

Differential expression of α B-crystallin causes maturation-dependent susceptibility of oligodendrocytes to oxidative stress

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Oligodendrocyte precursor cells (OPCs) are most susceptible to oxidative stress in the brain. However, the cause of differences in susceptibility to oxidative stress between OPCs and mature oligodendrocytes (mOLs) remains unclear. Recently, we identified *in vivo* that α B-crystallin (aBC) is expressed in mOLs but not in OPCs. Therefore, we examined in the present study whether aBC expression could affect cell survival under oxidative stress induced by hydrogen peroxide using primary cultures of OPCs and mOLs from neonatal rat brains. Expression of aBC was greater in mOLs than in OPCs, and the survival rate of mOLs was significantly higher than that of OPCs under oxidative stress. Suppression of aBC by siRNA transfection resulted in a decrease in the survival rate of mOLs under oxidative stress. These data suggest that higher susceptibility of OPCs than mOLs to oxidative stress is due, at least in part, to low levels of aBC expression. [BMB Reports 2013; 46(10): 501-506]

INTRODUCTION

Oligodendrocytes (OLs) are more susceptible to oxidative stress compared to any other cell types in the brain. OLs at all stages of differentiation have much lower levels of reduced-glutathione and glutathione peroxidase activity than astrocytes (1-5). These biochemical properties of OLs reflect their poor ability to scavenge peroxides, inducing oxidative stress. Several lines of evidence suggest that oxidative stress is a major cause of white matter injury, including multiple sclerosis

and periventricular leukomalacia (6, 7). Among OL-lineage cells, oligodendrocyte precursor cells (OPCs) are the most susceptible to oxidative stress (8, 9). However, the cause of maturation-dependent susceptibility of OL-lineage cells to oxidative stress remains unclear.

α B-crystallin (aBC) is well known as a lens protein and is considered as one of the small heat shock proteins. aBC is constitutively expressed in the cardiac muscle, skeletal muscle, the kidney, the central nervous system, and the lens at normal conditions (10-12). In the central nervous system, aBC is expressed in OLs of the white matter of the cerebral cortex, the cerebellum, and the brain stem (12). aBC expression in glial cells is increased in some neurodegenerative diseases, such as multiple sclerosis and Alexander disease (13, 14). Furthermore, aBC is also induced by hyperthermia, viral infection, ischemia, anoxia and oxidative stress (15, 16). Induced aBC increases cell survival to deleterious stimuli via binding to intracellular unfolding proteins and inhibiting protein aggregation (17).

Recently, we identified that aBC is exclusively expressed in mature oligodendrocytes (mOLs) and myelin sheaths, but not in OPCs of the developing avian retina (18). Therefore, we hypothesized that OPCs may be more susceptible to oxidative stress than mOLs partly due to low levels of intracellular aBC expression. Thus, this study was aimed at examining the relationship between aBC expression and susceptibility to oxidative stress in primary cells in two different developmental stages of OL-lineage cells from the neonate brain of the rat.

RESULTS

Identification of OPCs and mOLs

To evaluate the maturation of cultured cells, we identified the expression molecules of two cell types. Almost all cells in the OPC medium were labeled with anti-A2B5 antibody (98%). Most cells in the mOL medium were labeled with anti-MBP antibody (90%). These results indicate that the procedures for the isolation of OPCs and differentiation into mOLs were properly performed.

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aBC expression of OPCs and mOLs

To examine the level of aBC expression in OPCs and mOLs, we performed immunofluorescence and Western blot analysis after induction of oxidative stress by treatment with 100 μ M H_2O_2 . OPCs under normal condition did not express aBC (Fig. 1A). Under oxidative stress condition, a low level of aBC was expressed in the cell bodies of OPCs (Fig. 1B). In contrast, aBC expression in mOLs was high even under normal condition (Fig. 1C). Compared to normal condition, aBC expression was greatly increased in mOLs upon induction of oxidative stress (Fig. 1D).

