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Proteolysis of fibrinogen deposits enables hydrogen peroxide-stimulated polymorphonuclear leukocytes to spread in an acidified environment

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ABSTRACT

Polymorphonuclear leukocytes might be expected to employ functional regulatory systems adapted to an acidified environment, such as found in the inflammatory sites where polymorphonuclear leukocytes act in host defense. We previously reported the unusual characteristics of phorbol 12-myristate 13-acetate (PMA)-induced polymorphonuclear leukocyte spreading over immobilized fibrinogen at acidic pH, including extracellular Ca^{2+} requirement and independence of protein kinase C (PKC) activity. In the present study, we found that PMA-induced spreading was strongly inhibited at pH 6.0 by the serine protease inhibitor phenylmethanesulfonylfluoride at pH 6.0 but was only mildly inhibited at pH 7.2 and not inhibited at pH 8.0; furthermore, PMA-stimulated polymorphonuclear leukocytes markedly digested immobilized fibrinogen only at pH 6.0. In experiments without stimulation by PMA, we found that at pH 6.0 polymorphonuclear leukocytes were able to spread over fibrinogen surfaces pre-digested by neutrophil serine proteases; this process required extracellular Ca^{2+} and stimulation by hydrogen peroxide (H_2O_2). Pharmacological studies demonstrated the involvement of Src family protein tyrosine kinases, but not PKC, in H_2O_2-induced spreading over pre-digested fibrinogen surfaces; this was also the case for PMA-induced spreading at pH 6.0 but not at pH 7.2 or 8.0. These results suggest that PMA-induced polymorphonuclear leukocyte spreading depends on serine protease-mediated fibrinogenolysis in an acidic milieu, but that other mechanisms operate at neutral/alkaline pH.

1. Introduction

Polymorphonuclear leukocytes, most of which are neutrophils, play a critical role in host defense against infection. Nevertheless, polymorphonuclear leukocytes are also implicated in tissue-damaging inflammatory reactions leading to the pathogenesis and exacerbation of many inflammatory diseases, such as systemic inflammatory response syndrome and chronic obstructive pulmonary disease (Kaneider et al., 2006). At sites of infection, accumulated polymorphonuclear leukocytes express a variety of microbicidal-related functions such as phagocytosis, secretion of proteolytic enzymes, and hydrogen peroxide (H_2O_2) production (Graham et al., 1994; Kaneider et al., 2006).

β2-integrin-dependent firm adhesion (spreading) is an absolute prerequisite for activation of the microbicidal functions of polymorphonuclear leukocytes (Nathan, 1987; Yan et al., 1995). Leukocyte engagement of fibrinogen deposits via β_2-integrins (CD11b/CD18 and CD11c/CD18) leads to their spreading followed by activation (Lowell et al., 1996; Flick et al., 2004). Deposition of fibrinogen or fibrin is a universal feature of inflammatory sites and injured tissues (Flick et al., 2004). Notably, immobilized fibrinogen is bound with high affinity/avidity by the β_2-integrins, whereas soluble fibrinogen is a poor ligand (Lishko et al., 2002; Flick et al., 2004). Several epitopes have been identified within fibrinogen for the β_2-integrins, including P1 and/or P2 of the fibrinogen γ chain and a pattern recognition motif of negatively charged residues (Altieri et al., 1993; Ugarova et al., 1998; Vorup-Jensen et al., 2005). Although these epitopes are hidden in soluble fibrinogen, immobilization followed by denaturation or proteolysis of the molecule leads to their exposure (Hu et al., 2001; Lishko et al., 2002; Vorup-Jensen et al., 2005). Consequently, leukocyte integrins engage immobilized and denatured fibrinogen at inflammatory sites but not plasma fibrinogen within the intravascular space. Thus, in vitro experimental systems using fibrinogen-coated surfaces as a ligand for β2-integrin are helpful for an understanding of the regulatory mechanisms of polymorphonuclear leukocyte spreading and functional activation.

Polymorphonuclear leukocyte functions are largely affected by environmental pH (and thus intracellular pH). Thus, careful in vitro studies taking into account environmental pH are necessary for a precise understanding of the functional regulation of polymorphonuclear leukocytes in vivo, since acidification of the microenvironment often prevails at the sites of inflammation where they act in host defense (Sawyer et al., 1991; Nanda et al., 1992; Coakley et al., 2002). It

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might be expected that polymorphonuclear leukocytes would employ specific functional regulatory systems adapted to an acidified environment, although environmental or cytoplasmic acidification is reported to impair spreading over immobilized fibrinogen (Suzuki and Namiki, 2007) or fibronectin (Galkina et al., 2006), and glass surfaces (Demaurex et al., 1996).

In our previous study, we investigated the conditions under which phorbol 12-myristate 13-acetate (PMA)-stimulated polymorphonuclear leukocytes could undergo spreading over immobilized fibrinogen surfaces in an acidified and HCO_3^--free environment. We observed some unique characteristics of this spreading, as follows: 1) spreading at acidic pH, but not at neutral or alkaline pH, requires extracellular Ca^{2+}; 2) spreading at acidic pH does not require protein kinase C (PKC) activity; and 3) spreading at acidic pH is suppressed by H_2O_2 produced by activated NADPH oxidase or added exogenously (Suzuki et al., 2008). These findings indicate that the signaling pathway for PMA-stimulated polymorphonuclear leukocyte spreading at acidic pH is clearly distinct from that at neutral or alkaline pH.

In our previous studies, fibrinogen-coated surfaces were used for the spreading experiments either immediately after immobilization for one hour at room temperature (Suzuki et al., 2008) or after immobilization overnight at 4 °C (Suzuki and Namiki, 2007). Under these conditions, non-stimulated polymorphonuclear leukocytes could not undergo spontaneous spreading and cellular stimulation by PMA was necessary for induction of spreading irrespective of pH, possibly because the freshly immobilized fibrinogen was un-denatured and the β2-integrin epitopes described above remained hidden. However, it was recently found that, even in the condition of fibrinogen immobilization for 1 h at 37 °C, proteolysis of the immobilized fibrinogen by treatment with proteases, including plasmin, subtilisin and elastase, can expose epitopes for β_2-integrins, thereby enabling polymorphonuclear leukocytes to spread in the absence of stimulation (Vorup-Jensen et al., 2005). It is relevant that PMA induces release of fibrinogenolytic serine proteases such as elastase and cathepsin G from polymorphonuclear leukocytes in culture (Bangalore and Travis, 1994; Bos et al., 1997; Reynaud af Geijersstam et al., 2005) and that extracellular acidification enhances neutrophil degranulation stimulated by formyl-Met-Leu-Phe (FMLP) (Trevani et al., 1999).

