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Expression of α-synuclein, a presynaptic protein implicated in Parkinson's disease, in erythropoietic lineage

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Abstract

The present study investigated expression of α-synuclein (α-syn), a presynaptic protein involved in the pathogenesis of Parkinson's disease, in erythroid cells. Using various immunological procedures, immunoreactivity of α-syn was unambiguously demonstrated in erythroid lineage in murine bone marrows and peripheral erythrocytes. Expression of α-syn mRNA was also confirmed by *in situ* hybridization. Furthermore, flow cytometry analysis revealed that approximately 80% of erythroid cells in murine bone marrows expressed α-syn, while more than 90% of peripheral erythrocytes expressed α-syn. Nonetheless, α-syn null mice exhibited apparently no phenotypic changes in erythroid cells as was the case in their brains, suggesting that there might be underlying some redundant mechanisms. Together with previous reports showing the expression of α-syn in lymphocytes and platelets, the present finding supports a contention that α-syn might play some important functions in hematopoietic system.

Keywords: α-Synuclein; Bone marrows; DJ-1; Erythroblasts; Erythrocytes; Parkinson's disease

α-Synuclein (α-syn) is a presynaptic protein consisting of 140 amino acids and makes up the synuclein family of peptide along with two other members, β- and γ-syn [1]. Since the discovery of missense mutations in α-syn gene in rare familial Parkinson's disease (PD) cases, extensive studies have shown that this presynaptic protein may play a central role in the pathogenesis of synucleinopathy, including PD, diffuse Lewy body disease and multiple system atrophy [1–3]. Supporting this notion, α-syn was shown to be a major constituent of the Lewy body and glial inclusions [4–6]. In vitro, α-syn was induced to form amyloid-like fibrils [7,8]. Furthermore, overexpression of α-syn in transgenic mice recapitulated PD-like neuropathology [9]. Despite of the distinct role of α-syn for neurodegeneration, physiological roles of this molecule have been largely elusive.

Besides the central nervous system, expression of α-syn has been shown in a variety of tissues, including muscle, kidney, liver, lung, heart, testis, blood vessel and so forth [10–13]. However, in contrast to the abundant expression of α-syn in the brain during both fetal and adult stages, relatively high expression of α-syn during the developmental stage of non-neural tissues were significantly decreased in the adult stage [11]. In this context, the hematopoietic system may be regarded as exceptional populations since expression of α-syn in adult stage has been demonstrated in some lineages, including lymphocytes [14,15] and platelets [16–18]. Here, we add the erythroid lineage as a novel cell population expressing α-syn. As a result of investigating the expression of α-syn for both bone marrows and

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peripheral blood, we found that α-syn was expressed in erythroid cells, including erythroblasts, reticulocytes and erythrocytes in bone marrows. Immunoreactivity of α-syn was also confirmed in peripheral erythrocytes. Together, these results demonstrate that α-syn is abundantly expressed in the erythroid lineage, supporting a contention that α-syn might play some important roles in hematopoietic system.

Materials and methods

Reagents. Unless otherwise noted, all reagents were obtained from Sigma (St. Louis, MO). Recombinant α-syn and His-DJ-1 were produced as previously described [8,19].

Primary antibodies used were: monoclonal anti-α-syn syn-1 and monoclonal anti-β-syn (BD Biosciences, San Jose, CA), rabbit polyclonal anti-α-syn (C-terminal) [20], goat polyclonal anti-γ-syn (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal anti-DJ-1 (Stressgen, Ann Arbor, MI), rabbit polyclonal anti-mouse hemoglobin (MP biomedical, Solon, OH), and fluorescein isothiocyanate-labeled monoclonal TER-119 that recognizes mouse erythroid cell [21] (ImmunoTools, Friesoythe, Germany).

Mice and other tissue materials. C57BL/6 mice (four months old) and α-syn null mice were purchased from CLEA Japan (Tokyo, Japan) and Jackson Laboratories (Bar Harbor, ME), respectively. Human blood was from healthy men and brain was from a non-PD patient. Use of human materials was approved by the Human Ethics Committee of Fukushimura Hospital and Tokyo Metropolitan Institute for Neuroscience.

