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FULL PAPER

Expression of α-hemoglobin stabilizing protein and cellular prion protein in a subclone of murine erythroleukemia cell line MEL

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Abstract

α-Hemoglobin stabilizing protein (AHSP) functions as the erythroid-specific molecular chaperon for α-globin. AHSP gene expression has been reported to be downregulated in hematopoietic tissues of animals suffering from prion diseases though the mechanism remains to be clarified. Herein, we demonstrate that MELhipod8 cells, a subclone of murine erythroleukemia (MEL) cells, have prion protein (PrP^C) on the cell surface and have highly inducible expression of the AHSP and α- and β-globin genes, resembling the expression pattern of the PrP and AHSP genes in bipotential erythroid- and megakaryocyte-lineage cells followed by erythroid differentiation in normal erythropoiesis. Moreover, MELhipod8 cells exhibit greater effective erythroid differentiation with a population of hemoglobinized normoblast-like cells than that observed for the parental MEL cells. These findings suggest that MELhipod8 cells could provide a mechanism for downregulation of the AHSP gene in prion diseases.

Key Words: α-hemoglobin stabilizing protein, erythroid, murine erythroleukemia, prion protein

Introduction

α-Hemoglobin stabilizing protein (AHSP) is an erythroid-specific chaperon protein that specifically binds and stabilizes the α-subunit of hemoglobin[10]. AHSP neutralizes harmful effects of free α-hemoglobin (α-globin plus heme) in normal erythropoiesis and under disease conditions[10,12]. A study by Miele *et al.*[13] showed that the AHSP gene expression was specifically downregulated in the spleen, bone marrow and blood of animals suffering from transmissible spongiform encephalopathies (TSEs, prion diseases), suggesting that AHSP could be a diagnostic marker for the asymptomatic phase of TSEs[1,13] for which no tests are currently available in live animals[1]. However, a mechanism whereby TSE disease could affect AHSP expression levels remains to be proved.

A previous study also demonstrated that erythroid precursor cells expressed the prion protein (PrP) and AHSP genes at different levels with hematopoietic development[13]. *PrP* transcripts

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were expressed in a number of hematopoietic progenitor cells including the cells capable of erythroid, megakaryocyte and macrophage development and the bipotential cells capable of developing along either the erythroid or megakaryocyte lineage (E/Meg)[13]. On the other hand, *AHSP* expression is found in E/Meg cells and is thereafter confined to the erythroid lineage with higher levels of expression in blast-forming (BFU-E), colony-forming (CFU-E) and maturing erythroid cells[13]. The colocalization of *AHSP* and *PrP* expression in the bipotential E/Meg cells and the subsequent increase in *AHSP* expression thereafter indicate that the cells with characteristics of E/Meg cells might provide a clue to the mechanism by which TSEs could affect AHSP expression levels[13].

Murine erythroleukemia (MEL) cells resemble proerythroblasts morphologically and mature in culture to a normoblast-like stage[6]. MEL cell differentiation closely resembles the process of normal murine erythropoiesis[7]. MEL cells therefore might be useful as a model to investigate the mechanism for the downregulation of *AHSP* expression in TSEs if they express PrP protein. The purpose of the present study was to examine the expression of *PrP* and *AHSP* and the change in expression levels of these genes during terminal erythroid differentiation in MEL cells.

MEL cells differ in globin gene expression and thus have different responses between clones due to the phenotype of the clone's characteristic probability of differentiation, that is, the likelihood that a cell of this clone will undergo erythroid differentiation under given conditions[14]. Therefore, in the present study, we first isolated a clone of MEL cells that displayed hemoglobin synthesis at a higher level than parental cells. Then we analyzed *PrP* and *AHSP* expression induced in this clone, MEL-hipod, i.e. MEL cells with high probability of differentiation, by the chemical agent hexamethylene bisacetamide (HMBA)[16].

