Serum-free cell culture studies on the glial cell differentiation

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SERUM-FREE CELL CULTURE STUDIES ON THE GLIAL CELL DIFFERENTIATION

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ON THE GLIAL CELL DIFFERENTIATION

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Table of Contents

1. ABSTRACT

The mature nervous system contains a large number of terminally differentiated glial cells that developed from progenitors in the embryonic nervous system. Many aspects of the differentiation pathways leading to the formation of glial cells remain elusive because of the cellular and molecular complexity of the brain. One way to reduce the complexity is to study particular developmental stages in glial differentiation using cultured cells. This paper focuses on cultured glial cells from neonatal mouse cerebrum as one model to study glial differentiation. This model can be used to identify glial subtypes including their progenitors under serum-free conditions.

In Chapter 3, the methods of primary culture and cryopresevation of glial cells under serum-free conditions are described. Astrocytes, the most abundant glial subtype in the brain, were highly enriched by primary culturing cells from neonatal mouse cerebrum in a serum-free, chemically defined medium optimized for the selective growth of astrocytes. Furthermore, the enriched astrocytes can be cryopreseved in the presence of 10% dimethylsulfoxide and 0.1% methylcellulose. These methods should significantly enhance our ability to study the characteristics of astrocytes.

In Chapter 4, the characteristics of glial cultures starting from primary cultures of astrocytes are described.

The cells subcultured in a serum-free medium for 1 month consisted of four glial subtypes: type-1 astrocytes, oligodendrocyte-type-2 astrocyte (O-2A) progenitors, oligodendrocytes and type-2 astrocytes. Using the mixed glial cultures, the expression of fibronectin and laminin, which are considered to play important roles in the development of embryonic neurons, was examined. Immunofluorescence experiments revealed that the expression patterns of the molecules differ among the subtypes. Type-1 astrocytes appeared to be the most active producer of the molecules among the glial subtypes. In addition, Western blot analysis showed that glial fibronectin has a slightly higher molecular weight than mouse plasma fibronectin (230 kDa), and glial laminin is a variant with a 220 kDa B chain present and the 400 kDa A chain missing.

In Chapter 5, the characteristics of a newly established cell line of astrocyte progenitors (AP-16) are described. Cell line AP-16 was established by long-term culture of the mixed glial cells under serum-free conditions. Transforming growth factor-β (TGF-β) induced AP-16 cells to differentiate into phenotypically mature astrocyte. Furthermore, TGF-β increased the expression of fibronectin in AP-16 cells. This novel cell line should be useful for studying the roles of TGF-β in the differentiation of astrocyte progenitors.

In Chapter 6, cytokines affecting survival and differ-

entiation of the astrocyte progenitor cell line (AP-16) are described. Epidermal growth factor (EGF), transforming growth factor-α and basic fibroblast growth factor (bFGF) acted as survival factors that prevented death of AP-16 cells by apoptosis. The differentiation of AP-16 cells into astrocytes was induced by ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF). The results obtained with AP-16 cells suggest that these cytokines are involved in astrocyte development in the central nervous system.

In the present study, three types of glial cultures were developed using serum-free conditions: the primary culture, the mixed glial culture and the cell line. These cultures are considered to be valuable in vitro cellular systems to study the differentiation and functions of their in vivo counterparts. In particular, the novel cell line AP-16 has enabled us to increase our understanding of the astrocyte development. This is the first report describing cell death by apoptosis in astrocyte progenitors and involvement of the various cytokines in the astrocyte development.

2. INTRODUCTION

Most cells in the central nervous system are either neurons or glial cells. Glial cells are up to 10 times as numerous as neurons and can comprise up to 50% of the total cell volume in the cerebral cortex (Pope, 1978). Glial cells have traditionally been implicated in a variety of maintenance functions within the central nervous system (for review, Kimelberg and Norenberg, 1989). They wrap around and enclose the axons, dendrites, synapses and blood vessels. They control the composition of the interstitial medium which is fundamental to the operation of the system of computation and transmission that is dependent on membrane currents. Recent evidence indicates that glial cells are much more dynamic components than previously realized. In particular, they maintain specialized interactions with neurons through the production of specific cell-adhesion, extracellular matrix or neurotrophic molecules (for review, Lander, 1989). Therefore, it has become evident that the central nervous system will never be fully understood without an understanding of the properties and functions of glial cells.

In the past two decades, the characterization of glial cells has been improved considerably with cultured cells and immunological markers for cell identification. Booher and Sensenbrenner (1972) showed in their pioneering work that

cells dissociated from cerebral hemispheres during a particular period of development would yield nearly homogeneous cultures of astrocytes. This discovery stimulated extensive studies on the properties of astrocytes, studies that are difficult or impossible with the intact nervous system or bulk-isolated cells. For cell identification, the discovery of glial fibrillary acidic protein (GFAP) by Eng et al. (1971) was a significant advancement. GFAP is one component of the intermediate glial filaments found in the cytoplasm of astrocytes. GFAP is found only in astrocytes and has therefore proved to be an invaluable marker for identifying those cells in culture. Following the discovery of GFAP, numerous cell-type-specific markers have been discovered and used to identify cell types in mixed glial cultures (for review, Hansson, 1986).

Classically, from morphological studies using metal impregnation and electron microscopic methods, macroglial cells in the vertebrate central nervous system were divided into two types, oligodendrocytes and astrocytes, and the latter were subdivided into protoplasmic and fibrous types, found in the gray matter and white matter, respectively (for review, Privat and Rataboul, 1986). More recently, with immumocytochemical techniques, astrocytes from primary cultures of rat optic nerve, a white matter tract, were further subdivided into type-1 and type-2 derived from two distinct glial cell lineages (Raff et al., 1983 a, b). One comprises

oligodendrocytes, type-2 astrocytes and their common bipotential (O-2A) progenitors, and the other comprises type-1 astrocytes and their progenitors. Glial cell populations morphologically and antigenically similar to O-2A lineage cells and type-1 astrocytes are present also in primary cultures from neonatal rat cerebellum (Levi et al., 1986) and cerebral cortex (Bahar et al., 1988). This glial diversity has been one of the most challenging problems in neurobiology: how do the apparently homogeneous neuroepithelial cells of the neural tube lead to the variety of mature glial cells? It is not yet clear what factors influence the determination of these cell types.

To elucidate the factors influencing glial differentiation, serum-free culture systems are useful. Since the first report by Morrison and de Vellis (1981), there have been attempts to develop serum-free media specifically designed for glial cells in which the cells could be grown in a completely defined microenvironment. Such defined media have two potential advantages: (1) serum-free media can be formulated to select one specific glial cell type of the heterogeneous cell population of the central nervous system and (2) the effects on the glial differentiation and function of hormones, cytokines and extracellular matrix molecules can be studied without the variables introduced by unknown components in serum.

This paper focuses on cultured glial cells from neona-

tal mouse cerebrum as one model to study the differentiation and functions of glial cells. The cerebrum comprises the largest cellular mass in the mammalian brain; therefore, it is suitable for obtaining large quantities of metabolically-active, nontumorigenic glial cells. Furthermore, on postnatal days 1-3, the cerebrum is developmentally immature with respect to glial maturation, which allows for optimal mixed glial cultures. This model can be used to identify glial subtypes and to examine their functions, such as production of extracellular matrix molecules, under serum-free conditions. Also, serial passage of the mixed glial culture led to the establishment of a novel cell line (AP-16) of astrocyte progenitors.

Interestingly, AP-16 cells died by apoptosis after cytokine deprivation. Epidermal growth factor (EGF), transforming growth factor-α (TGF-α) and basic fibroblast growth factor (bFGF) acted as survival factors that prevented the apoptosis. In addition, transforming growth factor-β (TGF-β), ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) induced AP-16 cells to differentiate into phenotypically mature astrocytes. The factors that regulate survival and differentiation of astrocyte progenitors have been largely unknown to date. The results obtained with AP-16 cells suggest that these cytokines regulate the astrocyte development in the central nervous system.

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3. PRIMARY CULTURE AND CRYOPRESERVATION OF MOUSE ASTROCYTES UNDER SERUM-FREE CONDITIONS

3.1 Introduction

Primary cultures of enriched astrocytes have been developed in the last two decades (Booher and Sensenbrenner, 1972; Shapiro, 1973; Lim et al., 1973). Because these cultures are derived from normal tissue, such cells are considered to be a better model for astrocytes in vivo than transformed glial cell lines (Schousboe, 1977; Kimelberg et al., 1978). After the first report (Morrison and de Vellis, 1981) of a primary culture of astrocytes under serum-free conditions was published, several culture systems using serum-free media were developed (for review, Sensenbrenner et al., 1986). However, to our knowledge, there is only one paper describing a primary culture of astrocytes derived from mouse brain (Fischer et al., 1982). Fischer et al. reported a chemically defined medium consisting of transferrin, selenium, insulin, epidermal growth factor, aprotinin, hyaluronic acid and bovine serum albumin for astrocytes derived from newborn mouse cerebellum. They observed that astrocytes generated in the chemically defined medium remained immature with respect to expression of glial fibrillary acidic protein (GFAP), a protein which is specific for mature astrocytes (Bignami et al., 1972). These cells

expressed GFAP after being transferred to serum-supplemented medium. The first purpose of this study, therefore, was to establish a primary culture system for mouse mature astrocytes under serum-free conditions.

