Non-Vital Polymorphonuclear Leukocytes Express Myeloperoxidase on their Surface

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Key Words

Annexin V • Apoptosis • Flow cytometry • Granulocytes • Myeloperoxidase • Phosphatidylserine

Abstract

The heme-containing enzyme myeloperoxidase (MPO) becomes expressed to the cell surface of non-vital polymorphonuclear leukocytes (PMNs) as evidenced by flow cytometry analysis and confocal fluorescence microscopy. While only a very small percentage of freshly isolated cells was able to bind the MPO antibody, PMN suspensions cultured for 36 h or longer time periods contained an increasing number of cells able to interact with these antibodies. Two distinct patterns of fluorescence for the MPO antibodies were observed. Antibodies were localised either in surface patches or distributed over the whole cell body. The latter type dominated in cell samples cultured for more than three days, while the first type was predominantly found in samples cultured for lower time periods. We observed also two peaks for fluorescence distribution by flow cytometry after addition of MPO antibodies to PMNs. Myeloperoxidase was localised at phosphatidylserine epitopes at the

surface of non-vital PMNs as evidenced by coincubation with fluorescent MPO antibodies and FITC-labelled annexin V. Myeloperoxidase bound to the outer surface of PMNs uses hydrogen peroxide as a substrate as shown by appearance of an intense chemiluminescence using the impermeable luminescent protein Pholasin. Thus, myeloperoxidase becomes expressed to the surface of non-vital polymorphonuclear leukocytes colocalised with phosphatidylserine that may indicate a role of myeloperoxidase in apoptosis of PMNs.

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Introduction

Polymorphonuclear leukocytes (PMNs) are among the first cells attracted to inflammatory sites in our body. These cells release a number of proteins and mediators, that have specific functions in inactivation and killing of foreign pathogens, remodelling of damaged tissues as well as regulation of the inflammatory process.

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1015-8987/08/0214-0287\$24.50/0

Accessible online at:
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The heme protein myeloperoxidase (MPO) that is stored in huge amounts in azurophilic granules of PMNs is assumed to play an important role in pathogen defence by generation of halogenated products and other oxidants [1]. However, considering the real conditions in the phagosome, data on MPO-deficient individuals and detailed studies with MPO knock-out mice large doubt arose about the microbicidal action as the main function of myeloperoxidase. The activity of MPO released into the phagosome is mostly directed against other co-secreted proteins and the microbicidal effect of PMNs is realised by elastase and other proteins [2-4]. Patients suffering from MPO-deficiency have usually a normal health status. Only some of them have an increased susceptibility to infections caused by *Candida albicans* [5, 6]. Similarly, MPO knock-out mice exhibit a normal bactericidal activity against *Staphylococcus aureus*. However, they were affected by *Candida albicans* [7].

Experiments with MPO-deficient mice support the view, that myeloperoxidase products are involved in resolution of inflammatory states as these mice suffer from more severe inflammations than wild-type ones in experimental inflammation models [8-11]. These mice show also a suppression of apoptosis of inflammatory cells contained in the bronchoalveolar lavage fluid [10]. Moreover, apoptosis proceeds in MPO-deficient PMNs more slowly than in normal neutrophils [12].

We recently found that MPO adheres tightly to phosphatidylserine (PS) epitopes on the surface of apoptotic spermatozoa, while no binding of MPO occurred to vital cells [13]. It remains unknown whether myeloperoxidase released from PMNs becomes also attached to PS epitopes on apoptotic PMNs. To address this question, the model of spontaneous apoptosis of PMNs [14, 15] has been applied that allows us to obtain a time-resolved picture about the redistribution of MPO in relation to other PMN properties during storage. We were able to show that MPO can really be found on the surface of non-vital PMNs in close association with PS epitopes and that this enzyme is catalytically active in the presence of hydrogen peroxide.

Materials and Methods

Materials

Human neutrophil myeloperoxidase was a product of Planta GmbH (Vienna, Austria). Pholasin was obtained from Knight Scientific Ltd. (Plymouth, UK). Pholasin® is the registered trade mark of Knight Scientific Ltd. An aqueous stock solution of Pholasin (0.28 µM) was stored at -80°C. The con-

centration of a freshly prepared stock solution of hydrogen peroxide was determined using $\epsilon_{230} = 74 \text{ M}^{-1} \text{ cm}^{-1}$ [16]. All other chemicals were obtained from Sigma (Taufkirchen, Germany).