Materials and Methods

Preparation of recombinant murine AHSP and anti-mouse AHSP antibody: Full-length AHSP cDNA was amplified using the primer pair 5'-GGATCCATGGCCCCTTTTCAGAGCAATAAG-3' and 5'-CTCGAGTTATGAGGAGGGCAGTGTATTGC-3' designed based on the mouse *AHSP* sequence in a database (GenBank accession number, AF364516). These primers included *Bam* HI (underlined sequence) and *Xho* I (boldface sequence) restriction sites, respectively. The cDNA fragment obtained was cloned into the TA cloning vector pCRII (Invitrogen), and then subcloned into the pGEX-6P-1 vector (GE Healthcare Bioscience) using the *Bam* HI and *Xho* I restriction sites to create a pGEX-AHSP vector. *Escherichia coli* BL21 cells were transformed with the pGEX-AHSP plasmid and recombinant AHSP fused to glutathione S-transferase at the C-terminal side (GST-AHSP) was produced in the presence of 0.1 mM isopropyl-1-thio-D-galactopyranoside at 37℃ for 4 hours. The GST-AHSP was detected in the soluble fraction of bacterial cell lysates prepared in B-PER bacterial protein extraction reagent (Pierce Biotechnology). Recombinant protein captured in a glutathione-Sepharose column was digested with PreScission protease (GE Healthcare Bioscience) and recombinant AHSP was obtained in the eluates from the column. The recombinant AHSP was further applied to a gel permeation chromatography column of Superdex 75 HR 10/30 (GE Healthcare Bioscience). Figure 1A shows the protein contents in each purification step for recombinant mouse AHSP and demonstrates that recombinant AHSP was highly purified (Fig. 1A, *lane 4*). Mass spectrometric analysis using Bruker autoflex time-of-flight mass spectrometer (Bruker Daltonics) demonstrated that the molecular mass of the purified polypeptide was 12,237.6 ± 4.0 Da (n=4), in agreement with the theoretical mass of 12,243.6 Da for the recombinant murine AHSP (data not shown).

A female New Zealand white rabbit was immunized with 100 μg of purified mouse recombi-

nant AHSP emulsified with Freund's complete adjuvant (Rockland Immunochemicals) to produce anti-mouse AHSP polyclonal antiserum. The rabbit was boosted twice with 100 μg of recombinant AHSP mixed with Freund's incomplete adjuvant (Rockland Immunochemicals) every two weeks. The experimental protocols for the use of laboratory animals were approved by the Institutional Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University. The antibody was purified by affinity chromatography on a Protein G-Sepharose column (GE Healthcare Bioscience), followed by dialysis against phosphate-buffered saline (PBS, pH 7.4) supplemented with 0.05% NaN_3.

Immunoblotting analysis showed that the antibody obtained reacted with a 12-kDa polypeptide from a mouse bone marrow cell extract that was similar in size to the recombinant murine AHSP (Fig. 1B). The signal disappeared when the reaction was carried out in the presence of excessive amount of recombinant AHSP, indicating that the antibody specifically recognized murine AHSP with the size of 12 kDa. AHSP was also detected in homogenates from spleen and peripheral blood but not in the homogenates from liver, brain, heart and lung (data not shown).

Culture and cloning of MEL cells: Murine erythroleukemia (MEL) cells were grown in minimum essential medium (MEM, Sigma) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma) at 37℃ under 5% CO_2 in air. MEL cells were suspended in the medium at the density of 0.2 cells/200 μl/well, and cultured in 96-well plates for 14 days. Several sin-

