Immunocytochemical Localization of β -Citryl-L-glutamate in Primary Neuronal Cells and in the Differentiation of P19 Mouse Embryonal Carcinoma Cells into Neuronal Cells

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The immunocytochemical localization of β -citryl-L-glutamate (β -CG) in primary neuronal cells and in the differentiation of P19 cells was examined. 1: Cells with the morphological features of neurons in the primary culture were specifically stained with the anti- β -CG antibody both in neurites and in the cell body. 2: The neuronal cells differentiated from P19 cells were distinctly stained with the anti- β -CG antibody both in neurites and in the cell body, while the non-neuronal cells were not. 3: The concentration of β -CG was low in the P19 cells, but increased significantly with the differentiation of P19 cells into neurons. It was shown that β -CG was localized exclusively in neurons. These findings suggest that β -CG plays functional roles in the differentiation and growth of neuron.

Key words β -citryl-L-glutamate; neuron; P19 cell; cell differentiation; immunocytochemistry

We have isolated a novel compound containing glutamate from the newborn rat brain and identified it as β -citryl-L-glutamate (β -CG).^{1–3} This compound appeared at high concentrations during the period characterized by the growth and differentiation of neurons in the developing rat, guinea pig and chick, and then disappeared with maturation.^{2,3} The concentration of β -CG and its synthetic and hydrolytic activities in primary cultured neurons from chick optic tectum increased during the culture as well as in the optic lobes of the developing chick.⁴

β -CG was found in the testes of adult rats, being present predominantly in germinal cells, and accumulated at the stage of spermatogenesis coinciding with the appearance of late spermatocytes and early spermatids.⁵ The level of this compound was low in the epididymis and epididymis-ejaculated spermatozoa. We found also a novel enzyme responsible for deacylation of β -CG to citrate and glutamate in adult rat testis⁶ and purified it to homogeneity.⁷

Recently, β -CG was also found in the lens of various species, localized to the equator regions.⁸ During lens development, cell division in the proliferative zone is followed by migration of cells towards the equator where epithelial cells terminally differentiate to form lens fibers.⁹ The equatorial cortex (Bow area) showed the highest concentration of β -CG in bovine lens.⁸ Moreover, β -CG accumulated immediately before the differentiation of the bovine lens epithelial cells into lens fiber cells in primary culture.¹⁰ And the localization of β -CG in the primary cultured cells was immunocytochemically examined using anti- β -CG antibody. Cells around lentoid bodies were stained with anti- β -CG antibody, whereas cells in the bodies were stained strongly with anti- γ -crystallin antibody.¹⁰ These findings suggest that β -CG may play a role in regulating the differentiation of lens cells.

The cellular localization of β -CG in the brains and testis is unclear. Therefore, the cellular localization of β -CG in the developing brains was examined using primary cultured cells from embryonic chick and rat brain and retinoic acid (RA)-treated P19 cells. P19 is a cell line derived from mouse embryonal carcinomas (EC).¹¹ P19 cells provide an attractive

model for the study of early embryonic determination and differentiation. Under appropriate culture conditions, P19 cells differentiate into derivatives of three germ layers, endoderm, mesoderm and ectoderm.^{12,13} By treatment with a high concentration of RA, aggregated P19 cells can be stimulated to form neurons, glia and fibroblast-like cells.¹⁴ It is expected that β -CG would be localized in neurons and its concentration would increase during culture when P19 cells differentiate into neurons in response to RA, if β -CG is important for the differentiation of neuronal cells *in vivo*.

We report here the immunocytochemical and chemical changes of β -CG in primary cultured neurons and RA-treated P19 cells.