Isolation and culturing of human polymorphonuclear leukocytes

Polymorphonuclear leukocytes were isolated from freshly taken EDTA-blood from healthy volunteers by dextran-enhanced sedimentation of red blood cells, Ficoll-Histopaque density centrifugation, lysis of remaining erythrocytes with distilled water and washing of cells with Hank's balanced salt solution (HBSS) in the absence of any divalent cations. Finally, the cell pellet was suspended in 10 ml RPMI-1640-medium in the presence of 10% heat-inactivated foetal calf serum, 2 mM glutamine, 100 u/ml penicillin, and 0.1 mg/ml streptomycin. Cells were cultured in flat cell culture flasks at 37°C, 95% humidity, and 5% CO₂ content.

Detection of myeloperoxidase on the surface of PMNs

Polymorphonuclear leukocytes were incubated with the primary rabbit-anti-MPO-antibody (Calbiochem, Darmstadt, Germany, 1:100) for 1 h. After washing (three times with PBS, 400 × g, 2 min) PMNs were incubated with the secondary antibody (goat-anti-rabbit-CY-3 conjugated, Dianova, Hamburg, Germany, 1:100) for 45 min followed by three washing steps (PBS, 400 × g, 2 min). Binding of MPO antibodies to PMNs was examined either by the confocal laser scanning fluorescence microscope Leica DM IRBE (Leica, Wetzlar, Germany) using the software Leica Microsystems TCS SP2 or by flow cytometry using a Beckman-Coulter-Flowcytometer EPICS XL-MCL® and the EXPO 32 ACD® software (Beckman Coulter GmbH, Krefeld, Germany).

Detection of externalised phosphatidylserine on the PMN surface

Polymorphonuclear leukocyte samples were centrifuged (400 × g) and resuspended in a binding buffer (ApoAlert® Annexin V-FITC apoptosis kit, Clontech Lab., Mountain View, CA, USA). After a second centrifugation step and addition of 10 µl solution of FITC-conjugated annexin V samples were incubated 15 min in the dark at room temperature. To remove unbound annexin V samples were washed three times in PBS. Samples were used for flow cytometry analyses and fluorescence microscopy.

Assessment of cell vitality

Cell vitality was assessed by the trypan blue exclusion test [17] using a Neubauer counting chamber. This property was also measured using propidium iodide (0.9 µM, final concentration) in flow cytometry analysis.

Pholasin luminescence

All luminescence measurements were carried out at 37°C using the microplate luminometer MicroLumat LB 96 P (EG&G Berthold, Wildbad, Germany). Twenty five thousand PMNs per well were incubated with Pholasin (0.064 µM, final concentration) for five minutes. Then hydrogen peroxide (180 µM, final concentration) was added using an injection device. The

Fig. 1. Fluorescence profiles for the binding of MPO antibodies to PMNs as a function of incubation time. Human PMNs were isolated and cultured for 0.2 h (A), 19 h (B), 43 h (C), 47 h (D), 70 h (E) or 139 h (F). Then, the cells were incubated with MPO antibodies, washed and analysed by flow cytometry. Representative examples from four independent experiments are shown.

Fig. 2. (A) Alterations in percentage of PMNs with incubation time showing upon incubation with MPO antibodies only an autofluorescence (< 5 units, ■), a moderate fluorescence (from 5 to 40 units, ●) or a high fluorescence (> 40 units, ▲). (B) Changes in cell vitality as detected by the trypan blue exclusion test (filled symbols) or using propidium iodide (open symbols) as a function of incubation time of PMNs. Data from three different cell preparations are included.

final measuring volume per well was 275 μ l. Kinetics of luminescence were followed over the next 15 min. For calibration purposes, Pholasin (0.064 μ M) was also incubated with increasing amounts of MPO dissolved in PBS followed by injection of hydrogen peroxide (180 μ M).

