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Defensin-rich granules of human neutrophils: characterization of secretory properties

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Abstract

The various granule subtypes of the human neutrophil differ in propensity for exocytosis. As a rule, granules formed at late stages of myelopoiesis have a higher secretory potential than granules formed in more immature myeloid cells. Neutrophils contain four closely related α-defensins, which are stored in a subset of azurophil granules. These defensin-rich azurophil granules (DRG) are formed later than defensin-poor azurophil granules, near the promyelocyte/myelocyte transition. In order to characterize the secretory properties of DRG, we developed a sensitive and accurate ELISA for detection of the neutrophil α-defensins HNP 1–3. This allowed us to quantify the exocytosis of α-defensins and markers of azurophil (myeloperoxidase), specific (lactoferrin) and gelatinase (gelatinase) granules from neutrophils stimulated with different secretagogues. The release pattern of α-defensins correlated perfectly with the release of myeloperoxidase and showed no resemblance to the exocytosis of lactoferrin or gelatinase. This finding was substantiated through subcellular fractionation experiments. In conclusion, despite a distinct profile of biosynthesis, DRG are indistinguishable from defensin-poor azurophil granules with respect to exocytosis. Thus, in contrast to peroxidase-negative granules, azurophil granules display homogeneity in their availability for extracellular release.

Keywords: Defensin; ELISA; Subcellular fractionation; Exocytosis; Peroxidase-positive granule

1. Introduction

Granules of human neutrophils are formed sequentially during myeloid cell differentiation. Azurophil (peroxidase-positive) granules are the first to appear. These granules, traditionally defined by their content of myeloperoxidase, are formed at the promyelocyte stage of neutrophil development [1]. Peroxidase-negative granules are formed in myelocytes, metamyelocytes, band cells and segmented neutrophils [1,2], and can be divided into two subsets, which occur successively: specific granules, identified by a high content of lactoferrin, and gelatinase granules, identified by gelatinase [3]. The various granule subtypes differ in accessibility for exocytosis. Gelatinase granules are the most easily mobilized, followed by specific granules and azurophil granules [4]. Thus, as a rule, granules formed at later stages of myelopoiesis have a higher secretory potential than granules formed in more immature myeloid cells.

Alpha-defensins are important antimicrobial peptides [5]. The human neutrophil granulocyte contains four closely related α-defensins (HNP 1–4), which are stored in a subset of azurophil granules [6,7]. These defensin-rich azurophil granules (DRG) are formed later than defensin-poor azurophil granules, near the promyelocyte/myelocyte transition [7]. The late appearance of DRG is a consequence of the biosynthetic window of neutrophil α-defensins. Whereas other matrix proteins of azurophil granules like myeloperoxidase, proteinase-3, and elastase are synthesized throughout the promyelocytic stage, production of α-defensins is first initiated in late promyelocytes [8]. Furthermore, in contrast to other matrix proteins of azurophil granules, expression of α-defensins is dependent on the CCAAT/enhancer binding protein ε, a transcription factor that is also essential for the expression of a variety of specific and gelatinase granule matrix proteins [9,10]. Taken together, these features have led to speculations that DRG may have

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their own place in the functional hierarchy of neutrophil granules. The present study was undertaken in order to characterize the secretory properties of DRG.

2. Materials and methods

2.1. Isolation of neutrophils

Human neutrophils were isolated from freshly prepared buffy coats supplied by the hospital blood bank. Erythrocytes were sedimented for 45 min by addition of an equal volume of 2% Dextran T-500 (Amersham Pharmacia Biotech AB, Uppsala, Sweden) in 0.9% saline. The leukocyte-rich supernatant was siphoned off, and the cells centrifuged at $200 \times g$ for 10 min. Cells were resuspended in 0.9% saline and centrifuged through Lymphoprep (Nycomed Pharma AS, Oslo, Norway) at $400 \times g$ for 30 min to remove mononuclear cells. Remaining erythrocytes were lysed by hypotonic shock in ice-cold water for 30 s followed by restoration of tonicity by addition of 1.8% saline. The cells were subsequently washed once in saline and resuspended in the desired buffer. All steps except Dextran sedimentation were performed at 4 °C.

