Cytotrophoblasts of the chorionic plate and villi are essential for the exchange and passage of substances required for embryonic development [25], as well as for placental growth and development. For instance, ATP-binding cassette transporters such as P-glycoprotein (P-gp) and multidrug-resistance protein (MRP) have been shown to be localized in the brush-border membrane and the basement membrane of human placental trophoblasts and cytotrophoblasts also functioned as an efflux transport system for xenobiotics [26]. Also, this syncytial layer is thought to be the major placental barrier between maternal blood and fetal blood, although cytotrophoblasts can be an element of this barrier. Therefore, the localization of UCH-L1 and ubiquitin in the cytotrophoblasts of the chorion suggests that the ubiquitin–proteasome system creates the cell layer for the degradation of abnormal protein, which is exchanged or passed through the chorion. Furthermore, the localization of PCNA in cytotrophoblasts suggests that cytotrophoblasts are stem cells which proliferate rapidly and lead to placental growth. However, in the present study, cytotrophoblasts in the cell columns and cytotrophoblastic shell did not express UCH-L1, although they did express both ubiquitin and PCNA. Cytotrophoblasts in cell columns and cytotrophoblastic shell are known to play roles in infiltration and fixation into the decidua rather than exchanging substances and forming the blood–placenta barrier. Therefore, we suggest here that the ubiquitin–proteasome system in these areas functions with the aid of not UCH-L1, but rather other de-ubiquitinating enzymes.

The present study also determined the expression profiles of UCH-L1, ubiquitin, and PCNA in decidual cells in the decidua basalis of cynomolgus monkeys at three gestational phases. We previously investigated the expression of UCH-L1 protein in decidual cells of the mouse placenta, and our findings suggested that UCH-L1 plays a significant role in implantation and placental development [24]. Decidual cells, which are derived from endometrial stromal cells of the uterus following the establishment of pregnancy [9], play an important role in implantation and provide nutritional support for the embryo; they are also believed to protect the embryo from maternal immune rejection [27]. Therefore, we expect that the ubiquitin–proteasome system in decidual cells contributes to the degradation of unfavorable immunoproteins in order to enable fetal development.

In addition, the amount of UCH-L1 protein in the entire placenta increased in a stage-dependent manner. The number of chorionic villi increased in order to exchange or pass more effectively any necessary substances between mother and fetus with the passage of gestational days. Moreover, it was found that at GD 50, 80, and 120, the intensity and pattern of the UCH-L1 immunoreaction in the cytotrophoblasts remained unchanged. We considered that this finding was due to the increase in the number of UCH-L1-positive cytotrophoblasts, which occurred in concert with increments in the number of chorionic villi. The presence of UCH-L1 and the observed increase of the amount of UCH-L1 protein provide support for the idea that the contribution of the ubiquitin–proteasome system to fetal development increases as pregnancy progresses in primates.

In recent years, many studies have investigated placental function, as well as the developmental and reproductive toxicology of various toxic chemicals in rodents. However, rodents and primates have different gestation periods, and they also have different placental structures in the branches off the main projections of the chorionic plate. The primate placenta is of the villous type, and the chorionic villi maintain an arboreal pattern with innumerable branches. On the other hand, the branches off the main chorionic projections of the mouse labyrinth are much more interconnected and generate a maze-like pattern [28]. Thus, the intervillous space of primate placentas, which is filled with maternal blood, is more open than that of the mouse labyrinth. Although mouse placentas possess a three-layered labyrinth, primate placentas have double-layered chorionic villi, which are the analogue of the mouse labyrinth [28]. In our previous study, the mouse placenta was not found to have cytotrophoblasts stained with UCH-L1. Therefore, investigations of the rodent placenta may not accurately reflect human placental function. Thus, cynomolgus monkeys, which are non-human primates, could be a useful model for the further study of the various functions of the ubiquitin–proteasome system in human pregnancy.

In conclusion, we revealed the profiles of expression of UCH-L1 and ubiquitin protein in cytotrophoblasts and decidual cells. Our findings suggested that the ubiquitin–proteasome system is necessary for placental and fetal development in cynomolgus monkeys. In the future, further investigations of additional enzymes such as E1, E2, E3, proteasomes, and other de-ubiquitinating enzymes will be necessary to gain a better understanding of the role of the ubiquitin–proteasome system in the primate placenta.

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—Note—

Immunohistochemical Analysis of Protein Gene Product 9.5, a Ubiquitin Carboxyl-terminal Hydrolase, during Placental and Embryonic Development in the Mouse

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Abstract: *Protein gene product 9.5 (PGP9.5) is expressed at high level in the neural and neuroendocrine systems. We investigated the localization and degree of expression of PGP9.5 in the developing mouse placenta and embryo at 6.5, 10.5 and 14 days of gestation using an immunohistochemical technique. At 6.5 days of gestation PGP9.5 was detected at various levels in decidual and primary trophoblast giant cells in the placenta, and in embryonic ectodermal cells in the embryo. At 10.5 and 14 days of gestation PGP9.5 was expressed at moderate to strong levels in neurons in the embryo, but rarely in the placenta. These findings suggest that the protein may play a significant role in implantation and placental development, and differentiation of embryonic ectoderm.*

Key words: *embryo, mouse, PGP9.5, placenta*

The ubiquitin-proteasome system is a major pathway for selective protein degradation [4]. Ubiquitin attaches to the target proteins and forms a polyubiquitin chain, and the ubiquitinated proteins are recognized and degraded by a multi-subunit protease complex, called the proteasome [1]. Ubiquitin carboxyl-terminal hydrolases recycle ubiquitin from ubiquitin/protein complexes or polyubiquitin chains by cleaving the amide linkage neighbouring the C-terminal glycine of ubiquitin [19, 20]. The protein gene product 9.5 (PGP9.5) is equivalent to ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCHL1) [21]. PGP9.5 is one of the major proteins in the brain, constituting 1 to 5% of total soluble brain proteins [21]. Neuroaxonal dystrophy in the gracile nucleus of the medulla and gracile fascicules of the spinal cord occurs in the gracile axonal dystrophy (Gad) mouse due to deficiency of UCHL1 [13, 23]. The Gad mouse also exhibits deterioration of spermatogenesis with aging because PGP9.5/UCHL1 expression is defective in the spermatogonia and Sertoli cells [9].

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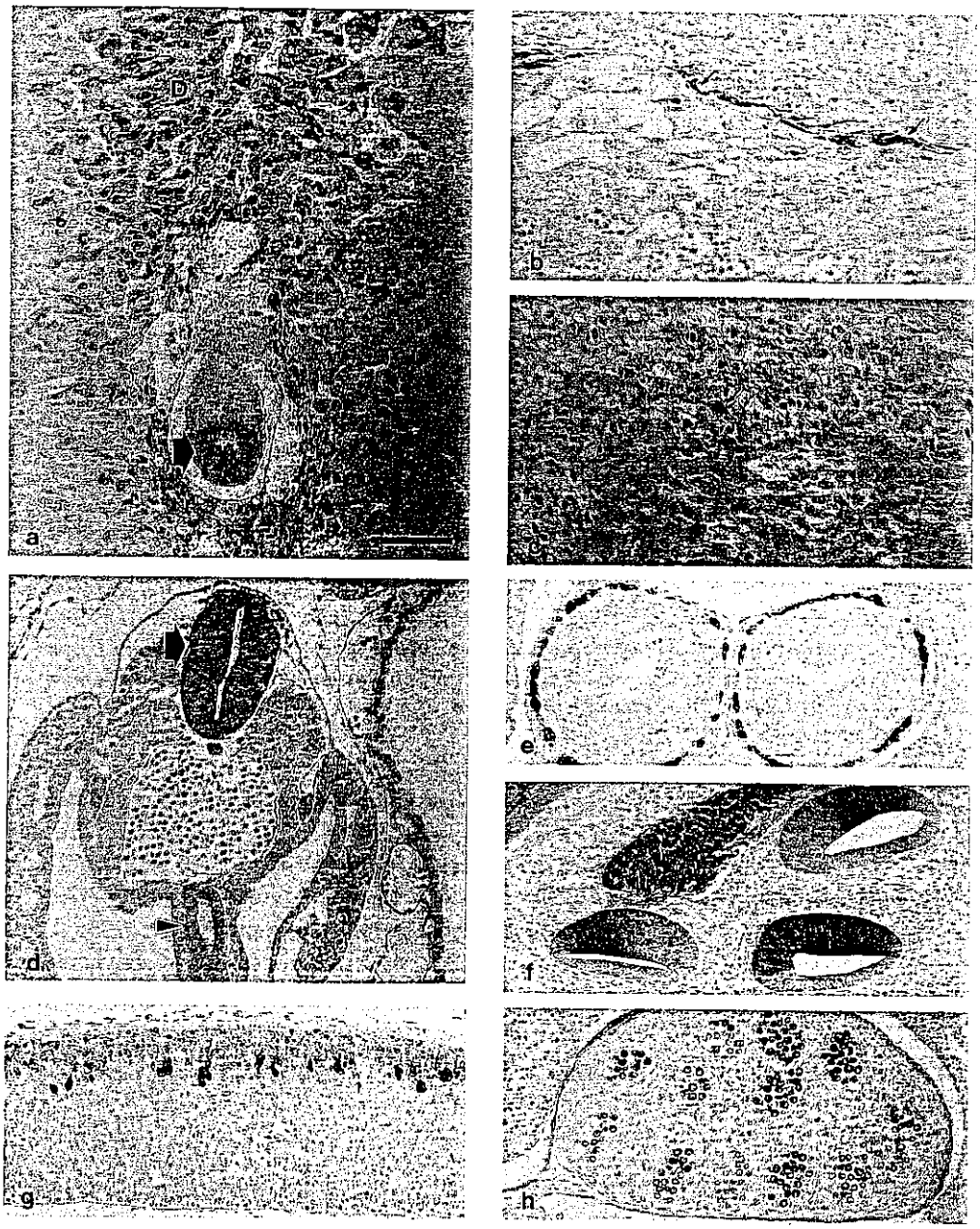

Fig. 1. Immunohistochemical reaction of PGP9.5. Hematoxylin counterstained, Bar = 100 μm. In placenta, PGP9.5 is detected at various levels in decidual cells (D) around the embryo at 6.5 days of gestation (a); in decidual cells (arrows) in the small area of basal decidua at 10.5 days of gestation (b); and at 14 days of gestation (c). In the embryo, PGP9.5 is detected at various levels in embryonic ectodermal cells (arrow in Fig. a) at 6.5 days of gestation; in most of neural tubular cells (arrow) and intestinal epithelial cells (arrow head) at 10.5 days of gestation (d); in neurons of the peripheral nervous system (e); in neurons of the ganglion (G) and epithelial cells of semicircular canals (f); migrated mature neurons of the mesencephalon (g); and in gonocytes of the testis at 14 days of gestation (h).

