

**INDUCTION OF HEAT SHOCK PROTEINS AND ITS EFFECTS ON GLIAL
DIFFERENTIATION IN RAT C6 GLIOBLASTOMA CELLS**

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KEY WORDS

heat shock protein 70 (HSP70); heme oxygenase-1 (HSP32); platelet-derived growth factor (PDGF); glial cells; differentiation; C6

ABSTRACT

Heat shock proteins (HSPs) are immediately expressed in neuronal and glial cells under various stressful conditions and play a protective role through molecular chaperones. We investigated the characteristics of the induction manner of heme oxygenase-1 (HO-1) and HSP70 in rat C6 glioblastoma cells. In heat treatment (42°C for 30 min), C6 cells expressed high level of HO-1 and HSP70 mRNAs Within 30-60 min, and their proteins at 3 hrs. Heat-induced expressions of HSPs mRNAs were completely inhibited with actinomycin D, suggesting the transcriptional regulation. Oxygen-glucose deprivation (OGD), cystine-free (inhibition of synthesis of glutathione), cyto-toxic (ethanol, sodium butyrate) treatments resulted in different expression manners between HO-1 and HSP70, which suggested that HO-1 and HSP70 play different protective roles against a variety kind of stressful conditions in glial cells.

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C6 cells can differentiate toward both astrocyte and oligodendrocyte directions. Treatment with dibutyryl cyclic AMP (cAMP) induces expression of glial fibrillary acidic protein (GFAP), a marker of astrocytes, and treatment with retinoic acid (RA) induces expression of myelin proteolipid protein (PLP), a marker of oligodendrocytes, respectively.

Heat treatment before the initiation of differentiation by RA reduced the RA-induced expression of PLP mRNA profoundly, but not in GFAP mRNA level induced by cAMP. Heat treatment after the initiation of differentiation by cAMP or RA accelerated the expression of GFAP or PLP mRNAs. Astroglial differentiation by cAMP reduced the heat-induced expressions of HSPs mRNAs, but no change with RA pre-treatment. These results suggested that HSPs may modulate the glial differentiation in the developing brain. On the contrary, glial differentiation may give influence on the stress-induced HSPs expression. The timing of stressful damages, resulting in the expression of HSPs, on the developing brain is critically important for the pathogenesis of glial lesion.

In the heat-treated C6 cells, the expression of platelet-derived growth factor (PDGF) receptor- α mRNA was significantly decreased. HSPs may have ability to induce the glial differentiation in part through down-regulation of the PDGF pathway.

INTRODUCTION

Heat shock proteins (HSPs) have attracted increasing attention in neuroscience because of their rapid induction in the central nervous system (CNS) following different stressful conditions. Heme oxygenase-1 (HO-1), an inducible form of HO, catalyzes the rate-limiting step in heme degradation yielding biliverdin which is subsequently reduced to form bilirubin. These products play powerful anti-oxidant effects, and HO-1 is thought as a possible candidate determining secondary damage formation. HO-1, with a heat shock element in the promoter region, has a character of HSP (also known as HSP32)¹⁾ Heat stress induces an array of different HSPs among which the HSP70 family is the most effective in chaperoning²⁾ and the most conserved in evolution. HSP70 plays a major role as 'molecular chaperones' in folding, assembly, and translocation of cytoplasmic proteins.^{2, 3)}

Both HO-1 and HSP70 have protective function to CNS against stressful conditions. Heat stress (hyperthermia) induces the expression of HO-1^{4, 5)} and HSP70⁶⁻⁸⁾ in vivo. They are also induced to express through ischemic treatment⁹⁻¹⁴⁾, traumatic injuries,^{15, 16)} and intracerebral hemorrhage.^{17, 18)} Also

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in cultured neuronal and glial cells HSPs are induced to express against heat,^{1, 7, 19-22)} oxidative stress,²³⁾ energy depletion,²⁴⁾ and cyto-toxic treatment.^{19, 25, 26)}

Besides the protective effects, HSPs are thought to have important roles in development and differentiation.²⁷⁾ The more cells have differentiated, the more cells are prone to lose the inducibility to express HSPs against stressful conditions.^{19, 28)} Protective effects of HSPs are in part through anti-apoptotic function, which may result in modulating the cellular differentiation.^{2, 3, 25, 29-32)}

In the present study, we analyzed the different characteristics of the induction manners of HO-1 and HSP70 in C6 cells. We further studied the effects of heat-induced HSPs on the expressions of glial genes, and found that HSPs can modulate glial differentiation. On the contrary, differentiated glial cells decrease the ability to express HSPs against heat. We opened discussion that HSPs are involved in perinatal brain damages through the modulation of glial differentiation.

