Journal of Cellular Biochemistry 60:550–559 (1996)

LFA-1/ICAM-3 Mediates Neutrophil Homotypic Aggregation Under Fluid Shear Stress

Masaki Okuyama, Jun-ichi Kambayashi, Masato Sakon, and Morito Monden

Department of Surgery II, Osaka University Medical School, Suita, Osaka 565, Japan

Abstract We found that human neutrophils undergo homotypic aggregation by loading the physiological range of fluid shear stress (12–30 dynes/cm^2). Under the fluid shear stress, an increase of intracellular Ca^{2+} concentration of neutrophils was observed. This increase of intracellular Ca^{2+} concentration was caused by Ca^{2+} influx, and the blockage of the flux by $NiCl_2$ suppressed the neutrophil homotypic aggregation. Furthermore, this neutrophil aggregation under fluid shear stress was completely inhibited by pretreatment with antibody against LFA-1 or ICAM-3. These results suggested that $NiCl_2$-sensitive Ca^{2+} channel played an important role in LFA-1/ICAM-3–mediated neutrophil homotypic aggregation under fluid shear stress. © 1996 Wiley-Liss, Inc.

Key words: shear stress, homotypic aggregation, LFA-1, ICAM-3, $NiCl_2$ sensitive Ca^{2+} channel, Ca^{2+} influx

Inflammatory response requires the recruitment of circulating neutrophils to the site of inflammation. The recruitment requires the ability of neutrophils to participate in a variety of adherence-related functions. Actually neutrophils use a number of different adhesion molecules to bind the endothelial cells in many different steps including rolling, stationary adhesion, and migration into the perivascular tissue. The first step, the rolling of neutrophils, is known to be mediated by the selectin family. The subsequent stationary adhesion and migration are mainly dependent on LFA-1/ICAM-1 interaction [Marlin and Springer, 1987; Lawrence et al., 1990; Abbassi et al., 1993; Beekhuizen and Furth, 1993; Buttrum and Nash, 1993; Jones et al., 1993]. In addition, LFA-1/ICAM-1 plays an important role of homotypic adhesion in various leukocytes including B cells, T cells, and monocytes. This homotypic aggregation of these cells has been observed under humoral stimuli such as PMA, but the mechanism of aggregation has not been clarified yet [Wuthrich, 1992; Bernard et al., 1994].

Recently, fluid shear stress has been reported to induce platelet aggregation, which is mediated by interactions of GPIb, GPIIb/IIIa, von Willebrand factor, and fibrinogen [Ikeda et al., 1993]. As neutrophils are also constantly exposed to a various degree of fluid shear stress in circulation, it is possible that neutrophils undergo homotypic aggregation under fluid shear stress. Since no such data is available, we attempted to study the effect of fluid shear stress on neutrophils, employing cone-plate shear loading apparatus developed for shear-induced platelet aggregation.

MATERIALS AND METHODS

Preparation of Neutrophils and Glutaraldehyde-Fixed 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 on 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 erythro-

Abbreviations used: BAPTA, 1,2-Bis (o-aminophenoxy) ethane-N, N, N′, N′-tetraacetic acid; tmLP, tormyl-meth: omyl-leucyl-phemylalanine; 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; VWF, von Willebrand factor.

Received July 17, 1995; accepted August 29, 1995.

Address reprint requests to Masaki Okuyama, MD, Department of Surgery II, Osaka University Medical School, 2-2 Yamada-oka, Suite, Osaka 565, Japan.

cytes 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. Glutaraldehyde-fixed neutrophils were prepared as follows: the neutrophil suspension was incubated with 2.5% glutaraldehyde for 1 h at 37°C. The cells were washed twice 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 modified Tyrode buffer (1×10^7/ml).

Monitoring of Shear-Induced Aggregation of Neutrophils

A neutrophil suspension (400 μl) was applied to the center of the plate in the cone-shape shear loading apparatus (Toray Industries Inc., Kanagawa, Japan) and then exposed to various shear stress by rotating the cone located 40 μm above the plate. Shear stress (τ) is calculated by the formula $\tau = \mu rw/d$, where τ 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.

Also, the homotypic aggregation of neutrophils was determined as follows: neutrophil aggregation (%) = log (Ia/Ib)/(Ipp/Ib) × 100, where Ia and Ib indicate transmitted light intensity of the neutrophil suspension after and before the application of shear stress, respectively, and Ipp is the transmitted light intensity of the neutrophil-free suspending buffer [Ikeda et al., 1993]. At the end of loading shear stress, the cells were subjected to morphological study by inverted phase microscopy.

The plate was read immediately after shear stress for 2 min in an inverted phase microscopy, and aggregation was scored on a semiquantitative scale ranging from 0 to +5 according to Rothlein and Springer [1986]: 0, no aggregation; +1, less than 10% of cells in aggregates; +2, 10–50% of the cells in aggregates; +3, 50–100% cells in small, loose clusters; +4, up to 100% of cells aggregated in large clusters; +5, nearly 100% of cells in large, very compact aggregates.

Effect of Inhibitors and Antibodies on Shear Stress–Induced Neutrophil Aggregation

Neutrophils were incubated in the presence of 10 μg/ml either anti-LFA-1 (CD11a) antibody 25.3.1 (Immunotech, Westbrook, ME), anti-ICAM-3 antibody HP2/19 (Immunotech, or nonimmune mouse IgG (Sigma, St. Louis, MO) for 30 min at 37°C in modified Tyrode buffer. Neutrophils were preincubated at 37°C with various agents, 10 ng/ml PGI_2, 300 ng/ml PGE_1, 2 mM EGTA, and 5 mM $NiCl_2$ for 5 min. In contrast to these agents, 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 and resuspended in modified Tyrode buffer in the presence of 1 mM $CaCl_2$. Pretreated neutrophils were exposed to 12 dynes/cm^2 of shear force for 2 min.