Immunohistochemical studies have demonstrated that PGP9.5 is highly expressed in neuronal and neuroendocrine tissues [3, 6, 10, 15, 24, 25], and PGP9.5 has also been shown to be expressed in the distal tubular epithelial cells, ova and corpus luteum cells of the ovary, Leydig cells and Sertoli cells of the testis of the mouse [8, 22], synoviocytes in the joints of the horse [7], and hair follicle cells of the rat [11]. Localization of PGP9.5

in the mouse embryo has also been described [6, 14]. PGP9.5 is present at high levels in the neuronal tissues in mouse embryo after 10.5 days of gestation [19], and in rat embryos after 11.5 days of gestation [6]. Though the presence of this protein has been revealed in the embryo, little is known about the placenta. The placenta fulfills indispensable functions such as metabolism, and provides an immunological barrier and endocrine secretion for embryonic development. In this paper we examined the localization and degree of the expression of PGP9.5 in the mouse placenta and embryo during the period of pregnancy.

C57BL/6J mice of both sexes were allowed to mate and midday when a vaginal plug was recognized was considered as day 0.5 of gestation. Two female mice at 6.5, 10.5 and 14 days of gestation, respectively, were sacrificed by cervical dislocation under ether anesthetization. Embryos and whole uterus were fixed in Bouin's solution for 5 to 6 h, then embedded in paraffin. Paraffin sections were cut at 5 to 6 μm in thickness, deparaffinized in xylene, and stained with haematoxylin and eosin.

Immunostaining was carried out by a labeled streptavidin-biotin (LsAB) method using rabbit anti-human PGP9.5 polyclonal antibody (Ultraclone Ltd. U.K.) as a primary antibody. Deparaffinized sections were pretreated with 0.3% H_2O_2 in methanol for 30 min and washed in phosphate buffer saline (PBS). In order to enhance immunoreactivity, sections were subjected to autoclave treatment for 10 min at 121°C. Non-specific binding of immunogloblins was blocked by incubation with Block Ace (Dainippon pharamaceutical Ltd. Japan) for 1 h at room temperature. After blocking, sections were incubated with primary antibody diluted at 1 : 8000 for 16 h at 4°C. Next, sections were incubated with biotinylated goat anti-rabbit IgG (DAKO Ltd. Denmark) diluted at 1 : 500 for 30 min at 37°C, and after washing in PBS, they were reacted with horseradish peroxidase conjugated streptavidin (DAKO Ltd. Denmark) diluted at 1 : 500 for 30 min at room temperature. After washing in PBS, the immunoreaction was visualized by incubation in 3,3'-diaminobenzidine tetrahydrochloride (DAB)-H_2O_2 solution and then counterstained with haematoxylin. Negative control sections were incubated with PBS or non-immunized rabbit serum as substitutes for the primary antibody. Taking into consideration which stage and what kind of cell expresses PGP9.5, the intensities of immunoreactions were classified into one of three grades: weak, moderate or strong.

The immunoreactivity of PGP9.5 was detected at various levels in decidual cells around the embryo and in primary trophoblastic giant cells at 6.5 days of gestation (Fig. 1a). The moderately or strongly positive decidual cells showed not only cytoplasmic but also nuclear staining. At 10.5 days of gestation, decidual cells with various levels of staining were seen in the smaller area of the basal decidua, and they also showed nuclear staining (Fig. 1b). At 14 days of gestation, a few cells stained positive at weak to moderate levels were present in the basal decidua (Fig. 1c , Table1). There was no difference in immunoreactivity of PGP9.5 among mouse placentas of the same litter mates.

At 6.5 days of gestation, moderate PGP9.5 immunoreactivity was detected in embryonic ectodermal cells (Fig. 1a). At 10.5 days of gestation, most neural tubular cells and intestinal epithelial cells were immunopositive at a moderate grade (Fig. 1d). In addition, otic vesicular cells, epithelial cells of the Wolffian duct, and notocord cells were weakly immunopositive. At 14 days of gestation, most of neurons were moderately or strongly positive. The neurons of the peripheral

Table 1. PGP9.5-positive cells in placenta of pregnant mouse

Grade	6.5 days	10.5 days	14 days
+	Decidual cells Primary trophoblastic giant cells	Basal decidual cells	Basal decidual cells
++	Decidual cells Primary trophoblastic giant cells	Basal decidual cells	Basal decidual cells
+++	Decidual cells	Basal decidual cells	

PGP9.5-positive reaction was classified into three grades: weak (+), moderate (++) and strong (+++). 6.5, 10.5 and 14 days mean gestation period.

Table 2. PGP9.5-positive cells in embryo of pregnant mouse

Grade	6.5 days	10.5 days	14 days
+		Neural tubular cells Otic vesicular cells Wolffian ductal cells Notocord cells Intestinal epithelial cells	Ependymocytes Adenohypophysis cells Epithelial cells of nasal pit bronchi mesonephric duct mesonephric tubule paramesonephric duct ureter hair follicle Gonocytes of testis Chondroblasts
++	Embryonic ectodermal cells	Neural tubular cells	Neurons of central nervous system
		Intestinal epithelial cells	Gonocytes of testis
+++			Neurons of central and peripheral nervous system Epithelial cells of retina and semicircular canals

PGP9.5-positive reaction was classified into three grades: weak (+), moderate (++) and strong (+++). 6.5, 10.5 and 14 days mean gestation period.

nervous system (Fig. 1e), ganglions (Fig. 1f), migrated mature neurons of the mesencephalon (Fig. 1g), and sensory epithelial cells of the retina and semicircular canals (Fig. 1f) were especially strongly stained. These strongly immunopositive cells also showed nuclear staining. Moreover, gonocytes of testis (Fig. 1h) were weakly or moderately stained, and a weak, positive reaction was seen in ependymocytes, adenohypophysis cells, epithelial cells of nasal pit, bronchi, mesonephric duct, mesonephric tubule, paramesonephric duct, ureter, hair follicle and some chondroblasts (Table 2). PGP9.5 expression in the embryo changed with embryogenesis, however, there was no difference in the immunoreactivity of PGP9.5 of mouse embryos among the same litter mates.

This study showed that PGP9.5 was expressed at various levels in decidual cells from the early stage of pregnancy, while the number of PGP9.5 positive cells decreased in the late stage. Generally, after implantation, the area occupied by decidual cells increases from 6 days to 10 days of gestation, and thereafter slightly decreases in the mouse [5]. The structure of the mouse placenta was unchanged after 14 days of gestation [17], and its weight and the number of the constituent cells reached a plateau by 14 days of gestation [5]. In fact, the decidual cells expressing PGP9.5 appeared and increased immediately after implantation, and thereafter most of them had disappeared by the time the placenta matured.

At 6.5 days primary trophoblastic giant cells were immunopositive at low to moderate levels, whereas at 10.5 and 14 days trophoblastic giant cells were all negative. Primary trophoblastic giant cells are derived from embryonal trophoblast and invade the maternal tissue on implantation [17]. This early expression of PGP9.5 in decidual and primary trophoblastic giant cells and the subsequent decreasing number of the positive cells suggest that the protein may play a significant role in implantation and placenta development Decidual cells are thought to function as a barrier to prevent immune rejection between the maternal body and embryo [18]. Therefore, they might degrade immunoproteins which are disadvantageous for embryo development.

In the embryo of the pregnant mouse PGP9.5 was already been expressed at a moderate level in embryonic ectodermal cells at 6.5 days of gestation, and the protein was expressed at moderate to strong levels in mature neurons at 10.5 and 14 days of gestation. The

nerves develop from the embryonic ectoderm [12]. This means that the protein plays an important role in degradation of unnecessary proteins in the process of embryonic ectodermal differentiation. Mature neurons expressed the protein more strongly than immature ones; therefore, we assume that the more neurons mature, the more active their metabolism becomes. This assumption is supported by the immunostaning of PGP9.5 in the nucleus as well as the cytoplasm in mature neurons.

At 10.5 days and 14 days of gestation PGP9.5 was demonstrated at low to moderate leves in the endodermal and mesodermal tissues, such as testis and bronchi. Although the distribution of PGP9.5 mRNA in developing gonads in the mouse was reported by Schofield *et al.* [14], this is the first description of the expression of PGP9.5 protein in the developing mouse embryo. The distribution of the protein in the developing lung was reported in human and rat [2, 16]. The significance of the expressions of PGP9.5 in the developing embryo remains unclear.

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Primary culture of cortical neurons, type-1 astrocytes, and microglial cells from cynomolgus monkey (*Macaca fascicularis*) fetuses

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Abstract

We established selective primary cultures of neurons, astrocytes, and microglial cells from cryopreserved fetal cerebral cortex of cynomolgus monkeys (*Macaca fascicularis*). At 14 days in serum-containing medium, the cell cultures of the fetal cerebral cortex consisted primarily of neurons, astrocytes, and floating microglial cells. At 21 days, we observed a small number of myelin basic protein (MBP)-positive oligodendrocytes. The addition of cytosine arabinoside (a selective DNA synthesis inhibitor) at 2 days in culture eliminated proliferative glial cells, allowing adequate numbers of neurons to survive selectively. A chemically defined serum-free medium successfully supported neuronal survival at a level equivalent to that supported by the serum-containing medium. Brain-derived neurotrophic factor (BDNF) significantly affected the survival of primate neurons. Glutamate induced a significant degree of neuronal cell death against primate neurons and MK-801, a selective *N*-methyl-D-aspartate receptor (NMDAR) antagonist, blocked cell death, which suggests that primate cortical neurons have NMDAR and the glutamate-induced cell toxicity is mediated by NMDAR. In the serum-free medium, type-1 astrocytes responded to dibutyryl cyclic AMP and showed a process-bearing morphology. The growth of type-1 astrocytes in the serum-free medium was stimulated by epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and hydrocortisone, which are known growth factors in rat type-1 astrocytes. Cultured microglial cells expressed CD68, a monocyte marker. Macrophage–colony stimulating factor (M–CSF) stimulated microglial cell growth in the serum-free medium. These selective primary culture systems of primate cerebral cortical cells will be useful in issues involving species specificity in neuroscience.

