# Biosynthetic profiles of neutrophil serine proteases in a human bone marrow-derived cellular myeloid differentiation model

Daniel Garwicz
Andreas Lennartsson
Sten Eirik W. Jacobsen
Urban Gullberg
Anders Lindmark

Background and Objectives. Human leukocyte elastase, proteinase 3 and cathepsin G are neutrophil granule proteins belonging to the hematopoietic serine protease superfamily. In addition to their established roles in inflammation, they have recently been implicated as regulators of granulopoiesis and mediators of apoptosis. We set out to characterize the individual biosynthetic profiles of these proteins in a neutrophil differentiation model.

Design and Methods. Bone marrow-derived $CD34^+CD38^+$ hematopoietic progenitor cells from 21 healthy human volunteers were cultured *in vitro* in the presence of recombinant human granulocyte colony-stimulating factor (G-CSF). Biosynthetic radiolabeling was performed in cells from 13 subjects after various periods of differentiation induction. Following protein extraction, the proteins were specifically immunoprecipitated from cell lysates and media and run in gel electrophoresis. Biosynthetic profiles of azurophil granule proteins, in particular members of the neutrophil serine protease family, were examined during myeloid differentiation.

Results. The onset of synthesis of myeloperoxidase, lysozyme, leukocyte elastase, and proteinase 3 occurred early after differentiation induction with G-CSF, while synthesis of cathepsin G, azurocidin, and bactericidal/permeability-increasing protein was detected somewhat later. Cathepsin G and proteinase 3 were retained intracellularly relatively efficiently, while leukocyte elastase and lysozyme were secreted to a greater extent. Cell morphology and positive immunocytochemistry for lactoferrin as well as flow cytometric analysis of selected surface antigens confirmed neutrophil-like maturation.

Interpretation and Conclusions. We demonstrate that azurophil granule proteins, including proforms of human leukocyte elastase, proteinase 3 and cathepsin G, are constitutively secreted to various degrees during *in vitro* myeloid differentiation of human hematopoietic progenitor cells, in addition to being stored intracellularly in active forms. These findings suggest protein-specific sorting mechanisms and may have implications for the regulation of granulopoiesis.

Key words: biosynthesis, neutrophils, serine protease, $CD34^+$ cells, G-CSF.

Haematologica 2005; 90:38-44


*From the Department of Hematology, Lund University, Lund, (DG, ALe, UG, AL); Division of Molecular Toxicology, Institute of Environmental Medicine, Karolinska Institutet, Stockholm, (DG); Hematopoietic Stem Cell Laboratory, Lund Stem Cell Center, Lund University, Lund, (SEWJ); Sweden.*

*Correspondence:*
*Daniel Garwicz, MD, PhD, Department of Hematology, Biomedical Center, C14, S-221 84 Lund, Sweden. E-mail: daniel.garwicz@hematologi.lu.se*

The differentiation of the pluripotent hematopoietic stem cell into a mature, functional neutrophil occurs in several well-defined morphological stages.[1] During this process, the neutrophil acquires its characteristic features, such as the ability to phagocytose and kill microorganisms[2] and the presence of different subsets of intracellular storage compartments; azurophil (primary), specific (secondary), and gelatinase (tertiary) granules as well as secretory vesicles.[3]

The azurophil granules characteristically contain enzymatically active myeloperoxidase (MPO), defensins, and a number of other potent microbicidal peptides and proteins, including the neutrophil serine proteases.[4] These proteins are stored in granules until exocytosed, by regulated secretion, in response to various stimuli. In addition to their proteolytic properties and involvement during inflammation, the neutrophil serine proteases have other important effects. Proteinase 3 (PR3; myeloblastin) has been suggested to play a role in growth and differentiation of hematopoietic cells during normal states and in leukemia.[5-8] Enzymatically active human leukocyte elastase (HLE; neutrophil elastase) has recently been implicated in negative feedback-regulation of granulopoiesis by cleavage of granulocyte colony-stimulating factor (G-CSF) and its receptor.[9,10] Moreover, cathepsin G (CatG),[11-13] PR3,[14] and HLE[14] have been implicated in decreased cell survival and mediation of apoptosis. The recent observation that many patients with cyclic and severe congenital neutropenia have heterozygous mutations in *ELA2*, the gene coding for leukocyte elastase,[15] suggests a potential role for this molecule in the pathogenesis of

these hematologic diseases. The neutrophil serine proteases are similar in many respects. They are considered to be synthesized and down-regulated in a co-ordinated manner during the promyelocytic stage of neutrophil differentiation in the bone marrow and their genes are similarly organized. With the exception of CatG (encoded on 14q11.2), the genes encoding the neutrophil serine proteases are clustered on the short arm of chromosome 19 (19p13.3).[16,17] Several of the promoter regions in the neutrophil serine protease genes have binding sites for the transcription factors PU.1, c-Myb, C/EBP, core binding factor,[18,19] and a cytidine-rich element, suggesting a common transcriptional regulation.