In the present study, therefore, we hypothesized that secretion of fibrinogenolytic proteases such as elastase and cathepsin G followed by proteolytic degradation of immobilized fibrinogen is involved in PMA-induced polymorphonuclear leukocyte spreading at acidic pH. Here we report that PMA-induced polymorphonuclear leukocyte spreading is strictly dependent on the activity of serine proteases at acidic pH, moderately dependent at neutral pH, but not dependent at alkaline pH. Furthermore, PMA stimulation of polymorphonuclear leukocytes at acidic pH, but not neutral or alkaline pH, leads to digestion of immobilized fibrinogen as detected by immunoblot analysis. Next, we demonstrate that polymorphonuclear leukocyte spreading at acidic pH requires both previous proteolysis of immobilized fibrinogen and cellular exposure to H_2O_2, whereas either proteolysis of immobilized fibrinogen or cellular stimulation with H_2O_2 alone can induce spreading at neutral/alkaline pH. Finally, we show that the characteristics of H_2O_2-stimulated polymorphonuclear leukocyte spreading over proteolyzed fibrinogen deposits are similar to those of PMA-induced spreading at acidic pH in terms of requirement for Ca^{2+}, PKC, and Src family tyrosine kinases.

2. Materials and methods

2.1. Reagents

Phenylmethanesulfonylfluoride (PMSF) and porcine fibrinogen were purchased from Sigma (St. Louis, MO, USA). Human neutrophil elastase, cathepsin G, 2-[1-(3-dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl)maleimide (Gö6983) and 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazole[3,4-d]pyrimidine (PP2) were from Calbiochem (San Diego, CA, USA). Methyl cellulose 25cP and PMA were from Wako Pure Chemical (Tokyo, Japan). Ficoll-Paque Plus was from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Dimethyl sulfoxide (DMSO), H_2O_2 and 3,3'-diaminobenzidine tetrahydrochloride dihydrate (DAB) were from Kanto Chemical (Tokyo, Japan). Fresh porcine peripheral blood was obtained from Tokyo Shibaura Zoki (Tokyo, Japan). Rabbit polyclonal antibody against porcine fibrinogen (RASw/Fbg) was obtained from Nordic Immunological Laboratories (Tilburg, the Netherlands). HRP-conjugated goat anti-rabbit IgG antibody was from Santa Cruz biotechnology (Santa Cruz, CA). All other chemicals were of the highest purity grade.

2.2. Preparation of immobilized fibrinogen surfaces

Culture plate with immobilized fibrinogen was prepared by incubation with fibrinogen (1 mg/ml) for 2 h (non-denaturing) or 24 h (denaturing) at room temperature (23 °C) followed by washing. If necessary, the fibrinogen-immobilized plate was pre-treated with proteases before experiments as described below.

2.3. Proteolysis of immobilized fibrinogen by exogenous proteases

In some experiments, proteolytic degradation treatment of immobilized fibrinogen by incubation with purified neutrophil cathepsin G or elastase was performed before assessment of spreading. After incubation with the proteases, the reaction mixtures were removed by aspiration and washing. Under our experimental conditions, it was confirmed that the naked plastic surface of the culture plate was not exposed as a consequence of the protease treatment, because there was no evidence of divalent cation- and β2-integrin-independent non-specific adhesion of resting polymorphonuclear leukocytes (K. Suzuki, unpublished), which is a characteristic of adhesion to naked plastic surfaces (Rainard, 1988; Aida and Pabst, 1991). It was pre-ascertained that polymorphonuclear leukocyte spreading over proteolyzed immobilized fibrinogen is unaffected by post-fibrinogenolysis blocking with BSA.

2.4. Polymorphonuclear leukocyte spreading

Polymorphonuclear leukocytes were isolated from porcine peripheral blood routinely (Suzuki et al., 2008). Polymorphonuclear leukocyte spreading over immobilized fibrinogen at different pH was assessed morphologically as described previously (Suzuki et al., 2008). Polymorphonuclear leukocytes suspended in pH-adjusted HEPES-NaCl buffer (10 mM HEPES, 140 mM NaCl, 5 mM glucose, 0.6 mM Mg^{2+} and 2 mM Ca^{2+}) were plated onto immobilized fibrinogen and incubated for 30 min at 37 °C in the presence or absence of PMA (10 ng/ml) and/or H_2O_2 at the indicated concentrations. In some experiments, cells were pretreated with inhibitors at the indicated concentrations before they were plated onto the fibrinogen surfaces. Photomicrographs were taken and cells were counted, and those that were phase dark, enlarged with irregular shapes were considered spread. Spreading data were shown as percentage of spread cells.

2.5. Detection of proteolytic digestion of immobilized fibrinogen

Fibrinogen digestion was assessed by immunoblotting with anti-whole porcine fibrinogen antibody (RASw/Fbg), which reacts with all three (α- β- and γ-) chains of the fibrinogen molecule. Fibrinogen immobilized on plastic surfaces (96-well microplate, as described above) was treated with culture supernatant of PMA-stimulated polymorphonuclear leukocytes and then recovered by mixing with heated SDS-PAGE sample buffer containing 2% SDS and 5%

