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CEREBELLAR ASTROCYTES SPECIFICALLY SUPPORT THE SURVIVAL OF PURKINJE CELLS IN CULTURE

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Summary: As a first step toward identifying the factor(s) that is/are produced by astrocytes and support(s) the survival of cerebellar Purkinje cells in dissociated culture, we compared the effect of astrocytes of cerebellar, hippocampal, and cerebral origin. A feeder coverslip of cerebellar astrocytes, which did not have cell-to-cell contact to neuronal culture, increased the percentage of Purkinje cells to about 8-9 fold with no change in the percentage of astrocytes. On the other hand, astrocytes of hippocampal or cerebral origin did not increase the percentage of Purkinje cells at low plating density, whereas they increased the number of astrocytes in neuronal culture. These results indicate that the factor(s), tentatively named as Purkinje-cell suvival factor was/were specifically produced by cerebellar astrocytes and affected directly on Purkinje cells.

Cerebellar Purkinje cells have been attracting much interest for their unique electrophysiological properties and for their critical role in cerebellar motor learning. Much of the important works on Purkinje cells has been performed *in vivo* or in cerebellar slice preparations (8, 9). However, analysis of channel activity in dispersed cells in culture has several advantages over studies *in vivo* or with brain slices. Better control of the external enviroment of neurons permits the recording the direct effect of

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drugs. Easy access to neurons, and better oxygenation permits the stable recording. In dispersed culture, Purkinje cells can be identified morphologically or immunocytochemically with specific markers in dispersed culture (3, 5, 16, 17, 18). These culture, however, consitsted of relativeley small populations of Purkinje cells. In adult rat cerebellum, the ratio of granule cells to Purkinje cells is reported to be 250-900 (6). Weber and Schachner (15) have reported that the survival of Purkinje cells in culture depends on the age of cerebella (from 14-day embryo to 1-day neonate), use of serum-free chemically defined medium, high plating density (2.3 x 10^5 cells/cm^2). The percentage of Purkinje cells in culture was still about 0.2%. Recently, Broson et al. (1) reported that the percentage of Purkinje cells could be increased up to 50-60% by co-culturing with cerebellar astrocytes. This effect seems to be mediated by diffusable factor(s) because neurons did not make contact with astrocytes in their culture method. It is still not known, however, whether the factor(s) is/are produced specifically by cerebellar astrocytes. It is also not clear that the factor(s) is/are acting directly on Purkinje cells because their culture contained about 10 % of astrocyte, and the factor(s) may have acted on the contaminated astrocytes which actually supported the survival of Purkinje cells. To answer these questions, we compared the effect of astrocytes of cerebellar, hippocampal, and cerebral origin and reduced the contamination of astrocytes in neuronal culture by prolonged use of cytosine arabinoside (AraC) in serum-free medium. We report that the factor(s) to support the survival of Purkinje cell is/are specifically produced by astrocytes of cerebellar origin and the factor(s) directly act(s) on Purkinje cells.

MATERIALS AND METHODS

One week prior to the neuronal culture, astrocyte culture was prepared on non-coated coverslips (diameter 1.5 cm, Matsunami) that are attached with three paraplast "feet" as described earlier (1, 12). Brains from postnatal day 1 pups (Wistar rat) were minced and treated with 1% trypsin (Difco) and 0.05% DNaseI (Sigma) in Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution (HBSS) (Sigma) for 5 min at 25 °C. The cells were washed with culture medium containing 10% horse serum, and dissociated by passing through a fine-tipped pipette in Ca^{2+}-free HBSS containing 0.05% DNaseI and 12mM $MgSO_4$. Dispersed cells were plated at a density of 4 x 10^3 cells/cm^2 onto the coverslips in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10 % fetal bovine serum, 0.1 mg/ml streptomycin (Meiji), and 100 U/ml penicillin (Banyu). After 1 week, the cultured cells consisted of mainly astrocytes

(about 95%) as judged by immunocytochemical staining for glial fibrillary acidic protein (GFAP).

