FEBS Letters 579 (2005) 2416–2420 FEBS 29472

Perchloric acid-soluble protein regulates cell proliferation and differentiation in the spinal cord of chick embryos

Emi Himeno[a], Kunitaka Yamazaki[a], Hiroaki Kanouchi[b], Mitsuharu Matsumoto[a], Yasushi Sugimoto[c], Tatsuzo Oka[a,*]

[a] *Department of Veterinary Medicine, Faculty of Agriculture, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan*
[b] *Department of Biochemistry, Kawasaki Medical School, 577 Matsushima, Kurashiki 701-0192, Japan*
[c] *The United Graduate School of Agricultural Sciences, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan*

Received 13 January 2005; accepted 28 February 2005

Available online 29 March 2005

Edited by Lukas Huber

Abstract The role of perchloric acid-soluble protein (PSP) was investigated in chick embryos. Fluorescently labeled anti-chick liver (CL)-PSP IgG was injected into the yolk sac in ovo at embryonic day 3, and became localized in neuroepithelial cells. Within 12 h, morphological changes were observed in 37.5% of anti-CL-PSP IgG-injected embryos, and the neuroepithelial cells formed a wavy line. No significant changes were observed in embryos injected with non-immune IgG or PBS. Increased expression of PCNA and decreased expression of neuronal class III β-tubulin were observed in the spinal cord after anti-CL-PSP IgG injection. These results suggest that PSP controls the proliferation and differentiation of neuroepithelial cells in chick embryos.

Keywords: Perchloric acid-soluble protein (PSP); Chick embryo; Spinal cord; Cell proliferation; Cell differentiation

1. Introduction

Perchloric acid-soluble protein (PSP) was originally isolated from rat liver (RL-PSP) [1]. Subsequently, we isolated and characterized PSP from rat kidney (RK-PSP) [2], rat brain (RB-PSP) [3], rat lung (RLu-PSP) [4], chick liver (CL-PSP) [5] and pig liver (PL-PSP) [6]. Proteins homologous to PSP were also characterized in mice (Hrp 12) [7], goats (UK-114) [8] and humans (p14.5) [9]. PSP and its homologs show high homology with a new hypothetical family of small proteins, the YER057c/YJGF family, from their cDNA sequences, and are highly conserved from *Escherichia coli* to humans [9]. The evolutionary conservation of these proteins may reflect an important role in cellular regulation. Indeed, the mRNA of p14.5 is significantly upregulated during differentiation from monocytes to macrophages. The expression of PSP in rat kidney increases from fetal day 17 to postnatal week 4 and then reaches a steady-state level. On the other hand, the expression of PSP in rat renal tumor tissues is decreased compared to that in normal tissue [2]. In addition, overexpression of recombinant PSP reduced cell proliferation of the normal rat kidney cell line NRK-52E, rat hepatocyte cell line RLN-10 and rat hepatoma cell line dRLh-84 [10]. Although the real function of PSP remains unknown, these results suggest that PSP is involved in cell differentiation and proliferation. If PSP is involved in the regulation of cell proliferation and differentiation, knock-out of the PSP gene would be lethal. In this study, we examined the effects of an anti-CL-PSP antibody in early stage chick embryos (E2 to E3.5) to clarify the function of PSP. Abnormal development of the spinal cord was observed accompanied by increased cell proliferation and decreased cell differentiation after immuno-neutralization of PSP.

2. Materials and methods

2.1. Polyclonal anti-CL-PSP antibody

PSP was purified from chick liver (male White Leghorn chicks; 90 days of age), according to a previously described method [5]. The purified CL-PSP was administered with an adjuvant (Difco, USA) to immunize two New Zealand white rabbits. Serum was collected from these rabbits at 60 days and the IgG was purified using a HiTrap Protein A HP column (Amersham Biosciences Corp., NJ, USA).

2.2. Injection of anti-CL-PSP IgG into the yolk sac in ovo

Fertilized White Leghorn eggs were incubated at 37.5 °C for 72 h. The top of the eggshell was then cut and about 1 ml of clear liquid was pumped out from the yolk sac. Next, 0.5 ml of anti-CL-PSP IgG (1 mg/ml in phosphate-buffered saline (PBS; pH 7.4)) was injected into the yolk sac. Non-immune IgG (1 mg/ml in PBS) and PBS were used as controls. After the injection, the holes were covered with plastic caps and the eggs were returned to a humidified incubator.

