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Superoxide dismutase (SOD) as a potential inhibitory mediator of inflammation via neutrophil apoptosis

KOZO YASUI[1], NORIMOTO KOBAYASHI[1], TAKASHI YAMAZAKI[1], KAZUNAGA AGEMATSU[1], SATOSHI MATSUZAKI[1], SUSUMU ITO[2], SETSUKO NAKATA[1], ATSUSHI BABA[1], & KENICHI KOIKE[1]

[1]*Department of Pediatrics, Shinshu University School of Medicine, Asahi 3-1-1, Matsumoto 390-8621, Japan, and* [2]*Blood Transfusion Service, Shinshu University Hospital, Matsumoto, Japan*

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Abstract
Superoxide dismutase (SOD) is supposed to be an effective agent for neutrophil-mediated inflammation in the area of critical medicine. We investigated the involvement of SOD in the regulation of neutrophil apoptosis. Exogenously added SOD effectively induced neutrophil apoptosis, and the fluorescence patterns determined using annexin-V and the 7-AAD were similar to those seen in Fas-mediated neutrophil apoptosis. Neutrophils are short-lived leukocytes that need to be removed safely by apoptosis. The clearance of apoptotic neutrophils from sites of inflammation is a crucial determinant of the resolution of inflammation. Catalase inhibited the neutrophil apoptosis and caspase-3 activation. Spontaneous apoptosis, hydrogen peroxide and anti-Fas antibody-induced apoptosis of neutrophils were accelerated in Down's syndrome patients, in whom the SOD gene is overexpressed. Hydrogen peroxide was thought to be a possible major mediator of ROS-induced neutrophil apoptosis in caspase-dependent manner. Neutrophil apoptosis represents a crucial step in the mechanism governing the resolution of inflammation and has been suggested as a possible target for the control of neutrophil-mediated tissue injury. SOD may be a potential inhibitory mediator of neutrophil-mediated inflammation.

Keywords: *Caspase, down's syndrome, tissue injury, hydrogen peroxide, reactive oxygen species*

Introduction

A vast amount of evidence indicates that oxygen-derived free radicals and other high-energy oxidants are important mediators of ischemia-reperfusion and/or septic shock-induced tissue injury [1–7]. Neutrophils play essential roles in host defense by engulfing and killing infectious microorganisms. Reactive oxygen species (ROS) and primary granule constituents, are secreted by activated neutrophils, and this sometimes leads to normal tissue injury. During circumstances of oxidative stress, superoxide dismutase (SOD) is an endogenous cellular defense system that degenerates superoxide (O_2^-) into oxygen and hydrogen peroxide [8,9], with the latter being further detoxified by glutathione peroxidase or catalase. There is increasing evidence that exogenous SOD has been a therapeutic potential agent in critical medicine for the treatment of septic shock [3,4,10–12]. It is proposed in part that SOD balances the production of excess superoxide anion.

An additional possible mechanism that contributes to the efficacy of SOD in critical care medicine is the regulation of neutrophil apoptosis. For the resolution

Correspondence: K. Yasui, Department of Pediatrics, Shinshu University School of Medicine, Asahi 3-1-1, Matsumoto 390-8621, Japan. Tel: 81 263 37 2642. Fax: 81 263 37 3089. E-mail: k-yasui@hsp.md.shinshu-u.ac.jp

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of inflammation, the activated neutrophils need to be safely removed by apoptosis [13–15], which suppresses the production of proinflammatory cytokines and proteinases such as neutrophil elastase. Several lines of evidence indicate that ROS by themselves are involved in apoptosis of neutrophils. First, neutrophil apoptosis is inhibited under hypoxic conditions, which cause marked decreases in the generation of ROS [16]. Second, neutrophils isolated from patients with chronic granulomatous disease, which is characterized by a genetic deficiency in production of superoxide and hydrogen peroxide with lack of NADPH oxidase complex, show decreased rates of spontaneous and CD95 (Fas)-mediated cell death [17] and of phagocytic clearance by macrophage [18]. ROS intermediates and antioxidants are possible regulators of the apoptotic caspases [19,20], but the mechanisms involved are still under debate and investigation.

In the present study, we demonstrate that an antioxidant SOD is *in vitro* involved in the regulation of neutrophil apoptosis (inflammatory sedation). In addition, we focused on the biochemical roles of ROS and antioxidant enzyme SOD in connection with apoptosis using neutrophils from patients with Down's syndrome (DS) (trisomy 21), in whom the copper-zinc superoxide dismutase (CuZnSOD) gene is overexpressed. Neutrophil apoptosis could be induced by the addition of exogenous SOD. SOD may be a potential inhibitory mediator and the treatment pathway of neutrophil-mediated inflammation via apoptosis.

