Signal Regulatory Protein Alpha Is Present in Several Neutrophil Granule Populations and Is Rapidly Mobilized to the Cell Surface to Negatively Fine-Tune Neutrophil Accumulation in Inflammation

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Key Words

Neutrophils · Inflammation · Signal regulatory protein alpha · Chemotactic factor · Skin window · Exudate

Abstract

Signal regulatory protein alpha (SIRPα) is a cell surface glycoprotein with inhibitory functions, which may regulate neutrophil transmigration. SIRPα is mobilized to the neutrophil surface from specific granules, gelatinase granules, and secretory vesicles following inflammatory activation *in vitro* and *in vivo*. The lack of SIRPα signaling and the ability to up-regulate SIRPα to the cell surface promote neutrophil accumulation during inflammation *in vivo*. © 2014 S. Karger AG, Basel

Introduction

Neutrophils constitute the frontline of defense in the innate immune system and migrate from blood into tissues in response to chemotactic and inflammatory factors [1]. This functional response relies on mobilization of membrane proteins to the plasma membrane, which takes place within minutes and is based on the exocytosis of cytoplasmic granules containing membrane proteins [2]. Neutrophils contain primary (azurophilic), secondary (specific), and tertiary (gelatinase) granules as well as a fourth subcellular compartment called secretory vesicles [2]. Granules and secretory vesicles are released following activation by chemotactic peptides, where the threshold for degranulation is such that secretory vesicles are released first, then tertiary granules, followed by secondary granules [2]. Primary granules are released by exocytosis only to a limited extent and rather fuse with phagosomes [2].

Signal regulatory protein alpha (SIRPα/SHPS-1/BIT/MFR/P84), a cell surface receptor of the immunoglobulin

(Ig) superfamily [3], is highly expressed in the brain and on myeloid cells, such as monocytes, macrophages, dendritic cells, and granulocytes [4, 5]. SIRP α seems to play an important role in the regulation of cell migration in several cell types [6] and has two known ligands – the cell surface glycoprotein CD47 and surfactant proteins A and D [7, 8]. The binding of SIRP α by specific antibodies, soluble CD47, or a SIRP α -binding CD47 peptide has been shown to inhibit neutrophil migration across epithelial monolayers or collagen-coated membranes [9, 10]. In neutrophils, SIRP α can be mobilized to the cell surface from intracellular storage compartments, supposedly the secondary granules, following stimulation with N-formyl-methionyl-leucyl-phenylalanine (fMLF) [9, 11]. However, the kinetics of mobilization and exact subcellular localization of SIRP α in neutrophils is not well understood. Therefore, here we investigated further the kinetics of mobilization, the identity of the intracellular compartment(s) from which SIRP α is mobilized, and its function in regulating neutrophil accumulation in inflammation *in vivo*.

Materials and Methods

Reagents

Polymorphprep was from Nycomed Pharma, Oslo, Norway. HRP-conjugated goat anti-mouse IgG was from Pierce, Rockford, Ill., USA. Fluorescein isothiocyanate (FITC)-conjugated anti-human CD11b (clone MEM-174) or anti-mouse CD11b (clone M1/70.15) were from ImmunoTools (Friesoythe, Germany). Dextran, Percoll and Ficoll-Paque were from Pharmacia (Uppsala, Sweden). The [^{57}Co]vitamin B12 was supplied by Amersham Laboratories (Amersham, UK). fMLF, FITC-conjugated goat anti-mouse IgG, and other reagents were from Sigma-Aldrich, St. Louis, Mo., USA. Anti-human SIRP α mAbs 142, 010, 031 and 070 (all mouse IgG1) or anti-mouse SIRP α mAb P84 (rat IgG1) were purified from hybridoma tissue culture supernatants by ammonium sulfate precipitation and protein A or protein G chromatography (Amersham Pharmacia, Piscataway, N.J., USA) [12, 13].

