Molecular Analysis of Myeloperoxidase Deficiency Shows Heterogeneous Patterns of the Complete Deficiency State Manifested at the Genomic, mRNA, and Protein Levels

By Michael E. Selsted, Carl W. Miller, Michael J. Novotny, Wendy L. Morris, and H. Philip Koeffler

Myeloperoxidase (MPO) is a glycosylated hemoprotein contained in the azurophil granules of human polymorphonuclear leukocytes (PMNs). MPO is thought to play a role in the oxidative antimicrobial activity of neutrophils by catalyzing the formation of hypochlorous acid, a potent microbicid, from hydrogen peroxide and chloride anions. Seven unrelated individuals with complete MPO deficiency, a relatively common heritable defect of neutrophils, were identified during routine blood tests. Molecular analyses were conducted to determine the level of the abnormality in these individuals. Western blot analysis showed that 6 of the 7 donors were devoid of immunoreactive MPO protein, while neutrophils from one individual contained only the 55-kd subunit. Northern analysis of bone marrow RNA from one MPO-deficient donor showed the presence of the normal-sized 3.3-kb transcript indicating that the defect in MPO biosynthesis in this case was posttranscriptional. Southern analysis of four MPO-deficient donors showed a normal Bgl II digestion pattern, whereas an abnormal restriction pattern was observed in a fifth individual. Although the Bgl II pattern was similar to that observed in an unrelated subject described by Nauseef (Blood 73:290, 1989), our study strongly suggests that the point mutation does not reflect a polymorphism. Taken together, these analyses show the existence of diverse abnormalities associated with MPO-deficiency that may be detected at the level of the MPO polypeptide, mRNA, and gene.

© 1993 by The American Society of Hematology.

MATERIALS AND METHODS

Patient samples. Seven healthy, adult MPO-deficient individuals were identified with the assistance of several Los Angeles area clinical laboratories. The MPO-deficiency of each individual was initially shown by automated leukocyte differential determinations performed during routine blood examination, and confirmed by immunohistochemical staining with aminocarbazole. Blood and bone marrow (BM) samples were collected after informed consent and approval from the institutional human subject protection committee.

Cells. Promyelocytic leukemia cell line HL-60, W138 embryonic lung fibroblasts (American Type Culture Collection [ATCC], Rockville, MD), and human lung adenocarcinoma cell line Lu-01 (a gift from L. Turner, Los Angeles, CA) were grown in α medium containing 10% fetal bovine serum (FBS) in a humidified atmosphere with 7% CO₂. Mononuclear cells were isolated from BM by centrifugation on Ficoll-Hypaque gradients (Pharmacia, Uppsala, Sweden), washed twice in phosphate buffered saline (PBS) and suspended in α medium containing 1% FBS and 7% CO₂.

From the Department of Pathology, University of California, Irvine; the Division of Hematology-Oncology, Department of Medicine, University of California, Los Angeles, and the Division of Hematology-Oncology, Cedars-Sinai Medical Center, Los Angeles, CA.

Submitted February 24, 1993; accepted April 19, 1993.

Supported in part by US Public Health Services Grants No. AI22931, CA26038, CA42710, and CA33936; The Weiz Family Foundation; The Leukemia Fund; and The Realtors of Real Estate Industry Division; and The Parker Hughes Fund.

Address reprint requests to Michael E. Selsted, PhD, MD, Department of Pathology, College of Medicine, University of California, Irvine, CA 92717.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1993 by The American Society of Hematology.

0006-4971/93/8204-002153.00/0
taining 10% FBS. PMNs were obtained by subjecting venous blood to dextran sedimentation and hypotonic lysis of the erythrocytes. Preparations from peripheral blood (PB) contained 95% granulocytes as determined by microscopic examination of Giemsa-stained cytospin preparations. Neutrophils were stored as frozen pellets in aliquots of 1 x 10^7 cells at -70°C.