Materials and methods

Reagents

Agonistic anti-Fas IgM MoAb (clone CH-11) was purchased from Medical & Biological Laboratories, Ltd. (Nagoya, Japan). The H_2O_2 and hypoxanthine were from Wako Pure Chemical (Osaka, Japan). The Annexin V-PE apoptosis kit containing annexin V-FITC and Via-probe (7-AAD) staining was from Becton and Dickinson Co. (San Jose, CA). DTT, CHAPS, HEPES, catalase, CuZnSOD (SOD from bovine erythrocytes; 3300 units/mg protein), and NAN_3 were from Sigma (St. Louis, MO). z-Val-Ala-Asp-chloromethylketone (zVAD-cmk), an inhibitor of caspase-3, was obtained from Peptide Institute, Inc (Osaka, Japan). A *Cypridina* luciferin analogue, 2-methyl-6-(*p*-methoxy-phenyl)-3,7-dihydrilmidazo[1,2-a] pyrazin-3-one hydrochloride (MCLA), was from Tokyo Kasei (Tokyo, Japan).

Preparation of neutrophils

Heparinized venous blood was obtained with informed consents from healthy volunteers and from DS patients and their parents. All DS patients (median age, 30 years; range 21–40 years) were cytogenetically diagnosed as having trisomy 21. Neutrophils were isolated by dextran sedimentation and centrifugation on a Histopaque gradient (without endotoxin; Sigma) as described previously [21,22]. Contaminating red blood cells were removed by cold hypotonic water lysis. The purity of the isolates was assessed by preparing cytocentrifuge smears and staining with May-Grünwald-Giemsa (Merck Ltd, Darmstadt, Germany). The purity of the neutrophil preparations was $>95\%$, and viability was greater than 98%, as determined by trypan blue dye exclusion (Sigma). Isolated neutrophils (10^6/ml) were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum with 2 mmol/l glutamine at 37°C in a humidified atmosphere containing 5% CO_2 (pH 7.4).

Assessment of neutrophil apoptosis

Neutrophil apoptosis was quantified morphologically and measured as the percentage of cells by membraneous binding of annexin V-FITC (FL1) and nuclear staining with a Via-probe 7-AAD (FL2) by flow cytometry. NaN_3 (1 mM) was added in the incubation buffer to prevent consumption of H_2O_2 by myeloperoxidase or catalase in some cases. Apoptotic cells were defined as phosphatidylserine exposure on the outer plasma membrane which binds annexin V (single-positive) and dead cells are stained with both of annexinV and 7-AAD (double-positive). Annexin V and 7-AAD fluorescence of individual nuclei were plotted and the data were registered on a logarithmic scale. Results were expresses as percentages of the total number of gated neutrophils by flow cytometry.

DNA fragmentation

First, 2×10^6 cells were lysed by incubation for 15 min in 400 μl of a cold mixture of 10 mM Tris–HCl, pH7.4, 0.2 mM EDTA, and 0.2% Triton X-100. The lysates were centrifuged for 5 min at 10,000*g*. The supernatant was extracted with chloroform/isoamyl alcohol/phenol, and the aqueous phase was collected. The DNA was precipitated with 50% 2-propranol and 0.5 M sodium acetate and left at −80°C overnight. After digestion with 50 μg/ml RNase A (Boehringer Mannheim, Mannheim, Germany) for 1 h at 37°C, the samples were electrophoresed through 1.2% agarose gels and stained with 0.5 μg/ml ethidium bromide [22].

Fluorometric assay for caspase-3-like activity

DEVD-7-amino-4-methylcoumarin (AMC) cleavage was measured using a modification of the fluorometric assay of Parvathenani et al. [23]. Neutrophils (5×10^6 cells) were placed in 100 μl of lysis buffer

[24] for 10 min on ice and then centrifuged (15,000*g*, 3 min). The protein concentrations of the lysates were determined by colorimetric analysis using protein assay reagents (BioDynamics Laboratory Inc., Tokyo, Japan). Cell lysate and the substrate DEVD-AMC (50 μM) were combined in a standard reaction buffer (10% sucrose, 0.1% CHAPS, 10 mM DTT and 50 mM HEPES; pH 7.5). Then, the enzymatic activity was determined spectrophotometrically (BioRad, Hercules, CA) for 60 min, using excitation and emission wavelengths of 355 and 460 nm, respectively.