Isolation of Human Neutrophils

Experiments on human neutrophils were approved by the regional ethics committees of Umeå (2012-327-31M) or Gothenburg (543-07). Heparinized venous blood was obtained from healthy adult volunteers attending the Blood Bank at Norrlands University Hospital, Umeå, Sweden after informed consent had been given, and buffy coats were obtained from healthy blood donors through the Blood Center at the Sahlgrenska University Hospital, Gothenburg, Sweden. The study was performed in accordance with the ethical standards of the 2008 Declaration of Helsinki. Blood neutrophils were isolated (>97% purity and viability) using Polymorphprep as previously described [14]. Neutrophils for subcellular fractionation were isolated from buffy coats, using dextran sedimentation at 1 g, hypotonic lysis of the remaining erythrocytes

and centrifugation in a Ficoll-Paque gradient [15]. *In vivo* transmigrated neutrophils were collected using an experimental skin chamber approach, which is an experimental model of acute inflammation. In this model, blisters are formed by negative pressure, the blister roofs are subsequently removed, and a collection chamber filled with autologous serum is applied [16, 17]. Neutrophils were allowed to accumulate over 24 h (more than 95% neutrophils). As controls, blood neutrophils were isolated from heparinized whole blood (obtained from the same person as the one who was carrying the skin chambers) as described above. When SIRP α expression was analyzed on skin chamber cells, 5 μl 7-aminoactinomycin D were added to the samples before analysis, and events positive for this nuclear dye (representing necrotic/leaky cells; <5%) were excluded from further analysis.

Subcellular Fractionation, Quantification of Granule Markers, and Preparation of Lipid Rafts

Subcellular fractionation was performed essentially according to the methods described by Borregaard et al. [18], Udby and Borregaard [19] and Dahlgren et al. [20]. In short, neutrophils isolated from buffy coats were treated with the serine protease inhibitor diisopropylfluorophosphate (8 μM), disintegrated by nitrogen cavitation (Parr Instrument Company, Moline, Ill., USA), and the postnuclear supernatant was centrifuged either on a three-layer Percoll gradient (to isolate the specific and gelatinase granules separately) or on a flotation gradient (to isolate the secretory vesicles from the plasma membranes). The gradients were collected in 1.5- or 1-ml fractions, respectively, by aspiration from the bottom of the centrifuge tube, and the localization of subcellular organelles in the gradients was determined by quantifying vitamin B12-binding protein (marker for the specific granules) with the cyanocobalamin technique, by quantifying gelatinase (marker for the gelatinase granules) and myeloperoxidase (marker for the azurophil granules) using ELISA methods, or alkaline phosphatase (marker for secretory vesicles and plasma membranes) measured as hydrolysis of *p*-nitrophenyl phosphate (2 mg/ml) in the presence or absence of Triton X-100 (0.4%). Lipid rafts or detergent-resistant membranes were isolated from neutrophil membranes as previously described [21]. The presence of SIRP α or the lipid raft marker stomatin in neutrophil subcellular fractions was determined by Western blot analysis as previously described [21, 22].

Mice and Isolation of Murine Neutrophils

Male and female SIRP α -mutant mice, which lack most of the SIRP α cytoplasmic domain, were previously described [23, 24]. SIRP α -mutant mice and their homozygous wild-type littermates were kept in accordance with local guidelines and maintained in a specific pathogen-free barrier facility. The experiments were performed in compliance with relevant Swedish and institutional laws and guidelines and approved by the Umeå research animal ethics committee (A14-12). Bone marrow neutrophils were isolated from femurs of 2- to 4-month-old mice, using a Percoll gradient as previously described [25] and were resuspended at $5 \times 10^6/\text{ml}$ in cold HBSS containing Ca^{2+} and Mg^{2+} (HBSS $^{++}$). These preparations routinely contained more than 85% neutrophils.

Murine Uric Acid-Induced Peritonitis

Peritonitis was induced by injecting 200 μl 10% (wt/vol) uric acid in saline as previously described [26]. At the indicated time points, the peritoneal cavity was lavaged with 5 ml PBS + 2% FCS.