How is the temporal expression of neutrophil serine proteases organized? Is it co-ordinated, as has been suggested for certain specific granule proteins,[20] or is there an individual and differential regulation, as indicated by expression of neutrophil granule proteins at the mRNA level in human acute promyelocytic leukemia cells[21] or cells from normal human bone marrow?[22] Conflicting reports exist on this matter depending on the experimental models used.[16,23,24] However, one drawback with these investigations on neutrophil serine proteases is that they are based on mRNA data, rather than protein biosynthesis. Recent reports have highlighted a sometimes poor correlation between mRNA expression and protein abundance, indicating that it may be difficult and perhaps even incorrect to extrapolate changes in mRNA to protein levels.[25] In addition, most previous studies on biosynthesis of neutrophil serine proteases at the protein level have utilized endogenous synthesis in human cell lines or transgenic expression in rodent cellular models.[4,26] In the present investigation, we set out to characterize the biosynthetic profiles of azurophil granule proteins, focusing on the neutrophil serine proteases, using radiolabeling of newly synthesized proteins in human hematopoietic progenitor cells subjected to *in vitro* differentiation with G-CSF. In addition, we examined whether differences in retention/constitutive secretion exist during neutrophil differentiation.

## Design and Methods

### *Study population, informed consent, and ethical approval*

Twenty-one healthy human adults (14 females aged 19-42 years and 7 males aged 21-49 years) formed the study group. The procedures followed were in accordance with ethical standards on human experimentation and with the Declaration of Helsinki and were approved by the Ethics committee at Lund University (reg. no. LU 390-96).

### *Collection of bone marrow and purification of CD34⁺CD38⁺ progenitor cells*

Bone marrow was aspirated from the iliac crest by licensed physicians at Lund University Hospital. Mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway), followed by positive selection for the CD34 antigen by magnetic cell sorting on MidiMACS (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of $CD34^+$ cells was typically greater than 90%, as determined by flow cytometric analysis. The cells were then stained with fluorescein isothiocyanate (FITC)-conjugated anti-human CD34 antibodies and phycoerythrin (PE)-conjugated anti-human CD38 antibodies allowing further purification of $CD34^+CD38^+$ cells by fluorescence activated cell sorting (FACS) on a FACSVantage (Becton Dickinson). The $CD34^+CD38^+$ cells, containing predominantly committed progenitor cells and constituting more than 90% of the $CD34^+$ population, were diluted in serum-free medium, X-vivo 15 (BioWhittaker, Verviers, Belgium), until cell culture was initiated. The remaining $CD34^+CD38^-$ cells, containing primitive hematopoietic stem cells, were used for other studies.

### *Cell culture*

Human bone-marrow $CD34^+CD38^+$ cells were cultured in Iscove's modified Dulbecco's medium (IMDM) with L-glutamine from GIBCO BRL (Life Technologies, Gaithersburg, MD, USA), 20% heat-inactivated fetal bovine serum (FBS) (BioWhittaker, Verviers, Belgium), 0.1 mM 2-mercapto-ethanol (Sigma, St. Louis, MO, USA) and 50 ng of recombinant human granulocyte colony-stimulating factor (G-CSF; Amgen, Thousand Oaks, CA, USA) per mL. G-CSF was added at the time of culture initiation and during prolonged pulse experiments. Cultures were kept in 5% $CO_2$ at 37°C in fully humidified air (relative humidity >95%). Cell viability exceeded 80% at all time points.

### *Cell morphology and assessment of differentiation*

Cytospin preparations of $CD34^+$ cells differentiated with G-CSF for various periods were fixated and stained, as per routine, with May-Grünwald Giemsa. Cells were classified according to morphological differentiation stage in a light microscope. At least 100 cells were counted on selected days.

### *Antibodies*

A polyclonal rabbit antiserum to MPO, a polyclonal sheep antiserum to HLE, a polyclonal rabbit antiserum to CatG, a polyclonal rabbit antiserum to human bactericidal/permeability-increasing protein (BPI), and a polyclonal rabbit antiserum to human lactoferrin were kindly supplied by Prof. Inge Olsson, Lund University,

Sweden. Monoclonal mouse antibodies to PR3 (4A3) were a generous gift from Prof. Jörgen Wieslander, Wieslab, Lund, Sweden. Monoclonal mouse antibodies to human azurocidin were a generous gift from Dr. Hans Flodgaard, Novo Nordisk, Bagsvaerd, Denmark. A polyclonal rabbit antiserum to human calreticulin (PA3-900), an apopro-MPO interacting protein, was from Affinity Bioreagents, Inc, Golden, CO, USA. Monoclonal mouse antibodies against the proenzyme of human cathepsin D (CatD, a lysosomal aspartyl proteinase) were a generous gift from Assoc. Prof. Gregory E. Conner, University of Miami, FL, USA. A polyclonal rabbit antiserum against human lysozyme (DAKO A0099) was from DAKO, Glostrup, Denmark. For immunocytochemistry, polyclonal rabbit antibodies to human lactoferrin (A 0186, DAKO) and alkaline phosphatase-conjugated swine anti-rabbit immunoglobulins (D 0306, DAKO) were utilized.

