The phagocyte NADPH oxidase and bacterial infections

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ABSTRACT Neutrophils play a crucial role in host defense against microbial infections. During phagocytosis of invading bacteria or fungi, the phagocyte NADPH oxidase produces superoxide. The importance of the oxidase is exemplified by the genetic disorder known as chronic granulomatous disease (CGD). The neutrophils of CGD patients cannot produce superoxide, with the result that affected infants and children suffer from severe recurrent bacterial and fungal infections. *Staphylococcus aureus* and *Aspergillus fumigatus* often cause life-threatening infections in these patients. The phagocyte NADPH oxidase is composed of the plasma membrane protein (cytochrome b_{558}) and cytosolic proteins. Cytochrome b_{558} is a heterodimer consisting of the gp91^{phox} and p22^{phox}. The cytosolic proteins are p47^{phox}, p67^{phox}, p40^{phox} and a small G-protein, Rac. The oxidase is also expressed in peripheral eosinophils, monocytes, B lymphocytes, and several cultured cell lines after differentiation other than neutrophils. Here, we will consider and discuss the oxidase in relation to CGD.

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Key words：**NADPH oxidase, Chronic granulomatous disease (CGD), Reactive oxygen species (ROS)**

A GENERAL VIEW OF BIODEFENSE

Skin, epithelia and mucosa that cover the human body, as well as respiratory and digestive organs are the primary shield against invading microorganisms. The surface cells are moving up and falling down according to their fate with incessant proliferation, which is the first defense to prevent infections[1)]. When pathogenic agents penetrate the surface barrier, neutrophils are in the front line in defense against these agents. It is generally known that the full of meshes by blood vessel does not overlook the invaders and facilitates a swift attack by the phagocytes[2)]. The most important function of neutrophils is containment of unwished visitors. The number of neutrophils is normally around 5,000 cells/μl. During bacterial infections, the number often rises to >10,000 cells. The rapid increase in neutrophils is considered to be one of the basic defense mechanism. When the number of neutrophils falls below 500 cells/μl, the risk of infectious diseases increases, as shown in patients with leukopenia, such as

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aplastic anemia, myelodysplastic syndrome and leukemia[3]. Monocytes/Macrophages reach areas of inflammation and cooperate with neutrophils in the fight against microorganisms. Antigen-presenting macrophages as well as B cells indicate the presence of invading organisms to T cells, via MHC-class-2-containing peptides derived from the digested microorganisms in the cells. Antigen-specific T cells increase their own activities and also induce antigen-specific B cells to secrete antibodies[4]. Ligand recognition sites and Fc portions contained in antibodies form a strong connection between the microorganisms and phagocytes, that is to say, the binding capability of the phagocytes to the antigens increases. Similarly, Complement system works as opsonization. Thus, white blood cells and serum coordinately eliminate invading microorganisms.

BACTERIAL KILLING BY NEUTROPHILS

Neutrophils as professional phagocytes play a crucial role in host defense against microbial infections. During phagocytosis of invading microorganisms or upon cell stimulation with the bacterium-derived peptide, formyl-methionyl-leucyl-phenylalanine (fMLP), phagocytes produce superoxide which is a precursor of microbicidal reactive oxygen species (ROS) by catalysis of activated phagocyte NADPH oxidase[5]. ROS derived from superoxide are essential for microbial killing. The importance of the phagocyte NADPH oxidase is underlined by the genetic disorder known as chronic granulomatous disease (CGD). The neutrophils of CGD patients do not produce superoxide, with the result that affected infants and children suffer from severe recurrent bacterial and fungal infections[6]. CGD was first reported in 1957 and was considered a rare genetic disease[7]. However, nowadays, there is approximately 1 case in 200,000 children in the USA, Europe and Japan. Neutrophils of some patients produce a little superoxide, but the relation between the quantity produced and the symptoms remained unclear until these days. Neutrophils also have a large number of defensive peptides, such as cathepsin, lysozyme and defensin in their granules. The peptides perform bacteriolysis after fusion to phagosomes containing bacteria[1]. After phagocytosis, the invaders are broken into pieces by ROS and the defensive peptides, together with several proteases.