Measurement of Intracellular Ca^{2+} Concentration

Neutrophils were incubated with 10 μM 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 2 min. The cell suspension was replaced into the cuvette, the fluorescence measurement started at least within 15 s, and intensity was continuously 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 Vandenberghe and Ceuppeno [1990].

RESULTS

Neutrophil Aggregation Under a Constant Shear Stress

Neutrophils were challenged by various degrees of shear stress ranging from 6–60 dynes/cm^2, and a marked aggregation was observed under a constant shear force from 12–30 dynes/cm^2 (Fig. 1). Under a shear force of 12 dynes/cm^2, the onset of aggregation was rapid, reaching the maximum aggregation rate (about 90%) within 2 min. To verify that the increased transmitted light intensity is the cause of the homo-

typic aggregation, an aliquot shows stress of the loaded neutrophil suspension was subjected to a morphological study by inverted microscopy. As shown in Figure 2, the neutrophil aggregation rate measured by a transmitted light intensity was comparable with the findings obtained by inverted phase microscopy.

Aggregation of Glutaraldehyde-Fixed Neutrophils and Calibration Beads

We exerted shear stress on glutaraldehyde-fixed neutrophils to investigate the surface event of the homotypic aggregation as glutaraldehyde fixation blocks intracellular signal transduction and conformational changes of membrane structure. Glutaraldehyde-fixed neutrophils under shear stress of 12 dynes/cm^2 for 6 min exhibited the identical aggregation pattern as intact neutrophils, measured by transmitted light intensity (Fig. 3a). Surprisingly, glutaraldehyde-fixed neutrophils that aggregated under shear stress were not aggregated at all in the inverted phase microscopy (Fig. 3b). We speculated that neutrophil interaction under shear stress measured by transmitted light intensity in our apparatus was physical and not a physiological phenomenon. To confirm the above speculation, calibration beads were applied to the apparatus. Calibration beads (6.5 μm, 1 × 10^7/ml) acted in the same manner as glutaraldehyde-fixed neutrophils under shear stress measured by transmitted light intensity, and no aggregation of beads was observed at the end of shear stress by inverted phase microscopy. (Fig. 4a,b).

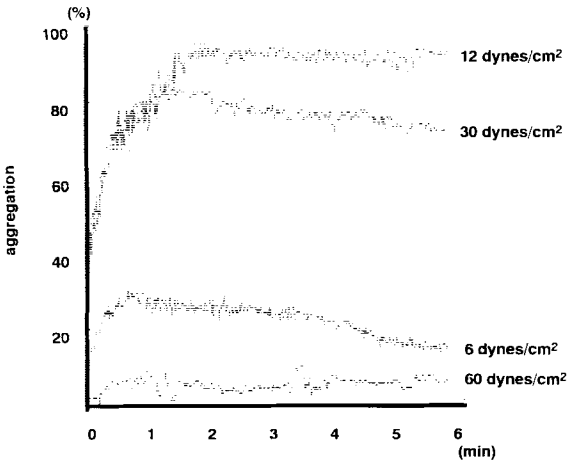


Fig. 1. Monitoring of shear-induced aggregation of neutrophils. Neutrophils (400 μl) in modified Tyrode buffer (1 × 10^7/ml) were exposed to a constant shear stress from 6–60 dynes/cm^2 for 6 min at room temperature. The aggregation rate was measured by transmitted light intensity. The figure shows a representative of three different experiments that had essentially the same results.

Effect of Fucoidin on Neutrophil Aggregation

Pretreatment of neutrophils for 10 min with fucoidin (10 μg/ml), a carbohydrate moiety that bound to and inhibited selectin function, had no effect on the neutrophil aggregation induced by shear stress determined by inverted phase microscopic observation (Fig. 5).

Effect of Anti-LFA-1 or ICAM-3 Antibody on Neutrophil Homotypic Aggregation Under Shear Stress

Next we examined the integrin family. So we examined the effect of antibodies against LFA-1 and ICAM-3 that are expressed on the neutrophil surface and that could interact with the LFA-1 molecule. Neutrophils were incubated in the presence of 10 μg/ml of either anti-LFA-1 (CD11a) antibody 25.3.1, anti-ICAM-3 antibody HP2/19, or nonimmune mouse IgG for 30 min at 37°C in modified Tyrode buffer. No aggregation was observed under pretreatment only with antibody. Neutrophils were exposed to a shear force of 12 dynes/cm^2 for 6 min in the presence of the antibody. Either anti-LFA-1 or anti-ICAM-3 antibody completely inhibited the neutrophil homotypic aggregation induced by shear stress observed by inverted phase microscopy, as shown in Figure 6, while the aggregation was not affected at all by pretreatment with nonimmune IgG.

Effect of Various Agents on Intracellular Ca^{2+} Concentration ($[Ca^{2+}]i$) and Shear Stress–Induced Aggregation of Neutrophils

We examined the effect of shear stress on the intracellular Ca^{2+} concentration of neutrophils. The $[Ca^{2+}]i$ of resting neutrophils was found to be 160 nM, and stimulation with 10^{-7} M fMLP caused a rapid increase of $[Ca^{2+}]i$ (about 1,000 nM) followed by a gradual decrease (Fig. 7, trace B). In contrast to the fMLP stimulation, a gradual increase of $[Ca^{2+}]i$ was observed under shear stress (Fig. 7, trace A). $[Ca^{2+}]i$ reached about 750 nM within 2 min after stimulation of 12 dynes/cm^2 shear stress for 2 min. This increase of $[Ca^{2+}]i$ was not inhibited by PGI_2 or PGE_1, which partially inhibited the increase of