MATERIALS AND METHODS

Materials

Rat glioblastoma cell line, C6, was obtained from Japanese Cancer Research Resources Bank, and used at the same cell-passages in an experiment. Cell culture medium RPMI 1640, and its modified medium depleting cystine, glutamine, and methionine (Sigma, R7513), dibutyryl cyclic AMP (CAMP), all-trans retinoic acid (RA), actinomycin D (ActD), cycloheximide (CH), L-glutamine were from Sigma (St. Louis, MO). Sodium butyrate was from Wako Pure Chemicals (Osaka, Japan).

Parts of cDNAs for rat HO-1 (736bp), HSP70 (922bp), glial fibrillary acidic protein (GFAP) (1102bp), myelin proteolipid protein (PLP) (907bp), platelet-derived growth factor (PDGF)-A chain (589bp), PDGF receptor-a (893bp) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (534bp) were designed with RT-PCR and subcloned into the pGEM-T vectors (Promega, Madison, WI). Partial cDNA fragments cut with appropriate restricting enzymes from the plasmids were used as probes for Northern blots.

Cell culture

C6 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and appropriate antibiotics and antimycotics at 37°C in humidified 5% CO₂-95% air atmosphere. Heat treatment was introduced using an incubator maintained at 42°C for 30 min. Hypoxia treatment was introduced

using an incubator containing less than 1% O₂. Glucose deprivation was introduced with replacement of culture medium to EBSS (Earle's Balanced Salt Solutions) buffer. Cystine-deprivation was introduced with replacement of culture media to the modified RPMI 1640 medium lacking cystine and methionine, but supplemented with 2 mM glutamine.

Northern blot

Total RNA fraction was obtained from cultured cells by the acid-guanidine-phenol-chloroform method. Northern blot analysis was performed using the ExpressHyb™ hybridization solution (CLONTECH, CA) with the manufacture's protocol. CDNAS Were labeled with [α -³²P]dCTP using the Megaprime DNA Labelling System (Amersham LIFE SCIENCE, Buckingham-shire, England). The hybridized membranes were washed appropriately and exposed to a Fuji RX-U film with an intensifying screen at -80°C. Quantification of the indicated bands of interests was performed using a TLC scanner densitometry (Shimadzu CS-930, Kyoto, Japan).

Western blot

Cultured C6 cells were collected with PBS containing 1 mM EDTA, and then lysed and homogenized in 5 x vols of lysis buffer (0.02% Tris-HCl, pH6.8, 0.04% EDTA, 1% Triton X-100, 1 mM PMSF). Aliquots were centrifuged at 12,000 g for 20 min at 4°C. The supernatants were subjected to 10% or 12.5% SDS/polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Immobilon™, Millipore, Bedford, MA). The membranes were incubated with a rabbit polyclonal anti-rat H0-1 antibody (StressGen, Victoria, BC, Canada), a mouse monoclonal anti-human HSP70 antibody (StressGen, Victoria, BC, Canada), and a rabbit polyclonal anti-human GFAP antibody (Progen Biotechnik, Heidelberg, Germany). After washes, the blots were incubated with a horseradish peroxidase-conjugated goat antibody against rabbit or mouse IgG, and immuno-complexes were visualized using an ECL chemiluminescence system (Amersham).

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RESULT

HSPs induction by heat treatment (Fig. 1)

HO-1 mRNA was detected as a monoband at 1.8-kb and HSP70 mRNA as double bands at 2.8- and 3.0-kb. Untreated control cells expressed low level of HO-1 mRNA, and the abundance of HO-1 mRNA increased to its peak at 60-120 min after heat treatment. HSP70 mRNA was hardly detectable in untreated cells, but the abundance of HSP70 mRNA increased rapidly at 30 min after heat treatment and reached its peak at 60-120 min. The abundances of HSPs mRNAs started to decline at 3 hrs. There was no apparent change in the abundance of GAPDH mRNA (1.6-kb) by heat treatment.