It is well known that defective function of neutrophils leads to infectious disease. Membrane-bound integrin-family is necessary for neutrophils to adhere to the vessel wall and migrate to the site of inflammation. CD18, one of the heterodimers comprising $\beta 2$ integrin, is defective in leukocyte adhesion deficiency (LAD)[8,9]. During a rolling in blood vessel, at first, neutrophils contact to the endothelial cells by way of sialic acid-dependent weak attachment. As a next step for extravasation, lymphocyte function associated antigen-1 (LFA-1; a heterodimer of CD18/CD11a), which is expressed by neutrophils, is necessary for strong adhesion to the vascular endothelial cells (Fig. 1). The counterpart of LFA-1 is intercellular adhesion molecule-1 (ICAM-1; CD54), which is expressed on the activated endothelial cells covering the inside of the blood vessel. Neutrophils of LAD patients do not undergo extravasation due to the lack of LFA-1. These neutrophils are also devoid of complement

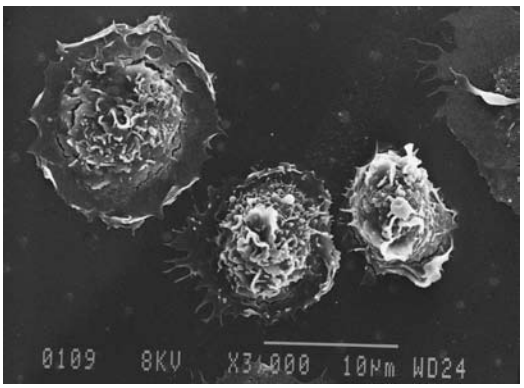

Fig. 1. Scanning electron micrograph of neutrophils is imaged at ×3,000 magnification. Scale bar represents 10 μm. Neutrophils are incubated at 37℃ for 30 min over slide glass.

receptor, CR3 constituted with CD18 and CD11b. Thus, the patients have defective activity of phagocytosis against opsonized bacteria in their neutrophils.

Both chemotaxis and bacterial killing activity by neutrophils are also disturbed in case of Chediak–Higashi syndrome, with sustained life-threatening infections caused by dysfunctions in lysosome-related organelle biogenesis. In other words, the defensive importance of neutrophils is highlighted by these hereditary diseases[10].

REACTIVE OXYGEN SPECIES (ROS)

Molecular oxygen is transported by red blood cells for respiration in daily life. The ultimate objective of respiration is to produce energy by aerobic metabolism within cells (Fig. 2a). Oxygen is also used by white blood cells as a defense purpose, which is the main theme of this article. Superoxide (O_2^- : superoxide anion) produced by the phagocyte NADPH oxidase is reduced by dismutation itself or catalyzed by superoxide dismutase (SOD) (Fig. 2b). Catalase catalyzes toxic hydrogen peroxide (H_2O_2) to water and oxygen (Fig. 2c). Thus, non-catalase producing bacteria such as *Streptococcus pyogenes* are not likely to become major infectious agents in CGD because they are killed by their own ROS in phagosomes. However, catalase-producing bacteria such as *Staphylococcus aureus* and the fungus *Aspergillus fumigatus* cause life-threatening infections in CGD patients[6].

a

$$C_6H_{12}O_6 + 6O_2 + 6H_2O \rightarrow 6CO_2 + 12H_2O$$

b

$$2H^+ + 2O_2^- \rightarrow H_2O_2 + O_2 \qquad (+/- \text{ SOD})$$

c

$$2H_2O_2 \rightarrow 2H_2O + O_2 \qquad (\text{Catalase})$$

d

$$H_2O_2 + Cl^- \rightarrow H_2O + OCl^- \qquad (\text{MPO})$$

Fig. 2. (a) Molecular oxygen is used for respiration in our daily life to extract energy. (b) Superoxide (O_2^-) is converted to hydrogen peroxide (H_2O_2) with or without enzyme, SOD. (c) Hydrogen peroxide is broken down by catalase into water and oxygen. (d) Hydrogen peroxide is converted to hypochlorous acid (OCl^-) in the phagosomes.

When we observe molecular oxygen structurally, it requires electrons to become a stable structure inevitably. It is a fate of oxygen to remove electrons from other atoms. Namely, oxygen facilitates oxidation for a partner of a chemical compound. Molecular oxygen is converted to water after reduction by four electrons. Thus, water is stable and may also be called inactive oxygen. Hydrogen peroxide is produced from superoxide, and hydroxy radical (·OH) is produced from hydrogen peroxide together with a reduced form of iron or superoxide. Superoxide itself is not thought to be too toxic for bacteria, but these derivatives act as strong disinfectants.