### *Enzymatic stainings and immunocytochemistry*

Staining for non-specific esterase as a marker for monocytic differentiation was performed on acetone/formaldehyde fixated cytospin preparations with α-naphthyl butyrate as substrate and hexazotized pararosaniline as the coupler.[27] Staining for peroxidase as a marker for early myeloid/neutrophil differentiation was performed on ethanol/formaldehyde fixated cytospin preparations with 3-amino-9-ethylcarbazole as substrate.[28] Staining for leukocyte alkaline phosphatase (LAP), as a marker for secretory vesicles/terminal neutrophil differentiation, was performed on methanol/formaldehyde fixated cytospin preparations with naphthol-AS-phosphate/Fast Blue BB salt (from Sigma) as substrate. Immunocytochemistry for detection of lactoferrin, a marker for specific granules, was performed essentially as described elsewhere.[29]

### *Flow cytometric analysis of cell surface antigens*

Cells were harvested on the days indicated, spun down in 96-well microtiter plates and resuspended in PBS at approximately $10^6$ cells/mL. Next, 5 μL of monoclonal antibodies were added to 50 μL of cell suspension and incubated at room temperature for 10 min. After washing twice in PBS, the cells were resuspended in a volume of 400 μL and analyzed on a FACScan flow cytometer (Becton Dickinson). Antibodies directed against the following surface antigens, and coupled to the fluorochrome indicated, were used: CD14-PE, CD15-FITC, CD66-FITC, (all from DAKO A/S, Copenhagen, Denmark).

### *Biosynthetic labeling and immunoprecipitation*

Biosynthetic radiolabeling was performed in $CD34^+$ $CD38^+$ cells from 13 of the 21 subjects in the study. Cells were starved for 30 min in RPMI 1640 medium with 1% dialyzed FBS but depleted of methionine and cysteine, whereupon they were pulse-labeled with $^{35}$S-methionine/$^{35}$S-cysteine (Tran$^{35}$S-label; ICN Pharmaceuticals, Irvine, CA, USA) at 100-200 μCi/mL for 30 min to 7 h at 37°C in the presence of G-CSF and in some cases other cytokines. Following protein extraction in lysis buffer containing protease inhibitors (Complete™, Boehringer Mannheim, Germany), biosynthetically labeled proteins, in lysed cells and media, were immunoprecipitated with protein A-sepharose CL4-B (Pharmacia, Uppsala, Sweden) and polyclonal antiserum or monoclonal antibodies directed towards the proteins. When monoclonal antibodies were used, protein G PLUS-agarose (Santa Cruz Biotechnology) was also added. Following immunoprecipitation overnight, the immunoprecipitates were washed three times and frozen at -20°C prior to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

### *SDS-PAGE and fluorography*

After addition of electrophoresis sample buffer, the immunoprecipitates were boiled for 5 min and the protein A-sepharose was spun down, whereupon 25 μL of the supernatant containing radiolabeled protein were applied to the gel (10-20% precast Tris-Glycine gel; Novex, San Diego, CA, USA) using a Hamilton syringe. Following electrophoresis, the gels were stained, destained, subjected to fluorescence enhancement and analyzed quantitatively for radioactivity or directly placed in film cassettes with Hyperfilm MP (Amersham). After exposure for various periods at -80°C, the fluorograms were developed and fixed.

### *Quantitative measurements of radiolabeled proteins*

Measurements of radioactivity in proteins derived from $CD34^+$ cells were performed in a Fluorescent Image Analyzer (FLA-3000; Fuji Photo Film Co, Ltd.) and data were analyzed (Image Reader FLA-3000 V 1.0 and Image Gauge V 3.12; Fuji Photo Film Co, Ltd). The numerical values representing specific protein bands were mathematically normalized so that the radiolabeled cell lysates of newly collected $CD34^+CD38^+$ cells (day 0) for each protein were given the arbitrary value of 1. Global background activities were subtracted from all values prior to normalization.

## Results

### *G-CSF-induced differentiation of human bone marrow $CD34^+CD38^+$ progenitor cells*

Following purification, approximately 98% of the cells were small, lymphocyte-like cells, representing $CD34^+CD38^+$ cells. Morphologically, the cells were fairly synchronized during *in vitro* culture with G-CSF. After 10-12 days, predominantly band cells or mature

neutrophils were seen. Terminal neutrophil differentiation was verified by typical trilobed nuclear morphology, positivity for peroxidase (approximately 90% of cells on day 14) and lactoferrin (75% on day 14) and emergence of LAP-positive cells from day 11 after differentiation induction with G-CSF. Moreover, FACS-analysis on day 14 revealed strong upregulation of the neutrophil-associated surface markers CD15 (95%) and CD66 (>75%), while high expression of CD14 and esterase-positivity, as seen in monocytes, were below 10%. During culture, the number of cells expanded on average 8-16 times during the two-week observation period. Cell viability was typically 100% prior to experiments and never went below 80% throughout the entire culture period, as determined by Trypan blue exclusion.

### *Biosynthesis of granule proteins during differentiation of CD34⁺CD38⁺ cells with G-CSF*

Biosynthesis was examined on consecutive days in cells subjected to differentiation induction with G-CSF (Figure 1). Biosynthesis of MPO was detected in newly collected CD34⁺CD38⁺ cells (i.e. on day 0) and throughout the entire period examined by means of biosynthetic labeling. Small amounts of lysozyme as well as HLE and PR3 were also detected on day 0/day 1, while the synthesis of CatG seemed to be initiated somewhat later. Clear differences in the tendency for intracellular retention vs. constitutive secretion were seen; while HLE was secreted to a great extent, the majority of PR3 and CatG was retained intracellularly. Biosynthesis of CatD and azurocidin was detected from day 2 (Figure 1), while expression of BPI was not seen until day 4 (*data not shown*) and 5 (Figure 2). The biosynthesis of most azurophil granule proteins was apparently maximal on days 3-4, although strong expression was still seen on day 5 in one individual (Figure 2). Synthesis of lactoferrin, a specific granule protein, could be demonstrated on day 8 in another individual (Figure 3).