2.3. Tissue localization of the injected anti-CL-PSP IgG

The anti-CL-PSP IgG was labeled with Alexa 488 carboxylic acid, succinimidyl ester mixed isomers (Molecular Probes, OR, USA) under light protection, according to the manufacturer's protocol. At 10 h after injection of the labeled anti-CL-PSP IgG, the embryos were fixed, embedded and sectioned at 4 μm. Sections were deparaffinized and observed under a confocal microscope (FLUOVIEW FV 500; Olympus, Tokyo, Japan).

2.4. Morphological examination of chick embryos

Morphological changes in the chick embryos were evaluated before fixation using a stereomicroscope (SZX 12; Olympus). Embryos showing a wavy spinal cord were scored as abnormal. The numbers of abnormal and normal embryos were analyzed using the χ^2 test. Embryos were fixed in Zamboni's solution, washed with 0.1 M phosphate buffer (PB; pH 7.4) at 4 °C, embedded in paraffin, sectioned at 4 μm, stained with hematoxylin and eosin (HE), and analyzed using an optical microscope (E 400; Nikon, Tokyo, Japan).

*Corresponding author. Fax: +81 99 285 8714.
E-mail address: oka@vet.agri.kagoshima-u.ac.jp (T. Oka).

0014-5793/$30.00
doi:10.1016/j.febslet.2005.02.081

2.5. Immunohistochemistry and analysis of cell proliferation and differentiation

Embryos were fixed in Zamboni's solution after incubation for 48, 72, and 84 h (E2, E3, and E3.5, respectively), embedded in paraffin and sectioned at 4 μm to confirm the expression of PSP. To analyze the cell proliferation and differentiation, embryos at 10 h after injection were fixed, embedded and sectioned as described above. Sections were deparaffinized and treated with 0.3% hydrogen peroxide in methanol for 30 min to remove the endogenous peroxidase. Sections were blocked with 1% BSA and 20% normal goat serum in PBS for 1 h at room temperature, and then incubated with the following antibodies: affinity-purified rabbit polyclonal anti-CL-PSP antibody (1:500); mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) (PC 10; Santa Cruz Biotechnology Inc., CA, USA; 1:5000) for cell proliferation; anti-neuronal class III β-tubulin (β-III tubulin) mouse monoclonal anti body (clone TuJ1; Covance, CA, USA; 1:500) for cell differentiation. The sections were incubated in the primary antibodies overnight at 4 °C followed by incubation in biotinylated secondary antibodies for 1 h at room temperature, and then visualized with diaminobenzidine using an ABC Kit (Vector, CA, USA). Anti β-III tubulin staining was performed after activation in 10 mM sodium acid citrate (pH 6.0) in a microwave oven for 15 min. Sections were examined using a microscope. The numbers of PCNA- and β-III tubulin-positive cells in the spinal cord were examined in five samples each. Data were analyzed by Student's *t* test.

3. Results

3.1. PSP expression in developing chick embryos

PSP expression was observed in the neuroepithelial cells of the ventricle (Fig. 1(a)) and spinal cord (Fig. 1(b) and (c)) at embryonic day 3 (E3) by immunohistochemistry. Similar localizations of PSP-positive cells were observed at E2 and E3.5 (data not shown). These data suggest that PSP is expressed in the central nervous system (CNS) in early stage chick embryos.

3.2. Effect of anti-CL-PSP IgG on the morphological changes of chick embryos

Anti-CL-PSP IgG was injected into the yolk sac to immuno-neutralize PSP and clarify its role in developing chick embryos.

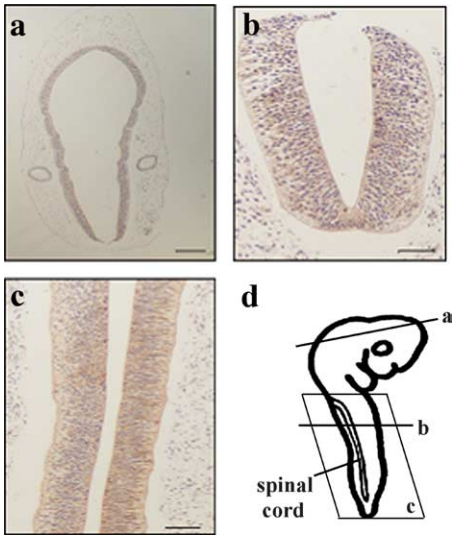

Fig. 1. Tissue localization of PSP in a chick embryo (embryonic day 3). The immunohistochemical analysis was performed as described in Section 2. PSP expression is observed in neuroepithelial cells of the brain ventricle (a) and spinal cord (b,c) of the chick embryo. The cut position is shown in (d). Scale bars: 200 μm (a), 100 μm (b,c).

