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Utilization of a serum-free primary culture of cortical neurons by using cyclodextrins in neurobiological research

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神経生物学的研究における基本的分析系の確立を目的として，シクロデキストリン（CD）を用いたラット大脳皮質神経細胞の初代培養を試みた。β-およびγ-CDは，無血清培地（ダルベッコ改変MEM／ハム培地）中で胎生16および18日目ラットの神経細胞を11日以上10%胎児ウシ血清を加えた培地中と同じ程度に生存させたが，α-CDには生存維持効果が無かった。β-CDはγ-CDより安定した生存維持効果を示したが，胎生21日目ラットの神経細胞を用いた場合は有意に生存率が低下し、新生児ラットでは生存維持効果が無かった。β-CDを用いた無血清培養では10%血清培地中と比べて神経突起の伸展が悪かったが，ときに顕著な突起伸展がみられ，これはCD分子に取り込まれた生理活性物質の作用と考えられた。また，β-CDを用いた無血清培養を利用してラット脳から精製したコンドロイチン硫酸プロテオグリカン（CSPG）の作用を検討し，CSPGがグルタミン酸による神経細胞死を防止すること，弱いながら培養神経細胞の生存を維持する作用をもつことを示した。以上の結果から，この無血清培養法は神経生物学的研究において有用な分析系となりうることを指摘した。

Key Words: β-cyclodextrins, γ-cyclodextrins, serum-free culture, cortical neuron, chondroitin sulfate proteoglycan

INTRODUCTION

The primary culture of neuronal cells is widely used in investigating cellular mechanism of proliferation, differentiation, and death of neurons. The serum as fetal calf serum (FCS) or newborn calf serum is commonly used for long-term survival of primary cultured neurons. Otherwise several kinds of nutritional supplements are required to survive cultured neurons as substitutes for serum. However it is difficult to rule out the effects of unknown molecules contained in the serum or these nutritional supplements on the results of experiments in these experimental conditions. In this context, we established a serum-free primary culture of cortical neurons using cyclodextrins (CDs) in which only β-CD is contained as a substitute for serum, and utilized it in examining the role of chondroitin sulfate proteoglycans in survival of primary cultured cortical neurons in addition to protective effect against excitotoxic neuronal death induced by glutamate.

CDs are cyclic oligosaccharides forming a hydrophobic cavity in their molecules which can incorporate water insoluble molecules[13]. α-, β-, and γ-CDs are consisted of 6, 7, and 8

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glucopyranose respectively, and the cavity diameters are different each other depending on the number of contained glucopyranose molecules. Although the nature forming inclusion compounds is widely used in industry and scientific research including tissue or cell culture, the type of most adequate CD is different depend on the cultured cell type or the culture condition. Hammami et al reported a successful culture of rat adrenocortical cells to induce corticosteroid biosynthesis in a serum-free, carrier protein-free medium by using a α-CD as a cholesterol carrier[4]. On the other hand, Ohmori et al. has shown that β-CD, but not α-or γ-CD, can be used as a substitute for FCS in evaluating primary antibody response to sheep erythrocyte by cultured murine lymphocyte in vitro[6),7)]. However, the role of CDs in neuronal cell culture has not been studied. In this paper, we report that β-and γ-CDs can be used as a substitute for serum in the primary culture of rat's cortical neurons, and that CDs may be a useful material for the investigation of molecules which modulate neurite outgrowth or neuronal survival.