Assay for SOD of isolated cells

The standard reaction mixture contained 2×10^{-7} M MCLA, 5×10^{-5} M hypoxanthine, 6.5 units of xanthine oxidase, either SOD standard or samples, and 50 mM Tris–HCl buffer at PH 7.8 containing 0.1 mM EDTA, in a total volume of 3.0 ml. Chemiluminescence measurement was initiated by adding MCLA to reaction mixture without XOD and continued for 2 min after the addition of XOD in the luminescence reader at 25°C. Inhibition of XOD-induced chemiluminescence by the SOD sample was calculated as a percentage and the concentration of SOD in the sample was determined by calibration with the standard curve [25]. SOD content was expresses as nanogram per 10^6 cells.

Statistical analysis

All data are presented as means ± SEM. The significance of differences between two groups was determined by Student *t*-test. For more than two experimental groups, analysis was performed by analysis of variance (ANOVA). In all cases, p values of < 0.05 were considered significant.

Results

Anti-Fas MoAb-triggered apoptosis and its evaluation

Apoptosis of cultured neutrophils was measured, the cells were treated with 100 ng/ml of anti-Fas antibody for the indicated periods. Neutrophils stained with May-Grünwald-Giemsa showed the characteristic morphology of apoptosis: one or more darkly stained pyknotic nuclei, and compacted but structurally intact cytoplasmic organelles. Important hallmarks of apoptotic cells are the exposure of PS on the outer leaflet of the plasma membrane stained with annexin V. The changes were evident in antibody-treated neutrophils on FACS examination (a representative data is shown in Figure 1), and the mean levels of apoptotic cells were increased in time-dependent manner and 39.1 ± 2.6% ($n = 4$) at 4 h. The post-apoptotic and necrotic cells (dead cells) were thought to be positive with both of annexin V and 7ADD stains (double positive).

ROS and neutrophil apoptosis

Culture of neutrophils resulted in accelerated spontaneous apoptosis in time-dependent manner, and the mean levels of apoptotic cells were about 20% at 6 h incubation (Figure 2). When the cells were incubated together with an excess of SOD (100 U/ml), the number of apoptotic neutrophils was increased. Exogenous hydrogen peroxide (0.1 mM) promoted neutrophil apoptosis, which is estimated by FACS (Figure 3), resulting in a rapid onset of nuclear condensation at 2 h (Figure 4A). When hydrogen peroxide was added together with SOD to induce H_2O_2 from superoxide anion, the number of apoptotic neutrophils showed a significant increase. Meanwhile, catalase (100 U/ml), a scavenger of hydrogen peroxide, significantly inhibited neutrophil apoptosis (Figure 3). These results support that neutrophil apoptosis is mainly mediated by the presence of H_2O_2. The morphological features of neutrophils incubated with H_2O_2 were quite similar to those with anti-Fas antibody (Figure 4A,B).

Activated caspase-3-like activity and DNA fragmentation in apoptotic neutrophils

Caspase-3-like activity was measured by cleavage of the fluorogenic substrate DEVD-AMC as described previously [23,24]. Preincubation with hydrogen peroxide (0.1 mM) significantly enhanced caspase activity (Figure 5A; $n = 3$, $p < 0.01$ by one-way factorial ANOVA). Clear induction of caspase activity was also seen in anti-Fas MoAb-treated neutrophils after 4 h of incubation (Figure 5B). Preincubation with SOD (100 U/ml) further enhanced the caspase activity ($n = 3$, $p < 0.01$ by ANOVA), but catalase decreased caspase activity ($n = 3$, $p < 0.0001$ by ANOVA). Agarose gel electrophoresis of DNA from the neutrophils showed characteristic ladder-like apoptotic patterns. The augmentation of laddering of DNA extracts by H_2O_2 and SOD treatment is visible (Figure 6A). DEVD-AMC is a specific substrate for caspase-3 protease, which mainly mediates cellular apoptosis and DNA fragmentation in neutrophils. The caspase-3 inhibitor ZVAD-cmk (10 μM) prevented the fragmentation of DNA (Figure 6B).

Neutrophil apoptosis in subjects with DS

Total SOD in neutrophils of DS subjects was significantly higher than in control cells (Table 1), and total SOD in serum was also higher in DS subjects. As shown in Figure 7A, the percentage of spontaneous apoptotic neutrophils in the control population ($n = 3$) was significantly lower than that in DS ($n = 3$,

