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Pharmacological analysis for mechanisms of GPI-80 release from tumour necrosis factor-α-stimulated human neutrophils

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1 GPI-80, a glycosylphosphatidylinositol (GPI)-anchored protein initially identified on human neutrophils, plays a role(s) in the regulation of β2 integrin function. Previous studies have shown that GPI-80 is sublocated in secretory vesicles. It is also found in soluble form in the synovial fluid of rheumatoid arthritis patients, and in the culture supernatant of formyl-methionyl-leucyl-phenylalanine-stimulated neutrophils. To understand the behaviour of GPI-80 under conditions of stimulation, we investigated the effects of tumour necrosis factor (TNF)-α on its expression and release. We also probed the mechanism of its release with various pharmacologic tools.

2 TNF-α induced the release of GPI-80 from human neutrophils in a concentration- and time-dependent manner (in the range of 1–100 u ml^{-1} and 30–120 min, respectively), but did not affect surface GPI-80 levels.

3 Cytochalasin B, genistein, and SB203580 but not PD98059 inhibited TNF-α-stimulated GPI-80 release and neutrophil adherence at the same concentration. In addition, TNF-α-induced GPI-80 release was inhibited by blocking monoclonal antibodies specific to components of Mac-1 (CD11b and CD18).

4 Antioxidants (pyrrolidine dithiocarbamate and N-acetyl-L-cysteine) inhibited GPI-80 release by TNF-α stimulation, but superoxide dismutase did not. Antioxidants but not superoxide dismutase reduced an intracellular oxidation state.

5 These findings indicate that TNF-α-stimulated GPI-80 release from human neutrophils depends upon adherence *via* β2 integrins. They also suggest that cytochalasin B, genistein, and SB203580 inhibit GPI-80 release by suppressing signals for cell adherence, rather than by a direct effect on its secretion. Finally, we suggest that GPI-80 release involves an intracellular change in a redox state. *British Journal of Pharmacology* (2002) **137,** 353–360. doi:10.1038/sj.bjp.0704860

Keywords: GPI-80; release; TNF-α; neutrophils; cytochalasin B; genistein; SB203580; antioxidants; adherence; β2 integrin

Abbreviations: FCS, foetal calf serum; fMLP, formyl-methionyl-leucyl-phenylalanine; GPI, glycosylphosphatidylinositol; MAP kinase, mitogen-activated protein kinase; NAC, *N*-acetyl-L-cysteine; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; PDTC, pyrrolidine dithiocarbamate; RA, rheumatoid arthritis; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α

Introduction

Neutrophils are known to play important roles in inflammation and innate immunity. They possess granules whose contents include enzymes, adhesion molecules, bactericidal proteins, etc. To infiltrate inflammatory sites, neutrophils use cellular adhesion molecules to cross endothelial cells. They are activated by various stimuli, such as bacterial components, cytokines, and chemical mediators. Activated neutrophils produce superoxide anion, inflammatory cytokines, and chemokines, and release granule components that contribute to defense against microorganisms. These functions mainly depend on cell adhesion (Williams & Solomkin, 1999).

In our series of studies on the role of adhesion in neutrophil function, we cloned the gene for GPI-80. This glycosylphosphatidylinositol (GPI)-anchored protein is expressed mainly on neutrophils and, to a lesser extent, on monocytes (Ohtake *et al*., 1997; Suzuki *et al*., 1999). It shows homology with biotinidase (Suzuki *et al*., 1999), Vanin-1 (Suzuki *et al*., 1999), and pig pantetheinase (Maras *et al*., 1999). Vanin-1, which is thought to participate in lymphocyte homing (Aurrand-Lions *et al*., 1996), has pantetheinase activity (Pitari *et al*., 2000), and it has been suggested that GPI-80 is related to pantetheinase because it contains the primary structure of the active site of the enzyme (Granjeaud *et al*., 1999). We previously showed that a monoclonal antibody against GPI-80 (3H9) modulates human neutrophil adhesion *via* β2 integrin (CD18) (Suzuki *et al*., 1999) and locomotion (Suzuki *et al*., 1997). During chemotaxis, GPI-80 was shown to move toward formyl-methionyl-leucyl-phenylalanine (fMLP), to the forward surface of neutrophil (Nakamura-Sato *et al*., 2000). In addition, GPI-80 is found on the plasma membranes and in the secretory vesicles of human neutrophils (Dahlgren *et al*., 2001). Aggregation of surface GPI-80 increases the level of β2 integrin on the surface (Yoshitake *et al*., 2002) of activated human neutrophils (Yu *et al*., 2000). These results suggest that GPI-80 regulates the function of β2 integrin to modulate neutrophil movement and adherence.