Keywords: Primate; Cryopreservation; Neuron; Glia; Serum-free

1. Introduction

Non-human primates are useful laboratory resources in experimental neurosciences because of their close phylogenetic relationships to humans (Czub et al., 2001; Kimura et al., 2001; Li et al., 1999; Mankowski et al., 2002; Sales et al., 1998; Sasseville et al., 1996; Westmoreland et al., 1998). Indeed, it is ethically difficult to use human samples in research that is fundamentally experimental. Previously, we reported on the cryopreservation and primary culture of primate brain cells, which in a serum-containing medium consisted mainly of neurons and astrocytes (Negishi et al., 2002b). When species specificity between rodents and primates becomes a subject, the primary culture of primate brain cells could be an effective tool in in vitro neuroscience. Cryopreservation of brain tissues would allow us to store and distribute these rare resources and enable us to plan well-designed studies (Negishi et al., 2002a).

Serum is known as an effective supplement for cultured brain cells, including neurons (Kaufman and Barrett, 1983). However, some experiments, such as those examining the weak potential effects of very small amounts of chemical substances, e.g. endocrine-disrupting chemicals (Nagel et al., 1997) should avoid serum supplements, since

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unknown factors and/or highly chemical-adsorbent proteins in the serum might affect the biological responses to the chemicals being tested.

Close anatomical and physiological associations between neurons and non-neuronal cells, such as astrocytes (Cotrina and Nedergaard, 2002) and microglial cells (Bruce-Keller, 1999), are well known. Even in an in vitro condition such as a mixed primary culture, there are still intricate interactions between neurons and non-neuronal cells. These structural and physiological complexities of the central nervous system (CNS) have often precluded definitive studies of each cell type in in vivo or in vitro conditions. In rodents, selective primary cultures of neurons, astrocytes, oligodendrocytes, and microglial cells have been established. These culture systems could be suitable for examinations of cell specific responses without the interactions among heterogeneous cells that occur in mixed in vitro condition.

Considering these contexts, we tried to establish selective primary cultures of neurons, type-1 astrocytes, and microglial cells, respectively, from the cerebral cortex of cynomolgus monkey fetuses in serum-free conditions. Although several studies have reported on the cryopreservation and primary mixed culture of fetal brain cells from non-rodents, such as humans (Mattson and Rychlik, 1990), monkeys (Negishi et al., 2002b), and bovine animals (Kitani et al., 2000), this is the first report to attempt selective culturing in serum-free conditions from a non-rodent cerebral cortex.

2. Materials and methods

2.1. Animals

Four fetuses of cynomolgus monkey (*Macaca fascicularis*) at 80 days of gestation were purchased from Shin Nippon Biomedical Laboratories (Kagoshima, Japan). All fetuses were obtained by Cesarean section and immediately killed by decapitation. This study was approved by the Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences, the University of Tokyo.

2.2. Cryopreservation of cerebral cortex

The fetal brains were transferred into an ice-cold isolation medium (IM) consisting of equal volumes of Ca^{2+} and Mg^{2+} -free phosphate-buffered saline (PBS) and Dulbecco's Modified Eagle's Medium (Sigma–Aldrich, St. Louis, MO, USA) with 1.2 mg/ml $NaHCO_3$, 110 μg/ml pyruvic acid, 25 μg/ml streptomycin, and 50 units/ml penicillin (mDMEM). After the meninges were carefully removed, the cerebral cortex was mechanically dissected and cut into small pieces in mDMEM with 10% fetal bovine serum (FBS) (serum-containing medium) supplemented with 10% dimethyl sulphoxide (DMSO) (Sigma–Aldrich) as a cryoprotectant (Negishi et al., 2002a). Aliquot tissue pieces in the cryoprotective medium (n = 24 from cortex of each fetus) were submitted for cryopreservation. The cortical tissue pieces were first frozen at −80 °C in the Cryo 1 °C Freezing Container (Nalge Nunc, Tokyo, Japan) at a rate of about −1 °C/min, and then stored in liquid nitrogen for more than 3 months.

2.3. Primary whole cortical cell culture

Cryopreserved cortical tissue pieces were thawed rapidly in a water bath at 32 °C, rinsed in IM to remove the serum and DMSO, and then digested in PBS containing 10 U/ml papain (Worthington Biochemical Corp., Lakewood, NJ), 0.1 mg/ml DNase I (Roche Diagnostics, Japan), 0.2 mg/ml cysteine, 0.2 mg/ml albumin and 5 mg/ml glucose at 32 °C for 30 min. The cells were dissociated gently by several passages through a disposable pipette and centrifuged three times in a serum-containing medium at 800 rpm for 5 min at a time at 32 °C. The cells were suspended in serum-containing medium, mDMEM supplemented with insulin–transferrin–selenium-X (ITS-X) (Invitrogen Corp., Carlsbad, CA) (sDMEM), or neurobasal medium (Invitrogen) with B-27 supplement (Invitrogen) (NBM), and were then plated onto culture dishes coated with 0.5% polyethylenimine (PEI) at 1000 cells/mm^2. All cultures were maintained at 37 °C in 95% humidified air and 5% CO_2.

2.4. Selective neuron culture

To eliminate proliferative cells, including astrocytes, 5 μM of cytosine arabinoside (AraC) (Sigma–Aldrich), a selective inhibitor of DNA synthesis, was added to the whole cortical cell culture at 2 days in culture.

2.5. Survival effect of growth factor on neurons cultured in serum-free medium

Basic fibroblast growth factor (bFGF) (10 ng/ml) (Invitrogen) and brain-derived neurotrophic factor (BDNF) (10 ng/ml) (Invitrogen) were examined as survival factors for neurons in the serum-free medium. Control cultures were treated with vehicle only. At 2 days in culture, the serum-containing medium was replaced by sDMEM containing each factor. In these experiments, AraC (5 μM) was added at 2 days in culture to prevent astrocyte growth. At 21 days in culture, the surviving neurons were counted following immunocytochemical identification (see Section 2.10).

2.6. Type-1 astrocyte culture

Type-1 astrocyte cultures were established according to the method previously described in rats (Murakami et al., 1999) with some modifications. At 14 days in culture, cultured whole cortical cells were dissociated by trypsin (0.025%) (Invitrogen). Dissociated cells were centrifuged two times in serum-containing medium, plated onto culture

dishes without additional chemical coating at 200 cells/mm^2, and maintained in serum-containing medium. When cultured cells became confluent (approximately 7 days), cells were dissociated by trypsin and plated again as before. This procedure was repeated several times, which allowed us to obtain a large number of type-1 astrocytes, while proliferative type-1 astrocytes were selected. Subsequently, fully purified type-1 astrocytes were dissociated by trypsin and prepared as described above. Purified type-1 astrocytes were suspended and plated onto culture dishes without additional chemical coating at 200 cells/mm^2 in the serum-free medium (sDMEM) to observe morphological conversion of type-1 astrocytes by dibutyryl cyclic AMP (dbcAMP) (Sigma–Aldrich), or in DMEM/F-12 (Invitrogen Corporation) supplemented with ITS-X (sDMEM/F-12) to examine growth responses to FBS (1–20%), the recombinant human epidermal growth factor (EGF) (10–100 ng/ml) (Invitrogen), bFGF (10–100 ng/ml) and hydrocortisone (10–10,000 nM) (Sigma–Aldrich). Control cultures were treated with vehicle only.

2.7. Microglial cell culture

At 14 days in culture, floating and weakly attached cells in each whole cortical cell culture were collected by shaking the culture flask (Giulian and Baker, 1986). The obtained cells were then plated at 200 cells/mm^2 onto a culture dish and maintained in sDMEM/F-12. Recombinant human macrophage–colony stimulating factor (M–CSF) (50 ng/ml) (Diaclone Research, Besançon, France) was examined as a growth factor for microglial cells. Control cultures were treated with vehicle only.

2.8. Quantification of cell growth

Cultured type-1 astrocytes and microglial cells were maintained in sDMEM/F-12. The alamarBlue™ assay (Wako Pure Chemical Industries, Osaka, Japan) for the detection of metabolic activity, was used to measure quantitatively the growth effect of the factors described above, according to the product manuals. A 1:10 dilution of alamarBlue in sDMEM/F-12 was warmed to 37 °C and applied to the cells. The cells were incubated for 4 h at 37 °C in 95% humidified air and 5% CO_2. The conversion of resazurin to resorufin was measured at absorbances of 570 and 600 nm. The results were indicated as percentages of control.

2.9. Quantification of neuronal cell death

Glutamate (Sigma–Aldrich) and MK-801 (Sigma–Aldrich), a selective *N*-methyl-D-aspartate receptor (NMDAR) antagonist, were first dissolved at 100 and 10 mM, respectively, in PBS. Selectively cultured neurons were exposed to glutamate and/or MK-801 for 24 h beginning at 14 days in culture. Control neurons were exposed to vehicle only. Glutamate (5, 50, and 500 μM)-induced neuronal cell death and its inhibition by MK-801 (50 μM) were evaluated by measuring the lactate dehydrogenase (LDH) activity released in the culture medium after 24-h glutamate exposure using CytoTox96 nonradioactive assay (Promega, Madison, WI) and quantified by measuring the wavelength absorbance at 490 nm. In this experiment, data were represented as percentages of full kill of the sample prepared by the lysis solution included in the kit.