Despite the importance of primary cultures of astrocytes, some problems arise when we use them. The major problems are in obtaining cells without differences among batches, maintaining of cells for an extended period of time and having cultured cells available when the need arises. To avoid these problems, cryopreservation is effective. Although cryopreservation is routinely used for many animal cell lines, it has not been used for the preservation of primary astrocytes maintained in a serum-free medium. Because serum has been shown to be effective in enhancing the survival of frozen cells (Dougherty, 1962), it is often added before freezing, even for cell lines maintained in serum-free media. If serum is used during freezing, many washings and long-term recultivation under serum-free conditions are required for complete removal of serum-derived factors. Recently Ohno et al. (1988) reported that methylcellulose is effective as a cryoprotectant for serum-free cultured cells lines. The second purpose of this study was to examine an effective method of cryopreservation of primary astrocytes under serum-free conditions.

3.2 Materials and Methods

Cell culture

Cerebra of 1-3-day-old C3H/He mice were dissected free of meninges aseptically. The cerebra were dissociated by passing them through a 320 μm nylon mesh with a rubber policeman. After washing with Hanks' balanced salt solution, the cell suspension was triturated with a Pasteur pipette and seeded onto poly-L-lysine-coated culture flasks (75 cm^2) at a density of two cerebra per flask in 20 ml of a culture medium. This culture medium consisted of Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS), glucose (2 mg/ml), insulin (5 μg/ml), penicillin (50 units/ml) and streptomycin (50 μg/ml). After three days, the attached cells were washed twice with MEM, and a serum-free, chemically defined medium was then added. The medium used was G5 medium which was developed by Michler-Stuke et al. (1984) for growth and differentiation of neonatal rat brain astrocytes. This medium consists of Dulbecco's modified Eagle's minimum essential medium (D-MEM) supplemented with glucose (4.5 mg/ml), 10 nM hydrocortisone, 30 nM sodium selenite, insulin (5 μg/ml), transferrin (50 μg/ml), biotin (10 ng/ml), fibroblast growth factor (FGF; Takara Shuzo Co., Ltd., Kyoto, 5 ng/ml) and epidermal growth factor (EGF; Takara Shuzo, 10 ng/ml). The cells were cultured in G5 medium with medium changes every 2-3 days for 3 weeks. All cultures were seeded on the surfaces coated with

poly-L-lysine and maintained in a humidified atmosphere of 5 % CO_2 in air at 37°C.

Immunofluorescence

To identify cell types in culture, the cells seeded on coverslips were immunolabelled with various antibodies. The cells were incubated at room temperature in each antibody solution for 30 minutes, followed by incubation in an appropriate fluorescein isothiocyanate (FITC)-conjugated second antibody solution. Antibodies were diluted in phosphate-buffered saline (PBS) containing 1% bovine serum albumin. After every incubation, cells were washed three times with PBS. The first antibodies used were: rabbit anti cow glial fibrillary acidic protein (anti-GFAP; Dako Co., Glostrup, diluted 1:100), rabbit anti bovine S-100 protein (anti-S-100; Advance Ltd., Tokyo, diluted 1:100), mouse monoclonal antibodies to human neurofilaments (anti-NF; Cosmo Bio Ltd., Tokyo, diluted 1:2), rabbit anti bovine galactocerebroside (anti-GalC; prepared by immunization with GalC, diluted 1:20) and rabbit anti mouse fibronectin (anti-fibronectin; Paesel GmbH, Borsigallee; diluted 1:100). Surface antigens (i.e. GalC and fibronectin) were immunolabelled on living cells and intracellular antigens (i.e. GFAP, S-100 and NF) were immunolabelled after fixation with 5% acetone in ethanol at -20°C for 15 minutes. As second antibodies, FITC-conjugated goat anti rabbit immunoglobulin (Cappel Laborato-

ries, Malveron, PA; diluted 1:200) or FITC-conjugated goat anti mouse immunoglobulin (Cappel Laboratories, diluted 1:200) was used. After additional washing, the coverslips were rinsed in distilled water and mounted on glass slides with 10% glycerol in PBS. The stained cells were observed under an Olympus BH-2 fluorescence microscope.

Freezing and thawing

The cells were harvested by trypsinization and suspended in G5 medium containing various cryoprotectants at a cell density of 2×10^6 cells/ml. The following cryoprotectants were used: (1) dimethylsulfoxide (DMSO, MW 78) at a final concentration of 10%; (2) methylcellulose (MC, 4000cp, MW 140,000, Wako Pure Chemicals Ltd., Osaka) at a final concentration of 0.1%; (4) hydroxyethyl starch (HES, MW 545,000, Sigma Chemical Company, MO) at a final concentration of 2%; (5) polyvinylpyrrolidone (PVP, K-90, MW 360,000, Wako Pure Chemicals) at a final concentration of 3%; and (6) FCS at a final concentration of 20%. Each 1 ml of cell suspension was dispensed into a glass ampoule and frozen to -80°C at a rate of 1°C/minute with a programmable controlled rate freezing unit (CM-MarkII, Taiyo Sanso Co. Ltd., Osaka). The frozen cells were stored in liquid nitrogen for 3 weeks. When the frozen cells were reconstituted, the ampoules were removed from the liquid nitrogen and rapidly plunged into a 37°C water bath until the contents were thawed. The thawed

cells were diluted tenfold with MEM and centrifuged at 180 g for 5 minutes. The pelleted cells were resuspended and recultivation in G5 medium was started.

Determination of viability and cell attachment

The viability of cells before freezing or after thawing was determined using a dye exclusion test. Unstained viable cells and stained dead cells were counted using a hemocytometer within 3-5 minutes after staining with 0.3% trypan blue solution. The viability of cells was calculated by dividing the number of viable cells by the number of total cells. After freezing and thawing, the number of cells attached to the dishes after a 16-hour incubation were counted by the dye exclusion test. The percentage of cell attachment was calculated by dividing the number of attached cells by the initial number of cells in the ampoule before freezing (i.e. 2×10^6). This calculation gives an index of the recovery of cells, those which are able to grow into subsequent generations attaching to a dish.

3.3 Results

Characterization of the primary culture

Two distinct cell types were recognized upon phase-microscopic examination of the cells grown for 3-14 days in the serum-free, chemically defined medium (G5); one was

fibroblast-like and the other was flat epithelial cell-like (Fig. 1a). When the culture was maintained for 3 weeks, the majority of cells were fibroblast-like (Fig. 1b). Figure 2 shows the growth of cells maintained in G5 medium. The cells grew in this medium as well as they did in serum-supplemented medium. The final cell density reached after 5 days was about 80% that of their serum grown culture.

To identify cell types in the primary culture generated in G5 medium for 3 weeks, immunofluorescence staining using antibodies against cell markers was carried out. The proportion of positively stained cells is shown in Table I. The great majority of the cells (>98%) in the culture contained GFAP which is a specific marker of mature astrocytes. The GFAP antigens were moderately filamentous, distributed in the cell bodies, and were not present in the nuclei of the cells (Fig. 3). About the same proportion of cells were stained with anti-S-100 which specifically binds to astrocytes (Hyden and McEwen, 1966). Cell types most likely to contaminate astrocytes include meningeal cells, endothelial cells, fibroblasts and oligodendrocytes (Stieg et al., 1980). The meningeal cells, endothelial cells and fibroblasts can be detected on the basis of their expression of fibronectin on the cell surface (Schachner et al., 1978). The cells stained with anti-fibronectin accounted for fewer than 1 % of the total cells. The cells stained with anti-GalC which binds to oligodendrocytes (Raff et al., 1978)

also accounted for fewer than 1% of the total cells. No neurofilament-positive neurons (Schlaepfer, 1977) were observed in the culture.

Besides immunocytochemical demonstration of GFAP-presence, a dibutyryl cyclic AMP (dBcAMP)-induced morphological conversion from a flat to a process-bearing cell has usually been employed to identify astrocytes (Manthorpe et al., 1979). The cell bodies decreased in size, and long slender processes were formed when 1 mM dBcAMP was added to the culture medium (Fig. 4). More than 90% of the total cells showed these morphological changes 5 days after the addition of dBcAMP. Based on these results, the primary culture maintained in G5 medium for 3 weeks is considered to be a highly enriched culture of astrocytes.

Cryopreservation

We examined the effects of cryoprotectants on the survival of enriched astrocytes under serum-free conditions. The cells were harvested by trypsinization and were then frozen in G5 medium containing various cryoprotectants at 1 °C/minute using a programmable controlled rate freezing unit. After the frozen cells were thawed in a 37°C water bath, the viability and percentage of cell attachment were determined as indexes of survival (Yamamoto et al., 1981). The experiments were replicated three times. The viability of the three cultures before freezing was 95 ± 2% (Mean ± standard

deviation, S.D.). The results are shown in Figure 5. The viability and percentage of cell attachment of thawed cells were 8% and 0%, respectively, when the cells were frozen and thawed without a cryoprotectant. No cryoprotective effect on the enriched astrocytes was observed when 0.1% MC, 2% HES or 3% PVP was added: the percentage of cell attachment was 0%. By adding of 10% DMSO, the viability and percentage of cell attachment increased to 45% and 12%, respectively. The combination of 0.1% MC, 2% HES or 3% PVP and 10% DMSO increased the viability to 72-76%. The percentage of cell attachment was highest when the cells were frozen with 0.1% MC and 10% DMSO. Using these two cryoprotectants, the viability and percentage of cell attachment were 74% and 23%, respectively, indicating that about 30% of the viable cells were able to attach to a dish.

We examined the characteristics of frozen cells in comparison with those of unfrozen cells. After the cells were frozen and thawed in the presence of 10% DMSO and 0.1% MC, we recultivated the cells in G5 medium for 7 days and then characterized them as follows. The growth rate of frozen cells was similar to that of unfrozen cells (data not shown). The proportion of cells stained positively with anti-GFAP and anti-S-100 revealed an almost pure (>98%) astrocyte culture after freezing and thawing. The intensity and patterns of the immunofluorescence profiles of the frozen cells stained with anti-GFAP were similar to those of

unfrozen cells as shown in Figure 3. No noticeable differences in morphology were found between those astrocytes subjected to the freezing-thawing procedures and those which were not. The cells recultivated in G5 medium appeared to be in good condition after more than 2 months.