2.2. Subcellular fractionation

Isolated neutrophils, resuspended at 3×10^7 cells/ml in 0.9% saline, were incubated for 5 min with 5 mM diisopropylflourophosphate (DFP, Aldrich Chemical Company, Milwaukee, WI, USA) and centrifuged at $200 \times g$ for 10 min. The pelleted cells were resuspended at 3×10^7 cells/ml in disruption buffer (100 mM KCl, 3 mM NaCl, 1 mM Na_2ATP, 3.5 mM $MgCl_2$, 10 mM PIPES, pH 7.2) containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and disrupted by nitrogen cavitaton (pressurized for 5 min) as described [11]. Nuclei and unbroken cells were pelleted by centrifugation at $400 \times g$ for 15 min. Ten milliliters of the postnuclear supernatant was applied on top of a three-layer Percoll gradient (1.05/1.09/1.12 g/ml) [12] containing 0.5 mM PMSF and centrifuged for 30 min at $37\,000 \times g$. This resulted in a gradient with four clearly visible bands: the bottom band (α-band) containing the azurophil granules, the low intermediate band (β_1-band) containing the specific granules, the high intermediate band (β_2) containing gelatinase granules and the top-band (γ-band) containing plasma membranes and secretory vesicles. The cytosol was present above the γ-band on top of the Percoll. The gradient was collected in fractions of 1 ml by aspiration from the bottom of the tube, and the content of granule markers in the different fractions was determined by ELISA as described below.

2.3. Purification of neutrophil α-defensins

Following subcellular fractionation, the α-band containing azurophil granules was harvested manually, and Percoll was removed by ultracentrifugation. Isolated granules were lysed in phosphate buffered saline (PBS; 136 mM $NaCl_2$, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.46 mM KH_2PO_4, pH 7.4) containing 40 mM *n*-octylglycosid-β-D-glucopyranoside (Calbiochem, La Jolla, CA, USA) and rotated overnight at 4 °C. The next day, membranes were pelleted by ultracentrifugation for 30 min at $20\,000 \times g$, and the supernatant, containing the granule matrix proteins, was dialysed against a 50 mM sodium-phosphate buffer, pH 6.7, with 20 mM *n*-octylglycosid-β-D-glucopyranoside. Isolated azurophil granule proteins were subjected to cation exchange chromatography on a MonoS column using ÄKTA-FPLC (Amersham Pharmacia Biotech). Most of the bound material was eluted with 1.0 M NaCl, pH 6.7. Alpha-defensins were subsequently eluted with 0.5 M NaOH. The purity of the eluted material was ascertained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which showed a single band in the expected low molecular weight zone after staining with Coomassie Blue (not shown). The defensins were dialysed into PBS using Slide-A-Lyzer® 3.5 K Dialysis Cassettes (Pierce, Rockford, IL, USA) and stored at −20 °C until further use. The concentration of α-defensins in the PBS suspension was determined spectrophotometrically using the specific extinction coefficients for HNP-1 and -3 at 280 nm.

2.4. Amino acid sequence analysis of purified α-defensins

For amino acid sequence analysis, an aliquot of the purified neutrophil α-defensins preparation was blotted onto a PVDF membrane as described below. The analysis was performed for 10 residues in a 494 A Procise Protein Sequencer (PerkinElmer, Palo Alto, CA) using the blot cartridge and PVDF cycles. All reagents and solvents were supplied by PerkinElmer. The results confirmed that the preparation contained a mixture of neutrophil α-defensins, HNP 1–3. HNP 4, which constitutes between 1% and 2% of the defensins in neutrophils [13], was not detected.