2.10. Immunocytochemistry

The cellular elements of cultured cells were identified by immunostaining. Antibodies against microtubule-associated protein-2 (MAP-2; 1:1000, Roche Diagnostics), glial fibrillary acidic protein (GFAP; 1:1000, DAKO, Kyoto, Japan), myelin basic protein (MBP; 1:500, DAKO) and the monocyte marker CD68 (1:500, Lab Vision, Fremont, CA) were used to identify neurons, type-1 astrocytes, oligodendrocytes and microglial cells, respectively. To identify neurons, type-1 astrocytes, and oligodendrocytes cultured cells were fixed by 2% paraformaldehyde and 8% sucrose in PBS for 1 h at room temperature, rinsed in PBS, and then incubated in 0.1% Triton-X for 5 min at room temperature as a pre-treatment. To identify microglial cells, cultured cells were fixed by −20 °C methanol for 5 min and then rinsed in PBS with no pre-treatment. Cultured cells were subsequently incubated overnight at 4 °C in primary antibodies diluted in PBS containing 2% bovine serum albumin and 4% horse serum. Fluorescein-conjugated anti-mouse IgG (1:1000, Vector Laboratories, Burlingame, CA) was used to detect anti-MAP-2, MBP, and CD68 antibodies, while Alexa-Fluor-568®-conjugated anti-rabbit IgG (1:1000, Molecular Probes Inc., Eugene, OR) was used to detect anti-GFAP antibody. The nuclei were stained by TO-PRO-3® (1:1000, Molecular Probes).

2.11. Statistical analysis

One-way analysis of variance (ANOVA) and post-hoc Fisher's protected least significant difference test were used to examine the statistical differences between treatments. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Whole cortical cell culture

Cryopreserved cortical tissues of 80-day-old fetuses yielded $2.8 \pm 0.4 \times 10^8$ viable cells/fetus (mean ± S.E.M., $n = 4$) just before plating. At 3 days in culture, cultured cells successfully attached to the PEI coating dish in serum-containing medium (Fig. 1A). sDMEM and NBM provided less support for cell attachment and neuronal process extension (Fig. 1B and C). The percentage of attaching cells in either of these serum-free media was

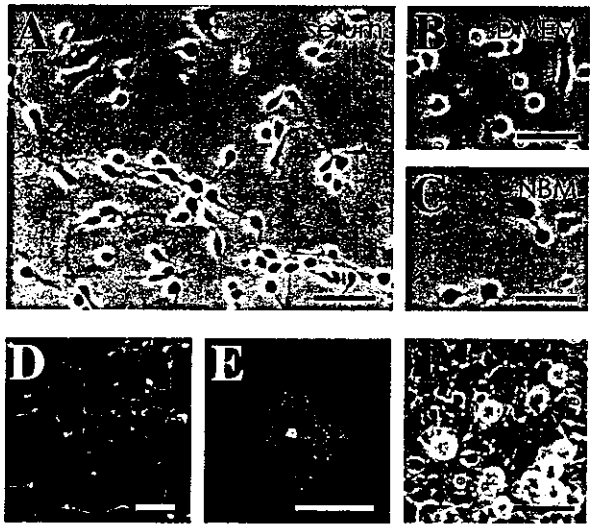

Fig. 1. (A–C) Phase-contrast microscopic observations of primate whole cortical cell culture prepared in serum-containing medium (serum) (A), serum-free medium (sDMEM) (B), and neurobasal medium with B-27 supplement (NBM) (C) at 3 days in culture. (D) Immunocytochemical characterization of primate neurons (MAP-2-positive; green) and astrocytes (GFAP-positive; red) in serum-containing medium at 3 days in culture. (E) Immunocytochemical characterization of primate oligodendrocyte (MBP-positive; green) at 21 days in culture. (F) Phase-contrast observation of microglial cells floating on flat type-1 astrocyte layer at 14 days in culture. Nuclei appear as blue images in 'D' and 'E'. Each scale bar represents 50 μm.

less than 30% that in serum-containing medium at 3 days in culture. At that stage, cultured cells consisted mainly of MAP-2-positive neurons and relatively small numbers (<10%) of GFAP-positive astrocytes (Fig. 1D). There were a few MBP-positive oligodendrocytes in serum-containing medium at 21 days in culture (Fig. 1E). The percentage of MBP-positive cells to total cells was less than 0.01% (240 fields from three separate experiments) at 21 days in culture. At 7 and 14 days in culture, there was no MBP-positive oligodendrocyte in serum-containing medium (240 fields from three separate experiments). At 14 days in culture, large floating cells, considered to be microglial cells, appeared on a mixed cell layer (Fig. 1F). The number of floating microglial cells was 178 ± 12 cells/mm^2 at 14 days in culture (mean ± S.E.M., $n = 120$ fields from three separate experiments). These cells were collected for selective microglial cell culture (see Section 3.4).

3.2. Selective neuron culture

In primary whole cortical cell culture, MAP-2-positive neurons and GFAP-positive astrocytes were observed at 14 days in culture in the serum-containing medium (Fig. 2A). The addition of 5 μM AraC at 2 days in culture successfully prevented proliferation of glial cells and allowed neurons to survive for at least 14 days in culture (Fig. 2B). After 14 days in culture, the number of MAP-2-positive neurons with AraC was significantly higher than that without AraC, while the number of GFAP-positive astrocytes was significantly suppressed by the addition of AraC (Fig. 2C). The percentages of MAP-2-positive neurons among total cells were 13.6 ± 1.2% without AraC and 92.4 ± 0.7%

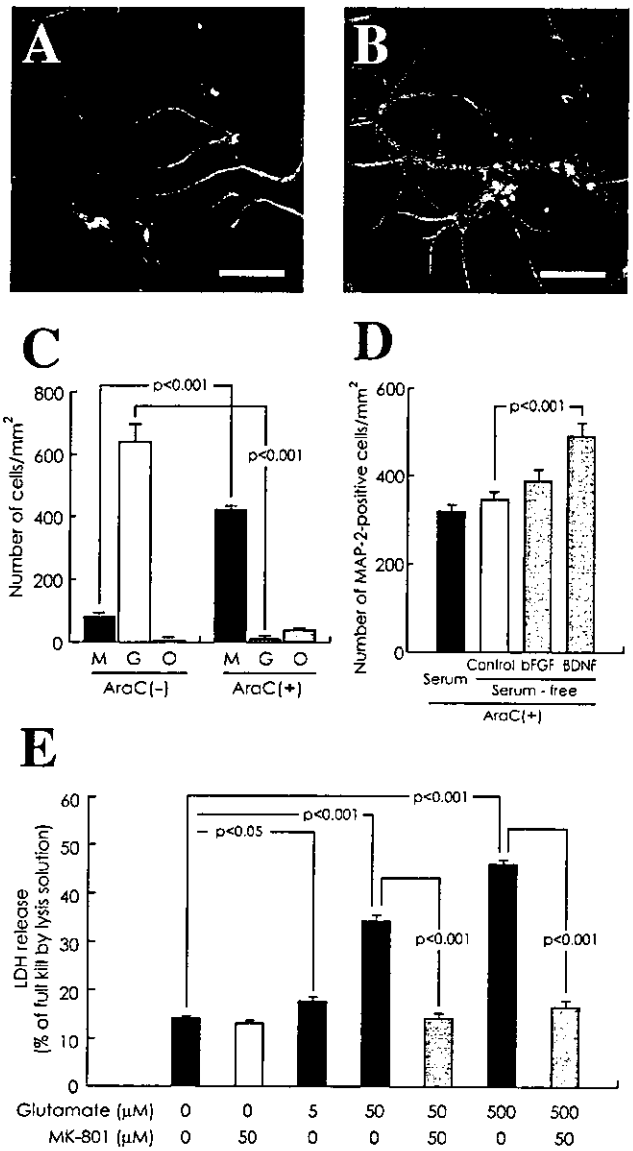

Fig. 2. (A and B) Immunocytochemical characterization of primate neurons (MAP-2-positive; green) and astrocytes (GFAP-positive; red) in serum-containing medium without AraC (A) and with AraC (B) at 14 days in culture. Nuclei appear as blue images. Each scale bar represents 50 μm. (C) The number of primate MAP-2-positive neurons (M), GFAP-positive astrocytes (G), and other cells (O) in serum-containing medium without AraC (AraC (−)) and with AraC (AraC (+)) at 14 days in culture. Note that floating microglial cells were lost during immunostaining procedures. (D) Survival effects of bFGF (10 ng/ml) and BDNF (10 ng/ml) in serum-free medium on the number of primate neurons at 21 days in culture. Each value represents the mean + S.E.M. ($n = 120$ fields from three separate experiments). (E) Glutamate-induced neuronal cell death and its inhibition by MK-801 in primate neurons at 14 days in culture. Each value represents the mean + S.E.M. ($n = 9$ from three separate experiments).

with AraC (mean ± S.E.M., n = 120 fields from 3 separate cultures). We immunocytochemically confirmed that there were no MBP-positive oligodendrocytes at 14 days in culture, regardless of the addition of AraC. Other cells indicated in Fig. 2C might be immature MBP-negative oligodendrocytes, microglial cells, flat capillary endothelial cells, or others. The serum-free medium (sDMEM) and the serum-containing medium supported neuronal survival equally (Fig. 2D) at 21 days in culture. bFGF (10 ng/ml) showed a slight and not statistically significant ($P < 0.1$) survival effect (Fig. 2D) at 21 days in culture. BDNF (10 ng/ml) showed a significant survival effect on primate neurons (Fig. 2D) at 21 days in culture. Glutamate showed significant neurotoxicity in primate neurons in a dose-dependent manner and MK-801 significantly blocked glutamate-induced LDH release (Fig. 2E). In all experimental conditions with AraC, the percentages of MAP-2-positive neurons were >90% of the total number of cultured cells.