3.4 Discussion

We were able to obtain a highly enriched culture of astrocytes from newborn mouse cerebrum with the serum-free, chemically defined medium (G5). Our results demonstrate that the cells generated in G5 medium are GFAP-positive, mature astrocytes. Fischer et al. (1982) reported that GFAP-negative, immature astrocytes were generated from newborn mouse cerebellum in their culture systems. The discrepancies between our results and those of Fischer et al. may be due to the differences in the chemically defined media or in brain area. Most investigators have used rat brain for primary cultures of astrocytes. G5 medium was developed for the enriched culture of astrocytes derived from rat brain (Michler-Stuke et al., 1984). Our results show that this medium is also effective for the enriched culture of astrocytes derived from mouse cerebrum. The great advantage of serum-free cultures is that fibroblast overgrowth is suppressed (Morrison and de Vellis, 1983). The fibroblasts amounted to 20-30% of the total cells when

we cultivated cells derived from mouse cerebrum in a serum-supplemented medium for 3 weeks (data not shown). In our serum-free culture system, the proportion of fibronectin-positive fibroblasts was less than 1%.

To preserve the enriched mouse astrocytes for an extended period of time without changing their characteristics, we examined a method of cryopreservation. Changes in the rates of cooling and thawing modify survival of cells but the most important factor influencing survival is the presence or absence of cryoprotectants (Mazur, 1970). Cryoprotectants are broadly divided on the basis of whether or not they permeate cells; cells are usually permeable to DMSO and glycerol but are impermeable to PVP, HES and serum proteins (Ashwood-Smith, 1987). MC used in our experiments must act as a non-permeating cryoprotectant because of its high molecular weight (MW; 140,000). The cryoprotective mechanisms of the two types of cryoprotectants are not yet clearly understood. Our results show that the separate cryoprotective effects of the permeating compounds and the non-permeating compounds on the survival of astrocytes are additive (Fig. 5). Among the three non-permeating cryoprotectants examined (i.e. MC, HES, PVP), MC was the most effective in the combination with DMSO. Ohno et al. (1988) reported that MC is effective for the cryopreservation of cultured cell lines. Our results confirmed their result using primary cultures of astrocytes.

In recent years, Fierz et al. (1985) found that astrocytes act as antigen-presenting cells. Many investigators are interested in the functions of astrocytes which mediate immune reactions in the brain. The astrocytes derived from inbred mice (C3H/He), which have been characterized their genetic background, should be more suitable for immunological studies than astrocytes derived from rats. Our methods of primary culture and cryopreservation for enriched mouse astrocytes will make it easier to perform various immunological and biochemical studies related to astrocytes.

3.5 Summary

The methods of primary culture and cryopreservation of mouse astrocytes under serum-free conditions were examined. Cerebra from newborn C3H/He mice were employed as the source of astrocytes. The cultured cells were able to grow in a serum-free, chemically defined medium containing transferrin, hydrocortisone, biotin, sodium selenite, insulin, fibroblast growth factor and epidermal growth factor. After the culture was maintained in the medium for 3 weeks, purity was assessed using immunofluorescence staining. The great majority of the cells (>98%) contained glial fibrillary acidic protein and S-100 protein which are cell markers of astrocytes. To cryopreserve the enriched astrocytes under serum-free conditions, various cryoprotectants were exam-

ined. The combination of 10% dimethylsulfoxide and 0.1% methylcellulose gave the highest survival rate. These methods of primary culture and cryopreservation will be useful in physiological and biochemical studies which require mouse astrocytes.

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Table I. Indirect immunofluorescence staining of primary astrocyte culture with various antibodies

Antibodies	Cell types	% cells positively stained
anti-GFAP	astrocyte	> 98
anti-S-100	astrocyte	> 98
anti-fibronectin	meningeal cell endothelial cell fibroblast	< 1
anti-GalC	oligodendrocyte	< 1
anti-NF	neuron	0

The data represent the percentage of cells expressing the cell marker in relation to the total cell population.

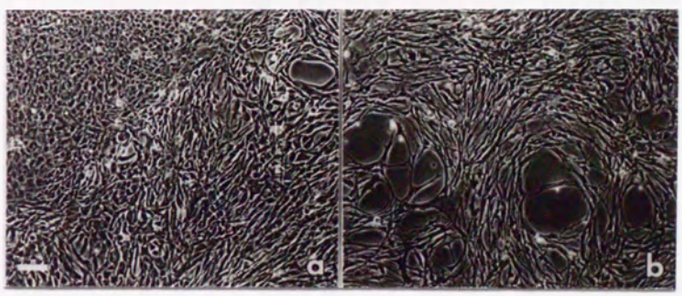

Fig. 1. Mouse cerebral cells cultured in G5 medium on poly-L-lysine-coated dishes. a; day 7, b; day 21
Bar, 20 μm

Fig. 2. Growth curves of primary cultures of astrocytes. Cells were plated at a cell density of 2 x 10^5/ 60-mm Petri dish in poly-L-lysine-coated dishes in G5 medium (open circles) or D-MEM supplemented with 10% FCS (closed circles). The numbers of cells were counted using the dye exclusion test.

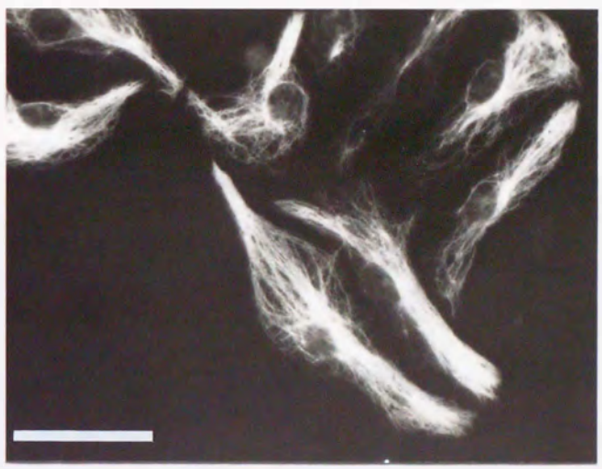

Fig. 3. Indirect immunofluorescence of astrocytes using anti-GFAP and FITC-conjugated anti rabbit immunoglobulin. Bar, 20 μm

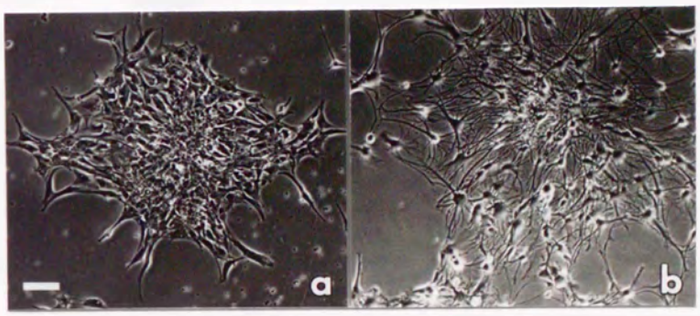

Fig. 4. Morphological change in enriched astrocytes treated with dBcAMP for 5 days. The enriched astrocytes maintained in G5 medium for 3 weeks were cultivated in the absence of dBcAMP (a) or in the presence of 1 mM dBcAMP (b). Bar, 20 μm

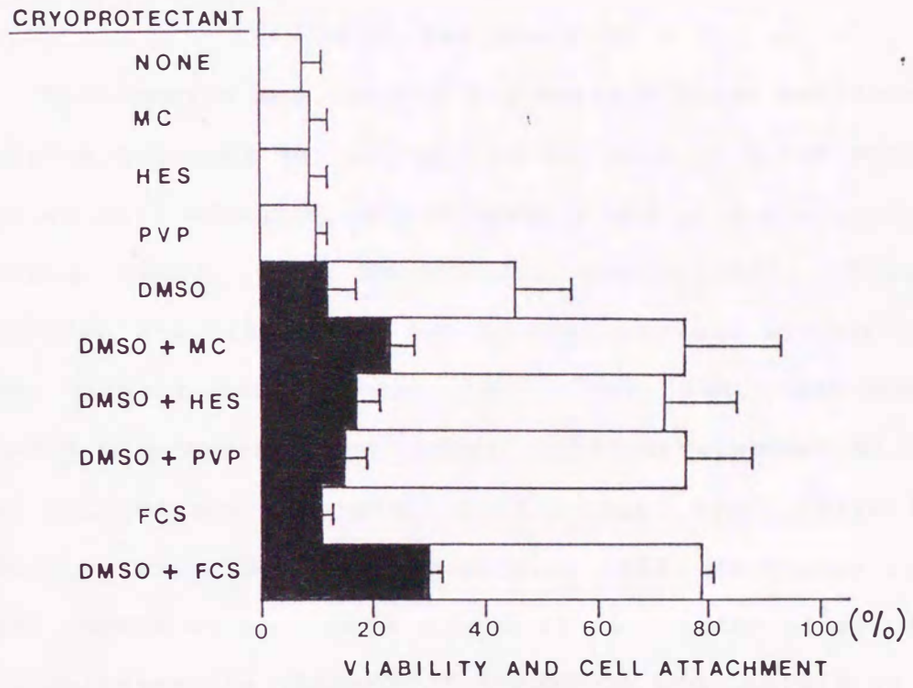

Fig. 5. Effects of various cryoprotectants on viability and cell attachment of the cryopreserved cells. The enriched astrocytes maintained in G5 medium for 3 weeks were frozen and thawed with various cryoprotectants. The viability (open bars) and percentage of cell attachment (closed bars) were determined according to the methods in Materials and Methods. Each value represents the mean ± S.D. of the three experiments.

