

Expression of gp91phox and p22phox, catalytic subunits of NADPH oxidase, on microglia in Nasu-Hakola disease brains

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Summary

The superoxide-producing nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex of phagocytes (phox) plays a key role in production of reactive oxygen species (ROS) by microglia. The catalytic subunits of the NADPH oxidase are composed of p22phox and gp91phox. Nasu-Hakola disease (NHD) is a rare autosomal recessive disorder caused by a loss-of-function mutation of either *TYROBP (DAP12)* or *TREM2*. Pathologically, the brains of NHD patients exhibit extensive demyelination designated leukoencephalopathy, astrogliosis, accumulation of axonal spheroids, and remarkable activation of microglia predominantly in the white matter of frontal and temporal lobes. However, a pathological role of the gp91phox-p22phox complex in generation of leukoencephalopathy in NHD remains unknown. We clarified the expression of gp91phox and p22phox in the white matter of the frontal cortex derived from five NHD and eight control subjects. We identified the expression of p22phox and gp91phox immunoreactivity almost exclusively on microglia. Microglia overexpressed gp91phox in NHD brains and p22phox in myotonic dystrophy (MD) brains, when compared with non-neurological control (NC) brains. These results suggest that the enhanced expression of gp91phox by microglia might contribute to overproduction of ROS highly toxic to myelinating oligodendrocytes, resulting in oligodendrocyte cell death that induces leukoencephalopathy in NHD brains.

Keywords: gp91phox, leukoencephalopathy, microglia, Nasu-Hakola disease, p22phox

1. Introduction

The superoxide-producing nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) complex of phagocytes (phox) plays a key role in the elimination of invading pathogens by phagocytic cells (1). The core catalytic enzymes called flavocytochrome b558 are composed of a membrane-associated heterodimeric complex, consisting of p22phox (α subunit) and gp91phox (Nox2, β subunit) (2). Upon

stimulation of phagocytes, regulatory subunits, including p40phox, p47phox, p67phox, and Rac1/2, are activated by posttranslational modification, conformational change, and protein-protein interaction, and translocates from the cytosol to the membrane, resulting in activation of the gp91phox-p22phox complex (2). Following the assembly of the oxidase complex, the flavocytochrome b558 transports electrons from intracellular NADPH to extracellular or phagosomal oxygen, leading to production of superoxide anion. Superoxide anion is short lived and reacts with various molecules to form reactive oxygen species (ROS), such as hydrogen peroxide, hydroxyl radicals, and hypochlorous acid, and peroxynitrite (1). An excessive amount of ROS causes oxidative stress and cell death by inducing oxidative modification of lipids, proteins, and nucleic acids, whereas a low level of ROS play a key role in redox-dependent intracellular

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signaling that amplifies the proinflammatory response (3,4).

Microglia, the resident myeloid cells in the central nervous system (CNS), have a capacity to constantly scavenge invading pathogens, apoptotic debris, unwanted synapses, and aggregated proteins by sensing them with a panel of pattern recognition receptors (PRRs) (5). Microglia serve as a major source of ROS through the expression of the phagocytic Nox in the lesions of cerebral ischemia, traumatic brain injury, multiple sclerosis (MS), and Alzheimer's disease (6-9). In these diseases, the microglial gp91phox-p22phox complex is promptly activated in response to neurotoxic stimuli, followed by ROS-mediated neuronal damage (3).

Nasu-Hakola disease (NHD), also designated polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOS; OMIM 221770), is a rare autosomal recessive disorder, characterized by progressive presenile dementia and formation of multifocal bone cysts, caused by genetic mutations of either *TYROBP(DAP12)* or *TREM2* (10). *TREM2* and *DAP12* constitute a receptor/adaptor signaling complex expressed exclusively on osteoclasts, dendritic cells, macrophages, and microglia. Although NHD patients are clustered in Japan and Finland, approximately 200 NHD cases are presently reported worldwide. Clinically, the patients with NHD show recurrent bone fractures during the third decade of life, and a frontal lobe syndrome during the fourth decade of life, and progressive dementia and death until the fifth decade of life (11). Pathologically, the brains of NHD patients exhibit extensive demyelination designated leukoencephalopathy, astrogliosis, accumulation of axonal spheroids, and remarkable activation of microglia predominantly in the white matter of frontal and temporal lobes and the basal ganglia (12). However, a pathological role of the gp91phox-p22phox complex with relevance to leukoencephalopathy in NHD remains largely unknown. In the present study, we have attempted to clarify the expression of gp91phox and p22phox in the white matter lesions of NHD brains by immunohistochemistry.

