PHAGOCYTES

Brief report

Arginase 1 is expressed in myelocytes/metamyelocytes and localized in gelatinase granules of human neutrophils

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Arginase 1 (ARG1) metabolizes arginine, thus reducing the availability of arginine as a substrate for nitric oxide synthase (NOS). The decreased production of nitric oxide (NO) by NOS and the production of ornithine by ARG1 affect immune responses and tissue regeneration at sites of infection, respectively. We here demonstrate that ARG1 is synthesized in myelocytes/metamyelocytes and is stored in gelatinase granules. In accordance with this, activated neutrophils coreleased ARG1 and gelatinase to the extracellular environment on stimulation with phorbol-12-myristate 13-acetate (PMA), formyl-methionyl-leucyl-phenylalanine (fMLP), or tumor necrosis factor α (TNF-α). Overall, these findings define ARG1 as a genuine gelatinase granule protein and support a model in which activated neutrophils release ARG1 at sites of infection to modulate immune responses and promote tissue regeneration. (Blood. 2007;109:3084-3087) © 2007 by The American Society of Hematology

Introduction

Arginase 1 (ARG1) metabolizes arginine to ornithine and thereby reduces extracellular arginine at sites of infection.1 By depleting nitric oxide synthase (NOS) of its substrate, ARG1 leads to reduced synthesis of nitric oxide (NO), which plays a pivotal role in wound healing and immune responses.1-6 Depletion of arginine by ARG1 released from myeloid cells has further been demonstrated to suppress T-cell immune responses.7 Finally, ARG1 generates the amino acid ornithine from arginine. Ornithine can be further metabolized to proline and polyamines, which enhance wound healing by supporting collagen synthesis and cell proliferation, respectively. Hence, the net effect of ARG1 activity at the site of infection is to regulate immune responses and to support tissue regeneration.

We have previously argued that the generation of human neutrophil granules, traditionally classified into azurophilic (primary or peroxidase-positive granules), specific granules (secondary granules), and gelatinase granules (tertiary granules), can be viewed as a continuum of partly overlapping subsets, which are formed during the entirety of granulocytic differentiation.8,9 No specific mechanism seems responsible for targeting individual granule proteins to a particular granule subset; therefore, granule proteins formed at the same time will be targeted to the same granule subset.10 Hence, different granule subsets arise as a consequence of differences in the biosynthetic windows of granule proteins. Those who are expressed primarily at the promyelocyte stage will consequently localize to azurophilic granules. Others expressed primarily at the myelocyte and metamyelocyte stages will localize to specific granules, and those expressed at the metamyelocyte and mature neutrophil stages will localize to gelatinase granules.11 This targeting by timing model explains the heterogeneity of human neutrophil granules and has been supported experimentally.9,12-14

We recently performed a comprehensive microarray analysis of human bone marrow populations representing successive stages of granulocytic differentiation, namely promyelocytes (PMs), myelocytes/metamyelocytes (MYs), and bone marrow neutrophils (BM-PMNs). The resulting microarray database enables us to test whether a given protein is expressed at the mRNA level during granulocytic differentiation, and, if so, whether, based on its expression profile, the protein will be targeted primarily to azurophil, specific, or gelatinase granules. So far, this has enabled us to demonstrate that, for example, α1 acid-glycoprotein and haptoglobin are major constituents of human neutrophil specific granules.15,16 Recently, Munder et al17 demonstrated the localization of ARG1 in azurophil granules, indicating that ARG1 is primarily synthesized in PMs.

Our microarray analysis revealed that ARG1 is expressed at the highest levels in MYs and BM-PMNs but only at low levels in PMs, which according to the targeting by timing model implicates that ARG1 is localized in gelatinase granules rather than azurophil granules of neutrophils. Because the findings by Munder et al17 challenge our model of how human neutrophil granules are formed, we decided to reexamine the localization of ARG1.

Materials and methods

Cell isolation

Peripheral blood (PB) and bone marrow (BM) samples were collected from healthy volunteers after informed consent was obtained in accordance with the ethics committee of the cities of Copenhagen and Frederiksberg as well as the Declaration of Helsinki. Neutrophils were isolated from peripheral...
isolated from samples of human BM by 3-layer density centrifugation and subsequent immunomagnetic depletion of nongranulocytic cells.\(^8,19\)

**Microarray analysis**

Microarray analysis was performed as described previously.\(^19\)

**Exocytosis studies**

Isolated neutrophils were stimulated by phorbol-12-myristate 13-acetate (PMA; 2.5 \(\mu\)g/mL; Sigma, Poole, United Kingdom), formyl-methionyl-leucyl-phenylalanine (fMLP; 100 nM; Sigma), or tumor necrosis factor \(\alpha\) (TNF-\(\alpha\); 50 ng/mL; Sigma). To prevent proteolytic degradation of ARG1, cells were incubated with diisopropyl-fluorophosphate (DFP; Sigma) immediately after stimulation. Granule proteins were analyzed by enzyme-linked immunosorbent assay (ELISA) or Western blot as described previously.\(^15\)

