Evaluation of the expression of NADPH oxidase components during maturation of HL-60 clone 15 cells to eosinophilic lineage

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Abstract. Objective: Superoxide-generating NADPH oxidase consists of the membrane-bound cytochrome b558 (gp91phox and p22phox) and the cytosolic components (p67phox, p47phox, p40phox and rac). In this study, we evaluated the superoxide-generating activity and the expression of NADPH oxidase components during eosinophilic maturation using HL-60 clone 15 cell line.

Materials and Methods: HL-60 clone 15 cells were matured to eosinophils by incubation with 0.5 mM butyrate for 7 days, and NADPH oxidase components were detected by Northern blot, Western blot analyses and immunocytochemical staining. Moreover, superoxide-generating activity was examined by nitro blue tetrazolium (NBT) assay.

Results: Northern blot and Western blot analyses revealed that mRNAs and proteins for gp91phox, p67phox and p47phox were expressed after eosinophilic myelocyte stages, whereas mRNAs and proteins for p40phox and rac-2 were expressed from the promyelocyte stage. Interestingly, p22phox mRNA was expressed from the promyelocyte stage, but its protein was expressed after eosinophilic myelocyte stages. Consistent with the results of Western blotting, immunocytochemical staining of butyrate-induced HL-60 clone 15 cells indicated that gp91phox, p22phox, p67phox and p47phox were detected after eosinophilic myelocyte stages (eosinophilic myelocytes, eosinophilic metamyelocytes, eosinophilic band cells and eosinophilic-segmented cells), whereas p40phox and rac-2 were expressed from the promyelocyte stage. Moreover, almost the same results as those with butyrate-treated HL-60 clone 15 cells were obtained using human bone marrow cells by immunocytochemical staining. Furthermore, nitro blue tetrazolium (NBT) assay indicated that superoxide could be produced after eosinophilic myelocyte stages but not produced before the promyelocyte stage.

Conclusions: Together these observations indicate that all the components for NADPH oxidase are expressed, and the superoxide-producing activity is obtained after myelocyte stages during eosinophilic maturation.

Key words: NADPH oxidase – Superoxide – Cytosolic factor – Eosinophil – HL-60

Introduction

Eosinophils play an important role in host defense against parasite infections, but have also been implicated in certain inflammatory and allergic diseases, such as asthma [1]. They are recruited to the sites of infection and inflammation, and activated in response to appropriate stimuli. During activation, eosinophils discharge an array of substances with tissue hyperreactivity and damage, including lipid mediators such as cysteinyl leukotrienes C4, D4, and E4, crystalloid granule proteins (major basic protein, eosinophil cationic protein, eosinophil-derived neurotoxin and eosinophil peroxidase), and products of superoxide anions (H2O2, hypochlorite, chloramines, and hydroxyl radicals) [1, 2]. As in neutrophils, the superoxide generation by eosinophils follows the activation and assembly of NADPH oxidase on the plasma membrane [3–7]. This enzyme is a multicomponent electron-transfer complex composed of the membrane-bound cytochrome b558 (gp91phox and p22phox) and the cytosolic components (p67phox, p47phox, p40phox and rac) [8, 9]. Upon activation, the cytosolic components translocate to the plasma membrane, where they associate with cytochrome b558, forming the active NADPH oxidase [8, 9]. It has been shown that eosinophils display a higher superoxide production upon stimulation as compared with neutrophils [4–7], and that the components of NADPH oxidase are expressed more abundantly in eosinophils than in neutrophils [6, 7].

The failure of phagocytes to produce superoxide is the functional basis for chronic granulomatous disease (CGD), an inherited disease characterized by recurrent and severe
infections, and granulomatous tissue reactions [10, 11]. Eosinophils from CGD patients are reported to be deficient in gp91phox, p22phox, p67phox, p47phox, and rac subunits of NADPH oxidase and unable to produce superoxide anion, similar to neutrophils from the same patients [6].

Recently, we have revealed that during neutrophil maturation the components for NADPH oxidase (gp91phox, p22phox, p67phox, p47phox, p40phox, and rac) are expressed and the superoxide-producing activity is obtained after myelocyte stages [12]. However, little information has been available concerning expression of NADPH oxidase components during eosinophil maturation. Lately, we have shown that superoxide-generating activity is obtained, and cytochrome b$_{55}$, p67phox, and p47phox are expressed during eosinophilic differentiation of butyrate-treated human promyelocytic leukemia HL-60 cells that had been cultured under alkaline condition [13]. However, it is not clear at which stage NADPH oxidase activity is acquired during maturation of eosinophil precursors. In this study, to clarify the stage(s) of respiratory-burst enzyme expression during eosinophilic maturation, we examined the superoxide-generating activity and the expression of NADPH oxidase components using HL-60 clone 15 cell line that could be matured to eosinophilic lineage cells by sodium butyrate treatment.