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We recently reported that soluble GPI-80 is present in the synovial fluid of rheumatoid arthritis (RA) patients (Huang *et al*., 2001). Although infiltrated and activated neutrophils are thought to be the source of this soluble GPI-80, the precise causes of GPI-80 release remain unclear. To address this issue, we investigated the behaviour of GPI-80 in neutrophils stimulated by TNF-α, a key modulator of RA (Feldmann *et al*., 2001). We also assessed the possible roles of adhesion and of a change in oxidative condition in GPI-80 release.

Methods

Reagents

Dextran 200 000, crystal violet and paraformaldehyde were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Ficoll-Paque from Pharmacia Biotech Inc. (Uppsala, Sweden), RPMI-1640 medium from Life Technologies (Grand Island, NY, U.S.A.), foetal calf serum (FCS) from ICN Pharmaceuticals, Inc. (Costa Mesa, CA, U.S.A.), TNF-α and Block Ace from Dainipponseiyaku Co. (Osaka, Japan), bovine serum albumin (BSA, fraction V) from Bayer (Kankakee, IL, U.S.A.), TMB substrate solution, cytochalasin B, SB203580, pyrrolidine dithiocarbamate (PDTC), superoxide dismutase (SOD), PD98059, 2′,7′-dichlorofluorescin diacetate from Sigma Chemical Co. (St. Louis, MI, U.S.A.), HRP-conjugated streptavidin from Dako (Glostrup, Denmark), *N*-acetyl-L-cysteine (NAC) from Merck KgaA (Darmstadt, Germany) and genistein from Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.).

Antibodies

Anti-GPI-80 monoclonal antibody (3H9, mouse IgG1) (Ohtake *et al*., 1997) and rabbit anti-GPI-80 polyclonal antibody (Huang *et al*., 2001) were produced as described previously. A monoclonal antibody, TCY-3 (mouse IgG1), specific for *Trypanosoma cruzi*, was used for control studies (Ohtake *et al*., 1997; Suzuki *et al*., 1999). An anti-CD18 monoclonal antibody producing hybridoma TS1/18.1.2.11 was purchased from American Type Culture Collections (Rockville, MD, U.S.A.). Anti-CD18 monoclonal antibody NHM23, anti-CD11b monoclonal antibody 2LPM19c, FITC-conjugated rabbit anti-mouse Ig $F(ab')_2$ antibody and biotinylated swine anti-rabbit IgG were purchased from Dako.

Preparation of human neutrophils

Heparinized venous blood from healthy volunteers was sedimented through Dextran 200 000. The leukocyte-rich supernatant (buffy coat) was centrifuged at $450 \times g$ for 5 min and washed with phosphate-buffered saline (PBS, pH 7.4). Neutrophils were isolated from the buffy coat using Ficoll-Paque, as described previously (Yakuwa *et al*., 1989), and residual erythrocytes were lysed by hypotonic shock. The neutrophils were resuspended in RPMI-1640 medium. The isolated neutrophils were more than 95% pure.

Stimulation of human neutrophils

Human neutrophils were stimulated with TNF-α in the RPMI-1640 medium containing 10% FCS in a 24-well plastic plate (Becton Dickinson Labware, Franklin Lakes, NJ, U.S.A.) at 37°C for the indicated times. In some experiments, neutrophils were stimulated in a microtest tube (Greiner Labortechnik, Frickenhausen, Germany). After stimulation, culture supernatants were collected after centrifugation at $450 \times g$ for 5 min.

Measurement of soluble GPI-80 in conditioned medium

GPI-80 released from human neutrophils was measured according to the methods described previously (Huang *et al*., 2001) with minor modifications. In brief, a 96-well microtiter plate (Nalge Nunc International, Rochester, NY, U.S.A.) was coated with monoclonal antibody 3H9 (10 μg ml^{-1}) at 4°C for 12 h. After blocking with a Block Ace, 50 μl of conditioned medium was added to each well and the plate was incubated at room temperature for 1 h. The plate was washed twice with PBS containing 0.05% Tween 20, and then 10 μg ml^{-1} rabbit anti-GPI-80 polyclonal antibody (Huang *et al*., 2001) was added, and the plate was incubated at room temperature for 30 min. This was followed by incubation with biotinylated goat anti-rabbit polyclonal antibody (1 : 2000 dilution), and with HRP-conjugated streptavidin (1 : 5000 dilution) under the same conditions as for the first polyclonal antibody. TMB substrate solution was added and enzymatic reaction proceeded at room temperature for 30 min. After 1N HCl was added to stop the reaction absorbence at 450 nM and 570 nM was measured. A standard curve was made using GPI-80 purified from human neutrophils (Huang *et al*., 2001). The concentration of GPI-80 was calculated using the standard curve. Low detectable limit is 3 ng ml^{-1}.