Preparation of cellular extracts. Neutrophil extracts were prepared by thawing 1 x 10^7 PMNs in 200 µL of ice-cold protease-inhibitor solution; 10 mmol/L NaPO₄, pH 7.4; 5 mmol/L iodoacetamide; 1 mmol/L phenylmethylsulfonyl fluoride; 1 mmol/L benzamidine; 1 mmol/L N-ethylmaleimide; 1 mmol/L ethyleneglycol-bis(N,N,N',N'-tetracetic acid; and 5 mmol/L leupeptin. The cell suspension was incubated on ice for 30 minutes after which 800 µL of ice-cold 12.5% acetic acid was added. The cell suspension was sonicated three times for 10 seconds at high power in a Biosonic IV sonicator fitted with a microprobe. After an additional 30 minutes incubation on ice, the suspension was centrifuged at 13,000g for 10 minutes at 8°C. The clarified supernatant was removed, and extracts (1 x 10⁶ cell-equivalents per aliquot) were stored at -70°C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. SDS-PAGE was performed essentially as described previously. Frozen extracts (1 x 10⁶ cell-equivalents) were lyophilized and suspended in SDS sample buffer containing 10 mmol/L dithiothreitol. After boiling for 5 minutes at 8°C, the clarified supernatant was removed, and extracts were electrophoresed for 6 hours at 150 V on a 12.5% polyacrylamide gel. Protein was transferred to nitrocellulose with an ABN Polyblot apparatus (Millipore, Bedford, MA) for 1,2 hours, washed, and exposed to Kodak ABN X-Omat film (Eastman Kodak, Rochester, NY). Aliquots of each donor sample were analyzed for MPO antigen by Western blot analysis using rabbit polyclonal anti-MPO antibody.

RESULTS

Histochemical expression of myeloperoxidase protein. Histochemical evaluation of whole blood and of buffy coat preparations from each MPO-deficient donor were used to confirm that neutrophil preparations were completely lacking in MPO enzymatic activity. Eosinophils, present in varying numbers in each sample, were strongly positive. Although no enzymatic activity was detectable by histochemistry in neutrophils from any of the donors, this method did not address the possibility that enzymatically-inactive MPO might be present in "MPO-negative" neutrophils. Therefore, Western blot analysis of cell lysates was performed using rabbit polyclonal anti-MPO antibody. As shown in Fig 1, this antibody detected 55-Kd and 14-Kd MPO subunits in lanes containing purified MPO, lysates of normal neutrophils, and HL-60 lysates. The antibody also detected a band of approximately 90 Kd, the size of the partially processed MPO precursor, in HL-60 cell lysates.

Six of the 7 MPO-deficient donor samples were devoid of MPO immunoreactive staining (Fig 1, lanes 5 through 10), while extracts of an equivalent number of normal cells consistently showed bands corresponding to the 55- and 14-Kd subunits. Therefore, in these six MPO-deficient donors, the absence of enzymatic activity appeared to be caused by the total absence of MPO protein. To address the possibility that the lack of MPO antigen in MPO-deficient cells might be caused by degradation, cell lysates from 4 MPO-deficient donors were analyzed by lysozyme by Western blot analysis. As shown in Fig 1B, extracts of normal and MPO-deficient PMN contained intact lysozyme, and all lysates contained comparable amounts of immunoreactive material. These data, and the lack of MPO-degradation products in Western blots of normal PMN, indicate that the procedure for producing PMN lysates adequately inhibited endogenous proteolytic activity, and indicate that the lack of peroxidase activity in 6 of 7 donors was caused by a major lesion in either production or accumulation of stable MPO protein.

A different pattern of immunoreactivity was observed in the Western blot analysis of PMN lysates from donor C.K. (Fig 1). In four separate determinations, neutrophil lysates from this individual contained a strong 55-Kd band, but the 14-Kd band was not detected. The smaller subunit was absent from Western blots even after a threefold increase in the loading of this sample (data not shown). This result raises the possibility that the MPO small subunit is required for normal MPO enzymatic function. However, it is equally possible that an abnormality of the large subunit renders the 14-Kd peptide unstable. Additional biochemical analysis of MPO protein from this donor's cells may provide new insights into the role of MPO subunits in the catalytic mechanism.