First, anti-CL-PSP IgG was labeled with a fluorescent dye and injected into the yolk sac to clarify the tissue localization of the injected antibody. The fluorescent signals were observed in the ventricle (Fig. 2(a) and (b)) and spinal cord (Fig. 2(c) and (d)), suggesting that the anti-CL-PSP IgG entered the tissues of the chick embryos and reacted with their PSP. Within 12 h after the anti-CL-PSP IgG injection, morphological changes of the spinal cord were observed in 37.5% of chick embryos (Table 1; Fig. 3). In contrast, no changes were observed in embryos injected with non-immune IgG or PBS. No morphological changes were observed in embryos that maintained normal development after the anti-CL-PSP IgG injection, suggesting that these embryos failed to take up the anti-CL-PSP IgG. On the other hand, the spinal cord of abnormal embryos showed remarkable changes, such as a wavy line (Fig. 3(c) and (e)), compared to the straight line of the controls (Fig. 3(d) and (f)). The excess neuroepithelial cells expanded into the ventricular zone of the tissue of the ventricle and spinal

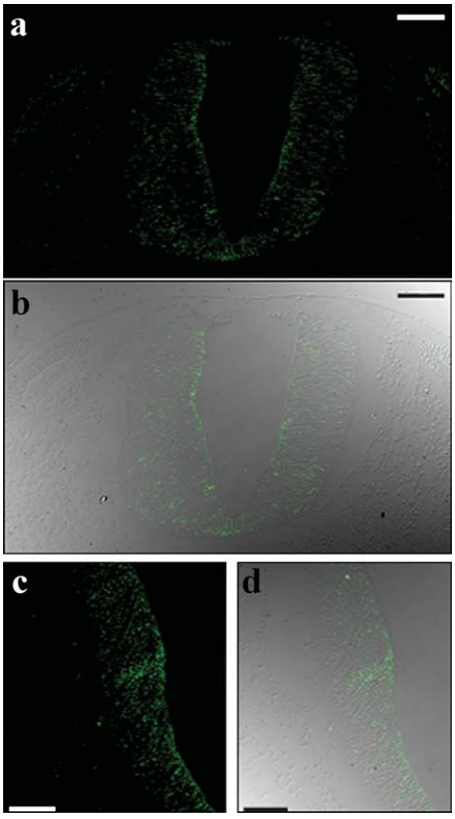

Fig. 2. Cellular localization of Alexa 488-labeled anti-CL-PSP IgG injected into the yolk sac. The fluorescent signals are present in neuroepithelial cells of the ventricle (a,b) and spinal cord (c,d). Scale bars: 100 μm.

Table 1
Induction of abnormal morphology by immuno-neutralization of PSP

Injected material	Abnormal morphology	Normal morphology	Total	Abnormal total (%)
Anti-PSP IgG	9	15	24	37.5*
Non-immuno IgG	0	10	10	0
PBS	0	20	20	0

*$P < 0.05$, χ^2 test.