3.3. Type-1 astrocyte culture

Almost all cells (>99.9%) obtained by the protocol for type-1 astrocyte culture expressed GFAP. Cultured type-1

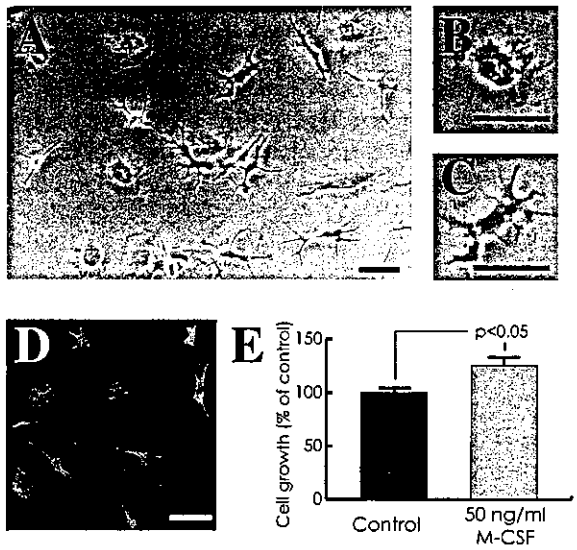

Fig. 4. (A–C) Phase-contrast observation of primate microglial cells (A) at 3 days in culture after selection. There were amoeboid (B) and ramified (C) forms in cultured microglial cells. (D) Expression of CD68 in primate microglial cells. Nuclei appear as blue images. Scale bar represents 50 μm. (E) Growth effect of M-CSF on primate microglial cells. Microglial cells were exposed to M-CSF for 72 h. Each value represents the mean+S.E.M. (n = 24 fields from three separate experiments).

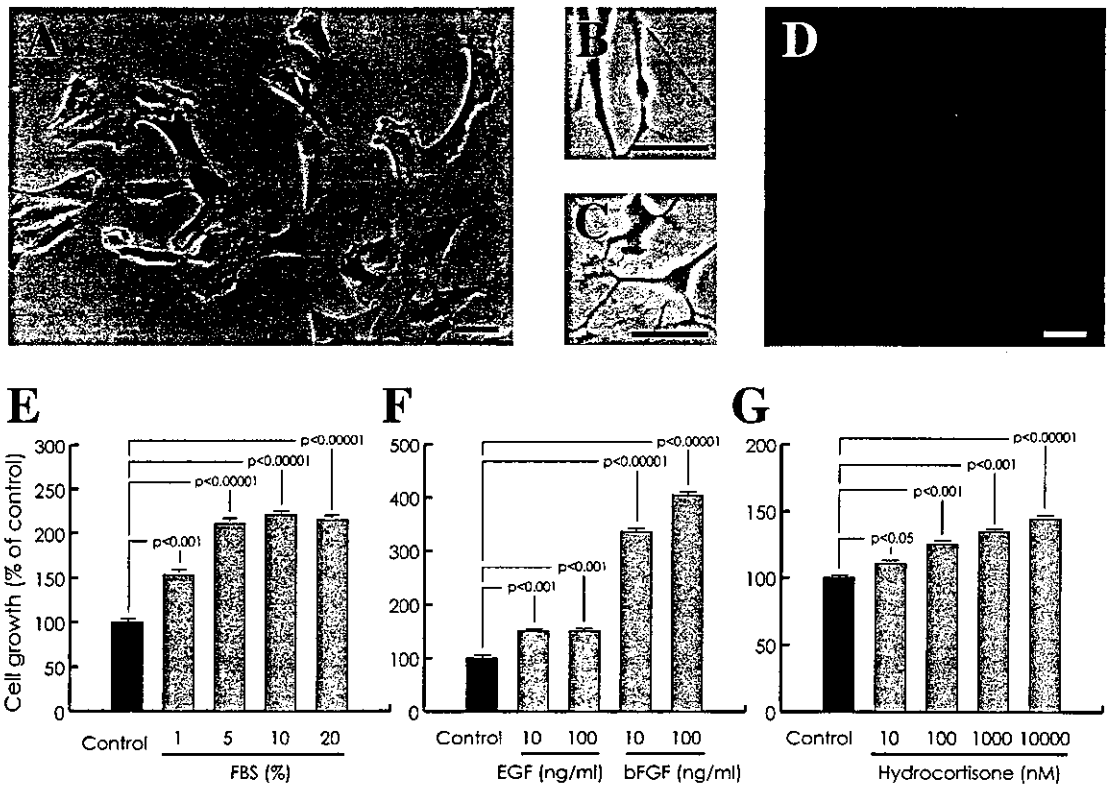

Fig. 3. (A) Phase-contrast microscopic observation of primate type-1 astrocytes cultured in serum-free medium at 7 days in culture after last passage. (B–D) Morphological conversions of primate type-1 astrocytes by dbcAMP (10 μM). Phase-contrast observations of bipolar (B) and process-bearing (C) type-1 astrocytes at 30 min after dbcAMP application. Expression of GFAP in both flat and process-bearing type-1 astrocytes (D). Nuclei appear as blue images. Scale bar represents 50 μm. (E–G) Growth effects of serum (FBS) (E), EGF and bFGF (F), and hydrocortisone (G) on primate type-1 astrocytes cultured in serum-free medium. Type-1 astrocytes were exposed to each factor for 72 h. Each value represents the mean + S.E.M. (n = 48 fields from 4 separate experiments).

astrocytes had a flat morphology in the serum-free medium (Fig. 3A). Type-1 astrocytes in the serum-free medium responded to 10 μM dbcAMP and showed bipolar (Fig. 3B) or process-bearing morphologies (Fig. 3C) at 30 min after the addition of dbcAMP. Both flat and process-bearing type-1 astrocytes expressed GFAP (Fig. 3D). At 30 min after the addition of dbcAMP, 30.2 ± 4.5% (mean ± S.E.M., $n = 60$ fields from three separate cultures) of the cells had morphologically changed. However, almost all cells had returned to the flat morphology at 12 h after the addition (data not shown). Serum prevented these morphological responses to dbcAMP (data not shown). Serum in sDMEM/F-12 stimulated the growth of type-1 astrocytes in a dose-dependent manner (Fig. 3E). EGF and bFGF stimulated the growth of type-1 astrocytes in sDMEM/F-12 (Fig. 3F). Hydrocortisone also stimulated their growth (Fig. 3G).

3.4. Microglial cell culture

At 14 days in culture, floating and weakly attached microglial cells in the whole cortical cell culture were collected by shaking the culture flask. After microglial cells were removed, the remaining microglial cells on the mixed cell layer repeatedly proliferated, and the number of floating microglial cells reached >100 cells/mm^2 after 4 days in culture. These cells could also be applied to the selective microglial cell culture (data not shown). At 3 days in culture after selection, cultured microglial cells (Fig. 4A) showed two morphologically distinct forms. One was an amoeboid form (Fig. 4B) and the other was a ramified, branched form (Fig. 4C). The percentage of amoeboid form cells among total microglial cells was 20.5 ± 3.2% (mean ± S.E.M., $n = 30$ fields from three separate cultures). Both amoeboid and ramified microglial cells expressed CD68 (Fig. 4D). M-CSF significantly stimulated the growth of microglial cells (Fig. 4E).

4. Discussion

In the present study, we established selective primary cultures of neurons, type-1 astrocytes and microglial cells from cryopreserved fetal primate cerebral cortex in chemically defined serum-free conditions. These culture systems offer several advantages. First, cryopreservation and long-term storage of the fetal cerebral cortex will enable us to plan extended and well-designed studies. Parallel cultures of purified neurons and other glial cells can be easily prepared from aliquot cryopreserved samples by shifting the durations of thawing and culturing for each cell type. Second, highly purified culture systems in a serum-free medium might make it easy to plan experiments and interpret their results, because there would be neither interactions with other cell types, e.g. neuron-astrocyte interactions, nor the effects of any unknown serum-derived factors. At the same time, physiological interactions among different types of brain cells through soluble factors, such as cytokines, could be clearly estimated by using a cell-conditioned medium. Third, even though the results were collected from different cell-type cultures, all cultures were prepared from the same brain region at an identical developmental stage, the 80-day-old fetal primate cerebral cortex, thus allowing standardized interpretations. Finally, these selective primary cultures could be useful tools for physiological, microbiological, and pathological studies having to do with species specificity.

We first prepared mixed cortical cell cultures and observed neurons, astrocytes, oligodendrocytes and microglial cells. Previous studies on the primary cultures of brain cells from non-rodent animals (Kitani et al., 2000; Mattson and Rychlik, 1990; Negishi et al., 2002b) focused mainly on neurons, if any, and astrocytes. The present report is substantially the first to refer to primary cultured oligodendrocytes and microglial cells of primate CNS. Our previous study (Negishi et al., 2002b) revealed that cerebral tissues of a cryopreserved 80-day-old fetus yielded about 3.6×10^8 cells. In the present study, we obtained 2.8×10^8 cells/fetus from cryopreserved fetal cortical tissues, and a value we consider sufficient for general cell culture studies.

Cytosine arabinoside successfully prevented primate glial cell proliferation and allowed primate neurons to survive, as was observed earlier in the primary culture of the rat neuron (Mao and Wang, 2001). Since it is considered that neurons and other cells have complicated interactions in in vitro, as well as in vivo conditions, these highly purified neurons would be an effective tool for the examination of restricted neuronal responses to experimental manipulations. The number of surviving neurons in the chemically defined serum-free condition was adequate when compared to the number in the serum-containing medium, although serum was shown to affect the survival of cultured rat neurons (Kaufman and Barrett, 1983). The present result suggested that the simple chemical supplements used in this study were equivalent to serum in primate neurons. As a serum-free medium for primary cultured neurons, Neurobasal medium with B-27 supplement is popular worldwide (Brewer et al., 1993). However, the B-27 supplement already contains several hormones, such as corticosterone, triiodothyronine and progesterone, as well as other nutrients to optimize neuronal survival (Brewer et al., 1993). To establish a simple serum-free condition for general use in various researches, including hormonal studies, we did not use the B-27 supplement in this study. Although we tried to culture primate neurons from the beginning of culture in the serum-free medium (sDMEM) and even in the Neurobasal medium with B-27, only a few neurons became attached to the culture dish, suggesting that some unknown factor(s) in the serum are essential for cell attachment to the culture dish in primate neurons. bFGF was found to be a survival factor in cultured rat neurons (Morrison et al., 1986; Walicke et al., 1986) and in human neurons (Mattson and Rychlik, 1990). However, we observed only a slight survival effect

of bFGF on cultured primate neurons. It is possible that the experimental condition in this study caused this inconsistent result. In serum-free conditions, the absence of surrounding glial cells may prevent an indirect effect of bFGF or reduce neuronal sensitivity to it. BDNF showed a significant survival effect on primate neurons, which was consistent with a previous report on rat cortical neurons (Frechilla et al., 2001). This result suggests that BDNF directly supports the survival of neurons and plays an important role in the development of the primate cortex in vivo. These primate neurons would be applicable to neurotoxicological studies relating to glutamate exposure, since glutamate showed a significant cytotoxicity in primate neurons, as it did in rat neurons (Harms et al., 2001). In addition, nearly full inhibition of glutamate-induced cell death by MK-801 suggests that primate cortical neurons express NMDAR even in in vitro condition and glutamate toxicity in primate neurons is caused mainly through NMDAR.

