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The glycosylation of myeloperoxidase

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ABSTRACT

The enzyme myeloperoxidase (MPO) is an important part of the neutrophil immune reaction and can be found in alfa granula. The presence of MPO can be used to distinguish acute myelogenous leukemia from acute lymphocytic leukemia. However, the methods employed to do so, such as flow cytometry and immunohistochemistry rely on antibody recognition, and therefore the characterization of the mature MPO, including post-translational modifications, must be considered as important as epitope mapping. MPO has 5 N-linked glycosylation sites, occupied by both high mannose and complex glycan structures. In this study we utilize intact glycopeptide MSMS analysis for site specific characterization of the glycan structures of MPO from a cancer patient. The identified glycan structures are compared to those of MPO from healthy donors, in order to probe for any potential differences that may have diagnostic use.

1. Introduction

MPO is an enzyme residing in the azurophilic granules of neutrophils and its main function is to produce hypochlorous acid (HOCl) that serves to kill pathogens as part of the immune response [1–3]. The mature MPO protein consists of 2 light and 2 heavy polypeptide chains with each heavy chain binding a prosthetic heme group [4,5]. Myeloperoxidase (MPO) has for some years received attention based on its application in the distinction between acute myelogenous leukemia (AML) and acute lymphocytic leukemia (ALL) [6–10]. For classification of leukemia cases the presence of MPO may be verified by flow cytometry or immunohistochemistry [11–15]. For these applications, it is important to verify that the antibodies used reliably detect myeloperoxidase in all samples where it is present, whether or not changes in MPO structure or post-translational modifications may have occurred. In this respect, it is necessary to know the structure of MPO as well as all possible post-translational modifications. In addition, it is important to obtain knowledge of the epitopes of the antibodies used, including their potential dependency on posttranslational modifications. Furthermore, MPO has been shown to be the target of autoantibodies in several autoimmune conditions, including several forms of vasculitis [16,17] making epitope mapping and characterization of the mature protein equally important tasks.

MPO has been shown to be highly glycosylated [18–24] and the extent and identity of the glycosylations may be of importance in terms of antibody recognition and epitope mapping. Moreover, it has been shown that protein glycosylation may change in cases of cancer [25–27], providing further means of distinction between cancer and non cancer cases.

Aside from one consensus site in the propeptide, MPO has 5 potential N-linked glycosylation sites. Previously X-ray crystallography and biochemical studies had indicated that only 4 (N323, N355, N391, N483) of these sites were glycosylated and only N483 had been assigned a full glycan structure ($GlcNAc_2Man_3Fuc_1$) [18–21,23]. Also, high mannose and complex structures had been suggested based on glycosidase treatment studies, with secreted precursor MPO containing more of the complex type structures than intracellular mature and precursor MPO [22,28]. However; a recent study by Van Antwerpen et al. [24], employing mass spectrometry, have demonstrated that all 5 N-linked glycosylation sites of both recombinant and human MPO are occupied by either complex or high mannose structures. In the same study it was found that the glycosylations were important for the enzyme activity of MPO.

Here we address the glycan structure of the 5 N-linked glycosylation sites of MPO by intact glycopeptide MSMS analysis. Furthermore, we compare the glycosylation of MPO between a sample from healthy donors and that of a cancer patient, in order to determine whether any potential differences might be of use for diagnostic purposes.

Abbreviations: ALL, Acute lymphocytic leukemia; AML, Acute myelogenous leukemia; DHB, 2,5-dihydroxybenzoic acid; FA, Formic acid; Fuc, Fucose; GlcNAc, N-Acetylglucosamine; Hex, Hexose; HexNAc, N-Acetylhexosamine; MALDI, Matrix assisted laser desorption ionization; Man, Mannose; MeCN, Acetonitrile; MeOH, Methanol; MPO, Myeloperoxidase; QTOF, Quadropole time of flight; Sia, Sialic acid; TFA, Trifluoroacetic acid