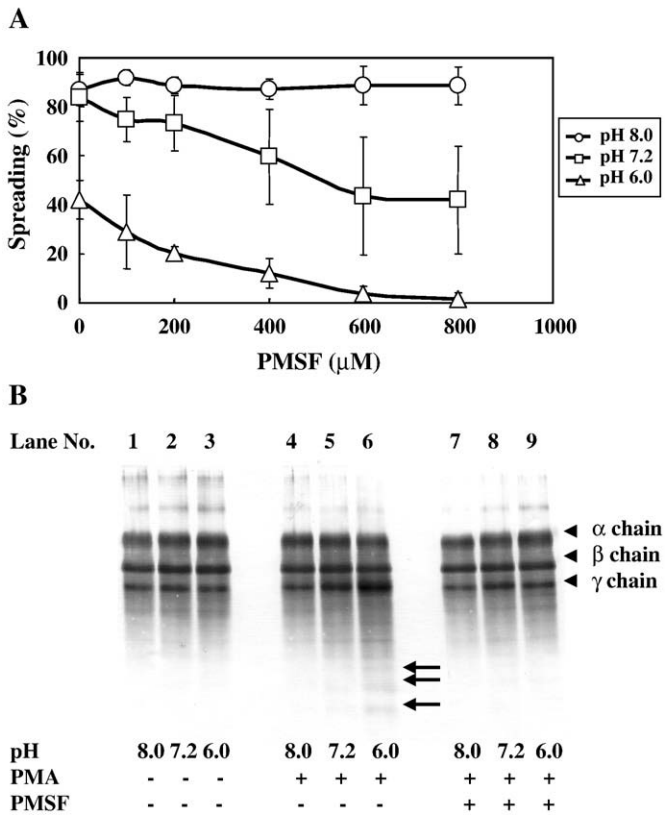

Fig. 1. (A) Effect of PMSF on PMA-induced polymorphonuclear leukocyte spreading over non-pre-denatured fibrinogen surfaces at pH 6.0, 7.2, and 8.0. Cells were pretreated with PMSF for 15 min at the indicated concentrations before plating onto the undenatured fibrinogen surfaces. Spreading was induced by stimulation with 10 ng/ml PMA for 30 min at 37 °C in the presence of 0.6 mM Mg^{2+} and 2.0 mM Ca^{2+}. All data are the mean ± SD. of at least three independent experiments. (B) Effect of pH on proteolytic degradation of immobilized fibrinogen by culture supernatants of PMA-activated polymorphonuclear leukocytes. Cells (5×10^6/ml) were stimulated with diluent (lanes 1–3) or 10 ng/ml PMA (lanes 4–9) for 30 min after pretreatment for 15 min with diluent (lanes 1–6) or 800 μM PMSF (lane 7–9) in the presence of Mg^{2+}/Ca^{2+} at the indicated pH. The culture supernatant was then recovered by centrifugation and incubated for 90 min at 37 °C with fibrinogen immobilized on a 96-well plastic microtiter plate. Fibrinogen was recovered and analyzed by immunoblotting with anti-whole porcine fibrinogen antibody, which reacts with all three chains (α- β-, and γ-chains, indicated by arrowheads) of the fibrinogen molecule, as described in the Methods section. Fibrinogen degradation was visualized as immunoreactive bands of low-molecular-weight fragments (arrows).

2-mercaptoethanol. A half amount of the fibrinogen sample recovered from each well was separated by SDS-PAGE using 12% polyacrylamide gel, and electrophoretically transferred to an Immobilon™-P membrane (Millipore, Bedford, MA, USA). Fibrinogen was then probed using rabbit anti-fibrinogen antibody described above after blocking of the membrane with 5% non-fat dried milk in phosphate-buffered saline (PBS). After washing, the membranes were incubated with HRP-conjugated anti-rabbit IgG secondary antibody, and the protein bands were detected by DAB color reaction (Suzuki and Namiki, 2007). Fibrinogen digestion was detected as immunoreactive bands of low-molecular-weight fragments.

3. Results

3.1. Serine protease inhibitor PMSF inhibits PMA-induced polymorphonuclear leukocyte spreading over un-denatured immobilized fibrinogen strongly at acidic pH, mildly at neutral pH but not at alkaline pH

We first attempted to examine whether endogenous serine proteases participate in PMA-induced polymorphonuclear leukocyte spreading over immobilized fibrinogen surfaces at different pH values, since it was recently reported that polymorphonuclear leukocytes respond to serine protease-digested fibrinogen deposits, resulting in adhesion (Vorup-Jensen et al., 2005). In agreement with our previous report (Suzuki et al., 2008), basal level of PMA-induced spreading was lower at pH 6.0 than those at pH 7.2 and 8.0 (Fig. 1A). PMSF, a serine protease inhibitor, inhibited PMA-induced spreading in a dose-dependent fashion at pH 6.0; at this pH, complete inhibition was observed at 600 μM or 800 μM PMSF. At pH 7.2, PMSF attenuated polymorphonuclear leukocyte spreading although did not show complete inhibition. At pH 8.0, PMSF had no effect on the spreading. These results suggest that endogenous serine protease activity is involved in PMA-induced polymorphonuclear leukocyte spreading over immobilized fibrinogen surfaces at acidic or neutral pH but not at alkaline pH.

3.2. PMSF-inhibitable proteolytic digestion of immobilized fibrinogen by conditioned medium of PMA-stimulated polymorphonuclear leukocytes at acidic pH

Digestion of immobilized fibrinogen by conditioned medium of PMA-stimulated polymorphonuclear leukocytes was assessed by immunoblot analysis using anti-fibrinogen antibody raised against the whole fibrinogen molecule, recognizing all three (α, β, and γ) chains (Fig. 1B). The protein level of immobilized fibrinogen recovered after incubation with culture supernatant of resting cells was similar in all the three pH conditions, indicating that there is no peeling off of adsorbed fibrinogen at any pH tested and that resting polymorphonuclear leukocytes do not break down the molecule. Incubation of

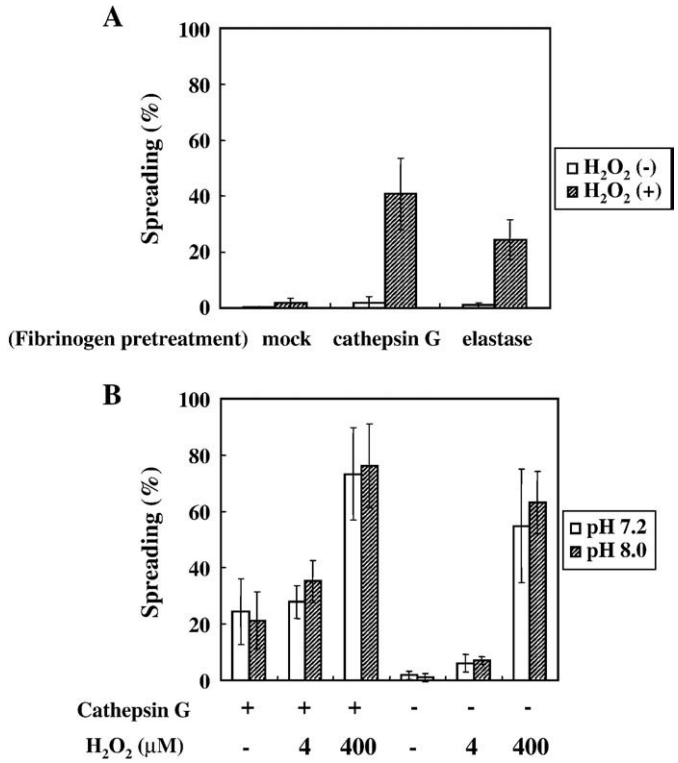

Fig. 2. Effect of H_2O_2 on polymorphonuclear leukocyte spreading over immobilized fibrinogen pre-digested with neutrophil-derived serine proteases at pH 6.0, 7.2 and 8.0. (A) Immobilized fibrinogen surfaces were pretreated with 6 mU/ml cathepsin G or 2 μg/ml elastase, or mock, for 90 min at 37 °C (pH 6.0) before further incubation with cells in the presence or absence of 400 μM H_2O_2 for 30 min at 37 °C (pH 6.0). (B) Immobilized fibrinogen surfaces were pretreated with 6 mU/ml cathepsin G or mock as described above before further incubation with cells in the presence of H_2O_2 at the indicated concentrations for 30 min at 37 °C (pH 7.2 or 8.0). All incubations were performed in the presence of Mg^{2+}/Ca^{2+}. All data are the mean ± SD of at least eight independent experiments.