MATERIALS AND METHODS

1. A SERUM-FREE CULTURE OF CORTICAL NEURONS USING CDs

The cerebral cortices and hippocampi were dissected from the brains of 18-day-old rat fetuses (Sprague-Dawley rat, Kurea, Osaka, Japan). The tissue was fragmented in ice-cold modified Leibovitz-15 medium (Dainippon Pharmaceutical, Tokyo, Japan), and centrifuged at 1,000 rpm for 5 min. The pellet was digested in 0.1% trypsin (from bovine pancreas, Sigma) solution supplemented with 0.002% deoxyribonuclease I (Sigma) at 37°C for 15 min. The trypsinization was halted by adding fetal calf serum (FCS, Gibco, Lot No. 34K5011) to the pellet after centrifugation at 1,000 rpm for 5 min. Following centrifugation, the dissociated cell was suspended in culture medium and aspirated gently through blue plastic tips for 20 times. This procedure was repeated three times. The culture medium was consisted of 1 : 1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (Dainippon Pharmaceutical, Tokyo, Japan) supplemented with 2.5 mM glutamine, 1.2 mg/ml of sodium bicarbonate, 50 U/ml penicillin G and 50μg/ml streptomycin (Dainippon Pharmaceutical, Tokyo, Japan), and 10% FCS. The dissociated cell suspension was passed through lens paper, and the cells were seeded onto polyethyleneimine-coated 24 well plates (Nunc, culture area 1.9cm^2,)at a density of 5×10^5 cells/cm^2 after the determination of cell viability by the ability of viable cells to exclude the dye Trypan blue. The polyethyleneimine (Sigma) coating was done by using a solution of 1mg/ml polyethyleneimine in 150 mM sodium borate buffer, PH8.4, for 2 hours at room temperature. The plate was washed twice with sterile water, and then twice with culture medium.

The cells were incubated in a humidified 5% CO_2-95% air atmosphere for 24 hours, and then the medium was replaced by serum-free DF medium supplemented with α-, β-, or γ-CD (all from Sigma). CDs were dissolved in PBS (-), and added to the culture medium at a final concentration of 500 μg/ml. On culture day 4, medium was replaced by serum-free medium containing 10 μM of cytosine arabinofuranoside (Ara-C, Sigma), and then half volume of the medium was replaced by serur-free DF medium without Ara-C on the next day. The half volume of medium was replaced twice a week thereafter. CDs were added at every replacement of the medium to maintain the concentra-

tion at 500 μg/ml. The same volume of PBS (-) was added instead of CD solution in the vehicle control. The number of neuron was counted on culture day 3, 5, 7, and 11 by a phase-contrast inverted microscope (olympus IMT-2, Olympus, Tokyo, Japan). The cells which had round-shaped, phase-bright somas were counted in five microscope fields/well at a magnification of 200×. Each experimental group was consisted of 8 wells, and the experiment was repeated three times.

2. PREPARATION OF CHONDROITIN SUIFATE PROTEOGLYCANS (CSPGs)

CSPGs were purified from 10-day-old SD rat's brain according to the method of Oohira et al[8),10),11)]. Briefly, brains were homogenized in PBS (-) containing 20 mM EDTA, 10 mM N-ethylmaleimide, and 2 mM phenylmethylsulfonyl fluoride (PMSF) followed by centrifugation at 4°C. The supernatant was lyophilized and suspended in PBS (-) containing 2 M urea, 50 mM Tris-HCl (pH 7.5) containing 0.35 M NaCl, 2 mM EDTA, 1 mM N-ethylmaleimide, and 0.2 mM PMSF. Following dialyzation against the same urea buffer, the material was applied to DEAE-Sephacel (Pharmacia, Uppsala, Sweden) column equilibrated with the same buffer. CSPGs were eluted with the same buffer containing 0.7 M NaCl after the elution of unbound materials with the same buffer containing 0.35 M NaCl. Hexuronate-rich fractions were collected and concentrated by centrifuzing on Centrimex KB D-01 tube (Sanko Junnyaku, Tokyo). The concentrated material was dialyzed against PBS (-) containing 1 mM sodium azide, and precipitated in 95% ethanol containing 1.3% potassium acetate. After the washing the pellet once with 70% ethanol containing 1.0% potassium acetate, CSPGs were lyophilized under sterile conditions and dissolved in sterile PBS (-). Hexuronate was measured by carbazole reaction according to Bitter and Muir[1)]. Core proteins and glycosaminoglycans (GAG) were also prepared by the method according to Oohira et al.[8),10),11)].