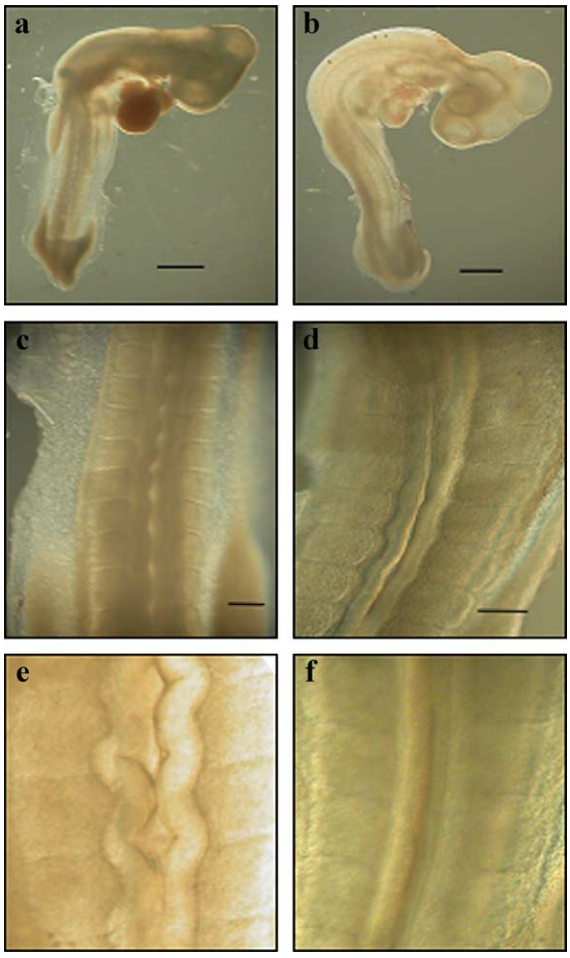

Fig. 3. Immuno-neutralization of PSP induces morphological changes in the spinal cord. Anti-CL-PSP IgG was injected as described in Section 2. The spinal cord forms a wavy line after the anti-CL-PSP IgG injection (c,e). In contrast, the spinal cord of the control is a straight line (d,f). Scale bars: 1 mm (a,b), 200 μm (c,d).

cord (Fig. 4(d)–(f)). Hence, these abnormal morphological changes were possibly induced by the immuno-neutralization of PSP.

3.3. Immuno-neutralization of PSP affects cell proliferation and differentiation

PCNA was reported to be associated with DNA replication [11–13] and has been used as a universal marker for cell proliferation. Therefore, the expression of PCNA was examined using the following three samples: (1) embryos fixed at the time of injection (Fig. 5Aa), (2) embryos fixed at 10 h after anti-CL PSP IgG injection (Fig. 5Ab), and (3) embryos fixed at 10 h after non-immune IgG injection (Fig. 5Ac). The number of PCNA-positive cells in the spinal cord of embryos injected with anti-CL-PSP IgG was significantly increased compared to those in the two control groups (Fig. 5B). In particular, high PCNA expression was observed in the region of the neuroepithelial masses (Fig. 5Ab, arrowheads). These results suggest that PSP was immuno-neutralized and that this induced cell proliferation, resulting in the induction of an irregular spinal cord structure.

In addition, the expression of β-III tubulin, a neuronal differentiation marker [14], was examined to clarify the influence

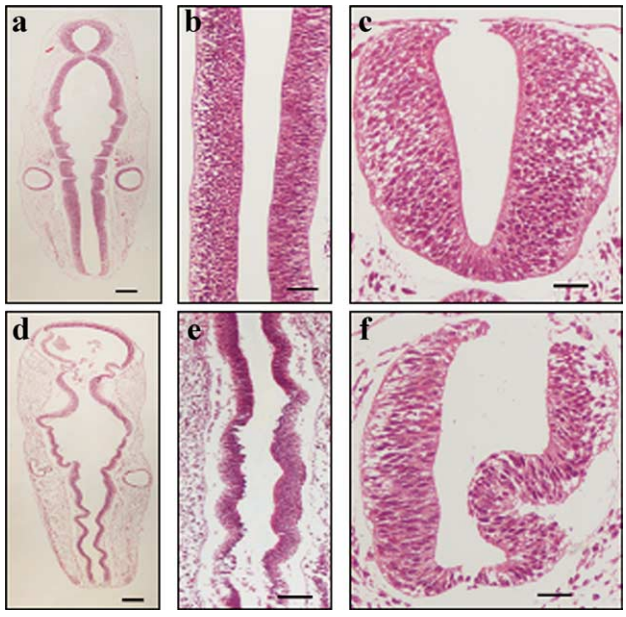

Fig. 4. HE staining of a chick embryo injected with anti-CL-PSP IgG. The staining was performed as described in Section 2. The neuroepithelial cells of the brain ventricle and spinal cord (d–f) show remarkable changes, such as a wavy line. The excess neuroepithelial cells have expanded into the ventricle compared to the control embryo injected with non-immune IgG (a–c). Scale bars: 200 μm (a,d), 100 μm (b,e), 50 μm (c,f).

of PSP immuno-neutralization on cell differentiation. The expression of β-III tubulin was reduced in immuno-neutralized embryos compared to embryos injected with non-immune IgG (Fig. 6Abc and Fig. 6B). Since PSP had already been expressed before the injection and could not increase after the injection, a similar level of PSP expression was observed (Fig. 6Aa and b).