THE PHAGOCYTE NADPH OXIDASE

It was well known that neutrophils consume oxygen abruptly just after phagocytosis of bacteria[11]. NADPH but not NADH is oxidized to transfer electrons from inside to outside of the plasma membrane at the same time with the consumption of oxygen[12]. NADH is not used by the oxidase at the physiological concentration in the cytosol because of the low affinity with the oxidase. NADPH is produced by way of pentose phosphate pathway from glucose, and historically, the oxidase was thought to be a simple enzyme that donated electrons to molecular oxygen. This enzyme is now believed to be a complex constituted by six proteins[13]. The consumption of oxygen accompanied with phagocytosis is not disturbed by cyanide, which disrupts the electron transport chain in mitochondria. It was found in the 1960s that stimulation of neutrophils leads to production of hydrogen peroxide and, in the 1970s, that superoxide is produced by

the same stimulation. The acidic conditions in the phagosomes with a large number of protons are ideal for superoxide to convert to hydrogen peroxide (Fig. 2b). Furthermore, myeloperoxidase (MPO) released from azurophilic granules after the fusion with phagosomes changes hydrogen peroxide to hypochlorous acid (HClO) (Fig. 2d). It is noteworthy that we are using hydrogen peroxide and hypochlorous acid as disinfectants on a daily basis and that our neutrophils are also utilizing the same agents in our body. MPO is present in large quantities and occupies >3% of all protein in neutrophils. Therefore, hypochlorous acid is produced more than hydroxy radicals in phagosomes under physiological conditions.

The phagocyte NADPH oxidase is composed of a membrane-spanning glycoprotein, cytochrome b_{558} and cytosolic proteins, p47^{phox}, p67^{phox}, p40^{phox} (*phox*: phagocyte oxidase), and a small G-protein, Rac[13,14]. Cytochrome b_{558} is a heterodimeric protein composed of gp91^{phox} (the 91-kDa glycoprotein) and p22^{phox}. Superoxide is produced by the active phagocyte NADPH oxidase of peripheral neutrophils, eosinophils, and monocytes[15]. In the 1990s, we found the oxidase in peripheral B lymphocytes and some B cell lines[16,17]. However, the exact function in B cells remains to be established. Obvious disorders of acquired immunity in CGD have not been clarified as yet, although hyper- γ globulinemia is sometimes present in the disease. Activation of the oxidase is strictly restricted because of its toxicity for humans as well as bacteria. When the phagocytes are stimulated, cytochrome b_{558} forms a complex with cytosolic proteins and is activated to produce superoxide (Fig. 3). p67^{phox} directly binds to p47^{phox} between the C-terminal src homology 3 (SH3) domain and the proline-rich region (PRR), respectively. p67^{phox} also directly binds to p40^{phox} via interaction between these PB1 domains. In the resting state, two SH3

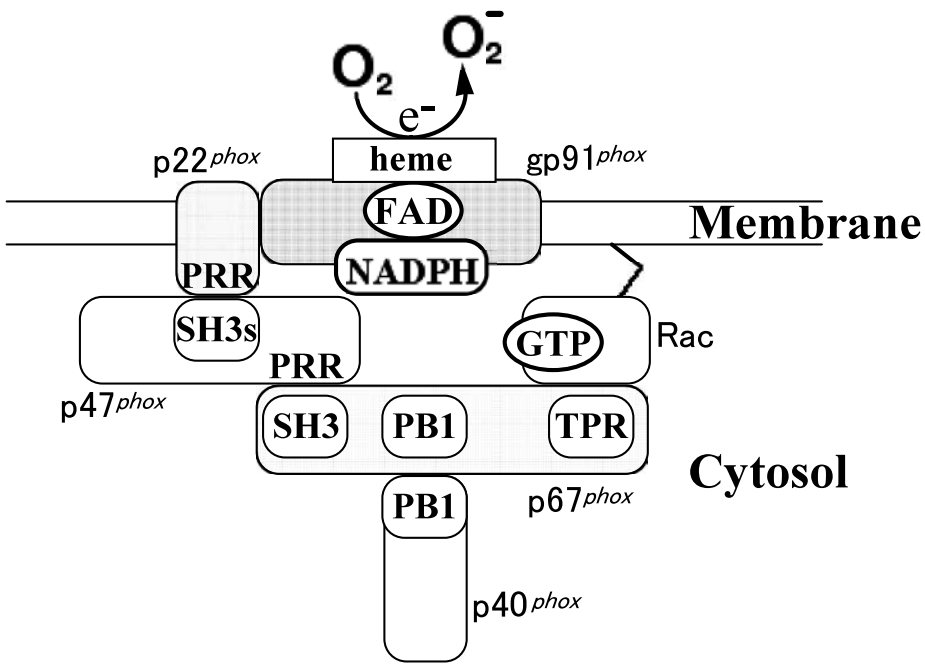

Fig. 3. Protein-protein interactions of the active phagocyte NADPH oxidase. p67phox contains a domain containing four units of TPR (tetratricopeptide repeat), PB1 domain (Phox and Bem1 domain) and SH3 domain. p47phox contains two SH3 domains (src homology 3 domain) in the center and the C-terminal PRR (proline-rich region). p40phox harbors PB1 domain.