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2. Materials and methods

2.1. Purification of MPO

A pool of MPO from six healthy donors was purified from a fraction obtained during purification of human neutrophil granulocyte proteinase 3 (PR3) [29]. Briefly, 6 buffy coats were used as a source to obtain neutrophil granulocytes by density gradient centrifugation. The granulocytes were disrupted by nitrogen cavitation and azurophilic granula isolated by density gradient centrifugation with Percoll (GE Healthcare, WI, USA). The granula were mixed 1:1 with 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-114 and sonicated on ice for 2×20 s. The extract was incubated at 37 °C and the water phase (containing MPO) was separated from the detergent phase (containing PR3). The water phase was dialysed against 50 mM Tris, pH 8 and loaded on a Mono S Sepharose column (GE Healthcare, WI, USA), which was eluted with a linear gradient to 1 M NaCl in 50 mM Tris, pH 8.0. MPO containing fractions were located by ELISA using rabbit anti human MPO A-0398 1:1000 (DAKO, Denmark). The fractions were pooled and concentrated using a centriprep cartridge, cut-off 10.000 Da (Millipore, MA, USA). Yield: 0.18 mg, (determined at A_{430}, with $\varepsilon_{430}=1.25$).

MPO from a patient with chronic myeloid leukaemia was obtained from Dr. Inge Olsson (University Hospital of Lund) and had been purified as described previously [30].

2.2. Identification of MPO glycopeptides by MALDI MS

One microgram of each MPO sample in 10 µl 50 mM NH_4HCO_3 was incubated with 10 mM dithiothreitol for 30 min at 56 °C, followed by alkylation with 30 mM iodacetamide for 30 min in the dark at room temperature. An additional 5 mM dithiothreitol was added to quench the reaction and the volume was adjusted to 40 µl with 50 mM NH_4HCO_3. In solution trypsinization was performed by incubating each of the reduced and alkylated samples with 2% trypsin (Promega, WI, USA) (W/W) overnight. at 37 °C. Glycopeptides were enriched on a graphite micro column custom made in a GELoader tip (Eppendorf, Germany), using graphite powder obtained from a prepacked 1 ml graphite column (Alltech, Australia) [31]. The column was equilibrated with H_2O, the sample added, and washed twice with H_2O. Glycopeptides were eluted on target with 0.6 µl 20% MeCN, 0.1% TFA and 0.6 µl 20 mg/ml DHB in 70% MeCN, 0.1% TFA was added. MS and MSMS were performed on a MALDI QTOF (Micromass, Waters, MA, USA).

2.3. SDS PAGE

Aliquots of 1 µg MPO were subjected to SDS PAGE using a precast 10% Tris-glycine polyacrylamide gel (Lonza, Switzerland) and 25 mM Tris, 192 mM glycine, 0.1% SDS as running buffer. Prior to loading on the gel, the samples were mixed in a 1:1 ratio with sample buffer containing 2-mercaptoethanol and bromophenol blue, and boiled for 5 min. Page Ruler™ Prestained Protein Ladder (Fermentas, Canada) was used as marker. The gel was run for 1 h at 160 V, stained in 0.25% Coomassie Brilliant Blue R250 in 45% MeOH, 10% acetic acid for 45 min and destained overnight in H_2O.

2.4. In-gel digestion and PNGaseF treatment

The bands containing MPO were cut from each lane in the gel and washed twice in 50% MeCN and once in 100% MeCN. The gel plugs were first incubated for 30 min at 56 °C with 10 mM dithiothreitol and then for 30 min with 55 mM iodacetamide in the dark at room temperature. The reduction and alkylation was followed by another washing step as described above. Protein digestion was achieved by adding 0.125 µg trypsin (Promega, WI, USA) in 15 µl 50 mM NH_4HCO_3 to each sample. The gel plugs were left on ice for 10 min, excess liquid was removed, 20 µl of NH_4HCO_3 was added and the samples left at 37 °C overnight. In one experiment the samples were redigested for 3 h at 37 °C with 0.02 µg AspN (Calbiochem, Merck, UK).