2.5. Generation of polyclonal anti-α-defensin antibodies

Purified neutrophil α-defensins were conjugated to ovalbumin (ICN Biochemicals Inc, Aurora, OH, USA) by *N*-succinimidyl 3-(2-pyridyldithio)propionate (Sigma, St. Louis, MO, USA), essentially as described [14]. Immunization was performed at Dako A/S (Glostrup, Denmark). Rabbits received subcutaneous injections of 50 μg α-defensin conjugated to 400 μg ovalbumin in 100 μl incomplete Freunds adjuvant four times at 2-week intervals and thereafter once a month. The IgG fraction of the obtained poly-clonal anti-defensin/anti-ovalbumin antiserum was isolated on a protein A column using ÄKTA-FPLC (Amersham Pharmacia Biotech). The antibodies were eluted with 3 M KSCN and immediately dialysed against PBS containing 0.1% sodium azide. A portion of

the IgG antibodies were biotinylated as described [15]. The biotinylated antibodies were dialysed into PBS and stored with 0.1% sodium azide, protected from light. Subsequently, the ovalbumin-reactive antibodies were removed from the protein A purified IgG fraction by affinity chromatography on a column with ovalbumin coupled to CNBr-activated Sepharose (Amersham Pharmacia Biotech).

2.6. SDS-PAGE and immunoblotting

SDS-PAGE [16] and immunoblotting [17] were performed with Mini-Protean 3 Cells and Mini Trans-Blot Electrophoretic Transfer Cells according to the instructions given by the manufacturer (Bio-Rad, Hercules, CA, USA). For immunoblotting, polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) were blocked for 1 h with 5% skimmed milk in PBS after transfer of proteins from the 14% polyacrylamide gels. The PDVF membranes were incubated overnight with rabbit polyclonal anti-defensin antiserum or protein A purified biotinylated IgG antibodies. The next day, membranes were incubated for 2 h with horseradish peroxidase-conjugated porcine antibodies to rabbit immunoglobulins (Dako P217) and visualized by diaminobenzidine–metal concentrate and stable peroxide substrate buffer (Pierce).

2.7. Generation of an ELISA for neutrophil α-defensins

An ELISA for the neutrophil α-defensins HNP 1–3 was generated using 96-well flat-bottom immunoplates (Nunc, Roskilde, Denmark). First, checkerboard titrations were performed with different concentrations of antigen, capture antibody, detecting antibody, and avidin–peroxidase. The dilutions mentioned below were found to be optimal in an ELISA with a detection limit of 3.0 ng/ml. The procedure was as follows:

1. Plates were coated overnight with anti-α-defensin IgG antibodies diluted 1/500 in carbonate buffer (50 mM $Na_2CO_3/NaHCO_3$, pH 9.6).
2. Additional binding sites were then blocked by incubation with 200 μl/well of buffer A (0.5 M NaCl, 3 mM KCl, 8 mM Na_2HPO_4/KH_2PO_4, 1% BSA, 1% Triton X-100, pH 7.2).
3. Samples and standards (purified neutrophil α-defensins ranging from 3125 to 200 ng/ml) were applied.
4. Biotinylated antibodies, diluted 1/125, were added.
5. Avidin–peroxidase (Dako P347), diluted 1/5000, was added.
6. Color was developed by a 30 min incubation period in buffer B (0.1 M sodium phosphate/0.1 M citric acid buffer, pH 5.0), containing 0.04% *o*-phenylenediamine (Kem-En-Tec, Copenhagen, Denmark) and 0.03% H_2O_2, and stopped by addition of 100 μl/well of 1 M H_2SO_4.

The plates were washed three times in buffer C (0.5 M NaCl, 3 mM KCL, 8 mM Na_2HPO_4/KH_2PO_4, 1% Triton X-100, pH 7.2) using a Skanwasher 410 (Skatron, Roskilde, Denmark) between each step. Before color development, an additional wash in buffer B was included. All incubations were performed at room temperature for 1 h after adding 100 μl of sample to each well. Samples and antibodies were diluted in buffer A. Absorbance was read at 492 nm in a Multiscan Ascent ELISA reader (Labsystems, Helsinki, Finland).

2.8. Additional ELISAs

Myeloperoxidase, lactoferrin, and gelatinase were measured by ELISA as described [18,19]. General ELISA procedures were as described above for the α-defensin ELISA.