Primate type-1 astrocytes were prepared according to a previous study on rats (Murakami et al., 1999) with some modifications. In the present study, primate type-1 astrocytes responded to dbcAMP, a cell-permeable cAMP analog, and showed morphological changes, as was also found in rat type-1 astrocytes (Gilad et al., 1990). This result suggests that the elevation of intracellular cAMP causes morphological conversion of primate type-1 astrocytes. Serum, EGF (Spina Purrello et al., 2002), and bFGF (Nakatsuji and Miller, 2001), which are growth factors for rodent type-1 astrocytes, also stimulated the growth of primate type-1 astrocytes. Hydrocortisone caused various physiological responses in rat type-1 astrocytes (Brookes, 1992; Cullingford et al., 1998; Loo et al., 1995; Morrison and de Vellis, 1981; Yarowsky et al., 1994). In the present study, hydrocortisone was a weak growth factor for primate type-1 astrocytes. These results suggest that astrocytes had few species differences between primates and rodents.

It is considered that, in rats, oligodendrocytes (Asou et al., 1995; Bogler and Noble, 1994) and type-2 astrocytes (Murakami et al., 1999) develop from O-2A progenitor cells. Although we also tried to establish primate oligodendrocyte and type-2 astrocyte culture by immunopanning A2B5-positive O-2A progenitor cells according to previous studies (Asou et al., 1995; Murakami et al., 1999), the amount of tissue needed to get 10^6 A2B5-positive cells was more than 1.0 g of cortical tissue, which is a sixth of the total weight of all cortices in the fetal primate brain. It may be difficult to consider this value a sufficient rate of cell recovery for large-scale cell biology. In addition, we could not differentiate O-2A progenitor cells to differentiate into oligodendrocytes even though the same protocol for rat oligodendrocytes (Asou et al., 1995; Bogler and Noble, 1994) were tried, while both A2B5- and GFAP-positive type-2 astrocytes were barely obtained (data not shown). The conditions necessary for O-2A progenitor cells into oligodendrocytes might differ between rodents and primates. We thought that the exceedingly small numbers of MBP-positive oligodendrocytes (<0.01%) found in cortical cell culture and the low recovery rate of the panning method might exclude the use of the cortical tissues of cynomolgus monkey fetus at 80 days of gestation as a suitable source of oligodendrocytes or type-2 astrocytes. Further examination is required on the optimal brain region and fetal stage for the primary culture of oligodendrocytes and type-2 astrocytes.

Primate microglial cell culture was established by collecting weakly attached and floating cells in a whole cortical cell culture, according to a previous study on rats (Giulian and Baker, 1986). In addition, we could repeat this procedure several times. We observed two distinct forms of cultured primate microglial cells: amoeboid and ramified. It is well known that activated rat microglial cells show the amoeboid form, while resting cells show the ramified form (Suzumura et al., 1991). We identified microglial cells by the expression of CD68, a monocyte marker of human microglial cells (de Groot et al., 2001; Wang et al., 2001), and by the growth response of microglial cells to M-CSF (Giulian and Ingeman, 1988). Microglial cells are capable of initiating and amplifying an inflammatory response in the human brain. This primate microglial cell culture would be useful for in vitro analysis of brain-specific inflammations in neurodegenerative disorders such as Alzheimer's disease (von Bernhardi and Ramirez, 2001) or viral encephalitis (Lane et al., 1996).

This is the first report to describe selective primary cultures of neurons, type-1 astrocytes and microglial cells from a non-rodent animal in serum-free conditions. In the future, selectively cultured primate brain cells might be applied to in vitro studies on CNS disorders such as Alzheimer's disease, virus-induced encephalitis, transmissible spongiform encephalitis (Sales et al., 1998) or others in which the species barrier becomes a subject of discussion. These systems might apply not only to pathological studies but also to toxicological studies in estimating the effects of endocrine-disrupting chemicals on the CNS, and to physiological studies of the CNS that consider species specificity.

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Astroglial responses against Aβ initially occur in cerebral primary cortical cultures: species differences between rat and cynomolgus monkey

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Abstract

In the present study, we investigated how amyloid beta (Aβ) peptides initially affect neuronal cells in primary cerebral cortical cultures from rat and cynomolgus monkey. In these cultures, complicated interactions between glial and neuronal cells occur; moreover, synaptic interactions similar to those observed in vivo also occur between neuronal cells in these cultures. In this study, we applied low concentrations of Aβ to these well-characterized primary cultures to investigate how Aβ initially affects neurons or astroglial cells. In both rat and monkey cortical cultures, treatment with low concentrations of Aβ failed to drastically change or damage of neurons. Aβ treatment, however, significantly activated astrocytes, resulting in increased apolipoprotein E (ApoE) production. Rat astrocytes were more sensitive to Aβ than monkey astrocytes, and responded to Aβ via a different mechanism. In monkey astrocyte cultures, only direct treatment with Aβ increased ApoE production. In rat astrocyte cultures, however, treatment with conditioned media from cortical cultures grown with Aβ increased ApoE production, indicating that some sort of neuron-derived soluble factor(s) was also involved in activating rat astrocytes. These species differences suggest that monkey cortical cultures would be more useful as an in vitro model system to understand the details of how Aβ accumulates in the human brain, since monkeys are phylogenetically more similar to humans.

Keywords: Amyloid beta peptides; Astrocyte; Monkey cortical culture; Rat cortical culture; Species difference

1. Introduction

Amyloid beta (Aβ) peptide consists of 40–43 amino acids and is derived from amyloid precursor protein (APP) (Citron et al., 1997). Aβ is the major protein component of senile plaques (SP), a characteristic feature of Alzheimer's disease (AD) (Glenner, 1988). Since Aβ is toxic to cultured nerve cells, some have argued that Aβ cytotoxicity is the major cause of brain damage observed in AD (Behl et al., 1992; Koh et al., 1990; Mattson et al., 1992; Yankner et al., 1990). Although Aβ toxicity has been thoroughly studied, the manner in which neuronal cells in vivo are initially affected by Aβ accumulation remains unknown.

Many studies also show that astrocytes have an important role in clearing Aβ from the brain (Funato et al., 1998; Matsunaga et al., 2003; Wyss-Coray et al., 2003). We previously showed that both neuronal and glial cells are found in primary cultures prepared from the cerebral cortices of either rat or cynomolgus monkeys (Negishi et al., 2002a,b). In these cultures, complicated interactions between glial and neuronal cells occur; moreover, synaptic interactions similar to those observed in vivo also occur between neuronal cells in these cultures. In the present study, we applied low concentrations of Aβ to these well-characterized primary cultures to investigate how Aβ initially affects neurons or astroglial cells. Since SP are conformed by the aggregation of low concentrations of Aβ with age, Aβ treatment in this study would reflects in vivo event of the early stage of Aβ accumulation. We also investigated how astroglial cells respond to Aβ when conditioned media are applied to the cultures. We were especially interested in determining whether species (rat versus monkey) differences emerged in the responses of neuronal and astroglial cells to Aβ.

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2. Materials and methods

2.1. Animals

Pregnant Sprague-Dawley rats were purchased from SLC Japan (Shizuoka, Japan). The animals were maintained under controlled conditions (temperature, 24 ± 1 °C; humidity,55 ± 5%) in plastic cages with sterilized wood shavings for bedding. They were fed a commercially available diet (CMF; Oriental Yeast, Tokyo, Japan) and had ad libitum access to food and tap water.

Six cynomolgus monkey (*Macaca fascicularis*) fetuses (80 days gestation) were used in this study. Four were purchased from Shin Nippon Biomedical Laboratories (Kagoshima, Japan), and two were obtained from the Tsukuba Primate Center, National Institute of Infectious Diseases, Japan. This experiment was conducted according to the guidelines of the Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences, The University of Tokyo.

2.2. Rat primary cerebral cortical cultures

Rat fetuses were removed on gestational day 18 by axillary exsanguination, and their brains were removed then transferred into ice-cold isolation medium (IM) consisting of equal volumes of Ca^{2+}-free phosphate-buffered saline (PBS), Mg^{2+}-free PBS, and Dulbecco's Modified Eagle's Medium containing 1.2 mg/ml $NaHCO_3$, 110 μg/ml pyruvic acid, 25 μg/ml streptomycin, and 50 U/ml penicillin (mDMEM). After bisecting the brains into cerebral hemispheres, the meninges, hippocampi, and other subcortical structures were carefully removed, and the cerebral cortices were rinsed in culture medium (CM: mDMEM with 5% fetal calf serum) and minced into small pieces ($<1\ mm^3$) in CM. The tissue pieces were digested at 32 °C for 30 min in PBS containing 1.5 U/ml papain (Worthington Biochemical Corporation, Lakewood, NJ, USA), 0.1 mg/ml DNase I (Roche Diagnostics, Japan), 0.2 mg/ml cysteine, 0.2 mg/ml albumin, and 5 mg/ml glucose. Cells were dissociated gently by passing the mixture several times through a disposable pipette, and then the mixture was centrifuged three times in CM at 800 rpm for 5 min at 32 °C. For TUNEL staining, cells were plated at 2.5×10^5 cells/cm^2 in CM onto a LAB-TEK chamber slide (Nalge Nunc, Tokyo, Japan) coated with 0.125% polyethylenimine. For other experiments, cells were plated at 4.2×10^5 cells/cm^2 onto culture dishes coated with 0.125% polyethylenimine. All cultures were maintained at 37 °C in a humidified chamber containing 95% air and 5% CO_2. Half the volume of culture supernatant was replaced with pre-warmed CM once per week.

