Biosynthesis of Granule Proteins in Normal Human Bone Marrow Cells. Gelatinase Is a Marker of Terminal Neutrophil Differentiation

By Niels Borregaard, Maxwell Sehested, Boye S. Nielsen, Henrik Søngeløv, and Lars Kjeldsen

Differentiation and maturation of myeloid cells is characterized by the sequential acquisition of two distinct cytoplasmic granule subsets, azurophil granules and specific granules. We recently showed the existence of a third granule subset, gelatinase granules. To investigate whether appearance of gelatinase granules marks a further step in maturation of myeloid cells beyond the appearance of specific granules, we sorted normal human bone marrow cells into one of three groups according to maturity by centrifugation on Percoll density gradients. The biosynthesis of myeloperoxidase (MPO) (an azurophil granule marker), lactoferrin and neutrophil gelatinase-associated lipocalin NGAL (specific granules markers) and gelatinase was then studied in each of these groups. We found that gelatinase was synthesized mainly in the group containing band cells and segmented cells. This contrasted with lactoferrin and NGAL, which were synthesized almost exclusively in the group containing myelocytes and metamyelocytes, and with MPO, which was mainly synthesized in the group containing myeloblasts and promyelocytes. Immunocytochemistry was in full agreement with the biosynthesis data, and showed that gelatinase appears in band cells, whereas NGAL and lactoferrin both appear in myelocytes. Thus, acquisition of gelatinase granules marks a step in neutrophil differentiation beyond the appearance of specific granules.

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negative granules), to the least dense and most easily mobilized, which contain the majority of gelatinase, but very little lactoferrin or NGAL (25% of total peroxidase-negative granules) with granules containing both located in between, in which the NGAL-gelatinase complex is found.11-13 Although gelatinase and lactoferrin colocalize in a substantial number of the peroxidase-negative granules, we believe that it is justified to apply the term "gelatinase granules" to the granule subset that contains a high concentration of gelatinase, and we reserve the term "specific granules" for lactoferrin-containing granules whether or not these also contain some gelatinase.12 We have recently been able to separate gelatinase granules from the lactoferrin-containing specific granules by subcellular fractionation13 and have shown that gelatinase granules are smaller than specific granules and are mobilized more easily than specific granules in response to stimulation with inflammatory mediators.13-14

Having defined gelatinase granules as a distinct granule subset (although representing the extreme of a continuum), we were interested in examining whether gelatinase granules are formed simultaneously with specific granules. If so, considerable sorting information must be incorporated in the nascent proteins desired for these granules during biosynthesis to target the proteins to the correct granule subset. However, if gelatinase granules are formed later than specific granules, this would imply a complex regulation of granule protein gene expression or mRNA translation to secure that these proteins are synthesized in a sequentially ordered way, but at the same time would require less complexity of the sorting apparatus and would add to the panel of markers that can be used for evaluation of myeloid cell differentiation and maturation.

MATERIALS AND METHODS

Isolation of bone marrow (BM) cells. Fifteen milliliters BM was aspirated from the posterior upper iliac crest of healthy volunteers. Anticoagulation was achieved by the immediate addition of 5 mL ACD (25 mmol/L sodium citrate, 126 mmol/L glucose). Erythrocytes were sedimented after addition of an equal volume of 2% Dextran T-500 (Pharmacia, Uppsala, Sweden) in saline, and the supernatant was aspirated and centrifuged at 200g for 10 minutes to sediment cells. The resulting supernatant was discarded and the pelleted cells were resuspended in 54 mL phosphate-buffered saline (PBS) and applied on top of four 50-mL centrifuge tubes each containing 9 mL Percoll, density 1.080 g/mL, layered below 9 mL Percoll of density 1.065 g/mL. The desired densities of Percoll were obtained by mixing 10× PBS (140 mmol/L NaCl, 10 mmol/L Na2HPO4/NaH2PO4, pH 7.4) with precalculated amounts of Percoll (Pharmacia) and distilled H2O. pH was adjusted to 7.4 by addition of 0.1 N HCl. The gradients were centrifuged at 1,000g for 20 minutes at 4°C. This resulted in fractionation of the BM cells into three groups that were procured by aspiration through a Pasteur pipette: band 1, a cell pellet at the bottom; band 2, all the cells between band 1 and a well-defined band on top of the gradient, band 3. Band 1 was contaminated with erythrocytes and, therefore, subjected to 30 seconds hypotonic lysis in distilled H2O. Cells from the three bands were then washed in saline and resuspended in 1 mL saline and counted. Cytospin preparations were made by applying 2×106 cells in 100 μL saline on glass slides.