Flow cytometry

GPI-80 expression on the surfaces of human neutrophils was measured by flow cytometry. In brief, 1×10^6 cells in 100 μl PBS containing 1% BSA and 0.05% NaN_3 were incubated for 30 min on ice with 1 μg ml^{-1} 3H9 and then fixed with 2% paraformaldehyde. After washing twice with PBS containing 0.1% BSA and 0.05% NaN_3, the fixed cells were incubated for 30 min on ice with FITC-conjugated anti-mouse Ig's $F(ab')_2$ antibody. After washing twice, fluorescence intensity was measured with a FACSCalibur (Beckton Dickinson Immunocytometry Systems, San Jose, CA, U.S.A.). Cell debris was excluded from the analysis by forward and side scatter gating.

Neutrophil adherence assay

Details of this assay have been described elsewhere (Yakuwa *et al*., 1989). Briefly, 96-well plastic plates (Becton Dickinson Labware, Franklin Lakes, NJ, U.S.A.) were coated with 20 μg ml^{-1} fibrinogen for 2 h at 37°C and then washed with PBS. Neutrophils (5×10^5 cells per well) were incubated onto the plates in RPMI-1640 containing 10% FCS, 10 u ml^{-1} TNF-α and various drugs for 30 min at 37°C. Non-adherent cells were thus discarded and stained with 0.05% crystal

violet solution in 3% formaldehyde. Stained cells were lysed by 1% SDS and absorbance at 570 nM was measured.

Measurement of an intracellular oxidative state

Determination of an intracellular oxidative state was performed according to the methods (Bass *et al*., 1983) with minor modifications. Briefly, human neutrophils (1×10^7 cells) were incubated in 10 ml in RPMI-1640 containing 10% FCS and 5 μM 2′,7′-dichlorofluorescin diacetate for 15 min at 37°C with horizontal agitation. After loading the fluorescent dye, the cells were incubated with 10 u ml^{-1} TNF-α and various drugs for 30 min at 37°C and then subjected to flow cytometry analysis as described above.

Statistical analysis

Analysis of the probability of statistical significance between two mean values was performed using Student's *t*-test. A probability of $P<0.05$ was considered statistically significant.

Results

Induction of GPI-80 release from human neutrophils by TNF-α

Soluble GPI-80 was detected in the medium when neutrophils were stimulated with 10 u ml^{-1} TNF-α. In contrast, GPI-80 release was very low in the absence of TNF-α stimulation (Figure 1a). TNF-α stimulation released GPI-80 from neutrophils in a time-dependent manner (Figure 1a). GPI-80 release was observed in a concentration (1 – 100 u ml^{-1})-dependent manner at 60 min after stimulation (Figure 1b).

Effect of TNF-α on GPI-80 expression

Since GPI-80 is detected externally (on the cell surface (Suzuki *et al*., 1999)) and internally (in secretory vesicles (Dahlgren *et al*., 2001)) in neutrophils, we used flow cytometry to examine whether TNF-α affects the expression of surface GPI-80 in non-permeabilized human neutrophils. We found that, unlike the expression of L-selectin (Allport *et al*., 1997; Griffin *et al*., 1990) and of the TNF receptor (Dri *et al*., 2000; Porteu & Hieblot, 1994), the level of GPI-80 on human neutrophils did not decrease with TNF-α stimulation (Figure 2). The surface GPI-80 levels on TNF-α-stimulated cells were almost comparable to those on non-stimulated cells at 30 and 60 min after stimulation (Figure 2).

Effects of various inhibitors on the process of GPI-80 release

We then examined the effects of cytochalasin B (an actin reorganization inhibitor), genistein (a protein tyrosine kinase inhibitor), SB203580 (a p38 mitogen-activated protein (MAP) kinase inhibitor) and PD98059 (an inhibitor of MEK (an activator of extracellular signal-regulated kinases) on TNF-α-stimulated GPI-80 release at 60 min from neutrophils. Cytochalasin B, genistein and SB203580 diminished GPI-80 release in a concentration-dependent manner (Figure 3a). For each agent, TNF-α-stimulated neutrophil adherence to plastic