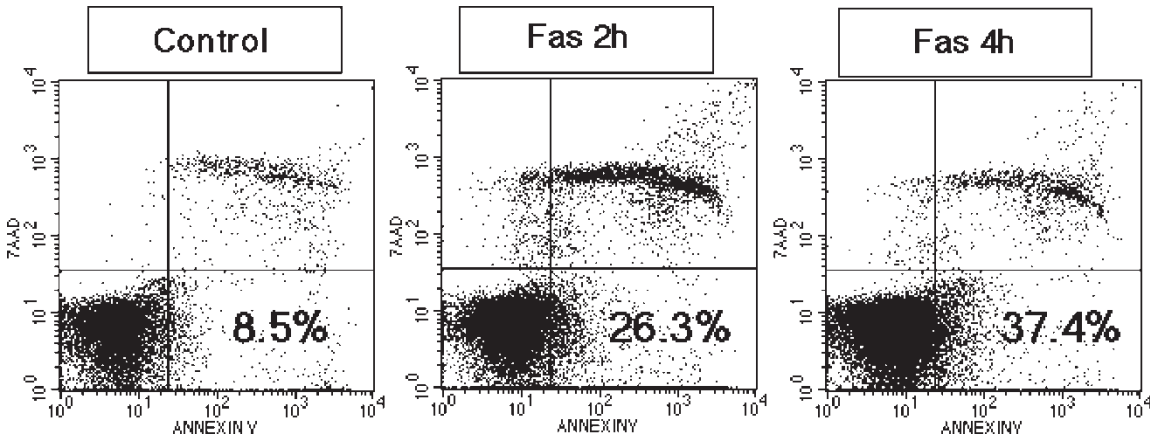

Figure 1. Flow cytometric analysis of annexin V and 7-AAD binding in neutrophils control (before incubation) and undergoing Fas-mediated (100 ng/ml CH11) apoptosis for various incubation periods. The percentages of Annexin V single-positive cells are shown. Representative data are shown.

$p < 0.0001$ by two-way factorial ANOVA). Similarly, mean percentage of apoptotic neutrophils in the controls was lower than that in DS subjects, when neutrophils were treated with the addition of hydrogen peroxide (Figure 7B; $n = 4$, $p < 0.001$ by two-way repeated measures ANOVA). Cellular apoptosis was confirmed by FACS analysis. The difference in neutrophil apoptosis between DS and normal subjects

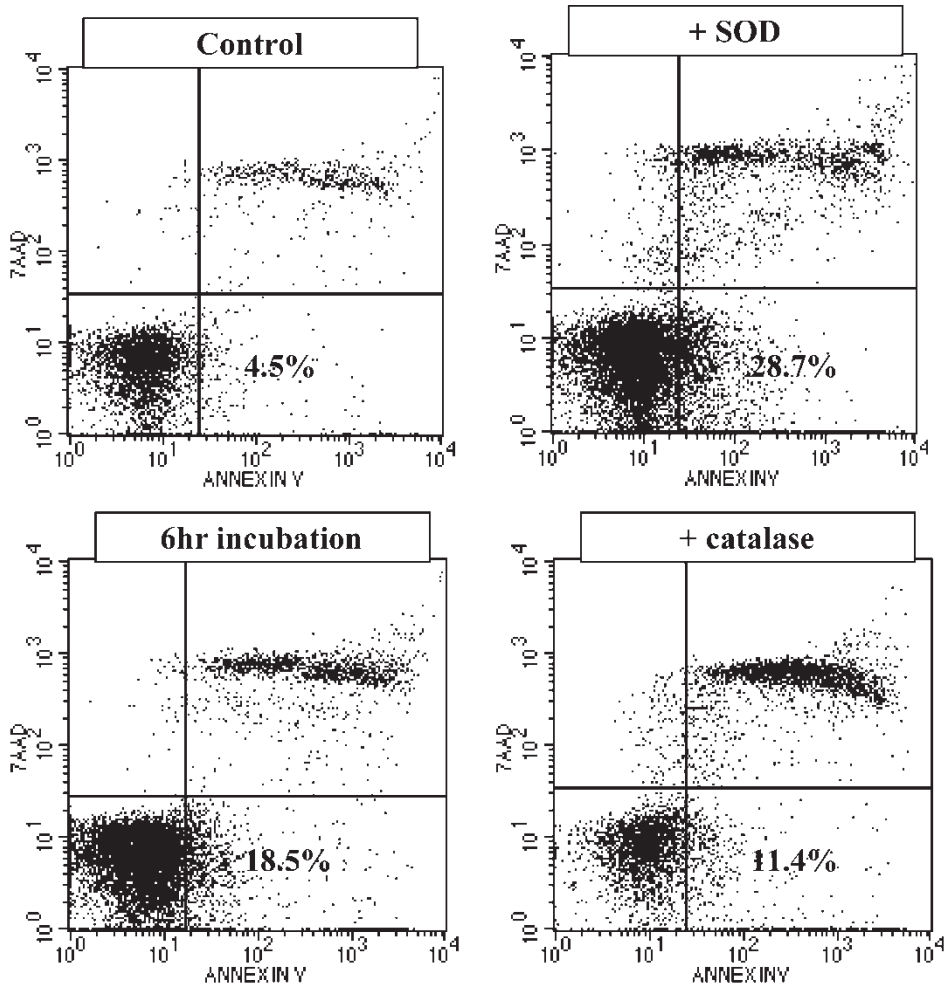

Figure 2. Flow cytometric analysis of annexin V and 7-AAD binding in neutrophils control (before incubation) and 6 h incubation. The cells were also incubated with with SOD (100 U/ml) or catalase (100 U/ml) for 6 h. The percentages of Annexin V single-positive cells are shown. Representative data are shown.