These results suggest that PSP regulates cell proliferation and differentiation in the CNS of chick embryos.

4. Discussion

The evolutionary conservation of PSP from prokaryotes to eukaryotes suggests that it may play an important role in cellular metabolism [9]. In this study, we examined the effect of an anti-CL-PSP IgG on chick embryo development. The neuronal tube consists of folded neuroepithelial cells. The neuropore closes and the neuronal tube is formed up to embryonic day 2 (corresponding to Hamburger and Hamilton stages 12–13 [15]). Furthermore, the cells in the brain vesicle continue to proliferate and differentiate. This regular cell proliferation is necessary for the correct development of chick embryos. PSP expression was observed in the CNS of developing chick embryos at E2, E3 and E3.5 (Fig. 1), and PSP was localized in neuroepithelial cells regardless of their dorsal or ventral position.

Alexa 488-labeled anti-CL-PSP IgG was injected into the yolk sac to clarify the function of PSP. The fluorescent signals revealed that the IgG became localized in neuroepithelial cells. Embryos take their nutrition from the yolk sac for development, and fluorescent signals were also observed in the epithelial cells of the stomach and duodenum (data not shown). In these organs, low PSP expression was observed (data not

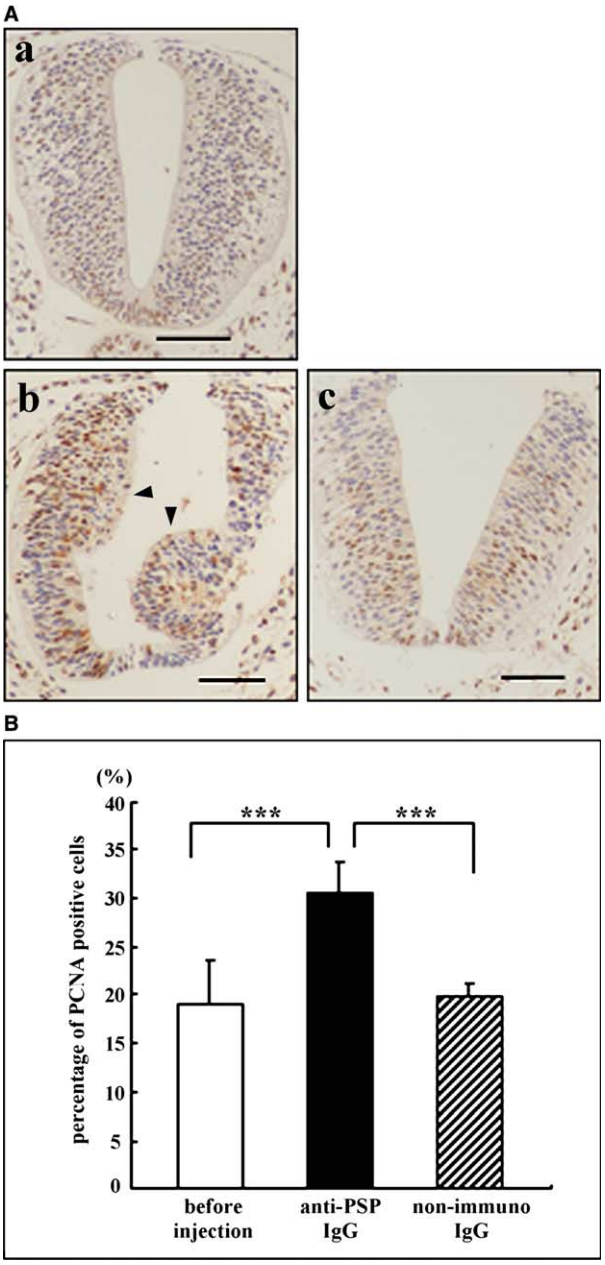

Fig. 5. (A) PCNA expression in the spinal cord. The immunohistochemical analysis was performed as described in Section 2. The PCNA expression in a chick embryo injected with anti-CL-PSP (b) is increased compared with embryos before the injection (a) or injected with non-immune IgG (c). High PCNA expression is observed in the region of the neuroepithelial masses (b, arrowheads). Scale bars: 100 μm. (B) Percentages of PCNA-positive cells. PCNA-positive cells are significantly increased in embryos injected with anti-CL-PSP IgG compared with control embryos. $^{***}P < 0.001$ (Student's t test).

