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Characterization of the unique regulatory mechanisms of phorbol ester-induced polymorphonuclear leukocyte spreading in an acidified environment

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ABSTRACT

In vitro studies have shown that acidic conditions impair spreading of polymorphonuclear leukocytes, which is prerequisite for activation of microbicidal functions. However, the mechanisms by which pH affects polymorphonuclear leukocytes functions remain obscure. Moreover, *in vitro* observations seem to contradict the fact that an acidic microenvironment often prevails at sites of inflammation where polymorphonuclear leukocytes must function for host defense. In the present study, we found three peculiar characteristics of porcine polymorphonuclear leukocyte that had been induced to spread over fibrinogen-coated surfaces by phorbol 12-myristate 13-acetate (PMA) in acidified medium. First, the PMA-induced spreading at acidic pH, but not at neutral/alkaline pH, was dependent on extracellular Ca^{2+}. Second, the spreading at acidic pH was independent of protein kinase C (PKC), whereas that at neutral/alkaline pH was strictly PKC-dependent. Finally, the spreading at acidic pH, but not at neutral/alkaline pH, was suppressed by H_2O_2 produced by activated NADPH oxidase or added exogenously. As a result, polymorphonuclear leukocyte spreading at acidic pH peaked at 30 min after PMA stimulation, and declined thereafter because of negative regulation triggered by accumulated H_2O_2, whereas that at neutral/alkaline pH was stable for at least 90 min. The NADPH oxidase inhibitor diphenyleneiodonium or the H_2O_2-degradation enzyme catalase consistently stabilized the spreading at acidic pH. We conclude that PMA-stimulated polymorphonuclear leukocytes spread in an acidic environment through a mechanism different from that under neutral/alkaline conditions. This H_2O_2-mediated negative regulation system in an acidic environment may be crucial for avoiding tissue-damaging inflammatory actions of accumulated polymorphonuclear leukocytes *in vivo*.

1. Introduction

Polymorphonuclear leukocytes, most of which are neutrophils, are the most abundant type of leukocyte and play a critical role in host defense against infection. However, polymorphonuclear leukocytes are also implicated in tissue-damaging inflammatory reactions resulting in the progression of many inflammatory diseases such as ulcerative colitis, Crohn's disease, systemic inflammatory response syndrome and others (Parkos et al., 1996; Kaneider et al., 2006; Lee et al., 2006). Accordingly, highly regulated activation of polymorphonuclear leukocytes is important for successful host defense. Polymorphonuclear leukocytes circulating in peripheral blood are quiescent and non-adherent, but become activated in response to various stimuli including cytokines, formyl-methionyl-leucyl-phenylalanine (fMLP), and phorbol esters (Graham et al., 1994). Activated polymorphonuclear leukocytes adhere to and spread over endothelial cells and extracellular matrix, followed by transmigration in a β2-integrin-dependent manner. At sites of infection, polymorphonuclear leukocytes express a variety of microbicidal-related functions such as phagocytosis, secretion of destructive enzymes, and H_2O_2 production (Nathan, 1987; Yan et al., 1995; Kaneider et al., 2006). Importantly, it has been shown that β2-integrin-dependent polymorphonuclear leukocyte adhesion/spreading is an absolute prerequisite not only for polymorphonuclear leukocyte recruitment in tissues but also for activation of their microbicidal functions (Nathan, 1987; Yan et al., 1995; Jenei et al., 2006).

Extensive *in vitro* studies have shown that soluble stimuli activate "inside-out signaling" leading to an increase in the affinity/avidity of integrins for their ligands, and that integrin engagement provides a second "outside-in signaling" mechanism leading to reorganization of the actin cytoskeleton (Lowell et al., 1996; Mócsai et al., 2002; Gakidis et al., 2004). Protein kinase C (PKC) has been implicated in both "inside-out" and in "outside-in" signaling (Merrill et al., 1990; Dumont and Bitonti, 1994; Lowell et al., 1996; Laudanna et al., 1998; Korchak et al., 1998; Takami et al., 2002; Mócsai et al., 2002; Jenei et al., 2006; Suzuki and Namiki, 2007).