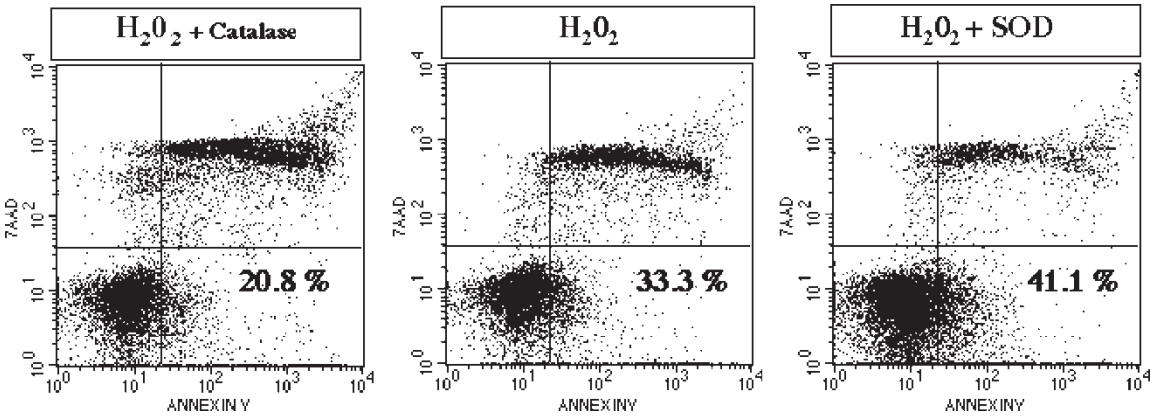

Figure 3. Flow cytometric analysis of annexin V and 7-AAD binding in neutrophils incubated with hydrogen peroxide (0.1 mM) for 2 h. The percentages of Annexin V single-positive cells are shown. The cells were also treated with SOD (100 U/ml) or catalase (100 U/ml). Representative data are shown.

was lost by the addition of exogenous SOD in excess (100 U/ml).

Discussion

Although a few conflicting observations have been reported [24,26,27], our results supported that neutrophil apoptosis is dependent on the production of hydrogen peroxide and caspase-3-dependent mechanism in agreement with the findings of several previous studies [16–20,28,29]. In the presence and absence of exogenous hydrogen peroxide (H_2O_2), the number of apoptotic neutrophils was increased together with SOD or NaN_3 to prevent consumption of H_2O_2 by catalase or myeloperoxidase, whereas the addition of catalase delayed neutrophil apoptosis. These observations supported a primitive role of hydrogen peroxide as a major mediator of neutrophil apoptosis.

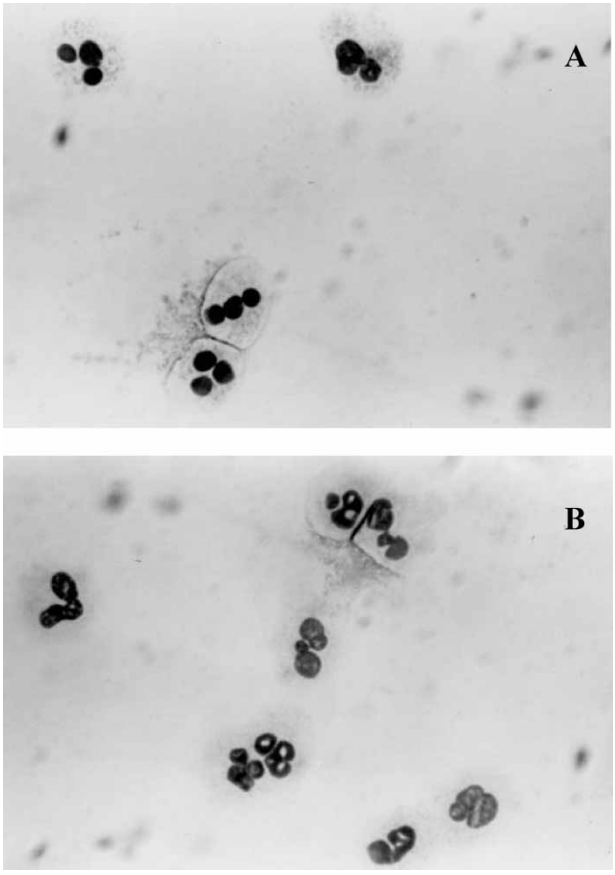

Figure 4. Morphological features of neutrophils incubated with hydrogen peroxide (0.1 mM) and 1 mM NaN_3 for 2 h (A) or with 100 ng/ml CH11 (anti-Fas antibody; B) for 4 h. May-Grünwald-Giemsa-stained preparations were photographed at 1000X.