Given the rather similar amounts of the various serine proteases in neutrophils,[30] the difficulties, in the present work, in detecting significant biosynthesis of azurocidin are puzzling. The functionality of the detection system was obvious, since antibodies were able to detect azurocidin on day 4 (*data not shown*) and day 5 (Figure 2) in two separate individuals. Furthermore, the antibodies have been successfully utilized, in a similar manner, for the detection of human azurocidin expressed in rodent cell lines.[31] Likewise, BPI had a very narrow biosynthetic window and was most difficult to detect (Figure 2), although the same antiserum[32] has been used previously to detect human BPI in HL-60 cells[33] and expressed in murine myeloid 32D cells.[34] Thus, the weak expression of azurocidin and BPI could depend either on the

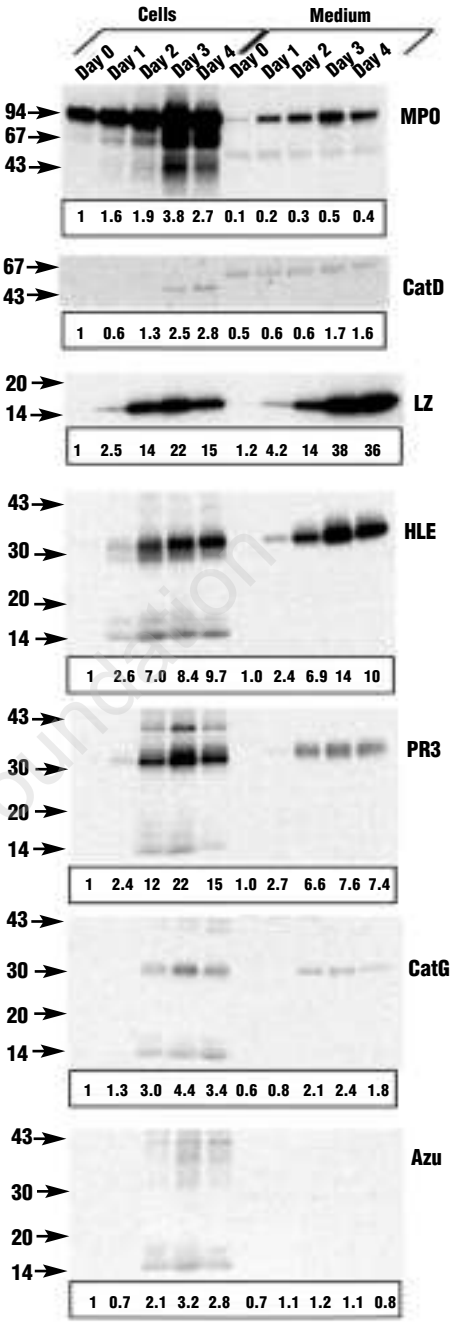


**Figure 1. Biosynthesis of azurophil granule proteins on days 0-4 of differentiation of human bone marrow CD34⁺CD38⁺ cells with G-CSF. Human CD34⁺CD38⁺ bone marrow cells were cultured in the presence of 50 ng G-CSF/mL for up to 4 days. During culture, 200×10³ cells were withdrawn daily and analyzed for active protein synthesis by 5 h of biosynthetic labeling with ³⁵S-methionine/³⁵S-cysteine in the presence of 50 ng G-CSF/mL, followed by protein extraction from cell lysates and pulse medium. The samples from each day were aliquoted, proteins were immunoprecipitated with specific antibodies against MPO, CatD, lysozyme (LZ), HLE, PR3, CatG, and azurocidin (Azu), respectively, and run on SDS-PAGE. The gels were dried and quantitative analysis of radioactivity was performed in a Fluorescent Imager Analyzer. Numbers in boxes represent the relative values, normalized for the first value, for the specific protein bands for each individual protein. The fluorograms were exposed for 4 days. Molecular weight markers are indicated with numbers (values expresssed in kDa) and arrows to the left. The depicted results are representative of three different individuals.**

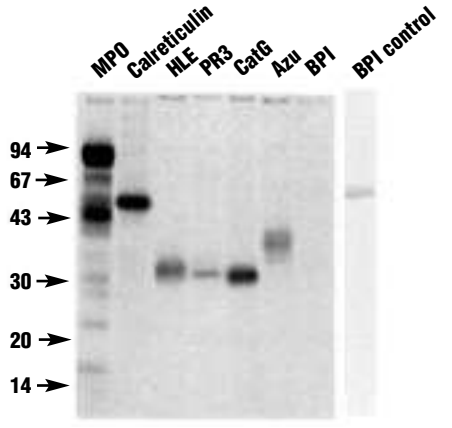


Figure 2. Biosynthesis of azurophil granule proteins on day 5 of differentiation of human bone marrow CD34⁺CD38⁺ cells with G-CSF. Human CD34⁺CD38⁺ bone marrow cells (1.8×10⁶ cells) were cultured in the presence of 50 ng G-CSF/mL. After 5 days of culture, 4×10⁶ cells were withdrawn and subjected to 2 h of biosynthetic labeling with $^{35}$S-methionine/$^{35}$S-cysteine in the presence of 50 ng G-CSF/mL, followed by protein extraction from cell lysates and immunoprecipitation with antibodies against MPO, calreticulin, HLE, PR3, CatG, Azu, and BPI, respectively. A positive control for the BPI antibody was provided by recombinantly expressed human BPI secreted from murine 32D cells.[34] The fluorogram was exposed for 8 weeks. Molecular weight markers are indicated with numbers (values expressed in kDa) and arrows to the left. The data depicted in the figure are from a single individual.