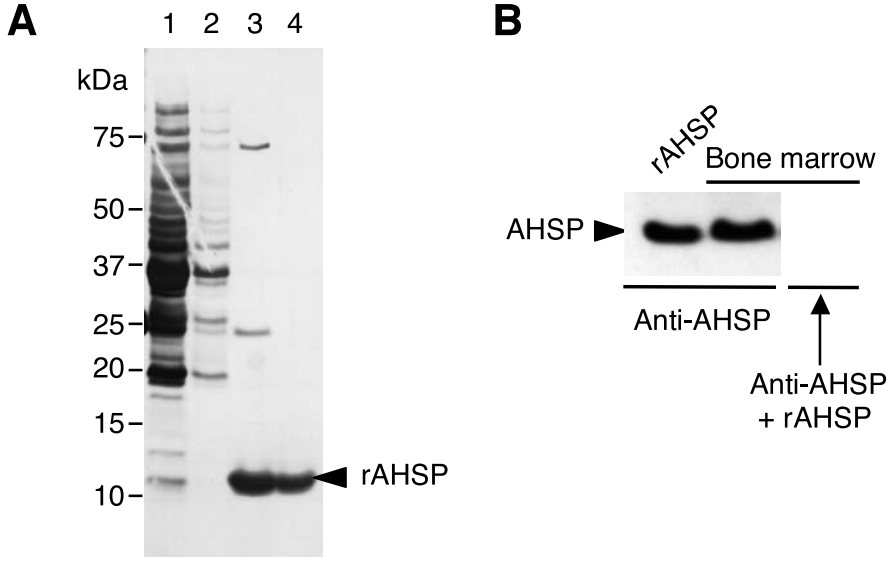

Fig. 1. Preparation of recombinant murine AHSP and the anti-AHSP antibody.
A, SDS-PAGE analysis for protein contents in each purification step for recombinant murine AHSP. Polypeptides in the bacterial lysates containing the GST-fused murine AHSP (*lane 1*) were applied onto a glutathione-Sepharose column and separated into the flow-through fraction (*lane 2*) and eluates after cleavage of polypeptides bound to the resin with the PreScission protease (*lane 3*). The recombinant AHSP (*rAHSP*) with the size of 12 kDa in the eluates was further purified on a gel permeation chromatography column of Superdex 75 HR (*lane 4*). Polypeptides were separated on 10% SDS-gel and visualized by silver staining. Migrating positions of marker proteins are indicated in kDa. B, immunoblotting detection of the recombinant AHSP and the AHSP in bone marrow cell extracts. Recombinant AHSP (*rAHSP, 10 ng/lane*) and proteins from mouse bone marrow cells (*Bone marrow, 60 μg/lane*) were separated by SDS-PAGE followed by immunoblotting detection for AHSP with the anti-AHSP antibody. The immunoreactive signal disappeared when the antibody was incubated with an excessive amount of recombinant AHSP (*Anti-AHSP + rAHSP*).

gle colonies were grown in 1 ml of MEM for 1 week and the isolated clones thus obtained were expanded in suspension cultures. The cells were incubated in the presence of 5 mM N,N'-hexamethylene bisacetamide (HMBA, Sigma) to induce erythroid differentiation. At 96 hours after induction, cells were stained with benzidine to detect hemoglobin[14]. The clones that exhibited marked hemoglobin production were selected and designated MELhipod cells for their characteristic high probability of differentiation. In the present study, one of these clones, MELhipod8, which showed that most potent synthesis of hemoglobin upon induction with HMBA, was used and analyzed. For morphological analysis, cells were collected onto slides using a Cytospin (Shandon) for Giemsa staining.

Quantitative PCR: Total RNA was isolated from MELhipod cells using an SV RNA isolation kit (Promega Corp.). cDNA was synthesized from 1 μg of total RNA using oligo$(dT)_{12\text{-}18}$ as primers and SuperScript III reverse transcriptase (Invitrogen) as described[9]. For quantitative PCR, SYBR Green PCR Master Mix (Perkin-Elmer Applied Biosystems) was used with 200 nM forward and reverse primers in a final volume of 25 μl for each reaction. The primers used for the quantitative PCR are listed in Table 1. Each sample was run in triplicate. The PCR was performed using the GeneAmp 5700 sequence detection system (Perkin-Elmer Applied Biosystems) with 40 cycles of 1 min at 95℃, 15 sec at 60℃, and 15 sec at 72℃ for all target genes. Copy numbers of the transcripts were estimated from standard curves obtained using plasmid clones of PrP, AHSP, and α- and β-globin cDNAs and were normalized with the copy number of the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) transcript.