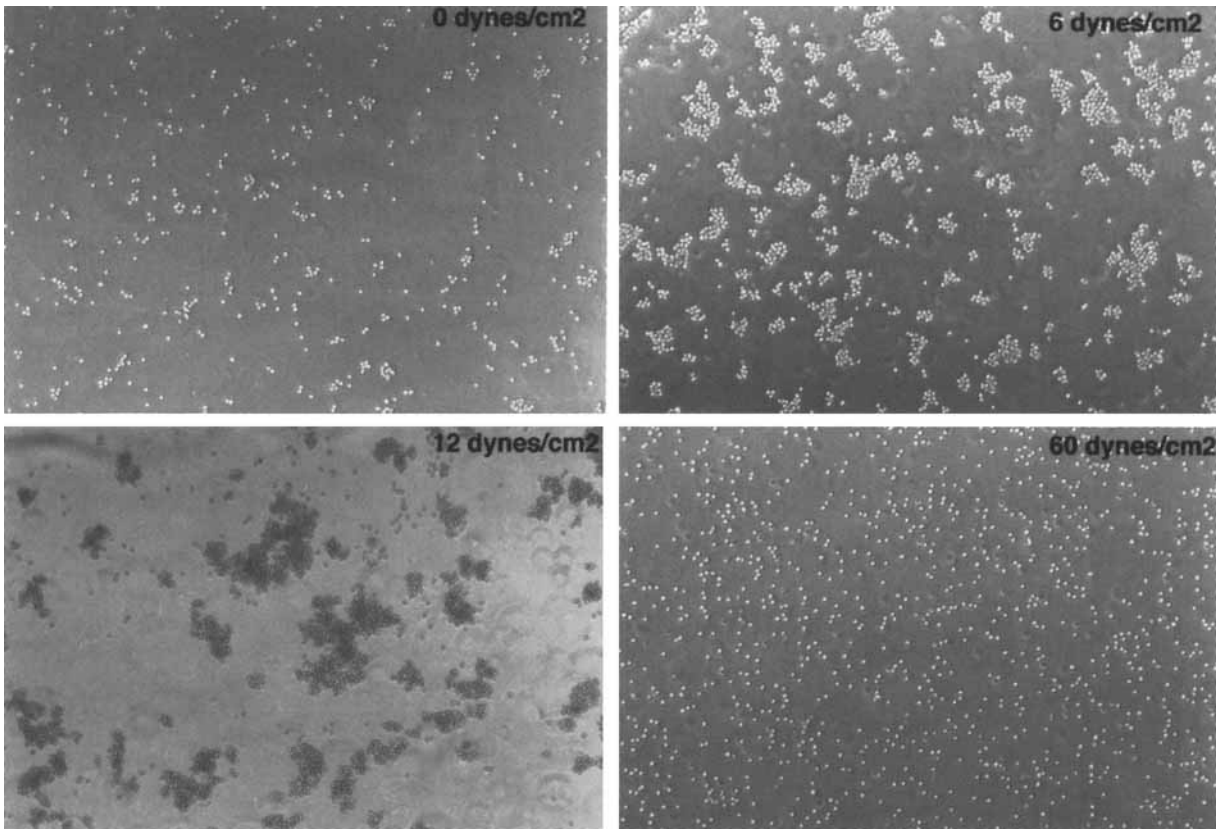
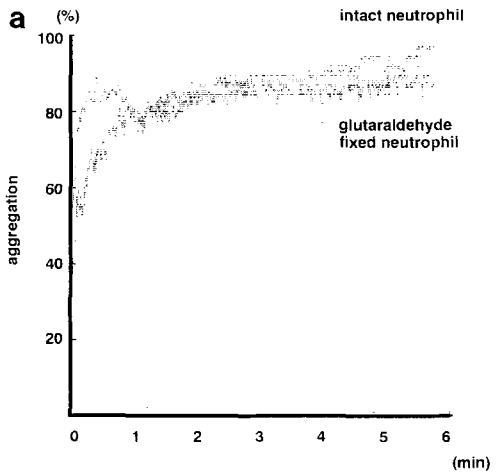


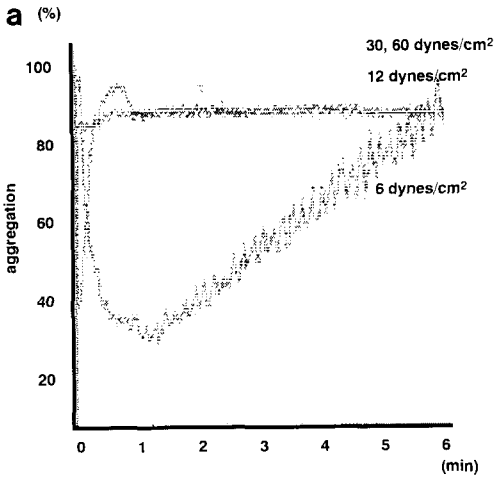
Fig. 2. Induction of neutrophil homotypic aggregation induced by shear stress. After neutrophils were exposed to a constant shear stress for 6 min, an aliquot was subjected to phase microscopic observation. The figure shows a representative of three different experiments that had essentially the same results.

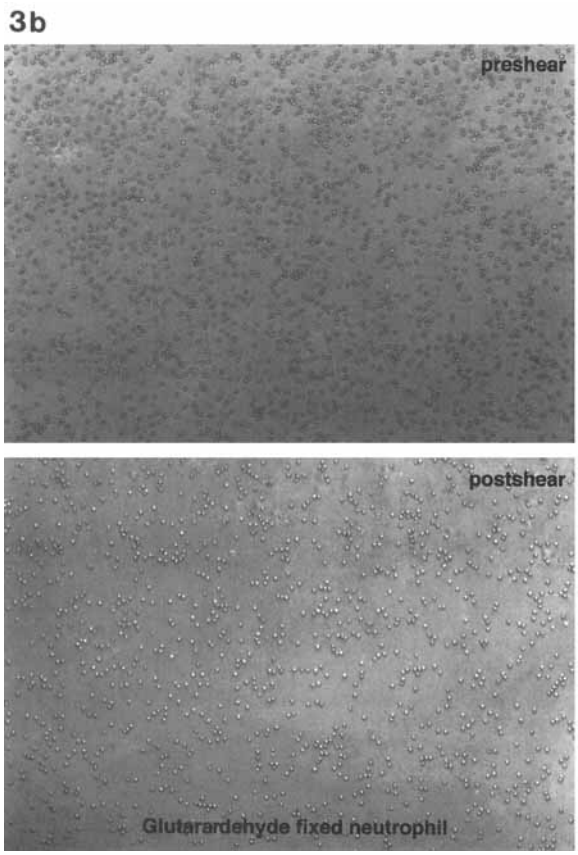
$[Ca^{2+}]i$ stimulated by fMLP. EGTA, BAPTA, or $NiCl_2$ inhibited both the increase of $[Ca^{2+}]i$ and aggregation induced by shear stress. On the other hand, anti-LFA-1 and anti-ICAM-3 antibodies failed to inhibit the increase of $[Ca^{2+}]i$, though these antibodies suppressed neutrophil aggregation under shear stress (Table I).