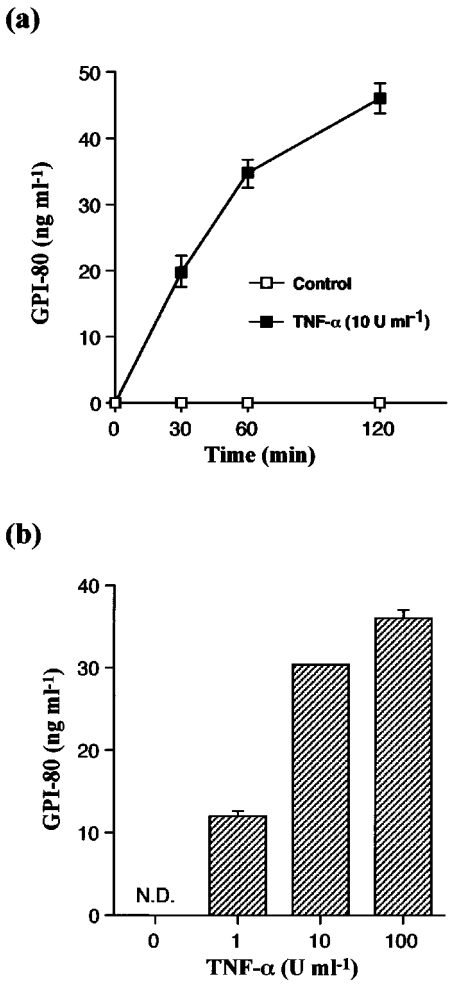


Figure 1 Induction of GPI-80 release from human neutrophils by TNF-α. Human neutrophils were stimulated with or without 10 u ml^{-1} TNF-α for the time indicated (a) and with various concentrations of TNF-α for 60 min (b). GPI-80 in the medium was measured by ELISA, using antibody specific to GPI-80. Vertical bars represent s.e.mean from triplicate measurements. Results are representative of three similar experiments.

plates and GPI-80 release were inhibited at the same concentration (Figure 3b). On the contrary, PD98059 did not affect either GPI-80 release (Figure 3a) or neutrophil adherence (Figure 3b). These drugs did not show any cytotoxic effect on neutrophil viability (data not shown). These results suggest that GPI-80 release have certain relations to neutrophil adhesion.

Involvement of adherence via *β2 integrin in GPI-80 release*

We hypothesized that these drugs inhibited GPI-80 release by suppressing neutrophil adherence. Therefore, we used blocking antibodies to a Mac-1 component to investigate whether GPI-80 release from TNF-α-stimulated human neutrophils is dependent on adherence *via* Mac-1. When neutrophils were stimulated with TNF-α, TS1/18 and NHM23 (blocking antibodies to CD18 (Arnaout *et al*., 1988; Tonnesen *et al*., 1989)) and 2LPM19c (a blocking antibody to CD11b (Wong & Luk, 1997)) inhibited GPI-80 release in a concentration-

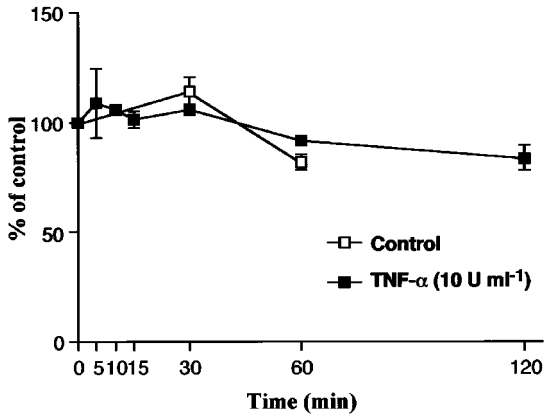


Figure 2 Expression of GPI-80 on the surface of human neutrophils during TNF-α stimulation. Human neutrophils were stimulated with or without 10 u ml^{-1} TNF-α for the time indicated, analysed by flow cytometry after treatment with 3H9, then stained with FITC-conjugated anti-mouse IgG. Values are relative to mean fluorescence intensity at 0 min. Vertical bars represent s.e.mean from three independent experiments.

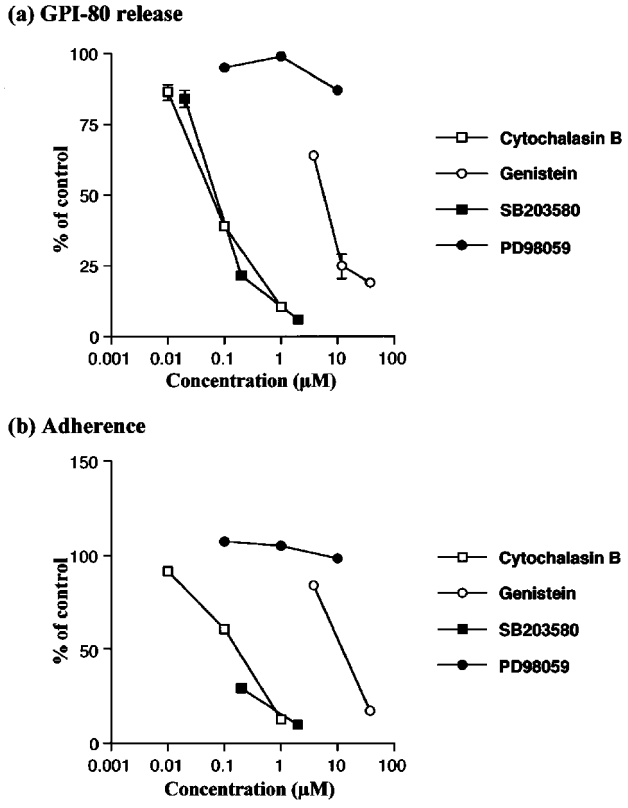


Figure 3 Inhibition of GPI-80 release and adherence in human neutrophils by cytochalasin B, genistein and SB203580. Human neutrophils were stimulated with 10 u ml^{-1} TNF-α for 60 min (a) or 30 min (b) in a medium containing 10% FCS and cytochalasin B, genistein, SB203580 or PD98059 at the concentrations indicated. (a) Effects of the drugs on GPI-80 release. GPI-80 in the medium was measured by ELISA, using antibody specific to GPI-80. The mean concentration of GPI-80 without drugs (control) was expressed as 100%. (b) Effects of the drugs on neutrophil adherence. OD570 was measured and followed by lysis with 1% SDS, after adherent cells were stained with crystal violet. The mean OD value without drugs (control) was expressed as 100%. Vertical bars represent s.e.mean from triplicate measurements. Results are representative of three similar experiments.