Immunoblotting and immunofluorescence microscopy analyses: MELhipod cells were washed in ice-cold PBS and lysed in lysis buffer containing 1% Zwittergent 3-14 (Calbiochem), 150 mM NaCl, 50 mM Tris/Cl, pH 7.5 supplemented with protease inhibitors (1 μg/ml pepstatin, 2 μg/ml leupeptin, 1 μg/ml aprotinin and 10 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, all from Sigma). Protein concentrations of the lysates were determined using a DC protein assay kit (Bio-Rad). Proteins in lysates were separated by Tricine SDS-PAGE[17] followed by immunoblotting as described previously[9]. For detection of AHSP and normal cellular PrP (PrP^C), an anti-AHSP polyclonal antibody and anti-mouse PrP monoclonal antibody 31C6[11] (kindly provided by Dr. M. Horiuchi, Hokkaido University) were used, respectively.

For immunofluorescence analysis, MELhipod cells were collected onto slides using Cytospin centrifugation. The cells fixed with 100% methanol were blocked with 1% (w/v) bovine serum albumin (BSA) in PBS for 1 hr at ambient temperature. Cells were incubated with the appropriate antibody in the same solution for 1 hr at ambient temperature, washed with PBS, and then incubated with the secondary antibody, Alexa Fluor 488 goat anti-rabbit IgG or Alexa Fluor 568 goat anti-mouse IgG (Invitrogen) for 1 hr at ambient temperature in the dark. After washing with PBS, cells were incubated with 300 nM 4'6-diamidino-2-phenylindole (DAPI) in PBS for nuclear staining, mounted in ProLong antifade reagent (Invitrogen), and then examined under a Nikon ECLIPSE E800 microscope.

Table 1. Primers for quantitative PCR

Genes	Forward primer (5'→3')	Reverse primer (5'→3')
PrP	GATCCATTTTGGCAACGACT	TCATCTTCACATCGGTCTCG
AHSP	GCTGGATCAGCAGGTCTTTGA	TGAGGAGGGCAGTGTATTGCT
α-globin	CTGAAGCCCTGGAAAGGATGT	GGCTCAGGAGCTTGAAGTTGA
β-globin	ACCCAGCGGTACTTTGATAGC	CCTGAAGTTCTCAGGATCCACAT
GAPDH	GAAGGTCGGTGTGAACGGATT	GAAGACACCAGTAGACTCCACGACATA

Results

Hemoglobin synthesis in MELhipod cells

We obtained several independent clones of MEL cells that exhibited different responses to erythroid differentiation induced by HMBA, with levels of hemoglobin synthesis higher than those observed for cells induced from the parental MEL cells. We designated those clones MELhipod for their high probability of differentiation[14] into hemoglobinized cells. We extensively characterized the expression of several genes in MELhipod8, the MELhipod clone with the highest level of hemoglobin production at 96 hr after HMBA induction.

MELhipod8 cells showed no obvious changes in α- and β-globin gene expression until 24 hr after induction with HMBA (Fig. 2A). Increases in the relative abundance of α- and β-globin gene transcripts were observed at 48 hr after induction and were accelerated thereafter. MELhipod8 cells were predominantly large cells, resembling proerythroblasts morphologically (Fig. 2B) as reported for parental MEL cells[6]. Few cells were positive for hemoglobin at this point, which was compatible with the finding that there was no detectable globin gene expression, as described above (Fig. 2A). Hemoglobin-positive cells were observed at 48 hr after induction with HMBA and increased exponentially (Fig. 2C) untill 96 hr after induction, whereas only a small population of the cells incubated in the absence of HMBA were positive for hemoglobin even at 96 hr of erythroid differentiation (Figs. 2B and 2C). The MELhipod8 cells at 96 hr of induction were smaller and had highly condensed nuclei, resembling normoblast morphology, whereas most cells without HMBA induction retained the proerythroblast feature (Fig. 2B). After 96 hr of erythroid differentiation, 70% of the MELhipod8 cells were positive for hemoglobin, which was more than twice the population of hemoglobin-positive cells derived from the parental MEL cells (Fig. 2C).