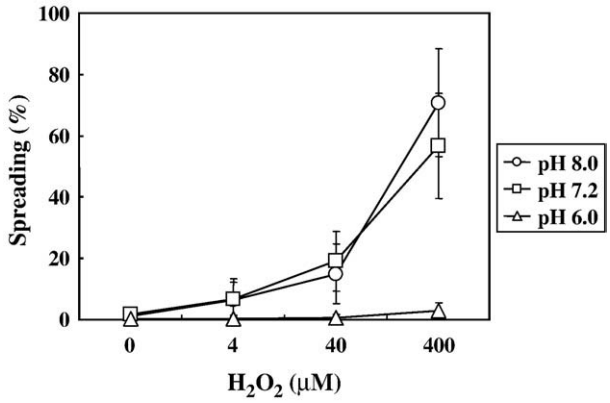

Fig. 3. Effect of H_2O_2 on polymorphonuclear leukocyte spreading over non-pre-denatured fibrinogen surfaces at pH 6.0, 7.2, and 8.0. Cells were plated onto undenatured fibrinogen surfaces and incubated with H_2O_2 at the indicated concentrations for 30 min at 37 °C. All incubations were performed in the presence of Mg^{2+}/Ca^{2+}. All data are the mean ± S.D. of at least five independent experiments.

immobilized fibrinogen with the conditioned medium of polymorphonuclear leukocytes stimulated by PMA at pH 6.0 resulted in marked digestion of the fibrinogen chains, which was confirmed by appearance of immunoreactive bands of low molecular weight fragments. Some faint bands of fibrinogen degradation products were observed also at pH 7.2. In contrast, however, little or no digestion was observed at pH 8.0. This fragmentation of fibrinogen was abrogated by PMSF.

3.3. Protease-digested immobilized fibrinogen enables H_2O_2-stimulated but not resting polymorphonuclear leukocytes to spread even at acidic pH

We therefore attempted to examine whether proteolytic digestion of immobilized fibrinogen surfaces enables polymorphonuclear leukocytes to undergo spreading at acidic pH. Polymorphonuclear leukocyte suspensions (pH 6.0) containing divalent cations were added onto wells in which un-denatured fibrinogen surfaces had been pretreated with purified neutrophil serine protease cathepsin G (6 mU/ml) or elastase (2 μg/ml) at pH 6.0 followed by removal of the enzymes. As shown in Fig. 2A, no spreading was observed when resting cells were merely incubated with pre-digested fibrinogen surfaces. We next examined whether exogenous H_2O_2 promotes the spreading of polymorphonuclear leukocytes over pre-digested fibrinogen surfaces. This experiment was based on reports that H_2O_2 can trigger polymorphonuclear leukocyte activation and adhesion by up-regulation of β_2-integrin expression (Fraticelli et al., 1996), that H_2O_2 boosts the activity of Src family tyrosine kinases required for polymorphonuclear leukocyte spreading (Yan and Berton, 1996), and that PMA-stimulated polymorphonuclear leukocytes perform degranulation and NADPH-mediated H_2O_2 release in parallel. Importantly, as shown in Fig. 2A, polymorphonuclear leukocytes displayed marked spreading over protease-treated fibrinogen surfaces at pH 6.0 when the cells were stimulated with 400 μM H_2O_2, whereas H_2O_2-stimulated cells showed poor spreading over mock-treated fibrinogen surfaces. Polymorphonuclear leukocytes failed to spread when plated onto un-denatured fibrinogen surfaces and incubated together with different concentrations of serine proteases and H_2O_2, possibly because exogenous proteases attack cell surface proteins as well as fibrinogen surfaces (data not shown). At pH 7.2 and 8.0, H_2O_2-stimulated polymorphonuclear leukocytes displayed spreading over both enzyme-treated and mock-treated fibrinogen surfaces (Fig. 2B). Notably, non-stimulated cells also showed spreading to some extent over enzyme-treated but not mock-treated fibrinogen surfaces at these pH values (Fig. 2B). At pH 7.2 and 8.0 but not 6.0, H_2O_2 alone

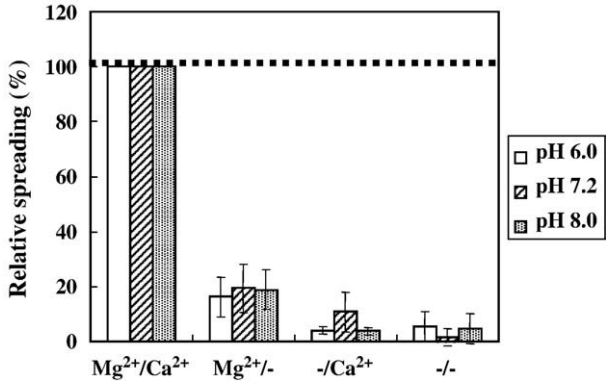

Fig. 4. Effect of divalent cations on the spreading of H_2O_2-stimulated polymorphonuclear leukocytes over pre-digested fibrinogen surfaces at pH 6.0, 7.2, and 8.0. Immobilized fibrinogen surfaces were pretreated with 6 mU/ml cathepsin G for 90 min at 37 °C before further incubation for 30 min at 37 °C with cells stimulated by 400 μM H_2O_2 in the presence or absence of Mg^{2+} (0.6 mM) and Ca^{2+} (2 mM). Data are expressed as the mean relative spreading ± SD of at least three independent experiments, in which the spreading in the presence of Mg^{2+} and Ca^{2+} at each pH was considered as 100%.