dependent manner (Figure 4a). Each antibody at highest concentration suppressed GPI-80 release in an undetectable level. All antibodies to CD11b and CD18 used in this study inhibited neutrophil adherence to fibrinogen-coated plates (data not shown). Furthermore, no GPI-80 release was detected when neutrophils were stimulated with TNF-α in suspension (Figure 4b). These results suggest that GPI-80 release from TNF-α-stimulated neutrophils is dependent on adherence *via* Mac-1. Neutrophils without TNF-α stimulation release slightly but obviously GPI-80 under adherent condition compared with suspension condition (Figure 4b), suggesting that adhesion by itself has a potential to induce GPI-80 release.

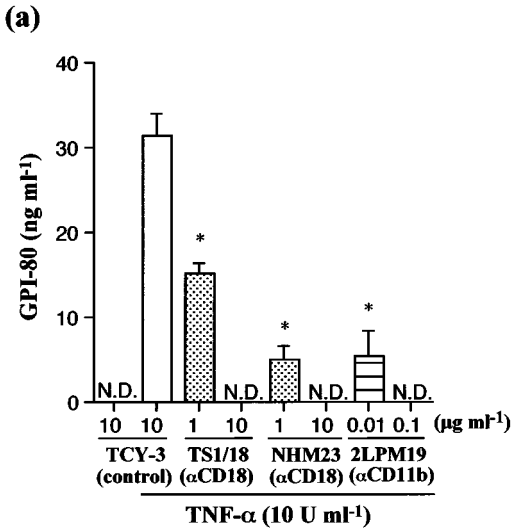
Effects of antioxidants on TNF-α-stimulated GPI-80 release

Because TNF-α elicits production of large amounts of superoxide anion from neutrophils (Figari *et al*., 1987; Tsujimoto *et al*., 1986), we examined the effects of the antioxidants pyrrolidine dithiocarbamate (PDTC) and N-acetyl-L-cysteine (NAC) on GPI-80 release from TNF-α-stimulated human neutrophils. PDTC (100 μM) significantly inhibited GPI-80 release from TNF-α-stimulated neutrophils as early as 30 min after stimulation (Figure 5); this suppression lasted to 120 min after stimulation. Cells incubated in the absence of TNF-α and PDTC (Figure 5) did not release GPI-80, suggesting that PDTC alone does not induce GPI-80 release. PDTC and NAC both inhibited GPI-80 release at 60 min in a concentration-dependent manner (Figure 6a), but PDTC was more effective than NAC: it completely suppressed GPI-80 release at lower concentrations. Furthermore, the lack of effect of these drugs on neutrophil adherence 30 min after stimulation (Figure 6b) suggests that antioxidants inhibition of GPI-80 release is independent of adherence, which is different from the action of cytochalasin B, genistein, and SB203580 (Figure 3). These drugs did not show any cytotoxic effect on neutrophil viability (data not shown).

We also used superoxide dismutase (SOD) to examine the effect of superoxide production on GPI-80 release: SOD had no effect on GPI-80 release (41.64 ± 2.2 ng ml^{-1} in the 10 u ml^{-1} TNF-α group, and 42.12 ± 4.54 ng ml^{-1} in the 10 u ml^{-1} TNF-α + 250 u ml^{-1} SOD group, respectively). Next, effects of these reagents on intracellular oxidative product formation were assessed using dichlorofluorescin diacetate. PDTC (100 μM) and NAC (1 mM) but not SOD (250 u ml^{-1}) reduced intracellular oxidative products 30 min after TNF-α stimulation (Figure 7). Therefore, we suggest that TNF-α-stimulated GPI-80 release from human neutrophils involves a change in intracellular oxidative state, but not superoxide production.