2. Materials and Methods

2.1. Human brain tissues

The brain autopsies were performed at the National Center Hospital, National Center of Neurology and Psychiatry (NCNP), Japan, Kohnodai Hospital, National Center for Global Health and Medicine (NCGM), Japan, and affiliated hospitals of Research Resource Network (RRN), Japan. The comprehensive examination by established neuropathologists (YS and TI) validated the pathological diagnosis. In all cases, written informed consent was obtained. The Ethics Committee of the NCNP for the Human Brain

Research, the Ethics Committee of the NCGM on the Research Use of Human Samples, and the Human Research Ethics Committee (HREC) of the Meiji Pharmaceutical University (MPU) approved the present study.

For immunohistochemical studies, serial sections containing the white matter of the frontal cortex were prepared from four subjects who died of non-neurological causes (NC), composed of a 63-year-old man who died of prostate cancer and acute myocardial infarction (NC1), a 67-year-old man who died of dissecting aortic aneurysm (NC2), a 57-year-old man who died of alcoholic liver cirrhosis (NC3), and a 61-year-old man who died of rheumatoid arthritis with interstitial pneumonia (NC4), four neuropsychiatric disease controls affected with myotonic dystrophy (MD), composed of a 68-year-old man (MD1), a 61-year-old man (MD2), a 60-year-old man (MD3), and a 53-year-old woman (MD4), and five NHD patients, composed of a 42-year-old man (NHD1), a 48-year-old woman (NHD2), a 44-year-old man (NHD3), a 32-year-old woman (NHD4), and a 38-year-old man (NHD5). The homozygous mutation of a single base deletion of 141G (141delG) in exon 3 of *DAP12* was identified in NHD1, NHD2, and NHD5, while the genetic analysis was not performed in NHD3 or NHD4, as described previously (13).

2.2. Immunohistochemistry

After deparaffination, tissue sections were heated in 10 mM citrate sodium buffer, pH 6.0 by autoclave at 110°C for 15 min in a temperature-controlled pressure chamber (Biocare Medical, Concord, CA, USA). They were treated at room temperature (RT) for 15 min with 3% hydrogen peroxide-containing methanol to block the endogenous peroxidase activity. They were then incubated with phosphate-buffered saline (PBS) containing 10% normal goat serum at RT for 15 min to block non-specific staining, followed by incubation in a moist chamber at 4°C overnight with rabbit polyclonal anti-p22phox antibody (sc-20781, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or with mouse monoclonal anti-gp91phox antibody (ab139371, Abcam, Cambridge, UK). After washing with PBS, tissue sections were incubated at RT for 30 min with horseradish peroxidase (HRP)-conjugated secondary antibodies (Nichirei, Tokyo, Japan), followed by incubation with diaminobenzidine tetrahydrochloride (DAB) substrate (Vector, Burlingame, CA, USA). They were processed for a counterstain with hematoxylin. Negative controls underwent all the steps except for exposure to primary antibody. In limited experiments, double immunolabeling of mouse anti-gp91phox antibody and rabbit antibodies against p22phox, Iba1 (Wako Pure Chemical, Tokyo, Japan; a marker of microglia/macrophages), GFAP (Dako, Tokyo, Japan;

a marker of astrocytes), or NeuN (Abcam; a marker of neurons), was performed, followed by incubation with HRP-conjugated or alkaline phosphatase-conjugated anti-mouse or anti-rabbit secondary antibody and exposure to DAB substrate and Warp Red chromogen (Biocare Medical).