3. THE ROLE OF CSPGs IN SURVIVAL OF PRIMARY CULTURED CORTICAL NEURONS.

The role of CSPGs in survival of cultured neurons was examined in primary culture of cortical neurons described above. Neurons were dissected from 18-day-old SD rat embryos, and plated on 24-well plastic plate at a density of 5×10^5 cells/cm^2. CSPGs or hyaluronate (the hexuronate-rich fractions of 0.35 M NaCl elution in DEAE-Sephacel chromatography, this fraction contained hyaluronate but not CSPGs) were added to the culture medium on culture day 4 at a concentration of 25 nmol/ml hexuronate, and viable neurons were counted on culture day 7, 14, 21, and 28. In another experiment, the cells were suspended in serum-free culture medium supplemented with 500 μg/ml of β-or γ-CD at the plating, and CSPGs were added to the medium just after the plating. Viable neurons were counted on culture day 3, 5, 7.

4. THE PROTECTIVE EFFECT OF CSPGs AGAINST GLUTAMATE NEUROTOXICITY

The role of CSPGs in glutamate neurotoxicity was examined on culture day 7. In this case, cerebral neocortex, but not hippocampus, was dissected from 18-day-old SD rat embryos. Cells were plated on 24-well plastic plate at a density of 5×10^5 cells/cm^2. The culture medium was replaced by serum-free medium supplemented with 500 μg/ml of β-CD on culture day 1. Three-fourth of the medium was re-

placed by the same serum-free medium on culture day 3 and 5. CSPGs, core proteins, or GAG were added at a concentration of 25 nmol/ml hexuronate following the change of medium on culture day 5. On culture day 7, glutamate dissolved in PBS (-) was added to the medium at a final concentration of 1 mM after washing once with serum-free DF medium. Neuronal death was quantified by measuring lactate dehydrogenase (LDH) activity in the culture medium according to the method of Koh and Choi[12] at 24 h later.

RESULTS

1. THE SERUM-FREE CULTURE OF CORTICAL NEURONS USING CDs.

The viability of dissociated cells assessed by trypan blue exclusion was 75.8 to 95.3%, and about 90% of attached cells were neurons. The number of viable neurons was around 4.5×10^5/cm^2 in all groups on culture day 3. However, more than 90% of neurons were lost in the groups cultured in serum-free medium supplemented with α-CD or PBS (-) on culture day 5, and nearly all neurons were disappeared on culture day 7 in these two groups (Fig. 1, left). In contrast, the number of viable neurons was over 3.2×10^5/cm^2 even on culture day 11 in the group supplemented with β-or γ-CD, and there was no difference in the number of viable neurons compared with the control cultured in medium containing 10% FCS. Almost 100% of viable cells were neurons in the CD groups while the mixture of glial and epithelial cells was inevitable in 10% FCS medium in spite of the use of Ara-C. It sometimes observed, however, a sudden complete loss of cells in some wells in CD groups after the fifth day in culture, whereas no sudden cell loss was observed in the