Discussion

In this study, we showed that a typical proinflammatory cytokine (TNF-α) involved in inflammatory disease (Feldmann *et al*., 2001; Wagner & Roth, 1999) induces GPI-80



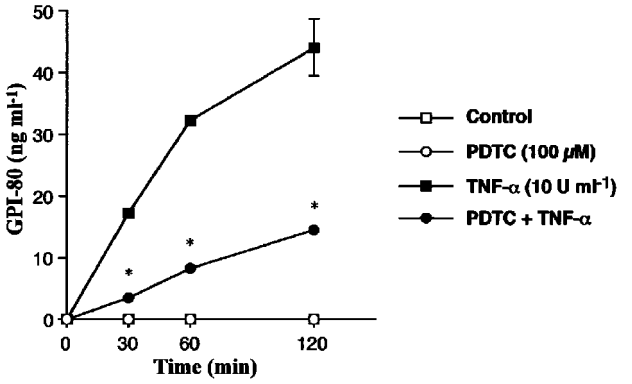


Figure 5 Inhibition of GPI-80 release from human neutrophils by PDTC. Human neutrophils were incubated without stimulation, with 10 u ml^{-1} TNF-α, with 100 μM PDTC, or with 10 u ml^{-1} TNF-α and 100 μM PDTC for the times indicated. GPI-80 in the medium was measured by ELISA, using antibody specific to GPI-80. Statistical significance: $^*P<0.05$ vs 10 u ml^{-1} TNF-α. Vertical bars represent s.e.mean from triplicate measurements. Results are representative of three similar experiments.

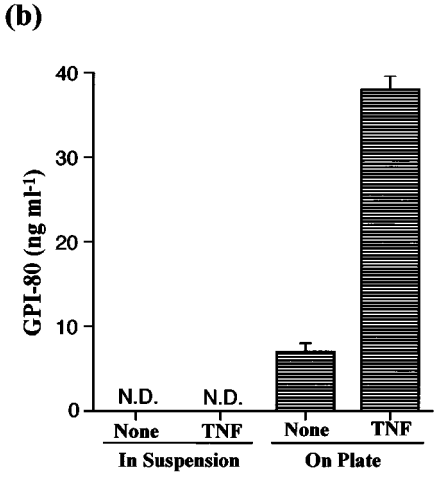


Figure 4 Requirement of adherence *via* β2 integrin for GPI-80 release from human neutrophils. (a) Inhibition of GPI-80 release by blocking antibodies to β2 integrin. Human neutrophils were stimulated with 10 u ml^{-1} TNF-α for 60 min in the presence of TCY-3 (control antibody), TS1/18 (anti-CD18), NHM23 (anti-CD18), or 2LPM19c (anti-CD11b). Statistical significance: $^*P<0.05$ vs 10 u ml^{-1} TNF-α + control antibody. (b) Failure to release GPI-80 in suspension. Human neutrophils were stimulated with 10 u ml^{-1} TNF-α for 60 min on a plastic plate (On plate) or in a plastic microtest tube (in suspension). GPI-80 in the medium was measured by ELISA, using antibody specific to GPI-80. Vertical bars represent s.e.mean from triplicate measurements. The results are representative of three similar experiments.

release from neutrophils. We also used pharmacological tools to investigate the mechanisms of GPI-80 release. Our group recently demonstrated that GPI-80 is located in secretory vesicles inside human neutrophils, as well as on their plasma membranes (Dahlgren *et al*., 2001). TNF-α has been reported to induce mobilization of secretory vesicles (Borregaard *et al*., 1990; 1992; 1994), yet TNF-α stimulation hardly changed GPI-80 expression on human neutrophil surfaces, even when significant amounts of GPI-80 were detected in the culture medium (Figure 2). We have reported that fMLP stimulation induce GPI-80 release as well as alkaline phosphatase expression (Dahlgren *et al*., 2001; Huang *et al*., 2001). In addition, examination using neutrophils with their surface labelled with biotin showed that the surface GPI-80 was internalized rather than release when the cells were stimulated (Huang *et al*., submitted). Therefore, it seems that unlike the release of L-selectin (Allport *et al*., 1997; Griffin *et al*., 1990) and TNF receptor (Dri *et al*., 2000; Porteu & Hieblot, 1994) from neutrophils, most of the GPI-80 in the culture medium originated inside the cells, in secretory vesicles, rather than from GPI anchored to the neutrophil surface. This hypothesis is also supported by reports that TNF-α induces human serum albumin (HSA) release (Borregaard *et al*., 1992) and mobilization of alkaline phosphatase (Borregaard *et al*., 1990; 1994), which are found in secretory vesicles, to neutrophil surfaces, and the finding that neutrophils store soluble GPI-80 beforehand (Huang *et al*., submitted).