Immediately after blood collection from humans and mice, smears were prepared and fixed in 4% paraformaldehyde (PFA). Erythrocytes were separated using Ficoll–Paque Plus (Amersham Biosciences, Uppsala, Sweden) and washed in phosphate-buffered saline (PBS) four times. Bone marrow cells were flushed out of the femurs with cold PBS through the cuts on epiphyses. For the histological analysis, the murine lumbar vertebra, sternum and brain were collected, fixed in Bouin's solution and processed for paraffin sections.

Histology, immunohistochemistry and immunofluorescence. For histological observation, sections were stained with hematoxylin and eosin. Immunohistochemistry was performed as previously described [22]. Briefly, sections were boiled in 0.01 M citrate buffer (pH 6.0) to retrieve antigens. They were immersed in 0.3% hydrogen peroxide in methanol as erythrocytes were a major source of endogenous peroxidase, and incubated in 5% normal goat or rabbit serum for blocking. Sections were incubated with the primary antibodies overnight at 4 °C, followed by the detection of antigen sites with biotinylated secondary antibodies and the avidin biotin complex kit (Vector Laboratories, Burlingame, CA). Nuclei were counterstained with hematoxylin. For immunofluorescence microscopy, sections and blood smears were first immunostained for α-syn or β-syn followed by the secondary antibody, Alexa Fluor 488® (Molecular Probes, Eugene, OR). Then, specimens were double stained for hemoglobin with the secondary antibody, Alexa Fluor 555®. Slides were observed by an Olympus FV1000 laser scanning confocal microscope.

In situ hybridization (*ISH*). Mouse α-syn cDNA fragments corresponding to the 327–825th of oligonucleotides (BC046764) were subcloned into pBlueScript SK(−). Riboprobes were prepared using a digoxigenin RNA labeling kit (Roche Diagnostics).

The deparaffinized sections were digested in proteinase K (20 μg/ml), post-fixed in 4% PFA, and acetylated. Hybridization was performed at 55 °C overnight. After rinse in 2× SSC, the sections were reacted with anti-digoxigenin antibody labeled with alkaline phosphatase, and visualized by nitro blue tetrazolium/5-bromo-4-chloro-3-indolylphosphate solution (Roche Diagnostic, Mannheim, Germany).

Immunoelectron microscopy. Immunoelectron microscopy was performed as previously described [16]. Washed mouse erythrocytes were fixed in 4% PFA and processed for embedding in LR White resin (Nisshin EM, Tokyo, Japan). Ultrathin sections were incubated with syn-1 antibody in PBS overnight, followed by the goat anti-mouse antibody labeled with 10 nm gold particles (BB International, Cardiff, UK). The sections were stained with uranyl acetate and lead citrate and observed by a Hitachi H-7500 electron microscope.

Flow cytometry. Isolated bone marrow cells and peripheral erythrocytes were fixed in 4% PFA and incubated with TER 119 [21] and anti-α-syn antibodies. Bone marrow cells were also stained for TER 119 and hemoglobin. α-syn and hemoglobin were detected with phycoerythrin-labeled anti-mouse IgG antibody (Abcom, Cambridge, MA) and anti-rabbit IgG antibody Alexa Fluor 555®, respectively. Cells incubated with a vehicle alone were used as a control. Samples (2×10^4 cells) were analyzed by a Beckman Coulter EPICS ALTRA flow cytometer (Fullerton, CA).

Immunoblot analysis. Detergent-soluble lysates were prepared from erythrocytes and brains. In some experiments, extracts were divided into cytosolic and particulate fractions [23]. Immunoblotting was performed as previously described [23]. For the aggregation assay, recombinant α-syn (50 ng) was incubated with human hemoglobin (0.13 μg) and hydrogen peroxide (0.1 mM) in 50 mM Tris–HCl (pH 7.4) buffer in the presence of recombinant DJ-1 (0, 0.25, 0.5 and 1.0 μg) for 18 h at room temperature. Samples were then analyzed by immunoblotting using syn-1.

Statistical analysis. Quantitative data were expressed as means ± standard error of the mean and compared between the wild type and α-syn null mice ($n = 3$–6 each genotype) using the Student's t-test. Differences were considered significant if p values were less than 0.05.