With the exception of HL-60 cell lysates, none of the samples analyzed contained bands larger than the MPO large subunit. In these and previous studies, we have failed to detect in normal or MPO-deficient PMNs the MPO-immunoreactive 90-Kd protein band corresponding to MPO precursor. That our antibody recognizes this form is shown by the 90-Kd immunoreactive band detected in lysates of HL-60 cells that actively synthesize MPO. These findings lead us to conclude that very little MPO precursor is present in normal PMN, and that the lesions in the MPO-deficient
Fig 1. Western blot analysis of MPO expression in normal and MPO-deficient cells. (A) PMNs and HL-60 cells were extracted, electrophoresed, blotted, and detected with polyclonal anti-MPO antibody as described in Materials and Methods. Lane 1, 3 μg purified MPO; lane 2, 1 × 10^6 HL-60 cells; lane 3, 5 μg purified EPO; lane 4 through 10, 1 × 10^6 PMNs from MPO-deficient donors; lane 11, 1 × 10^6 normal PMNs. (B) Selected donor samples (C.K., S.Q., L.J., L.B.) were analyzed by Western blot for lysozyme. Lysate from 1 × 10^6 PMNs was electrophoresed as in (A), blotted, and detected with polyclonal antilysozyme antibody. Lysozyme (LZM) standard was 0.1 μg of human leukemia lysozyme, and the control lane (CTRL) was a lysate of 1 × 10^6 PMN from a normal donor.

Donors of this study are not explained by abnormal post-translational processing as proposed by Nauseef et al. In characterizing three different anti-MPO antibody preparations, each generated with a separately purified sample of MPO antigen, we found that eosinophil peroxidase (EPO) cross-reacted with anti-MPO antibody in Western blots, though the antibody was nonreactive in immunodiffusion assays (data not shown). Though the lack of cross-reactivity of native EPO and MPO has been described previously, the immunoreactivity on Western blots is not unexpected given the high degree of primary structural similarity between human MPO and EPO polypeptides. Note that the signal of anti-EPO immunoreactivity is easily distinguished from that of MPO, as the heavy subunits of each protein migrate with different apparent molecular weights (Fig 1, lanes 1 and 3). Consistent with this immunoreactivity, the Western blot of cell lysates from two donors (S.Q. and E.H.) showed faintly positive bands at 49 Kd. This is explained by the fact that the granulocyte preparations from these two donors contained 7.3% and 5.2% eosinophils, respectively. Preparations from all other donors contained ≤1% eosinophils.

Accumulation of MPO mRNA. Expression of steady state MPO mRNA in a BM aspirate from one MPO-defi-
Fig 2. MPO RNA accumulation in MPO-deficient BM cells. Total RNA of various cells was isolated and analyzed by RNA blot using a 1.2-kb Sma I-Sma I pMPO2 cDNA probe. Lane 1, HL-60 cells; lane 2, WI38 embryonic fibroblasts; lane 3, cells from L.B., an MPO-deficient patient; lane 4, Lu-CSF-1, adenocarcinoma cell line of the lung; lane 5, PB leukemic cells from patient with AML, subtype M-2 by the French-American-British (FAB) classification. Each lane contained 10 μg of RNA; the autoradiogram was exposed for 12 hours. Panel below shows the 28S and 18S RNAs from the same gel used in electrophoresis before Northern blotting to confirm that the RNAs were intact.

Fig 3. Restriction enzyme analysis of DNA from PB lymphocytes of the MPO-deficient patients. DNA (10 μg/ lane) was isolated from mononuclear leukocytes, digested by various restriction enzymes and examined by Southern blotting using the 1.2-kb Sma I-Sma I pMPO2 cDNA probe. (Top panel) DNA digested with Bgl II (lane 1, father of C.K.); lane 2, C.K.; lane 3, S.Q.; lane 4, E.H.; lane 5, L.J.; lane 6, BV173 lymphocytic leukemia cell line from a non-MPO-deficient patient. (Lower panel) DNA double-digested with Bgl II and Kpn I: lanes 1 and 3, BV173; lanes 2 and 4, S.Q. (MPO-deficient patient). DNA was double-digested with Bgl II and Kpn I in lanes 1 and 2 and digested solely with Kpn I in lanes 3 and 4. Estimated fragment sizes are shown in kilobases.