Addition of 1 µg/ml of ActD at 30 min before heat treatment completely inhibited the increases in HO-1 and HSP70 mRNAs levels induced by heat treatment. Addition of 10 µg/ml of CH at 30 min before heat treatment decreased the abundance of HSPs mRNAs induced by heat treatment, but not complete inhibition. There was no apparent change in the abundance of GAPDH mRNA by treatment with ActD or CH.

HO-1 protein was detectable as a monoband at around 32-kD, and its abundance increased rapidly at 3 hrs, reaching its peak at 12 hrs after heat treatment. HSP70 protein was faintly detected in untreated control cells, but its abundance increased very rapidly at 3 hrs, reaching its peak at 12 hrs as a monoband at around 72-kD.

Effects of various stressful conditions on the expression of HSPs mRNA (Fig. 2)

Oxygen-glucose deprivation (OGD) induced the expression of HSP70 mRNA within 1 hr and reached the level of 200% compared with untreated control cells. The abundance of HO-1 mRNA did not change with OGD treatment. Oxygen or glucose deprivation only did not affect the HSPs mRNA levels (data not shown).

Replacement of culture medium to cystine-free prevents the synthesis of glutathione that is important as an anti-oxidant agent for viable cells. HO-1 mRNA level increased at 6 hrs with treatment of cystine-free medium. HSP70 mRNA level decreased profoundly at 3-6 hrs with cystine-free treatment.

Addition of ethanol to the culture medium increased the abundance of HO-1 and HSP70 mRNAs With 1%/vol dose at as quickly as 1 hr.

Sodium butyrate (1 mM) increased the abundance of HSP70 mRNA at 3-6 hrs. HO-1 mRNA level did not change with sodium butyrate.

The abundances of GAPDH did not change with these treatments. suggesting the equal loading of total RNAs.

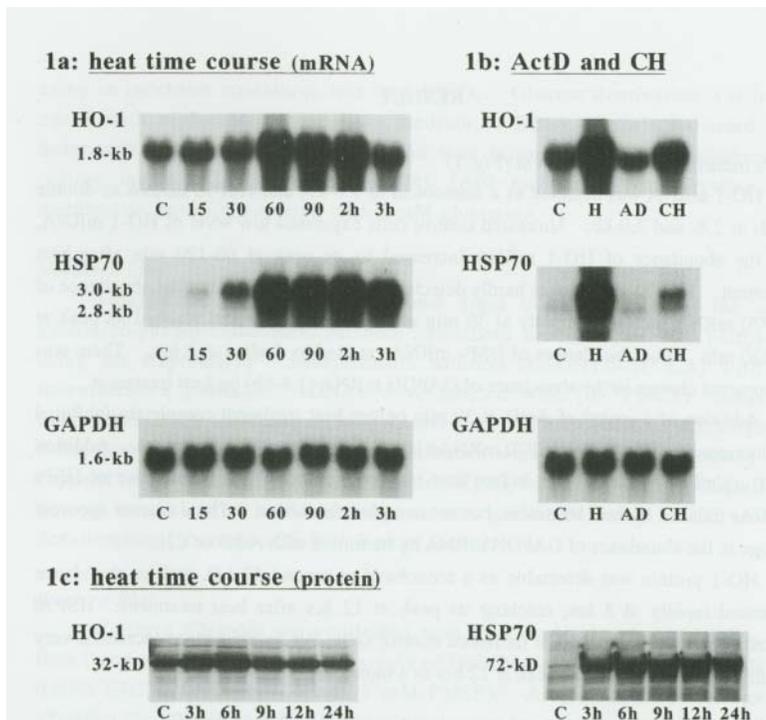


Fig. 1. HO-1 and HSP70 induction by heat treatment.

1a: Total RNA (20ug) extracted from C6 cells at the indicated time after the initiation of heat treatment (42°C, 30 min) was subjected to northern blot analyses using the HO-1, HSP70, and GAPDH probes.