Table 1. Biosynthesis of neutrophil granule proteins during differentiation.

| *Protein* | *Days of in vitro differentiation of CD34⁺CD38⁺ cells with G-CSF* | | | | | | |
|---|---|---|---|---|---|---|---|
| | *0* | *1* | *2* | *3* | *4* | *5* | *6* |
| MPO | + | ++ | ++ | ++ | ++ | ++ | + |
| HLE | –/(+) | + | ++ | ++ | ++ | ++ | + |
| PR3 | –/(+) | + | ++ | ++ | ++ | ++ | + |
| CatG | – | –/(+) | + | ++ | ++ | +/++ | – |
| Azu | – | – | (+) | (+)/+ | (+)/+ | +/++ | – |
| BPI | – | – | – | – | + | –/(+) | ND |
| CatD | – | – | (+) | + | ++ | + | ND |
| LZ | –/(+) | + | ++ | ++ | ++ | ND | ND |
| LF | ND | ND | ND | ND | – | ND | – |

*- : No detectable protein synthesis, (+): detectable, but weak, labeling, +: fairly strong labeling, ++: strong labeling, ND: not determined, G-CSF: granulocyte colony-stimulating factor, MPO: myeloperoxidase, HLE: human leukocyte elastase, PR3: proteinase 3, CatG: cathepsin G, Azu: azurocidin, BPI: bactericidal/permeability increasing protein, CatD: cathepsin D, LZ: lysozyme, LF: lactoferrin. The data in the table were derived from 12 individuals. Strong labelling of MPO and lactoferrin was detected on day 8 after differentiation induction in one individual (see Figure 3). For details on experimental design, see Design and Methods.*

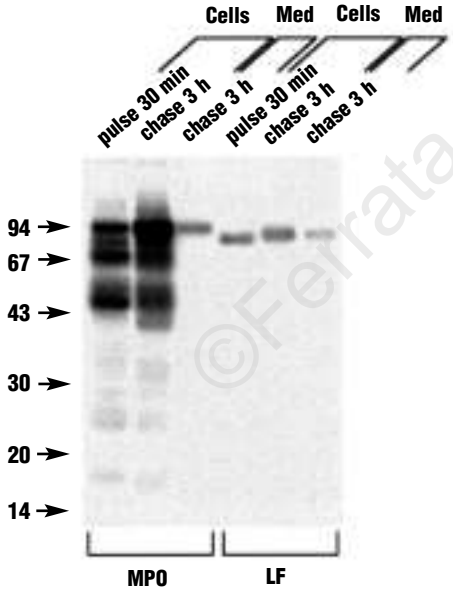


Figure 3. Biosynthesis of MPO and lactoferrin on day 8 of *in vitro* differentiation of human bone marrow CD34⁺CD38⁺ cells with G-CSF. Human CD34⁺CD38⁺ bone marrow cells (1×10⁶ cells) were cultured in the presence of 50 ng G-CSF/mL. After 8 days of culture, 1.3×10⁶ cells were withdrawn and subjected to 30 min of pulse-labeling with $^{35}$S-methionine/$^{35}$S-cysteine in the presence of 50 ng G-CSF/mL, followed by 3 h of chase of the label. After protein extraction from cell lysates and medium (Med), the samples were aliquoted and immunoprecipitation with antibodies directed against MPO and lactoferrin (LF) was performed. The fluorogram was exposed for 8 weeks. Molecular weight markers are indicated with numbers and arrows to the left. The data depicted in the figure are from a single individual.