The total number of cells in the lavages was determined, using Türk's reagent and a Bürker chamber, and the percentage of neutrophils was determined in cytospin preparations stained with May-Grünwald/Giemsa.

fMLF-Induced Receptor Expression and FACS Analysis

Neutrophils (2.5×10^5) in a volume of 500 μ l HBSS⁺⁺ with 0.25% HSA were incubated at 37°C in the presence or absence of fMLF or TNF α for the times indicated. In some experiments, neutrophils were preincubated for 15 min with inhibitors, followed by incubation in the presence or absence of fMLF for 20 min. Cells were then washed in ice-cold PBS and resuspended in ice-cold staining buffer (RPMI 1640 with 2% FCS and 1 mg/ml human IgG) with the indicated mAbs and incubated for 30 min on ice. If appropriate, cells were also incubated for 30 min on ice in the dark with FITC-conjugated goat anti-mouse IgG. The cells were finally washed, resuspended in cold PBS/1% BSA and analyzed by flow cytometry (FACScan or FACSCalibur, Becton Dickinson) and CellQuest software (Becton Dickinson).

Statistics

Statistical analyses were performed by one-parameter ANOVA and Tukey's post hoc test, or Student's *t* test for unpaired comparisons. All results are expressed as mean $\pm$ SEM.

Results and Discussion

Increased Neutrophil Cell Surface SIRP α Expression following Inflammatory Transmigration in vivo and following Inflammatory Activation in vitro

Due to their vast amount of granules, each containing a large array of membrane proteins, neutrophil activation and degranulation rapidly increase the cell surface levels of many proteins to reflect the shifting functional need of these cells [27]. The accumulation of neutrophils in human skin chamber exudates represents an aseptic model of inflammation to study in vivo activated neutrophils that have transmigrated from the blood into a skin chamber containing autologous serum factors, primarily C5a and IL-8 [17]. A large fraction of such neutrophils had higher levels of SIRP α on their cell surface as compared to peripheral blood neutrophils isolated from the same individual (fig. 1a). The population of exudate neutrophils with a reduced SIRP α expression in these experiments was proportional to the amount of apoptotic cells (data not shown), which is in line with our recent finding that SIRP α expression is downregulated during neutrophil apoptosis [13]. The bacterial chemotactic peptide fMLF can also induce mobilization of membrane proteins from intracellular granules to the neutrophil cell surface in vitro [22, 28]. We found that SIRP α was dose- and time-dependently increased by fMLF in human neutrophils in vitro (fig. 1b), a response evenly distributed with-

in the neutrophil population (fig. 1c). Since fMLF-stimulated neutrophil degranulation has been suggested to involve signaling by members of the mitogen-activated protein kinase (MAPK) family [29], we investigated whether fMLF-induced SIRP α upregulation was affected by specific inhibitors of p38 MAPK or ERK. The p38 MAPK inhibitor SB203580, but not the ERK inhibitor PD98059, used at concentrations shown to inhibit Fc γ R-mediated phagocytosis [30], had an inhibitory effect on fMLF-induced cell surface SIRP α expression (fig. 1d). Thus, these data are in line with the finding that degranulation of secondary granules is partly mediated by p38 MAPK but not by ERK [29]. In exudate neutrophils, it is unclear whether SIRP α is upregulated as a result of early activation during transendothelial migration or as a gradual response to increasing concentrations of chemotactic factors after completing the transmigration process. The latter hypothesis is supported by our in vitro data, showing that neutrophil SIRP α is upregulated dose-dependently by fMLF with rather modest effects observed at the lower chemoattractant concentrations that would be involved in initial neutrophil chemotactic recruitment from the blood in vivo (fig. 1b).

Subcellular Localization of SIRP α in Human Neutrophils

To get a detailed understanding of the intracellular localization of SIRP α , we investigated subcellular neutrophil fractions using immunoblot analysis and correlated the presence of SIRP α in these fractions with specific markers for azurophil granules (myeloperoxidase), specific granules (vitamin B12-binding protein), gelatinase granules (gelatinase but no vitamin B12-binding protein), and secretory vesicles/plasma membrane (latent/nonlatent alkaline phosphatase, respectively). Using a three-step Percoll gradient, allowing for separation of the specific granules from the gelatinase granules, we found that SIRP α colocalized with markers for specific granules, gelatinase granules, as well as light membranes (secretory vesicles/plasma membrane; fig. 2a). However, we could not detect SIRP α in azurophil granules (fig. 2a). Using a flotation gradient, where secretory vesicles are separated from the plasma membrane in the upper part of the gradient, we could separately confirm the presence of SIRP α in both the secretory vesicles and the plasma membrane (fig. 2b). Proteins of importance for signaling over the cell membrane have been shown to be often associated with so-called lipid rafts or detergent-resistant membrane domains. We explored the possibility that SIRP α is localized in such domains in neutrophils, knowing that these cells contain lipid rafts not