Results

Increased amount of myeloperoxidase is detected by antibodies during long-term incubation of PMNs

The overwhelming number of cells in a suspension of freshly isolated human PMNs did not show any MPO on their surface as shown by applying antibodies against MPO. There was only a single peak with a maximum at 2–3 fluorescence units in fluorescence distribution obtained by flow cytometry that can be attributed to the autofluorescence of cells (Fig. 1A). This peak dominated

Fig. 3. (A–C) Changes in the appearance of annexin V-positive epitopes on PMNs after a storage for 18 h (A), 43 h (B), or 75 h (C). Cells were incubated with FITC-labelled annexin V and assessed by flow cytometry. (D) Dot plots for the co-existence of MPO antibodies and annexin V at an incubation time for 43 h. Polymorphonuclear leukocytes were additionally incubated with MPO antibodies as described in the legend to Fig. 1. Selected examples from four independent experiments are shown.

also in a cell sample incubated for 19 h, but it developed a shoulder to higher fluorescence values indicating the appearance of a small fraction of cells able to bind the MPO antibody (Fig. 1B).

Total other fluorescence patterns were found in samples incubated for 43 h or longer time periods (Figs. 1C–1F). These distributions were characterised by appearance of two new fluorescence peaks with maxima at 10 and 100 – 200 fluorescence units. The first new peak was rather broad and ranged from about 5 – 6 to 40 fluorescence units. The lower limit of this peak could not well be determined as there was an overlapping with the distribution of cells not binding MPO antibodies. However, the second new peak was much better separated. It increased in intensity with increasing storage time.

A more quantitative picture of these changes is given for each cell fraction in Fig. 2A. During the first 24 h, more than 85% of cells did not bind the MPO antibody. Its percentage decreased continuously with increasing incubation time. There was an increase in the cell fraction able to bind moderate amounts of antibodies (5 – 40

units in flow cytometry) during the second day from about 10% to 50%. At longer incubation, the percentage of this fraction decreased slightly. Myeloperoxidase-positive cells with high fluorescence values (> 40 units) appeared only after storage for 42 h in PMN suspension. Then, its amount raised continuously.

These data were compared with the loss of vitality measured by the trypan blue exclusion test or using propidium iodide (Fig. 2B). Freshly isolated human PMNs contained only a small percentage of non-vital cells. The number of trypan blue positive cells raised continuously upon storage of these cells at 37 °C in the presence of antibiotics. After about 42 h, the same percentage of vital and non-vital cells was found. All cells were non-vital after 96 h. Similar results were obtained using propidium iodide in flow cytometry analysis. There was an increase in PI-positive cells starting during the second day of cultivation. Thus, the loss of vitality coincides well with the disappearance of cells unable to bind MPO antibodies from the cell suspension.

Fig. 4. Application of confocal fluorescence microscopy to determine the colocalisation of MPO and annexin V binding epitopes on non-vital PMNs. (A-C) A typical fluorescent cell from a cell suspension stored for 43 h stained with MPO antibodies (A), annexin V (B), and merge application (C). (D-F) Two fluorescent cells from a cell suspension stored for 69 h stained with MPO antibodies (D), annexin V (E), and merge application (F).

Binding of myeloperoxidase antibodies correlates well with annexin V binding on the surface of apoptotic PMNs in flow cytometry analysis

As we found recently a close association of MPO with phosphatidylserine epitopes on the surface of non-vital spermatozoa [13], we next examined the co-existence of annexin V and MPO on apoptotic neutrophils by flow cytometry. While in freshly isolated PMNs or cells incubated for one day most cells did not bind FITC-labelled annexin V (Fig. 3A), there was a clear shift in fluorescence distribution at higher incubation times indicating a binding of annexin V (Fig. 3B-C). A further small maximum with high fluorescence values at about 100 units appeared in samples incubated for 75 h (Fig. 3C).

The appearance of annexin V-binding epitopes on the surface of non-vital neutrophils correlated well with

the ability of these cells to bind MPO antibodies as visualised by dot plots (Fig. 3D). Older cells were characterised by higher fluorescence values for both parameters (data not shown).

Confocal fluorescence microscopy revealed the colocalisation of myeloperoxidase and annexin V on apoptotic PMNs

Confocal fluorescence microscopy was further used to analyse the existence of MPO on the surface of PMNs in dependence on incubation time. After addition of MPO antibodies, non-labelled cells dominated in freshly isolated PMN samples or samples stored for 20 h. Cells with fluorescence patches at the surface but without any staining in the cytoplasm were observed in samples cultured for more than 36 h (Fig. 4A) and in few cases

Fig. 5. Calibration for the determination of active myeloperoxidase by Pholasin luminescence. (A) Pholasin (0.064 μ M) was incubated with MPO (5 ng) in PBS for 5 min (only the last minute is shown) at 37°C. Then hydrogen peroxide (180 μ M) was added by means of an injection device. Luminescence was counted for every five seconds. All measurements were performed in triplicate. (B) Increase in luminescence values (30 s after the addition of hydrogen peroxide) addition is plotted as a function of the amount of myeloperoxidase.

also in samples stored for shorter times. In samples older than three days, there were concurrently two types of fluorescent cells, cells with surface patches and cells with an intense labelling of the whole cell body (Fig. 4D). These two types of staining by MPO antibodies correspond to the two newly formed fluorescence peaks in flow cytometry analysis.