2.9. Release experiments

Isolated neutrophils were resuspended in Krebs–Ringer phosphate (KRP, 130 mM NaCl, 5 mM KCl, 1.27 mM $MgSO_4$, 0.95 mM $CaCl_2$, 5 mM glucose, 10 mM NaH_2PO_4/Na_2HPO_4, pH 7.4) at a concentration of 3×10^7 cells/ml. For stimulation with either 1.0 μM Ionomycin (Calbiochem), 5 μg/ml phorbol myristate acetate (PMA, Sigma) or 10.0 nM *N*-formylmethionyl-leucyl-phenylalanine (fMLP, Sigma), neutrophils were preincubated for 5 min at 37 °C. After addition of the stimulatory agent, the cells were incubated at 37 °C for 15 min. For stimulation with Cytochalasin B/fMLP, the cells were preincubated for 5 min at 37° with 5.0 μM cytochalasin B (Sigma) followed by stimulation for 15 min at 37 °C with 1.0 μM fMLP. In all settings, stimulation was terminated by dilution with 2 volumes of ice-cold KRP and centrifugation at $200 \times g$ for 6 min. Control cells were kept on ice until dilution in KRP. The supernatant (S_0) containing exocytosed material was removed, and the pelleted cells were resuspended in saline. Release of α-defensins, myeloperoxidase, lactoferrin, and gelatinase was calculated as amount in S_0/amount in (S_0 + Pellet) and expressed as a percentage.

3. Results

3.1. Specificity of anti-defensin antibodies

The polyclonal rabbit anti-defensin antiserum was specific for neutrophil α-defensins as evidenced by Western blotting of a neutrophil homogenate (postnuclear supernatant), electrophoresed under reducing conditions (Fig. 1A). The IgG fraction of the antibodies retained the ability to bind α-defensins in immunoblotting after biotinylation (Fig. 1B).

3.2. ELISA accuracy and reproducibility

A standard curve for neutrophil α-defensins is shown in Fig. 2. Near parallelism between the standard curve and

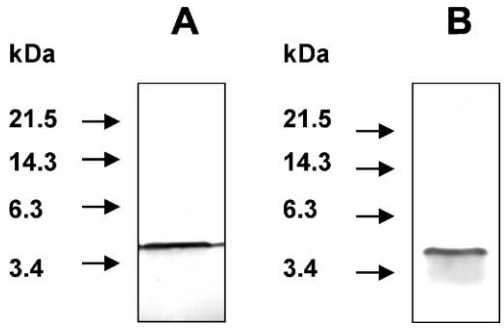

Fig. 1. Specificity of rabbit polyclonal antibodies against neutrophil α-defensins. SDS-PAGE was performed on a neutrophil homogenate (postnuclear supernatant) from 3×10^7 neutrophils/ml, diluted 10-fold in SDS sample buffer. (A) Immunoblotting with polyclonal anti-defensin antiserum. (B) Immunoblotting with affinity-purified IgG antibodies after biotinylation. In both lanes, a single band with the expected molecular weight is seen, indicating that the antibodies are specific and retain their reactivity against α-defensins after purification and biotinylation. Molecular weight markers are indicated on the left.

serial two-fold dilutions of either neutrophil homogenate or exocytosed material from neutrophils stimulated with 0.5 μM Ionomycin was observed (Fig. 2). Irrelevant neutrophil proteins are therefore unlikely to interfere with the ELISA. The accuracy of the assay was determined by adding different amounts of purified α-defensins to azurophil granule homogenate and exocytosed material from Ionomycin-stimulated neutrophils. The average recovery was 99.97% ± 4.14 (S.D.) (azurophil granule homogenate, $n = 4$) and 102.21 ± 1.07 (S.D.) (exocytosed material, $n = 4$). Reproducibility was estimated by repeated measurements on azurophil granule homogenate and exocytosed material. The average intra-assay coefficient of variation was 4.9% (4.06% for the azurophil granule homogenate ($n = 112$); 5.8% for the exocytosed material ($n = 122$)). The average day-to-day coefficient of variation was 8.6% (9.25% for the azurophil granule homogenate ($n = 5$); 8.1% for exocytosed material ($n = 6$)).

