no stimulant control). At the appropriate time, the fluorescence intensity was measured. Results shown are the mean of five experiments ± SE.

increasing of $[Ca^{2+}]i$ and neutrophil aggregation.

In fMLP stimulation, neutrophils formed pseudopods that contained the polymerized actin (Fig. 2). The formation of F-actin–rich pseudopods was observed even in the absence of an increase of $[Ca^{2+}]i$ (Fig. 4). It is generally accepted that fMLP-induced actin polymerization does not require an increase of $[Ca^{2+}]i$ [Sha'afi et al., 1986; Downey et al., 1990; Coates et al., 1992]. It seems that the mechanisms of action polymerization induced by shear stress were different from fMLP stimulation.

Figure 5 shows that shear stress–induced actin polymerization was inhibited by the suppression of an increase of $[Ca^{2+}]i$ using BAPTA. We previously reported that an increase of $[Ca^{2+}]i$ through a Ca^{2+} channel, which may be mechanoreceptor, occurred as a first event in the shear stress–induced neutrophil homotypic aggregation process [Okuyama et al., 1996b]. This increase of $[Ca^{2+}]i$ triggered the interaction of LFA-1 and ICAM-3 molecules because BAPTA completely blocked shear stress–induced aggregation. We had to decide whether actin polymerization required only an increase of $[Ca^{2+}]i$ in the LFA-1/ICAM-3–independent pathway or both Ca^{2+} increase and LFA-1/ICAM-3 interaction. To answer this question, we examined the effect of antibodies against LFA-1 and ICAM-3