C: control untreated cells, 15-90; min, h=hours.

The membrane of HO-1 experiment was re-probed with GAPDH probe.

1b: Total RNA (20ug) extracted from C6 cells at 60 min after the initiation of heat treatment (42°C, 30 min) was subjected to northern blot analyses as 1a.

C: control untreated cells, H: heat treatment only, AD: 1 µg/ml of actinomycin D was added to the culture medium at 30 min before heat treatment, CH: 10 µg/ml of cycloheximide was added to the culture medium at 30 min before heat treatment.

The membrane of HO-1 experiment was re-probed with GAPDH probe.

1c: Total protein (40 µg) extracted from C6 cells at the indicated time after the initiation of heat treatment was analyzed by western blot analyses using the anti-HO-1 and anti-HSP70 antibodies. C: control untreated cells, h=hours.

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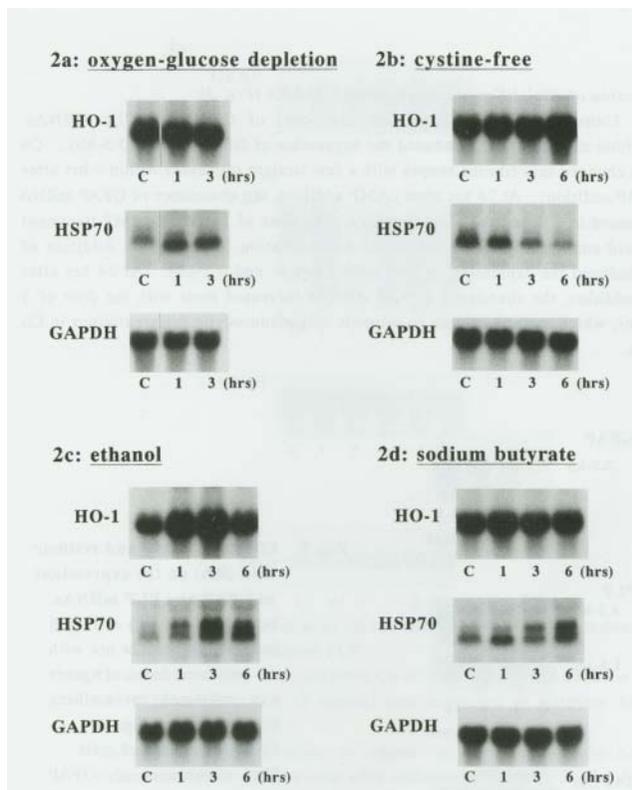


Fig. 2. Effects of various stressful treatments on the expressions of HO-1 and HSP70 mRNAs.

Total RNA (20 μ g) extracted from C6 cells at the indicated time with various treatments was subjected to northern blot analyses as Fig. 1.

C: control untreated cells.

The membrane of HO-1 experiment was re-probed with GAPDH probe.

Induction of glial differentiation by CAMP and RA (Fig. 3)

Untreated C6 cells expressed low level of GFAP and PLP mRNAs. Addition of 1 mM CAMP induced the expression of GFAP mRNA (3.5-kb). C6 cells changed into fibrous shapes with a few straight processes within 6 hrs after CAMP addition. At 24 hrs after CAMP addition, the abundance of GFAP mRNA increased in a dose dependent manner. The dose of 1 mM in CAMP treatment seemed enough to promote astrocytic differentiation in C6 cells. Addition of RA induced the expression of PLP mRNAs (1.6- and 3.2-kb). At 24 hrs after RA addition, the abundance of PLP mRNAs increased most with the dose of 5 µg/ml, which seemed enough to promote oligodendrocytic differentiation in C6 cells.

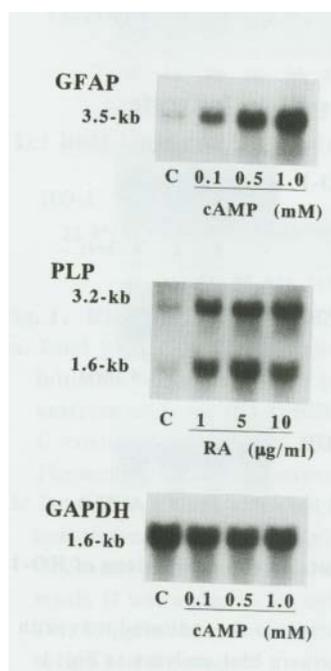


Fig.3.Effects of CAMP and retinoicacid (BA) on the expressions of GFAP and PLP mRNAs.