4. EXPRESSION OF FIBRONECTIN AND LAMININ BY DIFFERENT TYPES OF MOUSE GLIAL CELLS CULTURED IN A SERUM-FREE MEDIUM

4.1 Introduction

Fibronectin and laminin are extracellular matrix glycoproteins believed to be involved in many cellular functions such as cell adhesion, wound healing and cell migration (for reviews; Hynes, 1990; Martin and Timpl, 1987). These two molecules are present in the central nervous system (Liesi, 1985; Stewart and Pearlman, 1987; Zhou, 1990) and are considered to play important roles in the development of embryonic neurons and regeneration of damaged adult nerve fibers (Akers et al., 1981; Hatten et al., 1982; Manthorpe et al., 1983; Rogers et al., 1983; Liesi et al., 1984; Liesi, 1985). To understand the roles of fibronectin and laminin in brain development, it is necessary to identify the cell types that synthesize and deposit them in the extracellular matrix. In cell-culture studies, it is possible to analyze these molecules expressed by well-defined cell types. Previous reports have indicated that cultured astrocytes are able to express fibronectin (Price and Hynes, 1985; Liesi et al., 1986) and laminin (Liesi et al., 1983). However, there has been controversy whether astrocytes synthesize fibronectin (Paetau, 1988).

Raff et al. (1983a, b) have demonstrated two types of astrocytes in cultures of neonatal rat optic nerves, which are derived from two distinct developmental lineages. Evidence from various in vitro studies shows that oligodendrocytes and type-2 astrocytes are derived from common bipotential (O-2A) progenitors, whereas type-1 astrocytes develop from different progenitors. Although phenotypes of type-1 and type-2 astrocytes in other regions of the central nervous system are not fully established, type-1 and type-2 astrocytes differ in a number of morphological and functional properties (Raff, 1989). For example, most type-1 astrocytes are large, fibroblast-like, non-process-bearing cells. Functionally, type-1 astrocytes form the glial limiting membrane and may play a constitutional role in the formation of the blood-brain barrier (Janzer and Raff, 1987). In contrast, most type-2 astrocytes have a stellate, process-bearing morphology. Functionally, type-2 astrocytes ensheath exposed axonal plasma membrane at the nodes of Ranvier (ffrench-Constant and Raff, 1986).

In the present study, the first purpose was to determine whether glial cells can produce fibronectin and laminin using enriched glial cultures under serum-free conditions. The second purpose was to examine whether expression patterns of these molecules differ among glial subtypes. The third purpose was to examine molecular characteristics of glial fibronectin and laminin. We also examined the fate of

synthesized molecules: whether fibronectin and laminin are secreted as soluble forms into the culture medium or deposited as insoluble forms at the extracellular matrix.

4.2 Materials and Methods

Cell cultures

Primary glial cultures from neonatal C3H/He mouse cerebra were prepared as described in Section 3.2. Briefly, mechanically dissociated cells from cerebra of 1-day-old mice were maintained with Dulbecco's modified minimum essential medium (D-MEM) supplemented with 10% fetal calf serum (FCS). After 3 days, the culture medium was changed to a serum-free, chemically defined medium (G5 medium; Michler-Stuke et al., 1984) consisting of D-MEM, glucose (4.5 mg/ml), 10 nM hydrocortisone, 30 nM sodium selenite, recombinant human insulin (Wako Pure Chemicals Ltd., Osaka; 5 μg/ml), transferrin (50 μg/ml), biotin (10 ng/ml), recombinant human basic fibroblast growth factor (bFGF; Boehringer Mannheim GmbH, Mannheim; 5 ng/ml) and epidermal growth factor (EGF; Takara Shuzo Ltd., Kyoto; 10 ng/ml). After 7-10 days of primary culturing, with medium changes every 2-3 days, the cells were subcultured 3-4 times for 4 weeks. For subcultures the cells were incubated with 0.1% crystalline trypsin solution for 2-3 minutes at room temperature, and the trypsinization was terminated by replacing the

trypsin solution with G5 medium containing 0.5 mg/ml soybean trypsin inhibitor. All cultures were grown on poly-L-lysine-coated dishes at 37°C in a humidified 95% air/5% CO_2 atmosphere.

Reagents

Fibronectin purified from mouse plasma was purchased from Wako Pure Chemicals Ltd., Osaka, and laminin purified from Engelebreth-Holm-Swarm (EHS) sarcoma was purchased from Iwaki Glass Co., Tokyo. Antisera against fibronectin and against laminin were prepared using rabbits. Immunoglobulin G (IgG) fractions were purified from the antisera by ammonium sulfate precipitation followed by high pressure liquid chromatography on an adsorbent of protein A bound to cyanogen bromide-activated Sepharose 4B. Conjugation of anti-fibronectin IgG and of anti-laminin IgG to horseradish peroxidase (HRP) was performed using the two step methods of Ishikawa et al. (1983).

Immunofluorescence

An indirect immunofluorescent technique was used to identify surface and intracellular antigens to classify glial subtypes and non-glial cells. Cells seeded on poly-L-lysine-coated glass coverslips were incubated at room temperature in an antibody solution for 30 minutes followed by incubation in an appropriate fluorescein isothiocyanate

(FITC)-conjugated or tetramethylrhodamine isothiocyanate (TRITC)-conjugated second antibody solution. Antibodies were diluted in phosphate-buffered saline (PBS) containing 1% ovalbumin. After every incubation procedure, cells were washed three times with PBS. The first antibodies used were: rabbit anti-cow glial fibrillary acidic protein (anti-GFAP, Dako Co., Glostrup; diluted 1:100), rabbit anti-galactocerebroside (anti-GalC; prepared by immunization with GalC; 1:100), mouse monoclonal antibody to human neurofilaments (anti-NF; Cosmo Bio Ltd., Tokyo; 1:2), mouse monoclonal antibody (IgM) A2B5 (ascites; 1:4000) and mouse monoclonal antibody to Thy1.2 (Becton Dickinson Co., Mountain View, CA; 1:50). Surface antigens (i.e., GalC, A2B5 and Thy1.2) were immunolabelled on 2% paraformaldhyde-fixed cells and intracellular antigens (i.e., GFAP and NF) were immunolabelled after paraformaldehyde-fixing followed by permeabilization with 0.1% Triton X-100 in PBS. FITC-conjugated goat anti-rabbit IgG (Cappel Laboratories, Malvern, PA; 1:200), FITC-conjugated goat anti-mouse IgG (Cappel Laboratories, 1:200) or TRITC-conjugated goat anti-mouse IgM (Cappel Laboratories, 1:200) were used as the second antibodies. Glial subtypes were determined from double staining for A2B5/GalC and A2B5/GFAP.

The glial cultures were examined for anti-fibronectin IgG (1:200) and anti-laminin IgG (1:200) binding by double staining with monoclonal antibody A2B5 or mouse monoclonal

antibody to human GFAP (Labsystems, Helsinki; 1:100). At the time of staining, the culture medium was removed and the cells were gently washed in PBS. The cultures were fixed with 2% paraformaldehyde and permeabilized with 0.1% Triton X-100. To examine antigens on the cells, the first antibody was applied after paraformaldehyde-fixing without the permeabilization step. The stained cells were observed under an Olympus BH-2 fluorescence microscope.

In most experiments, the cells on 30 non-overlapping sequential fields with 3 glial cultures were visualized and counted. The total number of cells was determined using phase-contrast optics, and subsequently, the numbers of stained and non-stained cells were determined using fluorescein and rhodamine optics.

Western blot analysis

For Western blot analysis of fibronectin, the culture medium of glial cells was harvested and incubated with uncoupled Sepharose or gelatin-coupled Sepharose (Pharmacia Fine Chemicals, Uppsala) as described by Price and Hynes (1985). The Sepharose was washed and boiled in electrophoresis sample buffer to solubilize the bound materials. These samples were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). In the case of laminin analysis, the cellular proteins were extracted by the method of Liesi and Risteli

(1989) and then used for SDS-PAGE. These samples were fractionated under reducing conditions in 5% acrylamide gels with 3% stacking gels. Molecular weight markers were purchased from Bio-Rad Laboratories. The fractionated proteins were electrophoretically transferred to nitrocellulose sheets (Towbin, 1979). The nitrocellulose sheets were incubated in a blocking solution containing 3% ovalbumin and 0.05% Tween 20 in PBS for 1 hour at room temperature. The sheets were incubated with HRP-conjugated anti-fibronectin IgG (1:200) or HRP-conjugated anti-laminin IgG (1:200) for 1 hour at room temperature. After washing, immunoreactive bands were visualized by reacting with 4-chlore-1-naphthol.

Extraction of cell cultures

To examine the amounts of fibronectin and laminin in the culture medium, extracellular matrix and intracellular portion, cells were extracted as described by Ignotz and Massagué (1986). The cells were seeded (10^6 cells/35-mm well) and cultured in G5 medium. After culturing for 2 days, the medium was harvested and used for ELISA analysis. The remaining cells were washed with 0.15 M NaCl, 25 mM Tris-HCl (pH 7.4) and extracellular matrices were extracted with a solution containing 1 M urea, 1 mM dithiothreitol, 10 mM Tris-HCl (pH 7.4), 10 mM EDTA and 2 mM phenylmethylsulfonyl fluoride (PMSF). For extraction of intracellular speci-

mens, the cells harvested by trypsinization were lysed with Triton X-100 and the insoluble material was removed by centrifugation. The soluble extract was retained for ELISA analysis.