The present study clearly shows that inhibitors of actin polymerization, protein tyrosine kinases and p38 MAP kinases, all suppress TNF-α-stimulated GPI-80 release (Figure 3). However, these drugs also suppress adherence at the same concentrations (Figure 3), suggesting that they affect both adhesion and the GPI-80 secretion process. Among them, cytochalasin B is known to enhance stimuli-induced granule release whereas the drug inhibited GPI-80 release. Therefore, we considered that inhibition by cytochalasin B of actin rearrangement may force neutrophils not to adhere to plates and to release GPI-80. To explore the role of adhesion in GPI-80 release, we examined the effects of monoclonal antibodies to the components of β2 integrin on GPI-80 release. Blocking antibodies to CD11b and CD18 inhibited GPI-80 release (Figure 4a), and no GPI-80 was released in suspension cultures (Figure 4b). This suggests that GPI-80 release is essentially dependent on adhesion *via* Mac-1. To investigate whether adherent stimulus causes GPI-80 release, effect of cross-linking of CD18 was examined. Contrary to our prediction, cross-linking of CD18 did not cause GPI-80 release (Nitto, unpublished results). Moreover, simultaneous stimulation by TNF-α under CD18 cross-linking did not induce GPI-80 release at all (Nitto, unpublished results),

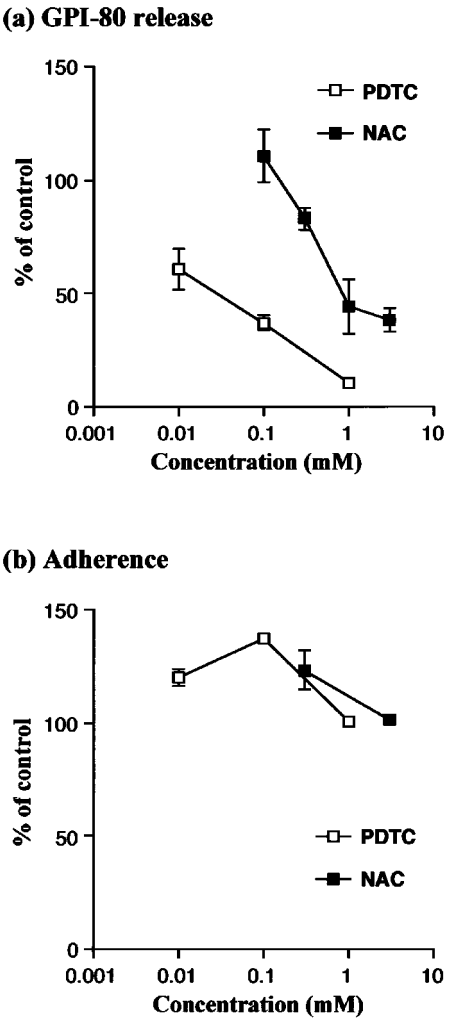


Figure 6 Effects of antioxidants on GPI-80 release and adherence in TNF-α-stimulated human neutrophils. Human neutrophils were stimulated with 10 u ml^{-1} TNF-α for 60 min (a) or 30 min (b) in a medium containing 10% FCS and PDTC or NAC at the concentrations indicated. (a) Inhibition of GPI-80 release by the antioxidants. GPI-80 in the medium was measured by ELISA, using antibody specific to GPI-80. (b) No effect of antioxidants on neutrophil adherence. OD570 was measured, and followed by lysis with 1% SDS, after adherent cells were stained with crystal violet. Data are expressed as per cent control. Vertical bars represent s.e.mean from three similar experiments.

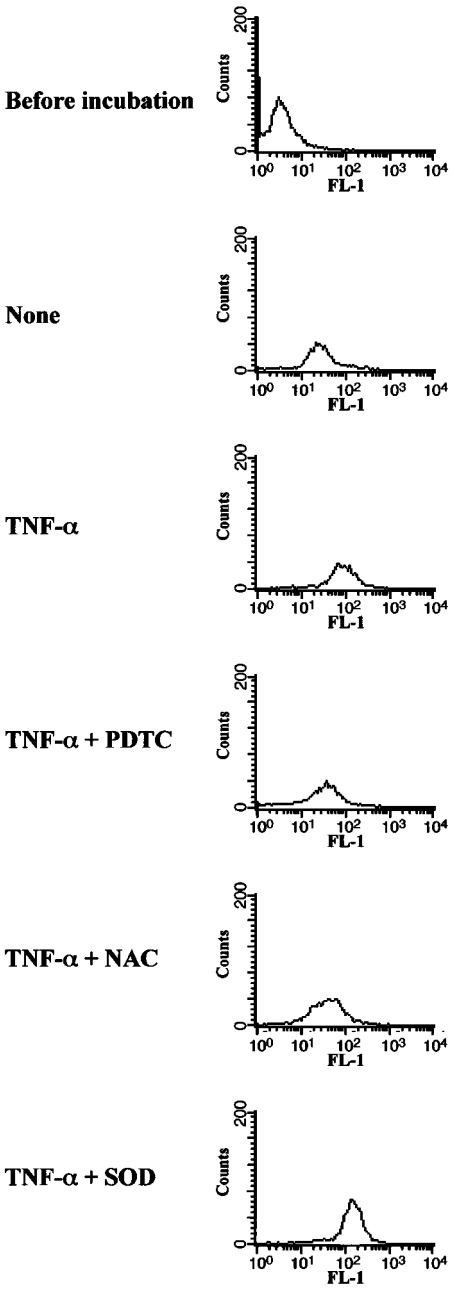


Figure 7 Effects of antioxidants on an intracellular oxidative state in TNF-α-stimulated human neutrophils. Human neutrophils were loaded with 2′, 7′-dichlorofluorescin diacetate (before incubation) and then incubated in a medium containing 10 u ml^{-1} TNF-α alone (TNF-α) or with 100 μM PDTC (TNF-α + PDTC), 1 mM NAC (TNF-α + NAC) or 250 u ml^{-1} SOD (TNF-α + SOD) for 30 min. None shows neutrophils incubated without stimulation. Fluorescence intensities with cells were measured by flow cytometry. Data shown are representative of three independent experiments.