Expression of PrP and AHSP in MELhipod cells

Next, we examined if the MELhipod cells could express the *PrP* and *AHSP* genes spontaneously or in response to induction of erythroid differentiation. Figure 3A demonstrates that MELhipod8 cells had PrP RNAs and that the levels of the transcripts were rapidly decreased once to about 20% and then arrested at 30% of the basal level observed for the cells without incubation by induction of erythroid differentiation, whereas those levels in uninduced cells showed a recovery to the basal level at 72 hr of incubation after a temporary reduction in the first 12 hr. In contrast, MELhipod8 cells contained a low level of AHSP RNA and exhibited a marked increase in the relative abundance of *AHSP* transcripts when incubated in the presence of HMBA. At 48 hr after induction, the *AHSP* expression reached a level similar to that observed at 72 hr, whereas no significant increase in the *AHSP* expression was noted in uninduced cells (Fig. 3A). Thus, the expression of *AHSP* in MELhipod8 cells appeared to precede the expression of the α- and β-globin genes, which showed remarkable increases from 48～72 hr after induction (Fig. 2A).

In immunoblotting, multiple polypeptides that reacted with the anti-PrP antibody were found in MELhipod8 cells and their sizes (27～39 kDa) were larger than those detected in brain tissues, presumably due to differences in the oligosaccharide structure (Fig. 3B). The contents of PrP judged by signal intensities showed a reduction during the induced differentiation for 5 days. Interestingly, there was a decrease in the signal intensities of 27-, 29-, and 37-kDa polypeptides and a subsequent increase in that of the 39-kDa band, suggesting a change in the process of glycan modification. Furthermore, immunofluorescence microscopy revealed that PrP localized to the plasma membrane of MELhipod8 cells (Fig. 3C-a). Incubation of living cells with the anti-PrP antibody caused condensation or agglutination of the fluorescence signals (Fig. 3C-b) that was similar to the "capping" phenomenon reported for PrP in lymphoid cells[21], indicating that the PrP actually localized at the cell surface in MELhipod8 cells.