3.3. Exocytosis of α-defensins from neutrophils during stimulation with various secretagogues

The release of α-defensins and classical markers of azurophil granules (myeloperoxidase), specific granules (lactoferrin), and gelatinase granules (gelatinase) in response to stimulation with Ionomycin, Cytochalasin B/fMLP, PMA, and fMLP is summarized in Fig. 3. The observed releases of myeloperoxidase, lactoferrin, and gelatinase are in full agreement with earlier findings [4,12] and confirm the different availability for exocytosis of neutrophil granule subsets (gelatinase granules>specific granules>azurophil granules). The release pattern of α-defensins correlated perfectly with the release of myeloperoxidase and showed no resemblance to the exocytosis of lactoferrin or gelatinase. Of note, α-defensins were exocytosed to a similar extend as MPO during stimulation with powerful agonists of azurophil granule release (Ionomycin, Cytochalasin B/fMLP). Furthermore, when cells were stimulated with PMA, which predominantly induces release of peroxidase-negative granules, the exocytosis of α-defensins was less than 5%, whereas the mean exocytosis of lactoferrin was 45.0%. These findings demonstrate that

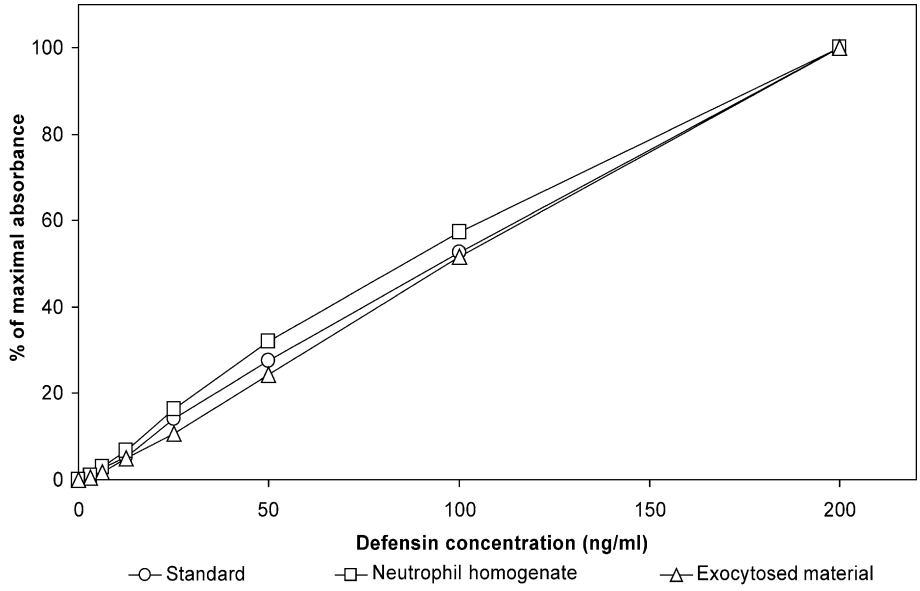

Fig. 2. Standard curve of neutrophil α-defensins and serial two-fold dilutions of a neutrophil homogenate and exocytosed material from Ionomycin-stimulated neutrophil granulocytes. Dilutions of neutrophil homogenate and exocytosed material were prepared to obtain approximately the same absorbance as observed with the lowest dilution of standard. Two-fold dilutions of samples are expressed in percentage of the absorbance obtained with the lowest dilution of the sample concerned (set to 100%).