domains in p47^{phox} are masked by itself and don't bind to PRR in p22^{phox}. When the cells are stimulated, p47^{phox} binds to PRR, which means translocation of cytosolic proteins to the plasma membrane. GTP-bound active Rac binds to TPR of p67^{phox} and anchors to plasma membrane with lipid attached to CAAX box in the C-terminus. The gene encoding gp91^{phox} (CYBB) alone is located on the X-chromosomes, and thus most of the patients are boys[6]. In the case of gp91^{phox} deficiency, expression level of p22^{phox} is also diminished, and the gp91^{phox} protein is hardly detected when the cause of CGD is loss of p22^{phox}. Gp91^{phox} harbors binding sites of heme, NADPH and FAD (Fig. 3)[18]. Thus, cytochrome b_{558} is thought to possess the core of the electron transport system. Indeed, the purified cytochrome b_{558} is sufficient for the production of superoxide when the protein is surrounded by certain lipid conditions[19].

DETECTION OF SUPEROXIDE AND DIAGNOSIS

Superoxide produced by neutrophils is detected by cytochrome *c* reduction (Fig. 4), nitro blue tetrazolium reduction, or calculated from cyanide-insensitive oxygen consumption[5, 11-13]. With the development of probes and machines to detect ROS by chemiluminescence (CL), this method has been used widely[13, 15]. CL with Lucigenin, one of the most sensitive probes to detect superoxide, is also dependent on the extent of adhesion by neutrophils, and therefore, reflects activities for both the phagocyte NADPH oxidase and cell adhesion[20]. Thus, Lucigenin-enhanced CL using a mixture of adhering and non-adhering cells in a conventional tube is adopted for screening both CGD and LAD. Either Luminol or Diogenes, other CL probes, can be used to avoid the effect by cell adhesion. A mix of the CL probes, Luminol and Diogenes, detects ROS more quantitatively and with higher sensitive than each probe alone, as well as Lucigenin[20].