**Materials and methods**

**Reagents**

Block Ace was obtained from Dainippon Pharmaceutical Co., Ltd., Tokyo, Japan. 4β-Phorbol 12-myristate 13-acetate (PMA), sodium butyrate, nitro blue tetrazolium (NBT) and L-glutamine were purchased from Sigma Chemical Co. (St. Louis, MO). Diisopropyl fluorophosphate (DFP) was purchased from Wako Pure Chemicals, Osaka, Japan.

**Animals**

New Zealand White rabbits weighing approximately 2000–2400 g were purchased from Sankylo Labo Service Corporation Inc., Tokyo, Japan, and used for the preparation of polyclonal antibodies. In accordance with the institutional guideline, rabbits received proper care and maintenance, and did not suffer unnecessary discomfort.

**Antibodies**

Rabbit anti-gp91phox and anti-p22phox polyclonal antibodies were prepared using synthetic peptides (corresponding to CISNSESGPRGVHFIFNKENF and CAGGPPGPQVNPIPVTDEVV, respectively) as described previously [14]. Mouse anti-gp91phox (7D5) monoclonal antibody was kindly provided by Dr. Michio Nakamura (Nagasaki University, Rochester, MN) [17]. Rabbit anti-gp91phox and anti-p22phox polyclonal antibodies were prepared using synthetic peptides (corresponding to CISNSESGPRGVHFIFNKENF and CAGGPPGPQVNPIPVTDEVV, respectively) as described previously [14]. Mouse anti-p40phox (PC10) monoclonal antibody was obtained from American Type Culture Collection (ATCC CRL-1964, Manassas, VA) [18]. The mouse anti-p40phox monoclonal antibody (7D5) was also obtained from American Type Culture Collection (ATCC CRL-1964, Manassas, VA) [18]. Rabbit anti-p40phox polyclonal antibody was raised in rabbits, and used for the preparation of polyclonal antibodies. In accordance with the institutional guideline, rabbits received proper care and maintenance, and did not suffer unnecessary discomfort.

**Preparation of cells**

After induction with butyrate, HL-60 clone 15 cells were collected on days 0, 1, 3, 5, and 7, and washed with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na$_2$HPO$_4$, 1.5 mM K$_2$HPO$_4$, pH 7.4). For Western blot analysis, the cells were treated with 5 mM DFP for 30 min at 4°C and washed in PBS. The cells were finally suspended in PBS at 10$^6$/ml and stored at −80°C. Human bone marrow cells were obtained by aspiration from the superior iliac crest of healthy volunteers. Each volunteer was fully informed of the procedures and gave written consent prior to taking part in the study, which was conducted according to the Declaration of Helsinki on biomedical research involving human subjects (Tokyo amendment). Bone marrow cells were immediately anticoagulated by an equal volume of heparin and diluted in an equal volume of PBS. After sedimentation of erythrocytes in 1% dextran and hypotonic lysis of residual erythrocytes, leukocyte-rich cells were obtained.

Cytocentrifuge preparations were made using Cytospin 2 (10$^4$ cells/slide; 340 rpm, 5 min; Shandon Instruments, Pittsburg, PA), stained with May/Grünwald/Giemsa or Discombe [20], and examined by light microscopy. Each morphological subtype of eosinophilic lineage cells was identified based on the conventional criteria (cytoplasmic granules, cell size, ratio of nucleus to cytoplasm, and characteristics of nuclear chromatin) [21, 22], and divided into four distinct stages (classes I, II, III, and IV). Myelocytes (class I cells) are characterized by a high nuclear to cytoplasmic ratio with spherical euchromatic nuclei. The cytoplasm of these cells has an intense basophilia and an absence of primary (azurophilic) and secondary (specific) granules. Promyelocytes and early myelocytes (class II cells) have a decreased nuclear to cytoplasmic ratio. The cytoplasm still retains a high degree of basophilia and frequently contains primary granules. Metamyelocytes (class III cells) are characterized by kidney-shaped nuclei. The cytoplasm is only slightly basophilic with evidence of both primary and secondary granules. Terminally differentiated eosinophilic band cells and segmented cells (class IV cells) are characterized by heterochromatic and lobed or ring-shaped nuclei. The cytoplasm of this cell type loses its basophilic staining properties, retaining only the coloration of the numerous and strongly eosin-staining secondary granules. In addition, MBP was used as a marker for the maturation of eosinophilic lineage, as described below.

**Assay of NADPH oxidase activity**

During maturation, acquisition of O$_2^·$-generating activity was determined by NBT assay, which detected the reduction of NBT to formazan by superoxide oxide. The assay was performed by incubating cells (1×10$^6$ cells/ml, 200 μl) with 0.04% NBT in PBS containing 1 mM CaCl$_2$ and 1 mM MgCl$_2$ in the presence or absence of 500 ng/ml PMA at 37°C for 30 min in a Lab-Tak chamber slide (Nalgene-Nunc International, Naperville, IL), and then formazan-containing cells were monitored, as described previously [12].