These primary rat cerebral cortical cultures consist mainly of neurons (more than 90%) with some astrocytes. We previously showed that these cultured neuronal cells have complicated interactions with glia and other neurons and make synaptic connections with other neurons similar to those in vivo (Negishi et al., 2002a). After 3 days in vitro, the total proteins of these cultures almost unchanged regardless of time course (data not shown).

2.3. Cynomolgus monkey primary cerebral cortical cultures

Monkey fetuses were removed on gestational day 80, and then digested and dissociated in the identical manner as for the rat cultures (above). Plating onto slides and culture dishes for TUNEL staining and other experiments was carried out in exactly the same way as for the rat cultures. As with the rat cultures, half the volume of culture supernatant was replaced with pre-warmed CM once per week.

As with the rat cultures, these monkey primary cerebral cortical cultures consisted mainly of neurons (more than 90%) with some astrocytes. We previously showed that these cultures also exhibit complicated interactions similar to those observed under in vivo conditions (Negishi et al., 2002b). After 3 days in vitro, the total proteins of these cultures almost unchanged regardless of time course such as rat (data not shown).

2.4. Rat and cynomolgus monkey astrocyte cultures

After 14 days of culturing, cerebral cortical cells were dissociated with 0.025% trypsin (Invitrogen, UK) and washed several times in CM. Proliferating type-1 astrocytes were quickly selected from this suspension. After one subculturing, cells were plated at 4.2×10^5 cells/cm^2 in CM onto uncoated culture dishes. Half the volume of culture supernatant was replaced with pre-warmed CM once per week (Negishi et al., 2003).

2.5. Amyloid beta treatment

Aβ peptides, Aβ1-40 (Aβ40) and Aβ1-42 (Aβ42) (Bachem, Torrance, CA, USA), were dissolved in 100% DMSO, then diluted in CM (0.45% DMSO final concentration). These Aβ40 and Aβ42 (i.e., no pre-aggregating) were ultimately added to primary cortical cultures and astrocyte cultures. After 3 days of culturing, CM containing Aβ peptides was added into the rat or monkey cortical primary cultures at a concentration of 2 μM or 5 μM. These cultures were maintained for 1, 3, 7, or 14 days. Aβ peptides (5 μM) were also added to confluent rat or monkey astrocyte cultures, and these were maintained for 3 days. The CM of control cultures contained the same concentration of DMSO (0.45%).

2.6. Treatment with conditioned media

To examine whether soluble factors produced by neurons affect astrocyte responses to Aβ peptides, the conditioned medium from the primary cortical cultures was collected and used to treat the astrocyte cultures. CM supernatant from the rat and monkey primary cultures was collected after 1, 3,

and 7 days of Aβ treatment (5 μM). Astrocyte cultures (see above) from rat or monkey were maintained in the respective supernatant for 3 days.

2.7. Antibodies

For Western blotting, the following antibodies were used: rabbit polyclonal anti-Caspase-3 (H277; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-Synaptophysin (SY38; DAKO, Denmark), rabbit polyclonal anti-APP (β-APP_{695}; Zymed Laboratories, San Francisco, CA, USA), mouse monoclonal anti-GSK3β (GSK; Transduction Laboratories, Lexington, KY, USA), rabbit polyclonal anti-phospho-GSK3β (S9; Cell Signaling Technology, Beverly, MA, USA), mouse monoclonal anti-Glial fibrillary acidic protein (6F2; DAKO, Denmark), and goat polyclonal anti-ApoE (APO-E; Chemicon). H277 reacts not only with p11, p17, and p20 subunits but also with the full-length precursor of caspase-3. β-APP_{695} reacts with all three forms of β-APP (β-APP_{695}, β-APP_{751}, β-APP_{770}) and recognizes the APP C-terminal fragment (βCTF) that results from the cleavage of APP by β-secretase. S9 recognizes the Ser-9-phosphorylated, inactive form of GSK3β.

2.8. Western blot analyses

To extract total cellular proteins from the cultured cells, the cells were bathed in a solution containing 9.85 mg/ml Tris–HCl, 0.774 mg/ml ethylenediaminetetraacetic acid (EDTA), 0.348 mg/ml ammonium persulfate, 0.5% (v/v) TritonX-100, and 2.3% (w/v) SDS in PBS. Total proteins were isolated by centrifugation, adjusted to 30 μg, then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE with 12.5% acrylamide gel). Separated proteins were blotted onto polyvinylidene fluoride membranes (Immobilon P, Millipore, Bedford, MA, USA). The membranes were blocked with 5% nonfat dried milk in 20 mM PBS (pH 7.0) and 0.1% Tween-20 overnight at 4 °C, then incubated with primary antibodies (H277, 1:2000; Syn, 1:5000; β-APP_{695}, 1:2000; GSK, 1:10,000; S9, 1:1000; GFAP, 1:10,000; APO-E, 1:3000) for 1 h at room temperature. They were then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG, mouse anti-rabbit IgG, or rabbit anti-goat IgG (1:6000, Jackson Immunoresearch Laboratories, West Grove, PA, USA) for 1 h at room temperature. Immunoreactive elements were visualized using enhanced chemiluminescence (ECLplus, Amersham, UK).

2.9. Data analyses

The effects of Aβ treatment on the expression of synaptophysin, APP-βCTF, GSK3β, capsase-3, GFAP, and ApoE were confirmed by quantifying the immunoreactive bands (obtained from the Western blots) with commercially available software (Quantity One, PDI, Inc, NY, USA). Data are shown as means ± S.D. For statistical analyses, one-way ANOVAs were performed followed by the Bonferroni/Dunn *post hoc* test.

3. Results

3.1. Western blot analyses of neuron- and astrocyte-related protein expression following aβ treatment

Western blot analyses were performed to determine whether Aβ treatment affected the expression of various neuron- and glia-related proteins. In rat cortical cultures, treatment with either Aβ40 or Aβ42, even at a concentration of 5 μM, did not significantly influence the expression of synaptophysin, APP-βCTF, or GSK3β (Figs. 1A and 2A–C). Aβ40 and Aβ42 also did not affect the expression of the full-length precursor of caspase-3 in these cells; moreover, expression of all caspase-3 subunits was absent, regardless of the duration of Aβ treatment (data not shown). Synaptophysin expression did not decrease even after 14 days of Aβ treatment; rather, synaptophysin expression

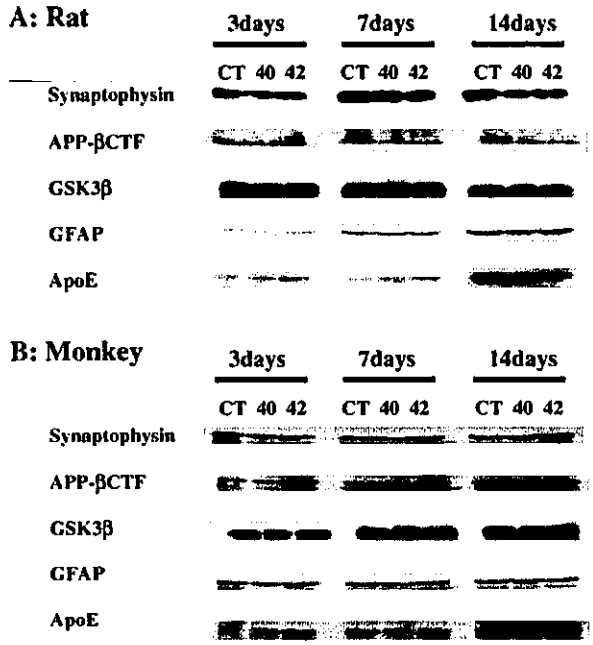

Fig. 1. Western blots showing the expression of neuron-related proteins (synaptophysin, APP-βCTF, and GSK3β) and astrocyte-related proteins (GFAP and ApoE) in rat primary cerebral cortical cultures (A) and monkey primary cerebral cortical cultures (B) following treatment with either 5 μM Aβ40 or 5 μM Aβ42. SY38 immunostained a 38 kDa band representing synaptophysin, β-APP_{695} immunostained a ~15 kDa band representing APP-βCTF, and GSK immunostained a 46 kDa band representing GSK3β. 6F2 immunostained a 52 kDa band representing GFAP, and APO-E immunostained a ~34 kDa band representing ApoE. CT—controls consisted of extracts from cultures grown in standard culture medium with DMSO; 40—extracts from cultures treated with Aβ40; 42—extracts from cultures treated with Aβ42; 3 days—after 3 days of Aβ treatment; 7 days—after 7 days of Aβ treatment; 14 days—after 14 days of Aβ treatment.

