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Antioxidant Defense Mechanisms in Human Neutrophils

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ABSTRACT

Neutrophils have a short half-life and high tendency to undergo apoptosis. One feature that may influence these characteristics is the antioxidant/oxidant balance of these cells. There are few studies on the levels of antioxidant enzymes in human neutrophils. We have analyzed by immunohistochemistry of paraffin-embedded cells and from cytospin preparations the most important antioxidant proteins in human neutrophils, and compared their levels with those in blood monocytes. Neutrophils showed moderate to high catalase, weak to moderate extracellular superoxide dismutase, and weak copper zinc superoxide dismutase and γ-glutamylcysteine synthetase immunoreactivities. There were no detectable levels of manganese superoxide dismutase, thioredoxin, and heme oxygenase 1. Some differences were observed between the samples prepared by embedding in paraffin or by cytospin. These results, in combination with a recent study from this laboratory, suggest that a prominent feature in neutrophils is their high catalase activity but lower level of glutathione-dependent antioxidant enzymes. The differences in antioxidant profiles in neutrophils and monocytes may have important effects on the life span of human neutrophils, in both healthy and diseased tissues. Antioxid. Redox Signal. 4, 27–34.

INTRODUCTION

NEUTROPHILS play a central role in inflammatory and infectious diseases. They generate superoxide by the respiratory burst through the activation of NADPH oxidase, but they also generate other toxic metabolites such as nitric oxide mainly by the inducible form of nitric oxide synthase and hypochlorous acid by myeloperoxidase. Neutrophils have a higher oxidant production than other inflammatory cells, which may contribute not only to protection of the tissues against parasites and bacteria, but possibly to the development of the injury of the target organ. A typical feature of neutrophils is their high turnover and tendency to undergo apoptosis (15). As antioxidant/oxidant balance has important effects on cell survival and apoptosis, it may also play a role in the life span of neutrophils and in the progression of neutrophilic inflammation. In spite of numerous studies on neutrophils and oxidants, very few studies have investigated antioxidant mechanisms of human neutrophils. These previous studies suggest the importance of glutathione (GSH) in protecting neutrophils against their own toxic metabolites (33, 35, 37).

Mammalian antioxidant enzymes (AOEs) scavenging superoxide include the superoxide dismutases (SODs), namely manganese superoxide dismutase (MnSOD), copper zinc superoxide dismutase (CuZnSOD), and extracellular superoxide dismutase (ECSOD) (5, 7, 8, 24, 25). MnSOD is expressed in the mitochondrial compartment of the cells (39) and is

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induced by a variety of oxidants and cytokines, such as tumor necrosis factor, interleukins, and changes in the cellular redox state (6, 10, 13, 40). CuZnSOD is constitutively expressed in the cytosol (36), and in contrast to MnSOD, oxidants and cytokines do not have any significant effects on the regulation of this enzyme (10, 13). ECSOD is expressed in the proximity of endothelial cells and collagen fibers, and ECSOD has been suggested to play a role in the defense of endothelial cells and extracellular matrix against exogenous oxidants (24). Oxidant and antioxidant processes in the extracellular lung compartment contribute to hyperoxia-induced lung damage, where overexpression of ECSOD has been shown to mediate protection (5). All these enzymes have been investigated mainly in cell culture models and experimental animals, whereas few studies have assessed their localization and expression in healthy and diseased human tissues.

Superoxide is decomposed to hydrogen peroxide (H_2O_2) both spontaneously and by SOD. H_2O_2 can diffuse through the plasma membrane, it has a longer half-life than superoxide, and it can contribute to the formation of other free oxygen radicals such as hydroxyl radicals (8). Most widely investigated H_2O_2-scavenging mechanisms include catalase (CAT), and GSH-dependent enzymes such as glutathione peroxidases (GPXs) and glutathione synthase (8). CAT is located mainly in peroxisomes, but it has also been detected in the cytosolic fraction of neutrophils (1). The rate-limiting enzyme of GSH synthesis is γ-glutamyl cysteine synthetase (γGCS). This enzyme constitutes two subunits, namely the heavy (catalytic; γGCSh) and light (regulatory; γGCSl) chains, which are synthesized by different genes. GSH synthesis is up-regulated by oxidative stress by transcriptional mechanism, and partly by increased mRNA stabilization (for review, see 29). It has been suggested that GSH is the most important antioxidant in human neutrophils, but also that CAT may be needed for the protection against oxidative injury of these cells (27, 33). No systematic studies have been conducted on γGCS in human neutrophils.