The results of the quantitative analysis were consistent with the immunofluorescence findings described above. The basal level of aBC expression in OPCs was almost absent under nor-

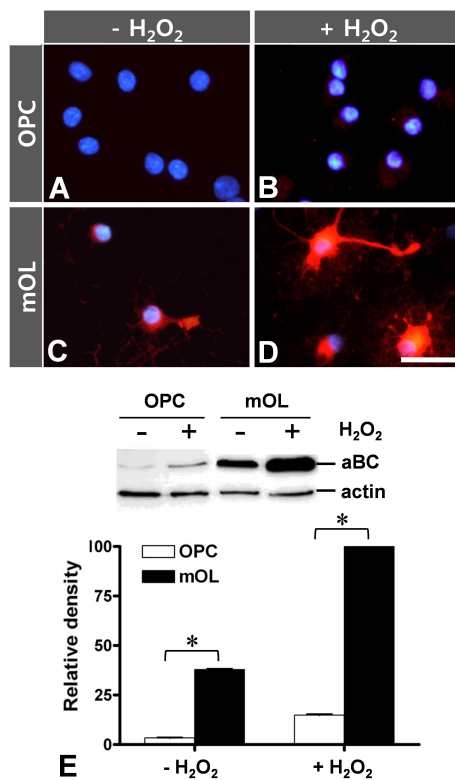


Fig. 1. α B-crystallin (aBC) expression in OPCs and mOLs. (A-D) Immunofluorescence. OPCs expressing aBC is not observed under normal condition without H_2O_2 treatment (A). Under oxidative stress condition induced by 100 μ M H_2O_2 treatment, aBC expression in OPCs is faintly observed, but still extremely low (B). In contrast, a large amount of aBC is expressed in mOLs under normal condition (C). In comparison with normal condition, aBC expression in mOLs is greatly increased under oxidative stress condition (D). (E) Quantitative analysis. In comparison with mOLs, expression levels of aBC are quite low in OPCs under normal and oxidative stress conditions (E). Note that oxidative stress induces a significant increase in aBC expression in mOLs, but not in OPCs. Asterisks denote significant differences between the two groups ($P < 0.01$). Scale bar = 50 μ m.

mal condition. Under oxidative stress condition, an insignificant rise of aBC expression was detected in OPCs. However, mOLs expressed large amounts of aBC even under normal condition. Furthermore, aBC expression in mOLs was significantly increased under oxidative stress condition (Fig. 1E).

Apoptosis and survival rate of OPCs and mOLs

Cell death was evaluated by Hoechst staining. Few or no OPCs and mOLs with condensed or fragmented nuclei were observed under normal condition (Fig. 2A, C). However, the number of OPCs with condensed nuclei was obviously increased under oxidative stress condition (Fig. 2B). In contrast, mOLs with condensed nuclei were less frequently observed than dead OPCs under oxidative stress condition (Fig. 2D). The survival rate of OPCs was decreased from 93% under normal condition to 47% under oxidative stress condition. In contrast, the survival rate of mOLs was decreased from 92% under normal condition to 78% under oxidative stress condition (Fig. 2E).

Suppression of aBC in mOLs and survival rate of aBC-suppressed mOLs

To examine the effect of aBC expression on cell survival, we performed gene knockdown by transfection with aBC siRNA

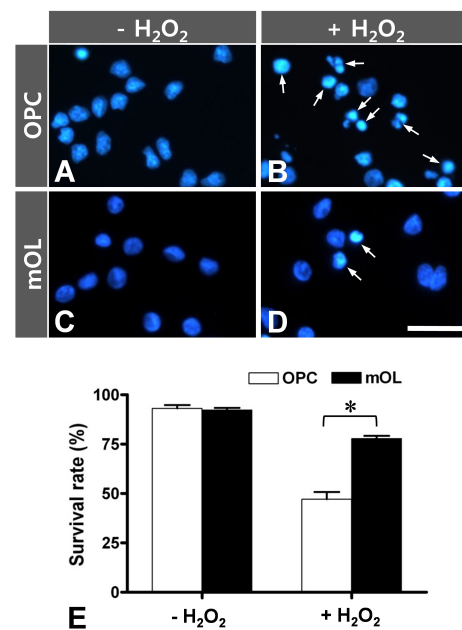


Fig. 2. Apoptosis and survival rate of OPCs and mOLs. (A, B) OPCs. In comparison with normal condition (A), OPCs with condensed nuclei are frequently observed under oxidative stress condition (arrows in B). (C, D) mOLs. In comparison with normal condition (C), a few mOLs shows dead cell features under oxidative stress condition (arrows in D). (E) Survival rates of OPCs and mOLs. Survival rate of OPCs is significantly lower than that of mOLs under oxidative stress condition. Asterisk denotes significant difference between the two groups ($P < 0.01$). Scale bar = 50 μ m.