Results

Expression of α-syn protein in murine bone marrows

Immunohistochemistry showed intense staining of α-syn in cells of erythroblastic islands in the mouse bone marrows (Fig. 1a). As detected by anti-α-syn syn-1, α-syn exhibited intense staining in erythroblasts, whereas the immunoreactivity of α-syn in reticulocytes and erythrocytes was less intense. Essentially similar results were observed by polyclonal α-syn C-terminal antibody (data not shown). Immunostaining of α-syn was also observed in megakaryocytes (Fig. 1a). To exclude the possibility that the peroxidase reaction was due to pseudo-positive staining by the heme, bone marrow sections were analyzed by immunofluorescence microscopy. Immunoreactivity of α-syn was detected in erythroid cells, including both erythroblastic cells and erythrocytes, which were identified by morphology (Fig. 1b) and double staining of hemoglobin (Fig. 1c and d). These results showed that α-syn protein was predominantly expressed in erythroid lineage in murine bone marrows. In contrast, there was no detectable immunoreactivity for β-syn (Fig. 1e). The antibody for γ-syn produced no immunoreaction in all histological preparations including the brain. No immunoreactivity was detected in control sections (data not shown).

Expression of α-syn mRNA in murine bone marrows

To determine whether or not α-syn protein is produced in erythroblastic cells, the expression of α-syn mRNA was determined by ISH. As a control observation, murine brain sections were hybridized with the antisense probe, giving rise to intense signals in neural cells, in particular those in the cerebral cortex (Fig. 1f) and hippocampus as