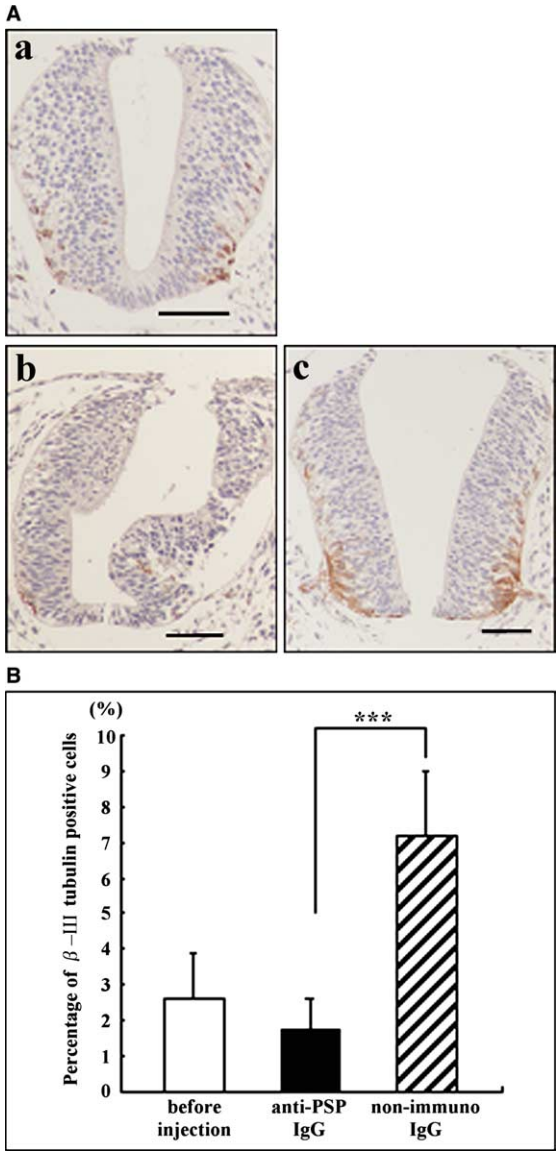

Fig. 6. (A) β-III tubulin expression in the spinal cord. The immunohistochemical analysis was performed as described in Section 2. The β-III tubulin expression in an embryo injected with anti-CL-PSP IgG (b) is decreased compared with embryos before the injection (a) or injected with non-immune IgG (c). Scale bars: 100 μm. (B) Percentage of β-III tubulin-positive cells. β-III tubulin-positive cells are significantly decreased in embryos injected with anti-CL-PSP IgG compared with control embryos injected with non-immune IgG. $^{***}P < 0.001$ (Student's t test).

shown) and the morphological changes were not as significant as those in the CNS. Furthermore, PCNA-positive cells in the stomach and duodenum hardly increased. Therefore, we focused the morphological changes in the CNS. It is interesting that IgG was absorbed into the embryo body through the epithelial cells of the foregut system. In addition, the IgG injected into the yolk sac became localized in the neuroepithelial cells of the CNS and induced cell proliferation by inhibiting the function of PSP.

In this study, 37.5% of chick embryos injected with the anti-CL-PSP IgG showed morphological changes (Table 1). These embryos showing morphological changes corresponded to stages 18 to 19 (Fig. 3(a)) of the incubation. Therefore, the timing of the IgG injection is an important factor for revealing the effect of PSP immuno-neutralization. Judging from the results, injection at stages 14 to 17 should be successful. The function of PSP was inhibited by the immuno-neutralization. The increase in cell proliferation after PSP immuno-neutralization is consistent with its reported role as an inhibitor of cell proliferation [10]. Furthermore, the decreased expression of the differentiation marker β-III tubulin suggests that PSP is also involved in cell differentiation. Thus, PSP may regulate cell

proliferation and differentiation of neuroepithelial cells, and may function to regulate the cell cycle.

On the other hand, it has been reported that PSP is expressed in astrocytes of the cerebrum, Bergmann cells and astrocytes of the cerebellum, oligodendrocytes of the corpus callosum, and ependymal cells of the choroid plexus in the adult rat brain. Furthermore, PSP expression increases from postnatal days 1 to 60 [3]. These data suggest that PSP is also involved in the nervous system after the birth.