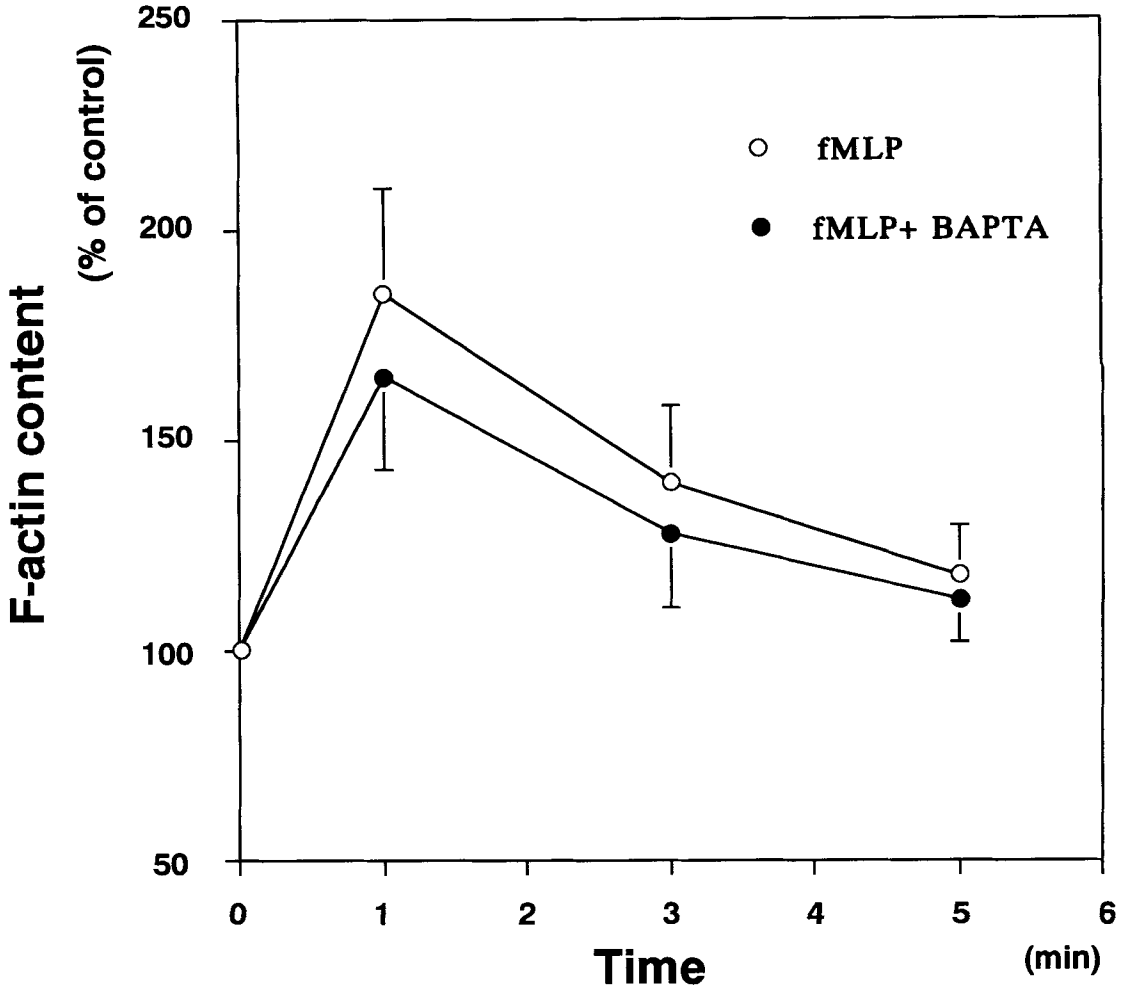

Fig. 4. Effect of Ca^{2+} chelation on fMLP-induced actin polymerization. Neutrophils with or without pretreated with BAPTA were stimulated by 10^{-7} M fMLP. Samples were taken at the indicated times and subjected to fixation and staining by rhodamine phalloidin. F-actin content was determined by a fluorescence spectrometer. Results shown are the mean of five experiments ± SE.

on shear stress–induced actin polymerization. We have reported that these antibodies inhibited shear stress–induced homotypic aggregation in the presence of an increase of $[Ca^{2+}]i$ [Okuyama et al., 1996b]. Neutrophils pretreated with these antibodies did not exhibit actin polymerization in spite of an increase of $[Ca^{2+}]i$ (Fig. 6). These results suggested that actin polymerization required LFA-1 and ICAM-3 interaction at least.

Recently the relationship between mechanisms of actin polymerization and the role of integrins has been extensively investigated. Löfgren et al. [1993] reported that β2 integrin CD18 mediated the actin polymerization in nonadhesion human neutrophils and the extracellular Ca^{2+} was not an absolute requirement for this actin polymerization. Altman et al. [1990] reported that cross-linking the T cell receptor complex with anti-CD3 mAb results in actin polymerization and colocalization with LFA-1. Antigen receptor triggering, by promoting phosphoinositide hydrolysis, also affected a transient intracellular Ca^{2+} increase. The ability of PMA alone to induce cytoskeletal rearrangement and LFA-1 and F-actin coclustering, in addition to the inability of the Ca^{2+} ionophore ionomycin to induce the same phenomenon, suggested that extracellular Ca^{2+} influx and/or Ca^{2+} redistribution from internal storage sites were not strictly required for these processes to occur [Pardi et al., 1992]. We hypothesized that actin polymerization induced Ca^{2+} influx through a Ca^{2+} channel or a so-called mechanoreceptor itself or that it was tightly coupled to a mechanoreceptor as a