These cell samples were also incubated with FITC-labelled annexin V (Figs. 4B and E). We detected the same fluorescence pattern as with MPO antibodies using confocal fluorescence microscopy. Merge pictures (Figs. 4C and F) show adequately that annexin V was bound at the same places where myeloperoxidase was detected by fluorescent antibodies.

Myeloperoxidase externalised to the PMN surface is in an active state able to use hydrogen peroxide as a substrate

We further proved whether myeloperoxidase bound to the surface of apoptotic PMNs is active. To address

this question, we applied Pholasin, the photoprotein of *Pholas dactylus*. Previous experiments showed that Pholasin emitted an intense luminescence upon incubation with the whole MPO-hydrogen peroxide system, but not with MPO alone [18-20]. Moreover, this photoprotein, which has a mass of 34 kDa, is unable to penetrate into cells and thus detects only extracellular events [19, 21].

For calibration, Pholasin (0.064 μ M, final concentration) was incubated with isolated MPO followed by addition of hydrogen peroxide (180 μ M, final concentration). Hydrogen peroxide caused an immediate increase of the basal luminescence level to a maximum value, that decreased slightly during the next 15 s to a luminescence that was constant over the next minute (Fig. 5A). Then this enhanced luminescence decreased slowly. The difference between the constant enhanced and the basal luminescence levels was plotted against the MPO amount and yielded a linear dependence between both parameters from 1 ng to 20 ng MPO (Fig. 5B). At lower amounts of MPO, there was a deviation from this linearity due to

Fig. 6. Application of Pholasin luminescence to detect active MPO expressed on the surface of PMNs in dependence on incubation time. (A) Selected examples for changes in Pholasin luminescence upon addition of hydrogen peroxide to a PMN sample cultured for 0.5 h (■), 70 h (●) or 120 h (▲). 25,000 cells were incubated with Pholasin (0.064 μ M) for 5 min (only the last three minutes are shown) at 37°C followed by addition of hydrogen peroxide (100 μ M). All measurements were performed in triplicate. (B) Increase in luminescence values (30 s after the addition of hydrogen peroxide) addition is given as a function of incubation time of PMNs.

side reactions of Pholasin with hydrogen peroxide.

In the next series of experiments, Pholasin was incubated with PMNs cultured for different times at 37°C followed by the addition of hydrogen peroxide. Concentrations of Pholasin and hydrogen peroxide were chosen the same as in calibration experiments. The increase in luminescence values upon addition of hydrogen peroxide depended on the incubation time of cells. Selected examples for kinetics are given in Fig. 6A for cells stored for 0.5 h, 75 h and 120 h. A more detailed picture is presented in Fig. 6B. While only small increases in luminescence were detected in freshly isolated cells, this increase raised continuously up to an incubation time of 72 h. Then the increase in luminescence decreased slightly.

Using the increase in Pholasin luminescence upon addition of hydrogen peroxide as a measure for externalised MPO, these data corresponded well with the binding of MPO antibodies to PMNs as a function of storage time. In addition, the long lasting luminescence of PMNs in the presence of Pholasin and hydrogen peroxide re-

flects that MPO expressed to the surface of apoptotic PMNs is really active in the presence of hydrogen peroxide.

Discussion

Myeloperoxidase becomes expressed to the cell surface during the loss of vitality of polymorphonuclear leukocytes as evidenced by flow cytometry analysis and confocal fluorescence microscopy. Moreover, this myeloperoxidase is an active state able to use hydrogen peroxide as a substrate as shown by the appearance of an intense, long lasting light emission using the cell impermeable luminescent protein Pholasin.