MATERIALS AND METHODS

Materials β -CG was synthesized as described previously.¹ Anti-microtubule associated protein 2 (MAP 2) monoclonal antibody, biotinylated donkey anti-mouse IgG and the biotinylated donkey anti-rabbit IgG were purchased from Amersham. Anti-neurofilament (NF) antibody from Sigma. Anti-glial fibrillary acidic protein (GFAP) polyclonal antibody from DAKO. Avidin-biotinylated horseradish peroxidase from Boehringer. 3-Amino-9-ethylcarbazole and Gel Tol from Lipshaw (U.S.A.). The polyclonal anti- β -CG antibody was prepared by us as described previously.⁸ The anti- β -CG antibody purified by ammonium sulfate precipitation, affinity chromatography and adsorption procedures reacted specifically to β -CG-conjugates among a series of β -CG related compounds.⁸ All other reagents were purchased from commercial sources and were of the highest grade available. P19 was obtained from the American Type Culture Collection (U.S.A.).

Primary Culture of Neurons from Chick and Rat Brain

The optic lobes of 7- or 8-day-old chick embryos or cerebral cortex of a newborn rat were dissected and placed in Ca^{2+} / Mg^{2+} -free Dulbecco's phosphate-buffered saline (PBS(–)). The dissociation and culture of neurons were carried out as described by Pettmann *et al.*,¹⁵ Skaper *et al.*,¹⁶ Adler *et al.*¹⁷

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Fig. 1. Typical Growth Profiles of Three Types of RA-Treated P19 Cells

(a) Under the conditions in the absence of cytosine arabinoside (CA-). (b) Under the conditions in the presence of cytosine arabinoside (CA+).

and Riopelle and Cameron¹⁸⁾ with a slight modification. In brief, the optic lobes or cerebral cortex were rinsed in PBS(-), minced with scissors, and then trypsinized with 0.025% trypsin-0.1 mM EDTA-0.008% DNase I for 10 min at 37°C in a 5% CO₂ atmosphere. Cells were washed and dispersed by *ca.* 20 passes through the flamed tip of a Pasteur pipette in DMEM containing gentamicin (40 µg/ml), and 10% fetal calf serum (FCS). The dispersed cells were passed through a lens paper and diluted with fresh culture medium. An aliquot (*ca.* 10 ml) of the cell suspension for immunocytochemical study (3×10^5 cells/well) was seeded on a glass multiwell plate (12-well culture plate, Iwaki) precoated with 0.1% gelatin and 0.1% poly-L-ornithine in PBS(-), at room temperature overnight. After incubation for 60 min at 37°C in a 5% CO₂ atmosphere, the medium was changed to serum-free DMEM containing N1 constituents (sigma), and cells were incubated for 2 d under the conditions described above. Thereafter, the culture medium was changed every 2 d to fresh DMEM supplemented with 10% FCS. Changes of morphological features of neurons in primary culture during growth *in vitro* were observed by phase-contrast microscopy.

Immunostaining Cells on the glass coverslips were washed with PBS(-) and fixed in a solution containing 4% carbodiimide and 10% formaldehyde in 50 mM phosphate buffer, pH 7.4, for 30 min at room temperature. The cells were washed with PBS(-), treated with 95% methanol containing 5% acetic acid for 5 min at -20°C and then treated with 0.1% Triton-X100, 0.15 mM NaCl in 0.05 M Tris-HCl (pH 7.6) and finally blocked for 15 min at room temperature with protein blocking agent (Lipshaw) after being washed with 20 mM Tris-HCl, pH 7.5, containing 200 mM NaCl and 0.05% Tween 20 (TTBS). The samples were incubated for 1 h at 37°C with anti-MAP 2 monoclonal antibody, anti-NF antibody, GFAP polyclonal antibody, or anti-β-CG polyclonal antibody⁸⁾ as first antibody, the biotinylated donkey anti-mouse IgG or the biotinylated donkey anti-rabbit IgG as second antibody and then avidin-biotinylated horse radish peroxidase as described by Hsu *et al.*¹⁹⁾ Following an addi-

tional rinse, the samples were incubated in 3-amino-9-ethyl-carbazole (0.5 mg/ml) containing 0.01% H₂O₂ for 5–10 min. The cells were counterstained with hematoxylin (Lipshaw). The samples were rinsed thoroughly with TTBS or water, mounted in the 20% glycerol, manicured and examined under a microscope (Olympus).