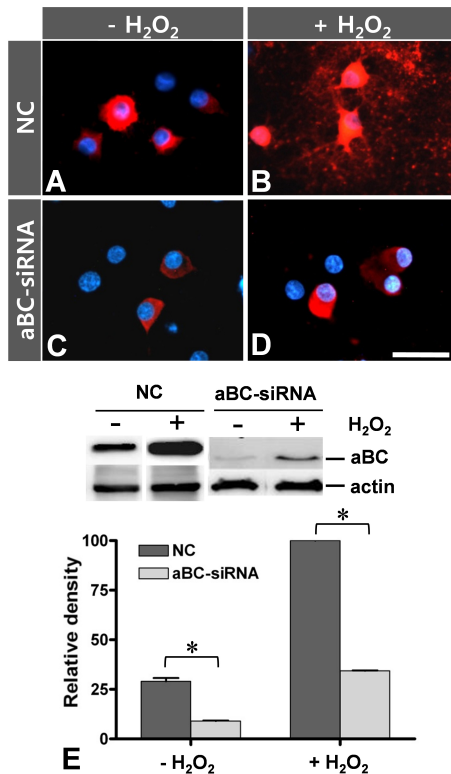


Fig. 3. Suppression of aBC expression in mOLs. (A-D) immunofluorescence. In comparison with the negative control (A, B), aBC expression in aBC-suppressed mOLs is decreased under normal and oxidative stress conditions (C, D). (E) Quantitative analysis. In comparison with the negative control, expression levels of aBC are significantly low in aBC-suppressed mOLs under normal and oxidative stress conditions. Note that oxidative stress induces a significant increase in aBC expression in the negative control mOLs, but not in aBC-suppressed mOLs. Asterisks denote significant differences between the two groups ($P < 0.01$). Scale bar = 50 μ m.

in mOLs. Compared with the negative control (Fig. 3A, B), aBC expression in mOLs transfected with aBC-siRNA was significantly suppressed under normal and oxidative stress conditions (Fig. 3C, D). Expression of aBC in aBC-suppressed mOLs was decreased to less than 30% of that in the negative control under oxidative stress condition (Fig. 3E).

Similar to the negative control (Fig. 4A), few aBC-suppressed mOLs with condensed nuclei were observed under normal condition (Fig. 4C). Under oxidative stress condition, cells with condensed nuclei were more frequently observed in aBC-suppressed mOLs (Fig. 4D) than in the negative control (Fig. 4B). The survival rate of aBC-suppressed mOLs was decreased from 89% under normal condition to 50% under oxidative stress condition, which was similar to that of OPCs (Fig. 4E). In summary, survival rates under oxidative stress condition were significantly higher in aBC-abundant mOLs than in aBC-insufficient OPCs or aBC-suppressed mOLs.