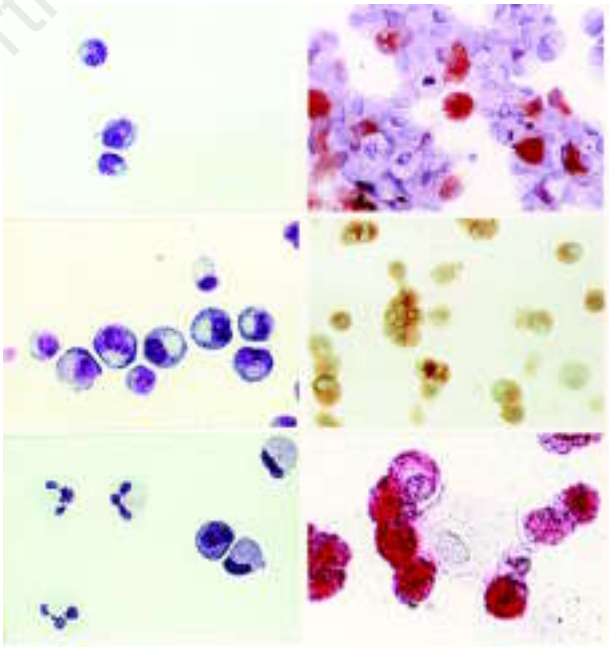

Figure 4. Cell morphology and special stainings in the human bone marrow-derived CD34⁺CD38⁺ *in vitro* G-CSF myeloid differentiation model. Human CD34⁺CD38⁺ bone marrow cells were cultured as decribed in *Design and Methods* for up to 14 days in the presence of 50 ng G-CSF/mL. Cell morphology on day 0 (upper left panel), day 4 (middle left panel) and day 14 (lower left panel). Non-specific esterase staining on day 7 of differentiation (upper right panel). Peroxidase staining on day 14 (middle right panel), immunocytochemistry for lactoferrin on day 14 (lower right panel). The data depicted in this figure were derived from three different individuals.

need for other cytokines, in addition to G-CSF, for maximal protein expression or on individual variations in the levels of these proteins. To examine the effect of single cytokines, $CD34^+CD38^+$ cells prestimulated for three days with G-CSF were pulse-labeled for 7 h in the presence of G-CSF in combination with either of human interleukin-1α (IL-1α; 20 ng/mL), GM-CSF (20 ng/mL), IL-6 (50 ng/mL), or tumor necrosis factor α (TNFα; 10 ng/mL). However, no evident effect on the biosynthesis of azurocidin or BPI was demonstrated (*data not shown*).

## Discussion

This is, to the best of our knowledge, the first comprehensive study on the biosynthesis of neutrophil serine proteases at the protein level adopting a straightforward myeloid differentiation model consisting of $CD34^+CD38^+$ human bone marrow progenitor cells subjected to *in vitro* differentiation with G-CSF. Our model uncovers the changes in granule protein biosynthesis over time as the myeloid progenitor cells differentiate (Table 1, Figure 4).

Biosynthesis of MPO, HLE, PR3, and lysozyme preceded the morphologically distinguishable appearance of promyelocytes, which is consistent with previous studies based on mRNA expression profiles.[22,35] In contrast, CatG, CatD, azurocidin and especially BPI were detected later, indicating that the biosynthesis of these proteins is initiated at the promyelocytic stage. For CatG this correlates well with its mRNA expression in cells derived from a patient with chronic myeloid leukemia.[36] These findings indicate that the expression of neutrophil serine protease family members is not entirely co-ordinated during differentiation, and suggests an individualized transcriptional or post-transcriptional control. We also demonstrate that the retention/secretion patterns differ among the azurophil granule proteins (Figure 1). While HLE and lysozyme were constitutively secreted to a relatively large extent in the $CD34^+$ cell differentiation model, PR3, CatG and CatD were retained intracellularly during synthesis to a greater extent. Substantial amounts of the newly synthesized neutrophil serine proteases are constitutively secreted immediately after synthesis. This conclusion is based on the findings that extracellular secretion of HLE, PR3, and CatG was not evident in conventional pulse-chase radiolabeling experiments, using a short pulse followed by a longer chase and subsequent analysis of the chase medium (*data not shown*). In contrast, the secretion was obvious after 5 h of continuous pulse-labeling and subsequent analysis of the pulse medium (Figure 1). Thus, as constitutive secretion is fast, with a short transit time from the Golgi apparatus to cell surface,[37] the most likely explanation for the observed discrepancy is that most of the constitutively secreted protein is released already during the pulse-labeling period. These findings may have functional and clinical implications since it has been proposed that some secreted neutrophil serine proforms can act as regulators of granulopoiesis.[6] It cannot be entirely excluded that the high, non-physiological, concentrations of G-CSF used in the present *in vitro* model may selectively increase the constitutive or regulated secretion and thus affect the balance between sorting to storage in granules versus secretion of granule proteins. However, since the long-term intracellular stability of PR3, as judged by Western blotting, was essentially unchanged after longer periods of culture with G-CSF, at times points when PR3 was no longer synthesized (*unpublished observation*), this explanation is less likely as G-CSF seemed to have a low impact on regulated secretion. Proteinase 3 is overexpressed in a variety of acute and chronic myeloid leukemia cells.[38] The findings of heterogenous mutations in *ELA2* gene, encoding leukocyte elastase, in patients with cyclic hematopoiesis and severe congenital neutropenia,[15] suggests that leukocyte elastase might play a role in the pathogenesis of these diseases, perhaps by affecting the delicate cell survival/apoptosis balance. The presented cellular myeloid differentiation model is expected to be useful in future biosynthesis studies of these clinical disorders with a disrupted myeloid differentiation and disturbances of neutrophil granule protein transcripts.[39]

*DG designed and co-ordinated the study, performed most of the experiments, analyzed and interpreted the data and wrote the manuscript draft. ALe contributed to the collection, analysis, and interpretation of data. AL, UG, and SEWJ designed and supervised the study, the sample and data collection, and contributed to the analysis and interpretation of the data. All investigators contributed to and reviewed the final report.*