One in-gel digested sample from a cancer patient was subjected to PNGaseF treatment by adding 0.5 U PNGase F (Roche, Germany) to the sample and incubating overnight at 37 °C.

2.5. LC-Orbitrap MS and ETD/CID MSMS

The digested MPO samples were prepared for LC-Orbitrap MS by purification on custom made micro columns. The columns were made in GELoader tips (Eppendorf, Germany) using a small piece of Empore C18 disk (3 M, MN, USA) as a plug with 1–2 mm oligo R3 resin (Applied Biosystems, CA, USA) on top. The columns were washed in 100% MeCN and equilibrated in 0.1% TFA before samples were loaded. The columns were then washed twice in 0.1% TFA and eluted with 60% MeCN, 0.1% TFA. The eluted samples were dried down and redissolved in 0.3 µl 100% FA followed by 5 µl 0.1% FA. The samples were run on an Easy LC system (Proxeon, Denmark) coupled in-line to an LTQ-Orbitrap instrument equipped with ETD (Thermo, Germany). Chromatographic separation was obtained using a C18 column, 3 µm Reprosil (Dr. Maisch Gmbh, Germany), of 20 cm in length with an inner diameter of 100 µm and a buffer system consisting of buffer A: 0.1% FA and B: 0.1% FA, 95% MeCN. The samples were loaded with a flow of 550 nl/min and a gradient of 0–34% buffer B in 30 min with a flow of 250 nl/min was applied, followed by 34–100% buffer B in 4 min. For each pre-scan in the Orbitrap the 5 most intense peaks were subjected to MSMS by either ETD or CID in the LTQ, while a high precision full-scan was obtained in the Orbitrap.

All together the sample obtained from a cancer patient and the pooled sample obtained from donors were each analyzed five times by in gel digestion and LC-Orbitrap MS and MSMS. Each of the following four experimental combinations were applied once; in gel trypsinization with or without AspN redigestion and MSMS performed by either CID or ETD, with the in gel trypsinization alone combined with CID for fragmentation applied twice. In addition to these five runs, the sample obtained from a cancer patient was analyzed once by in gel trypsinization followed by PNGase F treatment and CID MSMS, and once by in solution trypsinization and CID MSMS.

2.5.1. Antibody binding assays

For enzyme-linked immunosorbent assay (ELISA) 10 µg of MPO from a cancer patient was subjected to deglycosylation by incubation with 10 U PNGaseF (Roche, Germany) in 50 µl 50 mM NH_4HCO_3 at 37 °C overnight. The deglycosylation was confirmed by MALDI QTOF MS after trypsinization and enrichment on graphite as described above.

Polystyrene microtitre plates (Nunc, Denmark) were coated for 90 min at room temperature with 1 µg/ml untreated or deglycosylated MPO, 1 U/ml PNGaseF or 5 µl/ml NH_4HCO_3 in 100 µl 50 mM $NaHCO_3$ pH 9.6 pr well. The plates were washed 3 times and blocked for 15 min in TTN buffer; 50 mM Tris, 0,3 M NaCl 1% tween 20, pH 7.5. The monoclonal antibodies; ab25989 (Abcam, England), LS-C41696 (LifeSpan, WA, USA) and HYB194-01 (SSI, Denmark) were added for 1 h at room temperature in TTN buffer using the ratios 1:1000, 1:1000 and 1:10 respectively. The plates were washed 3 times in TTN buffer and incubated with alkaline phosphatase conjugated secondary antibody A-3688 (Sigma-Aldrich, MO, USA) at a concentration of 1:1000 in TTN buffer for 1 h at room temperature. After a final washing step, 1 mg/ml p-nitrophenylphosphate (Sigma-Aldrich, MO, USA) in 1 M diethanolamine, 0.5 mM $MgCl_2$ was added and the absorbance read at 405 nm, with background subtraction at 650 nm, after 5, 10 and 20 min on a VERSAmax microplate reader (Molecular Devices, CA, USA).