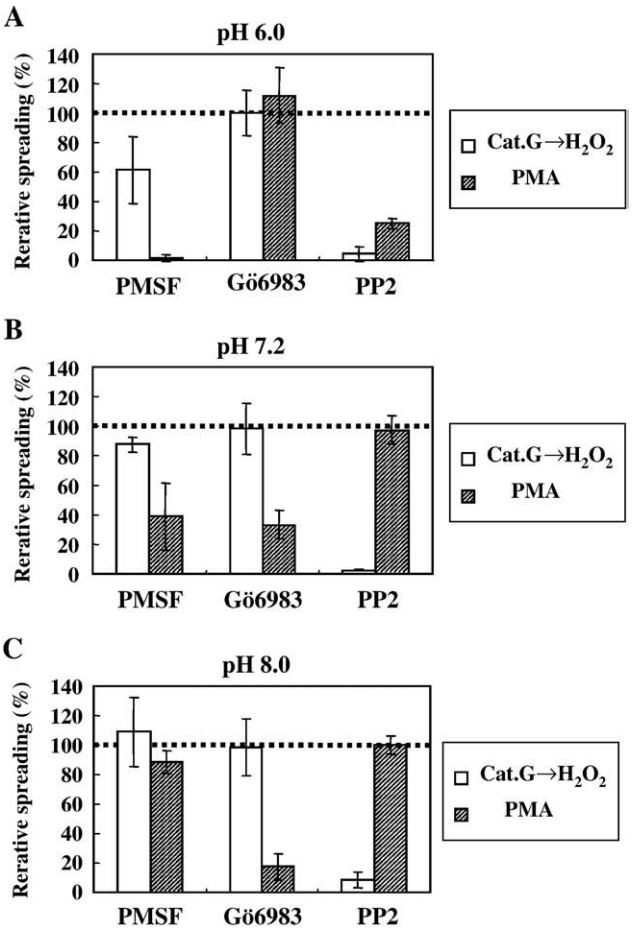

Fig. 5. Effect of pharmacological inhibitors on two patterns of polymorphonuclear leukocyte spreading at (A) pH 6.0, (B) 7.2, and (C) 8.0. Cells were pretreated with 800 μM PMSF, 10 μM Gö6983, and 30 μM PP2 respectively for 15 min (PMSF) or 60 min (Gö6983 and PP2) at room temperature before plating onto un-denatured or 6 mU/ml cathepsin G-treated fibrinogen surfaces. Spreading was induced by stimulation for 30 min with 10 ng/ml PMA (un-denatured fibrinogen surfaces) or 400 μM H_2O_2 (protease-treated fibrinogen surfaces) in the presence of Mg^{2+}/ Ca^{2+}. Data are expressed as the mean relative spreading ± SD of at least three independent experiments, in which the spreading in the absence of inhibitors at each pH was considered as 100%.

induced polymorphonuclear leukocyte spreading in a dose-dependent fashion (Fig. 3).

3.4. Spreading of H_2O_2-stimulated polymorphonuclear leukocytes over protease-treated fibrinogen surfaces requires extracellular Ca^{2+} as well as Mg^{2+} not only at pH 6.0 but also at pH 7.2 and 8.0

We tested the requirement for extracellular Ca^{2+} for the spreading of H_2O_2-stimulated polymorphonuclear leukocytes over immobilized and protease-treated fibrinogen surfaces, because we had previously found that PMA-induced polymorphonuclear leukocyte spreading over un-denatured (i.e. not pre-denatured) fibrinogen surfaces requires extracellular Ca^{2+} only at pH 6.0, and that extracellular Ca^{2+} is dispensable at pH 7.2 or 8.0 (Suzuki et al., 2008). As shown in Fig. 4, H_2O_2-stimulated polymorphonuclear leukocytes underwent little spreading over protease-treated fibrinogen surfaces in the presence of extracellular Ca^{2+} or Mg^{2+} alone under all three pH conditions.

3.5. Spreading of H_2O_2-stimulated polymorphonuclear leukocytes over protease-treated fibrinogen surfaces is more PMSF-resistant than is PMA-induced spreading over non-pre-denatured fibrinogen surfaces

To determine whether serine proteases have any role other than fibrinogen digestion in induction of spreading, the effect of PMSF on the spreading of H_2O_2-stimulated polymorphonuclear leukocytes over protease-treated fibrinogen surfaces was investigated. At pH 6.0 and 7.2, spreading was found to be more resistant to PMSF than was PMA-induced spreading (Fig. 5A and B). However, it is notable that PMSF still attenuated spreading over "pre-digested fibrinogen surfaces" at pH 6.0. PMSF hardly interfered with the spreading of H_2O_2-stimulated polymorphonuclear leukocytes over protease-treated fibrinogen surfaces, or with PMA-induced spreading over un-denatured fibrinogen, at pH 8.0 (Fig. 5C).

3.6. Src-family protein tyrosine kinases, but not PKC, are involved in the spreading of H_2O_2-stimulated polymorphonuclear leukocytes over protease-treated fibrinogen surfaces

One of our major interests is in whether the spreading of H_2O_2-stimulated polymorphonuclear leukocytes over protease-treated fibrinogen surfaces depends on PKC activity, because we previously found that PKC activity is required for PMA-induced spreading at pH 7.2 or 8.0, but not at pH 6.0 (Suzuki et al., 2008). The results of relevant experiments are shown in Fig. 5A–C. Consistently, the specific but not isozyme-selective PKC inhibitor Gö6983 markedly inhibited PMA-induced spreading at pH 7.2 or 8.0, but not pH 6.0. In contrast, H_2O_2-triggered spreading over protease-treated fibrinogen surfaces was completely resistant to Gö6983 under all three pH conditions.

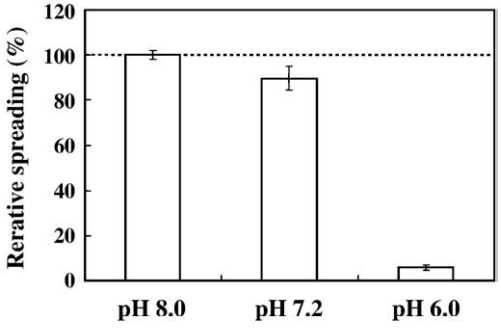

Fig. 6. Effect of PMA on the spreading of H_2O_2-stimulated polymorphonuclear leukocytes over protease-treated fibrinogen surfaces at pH 6.0, 7.2, and 8.0. Immobilized fibrinogen surfaces were pretreated by 6 mU/ml cathepsin G for 90 min at 37 °C before plating of cells. Spreading was induced by stimulation with 400 μM H_2O_2 and Mg^{2+}/Ca^{2+} for 30 min at 37 °C in the presence or absence of 10 ng/ml PMA. Data are expressed as the mean relative spreading ± SD of at least three independent experiments, in which the spreading in the absence of PMA at each pH was considered as 100%.