There are also other H_2O_2-scavenging pathways that are poorly characterized, but that may have an important role in neutrophils. Most potential of these other mechanisms are cysteine-containing proteins such as thioredoxin (TRX) and peroxiredoxins (9, 28, 31). TRX is a small protein that provides sulfhydryl groups for the TRX-dependent activity. TRX functions as a powerful antioxidant, protects cells against apoptosis by inhibiting apoptosis signal regulating kinase 1, and induces cell proliferation. TRX modulates the DNA binding activity of many transcription factors such as nuclear factor-κB, but also exhibits several cytokine-like activities (28). Due to the antiapoptotic properties of TRX, it may be hypothesized that the TRX system is not expressed in neutrophils or that its expression is very low.

In addition, there are other enzymes with efficient antioxidant capacity, one of them being heme oxygenase (HO). There are three isoenzymes of HO; among them, HO-1 is an inducible form of the enzyme that is up-regulated by oxidant stress and cytokines, and is associated with increased protection against oxidants (22). Experimental studies on rats treated with HO-1 transfection have exhibited attenuation of hyperoxia-induced neutrophil inflammation and apoptosis (23). That particular study suggested that HO-1 provides protection against hyperoxia-induced lung injury *in vivo* by modulation of neutrophil inflammation and apoptosis. We have recently detected HO-1 in human alveolar macrophages (14).

The present study aims to investigate the expression of various antioxidant pathways in human neutrophils. To assess the levels of the enzymes, their expression and/or activities were compared with those in monocytes, because both cell types represent inflammatory cells with different life spans and oxidant resistance.

MATERIALS AND METHODS

Cells from healthy volunteers

Cells were obtained from EDTA-anticoagulated venous blood of six voluntary healthy

nonsmoking subjects. Plasma was removed by centrifugation (3,500 rpm for 10 min at +4°C). The white cell fraction was collected, and contaminated red cells were lysed by hypotonic shock using ice-cold distilled water. Reconstitution of osmolarity was then done with concentrated phosphate-buffered saline (PBS). The cells were then washed once in PBS and resuspended in 1.5 ml of the same buffer. Cells were identified in cytocentrifuge preparations stained with May–Grünwald–Giemsa; at least 200 cells were counted.

Cytospin

Cells were fixed and permeabilized by FIX & PERM reagents (Caltag Laboratories, Inc., Burlingame, CA, U.S.A.) according to the manufacturer's instruction. Cytocentrifuge slides were done using a cytospin machine routinely used in hematology laboratory (SHANDON Sytospin 3). Two hundred microliters of cell suspension containing $\sim 4 \times 10^5$ cells was spun onto each slide. The slides were then dried through air flow and were thereafter ready for further analyses.

Cytoblocks

About 1.5×10^7 cells were fixed in 4% formalin for 12 h at +4°C, after which the cells were pelleted by centrifugation. The cell pellet was then suspended in melted 2% agarose. The agarose block was further embedded in paraffin. Thereafter 4-μm-thick sections were placed on slides, which were then ready for further analyses.

Immunocytochemistry

Before immunostaining, sections made from paraffin blocks were dewaxed in xylene and rehydrated through a series of ethanol solutions, whereas cytospin slides were fixed in 4% paraformalin for 10 min and washed in PBS thereafter. Then the samples were incubated in 2% H_2O_2 for 10 min to eliminate endogenous peroxidase activity. Prior to H_2O_2 treatment, samples were incubated in 10 m*M* citric acid monohydrate, pH 6.0, for 10 min and heated in a microwave oven, after which the samples were blocked by a blocking serum (Zymed Laboratories Inc., South San Francisco, CA, U.S.A.) for 10 min.