Southern blot analysis. DNA samples from lymphocytes of MPO-deficient donors and from the BV173 B-lymphocyte cell line (control), were digested with selected restriction enzymes and analyzed by Southern blotting using the Sma I-Sma I pMPO2 cDNA probe (Fig 3). Identical restriction patterns were obtained with Bgl II digests of DNA from FK (lane 1, father of MPO-deficient patient, C.K.), MPO-deficient donors C.K., E.H., L.J., L.B. (lanes 2, 4, 5, and 7, respectively) and BV173 cells (lane 6). In contrast, DNA from MPO-deficient donor S.Q. (lane 3) was missing the normal 2.5-kb Bgl II fragment that was replaced with a smaller 2-kb Bgl II fragment. DNA from S.Q. was then double digested with Bgl II and Kpn I (lane 3, lower panel) and found to lack the 1.6-kb fragment observed in digests of control DNA (lane 1). This resulted from the 1.6-kb fragment being digested into two smaller fragments that were not detectable on this Southern blot.

DISCUSSION

The results of this study show that the molecular abnormalities associated with MPO-deficiency may occur at mul-

Fig 3. Restriction enzyme analysis of DNA from PB lymphocytes of the MPO-deficient patients. DNA (10 μg/lane) was isolated from mononuclear leukocytes, digested by various restriction enzymes and examined by Southern blotting using the 1.2-kb Sma I-Sma I pMPO2 cDNA probe. (Top panel) DNA digested with Bgl II (lane 1, father of C.K.); lane 2, C.K.; lane 3, S.Q.; lane 4, E.H.; lane 5, L.J.; lane 6, BV173 lymphocytic leukemia cell line from a non-MPO-deficient patient. (Lower panel) DNA double-digested with Bgl II and Kpn I: lanes 1 and 3, BV173; lanes 2 and 4, S.Q. (MPO-deficient patient). DNA was double-digested with Bgl II and Kpn I in lanes 1 and 2 and digested solely with Kpn I in lanes 3 and 4. Estimated fragment sizes are shown in kilobases.
tiple levels. At the polypeptide level, neutrophils of six of seven MPO-deficient donors were devoid of MPO immunoreactive protein. The lack of protein may be caused by either a structural or regulatory lesion in the gene resulting in abnormal transcription or translation, or instability of mRNA or protein. Though the most common finding was a total lack of MPO protein, in one donor sample (C.K.) there was a total absence of the 14-kd peptide, whereas the 55-Kd subunit was present in approximately normal amounts (see below).

Consistent with results of this study, Nauseef et al. previously showed that MPO-deficient neutrophils typically lack both the 55-Kd and 14-Kd subunits of the enzyme. However, in contrast with our findings, they observed a 75- to 90-Kd MPO-immunoreactive protein in Western blots of lysates of MPO-deficient neutrophils as well as normal PMNs. Based on this finding, it was proposed that MPO deficiency in these patients was caused by aberrant processing of MPO-precursor polypeptide. In this and a previous study, we did not detect MPO precursor in either normal neutrophils or in cells from seven MPO-deficient individuals. However, the antibodies used in the present study did detect MPO precursor protein as shown with extracts of HL-60 cells (Fig 1, lane 2). While the basis for this discrepancy is not clear, it seems likely that the conflicting results are caused by differences in antibody specificities.

The unusual case of donor CK shows the diversity of MPO-deficiency at the protein level. While the Western blot signal corresponding to the MPO heavy subunit was approximately equivalent to that observed with control neutrophils, the 14-Kd subunit was not detected. Andrews and Krinsky have shown that hemimyeloperoxidase, a 55-Kd/14-Kd heterodimer, is enzymatically active; and Arnjots and Olsson showed that the heme group in MPO is incorporated into the heavy subunit precursor before its processing into mature enzyme. Together, these findings suggest that, like EPO, MPO can function enzymatically as a heterodimer in which the heme (chlorin) group is bound to the heavy subunit. In the present study, the absence of enzymatic activity in MPO from donor C.K. raises the possibility that the 14-Kd subunit may be required for enzymatic function. This is but one possible explanation of the role of the small subunit in the enzymatic activity of MPO. It is equally possible that the small subunit is actually produced normally, but that it is unstable because it does not associate normally with a structurally abnormal 55-Kd subunit. Reconstitution experiments using subunits from mutant and normal MPOs might provide useful information in this regard.