Total RNA (30 µg) extracted from C6 cells at 24 hrs with the indicated doses of agents was subjected to northern blot analyses as Fig. 1. C: control untreated cells. The membrane of GFAP experiment was re-probed with GAPDH probe.

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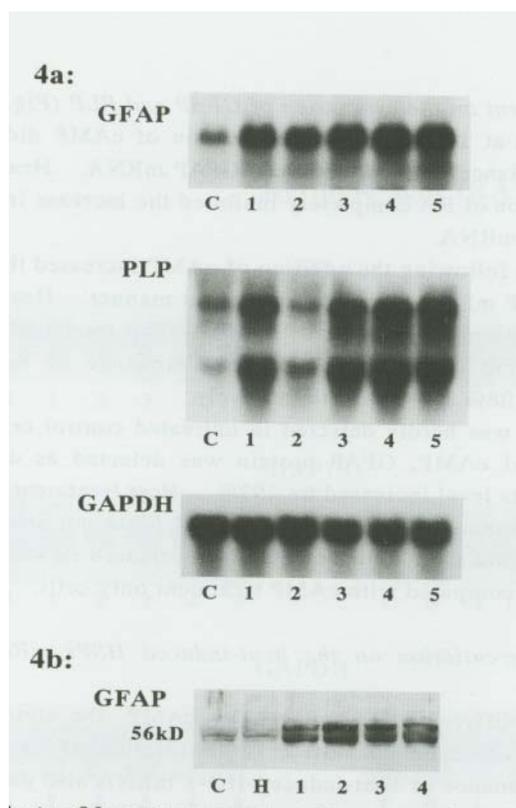


Fig. 4. Effects of heat treatment on the expressions of cAMP-induced GFAP and BA-induced PLP.

4a: Total RNA (30 μ g) extracted from C6 cells at 24 hrs after addition of CAMP (1 mM) or RA (5 μ g/ml) was subjected to northern blot analyses as Fig. 3. C: control untreated cells, 1: agents only, 2 - 5: 30-min heat treatment at the indicated time after addition of agents. 2: -1.5 hrs, 3: 0 hr, 4: +1 hr, 5: +6 hrs.

The membrane of GFAP experiment was re-probed with GAPDH probe.

4b: Total protein (80 μ g) extracted from C6 cells at 24 hrs after addition of CAMP (1 mM) was analyzed by western blot analysis using the anti-GFAP antibody. C: control untreated cells, H: heat treatment only, 1: CAMP only, 2 - 4: 30-min heat treatment at the indicated time after addition of cAMP. 2: 0 hr, 3: +6 hrs, 4: +12 hrs.

Effect of heat treatment on the expression of GFAP and PLP (Fig. 4)

Heat treatment at 1 hr prior to the addition of cAMP did not affect the increase in the abundance of cAMP-induced GFAP mRNA. Heat treatment at 1 hr prior to the addition of RA completely inhibited the increase in the abundance of RA-induced PLP mRNA.

Heat treatment following the addition of cAMP increased the abundance of cAMP-induced GFAP mRNA with time dependent manner. Heat treatment at 6 hrs after cAMP addition increased it by 122%. Heat treatment at 1 and 6 hrs following the addition of RA increased the abundance of RA-induced PLP mRNA (3.2-kb) by 108% and 119%, respectively.

GFAP protein was hardly detected in untreated control cells. At 24 hrs after the addition of cAMP, GFAP protein was detected as double bands at around 56 kD, and its level increased by 197%. Heat treatment before addition of cAMP did not change the GFAP protein level (data not shown). But heat treatment at 0 and 6 hrs after addition of cAMP increased its level by 122% and 124%, respectively, compared with cAMP treatment only cells.