P19 Cell Culture and Retinoic Acid Induction Routine culturing and induction were performed according to the methods of Rudnicki and McBurney²⁰⁾ with slight modification. In brief, induction was performed by absorbing cell aggregates at 1×10^5 cells/ml in 5×10^{-7} M all-*trans* RA (Sigma) for 96 h. After induction, cell aggregates were treated with 0.025% trypsin-0.1 mM EDTA in PBS(-) for 10 min at 37°C, and dissociated by pipetteing. The cell suspensions were washed 3 times with PBS(-) and plated at 3×10^5 cells/ml on a 100φ dish or glass multiwell plate (12-well culture plate, Iwaki) precoated with 0.1% gelatin and 0.1% poly-L-ornithine (Sigma). They were maintained in α-MEM containing serum (2.5% FCS+7.5% CS). The medium was changed every 2 d. The cells were cultured in the presence or absence of 20 µM cytosine arabinoside (CA). When cultured without CA (CA-), glial cells increased remarkably from day 4 of culture as shown in Fig. 1a. When cells were exposed to CA for 2 d (CA+) from day 2 after inoculation, the proliferation of glial cells was delayed and the cells increased gradually from about day 7 of culture (Fig. 1b).

Determination of β-CG in the Cultured Cells The β-CG in the cultured cells was determined with HPLC according to methods developed in our laboratory.⁴⁾ Cells were homogenated with 75% ethanol containing Tris base and the extracts were used for the determination. Values were corrected on the basis of the recovery rate obtained using β-[¹⁴C]CG as an internal standard. The residues were resuspended in ice-cold 10% trichloroacetic acid (TCA). The TCA extracts were used for the DNA determination and then the residues were solubilized with 1 M NaOH for protein determination. DNA and protein were determined by the diphenylamine method and Lowry's method,²¹⁾ respectively.

RESULTS AND DISCUSSION

Immunocytochemical Localization of β -CG in Neuronal Cells Cultured from Chick Embryo Optic Lobes

Neuronal cells were cultured from chick embryo optic lobes for 12 d and the cellular localization of β -CG was examined immunocytochemically with anti- β -CG antibody (Fig. 2). On day 3 of culture, the aggregation of cells and the formation of fine networks of neuronal processes became apparent. At 5 d after inoculation, "bundles" of neuronal processes connected with the adjacent cell aggregates, and the network of neuronal processes increased in complexity: a few thinly spread, phase-light non-neuronal elements ("flat" cells) migrated out of the clumps, their number gradually increasing. After day 9 of culture, the number of bundles of neuronal processes increased. There was little contamination by non-neuronal cells of the cultured cell population, assessed using anti-neurofilament (NF) antibodies, until day 5 after inoculation, but thereafter the population of non-neuronal cells in the culture dish increased gradually (data not shown).

On day 3 of culture, cells with the morphological features of neurons were specifically stained with the anti- β -CG antibody both in neurites and in the cell body (Fig. 2a), but not in nuclei. β -CG was found in cytosomal and crude mitochondrial fractions for the subcellular distribution of immature rat brain (Miyake, unpublished data). None of the cells were stained with preimmune serum of the rabbit (Fig. 2b). On day 7, 9, and 12 of culture, neurons were also stained with the anti- β -CG antibody both in neurites and in the cell body (data not shown). These staining results were similar to those at day 3 of culture. The intensity of the anti- β -CG antibody-staining in neuronal cells increased during culture. These changes were remarkably similar to the developmental increase in the concentration of β -CG in optic lobes of the developing chick brains and in primary neuronal cells from the chick embryo optic lobes as reported previously by us.⁴⁾

Immunocytochemical Localization of β -CG in Neuronal Cells Cultured from Neonatal Rat Cerebral Cortex