Immunostaining with various AOE antibodies was performed using the following primary antibodies: dilutions 1:1,000 for the rabbit antibody against human MnSOD, 1:100 for the rabbit antibody against CuZnSOD, 1:200 for the rabbit antibody against murine ECSOD, 1:200 for the rabbit antibody against human CAT, 1:1,000 for the rabbit antibody against human γGCSh, 1:1,000 for the rabbit antibody against human γGCSl, 1:1,000 for the rabbit antibody against human TRX, and 1:100 for the mouse antibody against HO-1. The MnSOD, CuZnSOD, ECSOD, and CAT antibodies had been generously provided by Prof. J. Crapo (Jewish Medical Center, Denver, CO, U.S.A.), γGCSh and γGCSl by Prof. T. Kavanagh (University of Washington, Seattle, WA, U.S.A.), and TRX by Prof. A. Holmgren (Karolinska Institutet, Stockholm, Sweden). HO-1 was purchased from Transduction Laboratories (Lexington, KY, U.S.A.). The immunostaining was performed using the Histostain-Plus Bulk Kit (Zymed Laboratories Inc.), and the chromogen used was aminoethylcarbazole (Zymed Laboratories Inc.). Negative control staining was carried out by substituting PBS and serum isotype controls (Zymed Laboratories Inc.) for the primary antibodies. The results of the immunostaining were evaluated by assessing the percentage of the positively stained neutrophils in the samples. Additionally, the intensity of the immunostaining reaction was assessed as no immunoreactivity or weak, moderate, or strong immunoreactivity.

RESULTS

The expressions of various AOEs in neutrophils were analyzed in cytospin preparations and paraffin-embedded cells. Cytospun neutrophils had moderate to high CAT, weak to moderate ECSOD, and weak to negative CuZnSOD and γGCS reactivities; no immunoreactivity for MnSOD, HO-1, or TRX could be found in any case (Table 1). When the intensities in the paraffin-embedded samples

TABLE 1. IMMUNOREACTIVITY OF CYTOSPIN PREPARATIONS OF HUMAN NEUTROPHILS

	Negative	*Weak*	*Moderate*	*Strong*
CAT	0 ± 0	0 ± 0	70 ± 20	30 ± 20
ECSOD	7 ± 5	32 ± 49	61 ± 48	0 ± 0
CuZnSOD	55 ± 38	45 ± 38	0 ± 0	0 ± 0
γGCSh	84 ± 14	16 ± 14	0 ± 0	0 ± 0
γGCSl	63 ± 30	37 ± 30	0 ± 0	0 ± 0
MnSOD	100 ± 0	0 ± 0	0 ± 0	0 ± 0
TRX	100 ± 0	0 ± 0	0 ± 0	0 ± 0
HO −1	100 ± 0	0 ± 0	0 ± 0	0 ± 0

Values are means ± SE from six healthy individuals.

and cytospin preparations were compared, γGCSh appeared to be slightly positive in the cytoplasm of neutrophils in the cytospin preparations, whereas no immunoreactivity was found in paraffin-embedded cells. The staining of ECSOD in the cytospin preparations appeared in both the cytosol and plasma membrane, whereas it was expressed only in the cytosol of paraffin-embedded cells. Representative stainings of CAT and ECSOD in the cytospin preparations are shown in Fig. 1. We have recently assessed the specific activities of CuZnSOD, MnSOD, CAT, GPX, and glutathione reductase (GR), and GSH concentrations in freshly isolated neutrophils and monocytes (27), and a summary of these results is gathered in Fig. 2. In addition, our recent observations have indicated that, in contrast to neutrophils, TRX and HO-1 can be detected in the macrophages of human lung (11, 14).