Biosynthesis of granule proteins. Cells from the individual bands were pelleted and resuspended at 107 cells/mL in methionine-free medium (GIBCO-BRL, Meda A/S, Herlev, Denmark) containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% (wt/vol) dialyzed fetal calf serum (FCS; GIBCO-BRL) and incubated for 45 minutes at 37°C. The cells were subsequently pelleted by centrifugation and pulsed by resuspending to a concentration of 3×107 cells/mL in medium as above to which [35S]methionine (New England Nuclear DuPont Danmark A/S, Brøndby, Denmark) had been added to a final concentration of 200 μCi/mL followed by incubation for 30 minutes at 37°C. The pulse was stopped by pelleting the cells, washing once by resuspension in saline and centrifugation, and finally resuspending the cells in RPMI-1640 (GIBCO-BRL) containing 10% dialyzed FCS at a concentration of 107 cells/mL. After incubation for 2 hours at 37°C, the cells were pelleted by centrifugation and resuspended to 3×107 cells/mL in lysis buffer (10 mmol/L HEPES, pH 7.4, 100 mmol/L KCl, 25 mmol/L N-octyl glucoside (Sigma Chemical Co, St Louis, MO), 0.2% cetyltrimethylammonium bromide (BDH Chemicals Ltd, Poole, UK) 1 mmol/L phenylmethyl-sulphonyl fluoride (Sigma), 200 U/mL aprotinin (TrasyloI; Bayer, Leverkusen, Germany), 100 μg/mL leupeptin (Sigma), 1 mmol/L EGTA) and incubated overnight at 4°C. Undissolved material was pelleted by centrifugation for 15 minutes at 30 PSI in an Airfuge (Beckman Instruments Co, Palo Alto, Ca) and the supernatants divided in two for immunoprecipitation with either two sets of antibodies coupled to Sepharose beads: Antigelatinase antibody followed by antilactoferrin antibody, or anti-NGAL antibody followed by anti-MPO antibody.

Immunoprecipitation. Affinity-purified rabbit antibodies against gelatinase, NGAL, MPO, and lactoferrin (not affinity purified) were coupled to CNBr-activated Sepharose 4B (Pharmacia) at 5 mg IgG/mL gel. Before use, unspecific protein binding sites present on the Sepharose beads were blocked by incubating with 5% Tven-20 in 10 mmol/L HEPES pH 7.4, 100 mmol/L KCl overnight. Immunoprecipitation was performed by adding 25 μL IgG coupled Sepharose beads/mL of lysis, incubating on ice for 2 hours followed by centrifugation, and washing of the Sepharose particles 5 times by centrifugation. It had been ascertained by repeated immunoprecipitation that all antigen was effectively precipitated by the Sepharose-bound antibody added. The lysis was reproprecipitated with Sepharose-bouill antibody against other granule proteins as described above. The immunoprecipitated proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by boiling the Sepharose particles in 100 μL SDS sample buffer and electrophoresis on 12% polyacrylamide gels under reducing conditions.13 After fixation, the gels were stained with Coomassie blue and destained. Molecular weight markers were identified and marked with traces of [35S]-methionine and the gels submerged in Amplify (Amersham International, Amersham, UK) for 1 hour, dried, mounted on Kodak X-omat AR films (Eastman-Kodak, Rochester, NY) with an intensifying screen, and exposed for 1 to 3 days at -80°C.