Enzyme-linked immunosorbent assay (ELISA)

Sandwich ELISAs were performed to quantify the amount of fibronectin or laminin in specimens prepared from glial cultures. The cell number at harvest was assessed by a trypan blue exclusion method. Each well of flat-bottom 96-well plates (Immunoplate I, Nunc, Poskilde) was coated overnight at 4°C with 100 μl of either anti-fibronectin or anti-laminin IgG at 10 μg/ml in 0.2 M bicarbonate buffer (pH 9.3). After coating, the IgG solutions were removed and the wells were washed with PBS. Subsequently, 150 μl of 1% ovalbumin in PBS was added to each well. After 2-hour incubation at room temperature, the ovalbumin solution was removed and the wells were washed with PBS containing 0.05% Tween 20 (PBS-Tween). The samples (50 μl/well) were added to the wells and authentic fibronectin and laminin (1,000-1 ng/ml) served as standards. The plates were incubated for 1 hour at room temperature. After washing with PBS-Tween, HRP-conjugated anti-fibronectin IgG or HRP-conjugated anti-laminin IgG (1:200, 50 μl/well) was added, and incubation continued for 1 hour at room temperature. The wells were washed with PBS-Tween and then a substrate for peroxidase,

2.2'-adino-di[3-ethelbenzothiazoline sulfonate(6)] (ABTS), was added. The optical density at 415 nm was measured using an ELISA reader.

4.3 Results

The characteristics of glial cultures

To identify cell types in the cultures prepared from neonatal mouse cerebra and subcultured in G5 medium for 4 weeks, immunofluorescence staining using antibodies against cell markers were carried out. The cultures contained both type-1 astrocytes and O-2A lineage cells (O-2A progenitors, oligodendrocytes and type-2 astrocytes). The proportion of each cell type is shown in Table II. In over 3 preparations, 77% of the cells were GFAP-positive and A2B5-negative and exhibited a fibroblast-like morphology (Fig. 6 a-c): features of type-1 astrocytes (Raff et al., 1983a, b). O-2A progenitors that are defined as A2B5-positive, GFAP-negative and of a process-bearing morphology (Raff et al., 1983a, b) accounted for 21% of the cells (Fig. 6 a-c). Less than 1% of the cells expressed GalC that is a cell marker of oligodendrocytes (Raff et al., 1978)(Fig. 6 f). A small number (<1%) of A2B5-positive, GFAP-positive and multipolar type-2 astrocytes (Raff et al., 1983a, b) was observed (Fig. 6 d, e). No Thy 1.2-positive or neurofilament-positive cells, which are classified as fibroblasts (Raff et al., 1979) or

neurons (Schlaepfer, 1977) were observed in the cultures.

Immunocytochemical analyses of fibronectin and laminin

The expression and cellular distribution of fibronectin and laminin by glial subtypes in the cultures were investigated immunocytochemically. To identify the subtypes double-labelling immunofluorescent experiments using monoclonal A2B5 antibody and monoclonal anti-GFAP antibody were performed. The staining with anti-fibronectin or anti-laminin was performed either with paraformaldehyde-fixed cells to detect the antigens on top of the cells or with paraformaldehyde-fixed cells subsequently subjected to a permeabilization step to detect the antigens in the intracellular and antigens between cells and substratum.

Almost all the type-1 astrocytes were strongly to moderately positive for fibronectin and laminin. Fibronectin and laminin were expressed by the type-1 astrocytes having flat and large morphology in a granular intracytoplasmic distribution confined to the perinuclear region (Fig. 7d, f; Fig. 8b, d). Careful focusing revealed that they were also deposited in a fibrillar matrix form underneath the cell monolayers (Fig. 7d, Fig. 8b). The staining pattern for fibronectin was mostly coincidental with the staining for laminin, although the laminin staining tended to have a more punctate appearance than fibronectin. Furthermore, very weak laminin matrices were present on top of

the monolayers (data not shown), whereas fibrillar fibronectin was found only at the outermost edges of the cell surface (Fig. 7b). Type-2 astrocytes were either weakly stained or not stained by anti-fibronectin and anti-laminin antibodies. The fluorescence was somewhat restricted to the cell body leaving many cell processes devoid of staining (Fig. 7f, Fig. 8d). In O-2A progenitors, both fibronectin and laminin appeared as diffuse intracytoplasmic staining (Fig. 7h, Fig. 8f). Neither fibronectin nor laminin was expressed on the cell surface of type-2 astrocytes and O-2A progenitors (data not shown). The staining with anti-fibronectin or anti-laminin on the whole decreased in the order: type-1 astrocytes, O-2A progenitors and type-2 astrocytes.

Western blot analyses of glial fibronectin and laminin

The molecular characteristics of the fibronectin and laminin produced by the glial cells were studied by Western blot analysis. A band at 230 kDa was identified as fibronectin by its binding to gelatin-sepharose (Fig. 9, lane 2). The fibronectin secreted by the glial cells (lane 2) had a slightly higher apparent molecular weight than that of mouse plasma fibronectin (230 kDa, lane 1). Anti-laminin IgG labelled only a band at 220 kDa (B chain, lane 5). The 400 kDa band (A chain) of the EHS laminin (lane 4) was not detected in the samples of glial cell extracts. Preimmune serum from rabbits stained no protein bands on the immuno-

blots (data not shown). The staining of the appropriate bands on the blots also confirmed the specificity of the antibodies used for immunochemical analysis.

The amounts of fibronectin and laminin detected by ELISA

Sandwich ELISAs were used to determine the amounts of fibronectin and laminin expressed by the glial cells. The amounts of fibronectin and laminin in the three portions of the glial cultures are shown in Table III. Both fibronectin and laminin existed in the intracellular and extracellular matrix portions in similar amounts. A large amount of fibronectin was detected in the culture medium, while laminin was not detected (less than 2 ng/ml). The fibronectin in the culture medium constituted 0.2% of the total proteins.

4.4 Discussion

This paper provides evidence for the expression of fibronectin and laminin by glial cells cultured in a serum-free, chemically defined medium using immunocytochemical and biochemical procedures. The glial cultures employed in our studies were of great advantage in the analysis of fibronectin and laminin expression for the following reasons. These cultures contained no detectable Thy 1.2-positive cells that are responsible for fibroblasts (Raff et al., 1979). Con-

tamination of cultures with a relatively small number of the fibroblasts that are active producers of fibronectin (Raff et al., 1979) can give very misleading results, whatever method of analysis is used. The oligodendrocytes that accounted for fewer than 1% of the total cells are considered to be negative for fibronectin production (Norton et al., 1983) and laminin production (Chiu et al., 1991). Also, these cultures were maintained under serum-free conditions. FCS contains 100 μg/ml of fibronectin (Price and Hynes, 1985), and its fibronectin can be incorporated into extracellular matrices (Hayman and Ruoslahti, 1979).

The glial cells subcultured in G5 medium consisted of type-1 astrocytes and O-2A lineage cells. O-2A progenitors can be induced to undergo continuous self-renewal in the absence of oligodendrocytic differentiation by exposure to a combination of platelet-derived growth factor (PDGF) and bFGF (Bögler et al., 1990; McKinnon et al., 1990). Type-1 astrocytes synthesize and secrete PDGF in vitro (Richardson et al., 1988). In primary cultures, we have shown that 98% of the cells are GFAP-positive type-1 astrocytes (Section 3.3). In our cell-culture systems using G5 medium, which contains bFGF, the proportion of O-2A progenitors was increased by subcultivation. Much work on the properties of each glial subtype has been carried out, but the expression of fibronectin and laminin in these cells has remained obscure. Recently, Gallo and Bertolotto (1990) have indi-

cated that type-1 astrocytes are stained with both anti-fibronectin and anti-laminin antibodies and that type-2 astrocytes are weakly stained with anti-laminin antibody and not stained with anti-fibronectin antibody. Our results are on the whole in the agreement with their report. Our experiments showed that type-2 astrocytes were weakly positive for fibronectin and that O-2A progenitors express fibronectin and laminin more abundantly than type-2 astrocytes. Both fibronectin and laminin were deposited in fibrillar matrices underneath type-1 astrocytes. This localization suggests that these molecules are involved in cell-substrate adhesion and may be responsible for the flattened morphology of type-1 astrocytes (Yamada et al., 1976). These differences in expression of the molecules among glial subtypes may correspond to various tasks that these cells perform during brain development.

We examined the molecular characteristics of fibronectin and laminin expressed by the mixed glial cultures using Western blot analysis. Price and Hynes (1985) showed that fibronectin produced by rat cortical astrocytes has a slightly higher molecular weight than that of fibronectin produced by rat fibroblasts. In our experiments we compared the molecular weights of glial fibronectin and mouse plasma fibronectin and showed glial fibronectin has a higher molecular weight than plasma fibronectin. In the case of laminin, we confirmed the results of Liesi and Risteli (1989)

and Wujek et al. (1990): glial laminin lacks the 400 kDa A chain. The molecular heterogeneity of glial fibronectin and laminin was not observed by Western blot analysis, although the cultures contained glial subtypes that expressed these molecules.

To ascertain whether glial cells secrete fibronectin and laminin, the three portions of cultures were examined using ELISAs. To our knowledge there are no reports describing quantitative analysis of the fibronectin and laminin produced by glial cells. These molecules were detected in the extracellular matrix at a similar concentration. The proportion of fibronectin that is secreted, as opposed to being bound to the extracellular matrix, varies with cell type: 20 % secretion by human embryo fibroblasts (Baum et al., 1977) and 100 % secretion by hepatocytes (Amrani et al., 1985). The high proportion of fibronectin secretion and the lack (or low degree) of laminin secretion by glial cells are of interest. The regulation mechanism for the biosynthesis of these molecules by glial cells is completely unknown. The effect of various cytokines on the production of fibronectin and laminin by glial cells is currently under investigation in our laboratory.