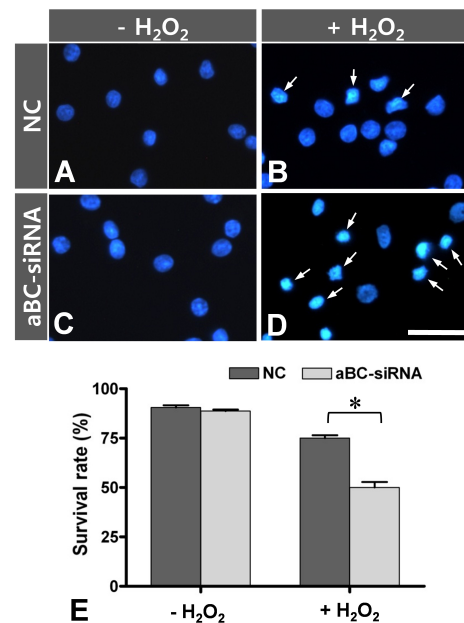


Fig. 4. Apoptosis and survival rate of aBC-suppressed mOLs. (A, B) Negative control. In comparison with normal condition (A), a few mOLs of negative control shows dead cell features under oxidative stress condition (arrows in B). (C, D) aBC-suppressed mOLs. In comparison with normal condition (C), aBC-suppressed mOLs with condensed nuclei are frequently observed under oxidative stress condition (arrows in D). (E) Survival rates of negative control and aBC-suppressed mOLs. Survival rate of aBC-suppressed mOLs is significantly lower than that of the negative control under oxidative stress condition. Asterisk denotes significant difference between the two groups ($P < 0.01$). Scale bar = 50 μ m.

DISCUSSION

In this study, we showed that the proportion of surviving mOLs was significantly higher than that of OPCs under oxidative stress condition by treatment with 100 μ M of H₂O₂. The different susceptibilities of OPCs and mOLs to oxidative stress were consistent with previously reported data, although we employed a different method of oxidative stress induction and cell survival or apoptosis measurement (8, 9, 19). The source of the difference in susceptibilities to oxidative stress between OPCs and mOLs is not fully understood. Nevertheless, we can, at least, explain that the difference is independent on the levels of intracellular reduced glutathione (GSH), a crucial scavenger of oxidative stress, in these two OL-lineage cells on the basis of convincing evidence that while depletion of intracellular GSH by cystine deprivation triggered death of OPCs, mOLs remained viable (8). This report implies that another protective mechanism to oxidative stress may exist in mOLs.

It was reported that aBC acts as a protective protein in various mammalian cells including retinal pigment epithelial cells (20), cardiac endothelial cells (21), myocytes (22), and as-

trocytes (23, 24). Our recent investigation of the retina showed that aBC was exclusively expressed in mOLs, but not in OPCs, of the developing as well as the adult avian retina (18). In the present study using separate primary cultures of OPCs and mOLs from the neonatal rat brains, we showed that the basal expression level of aBC in mOLs was high, while aBC expression was extremely low in OPCs. Under oxidative stress, aBC expression was significantly increased in mOLs, but aBC expression in OPCs was nearly unaffected. aBC expression in mOLs under oxidative stress condition was approximately ten-folds of that in OPCs. To confirm that aBC plays a protective role against oxidative stress, we suppressed aBC expression in mOLs via siRNA transfection. Suppression of aBC expression significantly diminished the survival rate of mOLs under oxidative stress condition, suggesting that aBC might favorably affect survival of mOLs under oxidative stress.

Details of the mechanism underlying the protective role of intracellular aBC on mOLs remain unclear. However, an accumulating body of evidence indicates that the inhibition of caspase 3, a representative apoptotic signaling molecule, may be involved in this process. aBC binding to partially processed caspase 3 was reported to inhibit caspase 3-dependent apoptosis in a human breast carcinoma cell line (25). Indeed, oxidative stress induced by H₂O₂ enhanced the level of active caspase 3 (p17) in OPCs, but no significant increase was detected in mOLs (19). A similar study was conducted in astroglia. Suppression of aBC over-expression in astroglia using aBC siRNA increased caspase 3-dependent apoptosis (23). Although further studies are needed to determine a direct correlation between aBC and caspase 3 in OL-lineage cells, our data, together with these reports, suggest that the high expression of aBC in mOLs inhibits H₂O₂-induced apoptosis of mOLs. This study demonstrated that the susceptibility of OPCs to oxidative stress is responsible for, at least in part, low levels of intracellular aBC.

OLs are easily exposed to oxidative stress, since these cells have high iron contents as well as oxidative metabolic activity. OPCs are more susceptible to oxidative stress than mOLs. Therefore, oxidative damage triggers death of OPCs prior to any other cell types in the brain. Indeed, oxidative stress is responsible for periventricular leukomalacia that mainly affects OPCs in the white matter of premature infants (7, 26). High susceptibility of OPCs to oxidative stress in this disease may be associated with low levels of aBC expression in OPCs. Clinical correlation between the level of aBC expression and maturation-dependent susceptibility of OL-lineage cells to oxidative stress should be further investigated.