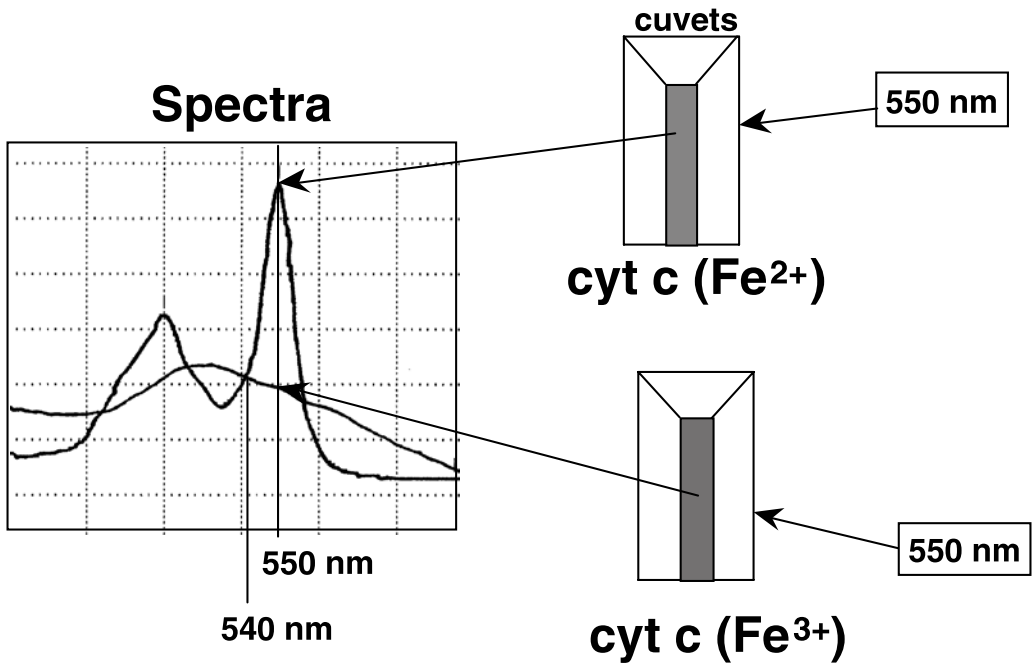

Fig. 4. Oxidized cytochrome *c* (Fe^{3+}) does not show absorbance at 550 nm. However, reduced form (Fe^{2+}) after receiving electron from superoxide absorbs light at the wave-length. Note that 540 nm is a specific wavelength where both reduced and oxidized cytochrome *c* indicate the same absorbance.

Recently, the fluorescent probe dihydrorhodamine 123 (DHR) has been widely used in flow-cytometry to quantify ROS produced by phagocytes. Superoxide produced outside the plasma membrane by the phagocyte NADPH oxidase is changed to hydrogen peroxide which in turn, passes through the membrane into the cytosol and oxidizes DHR. Oxidized DHR emits fluorescence around 525 nm by absorbing the energy of excitation at 488 nm[3, 21].

NEW INSIGHTS

Basic progress:

The transcriptional regulation of gp91^{phox} has been characterized in the past 15 years after it was discovered that CGD has a mutation in the promoter region of the gene. PU.1 is a key transcription factor for the expression of the gp91^{phox} gene in neutrophils but not in eosinophils[15, 22].

In 2011, we clarified epigenetic regulation of gp91^{phox} by GCN5, one of the most important histone acetyltransferase. GCN5-deficiency causes loss of activity to produce superoxide by repressing transcription of the gp91^{phox} gene. Chromatin immunoprecipitation assay reveals that both the association of GCN5 with the gp91^{phox} gene promoter and elevated acetylation levels of histones (H2B, Lys16 and H3, Lys9) around the promoter are important for the expression of gp91^{phox} [23].

Symptoms of CGD: Although CGD patients are susceptible to infections, the extent of the damage is diverse. Some patients do not have severe symptoms, and thus, a longer time is required for a definite diagnosis can be made. We have been thinking that patients with modest residual production of ROS in neutrophils have significantly less severe illness than those without any such production. In 2011, a study of 287 patients showed clearly that higher residual production of ROS is a predictor of longer survival[21]. The method for detection of ROS was based on DHR.

Patients with Mendelian susceptibility to mycobacterial diseases (MSMD), who are vulnerable to weakly virulent mycobacterial species as well as *Mycobacterium tuberculosis*, have a mutation in either of six genes (IFNGR1, IFNGR2, STAT1, IL12B, IL12BR1, and IKBKG), which results in impaired IL-12/23-IFN- γ -mediated signals[24]. However, the location of this genetic disorder has not been found in half of the MSMD patients. In 2011, it was reported that two relatives developed X-linked recessive MSMD, and each had a mutation leading to amino acid substitution (Thr178Phe or Gln231Phe) in gp91^{phox}, which has not previously been found in CGD patients. MSMD patients suffer from recurrent tuberculous mycobacterial disease and have impaired oxidase activity in monocyte-derived macrophages but not in monocytes or neutrophils[25].

Treatment for CGD: Bone marrow transplantation (BMT) still seems to be difficult for CGD patients because of the distinguish characteristics of the diseases, susceptible to infections. However, the conditioning regimens, including pretreatment according to the donor, certainly increase the success rate for BMT. BMT with infectious conditions found in several lymph nodes has been sometimes carried in CGD patients[26, 27]. Adenosine deaminase deficiency, X-linked severe combined immunodeficiency, Wiskott-Aldrich syndrome and CGD are the main targets for gene therapy with retroviral or lentiviral-vectors. Pretreatment with busulfan seems to be effective though myelodysplastic syndrome may be caused by some of the vectors[28]. It is still difficult to establish long-term effectiveness because of gene silencing of the introduced gp91^{phox}. This method seems to give us an assignment as to an improvement toward a prolonged and stable oxidase expression in neutrophils in patients with CGD. A transient recovery from infections, however, is hopefully possible.

Thalidomide was used as a sedative in West

Germany in 1957 and Japan in 1958, and malformations were reported in 1959. In the mid-1960s, its validity for multiple myeloma was confirmed, and the drug has been used in Japan since 2008 with sever restrictions. Thalidomide is thought to be suppressive of chronic inflammation, therefore, its future application for CGD might be possible[29. 30)].

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