2.3. Quantification of gp91phox and p22phox immunoreactivity

To quantify immunolabeled areas, the images derived from three fields of the white matter were captured at a 200 X magnification on the Olympus BX51 universal microscope. They were then processed for quantification by using ImageJ software (National Institute of Health, Bethesda, MD, USA). The gp91phox- or p22phox-immunolabeled area was calibrated by the Iba1-immunolabeled area of the identical field. The difference in the average of immunopositive areas between NHD and the controls was evaluated statistically by one-way analysis of variance (ANOVA) followed by post-hoc Tukey's test.

3. Results and Discussion

By immunohistochemistry, we identified the intense expression of gp91phox and p22phox immunoreactivity chiefly on microglia/macrophages in the white matter of the frontal cortex derived from NC, MD and NHD subjects (Figure 1, panels a-d and Figure 2, panels a-d). By double immunolabeling, the expression pattern of gp91phox overlapped with that of p22phox (Figure 3, panel a) and Iba1-positive microglia expressed gp91phox (Figure 3, panel b), whereas myelinating oligodendrocytes, GFAP-positive astrocytes and NeuN-positive neurons did not express gp91phox (Figure 3, panels c, d), suggesting that oligodendrocytes, astrocytes and neurons do not express discernible

levels of the gp91phox-p22phox complex in the human brain. By quantitative analysis, the immunolabeled area of gp91phox-positive microglia was increased significantly in NHD brains, when compared with NC brains but not with MD brains (Figure 4, panel a; $p = 0.0338$). In contrast, the immunolabeled area of p22phox-positive microglia was not significantly different between NC and NHD brains, whereas it was increased significantly in MD brains, when compared with NC brains (Figure 4, panel b; $p = 0.0096$).

The enhanced expression of the gp91phox-p22phox complex plays a key role in microglial production of superoxide anion and ROS (3,7). We previously found accumulation of numerous Iba1-positive microglia in NHD brains and showed that mRNA levels of microglia/macrophage marker genes, such as CD68, CD163, and MSR1, were elevated in NHD brains (12,14). The present study indicated that microglia overexpressed gp91phox in NHD brains and p22phox in MD brains, when compared with NC

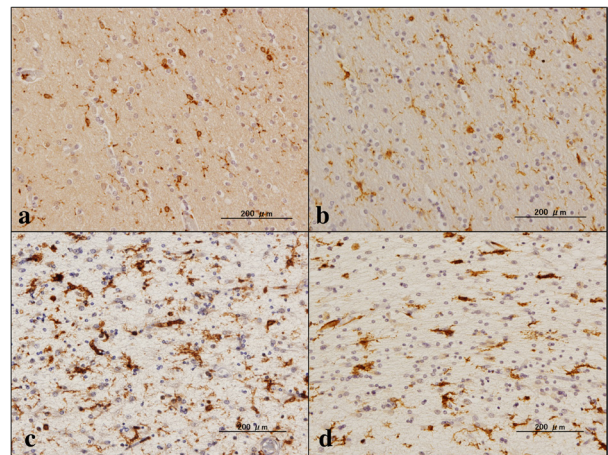


Figure 2. Expression of p22phox and Iba1. (a) NC, p22phox, (b) NC, the same area of (a), Iba1, (c) NHD, p22phox, and (d) NHD, the same area of (c), Iba1.

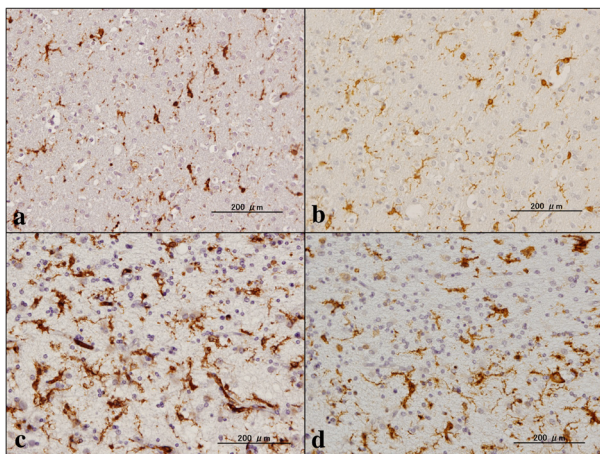


Figure 1. Expression of gp91phox and Iba1. (a) NC, gp91phox, (b) NC, the same area of (a), Iba1, (c) NHD, gp91phox, and (d) NHD, the same area of (c), Iba1.