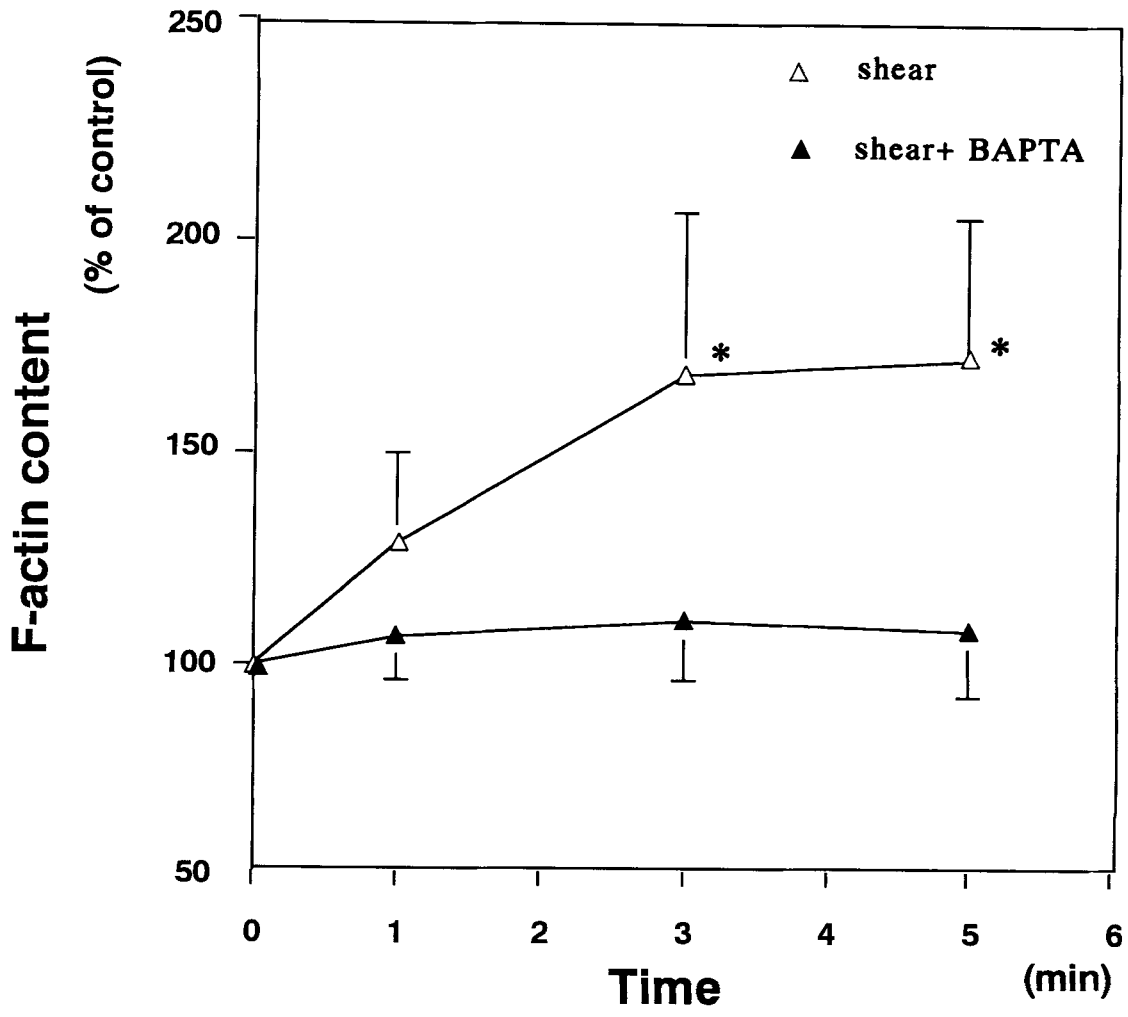

Fig. 5. Effect of Ca^{2+} chelation on shear-induced actin polymerization. Neutrophils with or without pretreated with BAPTA were stimulated by 12 dynes/cm^2 of shear force. Samples were taken at the indicated times and subjected to fixation and staining by rhodamine phalloidin. F-actin content was determined by a fluorescence spectrometer. Results shown are the mean of five experiments ± SE. *$P < 0.05$.

first event. Then LFA-1 and ICAM-3 interaction mediated by the increase of $[Ca^{2+}]i$ generated the intercellular signal in order to accumulate F-actin in the cell-cell contact regions.

Campanero et al [1993] reported that ICAM-3 interacted with LFA-1 and regulated the LFA-1/ICAM-1 cell adhesion pathway. Anti-ICAM-3 antibody HP2/19 was able to increase T lymphoblast attachment to ICAM-1, suggesting that T cell aggregation induced by this antibody could be mediated by increasing the avidity of LFA-1 for ICAM-1. In this study, we used the same antibody HP2/19. In contrast to T lymphoblasts, neutrophils did not undergo any aggregation during incubation with this antibody (Fig. 6). We considered, however, that neutrophils may not aggregate even though this antibody increased the affinity of LFA-1 for ICAM-1, because ICAM-3 was blocked by this antibody and there were no other ICAM molecules on the neutrophil surface.