Immunocytochemical localization of β -CG or two neuron-specific proteins and one glia-specific protein were examined in neuronal cells cultured from neonatal rat cerebral cortex (Fig. 3). The morphological changes in primary neurons from neonatal rat cerebral cortex during culture were similar to those in primary neurons from chick embryo optic lobes. Cells cultured for 12 d were examined with the anti- β -CG antibody or the antibodies specific to the neuron, anti-MAP 2²²⁾ and anti-NF antibody²³⁾ and the antibody specific to glia, anti-GFAP antibody.²⁴⁾ At day 5 of culture, the cells with the morphological features of neurons, which were strongly stained with anti-MAP 2 and anti-NF antibody, were distinctly stained with the anti- β -CG antibody both in neurites and in the cell body (Figs. 3a—c). Some of the cells with the morphology of clearly recognizable non-neuronal "flat" cells were stained with anti-GFAP antibody (Fig. 3d), but not with anti- β -CG antibody. None of the cells was stained with preimmune serum of the rabbit and normal serum of mouse (Fig. 3e). The changes in the intensity of the anti- β -CG antibody-staining for primary neurons from neonatal rat cerebral cortex during culture were also similar to the changes of β -CG concentration in cerebral cortex of the developing rat as reported previously by us.^{2,3)} These results indicate that β -

CG is localized exclusively in neurons.

Immunocytochemical Localization of β -CG in the Differentiation of P19 into Neuronal Cells in Response to RA P19 is an embryonic carcinoma cell, and it can be differentiated into neurons, glia, and fibroblast-like cells by treatment with retinoic acid (RA).¹⁴⁾ It is known that P19 is a good model system of the neuronal differentiation.

RA-treated P19 cells were cultured in the absence of cytosine arabinoside (CA) (CA(-); normal cell culture) or in the presence (CA(+); neuron-enriched culture). When cultured in the absence of CA (CA-), untreated P19 cells grew to be densely packed, displaying a characteristic cuboidal morphology evident on phase contrast microscopy. Treatment with RA resulted in a marked change in morphology. Within 24 h of plating, a flat layer of fibroblast-like cells migrated out from the periphery of the aggregate. Between 1 and 2 d after plating, neuron-like cells appeared whose processes grew rapidly from the aggregate over the fibroblast-like cell layer. The processes from these neuron-like cells were frequently arranged in bundles. After day 4—5 of plating, a population of glial cells initially appeared at the junction of the fibroblast-like monolayer and the aggregate and then increased rapidly. Thus RA-treated cultures contained three distinct cell types. The cells cultured for 12 d were examined with the anti- β -CG antibody, two antibodies specific to the neuron, anti-MAP 2 and anti-NF antibody and the antibody specific to the glia, anti-GFAP antibody. Figure 4 shows the results at day 5—7 of culture. The cells with the morphology of a neuron were stained with both anti-MAP 2 and anti-NF antibody and were strongly stained even with the anti- β -CG antibody both in neurites and in the cell body (Figs. 4a, b, c). However, the flat cells which are clearly morphologically unlike neurons, were stained with the anti-GFAP antibody (Fig. 4d), but not stained completely with anti- β -CG antibody. In addition, fibroblast-like cells were stained with neither anti- β -CG antibody nor other antibodies including anti-MAP 2, anti-NF and anti-GFAP antibody. None of the cells were stained with preimmune serum of the rabbit (Fig. 4e). Similar results to those at day 5—7 of culture were obtained at day 2, 4, and 9 of culture (data not shown). These immunocytochemical results with anti- β -CG antibodies suggest that β -CG is localized exclusively in neurons. When cultured in the presence of CA (CA+), the differentiated cell types appeared in a reproducible sequence after the plating of RA-treated aggregates. Fibroblast-like cells appeared initially (day 1), followed by neurons (day 2), and glial cells (day 6—7). The start of the proliferation of glial cells was delayed about 2 days and the degree of the proliferation was also considerably suppressed. Staining of the cells cultured for 12 d with various antibodies including anti- β -CG antibody was tried. However, the aggregated neuron-like cells bound to the glass coverslips were easily removed making staining difficult because of the many washing processes. Though the conditions for staining were not good, the neuron-like cells were stained with a neuron-specific marker, anti-MAP 2 antibody and even with anti- β -CG antibody (data not shown).