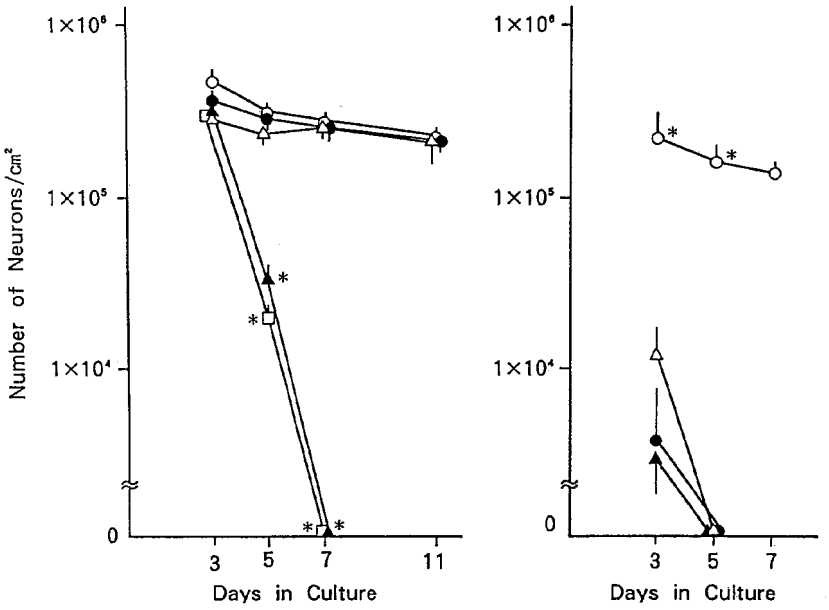

Fig. 1. Survival-promoting effect of CDs on 18-day-old rat fetus cortical neurons cultured in serum-free medium.
Open circle : 10% FCS control, closed circle : β-CD, open triangle : γ-CD, open triangle : α-CD, open square : vehicle control, left : Many neurons survived for more than 11 days in β-or γ-CD supplemented medium in contrast to almost complete disappearance of neurons in α-CD or PBS (-) supplemented medium. * : $P<0.01$ compared to 10% FCS, β-CD and γ-CD, right : Neurons did not survived more than 5 days when CD supplemented serum-free medium was used from the plating of cells. * : $P<0.01$ compared to CD supplemented serum-free medium.

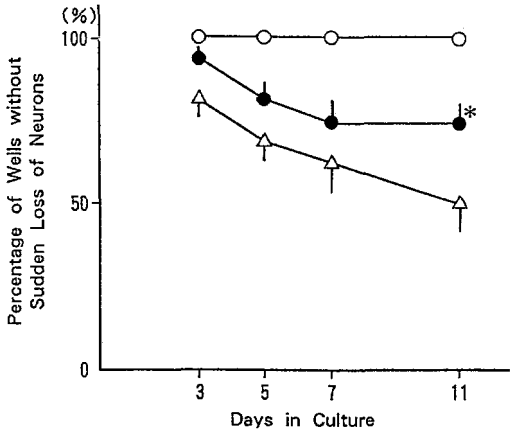

Fig. 2. Sudden cell loss observed in serum-free culture of cortical neuron.

Percentage of wells which was not effected by sudden cell loss is shown. open circle: control cultured in the medium containing 10% FCS, closed circle: β-CD, open triangle: γ-CD, * : $P<0.01$ compared to both the control and γ-CD.

control 10% FCS medium. The number of wells affected by the sudden cell loss was 25% in the β-CD group and 50% in the γ-CD group on culture day 11 (Fig. 2), but the number of viable neurons in healthy wells was equal to the control in the both groups. The percentage of wells affected by the sudden cell loss was not changed when the concentration of CDs was decreased less than 250μg/ml which is not toxic for mammalian cells[4),14),15)]. This result indicates that both β-and γ-CDs can be a serum-substitute in our serum-free culture system, but β-CD is better to maintain neuronal survival in serum-free conditions.

No further survival-promoting effect was observed when CDs were added to the medium containing 10% or 1% FCS, nor was there no dose dependence in survival-promoting effect of CDs in the serum-free condition within the dose examined (100-1000μg/ml, data is not shown). When serum-free medium containing CDs was used from the plating of cells, the number of viable neurons was less than 10% of seeded cells on culture day 3, and no viable cell was observed on culture day 5 even if β-or γ-CD was added (Fig. 1, right). This indicate that FCS is necessary for cell adhesion to culture plate, and that CDs promote survival of already attached neurons, but not promote the adhesion to plate.