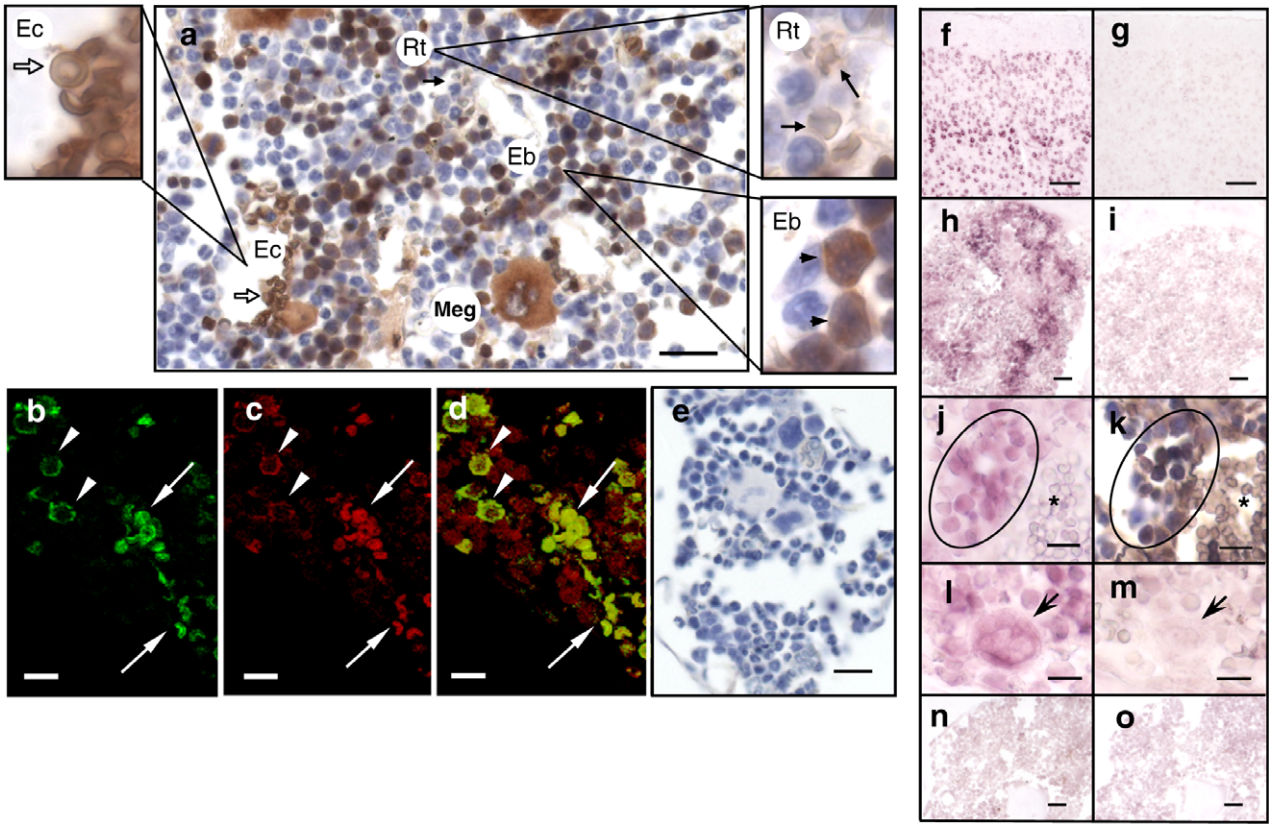

Fig. 1. Immunohistochemistry and *in situ* hybridization for syn in murine bone marrows. (a) Immunohistochemistry of α-syn in erythroblasts (Eb, arrowheads), reticulocytes (Rt, arrows), erythrocytes (Ec, open arrows) and megakaryocytes (Meg). Magnified images of Eb, Rt and Ec are shown aside. (b–d) Confocal microscope images of α-syn (b, green), hemoglobin (c, red), and merged in (d, yellow). Arrows and arrowheads indicate erythrocytes and erythroblasts, respectively. (e) Immunohistochemistry of β-syn. No immunoreaction of β-syn is seen. (f–o) *In situ* hybridization for α-syn mRNA in neural cells of the cerebral cortex (f, antisense; g, sense) and erythroblastic cells in bone marrows (h, antisense; i, sense). α-syn mRNA (j, encircled) is expressed in cells that are immunohistochemically positive for hemoglobin (k, encircled) of the erythroblastic island. The same blood vessels in neighboring sections are indicated by asterisks for orientation. Expression of α-syn mRNA in megakaryocyte (arrows) (l, antisense; m, sense). α-syn mRNA is absent in bone marrows of the α-syn null mouse (n, antisense; o, sense). Bars indicate 10 μm in b–d and j–m; 20 μm in a, e, h, i, n and o; 100 μm in f and g. (a–e) Lumbar vertebra, (f–o) sternum. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reported elsewhere [24–26,29–31], whereas no signal was detected with the sense probe (Fig. 1g). Under the same hybridization conditions, α-syn mRNA was detected in groups of cells in the bone marrows (Fig. 1h and i). Upon a high magnification, these cells were suspected to be erythroblasts in the erythroblastic islands (Fig. 1j), which were confirmed by immunostaining for hemoglobin in the neighboring sections (Fig. 1k). Consistent with the immunohistochemistry results, megakaryocytes also expressed the signal for α-syn mRNA at varying intensities (Fig. 1l and m). These signals were not detected in bone marrows of α-syn null mice (Fig. 1n and o). Taken together, α-syn is produced in erythroblasts in murine bone marrows.

Expression of α-syn protein in peripheral erythrocytes

Immunoreactivity of α-syn was also seen in murine peripheral erythrocytes, although compared to those in bone marrows, α-syn in peripheral erythrocytes varied greatly in staining intensity (Fig. 2a–c). A similar immunoreactive pattern of α-syn was observed for human erythrocytes (data not shown). Furthermore, it was confirmed that immunoreaction of α-syn was absent in erythrocytes from α-syn null mice (Fig. 2d and e). In contrast, no blood cells showed detectable staining for β-syn (Fig. 2f).

To further localize the intracellular expression of α-syn, immunoelectron microscopy was performed for murine erythrocytes. The result showed that gold particles representing α-syn were diffused throughout the cytoplasm (Fig. 2g) and also on the cell membrane of the erythrocytes (Fig. 2g, inset). By contrast, little particles were detected in the absence of the primary antibody (Fig. 2h).