4.5 Summary

The expression of fibronectin and laminin by cultured

glial cells was studied. The glial culture from neonatal mouse cerebra maintained in a chemically defined, serum-free medium consisted of type-1 astrocytes, oligodendrocyte-type-2 astrocyte (O-2A) progenitors, oligodendrocytes and type-2 astrocytes. Double-labelling immunofluorescent experiments performed using the mixed glial culture indicated that fibronectin and laminin are expressed in different patterns among the glial subtypes. The staining intensities with anti-fibronectin or anti-laminin antibodies decreased in the order: type-1 astrocytes, O-2A progenitors and type-2 astrocytes. Both molecules were deposited in a fibrillar matrix underneath type-1 astrocytes, whereas only intracytoplasmic localization of these molecules was observed with O-2A progenitors and type-2 astrocytes. Western blot analysis showed that glial fibronectin has a slightly higher molecular weight than mouse plasma fibronectin (230 kDa) and that glial laminin is a variant with a 220 kDa B chain present and the 400 kDa A chain missing. Using enzyme-linked immunosorbent assays (ELISA), these molecules were detected in the glial extracellular matrix at the concentration of 4 ng/10^6 cells. A large amount of fibronectin (82 ng/10^6 cells) was secreted into the culture medium, while secretion of laminin was not detected.

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Table II. Percentage of each cell type assessed by indirect immunofluorescence staining

Cell type	Cell markers	% cells
O-2A lineage		
Progenitors	$A2B5^+$ $GFAP^-$	21±7
Oligodendrocytes	$GalC^+$ $A2B5^-$	<1
Type-2 astrocytes	$A2B5^+$ $GFAP^+$	<1
Non-O-2A lineage		
Type-1 astrocytes	$A2B5^-$ $GFAP^+$	77±8
Neurons	$Neurofilament^+$	0
Fibroblasts	$Thy1.2^+$	0

Values represent the percentage of cells expressing the cell markers in relation to the total cell population (Mean±S.D.).

Table III. The amounts of fibronectin and laminin in three portions of glial cells

	Amount detected (ng/10^6 cells)		
Molecule	Intracellular	Matrix	Medium
Fibronectin	1.1±0.2	4.2±2.1	82±14
Laminin	3.3±0.2	3.5±2.1	<2

Two-day cultures were prepared and ELISAs were performed as described in Materials and Methods. The values were determined from a standard curve generated with mouse plasma fibronectin and mouse EHS sarcoma laminin. The values shown are means and standard deviations from three experiments.

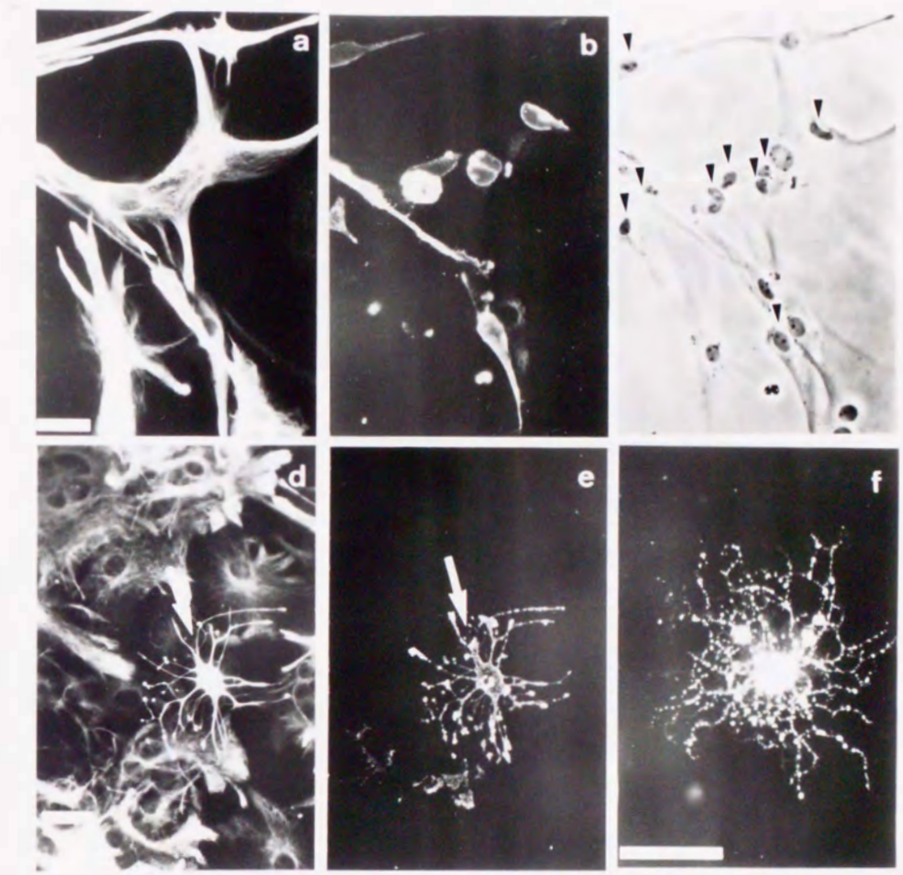

Fig. 6. Fluorescent and phase-contrast micrographs of four types of glial cells in serum-free cultures. Cells were cultured in G5 medium for 4 weeks as described in Materials and Methods. Cells were double-labelled with rabbit anti-GFAP (a, d; fluorescein optics) and mouse monoclonal A2B5 antibody (b, e; rhodamine optics). A phase contrast view of the same field seen in a and b is shown in c. Note that type-1 astrocytes are $GFAP^+$ (a, d) and $A2B5^-$ (b, e), while O-2A progenitors (arrowhead in c) are $GFAP^-$ (a) and $A2B5^+$ (b). A type-2 astrocyte (arrow in d, e) is shown to be $GFAP^+$ (d) and $A2B5^+$ (e). An oligodendrocyte is labelled with anti GalC (f; fluorescein optics). Bars; 40 μm.

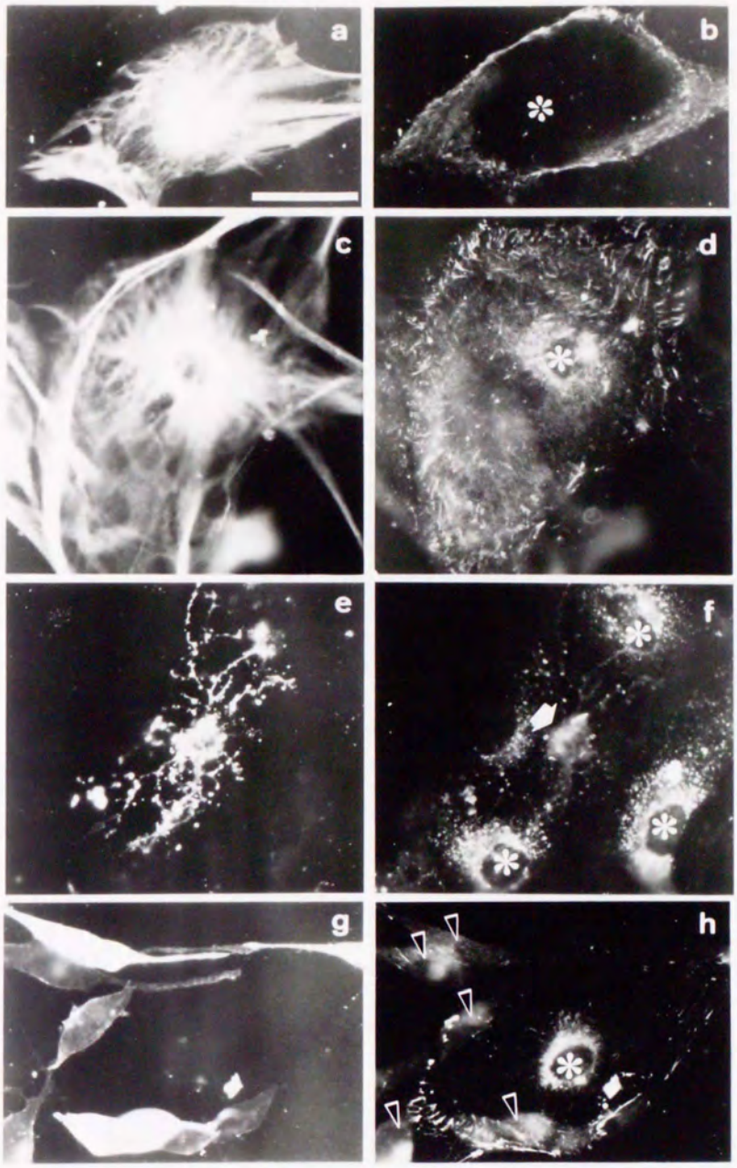

Fig. 7. Fluorescent micrographs showing the fibronectin expressed by glial subtypes. Cells were double-labelled with anti-GFAP (a, c; rhodamine optics) and anti-fibronectin (b, d; fluorescein optics) or with monoclonal A2B5 antibody (e, g; rhodamine optics) and anti-fibronectin (f, h; fluorescein optics). The staining with anti-fibronectin was performed on paraformaldehyde-fixed cells (b) to detect the antigen on cells or on paraformaldehyde-fixed cells subsequently subjected to a permeabilization step (d, f, h) to detect the antigen in the intracytoplasmic portion and antigens between cells and a substratum. The focus was adjusted to the level of the substrate level (d). Asterisks; type-1 astrocytes. Arrows; type-2 astrocytes. Arrowheads; O-2A progenitors. Bars; 40 μm.