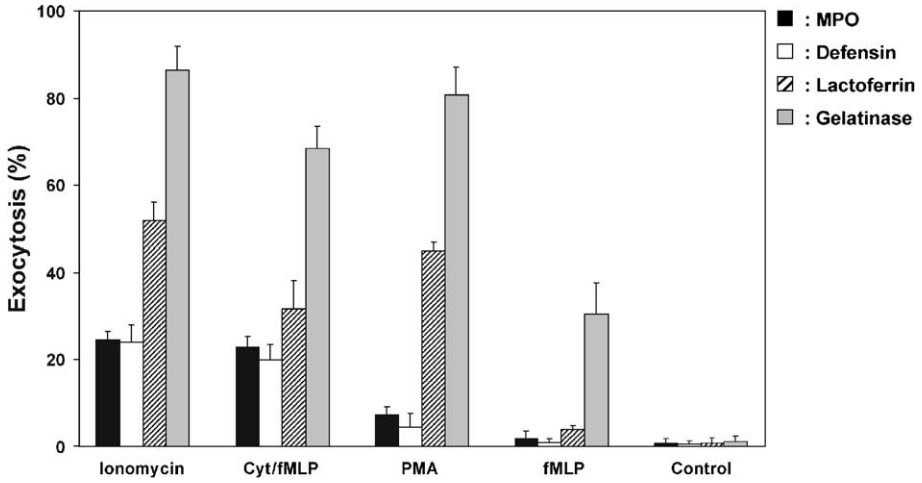

Fig. 3. Exocytosis of neutrophil granule markers during stimulation. Cells were stimulated as follows: Ionomycin, 1 μM; Cyt/fMLP (Cytochalasin B/fMLP), 5.0 μM/1.0 μM; PMA (phorbol myristate acetate), 5 μg/ml; fMLP (*N*-formylmethionyl-leucyl-phenylalanine), 10 nM. There was no significant difference between the exocytosis of α-defensins and myeloperoxidase (MPO) under any conditions ($P > 0.05$ in all settings; paired *t*-test). The release of lactoferrin was significantly higher than the release of α-defensins in response to all stimuli ($P < 0.02$), while gelatinase was exocytosed to a greater extent than both α-defensins and lactoferrin ($P < 0.05$). Bars are means of at least three experiments. Error bars represent S.D.

DRG do not have a unique release profile but share secretory properties with other azurophil granules.

3.4. Subcellular localization of α-defensins in mature neutrophils

The subcellular localization of α-defensins in unstimulated, mature neutrophils was determined in subcellular fractionation experiments using three-layer Percoll gradients (Fig. 4). High concentrations of α-defensins were detected only in the five higher density fractions (1–5) with a peak in the second fraction. In comparison, myeloperoxidase was found in relatively high concentrations throughout the fractions containing azurophil granule constituents with a peak in fraction 3. These findings are consistent with previous observations [6,7] and show that neutrophil α-defensins are confined to a subset of azurophil granules physically characterized by the highest density of all known granule subtypes. Subcellular fractionation of Ionomycin-stimulated neutrophils showed a uniform depletion of myeloperoxidase and α-defensins in fractions containing significant amounts of these markers (Fig. 4, fractions 1–7),

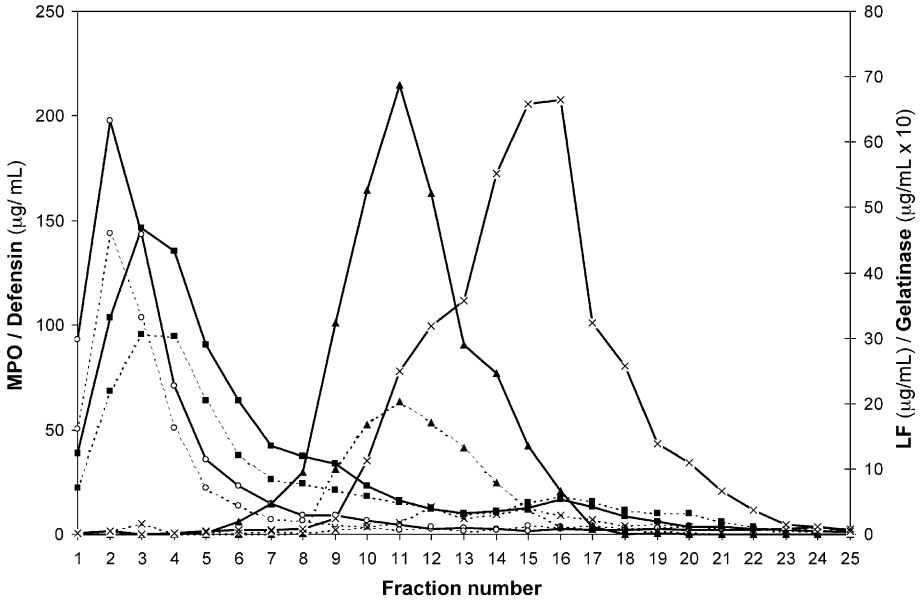

Fig. 4. Subcellular fractionation of control (—) and ionomycin-stimulated (- - - -) neutrophils on three-layer Percoll density gradients. Subcellular distribution of α-defensins (○), myeloperoxidase (MPO, ■), lactoferrin (LF, ▲) and gelatinase (×). Mean values of three independent experiments are shown.

clearly establishing that defensin-rich and defensin-poor azurophil granules are mobilized to the same extent during calcium ionophore-stimulation. The localization of lactoferrin (specific granules) and gelatinase (gelatinase granules) was as expected [12]. The distribution profiles of lactoferrin and α-defensins were without significant overlap. Thus, despite the almost overlapping biosynthetic windows of α-defensins and lactoferrin in myelopoiesis [8], these markers are restricted to late azurophil and specific granules, respectively.