DISCUSSION

Our study shows that human neutrophils express at least moderate levels of CAT, whereas they have weak or nondetectable levels of MnSOD, CuZnSOD, γGCS, TRX, and HO-1 when assessed by immunohistochemistry. Furthermore, our recent findings on freshly isolated neutrophils have demonstrated that CAT activity is significantly higher in neutrophils than in monocytes and that human monocytes express higher levels of GSH and GSH-dependent AOEs, GPX, and GR (27).

Cell isolation and sample preparation may have multiple effects on the final results obtained. In fact, our recent experiments with isolated cells indicated that the mRNA of MnSOD was higher in neutrophils than monocytes, whereas MnSOD specific activity in neutrophils was very low (27). Thus, up-regulation of the MnSOD mRNA in these cells might reflect transcriptional activation of the enzyme during cell isolation. This result is also one indication that neutrophils are activated during cell isolation with potential effects on the expression of oxidant-related genes. Therefore, results with isolated cells need to be interpreted with certain caution. On the other hand, enzyme activities decline rapidly after the isolation, and cell culture further causes down-regulation of several AOEs (12). That problem has been avoided in the present study by rapid preparation and

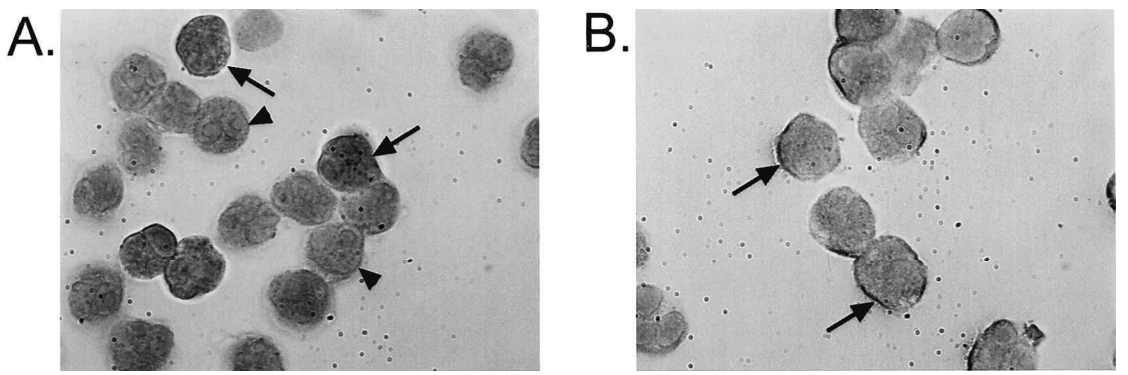

FIG. 1. Expression of CAT (A) and ECSOD (B) in the cytospin preparations. Strong and moderate intensities are demonstrated by arrows (strong) and arrowheads (moderate). Negative controls showed no immunoreactivity (data not shown).