Effect of glial differentiation on the heat-induced HSPs mRNAs expression (Fig. 5)

In astrocytic differentiation induced by cAMP, the abundance of heat-induced HSP70 mRNA decreased most at 12 hrs after cAMP by 41%. In HO-1 expression, the abundance of heat-induced HO-1 mRNA also decreased most at 12 hrs after cAMP by 56%.

In oligodendrocytic differentiation by RA treatment, there was no effect on the abundances of heat-induced HSPs mRNAs.

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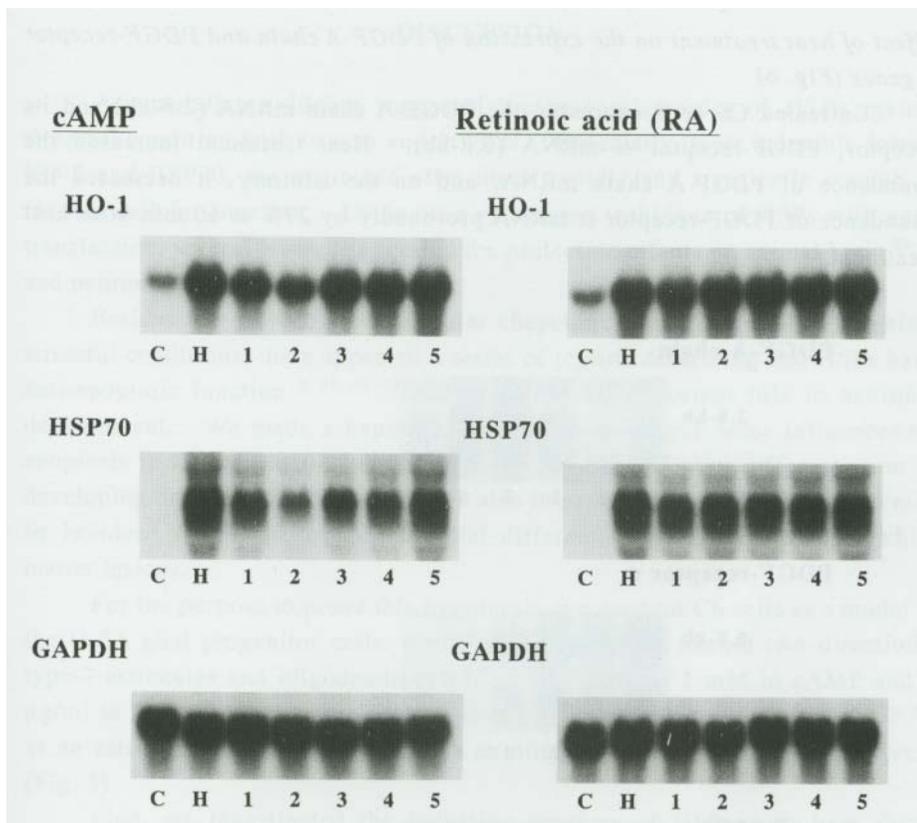


Fig.5. Effects of glial differentiation on the heat-induced HO-1 and HSP70 mRNAs expressions.

Total RNA (20 μ g) extracted from C6 cells at 60 min after the initiation of heat treatment (42°C, 30 min) was subjected to northern blot analyses.

C: control untreated cells, H: heat treatment only,

1 - 5: add CAMP or RA at the indicated time before heat treatment.

1: 24 hrs, 2: 12 hrs, 3: 6 hrs, 4: 3 hrs, 5: 0 hr.

The membrane of HO-1 experiment was re-probed with GAPDH probe.

Effect of heat treatment on the expression of PDGF-A chain and PDGF-receptor α genes (Fig. 6)

Untreated C6 cells expressed the PDGF-A chain mRNA (2.8-kb) and its receptor, PDGF-receptor α mRNA (6.8-kb). Heat treatment increased the abundance of PDGF-A chain mRNA, and on the contrary, it decreased the abundance of PDGF-receptor α mRNA profoundly by 27% at 60 min after heat treatment.