Quantitative analysis of α-syn positive erythroid cells in bone marrows and peripheral erythrocytes

Flow cytometry analysis showed that $22.5 \pm 4.0\%$ of bone marrow cells were recognized by TER-119, which was consistent with the previous report (Fig. 3a) [21]. Among these TER-119 positive cells, $80.0 \pm 6.8\%$ cells were positive for α-syn. In peripheral erythrocytes, α-syn positive cells were seen in $91.4 \pm 6.2\%$ of TER-119 positive

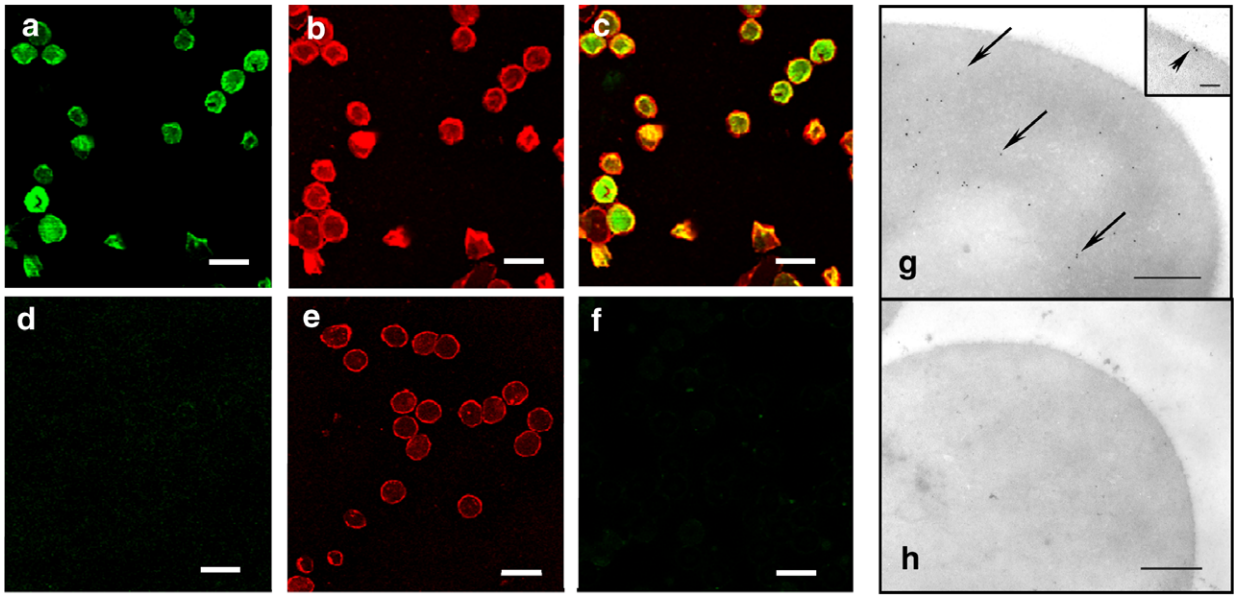

Fig. 2. Immunostaining of syn in the peripheral blood. (a–c) Expression of α-syn (a, green) in erythrocytes confirmed by double staining for hemoglobin (b, red) and the merged image (c, yellow). Note the difference in staining intensity of α-syn among erythrocytes. (d,e) Negative control. The smear from α-syn null mouse is double stained for α-syn (d) and hemoglobin (e). No α-syn (d) staining is detected. (f) Absence of β-syn in the peripheral blood. (g) Immunoelectron microscopy of α-syn in the erythrocyte. Note the cytoplasmic (arrows) and membranous (arrowhead in inset) localizations of α-syn. (h) No gold particle is seen in the control section. Bars indicate 10 μm in a–f; 500 nm in g and h; 100 nm in the inset. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