SOD is a key cellular defense system that degenerates O_2^- into oxygen and hydrogen peroxide, with the latter being further detoxified by glutathione peroxidase or catalase. Neutrophil apoptosis has been proposed to play a role in the control of inflammation [1,2]. Although SOD can hardly enter the cell, so we presented here that exogenous SOD in excess could induce neutrophil apoptosis in cooperation with ROS, our studies propose a novel therapeutic potential in normal tissues by inducing apoptosis of neutrophils. There were conflicting reports about this. Sato et al. [24] have reported that exogenous SOD failed to induce neutrophil apoptosis, however, they used SOD at the extremely low level (10 U/ml). Rollet-Labelle et al. [27] observed that SOD did not affect neutrophil apoptosis, however, the neutrophils were incubated for 24 h that is enough for complete spontaneous apoptosis *in vitro* and the hydrogen peroxide could be consumed by several enzymes (peroxidase and catalase). We demonstrated that spontaneous and hydrogen peroxide-induced apoptosis of neutrophils were augmented in excess of exogenous SOD. It is noteworthy that neutrophil apoptosis is accelerated in DS patients, in whom the CuZnSOD gene is overexpressed. The CuZnSOD gene is located on chromosome 21, and the total SOD activities in neutrophils and serum of DS subjects were significantly higher than controls. The present findings are in accordance with observations that DS subjects have increased susceptibility to severe neutropenia after