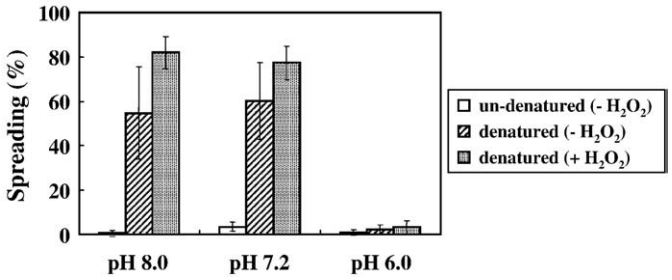

Fig. 7. Spreading of polymorphonuclear leukocytes over fibrinogen surfaces denatured by non-proteolytic long time incubation at pH 6.0, 7.2, and 8.0. Immobilization of fibrinogen was performed by incubation of fibrinogen solution (1 mg/ml in PBSG) with plastic well for 24 h at room temperature in order to denature by non-proteolytic process. As a negative control, un-denatured fibrinogen surfaces were also prepared by immobilization for 2 h at room temperature. Cells were then plated onto the fibrinogen surfaces and incubated for 30 min at 37 °C in the presence of Mg^{2+}/Ca^{2+}, and with or without of 400 μM H_2O_2. All data are the mean ± SD of at least three independent experiments.

It has been shown that H_2O_2-induced functional activation is mediated by Src-family protein tyrosine kinases in many types of cells, including polymorphonuclear leukocytes (Yan and Berton, 1996; Abe et al., 1997; Aikawa et al., 1997; Sato et al., 2001; Rosado et al., 2004; Catarzi et al., 2005; Saksena et al., 2008). Therefore, the inhibitory effect of a specific Src kinase inhibitor, PP2, on H_2O_2-triggered polymorphonuclear leukocyte spreading over protease-treated fibrinogen surfaces was assessed and compared with its effect on PMA-induced spreading over non-pre-denatured fibrinogen surfaces. As expected, PP2 impaired H_2O_2-triggered spreading over protease-treated fibrinogen surfaces under all three pH conditions (Fig. 5A-C). Importantly, PP2 also impaired PMA-induced spreading over non-pre-denatured fibrinogen surfaces at pH 6.0 (Fig. 5A). In contrast, PMA-induced spreading was unaffected by the inhibitor at pH 7.2 and 8.0 (Fig. 5B and C).

3.7. Spreading of H_2O_2-stimulated polymorphonuclear leukocytes over protease-treated fibrinogen surfaces is inhibited by PMA only at pH 6.0

We previously reported that PMA-induced polymorphonuclear leukocyte spreading over non-pre-denatured fibrinogen surfaces is inhibited by H_2O_2 at pH 6.0, but not at pH 7.2 or 8.0 (Suzuki et al., 2008), leading us to investigate whether spreading of H_2O_2-stimulated cells over protease-treated fibrinogen surfaces is inhibited by PMA. As shown in Fig. 6, H_2O_2-triggered spreading was strongly inhibited by PMA at pH 6.0, but was unaffected by PMA at pH7.2 and 8.0.

3.8. H_2O_2-stimulated polymorphonuclear leukocytes do not spread at pH 6.0 when plated onto immobilized fibrinogen denatured by non-proteolytic long-term incubation

It has been reported that the epitopes (specifically P1/P2) within fibrinogen for β2-integrins can also be exposed spontaneously by continuous incubation following immobilization at room temperature without proteolytic digestion (Hu et al., 2001). Consequently, we examined whether H_2O_2-stimulated polymorphonuclear leukocytes spread over immobilized fibrinogen pre-denatured by the non-proteolytic process. In our experiments, non-proteolytic denaturation following immobilization of fibrinogen was performed by incubation of fibrinogen solution within plastic wells for 24 h at room temperature. The results are shown in Fig. 7. At pH 7.2 and 8.0, non-stimulated cells showed marked spreading over the denatured fibrinogen surfaces, and H_2O_2-stimulated cells showed more stable spreading. At pH 6.0, however, neither resting nor H_2O_2-stimulated cells could spread over the denatured fibrinogen surfaces.

4. Discussion

Previously, we reported that PMA-induced polymorphonuclear leukocyte spreading over non-pre-denatured fibrinogen surfaces at pH 6.0 has characteristics distinct from those of spreading at pH 7.2 or 8.0 (Suzuki et al., 2008). We found in the previous study that 1) spreading at acidic pH, but not at neutral or alkaline pH, requires extracellular Ca^{2+}, 2) spreading at acidic pH does not require PKC activity, and 3) spreading at acidic pH is suppressed by H_2O_2 produced by activated NADPH oxidase or added exogenously. The objective of the present study was to clarify the unique mechanism of polymorphonuclear leukocyte spreading under acidic conditions.

The observations of Trevani et al. (1999) are relevant to the interpretation of our findings. Although environmental acidification has been thought generally to down-regulate many functions of polymorphonuclear leukocytes, such as spreading, phagocytosis, chemotaxis, and respiratory burst (Nasmith and Grinstein, 1986; Araki et al., 1991; Grinstein et al., 1991; Hofirek et al., 1995; Demaurex et al., 1996), Trevani et al. reported neutrophil activation by extracellular acidification. In particular, we focused on their observation that release of myeloperoxidase, a primary granule enzyme, is enhanced in neutrophils stimulated by formyl-Met-Leu-Phe (FMLP) under acidic conditions. Because some fibrinogenolytic serine proteases such as elastase and cathepsin G are also primary granule enzymes, their observation prompted us to hypothesize that release of these proteases in an acidified environment (pH 6.0) followed by proteolytic digestion of immobilized fibrinogen may cause exposure of the epitopes in fibrinogen for β_2-integrins, and that polymorphonuclear leukocyte spreading over immobilized fibrinogen at pH 6.0, but not at pH 7.2 or 8.0, may depend on proteolysis-mediated modification of fibrinogen. Our hypothesis is supported by the initial observations shown in Fig. 1A and B that PMSF inhibited PMA-induced spreading over non-pre-denatured fibrinogen surfaces at pH 6.0 more than at pH 7.2, and not at all at pH 8.0, and that by immunoblot analysis serine protease-mediated fragmentation of immobilized fibrinogen by PMA-stimulated polymorphonuclear leukocytes was detected clearly at pH 6.0 and faintly at pH 7.2, but not at pH 8.0.