Our data revealed a redistribution of myeloperoxidase during apoptosis of neutrophils. Significant amounts of this enzyme appeared at surface patches of dying cells. Phosphatidylserine epitopes were the apparent binding place for MPO as evidenced by

colocalisation of fluorescent MPO antibodies and annexin V. We reported recently, that MPO is able to bind to artificial PS liposomes as well as to mixed PS and phosphatidylcholine (PC) liposomes, but not to pure PC vesicles [13]. The binding of externally added MPO or of MPO released from coincubated PMNs to PS epitopes on apoptotic spermatozoa was also reported by our group [13]. Thus, the binding of MPO to the surface of apoptotic cells seems to be a common property that may take place at inflammatory loci where large amounts of PMNs are known to accumulate.

In addition to surface patches for bound MPO antibodies, a staining of whole cells was also observed. The appearance of both types of fluorescence staining was clearly separated in our experiments. Both types of fluorescence staining differ in their permeability for the MPO antibody as well as for annexin V. As PMNs contain large amounts of MPO, it is not surprising that an intense labelling of the whole cell body concurrent with high fluorescence values in flow cytometry occurred in samples older than three days. The high permeability of these samples reflects a higher degree of cell degradation. It is well known that apoptotic cells cleared not fast enough by professional macrophages undergo a secondary necrosis [22-24]. Likely, such necrosis events took place at higher incubation times in our cell samples.

The binding of MPO to PS epitopes is mostly driven by electrostatic interactions. MPO is a strong cationic protein with an isoelectric point higher than 10. To what extent, MPO is redistributed from the surface of apoptotic cells to other binding partners remains unknown at present. Myeloperoxidase is also known to form tight complexes with acidic plasma proteins such as albumin, α_1 -antiproteinase, fibronectin, apolipoprotein A1 and ceruloplasmin [25-31]. These interactions may favour site-specific reactions of MPO metabolites to selected targets and may contribute, thus, to a specific role of MPO during apoptosis events.

Polymorphonuclear leukocytes are known to accumulate in huge amount at inflammatory sites. Disturbance of apoptosis and/or clearance of apoptotic PMNs by macrophages is thought to be associated with appearance of severe and chronic inflammations as well as autoimmune disorders [24, 32, 34]. Apparently myeloperoxidase plays a role in apoptotic events in neutrophils. The possible involvement of myeloperoxidase in late phases of apoptosis of cells is further supported by the fact that MPO-deficient mice were more susceptible

to inflammations in different experimental models [8-11] and that their PMNs exhibited a suppressed apoptosis rate [10, 12].

Possibly myeloperoxidase plays a role of a bridging molecule between PS epitopes on apoptotic cells and the PS receptor of macrophages and facilitates in this way the internalisation of apoptotic cells by macrophages. A number of other proteins including annexin 1, thrombospondin and β_2 -glycoprotein 1 is known to arrange a close contact between PS epitopes and the PS receptor [34-36]. On the other hand, specific products of PS oxidation induced by the MPO-hydrogen peroxide-chloride system may also be involved in the apoptosis machinery as well as macrophage signalling. We showed here that myeloperoxidase expressed to PMN surface is able to use hydrogen peroxide as a substrate. Although enhanced levels of hydrogen peroxide exist at inflammatory sites, there will be a local burst of hydrogen peroxide production during by activation of the NADPH oxidase in macrophages [37]. Accumulation of oxidised phosphatidylserine with modified acyl chains is thought to promote recognition of apoptotic cells [38, 39]. To what extent modifications of the serine head group of PS by the MPO-hydrogen peroxide-chloride system play a role in the apoptosis process remains unsolved. Different α -amino acids, having the same structural elements at target site as PS, are known to be converted by the MPO-hydrogen peroxide-chloride system or the reagent hypochlorous acid to a chloramine derivative that decays within minutes to yield an aldehyde species with concomitant release of ammonia and carbon dioxide [40-42]. Principally, specific MPO-derived PS degradation products may be involved in regulation of signalling cascades. Chloramines such as taurine chloramine and monochloramine are known to impair signalling via the NF κ B pathway by oxidation of critical methionine residues in I κ B α , the inhibitory subunit of NF κ B [43-45].

Summarizing, our data revealed the novel finding that MPO is redistributed to the outer surface of PMNs during spontaneous apoptosis. MPO becomes colocalised with PS on the surface of non-vital PMNs.

Acknowledgements

This work was supported by the German Research Council (Project Nr. Gl 199/4-3) as well as by a grant from the company OXO Thai, Bangkok, Thailand.

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