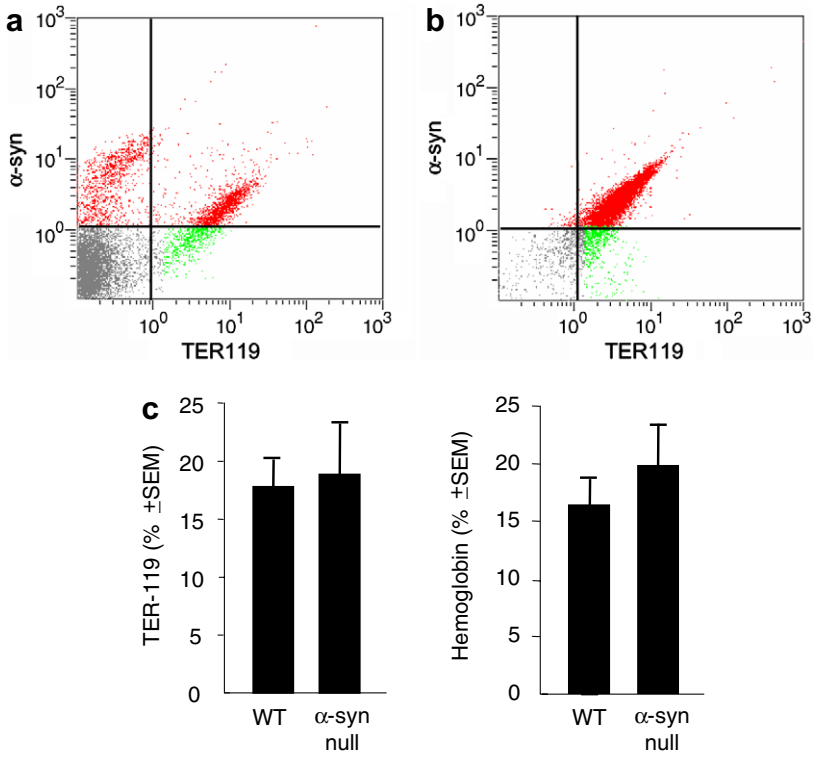

Fig. 3. Flow cytometry analysis of bone marrow cells and peripheral erythrocytes. (a,b) Flow cytormetry of bone marrow cells (a) and peripheral erythrocytes (b). Cells were double stained with anti-α-syn antibody and TER-119. Among TER-119 positive cells (the upper right and lower right compartments), approximately 80% (a) and 91% (b) cells (upper right compartment) are α-syn positive. (c) Flow cytometry of bone marrow cells. Cells were stained with TER-119 and anti-hemoglobin antibody and compared individually between wild type and α-syn null mice. There is no significant difference between α-syn genotypes in both staining.

cells (Fig. 3b). Frequencies of hemoglobin positive cells and TER-119 positive cells in bone marrows of WT mice did not differ significantly from those in α-syn null mice, respectively (Fig. 3c).

Peripheral erythrocyte profile, including erythrocyte count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration, in α-syn null mice showed no significant difference from those in wild type mice (data not shown).

Western blot analysis of α, β and γ-syn and DJ-1 in the peripheral erythrocytes

The α-syn expression in erythrocytes was further characterized by immunoblot analysis. A monomer of α-syn (20 kDa) was detected for both murine and human erythrocytes, with their intensities comparable to those of brains (Fig. 4a and b). Consistent with the localization of α-syn in both cytoplasm and plasma membrane by electron microscopic analyses, α-syn was detected in both cytosolic and particulate fractions (Fig. 4c). Expression levels of both β- and γ-syn in erythrocytes were negligible compared to those of the brain homogenates (Fig. 4d–f). We further investigated the expression of DJ-1 (Park 7), a familial PD-related molecule [27,28], and detected DJ-1 in erythrocytes (Fig. 4g–i).

Because DJ-1 acts as an anti-oxidant [28], it was predicted that DJ-1 might stabilize α-syn which otherwise would be prone to aggregate by the hemoglobin-mediated oxidative stress conditions in erythrocytes. To test this possibility, the effect of DJ-1 on the hemoglobin/hydrogen peroxide-induced aggregation of α-syn was evaluated under the cell-free conditions (Fig. 4i). α-syn was induced to aggregate by hemoglobin, which was stimulated by hydrogen peroxide (lanes 1–3). Co-incubation of DJ-1 dose-dependently inhibited the aggregation of α-syn (lanes 4–6).