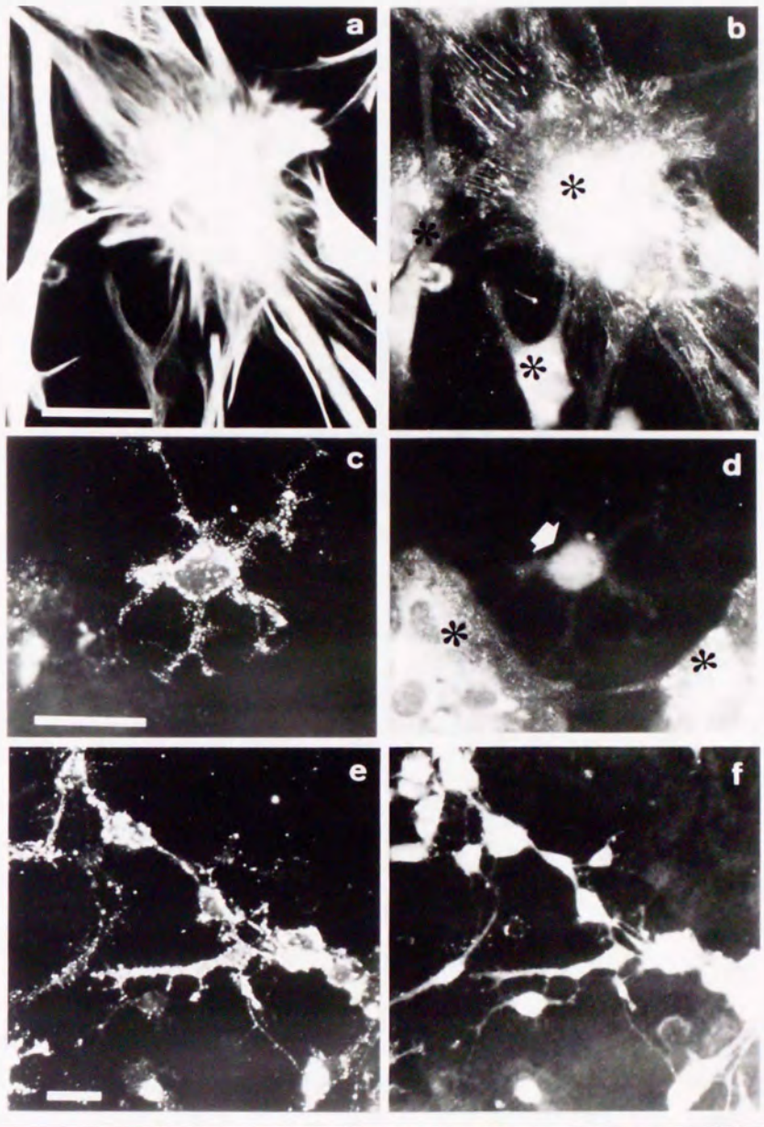

Fig. 8. Fluorescent micrographs showing the laminin expressed by glial subtypes. Cells were double-labelled with anti-GFAP (a; rhodamine optics) and anti-laminin (b; fluorescein optics) or with A2B5 (c, e; rhodamine optics) and anti-laminin (d, f; fluorescein optics). The staining with anti-laminin was performed on paraformaldehyde-fixed cells subsequently subjected to a permeabilization step. Asterisks; type-1 astrocytes. Arrows; type-2 astrocytes. Bars; 40 μm.

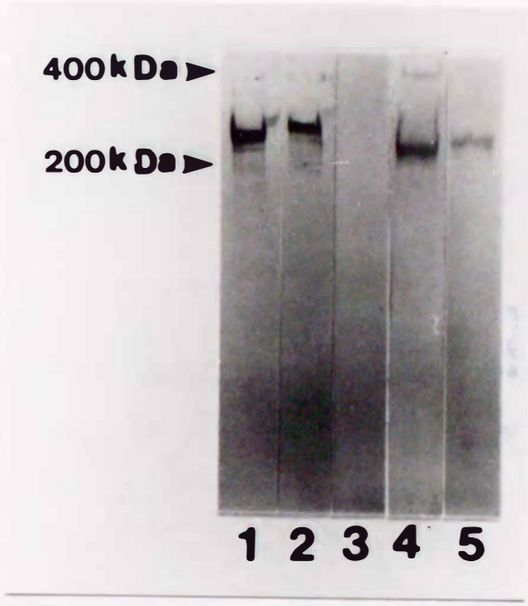

Fig. 9. Western blot analysis of fibronectin and laminin. Mouse plasma fibronectin (lane 1), the culture medium after incubation with gelatin-coupled Sepharose (lane 2), the culture medium after incubation with uncoupled Sepharose (lane 3), EHS laminin (lane 4) and the cell extracts of glial culture (lane 5) were electrophoresed (SDS-PAGE; 5%) under reducing conditions and transferred to nitrocellulose sheets. The sheets were immunostained with HRP-conjugated anti-fibronectin IgG (lanes 1, 2 and 3) or HRP-conjugated anti-laminin IgG (lanes 4 and 5). Molecular weights are indicated on the left-hand side.

5. ESTABLISHMENT OF AN ASTROCYTE PROGENITOR CELL LINE: INDUCTION OF GLIAL FIBRILLARY ACIDIC PROTEIN AND FIBRONECTIN BY TRANSFORMING GROWTH FACTOR-β1

5.1 Introduction

The use of primary cultures and the availability of immunological markers for cell type identification have improved the understanding of the gliogenesis considerably (for review, Cameron and Rakic, 1991). In primary cultures of neonatal rat optic nerve, three types of differentiated macroglial cells can be distinguished by their morphology and antigenic phenotype: type-1 astrocytes, type-2 astrocytes and oligodendrocytes (Raff et al. 1983a). These three types arise from two separate lineages: type-1 astrocytes develop from type-1 astrocyte progenitors (Raff et al., 1984), whereas oligodendrocytes and type-2 astrocytes develop from common bipotential O-2A progenitors (Raff et al., 1983b). Much information on O-2A progenitors is now available because of their availability in highly enriched cultures (Bahar et al., 1988; Hunter and Bottenstein, 1990). Less information is available on type-1 astrocyte progenitors since highly enriched cultures of these cells have not yet been obtained. Recently, the presence of distinct types of glial progenitors has been shown in cultures from other regions of the central nervous system (Miller and Szigeti,

1991; Vaysee and Goldman, 1992).

The factors controlling the differentiation and/or proliferation of glial progenitors would be accessible for molecular analysis if cell lines with progenitor properties could be established. Cell lines with the properties of O-2A progenitors in primary cultures have been established (Evrard et al., 1988; Aloisi et al., 1990; Louis et al., 1992). However, to our knowledge, there are no reports describing the establishment of other glial progenitors including type-1 astrocyte progenitors.

In the present study, we established a cell line (AP-16) from neonatal mouse cerebra by long-term, serum-free culturing. The antigenic and morphological features of AP-16 cells were found to be similar to those of O-2A progenitors, but the growth and differentiation properties of the cells resembled those of type-1 astrocyte progenitors. Interestingly, transforming growth factor-β1 (TGF-β1) induced AP-16 cells to differentiate into phenotypically mature astrocytes. Functionally, AP-16 cells were shown to synthesize fibronectin and laminin. The regulatory mechanism for the biosynthesis of these molecules by glial cells has been completely unknown. We also found that TGF-β1 increases the expression of fibronectin in AP-16 cells. These results show that AP-16 cells provide a useful in vitro system for analyzing the role of TGF-β1 in the differentiation of astrocyte progenitors.

5.2 Materials and Methods

Primary glial cultures and multipassages

Primary glial cultures were prepared from 1-day-old C3H/He mouse cerebra as described in Section 3.2. After 3 days of incubation in Dulbecco's modified minimum essential medium (D-MEM) supplemented with 10% fetal calf serum (FCS), the culture medium was changed to a serum-free, chemically defined medium (G5 medium) which has been optimized for the selective growth of mature astrocytes (Michler-Stuke et al., 1984). G5 medium consists of D-MEM supplemented with glucose (4.5 mg/ml), 10 nM hydrocortisone, 30 nM sodium selenite, recombinant human insulin (Wako Pure Chemicals Ltd., Osaka; 5 μg/ml), bovine plasma transferrin (holo type, 50 μg/ml), biotin (10 ng/ml), recombinant human basic fibroblast growth factor (bFGF, Boehringer Mannheim GmbH, Mannheim; 5 ng/ml) and mouse epidermal growth factor (EGF, Takara Shuzo Ltd., Kyoto; 10 ng/ml).

After 7 days of primary culturing, with medium changes every 2-3 days, the cells were passaged 30 times over 5 months. For subcultures the cells were incubated with 0.1% crystalline trypsin solution for 2-3 minutes at room temperature, and trypsinization was terminated by the addition of soybean trypsin inhibitor (0.5 mg/ml). The cultures were passaged every 3-7 days at a split ratio of 1:4. All cultures were grown on poly-L-lysine-coated dishes at 37°C in a humidified 95% air/5% CO_2 atmosphere.

Establishment of cell line AP-16

Cells at passage 30 were cloned by limiting dilution. A cell suspension was dispensed into 96-well plates in G5 medium supplemented with 10% conditioned medium of cultures at passage 4-6. Twelve clones having similar morphology grew after 10 days to 2 weeks. The conditioned medium was not used for further subcultures. One clone, designated AP-16, was selected for more extensive characterization. Hydrocortisone and bFGF did not affect the growth of AP-16 cells; therefore, G5 medium lacking bFGF and hydrocortisone was used as the standard culture medium for AP-16 cells.

AP-16 cells were tested and were shown to be free of mycoplasmal contamination by the microbiological culture method and the DNA fluorochrome staining method (Yoshida et al., 1988). Chromosome modal number and aberrations were determined on 50-well spread metaphases. For the tumorigenicity assay, cells (10^7 cells/mouse) were inoculated subcutaneously in 4-week-old C3H/He female mice (5 mice per dose). Mice were monitored for 4 months. Soft agar cultures were performed according to the standard protocol (MacPherson and Montagnier, 1964).

Antigenic phenotype analyses

An indirect immunofluorescent technique was used to examine antigenic phenotypes of cells. Cells were seeded at 2×10^5 cells/15-mm well on poly-L-lysine-coated coverslips.