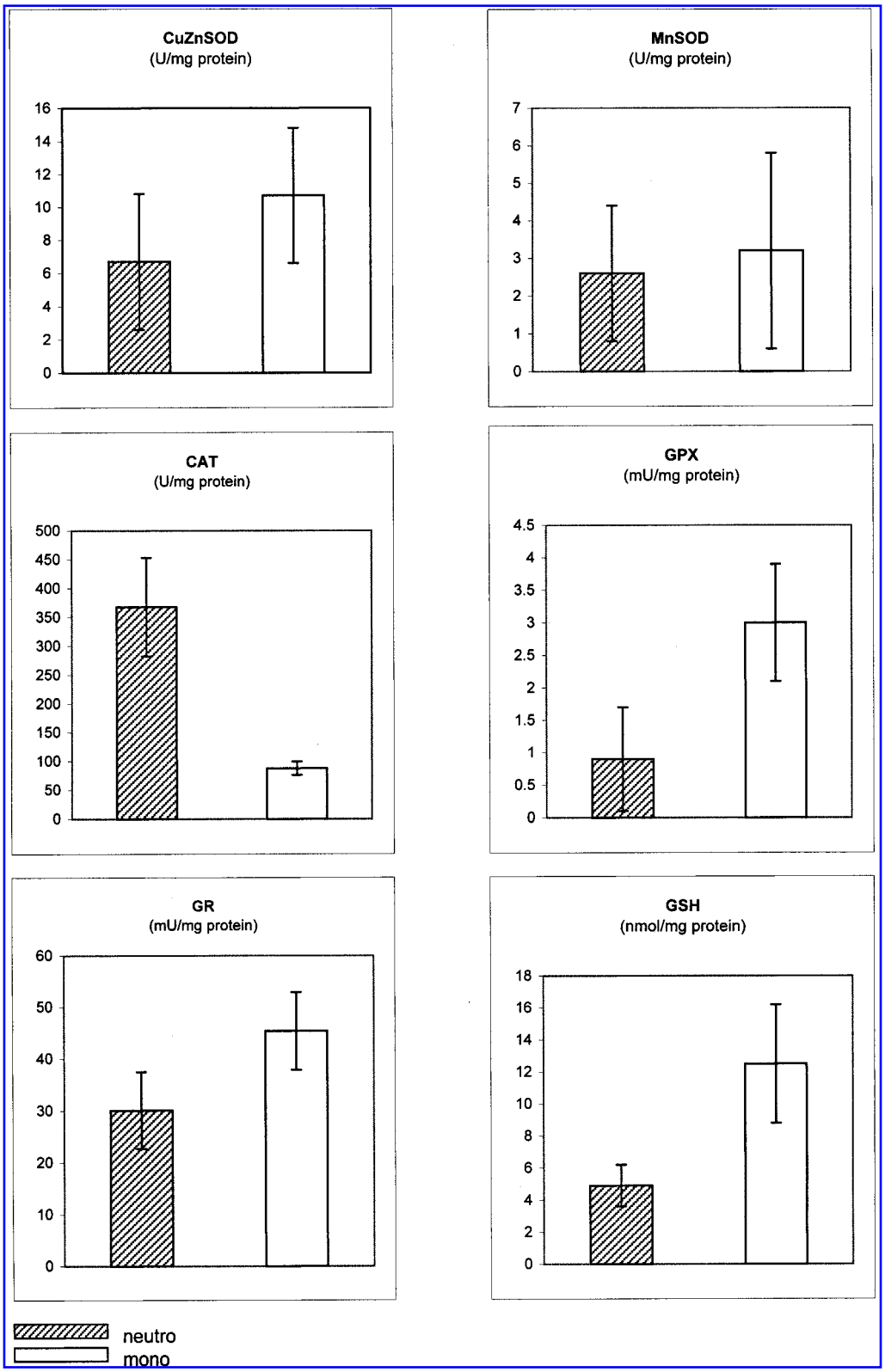

FIG. 2. Antioxidant enzyme activities and GSH levels in freshly isolated neutrophils and blood monocytes. The results are means ± SD from four to six separate experiments. The details and methods have been described earlier, and the figure represents a summary of those previously published results (27).

fixation of the cells. Knowing this caveat, we also prepared all samples using paraffin embedding of the cell pellets, but also fixation of the cytospin preparations. We also conducted the staining procedures by several modifications after numerous testing conditions (data not shown). These experiments, for instance, showed that citrate treatment in the microwave oven may have profound influence on the detection of various AOEs and needs to be tested carefully.