Immunocytochemistry. Cytospin preparations were fixed in 4% formaldehyde in 0.1 mol/L phosphate buffer pH 7.0 for 20 minutes at room temperature and permeabilized by incubation in 50 mmol/L TRIS/HCl pH 7.6, 150 mmol/L NaCl (TBS) containing 1.0 % Triton X-100 at room temperature for 30 minutes. Unspecific binding was blocked by incubating for 10 minutes, with TBS containing 1% bovine serum albumin (BSA; Sigma). Binding of primary antibody was performed during a 1-hour incubation at room temperature with antibodies diluted in TBS containing 0.25% BSA. The following rabbit antibodies were used: antigelatinase18 1.0 μg/mL, anti-NGAL19 2.5 μg/mL; antiactin (DAKO A 186) 8.6 μg/mL anti-MPO (DAKO A-398) 76 μg/mL; nonimmune serum (DAKO X-903) 16 μg/mL. The slides were then washed three times in TBS and incubated for 1 hour with alkaline phosphatase-conjugated swine antirabbit (DAKO D 306) diluted 50-fold in TBS 0.25% BSA, washed twice in TBS, and incubated in 100 mmol/L TRIS/HCl pH 11.5, 100 μg/mL nitroblue tetrazolium and 0.5 mg/mL 5-bromo-4-chloro-3-indolyl phosphate for 2 hours. After development, the slides were counterstained with methylene blue for 1 minute, washed, dehydrated, cleared, and mounted with Permount. The slides were examined under a Leica DMRB microscope (Leica, Wetzlar, Germany) at ×400 magnification. The number of positive cells was quantitated at ×400 magnification by using a square frame to delineate the area and averaging the count of the positive cells in eight 200×200 μm fields. The number of positive cells was determined by using the computer program Image Pro Plus version 2.0 (Media Cybernetics, Silver Spring, MD) for each of the experimental groups. The number of positive cells was compared using the Wilcoxon signed-rank test.
cells from band myeloid cell differentiation beyond the formation of specific tinase granules is an indicator of a further step in neutrophil granules.

As synthesized in the most mature cells: band cells at the same time, are routed to the same granule, whereas included that gelatinase, NGAL, and lactoferrin, if synthesized granules. This heterogeneity was observed both in granules containing lactoferrin and gelatinase in between. In these latter granules, the 135-kD complex of NGAL and lactoferrin (labeling only for gelatinase and not for lactoferrin in double-labeling immunoelectron microscopy) with granules containing both lactoferrin and gelatinase in between. In these latter granules, the 135-kD complex of NGAL and gelatinase is found and, thus, serves as a marker for these granules. This heterogeneity was observed both on immunoelectron microscopy and on subcellular fractionation.

We have recently been able to separate gelatinase granules from the lactoferrin-containing specific granules. Furthermore, we have shown that these granule subsets are functionally different. Gelatinase granules are released much more readily from neutrophils than granules containing both gelatinase and lactoferrin (measured as release of the NGAL-gelatinase complex). The granules that contain lactoferrin but no gelatinase are released only to a very limited extent when a panel of inflammatory mediators were used as stimuli. The fact that this functional difference between the

No biosynthesis of any of the marker proteins was detected when peripheral blood neutrophils were subjected to biosynthesis studies under the same conditions as the BM cells (data not shown). The demonstration of a sequential synthesis of granule proteins is in full agreement with immunodetection of the marker proteins in the cells of band 1, 2, and 3 (Table 2). Clearly, gelatinase is detected in band cells and in segmented cells, but not in the scattered metamyelocytes present in band 1, indicating that biosynthesis of gelatinase occurs in band cells. The most striking visualization of the separate appearance of lactoferrin and gelatinase is obtained by immunocytochemical staining of the cells from band 2 (Fig 3). Some myelocytes and most metamyelocytes identified stained positively for lactoferrin (and NGAL, not shown) indicating that biosynthesis of these proteins occurs at the myelocyte stage of differentiation (as confirmed by the lack of NGAL and lactoferrin from promyelocytes in bands 3 and 2, respectively). In contrast, gelatinase was absent from these cells and only observed in the few scattered band cells and segmented cells present in band 2. In addition, gelatinase was observed in the few monocytes present in band 3.