Of the several donors completely lacking MPO antigen, one (L.B.) was further studied by analyzing MPO transcripts in BM RNA. Northern analysis of total RNA showed a single RNA species of 3.3 kb, the size of the predominant MPO mRNA in normal BM. This contrasts with the RNA species detected in bone marrow of a previously studied MPO-deficient individual whose BM cells contained heterogeneous nuclear RNA of greater than 8 kb, and approximately 4 kb, but only trace amounts of the 3.3-kb MPO mRNA. The findings of this earlier investigation indicated that MPO-deficiency was caused by a pretranslational defect affecting the processing of nuclear RNA to mature RNA. Cells from the donor analyzed in the present study (L.B.) appear to contain appropriately processed MPO mRNA, suggesting that a different, posttranscriptional abnormality exists in this individual. In both cases, MPO-immunoreactive protein was absent, while normal restriction patterns for MPO were observed in genomic Southern blots (see below and report by Tobler et al). The results of Southern blot analyses further illustrate the heterogeneity of MPO deficiency. Bgl II digests of DNA from five donors were probed with the 1.2-kb Sma I-Sma I MPO cDNA fragment, showing normal restriction patterns of four individuals when compared with control DNA (Fig 3). DNA from one donor, S.Q., contained an abnormal homozygous Bgl II fragment of 2.0 kb. Sequence analysis of the MPO gene from this donor showed that the new Bgl II fragment was caused by the introduction of a new restriction site resulting from a single base substitution in codon 569 in exon 10. An analysis of DNA from 450 random donors was performed by digestion of polymerase chain reaction (PCR)-amplified exon 10, and none of these were found to contain the novel Bgl II site characteristic in the DNA of S.Q. These data strongly suggest that the point mutation in S.Q. does not represent a polymorphism.

Nauseef previously showed that DNA from three unrelated MPO-deficient individuals contained an abnormal 2.15-kb Bgl II fragment. These data suggest a strong correlation between the presence of the unique Bgl II fragment and the MPO-deficiency state. The similar sizes of the Bgl II fragments in the patients described by Nauseef and in this report suggests the possibility that genetic abnormalities may be similar. Further characterization of the DNA alterations in the former group of patients will be required if definitive comparisons are to be made.

The sequence of the EPO gene was recently reported, showing a high degree of genetic and protein structural similarity with MPO. Given that the coding sequences of the MPO and EPO genes are 72.4% homologous, it was necessary to analyze the specificity of the Sma I MPO cDNA probe used in these studies. An evaluation of the Northern blot in Fig 2 shows that the probe detects RNA of the correct size for MPO (3.3 kb), which is larger than 2.8-kb mature transcript of EPO detected in the 3 + C cosinophilic subline of HL-60. Further, the MPO cDNA probe did not detect any transcripts in this size range in BM RNA from a previous MPO-deficient individual who lacked the mature (3.3 kb) MPO transcript altogether. Finally, the specificity of the MPO cDNA probe for genomic sequences is further shown by the presence of expected restriction pattern obtained following Bgl II digestion of control DNA samples (Fig 3).

The studies described in this report show that MPO deficiency is a heterogeneous group of genetic disorders in which alterations have been characterized at the genomic DNA, mRNA, and protein levels. In this regard, the spectrum of molecular abnormalities is analogous to that of the thalassemias, and it is expected that additional variants will be described. Although much has been learned about the
structure of the MPO gene and protein, little is known of the mechanisms that account for the acquired MPO-deficiency state commonly associated with certain leukemias and myelodysplastic syndrome. An understanding of the molecular alterations that lead to these phenotypic changes may provide clues to the process of malignant transformation.

ACKNOWLEDGMENT

We thank Dr Michael Samoszuk for his assistance in identifying MPO-deficient blood samples and Patti McGuire for superb technical assistance.

REFERENCES

Molecular analysis of myeloperoxidase deficiency shows heterogeneous patterns of the complete deficiency state manifested at the genomic, mRNA, and protein levels

ME Selsted, CW Miller, MJ Novotny, WL Morris and HP Koeffler