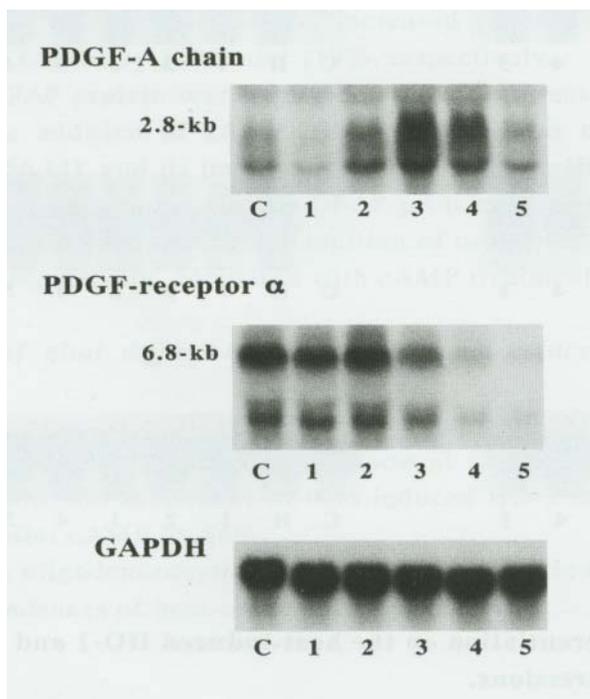


Fig. 6. Effect of heat treatment on the expressions of PDGF A chain and PDGF-receptor α mRNAs.

Total RNA (20 μ g) extracted from C6 cells with various treatments was subjected to northern blot analyses using the PDGF-A chain and PDGF-receptor α probes.

C: control untreated cells, 1: CAMP I mM 24 hts, 2: RA 5 μ g/ml 24 hrs, 3: at 60 min after the initiation of 30-min heat treatment, 4: at 2 hrs after the initiation of 30-min heat treatment, 5: after 2-hrs continuous heat treatment.

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DISCUSSION

Accumulating evidence suggested the preventive roles of HSPs against stressful condition both in vivo and in vitro. Especially, their inducible forms, HO-1 and HSP70, are expressed after insult rapidly and transiently enough to recover cellular function. Using over-expression technique of HSPs with gene transfection, several studies suggested the protective effects on animal brain³³⁻³⁵⁾ and neuronal cells.³⁶⁻³⁸⁾

Besides the protective, 'molecular chaperoning', effects of HSPs against stressful conditions, there appeared a series of reports describing that HSPs have anti-apoptotic function^{2, 25, 30, 32)} and may play an important role in neuronal development. We made a hypothesis that HSPs may give some influences on apoptosis in CNS formation and modulate neuronal and glial differentiation in developing brain. HSPs are expressed also in perinatal brain damages, and may be involved in the disturbance of glial differentiation leading to form white matter lesions.

For the purpose to prove this hypothesis, we used rat C6 cells as a model of the 0-2A glial progenitor cells, which can differentiate toward two directions, type-2 astrocytes and oligodendrocytes.³⁹⁾ The dose of 1 mM in CAMP and 5 µg/ml in RA treatment were adequate enough to induce the expression of GFAP as an astrocyte marker and of PLP as an oligodendrocyte marker, respectively (Fig. 3).

First, we investigated the induction manners of HSPs with heat shock (42°C) in C6 cells. Both HO-1 and HSP70 mRNAs were rapidly and strongly induced to express and also transiently, declining at 3 hrs after heat treatment (Fig. 1a). Untreated cells expressed faint levels of HO-1 and HSP70 mRNAs, and the heat-induced expressions of them were almost completely blocked by ActD, suggesting the transcriptional regulation (Fig. 1b).¹⁾ CH prevented the expressions of HSPs, however, the CH-block of expressions of HSPs were partial, which suggested that nuclear heat shock factor can activate the HSPs transcription without synthesis of de novo proteins. Western blots also showed that the proteins of HO-1 and HSP70 were synthesized rapidly within 3 hrs after heat treatment (Fig. 1c).