Cerebellar neurons were prepared as described previously (17, 18). Briefly, cerebella from Wistar rat embryos on gestational day 16 were treated with 0.1% trypsin and 0.05% DNaseI in Ca^{2+}/Mg^{2+}-free HBSS for 5 min at 25 °C. The cells were washed with culture medium containing 1 μg/ml of aprotinin (Sigma) and dissociated by passing through a fine-tipped pipette in Ca^{2+}-free HBSS containing 0.05% DNaseI and 12mM $MgSO_4$. Dispersed cells were plated onto poly-L-lysine (Sigma)-coated coverslips (diameter 1 cm) attached to the bottom of plastic dishes (Meridian) in modified serum-free defined medium (2): DMEM supplemented with 1 mg/ml bovine serum albumin (BSA), 10 mg/ml insulin, 0.1 nM L-thyroxine, 0.1 mg/ml transferrin, 1 mg/ml aprotinin (all from Sigma), 30 nM selenium (Merck), 0.1 mg/ml streptomycin, and 100 U/ml penicillin. Two hours after plating, the coverslips containing astrocytes were transferred and placed on to the neuronal culture with the astrocyte-side facing down to the neuronal culture. In this configuration, cell-to-cell contact was impossible and only diffusable factors could affect the neuronal culture. Two days after plating, 10 μM AraC was added to arrest the proliferation of contaminated astrocytes in neuronal culture. Half of the medium was changed twice a week. The culture was maintained in a humidified atmosphere of 5% CO_2 in air at 37 °C.

One week after plating of cerebellar neurons, cells were stained for inositol trisphosphate (IP_3) receptor, a specific marker for Purkinje cells, and for GFAP as described (17). Briefly, cells were fixed with 4 % paraformaldehyde in 0.1 M phosphate buffer for 10 min, permeabilized with 0.01 % Triton X-100 in phosphate-buffered saline (PBS) for 10 min, and blocked with 1 % non-fat milk in PBS for 60 min. Cells were incubated with monoclonal antibody against IP3 receptor, or with polyclonal antibody against GFAP, and stained by the avidin-biotin complex method with diaminobenzidine as a chromogen.

RESULTS AND DISCUSSION

In serum-free defined medium, the survival of Purkinje cells was totally dependent on the plating density (Fig.1, SFM), and the percentage of Purkinje cells increased to about 5 % at a plating density of 2.0 x 10^5 cells/cm^2 (Fig.2B) as reported previously (17). When the neurons were co-cultured with astrocytes of cerebellar origin, the percentage of Purkinje cells increased to 38-46 % and the survival of Purkinje cells was independent of plating density (Fig.1, SFM + Cbl; Fig.2B). Astrocytes of hippocampal (Fig.1, SFM + Hip.; Fig.2B) or cerebral origin (Fig.2B) increased the percentage of Purkinje cells to about 10-14 % in cultures plated at 5.0 x 10^4 and 2.0 x 10^5 cells/cm^2 (Fig.1, Fig.2B). However, they had no effect in increasing the percentage of Purkinje cells in cerebellar neurons plated at a density of 1.5 x 10^4 cells/cm^2. In cultures with hippocampal astrocytes or without any feeder coverslips of