DISCUSSION

In a vessel, the velocity of blood near the wall is lower than towards the center; this difference creates a shearing effect between adjacent layers of fluid moving at different speeds. This shear force can influence various cell functions in a vessel. In this study, we first reported that neutrophils undergo homotypic aggregation under fluid shear stress of the physiological range. The aggregation was detected by transmitted light intensity and inverted phase microscopy (Fig. 1, 2). The shear stress–induced aggregation of platelets is mediated by plasma adhesive proteins such as vWF and fibrinogen [Ikeda et al., 1993]. In contrast to platelets, neutrophils aggregated under shear stress in a simple buffer containing no plasma protein factors. It was pertinent to clarify whether this aggregation is provoked by physical events or not. Therefore, glutaraldehyde-fixed neutrophils were subjected to shear stress, as glutaraldehyde fixation blocks intracellular signal transduction and conformational change of adhesion molecules on the cellular surface. Figure 3 shows that glutaraldehyde-fixed neutrophils exhibited the same aggregation pattern as intact neutrophils measured by transmitted light intensity under shear stress, while at the end of shear stress glutaraldehyde-fixed neutrophils could not maintain the aggregation. These results indicated that metabolically active neutrophils are required in the neutrophil aggregation under shear stress. In addition, calibration beads acted in the same manner as glutaraldehyde-fixed neutrophils under shear stress, and no aggregation of beads was observed at the end of shear stress by inverted phase microscopy







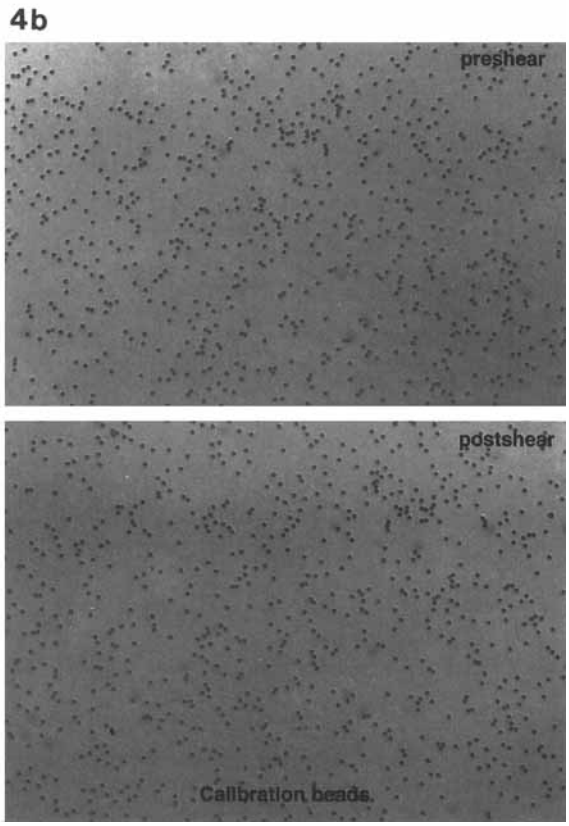


Fig. 3. Shear stress–induced aggregation of glutaraldehyde-*fixed neutrophils. Glutaraldehyde-fixed neutrophils* (see Materials and Methods) were resuspended in modified Tyrode buffer (1×10^7/ml) and exposed to a constant shear stress of 12 dynes/cm^2 for 6 min. Simultaneous recording of the aggregation measured by transmitted light intensity (**a**) and inverted phase microscopic observation (**b**) was performed. The figures show a representative of three different experiments that had essentially the same results.

Fig. 4. Shear stress–induced aggregation of calibration beads. Calibration beads (6.5 μm, 1×10^7/ml) in modified Tyrode buffer (1×10^7/ml) were exposed to a constant shear stress of 12 dynes/cm^2 for 6 min. Simultaneous recording of aggregation measured by transmitted light intensity (**a**) and inverted phase microscopic observation (**b**) was performed. The figures show a representative of three different experiments that had essentially the same results.