PSP is expressed not only in differentiated cells but also in high potential cells such as the neuroepithelial cells in developing chick embryos. PSP expression is necessary to control cell proliferation and differentiation. Further analysis of PSP is required to clarify the mechanism of chick embryo development.

References

[1] Oka, T., Tsuji, H., Noda, C., Sakai, K., Hong, Y.M., Suzuki, I., Munoz, S. and Natori, Y. (1995) Isolation and characterization of a novel perchloric acid-soluble protein inhibiting cell-free protein synthesis. J. Biol. Chem. 270, 30060–30067.

[2] Asagi, K., Oka, T., Arao, K., Suzuki, I., Thakur, M.K., Izumi, K. and Natori, Y. (1998) Purification, characterization and differentiation-dependent expression of a perchloric acid soluble protein from rat kidney. Nephron 79, 80–90.

[3] Suzuki, K., Nishida, T., Matsumoto, M., Kanouchi, H., Kaneki, K. and Oka, T. (2001) Purification, characterization and developmental expression of rat brain PSP protein. Biochim. Biophys. Acta 1527, 47–53.

[4] Matsumoto, M., Kanouchi, H., Suzuki, K., Kaneki, K., Kawasaki, Y. and Oka, T. (2003) Purification and characterization of perchloric acid soluble protein from rat lung. Comp. Biochem. Physiol. B 135, 255–262.

[5] Nordin, H., Matsumoto, M., Suzuki, K., Kaneki, K., Natori, Y., Kishi, K. and Oka, T. (2001) Purification, characterization and developmental expression of chick (*Gallus domesticus*) liver PSP protein. Comp. Biochem. Physiol. B 128, 135–143.

[6] Kaneki, K., Matsumoto, M., Suzuki, K., Akuzawa, M. and Oka, T. (2003) Purification, characterization and developmental expression of pig liver PSP. Comp. Biochem. Physiol. B 134, 571–578.

[7] Samuel, S.J., Tzung, S.P. and Cohen, S.A. (1997) Hrp12, a novel heat-responsive, tissue-specific, phosphorylated protein isolated from mouse liver. Hepatology 25, 1213–1222.

[8] Ceciliani, F., Faotto, L., Negri, A., Colombo, I., Berra, B., Bartorelli, A. and Ronchi, S. (1996) The primary structure of UK114 tumor antigen. FEBS Lett. 393, 147–150.

[9] Schmiedeknecht, G., Kerkhoff, C., Orso, E., Stohr, J., Aslanidis, C., Nagy, G.M., Knuechel, R. and Schmitz, G. (1996) Isolation and characterization of a 14.5-kDa trichloroacetic-acid-soluble translational inhibitor protein from human monocytes that is up regulated upon cellular differentiation. Eur. J. Biochem. 242, 339–351.

[10] Kanouchi, H., Tachibana, H., Oka, T. and Yamada, K. (2001) Recombinant expression of perchloric acid-soluble protein reduces cell proliferation. Cell Mol. Life Sci. 58, 1340–1343.

[11] Waseem, N.H. and Lane, D.P. (1990) Monoclonal antibody analysis of the proliferating cell nuclear antigen (PCNA). Structural conservation and the detection of a nucleolar form. J. Cell Sci. 96, 121–129.

[12] Bravo, R. and Macdonald-Bravo, H. (1987) Existence of two populations of cyclin/proliferating cell nuclear antigen during the cell cycle: association with DNA replication sites. J. Cell Biol. 105, 1549–1554.

[13] Woods, A.L., Hall, P.A., Shepherd, N.A., Hanby, A.M., Waseem, N.H., Lane, D.P. and Levison, D.A. (1991) The assessment of proliferating cell nuclear antigen (PCNA) immunostaining in primary gastrointestinal lymphomas and its relationship to histological grade, S + G2 + M phase fraction (flow cytometric analysis) and prognosis. Histopathology 19, 21–27.

[14] Lee, M.K., Tuttle, J.B., Rebhun, L.I., Cleveland, D.W. and Frankfurter, A. (1990) The expression and posttranslational modification of a neuron-specific beta-tubulin isotype during chick embryogenesis. Cell Motil. Cytoskeleton 17, 118–132.

[15] Hamburger, V. and Hamilton, H.L. (1951) A series of normal stages in the development of the chick embryo. J. Morphol. 88, 49–92.