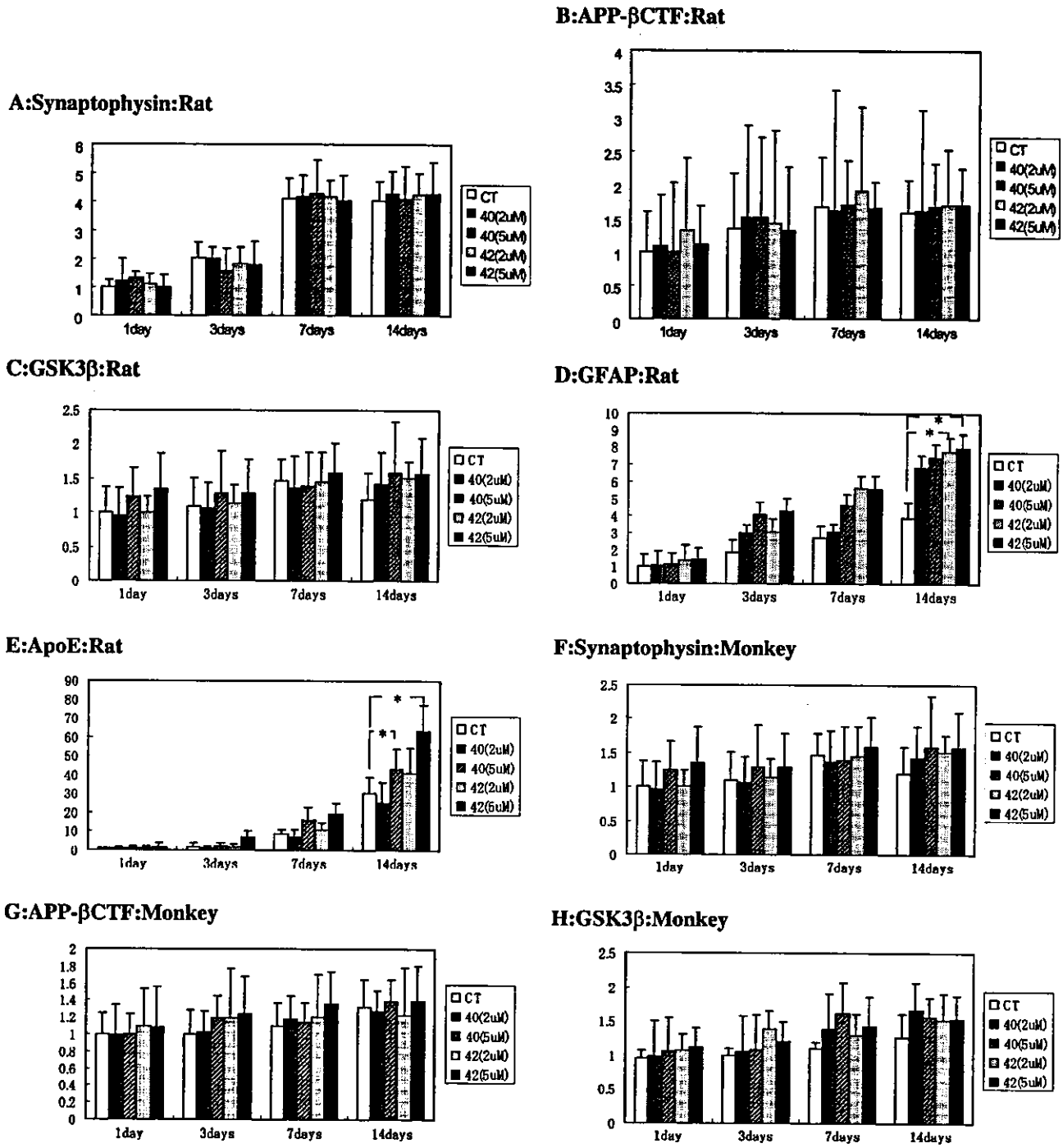

Fig. 2. Immunoreactive bands were quantified, and the resulting density data were compared to those measured from blots containing extracts from control cultures grown for 1 day (*$P < 0.02$). In rat cortical cultures ($N = 10$) (A–E): Aβ treatment did not significantly affect the expression of synaptophysin (A), APP-βCTF (B), and GSK3β (C). Interestingly, synaptophysin expression measured 7 and 14 days after Aβ treatment was slightly elevated and greater than that measured after 1 and 3 day(s) of Aβ treatment. In contrast to the neuron-related proteins, Aβ treatment induced increases in expression of astrocyte-related proteins. GFAP expression was significantly elevated in cultures treated with either 2 μM or 5 μM Aβ42 for 14 days (D). ApoE expression was significantly elevated in cultures treated with either 5 μM Aβ40 or 5 μM Aβ42 treatments (E). In monkey cortical cultures ($N = 6$) (F–J), Aβ treatment did not affect the expression of synaptophysin (F), APP-βCTF (G), and GSK3β (H). Although GFAP levels did not markedly increase in cultures treated with Aβ, even after 14 days of Aβ treatment (I), ApoE expression significantly increased in cultures treated with 5 μM Aβ42 for 14 days (J). Data are means and error bars are S.D.s.

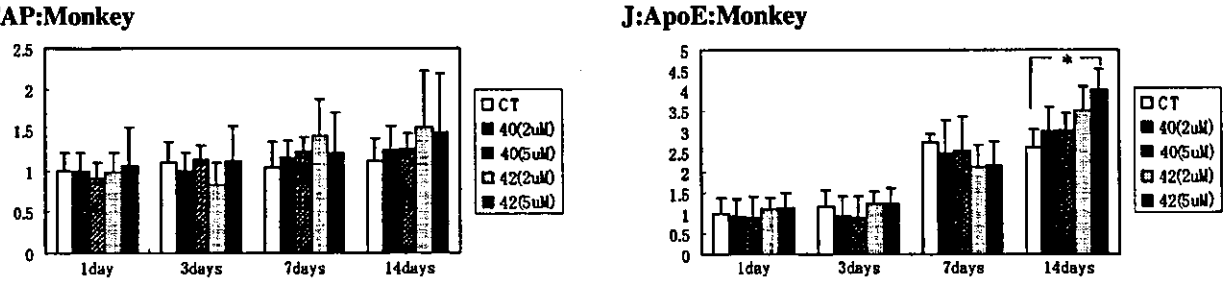

Fig. 2. (*Continued*).

was greater 14 days after treatment compared to that 3 days after treatment (Fig. 2A). APP-βCTF expression remained unchanged throughout the course of Aβ treatment, even after 14 days, indicating that Aβ treatment did not induce endogenous Aβ production from APP (Figs. 1A and 2B). GSK3β expression slightly increased in cultures treated with Aβ (Figs. 1A and 2C). However, levels of Ser-9-phosphorylated GSK3β, the inactive form of GSK3β, remained unchanged regardless of Aβ treatment length or concentration of Aβ (data not shown). Similarly, the expression of neuron-related proteins in monkey cortical cultures did not significantly decrease or increase during Aβ treatment (Figs. 1B and 2F–H).

In stark contrast to neuron-related proteins, we found that Aβ treatment profoundly affected astrocyte-related proteins in both rat and monkey cortical cultures (Figs. 1 and 2D,E,I,J), and the effects were different for the two species.

In rat cortical cultures, 14 days of Aβ42 treatment, even at a low concentration of 2 μM, increased GFAP expression significantly (Figs. 1A and 2D). Aβ40 treatment also increased GFAP expression, but not significantly (Fig. 2D). In addition, both Aβ40 and Aβ42 treatment (5 μM) significantly increased ApoE expression (Figs. 1A and 2E). On the other hand, in monkey cortical cultures, 14 days of Aβ42 treatment only slightly increased GFAP expression (Figs. 1B and 2I). Furthermore, only Aβ42 treatment (5 μM for 14 days) significantly increased ApoE expression; the magnitude of this increase was less than that observed in correspondingly treated rat cortical cultures (Fig. 2E,J).

3.2. Western blot analyses of astrocyte-related protein expression following treatment with conditioned media

To examine whether some kind of neuron-derived soluble factor(s) influences astrocytic responses to the different Aβ peptides in cortical cultures, we treated rat and monkey astrocyte cultures with either conditioned media from primary cultures treated with Aβ or media containing Aβ (i.e., direct Aβ treatment), then we compared the changes in the expression levels of astrocyte-related proteins (GFAP and ApoE). In rat astrocyte cultures, direct Aβ42 treatment significantly increased GFAP expression (Figs. 3A and 4A). On the other hand, conditioned-media treatment did not affect GFAP expression in astrocyte cultures, even when the conditioned medium was derived from cortical cultures treated with Aβ for 7 days (Figs. 3A and 4A). In monkey astrocyte cultures, direct Aβ treatment also increased GFAP expression, although not significantly (Figs. 3B and 4C). Similar to rat astrocyte cultures, conditioned-media treatment did not affect GFAP expression in monkey astrocyte cultures. Although direct Aβ treatment induced expression of GFAP and conditioned-media treatment had little effect in both rat and monkey astrocyte cultures, the opposite occurred for ApoE (Figs. 3 and 4B,D). In rat astrocyte cultures, direct Aβ treatment induced ApoE expression, and furthermore, treatment with conditioned medium from cortical cultures treated with Aβ42 for either 3 or 7 days also increased ApoE expression levels significantly (Figs. 3A and 4B). Treatment with conditioned media from cortical cultures treated with Aβ40 also induced ApoE expression, but not significantly (Fig. 4B). The degree of ApoE increase depended on the source of the conditioned medium (i.e., media collected from cortical cultures treated with Aβ for 1, 3, or 7 days) (Fig. 4B). In contrast to rat astrocyte cultures, in monkey astrocyte cultures, only direct treatment with Aβ42 significantly increased ApoE expression levels (Figs. 3B and 4D). Direct Aβ40 treatment, as well as any

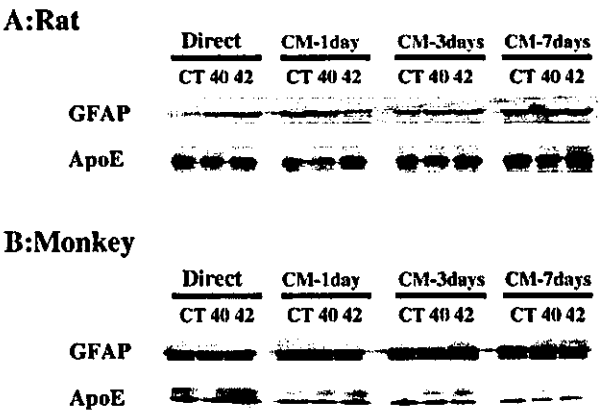

Fig. 3. Western blots showing the expression of GFAP and ApoE in extracts from rat astrocyte cultures (A) and monkey astrocyte cultures (B) following direct treatment with Aβ or treatment with conditioned media from cortical cultures treated with Aβ for 3 days. Direct—direct Aβ treatment; CM—treatment of astrocyte cultures with conditioned medium collected from cortical cultures exposed to Aβ for 1, 3 or 7 days; CT—control; 40—Aβ40 treatment; 42—Aβ42 treatment.