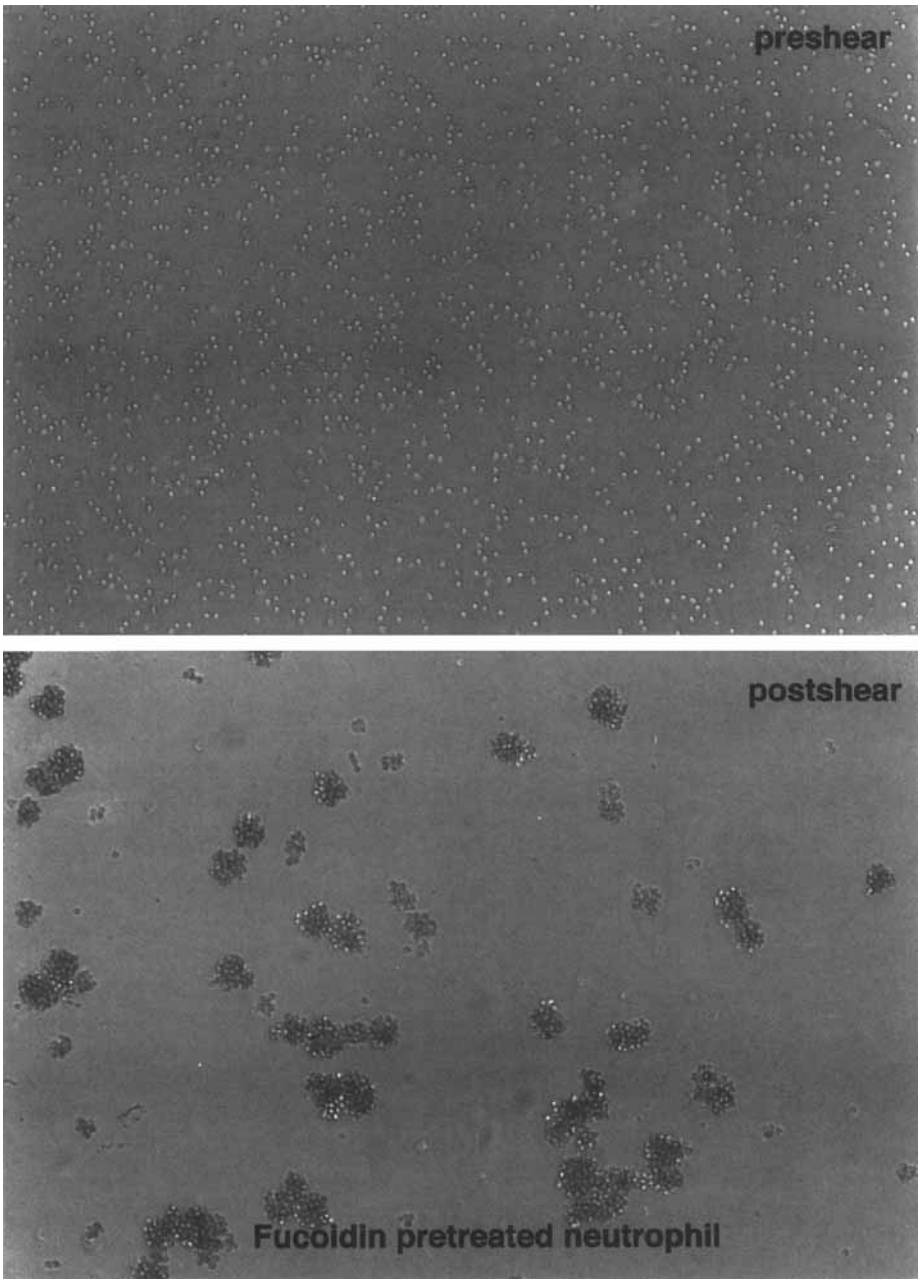


Fig. 5. Effect of fucoidin on shear stress–induced neutrophil homotypic aggregation. Neutrophils were pretreated with 10 μg/ml fucoidin for 10 min. After pretreatment, neutrophils were exposed to a constant shear stress of 12 dynes/cm^2 for 6 min, and inverted phase microscopic observation was performed. The figure shows a representative of three different experiments that had essentially the same results.

(Fig. 4). Therefore, we concluded that under certain conditions neutrophils aggregate by physical force in our apparatus. However, the sustained aggregation triggered by the shear stress may be mediated by the intracellular signal transduction as a physiological phenomenon, because glutaraldehyde-treated neutrophils and calibration beads failed to sustain the aggregation at the end of shear stress.

Neutrophils needed to have both receptors and ligands on their surface because this shear stress–induced aggregation occurred in a simple buffer containing no plasma protein factors or the other cells. Recently, the identity of the