Discussion

The present study investigated expression of α-syn in the erythroid lineage in bone marrows and peripheral erythrocytes. α-syn protein was demonstrated in erythroblasts and erythrocytes using various immunological procedures,

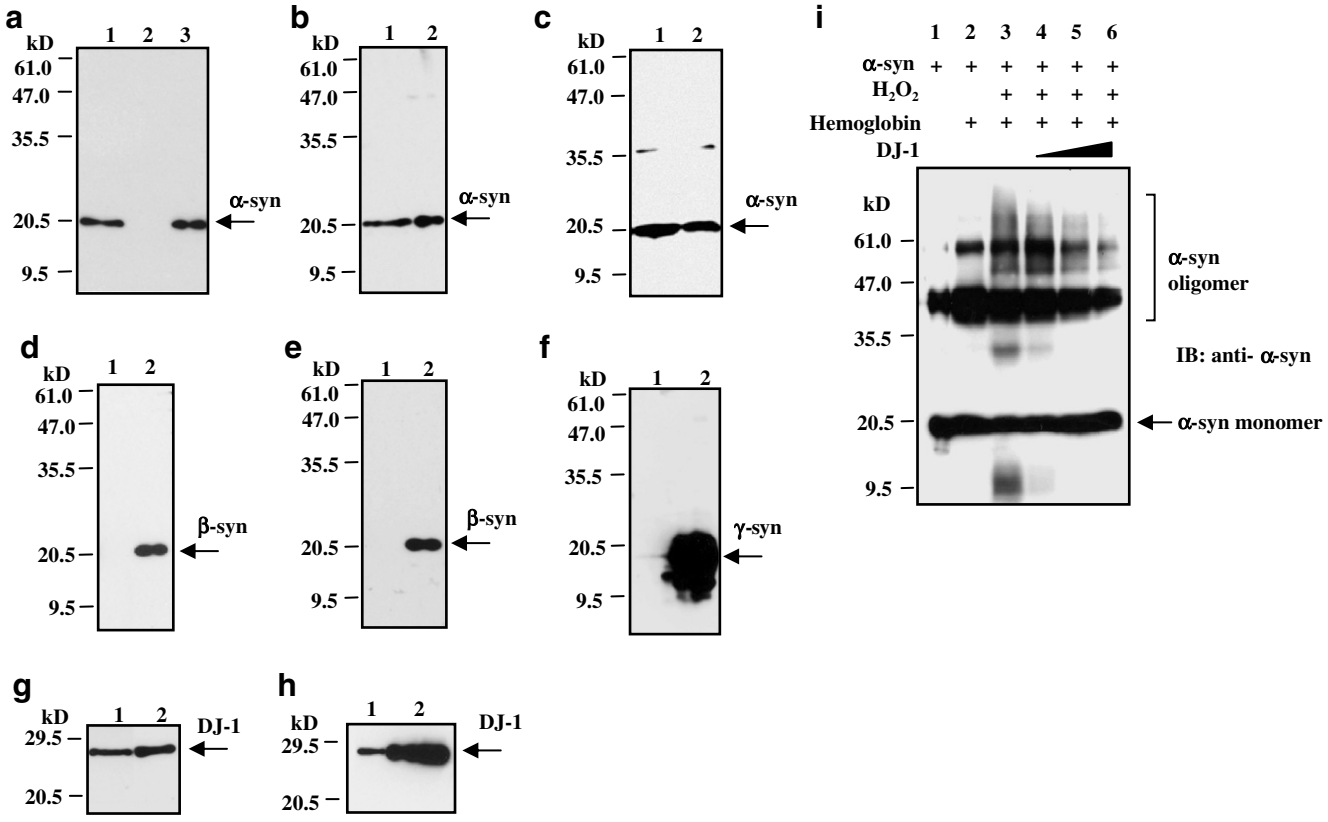

Fig. 4. Immunoblot analyses of peripheral erythrocytes. Immunoblotting was performed for the detergent-soluble lysates prepared from erythrocytes and brains (a, b, e–h), and for the cytosolic and particulate fractions of erythrocytes (c). (a) α-syn in the wild mouse erythrocytes (lane 1) and brain (lane 3) and α-syn null mouse erythrocytes (lane 2). (b) α-syn of human erythrocytes (lane 1) and brain (lane 2). (c) α-syn in the cytosolic (lane 1) and particulate (lane 2) fractions of erythrocyte of wild mice. (d) β-syn in wild mouse erythrocytes (lane 1) and brain (lane 2). (e) β-syn in human erythrocytes (lane 1) and brain (lane 2). (f) γ-syn in human erythrocytes (lane 1) and brain (lane 2). (g) DJ-1 in wild type mouse erythrocytes (lane 1) and brain (lane 2). (h) DJ-1 in human erythrocytes (lane 1) and brain (lane 2). (i) Suppressive effects of DJ-1 on α-syn aggregation induced by hemoglobin-mediated oxidative stress. Recombinant α-syn (50 ng) was incubated in the presence of hydrogen peroxide (0.1 mM) and hemoglobin (0.13 μg) with different amount of His-DJ-1 (0.25 μg in lane 4, 0.5 μg in lane 5, and 1 μg in lane 6).

including light and fluorescence microscopy (Figs. 1 and 2), immunoelectron microscopy (Fig. 2) and immunoblot analyses (Fig. 4). Consistent with this, expression of α-syn mRNA was detected in erythroblasts by *in situ* hybridization (Fig. 1). Furthermore, quantitative analysis by flow cytometry revealed that approximately 80% of erythroid cells in murine bone marrows expressed α-syn, while more than 90% of peripheral erythrocytes expressed α-syn (Fig. 3). Thus, these results unambiguously demonstrated that α-syn was expressed in the erythroid lineage in bone marrows and peripheral erythrocytes.

Then, what is the physiological function of α-syn for the erythroid cells? Although we compared cell populations of hemoglobin and TER-119 positive cells in bone marrows between wild type and α-syn null mice, there was no difference between genotypes (Fig. 3). In addition, analyses of peripheral erythrocyte profile indicated no detectable difference between wild type and α-syn null mice (data not shown). Thus, the apparent lack of phenotype in erythroid cells of α-syn null mice is similar to the