In this study, the mechanisms of actin polymerization after the intracellular signal generated by LFA-1 and ICAM-3 interaction remained unknown. LFA-1 and ICAM-3 interaction induced by physiological shear stress created intracellular signal following actin polymerization. The physiological and clinical role of neutrophil homotypic aggregation and actin polymerization was still unclear. In circulation, the influence of shear stress affected not only neutrophils but also many cells, including platelets, erythrocyte, and endothelial cells. The examination of the relationship between neutrophil and

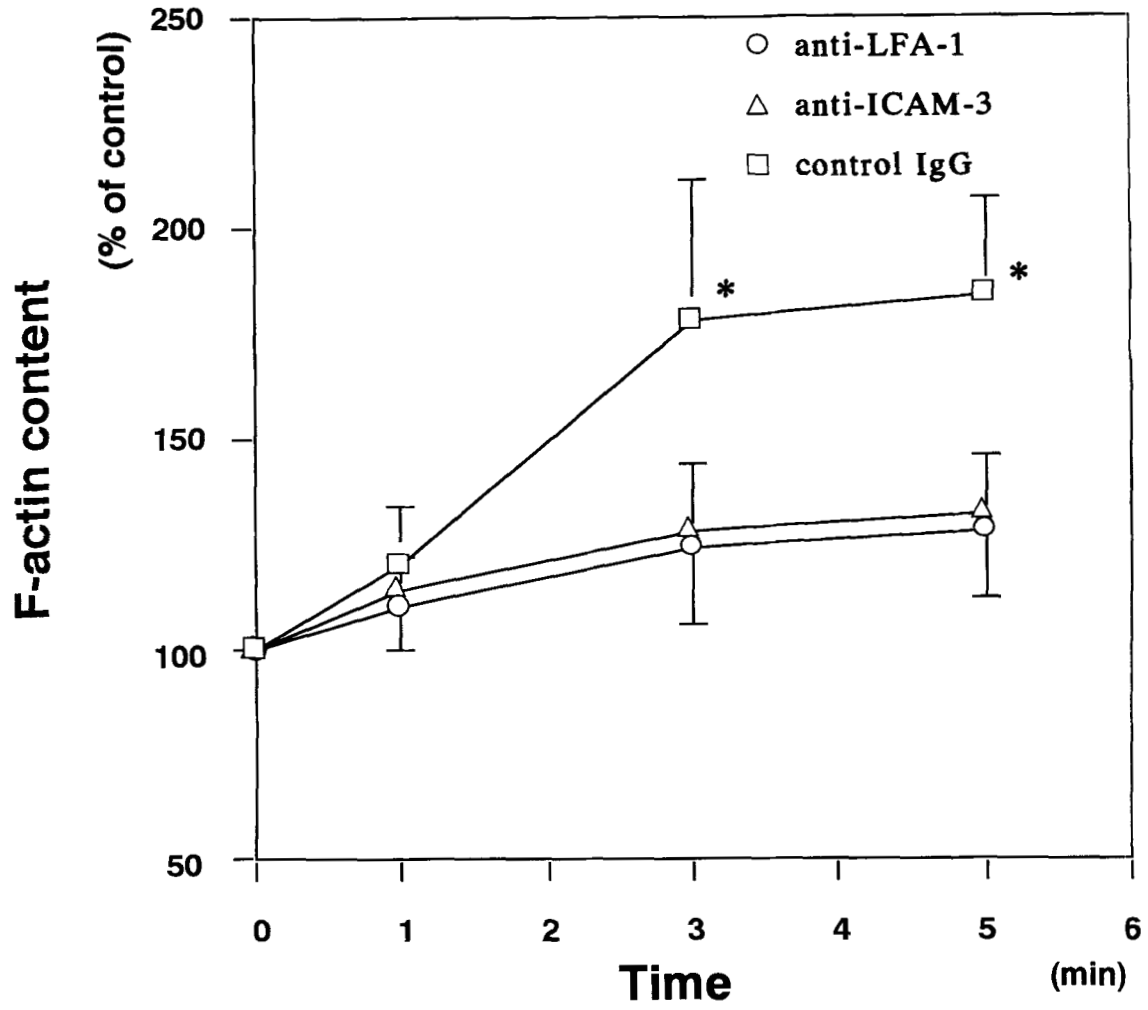

Fig. 6. Effect of antibody against LFA-1 or ICAM-3 or shear-induced actin polymerization. Neutrophils pretreated with anti-LFA-1, anti-ICAM-3 antibody, or nonimmune mouse IgG were stimulated by 12 dynes/cm^2 of shear force. Samples were taken at the indicated times and subjected to fixation and staining by rhodamine phalloidin. F-actin content was determined by a fluorescence spectrometer. Results shown are the mean of five experiments ± SE. *$P < 0.05$.

these cells under shear stress was needed. In conclusion, a physiological range of shear stress induces actin polymerization mediated by an intracellular signal generated from LFA-1 and ICAM-3 interaction.

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