*We are grateful to Ingbritt Åstrand-Grundström, Per Anders Bertilsson, and Sverker Segrén for purification and sorting of $CD34^+$ cells, Tor Olofsson for FACS-analyses, Gunilla Naumann for enzymatic stainings and Ann-Maj Persson for technical assistance.*

*We also thank the human subjects for participating in the study and the reviewers and colleagues for valuable comments regarding the manuscript. This work was supported by grants from the Faculty of Medicine at Lund University and Lund University Hospital (ALF-funding), the Swedish Society for Medical Research, the Swedish Medical Association, the Foundation for Combating Blood Diseases, the Swedish Society for Hematology (travel grant), the Swedish Medical Research Council (project no. 11546), the Crafoord Foundation, the Swedish Cancer Foundation, the Swedish Childhood Cancer Foundation, the Vera and Carl J Michaelsen Foundation, the John Persson Foundation, the Greta and Johan Kock Foundation, the Alfred Österlund Foundation, and the Georg Danielsson Foundation. The authors declare that they have no potential conflicts of interest.*

*Part of this work was presented at the 40th Annual Meeting of The American Society of Hematology, Miami Beach, FL, USA, Dec 4-8 1998[40] and at the 41st Annual Meeting of The American Society of Hematology , New Orleans, LA, USA, Dec 3-7, 1999.[41]*