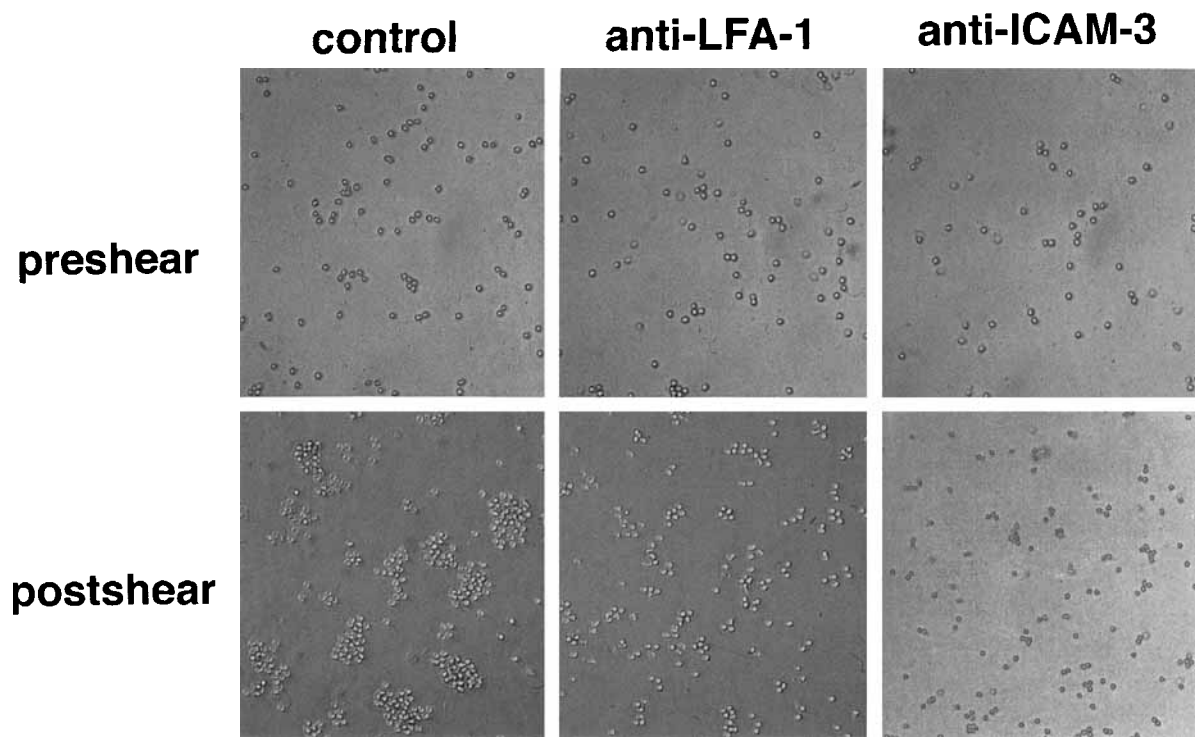


Fig. 6. Effect of anti-LFA-1 or anti-ICAM-3 antibody on shear stress–induced neutrophil homotypic aggregation. Neutrophils were incubated in the presence of 10 μg/ml anti-LFA-1 (CD11a) antibody 25.3.1, anti-ICAM-3 antibody HP2/19, or nonimmune mouse IgG for 30 min at 37°C. After incubation, neutrophils were exposed to 12 dynes/cm^2 of shear force for 6 min. Inverted phase microscopic observation was performed before and after shear stress. The figure shows a representative of five different experiments that had essentially the same results.

selectin, the integrin, and the immunoglobulin superfamily (three groups of the adhesion molecules) [Marlin and Springer, 1987; Lawrence et al., 1990; Abbassi et al., 1993; Beekhuizen and Furth, 1993; Buttrum and Nash, 1993; Jones et al., 1993]. First, we examined whether the sustained neutrophil aggregation was mediated by selectins. L-selectin was expressed on the neutrophil surface. L-selectin has been suggested to recognize SLe^a- and SLe^x-bearing glycoproteins, like E-selectin [Beekhuizen et al., 1993]. We examined the effect of fucoidin, a carbohydrate moiety that bound to and inhibited selectin function [Gaboury and Kubes, 1994], on neutrophil aggregation induced by shear stress (Fig. 5). Fucoidin did not affect neutrophil homotypic aggregation under shear stress. This indicated that the neutrophil aggregation was not mediated by the selectin family.

Then we examined the possible involvement of the integrin and the immunoglobulin superfamily in this aggregation. Several surface molecules have been shown to trigger homotypic

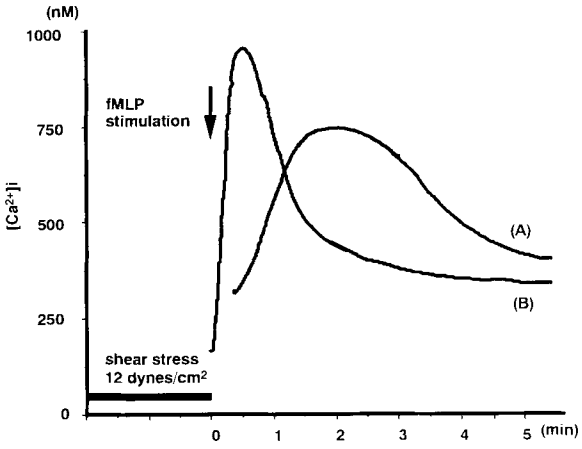


Fig. 7. Effect of intracellular Ca^{2+} concentration stimulated by fMLP or shear stress. Fluo-3–loaded neutrophils stimulated by 10^{-7} M fMLP (*arrow*) were subjected to measurement of intracellular Ca^{2+} concentration by a fluorescence spectrometer (*trace B*). Fluo-3–loaded neutrophils were exposed to a constant shear force of 12 dynes/cm^2 for 2 min (*underline*). At the end of shear stress, the cell suspension (400 μl) was replaced in the cuvette, the fluorescence measurement started at least within 15 s, and intensity was continuously monitored by a fluorescence spectrometer (*trace A*).

TABLE I. Effect of Various Agents on Shear Stress–Induced Neutrophil Homotypic Aggregation and Intracellular Ca^{2+} Concentration

Agents	Aggregation score (grade 0–+5)	$[Ca^{2+}]i$ (nM)
Control (no shear stress)	+1	188 ± 32
12 dynes/cm^2		
Control (no addition)	+4	754 ± 62
EGTA (2 mM)	0	123 ± 37
PGE_1 (300 ng/ml)	+4	760 ± 43
PGI_2 (10 ng/ml)	+4	771 ± 51
BAPTA-AM (100 μM)	+1	139 ± 20
$NiCl_2$ (5 mM)	+1	224 ± 28
Anti-LFA-1 Ab (10 μg/ml)	+1	746 ± 57
Anti-ICAM-3 Ab (10 μg/ml)	+1	758 ± 82

Neutrophils were suspended in modified Tyrode buffer in the presence of 1 mM $CaCl_2$ and then mixed with the following agents: 2 mM EGTA, 300 ng/ml PGE_1, 10 mg/ml PGI_2, and 5 mM $NiCl_2$ for 5 min and 10 μg/ml anti-LFA-1 antibody and 10 μg/ml anti-ICAM-3 antibody for 30 min. 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 same buffer in the presence of 1 mM $CaCl_2$. Incubated neutrophils were exposed to 12 dynes/cm^2 of shear force for 2 min, and inverted phase microscopic observation was performed. Fluo-3–loaded neutrophils were incubated with various agents as described above. Incubated neutrophils were exposed to 12 dynes/cm^2 of shear force for 2 min. The fluorescence measurement started at least within 15 s, and intensity was oontinuously monitored.