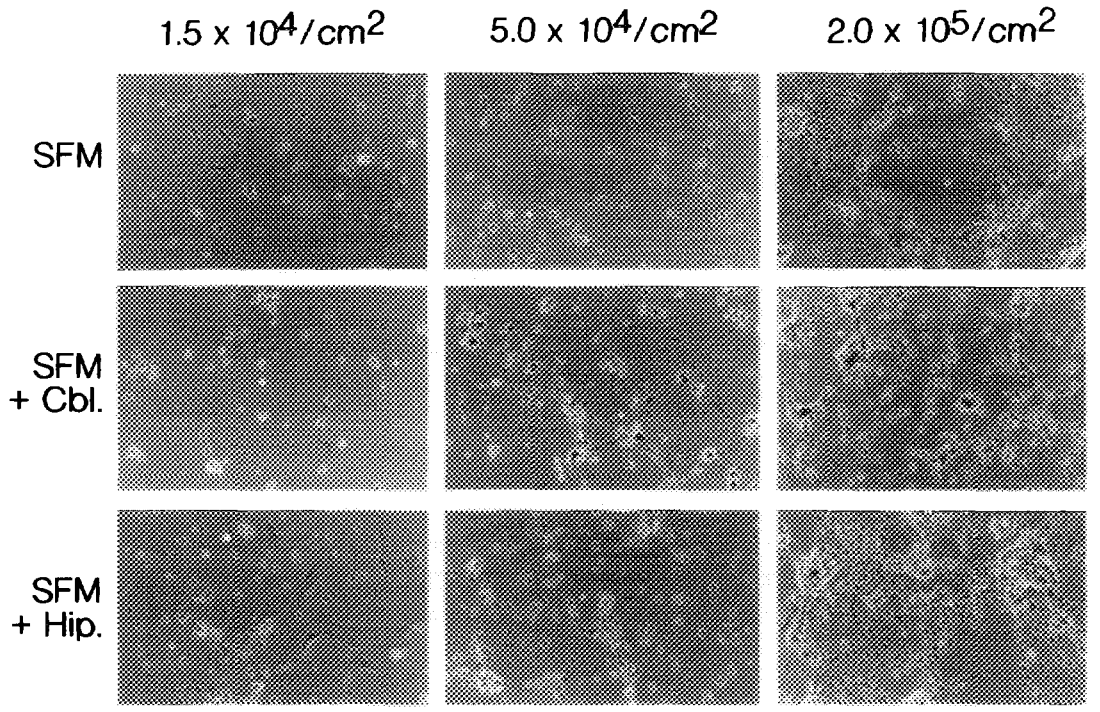

Fig.1. Immunocytochemical detection of Purkinje cells in different culture conditions. Cerebellar neurons were cultured in serum-free medium (SFM), feeder coverslips of cerebellar astrocytes (SFM + Cbl.), or feeder coverslips of hippocampal astrocytes (SFM + Hip.) at plating density of 1.5 x 10^4, 5.0 x 10^4, or 2.0 x 10^5 cells/cm^2 as described in materials and methods. On day 9 of culture, cells were reacted with an antibody against inositol trisphosphate receptor and stained by avidin-biotin-peroxidase method using diaminobenzidine as a chromogen. Scale bar at the right bottom corner indicates 50 μm.

astrocytes, Purkinje cells were always surrounded by many granule cells and some contaminated astrocytes and never survive as single neurons (see Fig.1 SFM+Hip. at 2.0 x 10^5, for example). In cultures with cerebellar astrocytes, Purkinje cells often lived alone (Fig.2A). These data indicate that the diffusable factor(s) that supported the survival of Purkinje cells was/were preferentially produced by cerebellar astrocytes.

It was difficult to estimate the effect of feeder coverslips of astrocytes on other constituents of cerebellar neuronal cultures, such as granule, Golgi, basket, and stellate cells, due to a lack of specific markers for discrimination of these cells. There were little differences in the total number of survived neurons, which was counted after immunostaining for astrocytes by GFAP as described later, among the various