DISCUSSION

The known order in which granulopoiesis occurs during differentiation and maturation of normal myeloid cells has previously been described by immunocytochemistry and electron microscopy. This is now confirmed by studying the biosynthesis of granule markers. More importantly, it is shown that gelatinase is synthesized later than lactoferrin and, thus, is a marker for myeloid cells differentiated beyond acquisition of specific granules. By itself, this observation adds one parameter more to be used in assessing myeloid cell differentiation. The usefulness of gelatinase immunocytochemistry in evaluating dys-differentiation and dys-maturation in disorders of myelopoiesis (in particular, the myelodysplastic syndromes) should be evaluated.

Another aspect of this study concerns the targeting of proteins to granules during granulopoiesis. We have recently shown that peroxidase-negative granules exist as a continuum from granules containing lactoferrin and NGAL, but no gelatinase, to granules rich in gelatinase and poor in lactoferrin (labeling only for gelatinase and not for lactoferrin in double-labeling immunoelectron microscopy) with granules containing both lactoferrin and gelatinase in between. In these latter granules, the 135-kD complex of NGAL and gelatinase is found and, thus, serves as a marker for these granules. This heterogeneity was observed both on immunoelectron microscopy and on subcellular fractionation.

9.6 for 20 minutes with Fast-Red (Kem-En-Tec, Copenhagen DK) as recommended by the manufacturer. After washing in running tap water, the slides were counter stained in Mayer's hematoxylin for 3 minutes, washed, and mounted.

RESULTS

The association between cell maturation and specific cell density described by Boyum was used to sort normal BM cells into three groups: band 1, containing the most mature cells (polymorphonuclear leukocytes and band cells); band 2, containing myeloid cells of intermediate maturity (metamyelocytes and myelocytes; and band 3, containing myeloblasts and promyelocytes (in addition to megakaryocytes and cells from the erythrocyte lineage) (Fig 1, A through C) and Table 1.

Biosynthesis experiments were performed as a 45-minute starvation in methionine-free medium followed by a 30-minute pulse in medium containing [³⁵S]-methionine and a 2-hour chase in tracer-free medium. Subsequent immunoprecipitation showed that MPO is synthesized mainly in cells from band 3 and that no synthesis of the specific granule markers NGAL and lactoferrin or gelatinase takes place in these cells (Fig 2). This confirms previous observations on the sequential acquisition of azurophil and specific granules. More important is the clear-cut separation between biosynthesis of gelatinase, on the one hand, and lactoferrin and NGAL on the other (Fig 2). Whereas lactoferrin and NGAL were synthesized almost exclusively in cells from band 2 (myelocytes and metamyelocytes), the main part of gelatinase was synthesized in the most mature cells: band cells and segmented cells. These are cells not previously recognized as active in granulopoiesis. Thus, acquisition of gelatinase granules is an indicator of a further step in neutrophil myeloid cell differentiation beyond the formation of specific granules.

Interestingly, the small part of gelatinase synthesized in cells from band 2 was (partly) associated with NGAL as evident from the coinmunoprecipitation of gelatinase with NGAL in these cells (Fig 2B). This shows that the complex between NGAL and gelatinase is formed only when these are synthesized simultaneously. If already synthesized NGAL could associate with newly synthesized gelatinase, then the anti-NGAL antibodies should be able to precipitate some labeled gelatinase in cells from band 1. This was not observed. The fact that the main part of gelatinase is located in granules different from the NGAL containing specific granules fully explains this fact. Therefore, it can be concluded that gelatinase, NGAL, and lactoferrin, if synthesized at the same time, are routed to the same granule, whereas gelatinase synthesized later is routed into separate gelatinase granules.