Changes of β -CG Concentration in the Differentiation of P19 into Neuronal Cells in Response to RA The cells were collected during culture and the level of β -CG was determined with HPLC according to methods developed in our laboratory.⁴⁾ Changes in β -CG concentrations in RA-treated

Fig. 2. Immunocytochemical Localization of β -CG in Neuronal Cells Cultured from Chick Embryo Optic Lobes

(a), (b) Immunostaining of neuronal cells cultured for 3 d. (a) Anti- β -CG antibody, (b) preimmune rabbit serum. Bar=50 μ m.

Fig. 3. Immunocytochemical Localization of β -CG in Neuronal Cells Cultured from Neonatal Rat Cerebral Cortex

(a)–(e) Immunostaining of the neuronal cells cultured for 5 d ($\times 200$). (a) Anti- β -CG antibody, (b) anti-MAP 2 antibody, (c) anti-NF antibody, (d) anti-GFAP antibody, (e) preimmune rabbit serum. Bar=50 μ m.

Fig. 4. Immunocytochemical Localization of β -CG in the RA-Treated P19 Cells

(a)–(d) Immunostaining of cells cultured for 7 d. (e) Immunostaining of cells cultured for 5 d. (a) Anti- β -CG antibody, (b) anti-MAP 2 antibody, (c) anti-NF antibody, (d) anti-GFAP antibody, (e) preimmune rabbit serum. Bar=50 μ m.

P19 during culture are shown in Fig. 5. As estimated from DNA content per dish, the cell number decreased significantly with incubation time, so the β -CG concentrations were expressed as nanograms β -CG/micrograms of DNA. When cultured in the absence of CA (CA $-$), β -CG concentrations increased gradually in RA-treated cultures of P19 cells and were highest when the cultures contained the largest numbers of morphologically identifiable neurons. β -CG increased markedly in the neuron-enriched culture (CA $+$) after day 7 of culture and then slightly decreased. These changes agreed with the growth of neurons in the culture dish and the increased intensities of the staining with anti- β -CG antibody. The β -CG concentration was low in the RA-untreated P19 cells. A few populations of cells in RA-untreated P19 were stained with anti- β -CG antibody. This may mean that a few cell populations in P19 spontaneously differentiate into neurons.

It is known that PC12²⁵⁾ rat pheochromocytoma cells re-

spond to nerve growth factor (NGF) and shift from a chromaffin-cell-like phenotype to a neurite-bearing sympathetic-neuron-like phenotype. The β -CG level was determined during the differentiation of PC12 cells into neurons. But β -CG could not be chemically detected in the neurons treated with NGF. Also, none of the neurons exposed to NGF were stained with anti- β -CG antibody (data not shown). β -CG could not be chemically detected in the adrenal medulla, sciatic nerve and sympathetic ganglia of the adult rat (Miyake, unpublished data). More detailed studies are necessary to clarify these subjects.

It became clear by this study that β -CG was localized exclusively in neuronal cells both in the primary culture and RA-treated P19 cells (Figs. 2–4). The concentration of β -CG increased with the differentiation of neurons from RA-treated P19 cells and then decreased slightly (Fig. 5). Previously we have shown that β -CG increased with the differentiation and growth of neurons both *in vivo*^{3,4)} and *in vitro*⁴⁾ and

Fig. 5. Changes of β -CG in the RA-Treated P19 Cells

The β -CG level in cultured P19 cells was determined by HPLC. Values were corrected on the basis of the recovery rate. Data are expressed as the mean $\pm$ S.E.M (bar values for three samples). $\bullet$, CA+; $\circ$, CA-.

then decreased with maturation. These findings suggest that β -CG is necessary for differentiation and growth of neuronal cells. Recently we found that β -CG increased immediately before the differentiation of the bovine lens epithelial cells into lens fiber cells, and then decreased slightly.¹⁰⁾ β -CG was present predominantly in germinal cells of various animals including mammals, amphibians and fish, and increased at the stage of spermatogenesis coinciding with the appearance of late spermatocytes and early spermatids in rat testis, while it decreased remarkably by seminiferous tubule failure such as experimental cryptorchidism.⁵⁾ All these results suggest that β -CG may play roles in differentiation and growth of neurons, lens epithelial cells and spermatocytes.

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