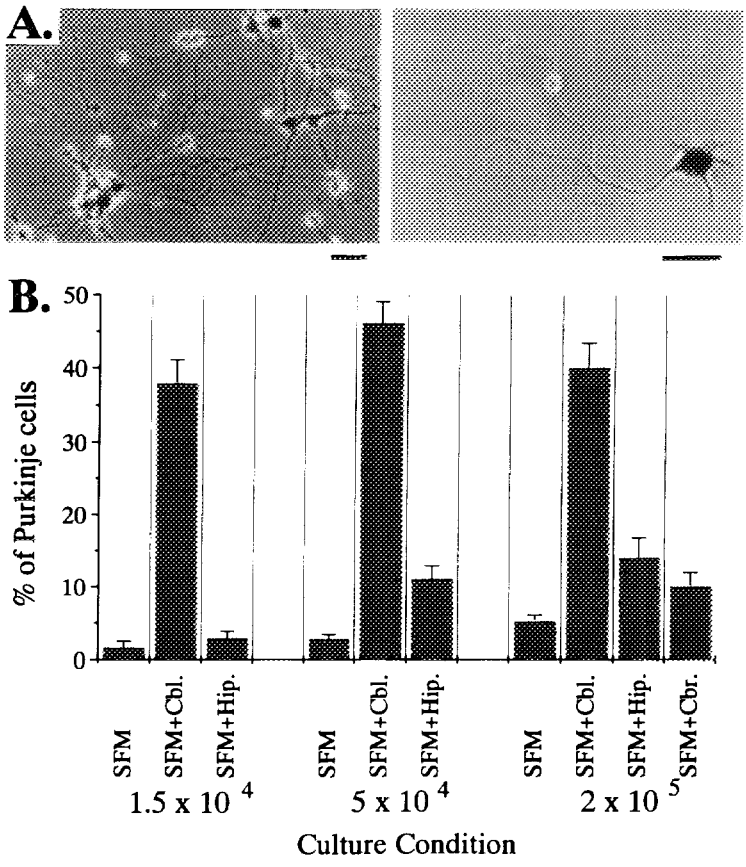

Fig.2. Purkinje cells at higher magnification and the percentage of Purkinje cells in each culture condition. (A) Purkinje cells in culture at plating density of 5.0×10^4 cells/cm^2 with feeder coverslips of cerebellar astrocytes were stained with an antibody against inositol trisphosphate receptor. Scale bars indicate 50 μm. (B) Percentage of Purkinje cells in different culture condition. SFM: serum-free medium only, SFM+Cbl.: co-cultured with cerebellar astrocytes, SFM+Hip.: co-cultured with hippocampal astrocytes, SFM+Cbr.: co-cultured with cerebral astrocytes. Columns and bars represent means ± standard error of means (n=3).

culture conditions (Fig.1). As granule cells are the major constituents (about 95%) of neuronal cultures (2, 15), the increase in the percentage of Purkinje cells in culture fed by cerebellar astrocytes suggest that granule cells decreased in number during early stages in culture. As Purkinje cells cease to divide at early embryonic stage (6), we suggest that the factor(s) produced by cerebellar astrocytes supported the survival of Purkinje cells specifically. We thus tentatively refer to the factor(s) as Purkinje cell survival factor(s) (PSF).

PSF(s) produced by cerebellar astrocytes may have acted on the contaminated astrocytes that actually supported the survival of Purkinje cells. We next investigated the contamination of astrocytes in each neuronal culture. Contamination of astrocytes was minimized by use of serum-free defined medium and prolonged use of AraC. The percentage of astrocytes, however, tended to increase at higher plating density. It was 1 % in cultures plated at 1.5 x 10^4 cells/cm^2 and increased to 3 % in cultures plated at 2.0 x 10^5 cells/cm^2 in serum-free medium. The percentage of astrocytes in neuronal cultures co-cultured with astrocytes of cerebellar origin was very similar: 2 % at 1.5 x 10^4 cells/cm^2 and 4% at 2.0 x 10^5 cells/cm^2. In contrast, the percentage of astrocytes in neuronal cultures co-cultured with astrocytes of hippocampal or cerebral origin was very high. It was 29 % at 1.5 x 10^4 cells/cm^2 and 47% at 2.0 x 10^5 cells/cm^2 for hippocampal co-culture, and 38 % at 2.0 x 10^5 cells/cm^2 for cerebral co-culture. These results suggest that the diffusable factor(s) produced from astrocytes of hippocampal and cerebral origin promotes the survival or proliferation of astrocytes contaminated in neuronal cultures. The partial effect of astrocytes of hippocampal and cerebral origin in supporting the survival of Purkinje cells (Fig.2B) may be attributed to the PSF(s) produced by the contaminated cerebellar astrocytes that were activated by the diffusable factor(s) from astrocytes of hippocampal and cerebral origin.

The intensity of staining for IP_3 receptor was weaker in Purkinje cells plated at a density of 1.5 x 10^4 cells/cm^2 than those plated at higher densities (Fig.1 SFM+Cbl.). As electrical activity in cerebellar cultures affect the differentiation of Purkinje cells (11), this may be due to the lack of electical activity in a culture plated at low density.

Several neurotrophic factors are known to support the survival of neurons (14) and some of them are produced by astrocytes (4, 7, 10). In our preliminary experiments, nerve growth factor (NGF) had no effect in supporting the survival of Purkinje cells (data not shown). Segal et al. (13) reported that brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) supported the survival of granule cells but not that of Purkinje cells. In any case, factors that specifically support the survival of GABAergic neurons such as Purkinje cells have never been reported. Purkinje cells are specifically degenerated in some types of cerebellar

atrophic diseases. It is therefore important to identify PSF(s) for potential therapeutic use and for elucidation of the mechanism of such diseases. For this purpose, differential study using cerebellar astrocytes and hippocampal astrocytes, as we have shown here, may be of much value.

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