Release of H_2O_2 from adherent polymorphonuclear leukocytes is accomplished by activation of NADPH oxidase complex (Korchak et al., 1998; Ago et al., 1999; Zhan et al., 2004). During the activation process,

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extensive phosphorylation of a cytosolic component $p47^{phox}$ enables it to translocate to the plasma membrane, bringing the other cytosolic components to the $p22^{phox}$ subunit of the catalytic core flavocytochrome b_{558}.

Polymorphonuclear leukocyte functions are affected or regulated by changes in intracellular pH (pHi). Generally, cytoplasmic acidification impairs cell spreading, H_2O_2 production, chemotaxis, and aerobic bacterial killing (Nasmith and Grinstein, 1986; Grinstein et al., 1988, 1991; Araki et al., 1991; Hofirek et al., 1995; Demaurex et al., 1996; Suzuki and Namiki, 2007). On the other hand, cytoplasmic alkalization induced by an increase of extracellular pH or by cytokine-induced priming up-regulates these polymorphonuclear leukocyte functions (Araki et al., 1991; Sullivan, 1992; Yuo et al., 1993; Saeki et al., 1998; Suzuki and Namiki, 2007). The molecular mechanisms by which changes in pHi affect polymorphonuclear leukocyte functions have long been obscure. We previously reported that in polymorphonuclear leukocytes suspended in pH-adjusted HEPES–saline containing Mg^{2+} but not Ca^{2+}, cytoplasmic pre-alkalization per se up-regulates PKC membrane distribution and F-actin assembly, providing a potential explanation for the pH-regulation of polymorphonuclear leukocyte spreading (Suzuki and Namiki, 2007).

Here, a problem appears to arise. The homeostasis of pHi in polymorphonuclear leukocytes is often challenged *in vivo*. Acidification of the microenvironment often prevails at sites of inflammation or tumors where polymorphonuclear leukocytes must function for host defense (Sawyer et al., 1991; Nanda et al., 1992; McLean et al., 2000; Coakley et al., 2002; Verrière et al., 2005). Environmental acidification can be caused mainly by metabolic acid released by surrounding host cells, tumor cells, and infecting bacteria. Furthermore, accumulated polymorphonuclear leukocytes themselves generate large amounts of acid equivalents by NADPH oxidase-induced oxidation of NADPH. Polymorphonuclear leukocytes regulate their pHi by bicarbonate-independent Na^+/H^+ exchange (NHE) and by bicarbonate-dependent Cl^-/HCO_3^- exchange (Grinstein et al., 1991). In the acidic microenvironment, however, the activity of NHE can be severely impaired due to competition between H^+ and Na^+ for binding to the external face of the transport site (Simchowitz, 1985; Nanda et al., 1992). Moreover, pHi regulation through Cl^-/HCO_3^- exchange is also impeded because of reduced external HCO_3^- (Nanda et al., 1992; McLean et al., 2000). Although polymorphonuclear leukocytes have other pHi-regulating system "vacuolar-type H^+ pumps" (V-type ATPases) (Grinstein et al., 1991; Nanda et al., 1992; Coakley et al., 2002; Galkina et al., 2006), the ability of the various pHi regulatory systems may be overcome, leading to cytoplasmic acidification, particularly in the microenvironment associated with abscesses (Grinstein et al., 1991). These circumstances seem to prevent accumulated polymorphonuclear leukocytes from becoming appropriately active, so that they are unable to kill microbes or tumor cells. In some cases, dysregulation of polymorphonuclear leukocyte responses may actually occur, resulting in necrosis and tissue destruction (Coakley et al., 2002).

Because the field in which polymorphonuclear leukocytes exert their anti-microbial action *in vivo* is not the well-buffered intravascular milieu but acidified and weakly buffered extravascular inflammatory tissues, it might be expected that polymorphonuclear leukocytes employ some peculiar functional regulation systems adapted to the acidified environment. Clarification of the true regulatory mechanisms of polymorphonuclear leukocyte functional activation considering the environmental pH is therefore required in order to precisely understand the polymorphonuclear leukocyte-mediated inflammatory responses *in vivo* and to therapeutically target polymorphonuclear leukocytes for control of inflammation and tissue injury.