lack of major phenotype in the brain of α-syn null mouse [29]. Also, no phenotypic changes were observed in α- and β-syn double knockout mouse [30]. However, these results may not necessarily imply that α-syn does not have physiological functions. Of a particular note is that α-syn was shown to rescue neurodegeneration caused by deletion of cysteine-string protein-α, the presynaptic co-chaperone to Hsc70 in mice [31], suggesting that α-syn might play a protective role for during the early development in the nervous system. In the similar context, one may speculate that α-syn might function in the early stage of hematopoietic differentiation. Since α-syn is expressed in various type of hematopoietic cells, including lymphocytes [14,15] and platelets [16–18] and since α-syn has been characterized by a long half-life time [32], expression of α-syn in multiple hematopoietic lineages might reflect the possible protective role of α-syn for their common progenitors. Alternatively, α-syn might be at a play for cellular event that takes place (e.g. denucleation or hemoglobin accumulation) before the reticulocyte formation because our result showed intense immunostaining of α-syn in erythroblasts and to a lesser extent in reticulocytes and erythrocytes (Fig. 1).

Several pieces of evidence further suggest that the regulatory mechanisms of α-syn expression in erythrocytes might in some respects overlap with those in presynapse. First, as is the case of α-syn in presynapse, expression of α-syn in denucleated erythrocytes may be regulated by the posttranslational mechanism, such as degradation and release of α-syn. Second, as is the case of the intracellular distribution of α-syn in presynapse, α-syn is detected in the cytoplasm and is concomitantly associated with plasma membrane of erythrocytes (Figs. 2 and 4). In this regard, the membrane-associated form of α-syn is prone to aggregate in neuronal cells [33]. Third, erythrocytes are chronically exposed to oxidative stress caused by hemoglobin as doperminergic neurons in substantia nigra is susceptible to iron-mediated oxidative stress in PD. Finally, as DJ-1 exerts anti-oxidative effects on the brain [27], this molecule may serve to protect α-syn against oxidative stress in erythrocytes (Fig. 4). Collectively, these results raise a possibility that erythrocytes could be useful for the model to mimic some aspects of pathogenesis in PD and related neurodegenerative disease.

Acknowledgments

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