4. Discussion

The differences in propensity for exocytosis of neutrophil granule subsets provide the neutrophil with the ability to differentiate its release of antimicrobial proteins in response to inflammatory challenge. Furthermore, the functional hierarchy of granules allows a sequential translocation of adhesion molecules to the plasma-membrane of the activated neutrophil. Membrane-bound proteins needed for neutrophil extravasation and diapedesis are localized to the most readily mobilizable intracellular compartments, i.e. secretory vesicles, gelatinase granules and specific granules [20].

Among peroxidase-negative granules, a hierarchy of mobilization has been convincingly demonstrated. Specific granules, which are formed in myelocytes and metamyelocytes, have a lower secretory potential than gelatinase granules, which are formed in band cells and segmented neutrophils [2,12]. The molecular mechanisms underlying the differential secretion of peroxidase-negative granule subsets are incompletely understood, but probably involve a lower density of docking/fusion proteins on granules formed at early stages of neutrophil development than on granules formed late in myelopoiesis [21,22].

No docking/fusion molecules have been identified in the membranes of azurophil granules so far, and these granules undergo very limited exocytosis during stimulation with most inflammatory mediators [4,20,23]. It is therefore believed, that azurophil granules are mobilized primarily to phagosomes after engulfment of opsonized targets. However, a recent study has challenged this simplistic view on azurophil granules by showing that the specific granule protein hCAP-18 is processed to the antimicrobial peptide LL-37 by extracellular cleavage with the azurophil matrix protein proteinase-3 [24]. Furthermore, neutrophil α-defensins induce selective chemotaxis of CD45RA/CD4 T cells, CD8 T cells, and immature dendritic cells at nanomolar concentrations [25,26]. Together, these observations clearly demonstrate a physiological role for azurophil granule proteins after exocytosis.

Limited information is available regarding the secretory properties of azurophil granule subsets [27]. Based on morphology, density, content, and timing of formation, at least two populations of azurophil granules can be identified: early-appearing defensin-poor and late-appearing defensin-rich granules [6,7]. In order to compare the mobilization kinetics of these granules, we developed a sensitive and accurate ELISA for detection of the neutrophil α-defensins HNP 1–3. This allowed us to quantify the extracellular release of α-defensins from neutrophils stimulated with different secretagogues and to study the exocytosis of defensin-rich and defensin-poor azurophil granules in subcellular fractionation experiments. Our investigations show, that DRGs are indistinguishable from other azurophil granules with respect to exocytosis. This finding raises the question of why azurophil granule heterogeneity exists, when, in contrast to peroxidase-negative granules, all azurophils are exocytosed to the same extent during stimulation. It has previously been shown, that the specific granule protein NGAL will be targeted to azurophil granules if the biosynthetic window of the protein is changed from the myelocyte stage to the promyelocyte stage [28]. However, such retargeting results in a slow intragranular degradation of NGAL, probably mediated by one of the many proteases contained within azurophil granules. This observation demonstrates, that not all granule proteins can exist together. Similarly, it could be speculated, that α-defensins are confined to DRG in order to prevent some inappropriate interaction between α-defensins and constituents of defensin-poor azurophil granules. Alternatively, α-defensins may be targeted to late-appearing azurophil granules simply as a consequence of their biosynthetic window and a dependency on azurophil granule proteases for their correct processing and intracellular retention [29].

In conclusion, this study provides a characterization of the secretory properties of DRG. Despite a distinct profile of biosynthesis, DRG are indistinguishable from defensin-poor azurophil granules with respect to exocytosis. Thus, unlike peroxidase-negative granules, azurophil granules display homogeneity in their availability for extracellular release.

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