We further examined the effect of β-CD in primary culture of hippocampal and cerebral cortical neurons of 16-, 21-day-old fetus, and new-born SD rats (Fig. 3). The survival promoting effect was most remarkable in 16-day-old fetus neurons, and the effect became less apparent along with maturation of the rat brain. It was actually impossible to culture new-born rat's

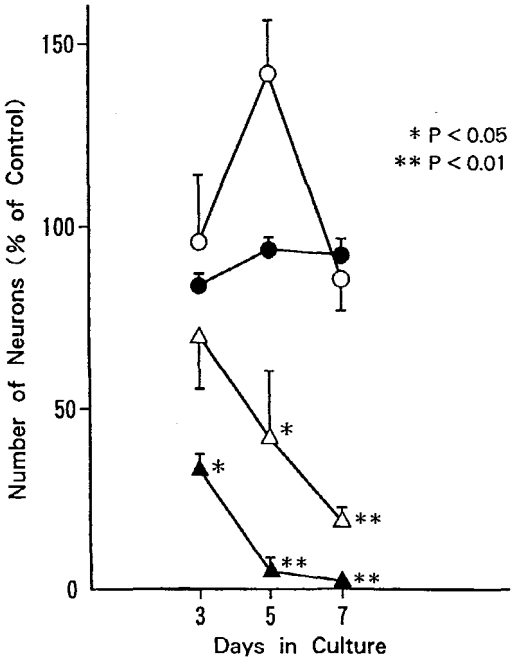

Fig. 3. Decreasing efficacy of β-CD in supporting survival of cultured neurons along with the increase of fetal age.

Values are expressed as percent of the mean number of survived neurons from the same fetal age cultured in 10% FCS supplemented medium. open circle: 16-day-old fetus, closed circle: 18-day-old fetus, open triangle: 21-day-old fetus, closed triangle: new-born rat, * $P<0.05$ * * : $P<0.01$ compared to 10% FCS control.

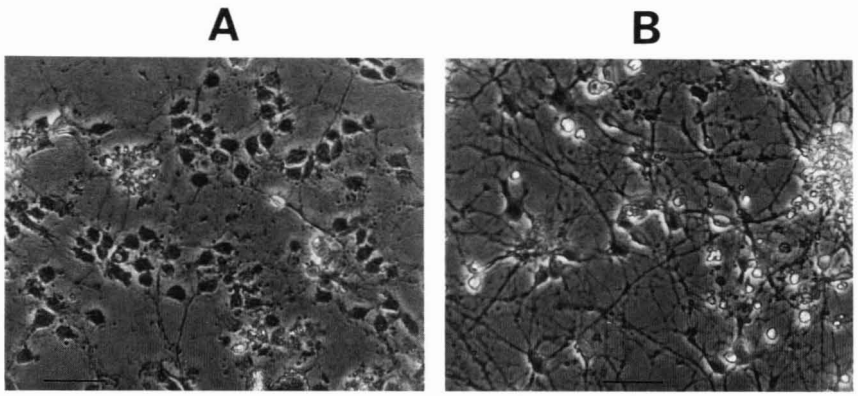

Fig. 4. Phase contrast microscopy of cortical neurons cultured in serum-free medium supplemented with β-CD (culture day 7). A : All neurons have only short neurites. B : Many neurons have long, branched neurites. Calibration : 20μm for both A and B.

neurons in serum-free CD containing medium. Neurite outgrowth of the cultured neurons was generally poor in CD containing serum-free medium even on culture day 7 when most neurons have matured long neurites in the control 10% FCS medium (Fig. 4A). However, neurons bearing long, branched neurites were observed in some wells (Fig. 4B). There was no relation between the degree of neurite extension and survival rate of neurons.