MATERIALS AND METHODS

Animals and dissection

Approximately 10-12 postnatal day 1 (P1) Sprague-Dawley rats (DBL, Korea) were used at a time in this study. P1 rats were cryoanesthetized and decapitated. The skulls were then

carefully cut by micro-dissecting scissors, and the brains were excavated with cold Hanks balanced salt solution (HBSS) containing D-glucose and sucrose. This work was approved by the Institutional Animal Care and the Use Committee of Chungbuk National University (Approval No. CBNUA-092-0906-01).

Culture of neonate rat cerebra and isolation of OPCs

Culturing of the neonate rat cerebra and OPC isolation were performed according to the method by Chen et al. (27). Briefly, the meninges of the brains were carefully removed in cold HBSS. The cerebra of the brains free of meninges were mechanically dissociated using a pipette until nearly homogenous. Then, the dissociated cells were centrifuged for 10 min at 500 g, 4°C. After discarding the supernatant, the settled cells were resuspended by 5 ml DMEM20S medium (DMEM, 20% fetal bovine serum, 4 mM L-glutamine, 1mM sodium pyruvate, 1×antibiotic-antimycotic). Ten ml DMEM20S medium containing 0.5 ml cell suspension was plated and maintained in a 75T-flask coated with 0.01% poly-L-lysine for 7 to 10 days. Medium was refreshed every 3 days. When mixed glial cells reached a confluence of 80-90%, the flask was shaken on the shaking incubator for 1 hr at 200 rpm, 37°C. After discarding the media that included microglial cells, 10 ml DMEM20S medium was added to the flask. The flask was shaken in a shaking incubator for 18-20 hrs at 200 rpm, 37°C to get OPCs from mixed glial cells. The supernatant (DMEM20S medium) containing OPCs were used in this experiment.

OPC culture

OPCs were maintained according to the method by Chen et al. (27). Briefly, DMEM20S medium was changed with OPC medium (DMEM, 4 mM L-glutamine, 1 mM sodium pyruvate, 0.1% bovine serum albumin, 50 µg/ml apo-transferin, 5 µg/ml insulin, 30 nM sodium selenite, 10 nM D-biotin, 10 nM hydrocortisone, 10 ng/ml PDGF-AA, 10 ng/ml b-FGF) and cells (2×10⁵ cells/well) were plated in a 24-well plate containing culture glasses coated with poly-L-lysine. The OPC culture was maintained in the OPC medium for 9-12 days at 37°C in a 5% CO₂ incubator. The medium was refreshed every 2 days.

mOL culture

The mOLs culture was maintained according to the method by Bo et al. (28). Briefly, the DMEM20S medium was changed with the mOL medium (DMEM, 4 mM L-glutamine, 1 mM sodium pyruvate, 0.1% bovine serum albumin, 50 µg/ml apo-transferin, 5 µg/ml insulin, 30 nM sodium selenite, 10 nM D-biotin, 10 nM hydrocortisone, 40 ng/ml thyroxine, 40 ng/ml triiodothyronine) and cells (8×10⁵ cells/well) were plated in a 24-well plate containing the culture glasses coated with 0.01% poly-L-lysine. The mOLs culture was maintained in the mOL medium for 10-12 days at 37°C in a 5% CO₂ incubator. The medium was refreshed every 2 days.

Identification of OPCs and mOLs

Nine days after starting the primary culture, immunofluorescence with specific markers for OPCs (anti-A2B5 antibody) and mOLs (anti-MBP antibody) was performed for confirmation of cell types.