adhesion of B cells, T cells, or monocytes [Wuthrich, 1992; Bernard et al., 1994]. In many cases, these homotypic adhesion events are mediated by the LFA-1/ICAM-1 pathway [Koopman et al., 1990; Lauener et al., 1990; Kansas et al., 1991; De Smet et al., 1993]. However, ICAM-1 has not been reported to be expressed on the neutrophil surface. Recently, ICAM-3, a third adhesion counterreceptor for LFA-1, has been found, which is well expressed on all leukocytes and is absent in endothelial cells [Antonin et al., 1992, 1994; Fawcett et al., 1992; Campanero et al., 1993; Hernandes-Caselles et al., 1993; Cid et al., 1994]. We examined the influence of anti-LFA-1 or anti-ICAM-3 antibody on the neutrophil homotypic aggregation induced by shear stress. Neutrophils were incubated in the presence of either the anti-LFA-1 antibody 25.3.1 [Fischer et al., 1986] or anti-ICAM-3 antibody HP2/19 [Hernandez-Caselles et al., 1993]. No aggregation of neutrophils was observed under pretreatment with only these antibodies. Anti-ICAM-3 antibody HP2/19 was reported to be able to increase T lymphoblast homotypic aggregation, suggesting that T cell aggregation induced by this antibody could be mediated by increasing the avidity of LFA-1 for ICAM-1 [Campanero et al., 1993]. We speculated that HP2/19 pretreated neutrophils could not aggregate, though this antibody increased the avidity of LFA-1 for ICAMs including ICAM-3, because ICAM-3 receptor was blocked by this antibody and other ICAMs receptors were absent on the neutrophil surface. These pretreated neutrophils were exposed to shear stress. Either anti-LFA-1 or anti-ICAM-3 antibody completely inhibited the neutrophil homotypic aggregation induced by shear stress observed by inverted phase microscopy, as shown in Figure 6. These findings suggested that neutrophil aggregation induced by shear stress was mediated by an LFA-1/ICAM-3–dependent pathway and LFA-1 might be activated by shear stress. Whether the LFA-1 molecule is directly activated by shear stress or indirectly activated by physical cell-cell interaction under shear stress remains to be elucidated.

Platelet aggregation under shear stress has been well characterized [Ikeda et al., 1993]. This platelet aggregation is mediated by a Ca^{2+} influx through a Ca^{2+} channel. So we examined $[Ca^{2+}]i$ under shear stress by using fluo-3–loaded neutrophils. We could not measure $[Ca^{2+}]i$ at the same time during shear stress in our apparatus, but $[Ca^{2+}]i$ was monitored within 15 s at the end of shear stress. We detected a gradual increase in $[Ca^{2+}]i$ stimulated by 12 dynes/cm^2 for 2 min (Fig. 7, trace A). This increase of $[Ca^{2+}]i$ was observed in shear stress–induced platelet aggregation, but shear force was higher than that of neutrophil aggregation [Ikeda et al., 1993]. We examined the effects of various agents in order to investigate the mechanism of increase of $[Ca^{2+}]i$ (Table I). PGI_2 or PGE_1 had no effect on neutrophil aggregation and the concomitant increase of $[Ca^{2+}]i$, though these agents inhibited the increase of $[Ca^{2+}]i$ stimulated by fMLP (data not shown). We speculated that the mechanism of the increase of $[Ca^{2+}]i$ induced by shear stress was different from that of fMLP stimulation. EGTA inhibited neutrophil aggregation and the increase $[Ca^{2+}]i$. This finding indicated that neutrophil aggregation and the increase of $[Ca^{2+}]i$

under shear stress required extracellular Ca^{2+}. Many authors reported that the LFA-1 molecule requires extracellular Ca^{2+} in order to sustain the molecular conformation to bind the ligands [Rothlein Springer, 1986; Marlin Springer, 1987]. The question remained whether the inhibitory effect of EGTA was due to suppression of Ca^{2+} influx or to inhibition of the sustaining of LFA-1 molecular conformation. So we tried to examine the effect of BAPTA that potentially chelates the intracellular Ca^{2+}. Preloading of BAPTA inhibited the shear stress–induced neutrophil aggregation, indicating that the increase of $[Ca^{2+}]i$ was required for shear stress–induced neutrophil aggregation. In general, there are two established pathways for the increase of $[Ca^{2+}]i$, namely Ca^{2+} influx and Ca^{2+} mobilization. We used $NiCl_2$ that was reported to inhibit the Ca^{2+} channel [Marriott et al., 1994]. Pretreatment with $NiCl_2$ inhibited both the aggregation and the increase of $[Ca^{2+}]i$ induced by shear stress in the presence of extracellular Ca^{2+}. Thapsgargin, which inhibits Ca^{2+} mobilization from Ca^{2+} storage sites, had no effect on shear stress–induced neutrophil aggregation and the increase on $[Ca^{2+}]i$ (data not shown). These results suggested that Ca^{2+} influx, which may be mediated by a $NiCl_2$–sensitive Ca^{2+} channel, played an important role in the increase of $[Ca^{2+}]i$ and neutrophil aggregation under shear stress. In contrast to $NiCl_2$, verapamil, an inhibitor of a voltage-dependent Ca^{2+} channel, had no effect on the increase of $[Ca^{2+}]i$ or on shear stress–induced aggregation (data not shown). This $NiCl_2$-sensitive Ca^{2+} channel would act as a mechanoreceptor. The increase of $[Ca^{2+}]i$ was found to be essential for shear stress–induced neutrophil aggregation, but the time sequence of the increase by shear stress remained to be elucidated. $[Ca^{2+}]i$ increased even in shear-loaded neutrophils pretreated with anti-LFA-1 or anti-ICAM-3 antibody, which did not aggregate. These results suggested that the increase of $[Ca^{2+}]i$ might be the initial event. The increase of $[Ca^{2+}]i$ likely caused by Ca^{2+} influx through $NiCl_2$-sensitive Ca^{2+} channel induced LFA-1/ICAM-3–dependent neutrophil aggregation under fluid shear stress.

The physiological implication of this novel phenomenon may not be answered until further detailed investigation is done. In conclusion, we found for the first time that human neutrophils undergo homotypic aggregation under fluid shear stress of the physiological range. This aggregation was mediated by LFA-1/ICAM-3 pathway which was activated by the increase of $[Ca^{2+}]i$ through a Ni^{2+}-sensitive Ca^{2+} channel.