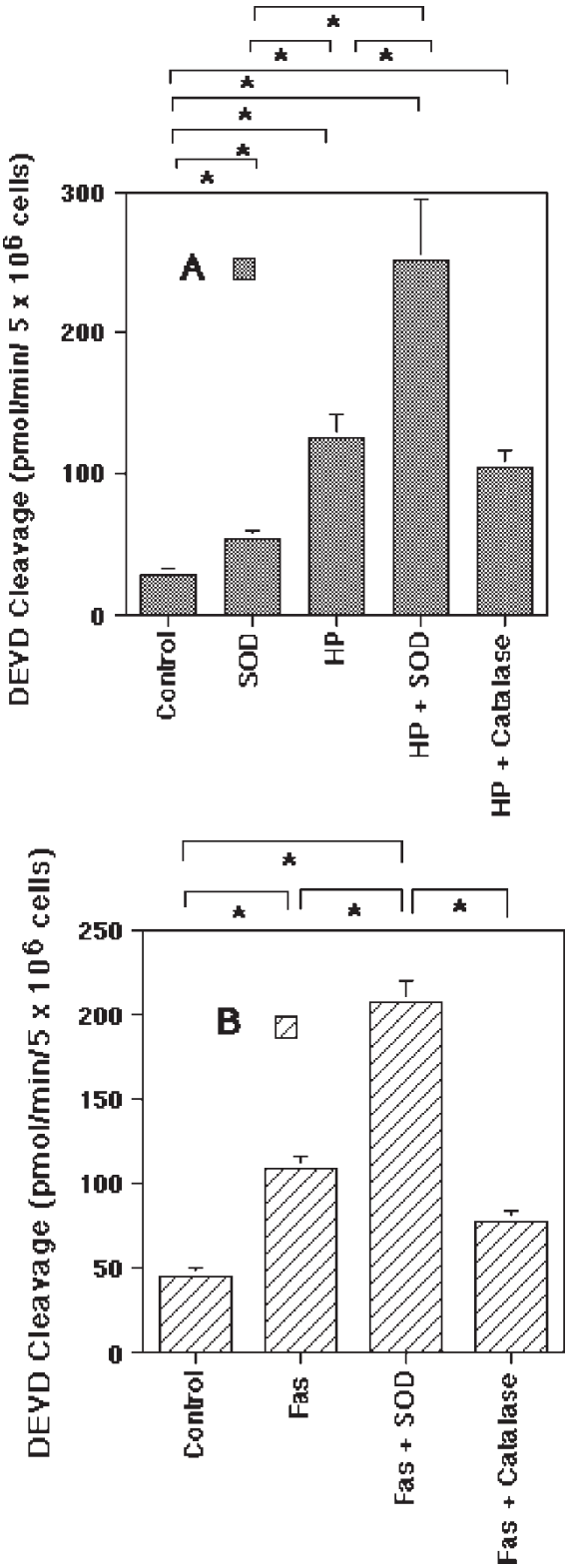

Figure 5. Activity of caspase-3 in neutrophils undergoing without (A) and with anti-Fas Ab (100 ng/ml CH11) treatment (B). The neutrophils were incubated for 2 h for the assay of enzyme activity with hydrogen peroxide (HP; 0.1 mM), SOD (100 U/ml) or catalase (100 U/ml) ($n = 3$). The cells were incubated for 4 h in the experiments with anti-Fas Ab ($n = 3$). Data are presented as means ± SEM. Significant differences are asterisked (by ANOVA). *$p < 0.05$ compared with the other three groups.

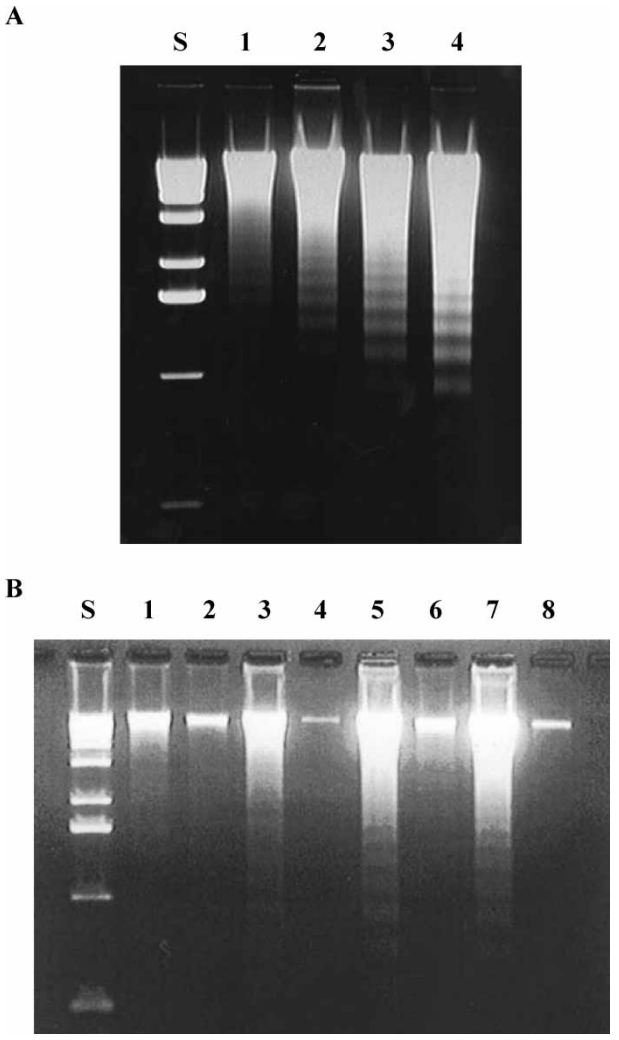

Figure 6. (A) Agarose gel electrophoresis of DNA extracted from neutrophils incubated for 2 h (lane 1) without or (lane 2–4) with hydrogen peroxide (HP; 0.1 mM) and (lane 2) SOD (100 U/ml) or (lane 4) catalase (100 U/ml). Left lane (S) shows molecular weight standards. (B) Agarose gel electrophoresis of DNA extracted from neutrophils after culture for 2 h under various conditions: (lane S) molecular weight standards, (lane 1) with no addition, (lane 2) with catalase (100 U/ml), (lane 3) with SOD (100 U/ml), (lane 4,6) with ZVAD-cmk (10^6 M), (lanes 5–7) with anti-Fas Ab (100 ng/ml CH11), (lane 7) with SOD, (lane 8) freshly isolated neutrophils as controls. Representative data are shown.

the anti-cancer chemotherapy [30,31]. We concluded that SOD may have a therapeutic potential for the sedation of neutrophil-mediated inflammation via neutrophil apoptosis.

Table I. Superoxide dismutase (SOD) in the serum and neutrophils.