It should be noted that the immunoprecipitations were performed on all cells present in bands 1, 2, and 3. Because the number of cells increases with maturity, band 1 contains the most cells and band 3, by far, the least (Table 1). Despite this, MPO synthesis was most pronounced in band 3. Therefore, this means that on a cell basis, the MPO synthesis is far more efficient in cells from band 3 than in cells from the other bands.

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Fig 1. Sorting of BM cells. Giemsa-stained cytopsin preparations of BM cells from band 1 (A), band 2 (B), and band 3 (C) obtained by separation on Percoll density gradients.

Fig 3. Immunohistochemistry of BM cells. Cytospin preparations of cells from bands 1, 2, and 3 were labeled with antibodies against lactoferrin, gelatinase, or myeloperoxidase. (A), gelatinase band 3; (B and C), gelatinase band 2 (two different magnifications); (D), gelatinase band 1; (E and F), lactoferrin band 2 (two different magnifications); (G), lactoferrin band 1; (H), myeloperoxidase band 2.
The distribution of myeloid cells in the three bands obtained from density gradient centrifugation of normal BM on a two-layer Percoll density gradient. The total number of each cell type in each band was obtained by multiplying the fraction of any cell type (as determined by differential counting of 300 cells in cytospin preparations) by the total number of cells in the band. The results are mean of three independent experiments.

The same hypothesis may also explain the heterogeneity of peroxidase-positive granules that have been observed in several groups. Although a recent report on the transcription of lactoferrin and gelatinase genes in a mouse myeloid cell line did not add credence to this hypothesis regarding peroxidase-negative granules, the biosynthesis studies presented here prove the hypothesis correct. In fact, a difference of 2 days between the peak of lactoferrin gene transcription and gelatinase gene transcription (both measured as mRNA content) was observed in the mouse model, and this may be significant. Furthermore, the observation that gelatinase but not lactoferrin gene transcription can be induced in NB4 cells and HL-60 cells when these are differentiated along the monocyte lineage indicates a separate regulation of gene transcription of these proteins in myeloid cells.

Fig 2. Biosynthesis of granule marker proteins. BM cells from band 1 (16.6 x 10^7 cells), band 2 (6.4 x 10^7 cells), and band 3 (2.8 x 10^7 cells) were incubated with [35S]-methionine for 30 minutes and chased for 2 hours. Cell lysates were immunoprecipitated with anti-NGAL, anti-gelatinase, anti-MPO, and anti-lactoferrin. The immunoprecipitates were analyzed by 12% SDS-PAGE and fluorography. (B) is a separate experiment which shows some coprecipitation of gelatinase (arrow) with the anti-NGAL antibodies in cells from band 2 (4.6 x 10^7 cells).
BIOSYNTHESIS OF GRANULE PROTEINS

Table 2. Immunohistochemistry of Cytospin Preparations of BM Cells

<table>
<thead>
<tr>
<th>Gelatinase (--)</th>
<th>NGAL (--)</th>
<th>Lactoferrin (--)</th>
<th>Myeloperoxidase (--)</th>
<th>NI (--)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Band 1</td>
<td>15/85</td>
<td>10/90</td>
<td>10/90</td>
<td>5/95</td>
</tr>
<tr>
<td>Band 2</td>
<td>62/18</td>
<td>28/72</td>
<td>33/67</td>
<td>4/96</td>
</tr>
<tr>
<td>Band 3</td>
<td>98/4</td>
<td>91/9</td>
<td>94/6</td>
<td>56/44</td>
</tr>
</tbody>
</table>

Immunohistochemistry of cytospin preparations of BM cells labeled with antibody against gelatinase, NGAL, lactoferrin, myeloperoxidase, or control with nonimmune serum (NI). The number of --/+ cells in each band is given. One hundred cells were counted for each condition. Band 1 contains the most mature cells (Table 1).

Thus, sorting of granule proteins is controlled by timing of biosynthesis and not by targeting to individual granule subsets. Although it cannot be ruled out that this control is exerted on mRNA, it is most likely to occur at the level of gene transcription. Identification of the timers that control transcription of genes coding for granule proteins will be important for the understanding of mechanisms controlling differentiation of myeloid cells.

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