REFERENCES

Abbassi O, Kishimoto TK, Mcintire LV, Smith CW (1993): Neutrophil adhesion to endothelial cells. Blood Cells 19: 245–260.

Antonin R, Fougerolles D, Springer TA (1992): Intercellular adhesion molecule 3, a third adhesion counter-receptor for lymphocyte function-associated molecule 1 on resting lymphocytes. J Exp Med 175:185–190.

Antonin R, Fougerolles D, Qin X, Springer TA (1994): Characterization of the function of intercellular adhesion molecule (ICAM)-3 and comparison with ICAM-1 and ICAM-2 in immune responses. J Exp Med 179:619–629.

Beekhuizen H, Furth RV (1993): Monocyte adherence to human vascular endothelium. J Leukoc Biol 54:363–378.

Bernard G, Zoccola D, Ticchioni M, Breittmayer JP, Aussel C, Bernard A (1994): Engagement of the CD45 molecule induces homotypic adhesion of human thymocytes through a LFA-1/ICAM-3–dependent pathway. J Immunol 152: 5161–5170.

Buttrum SMB, Nash GB (1993): Effect of activation on adhesion of flowing neutrophils to cultured endothelium: Time course and inhibition by a calcium channel blocker (nitrendipine). Br J Pharmacol 110:1630–1634.

Campanero MR, Pozo MAD, Arroyo AG, Sanchez-Mateos P, Hernandez-Caselles T, Craig A, Pulido R, Sanchez-Madrid F (1993): ICAM-3 interacts with LFA-1 and regulates the LFA-1/ICAM-1 cell adhesion pathway. J Cell Biol 123: 1007–1016.

Cid MC, Esparza J, Juan M, Miralles A, Ordi J, Vilella R, Urbano-Marques A, Gaya A, Vives J, Yague J (1994): Signaling through CD50 (ICAM-3) stimulates T lymphocyte binding to human umbilical vein endothelial cells and extracellular matrix proteins via an increase in β1 and β2 integrin function. Eur J Immunol 24:1377–1382.

De Smet W, Walter H, Hove LV (1993): A new CD43 monoclonal antibody induces homotypic aggregation of human leukocytes a CD11a/CD18-dependent and -independent mechanism. Immunology 79:46–54.

Fawcett J, Holness CLL, Needham LA, Turly H, Gatter KC, Mason DY, Simmons DL (1992): Molecular cloning of ICAM-3, a third ligand for LFA-1, constitutively expressed on resting leukocytes. Nature 360:481–484.

Fischer A, Blanche S, Veber F, Delaage M, Mawas C, Griscelli C, Le Deist F, Lopez M, Olive D, Janossy G (1986): Prevention of graft failure by an anti-HLFA-1 monoclonal antibody in HLA-mismatched bone-marrow transplantation. Lancet 1058–1061.

Gaboury JP, Kubes P (1994): Reductions in physiologic shear rates lead to CD11/18-dependent, selectin-independent leukocyte rolling in vivo. Blood 83:345–350.

Hernandez-Caselles T, Rubio G, Campanero MR, Pozo MAD, Muro M, Sanchez-Madrid F, Aparicio P (1993): ICAM-3, the third LFA-1 counterreceptor, is a co-stimulatory molecule for both resting anf activated T lymphocytes. Eur J Immunol 23:2799–2806.

Ikeda Y, Handa M, Kamata T, Kawano K, Kawai Y, Watanabe K, Kawakami K, Sakai K, Fukuyama M, Itagaki I,

Yosioka A, Ruggeri ZM (1993): Transmembrane calcium influx associated with von Willebrand factor binding to GP Ib in the initiation of shear-induced platelet aggregation. Thromb Haemost 69:496–502.

Jones DA, Abbassi O, McIntire LV, McEver RP, Smith CW (1993): P-selectin mediates neutrophil rolling on histamine-stimulated endothelial cells. Biophys J 65:1560–1569.

Kansas GS, Wood GS, Tedder TF (1991): Expression, distribution, and biochemistry of human CD39. J Immunol 146:2235–2244.

Koopman G, Kooyk YV, Graaff MD, Meyer CJLM, Figdor CG, Pals ST (1990): Triggering of the CD44 antigen on T lymphocytes promotes T cell adhesion through the LFA-1 pathway. J Immunol 145:3589–3593.

Lauener RP, Geha RS, Vercelli D (1990): Engagement of the monocyte surface antigen CD14 induces lymphocyte function-associated antigen-1/intercellular adhesion molecule-1–dependent homotypic adhesion. J Immunol 145:1390–1394.

Lawrence MB, Smith CW, Eskin SG, McIntire LV (1990): Effect of venous shear stress on CD18-mediated neutrophil adhesion to cultured endothelium. Blood 75:227–237.

Marlin SD, Spriger TA (1987): Purified intracellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). Cell 51:813–819.

Marriott I, Bost KL, Mason MJ (1994): Role of intracellular Ca^{2+} stores in the regulation of electrogenic plasma membrane Ca^{2+} uptake in a B-lymphocytic cell line. 161:441–448.

Rothlein R, Springer TA (1986): The requirement for lymphocyte function-associated antigen 1 in homotypic leukocyte adhesion stimulated by phorbol ester. J Exp Med 163:1132–1138.

Vandenbergh PA, Ceuppens JL (1990): Flow cytometric measurement of cytoplasmic free calcium in human peripheral blood T lymphocytes with fluo fluo-3, a new fluo-3, a new fluorescence calcium indicator. J Immunol Methods 127:197–205.

Wuthrich RP (1992): Monoclonal antibodies targeting murine LFA-1 induced LFA-1/ICAM-1–independent homotypic lymphocyte aggregation. Cell Immunol 144:22–31.