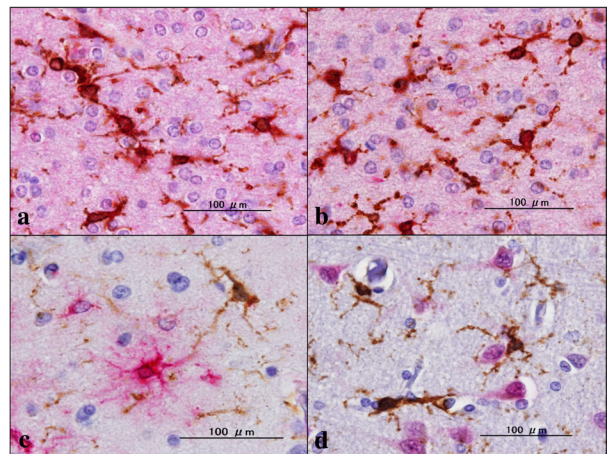


Figure 3. Double labeling of gp91phox and cell type-specific markers. (a) MD, gp91phox (brown), p22phox (red), (b) MD, gp91phox (brown), Iba1 (red), (c) MD, gp91phox (brown), GFAP (red), and (d) gp91phox (brown), NeuN (red).

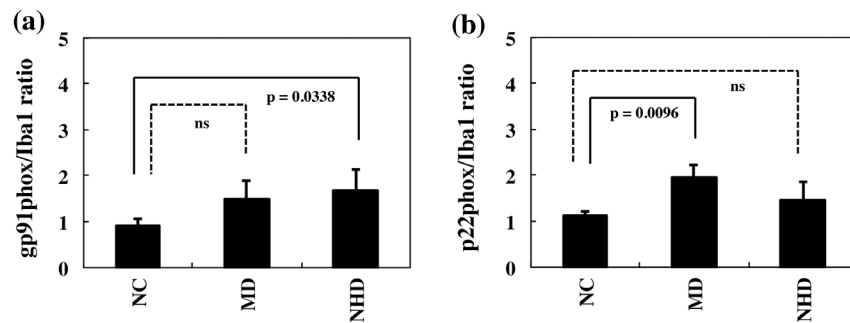


Figure 4. Quantitative analysis of gp91phox and p22phox expression on microglia in NC, MD, and NHD brains. (a) The ratio of immunolabeled area of gp91phox/Iba1, and **(b)** The ratio of immunolabeled area of p22phox/Iba1.

brains. The elevation of p22phox expression levels in MD brains is attributable to a high prevalence of white matter damage in this disease (15). More importantly, our observations raise a possible scenario that the enhanced expression of gp91phox in NHD brains contributes to overproduction of ROS highly toxic to myelinating oligodendrocytes (16,17), resulting in oligodendrocyte cell death, followed by development of leukoencephalopathy. Supporting this, previous studies showed that the expression of gp91phox and p22phox was upregulated in activated microglia accumulating in initial lesions of MS, an inflammatory demyelinating disease in the CNS, suggesting a role of oxidative tissue damage in progression of demyelination (8,18).

Notably, a population of activated microglia expressed gp91 in the penumbra of transient cerebral ischemia (6). The expression of gp91 was upregulated in amoeboid microglia, which generate superoxide anion and peroxynitrite, distributed in the pericontusional area following traumatic brain injury (7). Microglia play a key role in lipopolysaccharide (LPS)-induced dopaminergic cell death in a manner dependent on the expression of gp91phox (19). All of these observations suggest that microglia contain a functionally active NADPH oxidase capable of generating ROS. Microglia, promptly activated by proinflammatory cytokines, disease-associated proteins, and environmental toxins, express high levels of gp91phox and produce a large amount of ROS that cause oxidative stress leading to neuronal and myelin damage (3,16,17).

In conclusion, the enhanced expression of gp91phox on microglia, a central component of the NADPH oxidase, might be involved in development of leukoencephalopathy in NHD brains *via* myelin damage caused by superoxide anion-inducible ROS.

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