Fig. 1. Neutrophil cell surface SIRP α expression is increased following in vivo transmigration or stimulation with fMLF in vitro. **a** Cell surface SIRP α expression was increased on human skin chamber exudate neutrophils (bold line) as compared to that on peripheral blood neutrophils (grey histogram). The fluorescence intensity of exudate neutrophils (dotted line) or peripheral blood neutrophils from the same donor (dashed line) incubated with control IgG is also shown. The data are representative of 3 separate experiments with virtually identical results. **b** Dose- and time-dependent upregulation of SIRP α on human neutrophils following incubation for 5–20 min at 37°C in the absence or presence of 1–100 nM fMLF. Values are means $\pm$ SEM for 3 separate experiments. **c** Flow-cytometric profiles of SIRP α expression in unstimulated human neutrophils (grey histogram, control) or following incubation for 20 min at 37°C with 100 nM fMLF (bold line). The thin line indicates cells labeled with an isotype control antibody. Data from 1 representative experiment presented in **b** are shown. **d** Neutrophils were preincubated for 15 min with the inhibitors SB203580 (SB; p38 MAPK inhibitor) or 50 μ M PD98059 (PD; ERK inhibitor) followed by incubation in the presence of 100 nM fMLF for 15 min at 37°C. Cells were then labeled with anti-SIRP α mAb and analyzed by flow cytometry. Neutrophil SIRP α expression was calculated as percent of that in neutrophils incubated only in vehicle (DMSO). Values are means $\pm$ SEM of 3–5 separate experiments. * $p < 0.05$, one-parameter ANOVA and Tukey's post hoc test.

only in the plasma membrane but also in intracellular granule membranes [21]. Detergent-resistant fractions were isolated from the light membrane fraction of neutrophils (secretory vesicles/plasma membranes), as well as from the specific and gelatinase granule membranes, by flotation in a Percoll gradient. In both these compartments, SIRP α was to some extent present in the lipid rafts (fig. 2c, d), colocalizing with the raft marker stomatin (fig. 2c, d). Thus, our data confirmed the presence of SIRP α

in neutrophil-specific granules as suggested previously [11], but could in addition show SIRP α to be present in the gelatinase granules and the secretory vesicles.

SIRP α Mediates Negative Fine-Tuning of Neutrophil Accumulation in Inflammation

The functional significance of a gradually increased SIRP α expression in relation to neutrophil migration is not entirely clear. However, ligation of SIRP α , either by

Fig. 2. Subcellular localization of SIRP α in neutrophils. Human neutrophils were fractionated on a three-step gradient **(a)** or a flotation gradient **(b)** as described in Materials and Methods. The localization of neutrophil granules in the gradient was determined by marker analysis of the fractions; arrows indicate peak fractions of the different markers. Isolated membranes from plasma membrane/secretory vesicles **(c)** or specific/gelatinase granules **(d)** were resuspended in relaxation buffer containing Triton X-100, layered under Percoll and centrifuged as described in Materials and Methods. The collected fractions were analyzed for stomatin (lipid raft marker) by immunoblotting and the results show that Triton-insoluble membrane domains have floated upwards to gradient fractions 16 and 17. The localization of SIRP α in the fractions is shown by immunoblotting, using a combination of mAbs 010, 031, and 070.

Fig. 3. Lack of SIRP α signaling and its upregulation to the cell surface enhance neutrophil accumulation in inflammation. **a** Increased expression of SIRP α by peritoneal exudate neutrophils in wild-type mice following injection of uric acid (bold line) as compared with that on murine peripheral blood neutrophils (grey histogram). The data are representative of 3 separate mice showing virtually identical results. **b** Dose- and time-dependent upregulation of SIRP α on murine neutrophils following incubation for 5–20 min at 37°C in the absence or presence of fMLF. The data are expressed as a percent increase from the SIRP α expression on cells kept on ice (0 min). Values are means $\pm$ SEM of 3 separate experiments. **c** Neutrophil SIRP α expression in wild-type or SIRP α mutant neutrophils, either kept on ice or incubated for 20 min at 37°C