Recently, Vorup-Jensen et al. reported that protease digestion of fibrinogen surfaces results in exposure of epitopes for β_2-integrins, followed by induction of β_2-integrin-dependent adhesion of non-stimulated neutrophils (Vorup-Jensen et al., 2005). In agreement with their observation, we also found that fibrinogen surfaces pre-digested by neutrophil serine proteases induced spreading of non-stimulated polymorphonuclear leukocytes to some extent at pH 7.2 and 8.0 (Fig. 2B). Importantly, polymorphonuclear leukocytes were found to spread over the protease-treated fibrinogen surfaces also at pH 6.0 when the cells were further stimulated with H_2O_2, although neither fibrinogenolysis nor cellular stimulation with H_2O_2 were alone sufficient to induce spreading at acidic pH (Fig. 2A). It was also found that neither non-stimulated nor H_2O_2-stimulated cells were able to spread at pH 6.0 when fibrinogen was denatured spontaneously by non-proteolytic long-term incubation at room temperature, whereas both non-stimulated and H_2O_2-stimulated cells were able to spread on this substrate at pH 7.2 and 8.0 (Fig. 7). The difference in efficiency of cell spreading at pH 6.0 over protease-treated fibrinogen surfaces or over those denatured without proteolysis may result from exposure of different epitopes for β_2-integrins. P1 and P2 of the fibrinogen γ chain are the principal epitopes, and have been reported to be exposed by immobilization-induced denaturation as well as by proteolytic digestion (Ugarova et al., 1998; Hu et al., 2001; Lishko et al., 2002). However, several novel binding sites for β_2-integrins have recently been reported (Lishko et al., 2001; Lishko et al., 2004; Vorup-Jensen et al., 2005). Among these, the study of Vorup-Jensen et al. described above is of particular relevance to our proposal. An intriguing finding of their study is that guanidine-treated (denatured without proteolysis) fibrinogen supports adhesion of non-stimulated β_2-integrin-expressing K562 cells, whereas integrin activation with Mn^{2+} is required for adhesion to protease-treated fibrinogen surfaces, further suggesting proteolysis-induced exposure of epitopes that are distinct from those exposed by denaturation via non-proteolytic processes. Identification of the epitopes in the fibrinogen molecule responsible for spreading of H_2O_2-stimulated cells over protease-treated fibrinogen surfaces as well as for PMA-induced spreading over non-pre-denatured fibrinogen surfaces was not performed in this study.

Extracellular Ca^{2+} has been shown to be dispensable for polymorphonuclear leukocyte adhesion and/or spreading (Kruskal et al., 1986; Yan et al., 1995; Suzuki and Namiki, 2007). Our present data (Fig. 4) clearly show that extracellular Ca^{2+} as well as Mg^{2+} is required for spreading over protease-treated fibrinogen surfaces, irrespective of pH. It is noteworthy that Ca^{2+}-containing medium has been used in experiments to induce polymorphonuclear leukocyte adhesion to immobilized fibrinogen fragments (Ugarova et al., 1998; Yakubenko et al., 2001; Vorup-Jensen et al., 2005). Especially, Vorup-Jensen et al. showed that binding of the I domains in β_2-integrin α subunit to immobilized fibrinogen with or without proteolytic denaturation requires Mg^{2+} but not Ca^{2+} in cell-free experiments, whereas both divalent cations are required for adhesion of intact cells. On the other hand, Pettit and Hallet (1996) reported that polymorphonuclear leukocyte spreading is preceded by Ca^{2+} influx from the extracellular milieu following integrin engagement. They further reported that photolytic generation of a Ca^{2+} rise in neutrophils after integrin engagement by use of the intracellular Ca^{2+} chelator nitr5 rapidly triggered spreading (Pettit and Hallet, 1998). Together, these observations suggest that extracellular Ca^{2+} may be required for influx into cells following integrin engagement and may be the cause of cell shape change rather than integrin binding itself, and that PMA may bypass the signaling step or trigger distinct signaling pathways leading to spreading at pH 7.2 and 8.0. Requirement for extracellular Ca^{2+} is therefore not necessarily peculiar to polymorphonuclear leukocyte spreading at acidic pH.

In order to further characterize the spreading of H_2O_2-stimulated polymorphonuclear leukocytes over protease-treated fibrinogen surfaces, sensitivity of the spreading to several pharmacological inhibitors was assessed and compared with that of PMA-induced spreading over non-pre-denatured fibrinogen surfaces under each condition of pH (Fig. 5A–C). Even though H_2O_2-induced spreading over protease-treated fibrinogen surfaces at pH 6.0 was attenuated by PMSF, it was clearly more resistant to PMSF than was PMA-induced spreading over non-pre-denatured fibrinogen surfaces at the same pH. It is therefore possible that, at pH 6.0, fibrinogenolytic serine proteases such as elastase and cathepsin G released by cellular activation are the major participants in PMA-induced spreading. It is possible that H_2O_2-stimulated polymorphonuclear leukocytes plated onto protease-treated fibrinogen surfaces newly release serine proteases and help further digestion of fibrinogen, although serine protease(s) may also be involved in processes other than fibrinogenolysis.

Another important finding in this comparative study is that H_2O_2-induced spreading over protease-treated fibrinogen surfaces was not inhibited by the PKC inhibitor Gö6983 irrespective of pH, whereas PMA-induced spreading was resistant against the inhibitor only at pH 6.0 but was suppressed at pH 7.2 or 8.0. Furthermore, the Src-family protein tyrosine kinase inhibitor PP2 abrogated H_2O_2-induced spreading over protease-treated fibrinogen surfaces irrespective of pH, whereas it inhibited PMA-induced spreading only at pH 6.0 but had no effect at pH 7.2 and 8.0. These data suggest that Src, but not PKC, participates in H_2O_2-induced spreading over protease-treated fibrinogen surfaces at all pH values, and in PMA-induced spreading only at pH 6.0; conversely, PKC, but not Src, participates in PMA-induced spreading at pH 7.2 and 8.0. The results of these experiments with PP2 were in accordance with our predictions and are consistent with previous reports that Src is required for β_2-integrin-mediated

outside-in signaling leading to spreading, whereas it is dispensable for integrin-activating inside-out signaling (Giagulli et al., 2006), that H_2O_2 stimulates the activity of Src in polymorphonuclear leukocytes (Yan and Berton, 1996), and that Src is dispensable for PMA-induced polymorphonuclear leukocyte spreading (Lowell et al., 1996).