2. THE EFFECT OF CSPGs ON SURVIVAL OF CULTURED CORTICAL NEURONS.

The biochemical properties of CSPGs were corresponded to those described by Oohira et al[8),10),11)]. CSPGs did not promote the survival of cortical neurons when neurons were cultured in

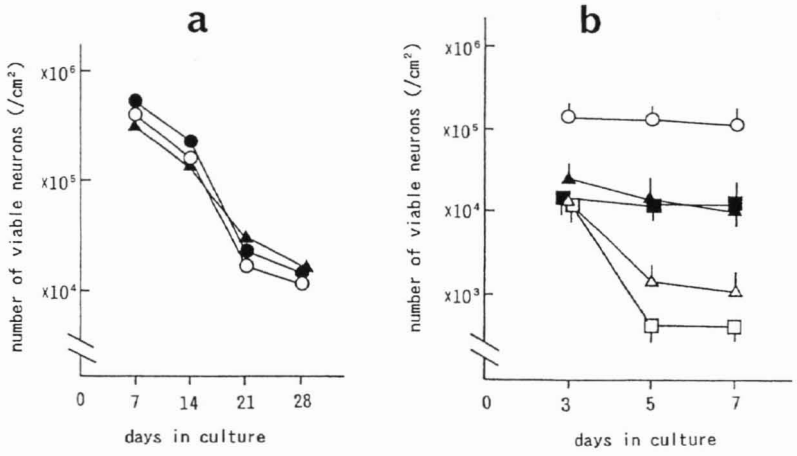

Fig. 5. Effects of CSPGs on survival of cultured cortical neurons.

a. Neurons were cultured in the medium containing 10% FCS. CSPGs or hyaluronate was added to the medium on culture day 4. open circle : PBS (-) control, closed circle : CSPGs, closed triangle : hyaluronate b. Neurons were cultured in serum-free medium containing CDs. CSPGs were added to the medium at plating neurons. open circle : control cultured in the medium containing 10% FCS, closed and open triangles : the serum-free culture using β-CD with and without CSPGs respectively, closed and open squares : the serum-free culture using γ-CD with and without CSPGs respectively, More neurons were survived in the serum-free medium containing CSPGs than in the medium without CSPGs on culture day 5 and 7. * : $P<0.01$ compared to the cultures without CSPGs.

the medium containing 10% FCS (Fig. 5A). However, they showed an apparent survival-promoting effect when neurons were cultured in serum-free medium supplemented with β-or γ-CD. The number of viable neurons was less than 10% of the control in which neurons were cultured in the medium containing 10% FCS on culture day 3. However, the number of viable neurons was not decreased on culture day 5 and 7 when CSPGs were added to the culture medium, in contrast to the rapid disappearance of neurons in the wells where PBS (-) was added to the medium instead of CSPGs (Fig 5B). This indicate that CSPGs may have an neurotrophic action under the conditions without FCS but it's effect may be covered when FCS is present in the culture medium.

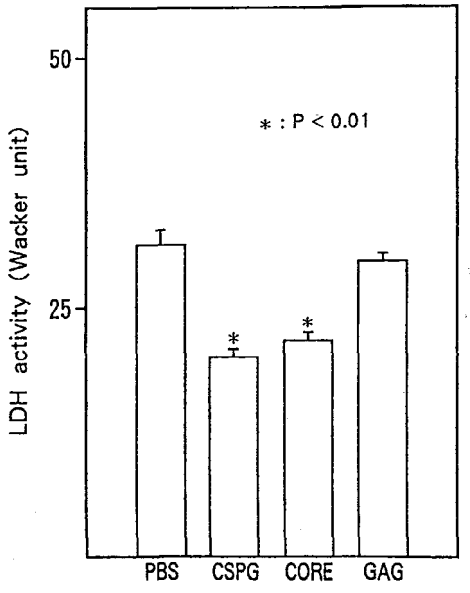

Fig. 6. Protective effect of CSPGs against excitotoxic neuronal death induced by glutamate.