Most previous studies on AOEs on human neutrophils have assessed them in the diseased tissues. It has been suggested that oxidant/antioxidant balance and low total SOD activity in neutrophils may further hasten the onset of neutrophil apoptosis (20, 21). It has also been suggested that MnSOD has no effect on the apoptotic cascade of neutrophils (2). Our study shows that neutrophils contain very low levels of MnSOD, but neutrophils did express CuZnSOD and ECSOD. Interestingly, ECSOD was expressed on the outer membrane of the neutrophils of cytospin preparations possibly due to the heparin-binding properties of this enzyme. It may be possible that the lack of ECSOD staining on the plasma membrane of paraffin-embedded cells may be associated with the fixation procedure of the cells. A recent study on rat lung could not detect ECSOD in neutrophils of the circulating blood (16). This study also suggested that neutrophils are capable of binding ECSOD, which is consistent with our results. It has to be noted that immunocytochemistry is relatively insensitive, and that a negative finding does not rule out the presence of minor levels of AOEs in the cells.

Our present findings showed that CAT was the only AOE to be highly expressed in neutrophils. In agreement, we recently observed that the mRNA of CAT was 50-fold and its specific activity fourfold higher in neutrophils than in monocytes (27). High CAT activity in neutrophils may explain their resistance to high exogenous concentrations of oxidants during acute exposures as we also demonstrated (27). Thus neutrophils undergo apoptosis by mechanisms that may at least partly be associated with antioxidant pathways other than CAT.

Our earlier findings showed that GSH content and GPX activity were about one third of the corresponding values in monocytes (27). In the present study, immunohistochemical staining of γGCS, the rate-limiting enzyme in GSH synthesis, revealed weak or absent immunoreactivity in neutrophils. GSH may, however, have an important biological role in neutrophils. For instance, glutathione synthase deficiency leads to 80–90% decrease in the GSH content of neutrophils and to structural and functional abnormalities, one of those being impaired bacterial killing by neutrophils (37). We have also observed that neutrophil viability is decreased by depletion of GSH with buthionine sulfoximine *in vitro* (27), which result is in agreement with the effects of the manipulation of intracellular thiols by GSH-depleting agents (38). Neutrophils undergoing apoptosis have also other abnormalities in their oxidant/antioxidant balance, for instance, in the production of nitric oxide and activity of CuZnSOD; both of these phenomena have also been shown to be associated with decreased GSH content in these cells (18, 21). Furthermore, it has been suggested that GSH not only contributes to the regulation of neutrophil apoptosis, but also to the resolution of inflammation in the lung (18).

There are also studies that have shown that antioxidant levels of human neutrophils decrease in systemic diseases. Total SOD, GPX, and GR activities appear to be lower in neutrophils from patients with type 2 diabetes than in controls (19). Total SOD, GPX, and CAT activities are lower in the neutrophils of hyperlipoproteinemia patients than in the healthy controls (3). Neutrophil antioxidant capacity is also down-regulated in bronchiolitis obliterans syndrome of lung transplant patients (32). Furthermore, liver cirrhosis and hepatitis are associated with defective neutrophil phagocytosis, which has been suggested to result from reduced neutrophil GSH levels (30). Chronic granulomatous disease has also been reported to have declined activities of neutrophil GR and GPX (34). On the other hand, higher GPX expression in eosinophils than in neutrophils of asthmatic patients may be related to the survival of

eosinophils at the sites of inflammation (17). Although these studies show that systemic illnesses may be associated with decreased antioxidant defense in neutrophils, the significance of these findings in various clinical conditions with multiple other abnormalities remains unclear.

In conclusion, typical features of the antioxidant profile of neutrophils are their high CAT content, low levels of GSH-dependent enzymes, and nondetectable levels of TRX and HO-1 by immunohistochemistry. These differences may have important consequences, including the effects of antioxidant enzymes on the life span and apoptosis of these cells.

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ABBREVIATIONS

AOE, antioxidant enzyme; CAT, catalase; CuZnSOD, copper zinc superoxide dismutase; ECSOD, extracellular superoxide dismutase; γGCS, γ-glutamyl cysteine synthetase; γGCSh and γGCSl, heavy and light chain subunits, respectively, of γGCS; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; HO, heme oxygenase; H_2O_2, hydrogen peroxide; MnSOD, manganese superoxide dismutase; PBS, phosphate-buffered saline; SOD, superoxide dismutase; TRX, thioredoxin.

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