Collectively, our present findings suggest that PMA activation of polymorphonuclear leukocytes at pH 6.0 induces release of serine proteases followed by fibrinogenolysis and production of H_2O_2 in parallel, leading to cell spreading in a Src-dependent but PKC-independent fashion. This mechanism may be less involved in PMA-induced spreading at pH 7.2 and 8.0, in which another pathway mediated by PKC but not by Src may operate. However, two important questions remain unresolved. First, do polymorphonuclear leukocytes really mediate fibrinogenolysis in a PKC-independent fashion at pH 6.0, leading to exposure of distinctive epitopes to which they can respond? In preliminary experiments, Gö6983 appeared to suppress visible fibrinogen digestion by PMA-activated polymorphonuclear leukocytes at pH 6.0, apparently in conflict with the proposal above (K. Suzuki, unpublished). Unfortunately, we failed to determine whether H_2O_2-stimulated polymorphonuclear leukocytes can spread over immobilized fibrinogen pretreated with proteases released by PMA-activated cells, since residual PMA in the culture supernatant was adsorbed by the fibrinogen surface and could not be removed completely in spite of extensive washing. Furthermore, PMA pre-removal by ultrafiltration was accompanied by severe loss of protease activity. Establishment of a direct detection method for, following definitive identification of, the distinctive epitope exposed by fibrinogen proteolysis that is responsible for polymorphonuclear leukocyte adhesion under acidic conditions will be important for our future studies. Nevertheless, PKC activity is likely be dispensable for the release of serine proteases from PMA-activated polymorphonuclear leukocytes required for minimal proteolytic modification of fibrinogen surfaces enabling them to spread, since there is evidence that PKC has little or no involvement in exocytosis of primary granules or serine proteases from polymorphonuclear leukocytes (Dewald et al., 1989; Cabanis et al., 1996; Abdel-Latif et al., 2005; Korchak et al., 2007). Secondly, do polymorphonuclear leukocytes really produce sufficient amounts of H_2O_2 to induce spreading when activated by PMA at pH 6.0? We previously estimated that PMA-induced H_2O_2 release into the culture medium at pH 6.0 is about 7.4 μM/30 min, which is 30-35% of the release at pH 7.2 or 8.0 (Suzuki et al., 2008). Furthermore, Gö6983 strongly inhibits PMA-induced H_2O_2 release irrespective of pH, indicating a PKC-dependent phenomenon, which is consistent with other reports (Dewald et al., 1989; Chen et al., 2003; Abdel-Latif et al., 2005; Korchak et al., 2007). Our observation that Gö6983 did not impair PMA-induced spreading at pH 6.0 may therefore conflict with the proposal that PMA-activated polymorphonuclear leukocytes at pH 6.0 undergo spreading in a "PKC-independent" fashion by utilizing H_2O_2 they produced in a "PKC-dependent" fashion. However, it is also possible that much higher concentrations of H_2O_2 are present in the local microenvironment of the target molecule which triggers the signaling pathway leading to spreading. Moreover, H_2O_2 may be produced by another mechanism than the conventional PKC-dependent NADPH oxidase, because we have observed previously that PMA provokes some intracellular H_2O_2 accumulation that is resistant to the NADPH oxidase inhibitor diphenylene iodonium (DPI), even though the inhibitor completely abrogates extracellular H_2O_2 release (Suzuki et al., 2008). At present, we cannot exclude the other possibility that PMA stimulation of polymorphonuclear leukocytes at pH 6.0 triggers an alternative non-H_2O_2-mediated intracellular signaling mechanism leading to Src activation.

We also reported previously that PMA-induced polymorphonuclear leukocyte spreading is down-regulated by H_2O_2 produced by NADPH oxidase or added exogenously only at pH 6.0, suggesting that H_2O_2 is responsible for negative feedback regulation of spreading (Suzuki et al., 2008). In the present study, we found that H_2O_2 acts as an inducer but not as a suppressor of spreading over protease-treated fibrinogen surfaces at pH 6.0. Importantly, it was also found that PMA strongly inhibited H_2O_2-induced spreading only at pH 6.0 (Fig. 6). These observations may be explained by a biphasic effect of H_2O_2 on polymorphonuclear leukocyte spreading: although H_2O_2 is a robust trigger of a pH-independent signaling pathway leading to spreading, PMA and possibly other appropriate stimuli may enable another acidic pH-specific and H_2O_2-mediated pathway that operates to inhibit spreading.

It also seems curious that the level of spreading at pH 6.0, induced by digested fibrinogen/H_2O_2 as well as by PMA, was substantially lower than those at pH 7.2 and 8.0 (Figs. 1 and 2). At present, the difference in spreading efficiency between under acidic and neutral/alkaline pH conditions remains to be explained. One possibility is that "senescent" cells are difficult to undergo spreading under acidified, severe conditions. Polymorphonuclear leukocytes have the shortest lifespan of all leukocytes, and apoptotic (senescent) cells are known to lose their ability of functional activation, including degranulation and spreading (Lee et al., 1993; Haslett et al., 1994). We expect that, in order to avoid uncontrolled inflammatory responses of senescent cells, functional longevity of them may be severely restrained in an acidic milieu such as inflammatory sites. Causal relationship between progression of apoptosis and ability to undergo spreading in each pH conditions is currently under investigation.

In conclusion, we found for the first time that polymorphonuclear leukocytes are able to spread over fibrinogen surfaces in an acidic environment when two conditions are fulfilled: the fibrinogen should be digested by proteases to expose the relevant epitopes for β2-integrins, and the cells should be stimulated by H_2O_2. This mechanism also appears to operate in spreading at neutral/alkaline pH, but contributes less to PMA-induced spreading under these pH conditions. We have attempted to confirm the hypothesis that polymorphonuclear leukocytes employ distinctive functional regulatory systems adapted to an acidified environment, because the setting in which they exert their microbicidal action in vivo is acidified and weakly buffered extravascular inflammatory tissues rather than the well-buffered intravascular milieu. The present findings are important for understanding of the regulatory mechanisms of polymorphonuclear leukocyte functional activation in the acidified environment typical of inflammatory sites. It is now clear that there is a causal relationship between formation of fibrinogen deposits and promotion of polymorphonuclear leukocyte-mediated inflammatory responses (Tang et al., 1996; Hu et al., 2001; Vorup-Jensen et al., 2005; Flick et al., 2007). Moreover, it has been reported that polymorphonuclear leukocytes release destructive proteases leading to fibrinogen degradation in inflammatory tissues in vivo (Bos et al., 1997; Trevani et al., 1999; Coakley et al., 2000). We anticipate that our demonstration of polymorphonuclear leukocyte functional activation in an acidified milieu mediated by digested fibrinogen deposits and H_2O_2 will form the basis of numerous future studies, and will contribute to new strategies aimed at controlling polymorphonuclear leukocyte-mediated inflammation and tissue injury.

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