The culture medium was replaced by serum-free medium containing β-CD on culture day 1, and CSPGs were added to the medium on culture day 5. Neurons were exposed to 1 mM glutamate on cultured day 7 and LDH activity of the medium was measured 24 h later. PBS : PBS (-) control, CSPG : CSPGs, CORE : core proteins, GAG : glycosaminoglycans, CSPGs and core proteins, but not GAG, significantly protected cultured neurons from excitotoxic death induced bo glutamate.

3. THE PROTECTIVE EFFECT OF CSPGs AGAINST GLUTAMATE NEUROTOXICITY.

The death of cultured neurons induced by excitatory amino acids is considered to be an in vitro experimental model of neuronal injury in hypoxia-ischemia, hypoglycemia, epilepsy and chronic degenerative diseases[2),3)]. A method to quantify the excitotoxic neuronal death is to measure LDH activity in the culture medium which has been reported to reflect the death of neuronal cell even in a mixed cell culture as in our primary culture of cortical neurons[12)]. Serum-free media with several nutritional supplements are commonly used in measuring LDH activity in the medium to exclude the effect of serum on the LDH activity[12)]. In this experiment, we utilized a serum-free medium supplemented with only β-CD instead of the commonly used nutritional supplements. In the latter, an extensive washing of wells is required to washout the serum to exclude the effect of it on LDH activity, while no extensive washing was required when neurons were cultured in serum-free medium containing β-CD. CSPGs and core proteins, but not GAG, significantly protected cultured neurons from excitotoxic death induced by glutamate. This finding was corresponded to the results obtained by experiments in which protective effects of CSPGs were examined in neuronal cultures using serum-containing medium or serum-free medium with commonly used nutritional supplements[8),9)].

DISCUSSION

In this experiment, we showed the usefulness of serum-free culture of cortical neurons using

CDs in neurobiological research. CSPGs did not show a neurotrophic effect on cultured cortical neurons in serum-containing culture medium. However, they promoted survival of neurons in the serum-free medium supplemented with β-CD, although their survival-promoting effect was not so prominent. This finding indicates that CSPGs may have a neurotrophic action on cortical neurons, and that our serum-free culture using CDs may be an useful strategy to investigate neurotrophic substances. Our serum-free culture was also useful in neurochemical experiment. CSPGs showed neuroprotective action against glutamate toxicity when neuronal cell death edath, was quantified by measuring LDH activity in the medium supplemented with β-CD. The result was consistent with the results of the experiments where serum-containing medium or serum-free medium supplemented with commonly used nutrients was used to investigate the neuroprotective action of CSPGs[8),9)]. The advantage of serum-free medium containing β-CD is to be able to omit the extensive washing of wells inevitable to avoid the unknown effect of serum on LDH activity.

The mechanism of survival-promoting effect of CDs is not clear. However, it seems likely that CDs incorporate toxic substances produced during culture[5)] or trace substances released from neurons or glial cells into medium which support neuronal survival or neurite extension[16)], rather than that they have a direct neurotrophic effect on cultured neurons. β-CD, among three CDs, is most commonly used as a complexing agent in tissue and cell culture because the cavity diameter is wellsuited for the size of hormones, vitamins and many other compounds frequently used in culture. This property of β-CD is consistent with our findings that β-CD was most useful as a substitute for serum in maintaining survival of cultured neurons, and may explain why the survival-promoting effect of β-CD is different depend on the maturity of rats brain, and why the neurite extension is poor in most wells but not in few wells. A purpose of neuroscience researches using the cultured neuron is to investigate molecules which modify the development or survival of neurons. Our serum-free culture using CDs may be a useful method to investigate such molecules, especially hydrophobic ones. However, a sudden loss of neurons observed in our serum-free culture limit it's use in neurobiological experiments. It is necessary to investigate the cause of sudden neuronal death and improve the stability of our method as a serum-free culture of neurons.

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