in the absence or presence of 1,000 nM fMLF. Data are means $\pm$ SEM of 4 separate mice per genotype. **d** Neutrophil CD11b expression in wild-type or SIRP α mutant neutrophils, either kept on ice or incubated for 20 min at 37°C in the absence or presence of 1,000 nM fMLF. Data are means $\pm$ SEM of 4 separate mice per genotype. **e** Wild-type or SIRP α -mutant mice were injected i.p. with 10% uric acid, and peritoneal cells were harvested 6 h later. The total number of cells was determined using a Bürker chamber, and the fraction of neutrophils was determined from cytospin preparations stained with May-Grünwald-Giemsa. Data represent means $\pm$ SEM of 5 mice per group. ** $p < 0.01$, using Student's *t* test for unpaired comparisons. MFI = Mean fluorescence intensity; Wt = wild-type; Mut = mutant; unstim. = unstimulated.

antibodies, by a soluble form of its ligand CD47, or by using a CD47-derived peptide, was reported to inhibit neutrophil migration across epithelial cells or cell-free collagen-coated filters *in vitro* [9, 10]. This suggests an active role played by SIRP α in negatively regulating neutrophil migration. To investigate this further, we studied SIRP α -mutant mice, which only express the extracellular do-

main of SIRP α , while the cytoplasmic signaling ITIM domain is deleted [23]. Similar to that in human exudate neutrophils, we found that SIRP α was upregulated on the surface of wild-type neutrophils accumulating in urate-induced peritonitis (fig. 3a) or when stimulated with fMLF *in vitro* (fig. 3b). However, in SIRP α -mutant neutrophils, we found a more than 50% reduction in the

amount of the SIRP α extracellular domain on the cell surface of nonstimulated cells as compared with wild-type neutrophils (fig. 3c). In addition, mutant cells were unable to upregulate SIRP α on the cell surface in response to fMLF (fig. 3c). It was unlikely that this was a result of defects in degranulation, since CD11b was found to be upregulated normally in mutant neutrophils stimulated with fMLF (fig. 3d). The fact that SIRP α can neither signal nor be increased on the cell surface in response to inflammatory activation in SIRP α -mutant neutrophils suggested that these mice would represent a good model to investigate a functional role of SIRP α in vivo. For this, we used the uric acid-induced peritonitis model of sterile inflammation, in which acute neutrophil influx into the peritoneal cavity is mediated by IL-1 β , IL-6, and TNF α produced by resident peritoneal macrophages [31]. In addition, a role for macrophage inflammatory protein-2 and peritoneal mast cells has also been suggested in this model [32]. We found that significantly more neutrophils were recovered in peritoneal lavages from SIRP α -mutant mice, as compared with that in wild-type controls at 6 h after uric acid injection ($p < 0.01$; fig. 3e). This was neither the result of an increased number of peritoneal neutrophils in naïve SIRP α -mutant mice (0 h; fig. 3e) nor the result of increased numbers of circulating neutrophils, since wild-type and mutant mice had similar numbers of neutrophils in blood (795 ± 70 vs. 723 ± 112 cells/ μ l, respectively; $p > 0.05$). These data therefore suggest that an increased level of SIRP α on the neutrophil cell surface could serve to negatively fine-tune neutrophil accumulation at sites of inflammation. While preparing this manuscript, Zen et al. [33] presented data confirming our findings by showing that SIRP α -mutant mice have an in-

creased accumulation of neutrophils in a model of zymosan-induced peritonitis. Since CD47 can function as a ligand for SIRP α [34] and CD47 is expressed by virtually all cells in the host [35], the ability of SIRP α to negatively fine-tune neutrophil accumulation in inflammation is possibly mediated by the interaction between neutrophil SIRP α and CD47 on other cells.

In conclusion, we have shown that SIRP α is rapidly mobilized to the cell surface from neutrophil secretory vesicles, gelatinase granules, and specific granules, following stimulation with fMLF in vitro or in vivo transmigration into tissue. The enhanced accumulation during inflammation in vivo of murine neutrophils lacking both the signaling SIRP α ITIM domain and the ability to increase the level of the SIRP α extracellular domain in response to inflammation suggests that SIRP α negatively fine-tunes the accumulation of inflammatory-activated neutrophils.

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Disclosure Statement

The authors declare no competing financial interests.

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