**Subcellular fractionation**

Subcellular fractions highly enriched in azurophil, specific, and gelatinase granules were isolated by disruption of isolated neutrophils, pelleting of nuclei, and subsequent centrifugation of the postnuclear supernatant on 3-layer Percoll density gradients.\(^16,20\)

**Western blot analysis**

Western blot analysis were performed as described previously using the following primary antibodies: mouse anti–human ARG1 (1:200, HM2163; HyCult Biotechnology, Uden, The Netherlands), rabbit anti–human myeloperoxidase (MPO; 1:2000, A0398; DakoCytomation, Glostrup, Denmark), rabbit anti–human lactoferrin (LF; 1:20 000; DakoCytomation), and rabbit anti–human gelatinase (GEL; 1:100016).\(^20\) Recombinant ARG1 was used to test the specificity of the ARG1 antibody at a concentration of 6.6 \(\mu\)g/mL (ALX-201-081; Alexis Biochemicals, San Diego, CA).

**Results and discussion**

A hallmark of neutrophil granule formation is the sequential emergence of azurophil, specific, and gelatinase granules and their constituent proteins during granulocytic differentiation. To define the time of ARG1 synthesis during granulocytic differentiation, we analyzed the mRNA and protein expression profiles for ARG1 and the marker proteins of azurophil (MPO), specific (LF), and gelatinase (GEL) granules in populations highly enriched in PMs, MYs, bm-PMNs, and pb-PMNs. This analysis demonstrated that ARG1, like the specific/gelatinase granule proteins LF and GEL, is synthesized in MYs and to some extent in bm-PMNs, but not in PMs like the azurophil granule protein MPO (Figure 1Ai-ii,B). Subsequent sequence analysis revealed that ARG1 transcripts expressed in MYs were 100% identical to the ARG1 transcripts expressed in the liver (GenBank locus NM_000045; data not shown). The finding that ARG1 is synthesized in MYs, indicates that ARG1 is stored in specific/gelatinase granules and not in azurophil granules.

To determine the subcellular localization of ARG1 in neutrophils, we isolated subcellular fractions from disrupted neutrophils by 3-layer Percoll density gradient centrifugation. As depicted in Figure 1Aiii, ARG1 protein was found exclusively in the subcellular fraction highly enriched in gelatinase granules, which are defined by a high content of GEL and a low content of LF. Importantly, ARG1 protein was not detectable in subcellular fractions enriched in azurophil granules (high content of MPO) and specific granules (high content of LF, very low content of GEL) (Figure 1Ai-iii). Despite numerous attempts, immunoelectron microscopy failed to give sufficient specific labeling to allow visualization of ARG1 in neutrophils.

Subsequent exocytosis studies revealed that neutrophils release ARG1 and GEL at higher levels in response to PMA, fMLP, and
ARG1 must be released from its intracellular compartment to the extracellular environment to effectively deplete extracellular arginine and thus to suppress T-cell immune responses. Because azurophil granule proteins are primarily released to phagosomes by activated neutrophils, Munder et al.7 argued that ARG1 contained in azurophil granules would be liberated by neutrophils at sites of infection as a consequence of necrotic cell lysis. Although necrosis of neutrophils may occur, it is not the major default pathway of neutrophils, because necrotic cell lysis liberates a plethora of toxic compounds that promote tissue destruction and inflammation. Hence, neutrophils that have phagocytosed microorganisms primarily undergo apoptosis and are removed by macrophages to facilitate resolution of inflammation.21,22 In this context, the presence of ARG1 in gelatinase granules rather than azurophilic granules is physiologically meaningful, because activated neutrophils readily mobilize gelatinase granules to the extracellular environment.15,16,23-25

Overall, our study indicates that neutrophils migrate to sites of infection and release ARG1 stored in gelatinase granules. ARG1 generates ornithine from arginine and thereby competes with NOS for arginine as a substrate. As a consequence, ARG1 reduces the synthesis of NO by endothelial cells and activated macrophages locally. Moreover, depletion of arginine by ARG1 might potentially suppress early T-cell immune responses. Finally, ornithine generated by ARG1 can be further metabolized into proline and polyamines, which support wound healing. In this context, our study supports a model in which activated neutrophils regulate early immune responses and support tissue regeneration by release of ARG1.

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Authorship

Contribution: L.C.J. designed and performed the experiments and wrote the manuscript; K.T.-M. assisted in designing the experiments and wrote the manuscript; E.I.C. performed the immunoelectron microscopy experiment; and N.B. assisted in designing the experiments and writing the manuscript.

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References


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