suggesting that signalling through subsequent activation of β2 integrin by TNF-α stimulation is important for GPI-80 release. From these findings, the mechanism of TNF-α-stimulated GPI-80 release in human neutrophils can be explained as follows: when TNF-α binds to its receptor, it activates protein tyrosine kinases and p38 MAP kinases, then induces actin reorganization. After these events, neutrophils use β2 integrin to adhere to a matrix (β2 integrin ligands such as fibrinogen), which leads to GPI-80 release. Indeed, some investigators have demonstrated that induction of the respiratory burst (Nathan, 1987), degranulation (Richter *et al*., 1990), and cytotoxicity (von Asmuth *et al*., 1991) by TNF-α are mostly dependent on adherence *via* β2 integrin. This may also be the case for GPI-80 release. It may be also possible that adhesion through another integrin such as β1 integrin is involved in GPI-80 release.

Since it has been reported that TNF-α stimulation induces an oxidative burst in human neutrophils (Figari *et al*., 1987; Tsujimoto *et al*., 1986), we assessed the effects of PDTC, NAC and SOD as antioxidants. SOD (250 u ml^{-1}), PDTC (100 μM), and NAC (1 mM) eliminated the superoxide anion

produced by human neutrophils in response to phorbol myristate 13-acetate (Nitto *et al*., unpublished observations). Therefore, extracellular superoxide production or release do not seem to be involved in GPI-80 release from TNF-α-stimulated neutrophils. Since PDTC and NAC reduced an intracellular oxidation state in TNF-α-stimulated cells (Figure 7), a change in an intracellular redox state is likely to be important for GPI-80 release. Furthermore, since PDTC and NAC inhibit GPI-80 release without affecting neutrophil adherence (Figure 6), an intracellular redox change probably causes GPI-80 release that is independent of neutrophil adherence. It cannot, however, be ruled out that the redox change is caused as a consequence of neutrophil adherence, which then leads to GPI-80 release.

On the other hand, those drugs can inhibit nuclear factor (NF)-κB activation by TNF-α (Schreck *et al*., 1992). It is unlikely, however, that they suppress GPI-80 release by inhibiting NF-κB, because neither actinomycin D (a transcription inhibitor) nor cycloheximide (a protein synthesis inhibitor) suppress GPI-80 release (Nitto *et al*., unpublished observations).

The finding that an antioxidant suppressed GPI-80 release reveals the possibility that GPI-80 release also plays a role in cellular redox regulation. GPI-80, which belongs to a pantetheinase family, has conserved amino acid sequences of the enzymatic active site (Granjeaud *et al*., 1999). Pantetheinase (also called Vanin-1/VNN-1 (Aurrand-Lions *et al*., 1996)) catabolizes pantetheine into pantothenate and cysteamine (Dupre *et al*., 1970), and the latter has a potent anti-oxidative activity (Aruoma *et al*., 1988). On the other hand, expression of VNN-3, a related molecule involved in detoxification of heavy metals and intracellular redox regulation, is significantly increased in methalothionein-deficient mice (Kimura *et al*., 2000). These observations lead us to hypothesize that the actions of molecules belonging to a pantetheinase family have some role in redox regulation.

We recently reported high levels of GPI-80 in synovial fluid from RA patients, despite low serum levels in these patients and in normal volunteers (Huang *et al*., 2001). Large amounts of TNF-α and large numbers of neutrophils in synovial fluid have been reported in RA patients (Hopkins & Meager, 1988; Saxne *et al*., 1988). Based on our findings, it seems possible that TNF-α-stimulated neutrophils at the affected joint are one source of GPI-80 in the synovial fluid.

In conclusion, we demonstrated that TNF-α induces GPI-80 release from human neutrophils, and that this release depends on two factors: adherence *via* β2 integrin, and a potential change in an intracellular redox state. Given that GPI-80 is found in secretory vesicles and on the plasma membrane, and that GPI-80 levels on plasma membrane did not change, GPI-80 likely is released mainly from secretory vesicles. Therefore, like alkaline phosphatase (Borregaard *et al*., 1990; 1994) and HSA (Borregaard *et al*., 1992), GPI-80 release may reflect secretory vesicle mobilization.

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