In this study, we searched for the conditions in which phorbol 12-myristate 13-acetate (PMA)-stimulated polymorphonuclear leukocytes can undergo spreading over fibrinogen-coated surfaces in acidified, nominally HCO_3^--free medium. Here we report the unique mechanisms of induction of polymorphonuclear leukocyte spreading in an acidic milieu, which are distinct from neutral or alkaline pH in their dependency on extracellular Ca^{2+} and PKC, and negative regulation by H_2O_2 produced by NADPH oxidase.

2. Materials and methods

2.1. Reagents and antibodies

Phenylmethanesulfonicfluoride (PMSF), porcine fibrinogen, scopoletin, 2′,7′-dichlorofluorescein (DCF), diphenyleneiodonium chloride (DPI) and catalase were purchased from Sigma (St. Louis, MO, USA). 2-[1-(3-dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1*H*-indol-3-yl)maleimide (Gö6983), and 2′,7′-dichlorofluorescin diacetate (DCFH-DA) were from Calbiochem (San Diego, CA, USA). Methyl cellulose 25cP, horseradish peroxidase (HRP) and PMA were from Wako Pure Chemical (Tokyo, Japan). EDTA-free Complete Mini protease inhibitor cocktail (tablets) was from Roche Diagnostics (Mannheim, Germany). 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). Goat polyclonal antibody against $p47^{phox}$, rabbit polyclonal antibody against $p22^{phox}$, and HRP-conjugated goat anti-rabbit IgG antibody were from Santa Cruz biotechnology (Santa Cruz, CA). HRP-conjugated horse anti-goat IgG antibody was from Vector Laboratories (Burlingame, CA).

2.2. Isolation of polymorphonuclear leukocytes

Polymorphonuclear leukocytes were isolated routinely from porcine peripheral blood by hypotonic lysis following methyl cellulose sedimentation and centrifugation through Ficoll-Paque as described previously (Suzuki and Namiki, 2007). Cell viability was checked by trypan blue exclusion. More than 95% were polymorphonuclear leukocytes and more than 98% were viable. Polymorphonuclear leukocytes were suspended in HEPES-buffered saline (named "HEPES–NaCl" containing 10 mM HEPES (pH 7.2), 140 mM NaCl and 5 mM glucose) and used for experiments immediately. The polymorphonuclear leukocyte isolation was performed under strict control of temperature and time of work in order to avoid non-specific polymorphonuclear leukocyte activation during the isolation process.

2.3. Cell spreading

Fibrinogen-coated culture plate was prepared by incubation with fibrinogen (1 mg/ml) for 1 h at room temperature followed by washing. Polymorphonuclear leukocyte spreading on fibrinogen-coated surfaces was assessed morphologically as described previously (Suzuki and Namiki, 2007). Polymorphonuclear leukocytes (1.0×10^6 cells/ml) were re-suspended in HEPES–NaCl (pH was adjusted at the indicated values) containing 0.6 mM Mg^{2+} and plated onto fibrinogen-coated culture plate. Cells were pre-incubated in the presence or absence of 2 mM Ca^{2+} and with pharmacological inhibitors or diluent. Subsequently the cells were stimulated with PMA at the indicated concentrations and for the indicated times. Photomicrographs were taken and cells were counted, and those that were phase dark, enlarged, and with irregular shapes were considered spread (see Fig. 1E). Percentages of spread cells were calculated.

2.4. Measurement of extracellular H_2O_2 released by polymorphonuclear leukocytes

Extracellular H_2O_2 was quantified as the HRP-catalyzed oxidation of the fluorescent dye scopoletin (Waddell et al., 1994), with minor modifications. Polymorphonuclear leukocytes (1.0×10^6 cells/ml in pH-adjusted HEPES–NaCl) were stimulated with PMA (10 ng/ml) following