We next investigated the effects of other stressful conditions on the inducibility of HSPs (Fig. 2). As the model of hypoxic-ischemic encephalo-pathy, we used OGD and cystine-free medium, which resulted in depletion of glutathione, one of the most important anti-oxidant agents. Treatment with less than 1% oxygen reduced the pO₂ level from 170 mmHg to less than 70 mmHg in

culture medium, which was enough to reduce the cell viability at 6 hrs after OGD (data not shown). The expression of HSP70 mRNA was slightly induced, while the level of HO-1 mRNA did not change at all. Under the condition of anaerobic glycolysis, HSP70 may have a more important role than HO-1 in glial cells.^{38, 40)} Treatment with cystine-free medium induced the expression of HO-1 mRNA. While, the level of HSP70 mRNA did not increase but decreased profoundly. HO-1 can produce anti-oxidants efficiently so that there may exist rational mechanism to enhance the expression of HO-1 gene against oxidative stress.²³⁾

Under the cyto-toxic condition, both ethanol (1%/vol) and sodium butyrate (1 mM), HO-1 and HSP70 mRNAs increased their levels within 1-3 hrs.¹⁹⁾ Both HO-1 and HSP70 have various routes to enhance their expressions of the genes, which are in part dependent on their own enhancer structures on their genes.^{1, 7)} In vivo also glial cells may have HSPs inducibility against a variety of stressful conditions. Over-expression technique proved that expressed HSPs have efficient protective effects on CNS against various stress in vivo, such as ischemic (infarction),³³⁻³⁵⁾ and epileptic damages.³⁴⁾

There appeared a series of reports recently describing the anti-apoptotic function of HSPs in part through chaperoning mechanism. HSPs inhibited the apoptotic cell death of various cell types in *in vitro* studies. HSP70 may rescue cells from apoptosis later in the death signaling pathway.^{30, 32)} Carbon monoxide generated by HO-1 also has anti-apoptotic activity.³¹⁾ They may also show anti-apoptotic function against the programmed cell death occurred in the early stage of CNS development and influence the developmental course of neuron-glia interaction.

We used the heat-shock treatment as a model of transient stressful damage that occurred in developing brain. Heat-shock before the addition of CAMP (astrocytic induction) did not affect the induction of GFAP mRNA and protein. On the contrary, heat-shock before the addition of RA (oligodendrocytic induction) profoundly reduced the level of PLP mRNA (Fig. 4). Heat-shock after the addition of CAMP increased the level of GFAP mRNA, and its effect was the strongest when heat-shock was introduced at 6 hrs after cAMP. PLP mRNA also increased its level when heat-shock was introduced after RA treatment. After the astrocytic differentiation the heat-induced expression of HSP70 mRNA decreased profoundly, and the heat-induced expression of HO-1 mRNA also decreased (Fig. 5). Several studies have reported that HSPs production against stress decreased as the host cells underwent differentiation.^{19, 28)} Differentiation from the 0-2A progenitor cells into the oligodendrocytes

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may be inhibited *in vivo* when stressful damage occurs before the initiation of glial differentiation. Inappropriate induction of the glial differentiation by the stress-induced HSPs may result in imbalanced glial population more toward astrocytes, which may lead to oligodendrocyte depletion in the deep white matter causing periventricular leukomalacia in premature infants. Induction of excessive HSPs against stress in the developing brain may also affect the glial cell integration through anti-apoptotic effect.

Heat-shock decreased the level of PDGF-receptor α mRNA profoundly in C6 cells. PDGF, especially PDGF-A chain and its receptor α have important roles in glial proliferation and differentiation.⁴¹⁾ The bipotential glial progenitor cells (0-2A progenitors), which give rise to oligodendrocytes and type-2 astrocytes, are stimulated to proliferate and controlled the timing of differentiation by PDGF secreted mainly from type-1 astrocytes.⁴²⁾ 0-2A progenitor cells can continue to proliferate with the presence of PDGF-A chain and basic FGF. Down-regulation of PDGF-receptor α may lead to glial differentiation also in C6 cells. HSPs may have function to organize glial differentiation in part through PDGF pathway.

APPENDICES

The abbreviations used are: ActD, actinomycin D; CH, cycloheximide; CNS, central nervous system; cAMP, dibutyryl cyclic adenosine monophosphate; FGF, fibroblast growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; H0-1, heme oxygenase-1; HSP70, heat shock protein70; PDGF, platelet-derived growth factor; PLP, proteolipid protein; RA, all-trans retinoic acid; RT-PCR, reverse transcription-polymerase chain reaction.

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