In contrast, AHSP was detected at 48 hr after

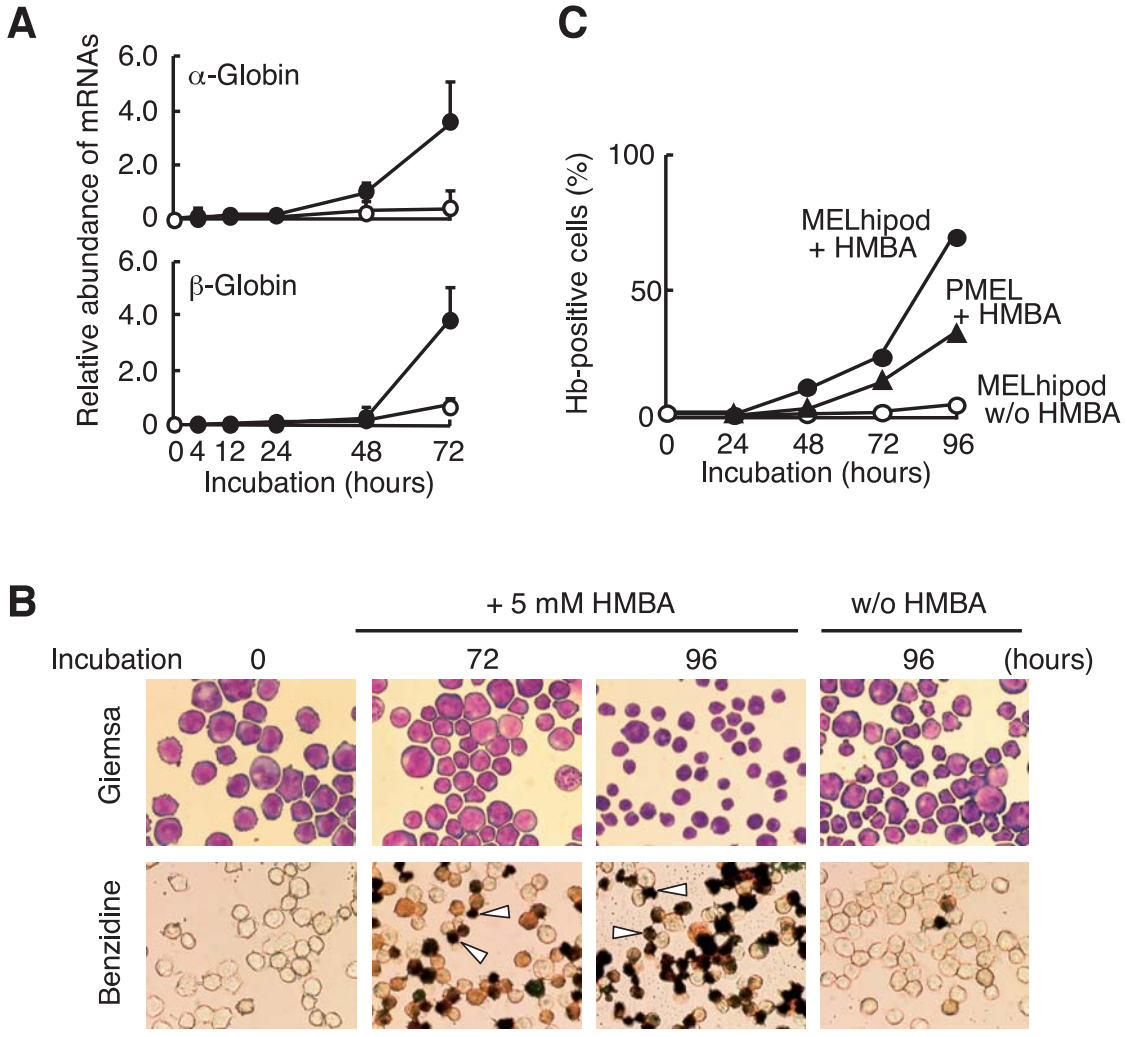

Fig. 2. Characterization of HMBA-induced erythroid differentiation of MELhipod8 cells.
A, the levels of α- and β-globin gene transcripts in MELhipod8 cells incubated for the indicated time periods in the presence (●) or absence (○) of 5 mM HMBA. The α- and β-globin RNAs were examined by quantitative PCR and are expressed as the abundance relative to that of GAPDH (mean ± S.D., n=4). B, changes in morphology and hemoglobin production of MELhipod8cells were examined by Giemsa and benzidine staining, respectively, after incubation in the presence (*+ 5 mM HMBA*) or absence (*w/o HMBA*) of HMBA for the indicated periods. C, the populations of hemoglobin (*Hb*)-positive MELhipod8 cells (*MELhipod*) and parental cells (*PMEL*) incubated in the presence (*+HMBA*) or in the absence (*w/o HMBA*) of HMBA for the indicated periods were counted as described above and are shown in %.

induction and the signal intensity of the 12-kDa band was remarkably increased, reflecting the elevated expression level (Fig. 3B). Compatible with this observation, immunofluorescence signals for AHSP were observed in the cytoplasm of MELhipod8 cells that were incubated for 48～72 hr in the presence of HMBA, whereas no such signals were detected in the cells without induction to erythroid differentiation (Figs. 3C-c and 3C-d). These results were in agreement with the previous findings that expression of AHSP was found in the bipotential E/Meg cells and with higher levels in BFU-E and CFU-E cells[13] preceding expression of the globin genes.