	Sample	
Subjects	Serum SOD (ng/ml)	Neutrophils (ng/10^6 cells)
Controls ($n = 4$)	79.5 ± 12.2	11.8 ± 1.5
Down's syndrome ($n = 4$)	112.3 ± 7.9 ($p = 0.0041$)	16.0 ± 1.0 ($p = 0.0034$)

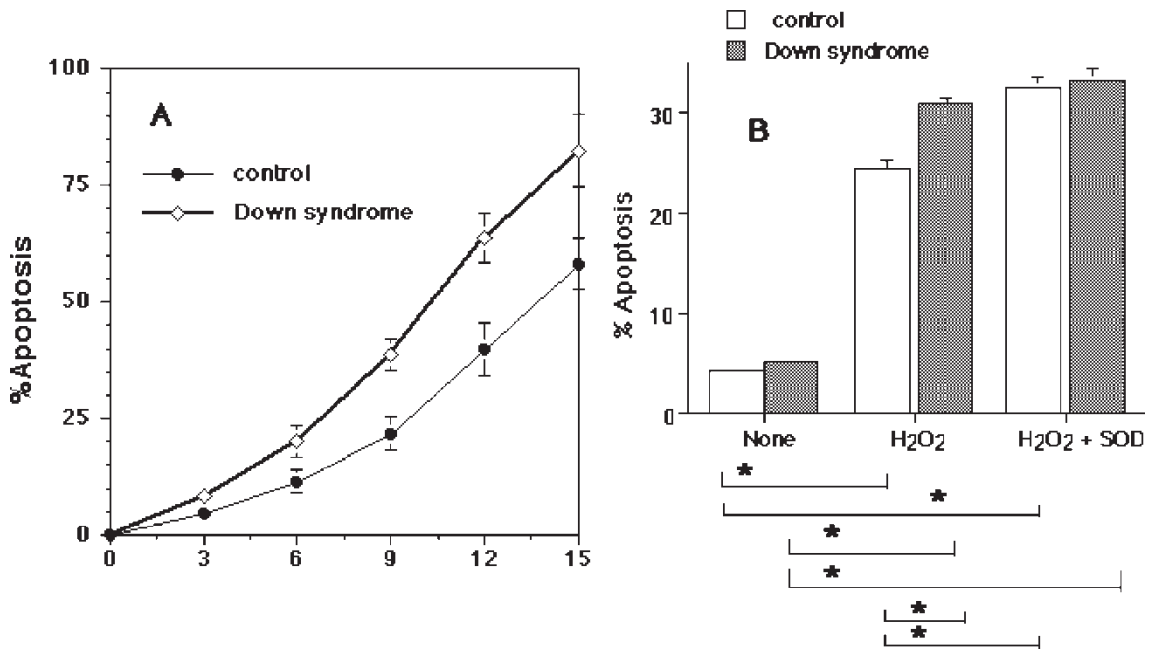

Figure 7. (A) Spontaneous apoptosis of neutrophils in DS subjects ($n = 3$) and in controls ($n = 3$). Percentages of apoptotic neutrophils are shown. Data are presented as means ± SEM. (B) Effects of exogenous hydrogen peroxide and SOD on neutrophil apoptosis. Exogenous SOD (100 U/ml) was added to cell suspensions immediately after exposure of neutrophils to 100 μM hydrogen peroxide, and incubated for 2 h. Neutrophils were from DS subjects ($n = 4$) and from controls ($n = 4$). Data are presented as means ± SEM. Significant differences are asterisked (by ANOVA).

SOD from epithelial cells would eliminate potentially toxic neutrophils or microbacteria-infected cells in a controlled manner via generation of hydrogen peroxide and induction of neutrophil apoptosis, thereby contribute to resolution of inflammation. On the contrary, during the acute phases of microbial invasion, bactericidal ability needs to be preserved and the apoptotic process should be retarded. Human macrophages generate GM-CSF and/or express high levels of catalase activity [32], which may protect apoptotic signals in neutrophils and then exhibit profound neutrophil-mediated defence mechanism.

Neutrophils are short-lived leukocytes that need to be removed safely by apoptosis. Our observations suggested that both of ROS- and Fas-mediated apoptosis of human neutrophils may occur in caspase-3-dependent manner, and that hydrogen peroxide is a possible major mediator of ROS-mediated neutrophil apoptosis augmented by extracellular SOD. The generation of superoxide anions possibly may not be a direct mediator of neutrophil apoptosis. It is possible that apoptosis could be processed not only by microbial invasion and primary bactericidal action but also by enzymal modification, involving SOD activation. Neutrophil apoptosis represents a crucial step in the mechanism governing the resolution of inflammation and has been suggested as a possible target for the control of neutrophil-mediated tissue injury. SOD may be a potential inhibitory mediator of neutrophil-mediated inflammation. Although further studies are needed to elucidate the mechanisms of this pathway and the pathology of various inflammatory conditions, the SOD may represent a novel therapeutic approach for the ROS-dependent tissue damage induced by neutrophils by several mechanisms.

Acknowledgements

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