H₂O₂ treatment and Hoechst staining

To induce oxidative stress, OPCs and mOLs were treated with 100 μ M H₂O₂ diluted in media for 1 hr at 37°C in a 5% CO₂ incubator. Then, OPCs and mOLs were cultured for 12 hrs in OPC and mOL media for recovery, respectively. Sequentially, cells were fixed with 4% paraformaldehyde for 30 min and rinsed with PBS 2 times for 5 min. Cells were incubated with 2 μ g/ml Hoechst 33258 (Sigma, USA) for 15 min at 37°C and rinsed with PBS (29). Stained cells were observed under a multipurpose microscope with an epifluorescence attachment (DMLB, Leica, Germany). Three to four wells per each condition were observed, and images were obtained from four areas (0.4 mm²/area) for each well. Cells with condensed or fragmented nuclei in Hoechst stain were determined as dead cells.

Transfection with aBC siRNA

To suppress aBC expression in mOLs, siRNA duplex specifically targeting aBC (siRNA no. 1627041, accession no. NM_012935.2, Bioneer, Korea) was transfected using Lipofectamine (Invitrogen, USA) according to the manufacturer's instruction. Before transfection, mOLs in each well (8 \times 10⁵ cells/well) were incubated in the mOL medium without serum and antibiotic-antimycotic for 24 hrs. siRNA and Lipofectamine complexes (100 nM siRNA in 3.5 μ l Lipofectamine) were introduced into each well for 4 hrs in 37°C CO₂ incubator. Then the transfected mOLs were re-incubated in mOL medium for 24 hrs in 37°C in a 5% CO₂ incubator. For negative control, mOLs were transfected with scrambled siRNA (Bioneer) according to the manufacturer's instructions.

Immunofluorescence

OPCs and mOLs on cover slips were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 30 min. To block for nonspecific staining, they were incubated in PBS containing 1% normal goat serum for 30 min. The cells were incubated overnight at 4°C with a mouse anti-aBC (1 : 500, Enzo Life Sciences, USA), mouse anti-A2B5 (1 : 100, Abcam, USA) and a rat anti-MBP (1 : 300, Chemicon, USA) antibodies, respectively. Then, the cells were incubated for 2 hrs at room temperature with secondary antibodies: Cy3-labeled goat anti-mouse IgG for anti-aBC antibody (1 : 500, Jackson ImmunoResearch Laboratories, USA), Cy3-labeled goat anti-mouse IgM for anti-A2B5 antibody (1 : 500, Jackson ImmunoResearch Laboratories) and Cy2-labeled goat anti-rat IgG for anti-MBP antibody (1 : 500, Jackson ImmunoResearch Laboratories). For counterstaining, the nuclei of the cultured

cells were stained with 0.05% 4',6-diamidino-2-phenylindole (DAPI, Sigma, USA) for 3 min. Between each step, cover slips were washed with PBS 3 times for 5 min. Labeled cells were observed under a multipurpose microscope with an epifluorescence attachment (DMLB, Leica, Germany).

Western blot

OPCs and mOLs in each well were lysed at 4°C for 20 min in RIPA buffer (Elpis Biotech, Korea). Lysates were centrifuged for 5 min at 14,000 rpm, 4°C and denatured by boiling in sample buffer (Elpis Biotech) for 5 min. Then, the denatured proteins were separated on a 10-15% polyacrylamide gel by electrophoresis according to the method of Laemmli (30). The separated proteins were transferred to polyvinylidene difluoride membrane (Immun-Blot, Bio-Rad, USA). Membranes were blocked with 5% skim milk in PBS for 30 min. The membranes were then incubated with primary antibodies: mouse anti-aBC (1 : 500, Enzo Life Sciences, USA) and mouse anti-actin (JLA20, 1 : 300, Developmental Study Hybridoma Bank, USA). Horseradish peroxidase-conjugated horse anti-mouse IgG (1 : 500, Vector, USA) as a secondary antibody was used. Between each step, membranes were washed with PBS 3 times for 10 min. The immunoreactive proteins were detected using EPD western reagent kit (Elpis Biotech, Korea), and the membrane was exposed to LAS-3000 mini (Fuji Film, Japan) for analysis.

Statistical analysis

Data were expressed as mean \pm standard error of the mean (SEM). The statistical differences were analyzed by analysis of variance (ANOVA) followed by the Newman-Keuls method as post-hoc analysis with the Prism 4 program (GraphPad, USA). P values less than 0.01 were defined as statistically significant.

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