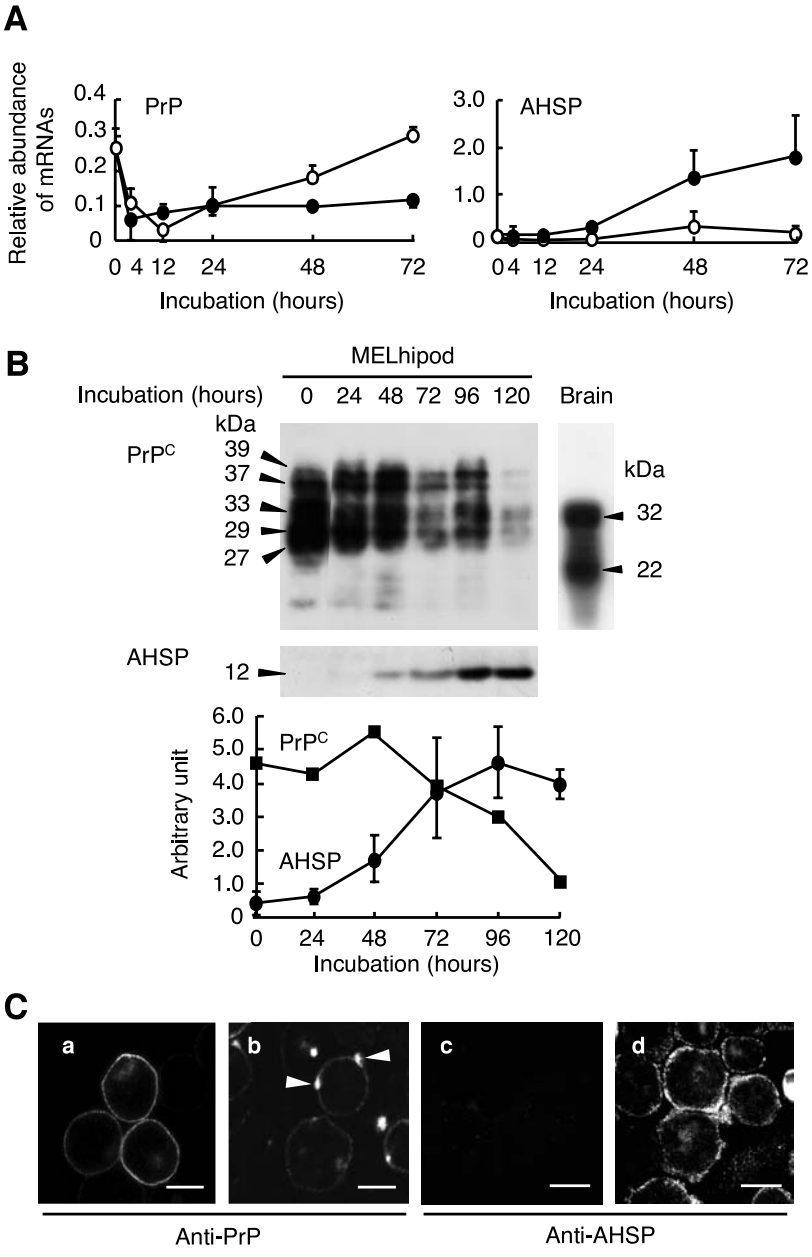

Fig. 3. Expression of *PrP* and *AHSP* in MELhipod8 cells.
A, relative levels of PrP and AHSP RNAs in MELhipod8 cells incubated with (●) or without (○) 5 mM HMBA for the indicated periods were estimated by quantitative PCR and are expressed as the abundance relative to that of GAPDH (mean ± S.D., n=4). B, expression of cellular PrP (*PrP*^{c}) and AHSP in MELhipod8 cells incubated in the presence of HMBA for the indicated periods were analyzed by immunoblotting. An immunoblot for PrP^{c} in murine brain tissue is also shown. The sizes of multiple PrP^{c} polypeptides and AHSP are shown in kDa. The lower panel shows the changes in relative contents of PrP^{c} and AHSP during erythroid differentiation determined by densitometric scanning of the immunoblots. The values in arbitrary units are expressed as the mean values of two independent experiments for PrP^{c} (■) and the means ± S. D. from four independent experiments for AHSP (●). C, intracellular localization of PrP^{c} (*a* and *b*) and AHSP (*c* and *d*) was examined by immunofluorescence microscopy in MELhipod8 cells after 72 hr of incubation with (*a, b,* and *d*) or without (*c*) HMBA. Incubation with the anti-PrP or anti-AHSP antibodies was carried out for cells fixed with methanol (*a, c,* and *d*) and live cells (*b*). Arrowheads in *b* indicate the "capping"-like condensation of PrP^{c}. Bars = 10 μm.