*Manuscript received July 8, 2004. Accepted September 13, 2004.*

## References


1. Bainton DF. Developmental biology of neutrophils and eosinophils. In: Gallin JI, Goldstein IM, Snyderman R, eds. Inflammation: Basic principles and clinical correlates, 2nd Ed. New York: Raven Press; 1992. p. 303-24.
2. Hammond WP. Neutrophils. In: Spivak JL, Eichner ER, eds. The fundamentals of clinical hematology, 3rd ed. Baltimore and London: The Johns Hopkins University Press; 1993. p 129-40.
3. Borregaard N, Cowland JB. Granules of the human neutrophilic polymorphonuclear leukocyte. Blood 1997;89: 3503-21.
4. Gullberg U, Andersson E, Garwicz D, Lindmark A, Olsson I. Biosynthesis, processing and sorting of neutrophil proteins: insight into neutrophil granule development. Eur J Haematol 1997; 58:137-53.
5. Bories D, Raynal M-C, Solomon DH, Darzynkiewicz Z, Cayre YE. Down-regulation of a serine protease, myeloblastin, causes growth arrest and differentiation of promyelocytic leukemia cells. Cell 1989;59:959-68.
6. Sköld S, Rosberg B, Gullberg U, Olofsson T. A secreted proform of neutrophil proteinase 3 regulates the proliferation of granulopoietic progenitor cells. Blood 1999;93:849-56.
7. Lutz PG, Moog-Lutz C, Coumau-Gatbois E, Kobari L, Di Gioia Y, Cayre YE. Myeloblastin is a granulocyte colony-stimulating factor-responsive gene conferring factor-independent growth to hematopoietic cells. Proc Natl Acad Sci USA 2000;97:1601-6.
8. Lutz PG, Houzel-Charavel A, Moog-Lutz C, Cayre YE. Myeloblastin is an Myb target gene: mechanisms of regulation in myeloid leukemia cells growth arrested by retinoic acid. Blood 2001;97:2449-56.
9. El Ouriaghli F, Fujiwara H, Melenhorst JJ, Sconocchia G, Hensel N, Barrett AJ. Neutrophil elastase enzymatically antagonizes the in vitro action of G-CSF: implications for the regulation of granulopoiesis. Blood 2003;101;1752-8.
10. Hunter MG, Druhan LJ, Massullo PR, Avalos BR. Proteolytic cleavage of granulocyte colony-stimulating factor and its receptor by neutrophil elastase induces growth inhibition and decreased cell surface expression of the granulocyte colony-stimulating factor receptor. Am J Hematol 2003;74;149-55.
11. Zhou Q, Salvesen GS. Activation of pro-caspase-7 by serine proteases includes a non-canonical specificity. Biochem J 1997;324:361-4.
12. Garwicz D, Lindmark A, Persson A-M, Gullberg U. On the role of the proform-conformation for processing and intracellular sorting of human cathepsin G. Blood 1998;92:1415-22.
13. Biggs JR, Yang J, Gullberg U, Muchardt C, Yaniv M, Kraft AS. The human brm protein is cleaved during apoptosis: the role of cathepsin G. Proc Natl Acad Sci USA 2001;98:3814-9.
14. Yang JJ, Kettritz R, Falk RJ, Jenette JC, Gaido ML. Apoptosis of endothelial cells induced by the neutrophil serine proteinase 3 and elastase. Am J Pathol 1996; 149:1617-26.
15. Dale DC, Person RE, Bolyard AA, Aprikyan AG, Bos C, Bonilla MA et al. Mutations in the gene encoding neutrophil elastase in congenital and cyclic neutropenia. Blood 2000;96:2317-22.
16. Zimmer M, Medcalf RL, Fink TM, Mattmann C, Lichter P, Jenne DE. Three human elastase-like genes coordinately expressed in the myelomonocytic lineage are organized as a single genetic locus on 19pter. Proc Natl Acad Sci USA 1992;89:8215-9.
17. Jenne DE. Structure of the azurocidin, proteinase 3, and neutrophil elastase genes. Implications for inflammation and vasculitis. Am J Respir Crit Care Med 1994;150:S147-54.
18. Clarke S, Gordon S. Myeloid-specific gene expression. J Leukoc Biol 1998;63: 153-68.
19. Ward AC, Loeb DM, Soede-Bobok AA, Touw IP, Friedman AD. Regulation of granulopoiesis by transcription factors and cytokine signals. Leukemia 2000; 14:973-90.
20. Graubert T, Johnston J, Berliner N. Cloning and expression of the cDNA encoding mouse neutrophil gelatinase: demonstration of coordinate secondary granule protein gene expression during terminal neutrophil maturation. Blood 1993;82: 3192-7.
21. Hirata RK, Chen S-T, Weil SC. Expression of granule protein mRNAs in acute promyelocytic leukemia. Hematol Pathol 1993;7:225-38.
22. Cowland JB, Borregaard N. The individual regulation of granule protein mRNA levels during neutrophil maturation explains the heterogeneity of neutrophil granules. J Leukoc Biol 1999; 66:989-95.
23. Labbaye C, Zhang J, Casanova JL, Lanotte M, Teng J, Miller Jr WH, et al. Regulation of myeloblastin messenger RNA expression in myeloid leukemia cells treated with all-trans retinoic acid. Blood 1993;81:475-81.
24. Seale J, Delva L, Renesto P, Balitrand N, Dombret H, Scrobohaci ML, et al. All-trans retinoic acid rapidly decreases cathepsin G synthesis and mRNA expression in acute promyelocytic leukemia. Leukemia 1996;10:95-101.
25. Lian Z, Wang L, Yamaga S, Bonds W, Beazer-Barclay Y, Kluger Y, et al. Genomic and proteomic analysis of the myeloid differentiation program. Blood 2001;98:513-24.
26. Gullberg U, Bengtsson N, Bülow E, Garwicz D, Lindmark A, Olsson I. Processing and targeting of granule proteins in human neutrophils. J Immunol Methods 1999;232:201-10.
27. Li CY, Lam KW, Yam LT. Esterases in human leukocytes. J Histochem Cytochem 1973;21:1-12.
28. Schaefer HE, Fischer R. Der Peroxydasenachweis an Ausstrichpräparaten sowie an Gewebsschnitten nach Entkalkung und Paraffineinbettung. Klin Wschr 1968;46:1228-30.
29. Borregaard N, Sehested M, Nielsen BS, Sengeløv H, Kjeldsen L. Biosynthesis of granule proteins in normal human bone marrow cells. Gelatinase is a marker of terminal neutrophil differentiation. Blood 1995;85:812-7.
30. Gabay JE, Almeida RP. Antibiotic peptides and serine protease homologs in human polymorphonuclear leukocytes: defensins and azurocidin. Curr Opin Immunol 1993;5:97-102.
31. Lindmark A, Garwicz D, Rasmussen PB, Flodgaard H, Gullberg U. Characterization of the biosynthesis, processing, and sorting of human HBP/CAP37/ azurocidin. J Leukoc Biol 1999;66:634-43.
32. Weiss J, Elsbach P, Olsson I, Odeberg H. Purification and characterization of a potent bactericidal and membrane active protein from the granules of human polymorphonuclear leukocytes. J Biol Chem 1978;253:2664-72.
33. Weiss J, Olsson I. Cellular and subcellular localization of the bactericidal/permeability-increasing protein of neutrophils. Blood 1987;69:652-9.
34. Bülow E, Gullberg U, Olsson I. Structural requirements for intracellular processing and sorting of bactericidal/permeability-increasing protein (BPI): comparison with lipopolysaccharide-binding protein. J Leukoc Biol 2000;68:669-78.
35. Fouret P, Du Bois RM, Bernaudin J-F, Takahashi H, Ferrans VJ, Crystal RG. Expression of the neutrophil elastase gene during human bone marrow cell differentiation. J Exp Med 1989;169: 833-45.
36. Hanson RD, Connolly NL, Burnett D, Campbell EJ, Senior RM, Ley TJ. Developmental regulation of the human cathepsin G gene in myelomonocytic cells. J Biol Chem 1990;265: 1524-30.
37. Kelly RB. Pathways of protein secretion in eukaryotes. Science 1985;230: 25-32.
38. Dengler R, Münstermann U, al-Batran-S, Hausner I, Faderl S, Nerl C, et al. Immunocytochemical and flow cytometric detection of proteinase 3 (myeloblastin) in normal and leukemic myeloid cells. Br J Haematol 1995;89: 250-7.
39. Kawaguchi H, Kobayashi M, Nakamura K, Konishi N, Miyagawa S, Sato T, et al. Dysregulation of transcriptions in primary granule constituents during myeloid proliferation and differentiation in patients with severe congenital neutropenia. J Leukoc Biol 2003;73: 225-34.
40. Garwicz D, Lindmark A, Jacobsen SEW, Gullberg U. Biosynthesis of neutrophil granule proteins during differentiation of CD34+ progenitor cells. Blood 1998;90 Suppl 1:163a:657 [abstract].
41. Lindmark A, Garwicz D, Jacobsen SEW, Gullberg U. Expression of granule proteins and surface antigens in human neutrophil differentiation. Blood 1999; 94 Suppl 1:210a:925[abstract].