Discussion

A study by Miele *et al.*[13] showed a progressive reduction in transcript levels of AHSP, originally reported as EDRF, erythroid differentiation-related factor, in mice after exposure to TSEs as well as decreased levels of *AHSP* expression in infected cattle and sheep. That was the first demonstration of a TSE-induced effect on gene expression outside the central nervous system, and may be relevant to some reports on the detectable infectivity in bone marrow from cattle in the clinical stage of BSE[24] and in whole blood from BSE-infected sheep in the preclinical phase[8]. However, the mechanism by which TSE diseases could affect the expression levels of AHSP, which is specific to erythroid lineage cells, is unclear. It is also unknown whether *AHSP* downregulation is a prion-specific phenomenon, or whether it occurs in other diseases *via* nonspecific inhibition of marrow erythroid progenitors. One possibility is that the cells expressing AHSP play some role in replication of TSE agents or TSE disease pathogenesis. This may require the expression of cellular PrP (PrP^C) in AHSP-expressing cells, because the presence of PrP^C is a prerequisite for TSE pathogenesis[3].

Our present study demonstrated that MELhipod8 cells expressed PrP^C at the cell surface and had highly inducible expression of the AHSP gene with HMBA (Fig. 3), resulting in a high population of differentiated normoblast-like cells containing hemoglobin (Fig. 2). A previous study demonstrated that expression of *AHSP* and *PrP* was colocalized in bipotential E/Meg cells in mice[13], followed by upregulation of *AHSP* expression by the essential erythroid transcription factor GATA-1 in late erythroid precursor cells[10,19]. Taken together, our data indicate that MELhipod8 cells possess characteristics of gene expression similar to those in cells at the E/Meg stage, in agreement with MEL cells constitutively and inducibly expressing megakaryocyte-specific genes[22], although MEL cells resemble proerythroblasts morphologically[6]. Our findings therefore suggest that MELhipod8 cells are suitable to investigate the replication or the effect of TSE agents on the expression of *AHSP*.

An alternative mechanism to be examined is that TSEs affect erythroid cells and *AHSP* expression through interaction of the TSE agent with macrophages that are involved in maturation of erythroid cells[2] and capture of the TSE agent[4]. A possible approach involves determination of the effects of several proinflammatory cytokines that have been shown to be elevated in TSEs[18,20,23] on the expression of *AHSP* in MELhipod cells.

The MELhipod8 cell clone was one of several clones, each of which had a distinct response to erythroid differentiation induced by the compound HMBA. Some of these clones displayed larger populations of hemoglobinized cells than that observed for the parental MEL cells (Fig. 2), whereas several other clones exhibited no detectable hemoglobin production with negligible expression of erythroid-specific genes, including the AHSP and β-globin genes (data not shown). The phenotype of MEL cells transformed with Friend virus[6] was recognized as the clone's probability of differentiation[14]. Subsequent studies have demonstrated that several genes, including *myc, myb,* and *PU.1,* are involved in terminal erythroid differentiation and phenotypic differences of MEL cells[5,15]. However, the exact regulatory mechanisms have not been clarified yet.

In conclusion, the present study established a subclone of MEL cells, MELhipod8 and has demonstrated that MELhipod8 cells are suitable to investigate the mechanism for downregulation of *AHSP* expression in prion diseases.

Acknowledgements

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Excellence of Zoonosis Control (M. I.).

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