The first antibodies used were A2B5 mouse monoclonal antibody (IgM class; used as a dialyzed ammonium sulfate fraction of ascites fluid): rabbit anti-cow glial fibrillary acidic protein (anti-GFAP; Dako Co., Glostrup): and rabbit anti-bovine galactocerebroside (anti-GalC, prepared by immunization with GalC). As the second antibodies, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Cappel Laboratories, Malveron, PA) and Texas red-conjugated goat anti-mouse IgM (E.Y Laboratories, Inc. San Mateo, CA) were used. Fixation and staining procedures were as described in Section 4.2. Briefly, surface antigens (A2B5 and GalC) were immunolabelled on 2% paraformaldehyde-fixed cells, and an intracellular antigen (GFAP) was immunolabelled after paraformaldehyde-fixing followed by permeabilization with 0.1% Triton X-100. The stained cells were observed under an Olympus BH-2 fluorescence microscope.

Expression of A2B5 on AP-16 cells was examined by flow cytometry. Trypsinized cells were stained and analyzed on a FACStar (Becton Dickinson, Mountain View, CA). Expression of GFAP was examined by Western blot analysis as described by Sakai et al. (1990).

Assays of differentiation

Cells were cultured in the standard culture medium supplemented with 10% FCS, porcine TGF-β1 (10 ng/ml; R & D Systems Inc., Minneapolis, MN), TGF-β2 (10 ng/ml), recombi-

nant human platelet-derived growth factor-AA (PDGF-AA; 10 ng/ml; Collaborative Research Inc., Bedford, MA), bFGF (5 ng/ml), dibutyryl-cAMP (1 mM) or hydrocortisone (10 nM). After 2 days, the morphological and antigenic features of the cells were examined.

Enzyme-linked immunosorbent assay (ELISA)

The amounts of fibronectin and laminin were assessed by a sandwich ELISA as described in Section 4.2. Briefly, cells were seeded (5 x 10^5 cells/35-mm dish) and cultured with or without TGF-β1 (10 ng/ml). After 3 days, three portions: intracellular, extracellular matrix and culture medium, were extracted from the cultures as described by Ignotz and Massagué (1986). The specificity of anti-fibronectin and anti-laminin has been confirmed by Western blot analysis (Section 4.3). The amounts of fibronectin and laminin were determined from standard curves generated with mouse plasma fibronectin (Wako Pure Chemicals Ltd.) or mouse EHS laminin (Iwaki Glass Co., Tokyo).

5.3 Results

Establishment of cell line AP-16 from glial cultures

Mixed glial cultures from neonatal mouse cerebra were maintained in serum-free G5 medium that contains bFGF and EGF as supplements. The cellular composition of the cul-

tures was analyzed immunocytochemically over a period of 5 months. The proportion of cell population in the cultures changed with increasing passages (Fig. 10). Double immunofluorescent staining using A2B5 monoclonal antibody and anti-GFAP revealed that the percentage of $A2B5^-/GFAP^+$ cells decreased and that the percentage of $A2B5^+/GFAP^-$ cells increased. The $A2B5^-/GFAP^+$ cells displayed a fibroblast-like morphology, like type-1 astrocytes (Raff et al., 1983a). The $A2B5^+/GFAP^-$ cells exhibited a process-bearing morphology, like O-2A progenitors (Raff et al., 1983b). In the early passage cultures (passage 9-12), a small population of type-2 astrocytes ($A2B5^+/GFAP^+$, multipolar) and oligodendrocytes ($GalC^+$, multipolar) were observed. The later passage cultures (over passage 20) contained neither type-2 astrocytes nor oligodendrocytes, even though the cultures contained a large population of cells with morphological and antigenic features of O-2A progenitors.

The mixed glial culture grew for 5 months without an obvious cell crisis period. When the culture reached passage 30, cell cloning was achieved by limiting dilution. One clone (AP-16) was expanded to mass cultures in the standard culture medium, consisting of D-MEM supplemented with insulin, transferrin, sodium selenite, biotin and EGF. Most AP-16 cells, like O-2A progenitors (Temple and Raff, 1986), were round, bipolar or multipolar in this medium (Fig. 11). As the cells increased in number, many of them

aggregated and appeared to be less strongly attached to the poly-L-lysine-coated dishes.

AP-16 cells were phenotypically nontumorigenic on the basis of the classical criteria: absence of tumorigenicity in C3H/He mice and inability to grow in soft agarose medium. AP-16 cells exhibited aneuploid karyotypes; the chromosome numbers ranged from 49 to 72, and the modal number of chromosomes was 69.

Antigenic and growth characteristics of AP-16 cells

To examine the antigenic features of AP-16 cells, immunofluorescent staining using antibodies was performed. Most cells expressed A2B5, a marker for O-2A progenitors and type-2 astrocytes (Raff et al., 1984), with intense plasma membrane fluorescence (Fig. 12 A). GFAP, a marker for mature astrocytes (Bignami et al., 1972), and GalC, a marker for oligodendrocytes (Raff et al., 1978), were not detected (Fig. 12 B, C).

To examine the growth properties of AP-16 cells, the cells were cultured in various media. In the standard culture medium, a logarithmic increase in the number of cells was observed (Fig. 13). The population doubling time of the cells was about 23 hours. AP-16 cells depended strikingly on EGF for survival: greater than 80% of the cells were dead within 2 days in the culture medium lacking EGF. AP-16 cells grew at a much lower rate in the presence

of 10% FCS than in the standard culture medium. PDGF (10 ng/ml) and bFGF (5 ng/ml), mitogens for O-2A progenitors (Noble et al., 1988; Bögler et al., 1990; McKinnon et al., 1990), had no effect on the growth of AP-16 cells.

Differentiation properties of AP-16 cells

To determine whether AP-16 cells could differentiate into mature glial cells, the cells were characterized by morphological and antigenic criteria after 2 days of exposure to various factors. TGF-β1 (10 ng/ml) and FCS (10%) induced morphological changes in AP-16 cells: a flattened morphology and an epithelial-like morphology, respectively (Fig. 14). TGF-β1 did not affect the growth of the cells. As judged by double staining with A2B5 monoclonal antibody and anti-GFAP, treatment of the cells with TGF-β1 or FCS for 2 days induced the expression of GFAP with a decrease in A2B5-positive cells (Fig. 15). Of the 1,000 cells determined, the proportion of $A2B5^-/GFAP^+$ cells was 71% and 95% in TGF-β1-treated cells and FCS-treated cells, respectively. The decrease in A2B5 expression and GFAP appearance was confirmed by flow cytometry and Western blotting (Fig. 16). TGF-β2 (10 ng/ml), which has been described as a secreting product of astrocytes (Constam et al., 1992), also induced the morphological and antigenic changes in AP-16 cells. No GalC-positive cells were observed in the presence of TGF-β1, TGF-β2 or FCS. The treatment of AP-16 cells with other

agents known to influence glial differentiation: PDGF-AA, hydrocortisone, bFGF and dibutyryl-cAMP, had no effect on the expression of A2B5, GFAP or GalC.

Increase in fibronectin expression by TGF-β1

One of the functional properties of glial cells is the synthesis of fibronectin and laminin (Price and Hynes, 1985; Liesi et al., 1983). An immunofluorescent staining showed that AP-16 cells expressed both fibronectin and laminin (data not shown). TGF-β1 has been shown to stimulate production of fibronectin and to increase its deposition in the extracellular matrix portion in a variety of cell types (Ignotz and Massagué, 1986). To determine whether TGF-β1 could modulate the expression of fibronectin in AP-16 cells, the cells were cultured for 3 days in the standard culture medium with or without TGF-β1 (10 ng/ml). At the end of the incubation period, the amounts of fibronectin and laminin in the three portions of the cultures: intracellular, extracellular matrix and cultured medium, were assessed using an ELISA. Table IV shows that treatment of AP-16 cells with TGF-β1 increased the expression of fibronectin. The increase was observed in both the extracellular matrix portion and cultured medium. The cultured medium of the cells treated with TGF-β1 contained 8 times more fibronectin per 10^6 cells than found in control. No significant increase in laminin was observed.

To determine whether the induction of GFAP by TGF-β1 in AP-16 cells was mediated by the increased fibronectin, cells were cultured on dishes precoated with mouse plasma fibronectin or in medium supplemented with fibronectin (10 μg/ml). Mouse plasma fibronectin had no effect on the expression of GFAP in AP-16 cells.

5.4 Discussion

The present study shows that serial passaging of mixed glial cultures in serum-free G5 medium can lead to the spontaneous immortalization of the cells. The AP-16 cells retained the properties of astrocyte progenitors. The morphological and antigenic features of AP-16 cells were similar to those of O-2A progenitors and can be distinguished from those of type-1 astrocyte progenitors in primary cultures: A2B5$^-$, a flattened, epithelial-like morphology (Abney et al., 1981; Raff et al., 1984; da Cunha and Vitković, 1990). The growth properties of AP-16 cells were similar to those of type-1 astrocyte lineage cells. AP-16 cells depended on EGF for survival. EGF is known to be a mitogen for type-1 astrocytes (Raff et al., 1983a) and immature astrocytes (Fisher et al., 1982) but not for O-2A progenitors (Hunter and Bottenstein, 1990) or type-2 astrocytes (Raff et al., 1983a). In addition, PDGF and bFGF, both mitogens for O-2A progenitors, did not stimulate the

proliferation of AP-16 cells in the presence of EGF. The differentiation properties of AP-16 cells resembled those of type-1 astrocyte progenitors: (1) AP-16 cells proliferated without differentiation into oligodendrocytes in the absence of bFGF and PDGF and (2) in the presence of FCS, AP-16 cells differentiated into epithelial-like, $A2B5^{-}/GFAP^{+}$ cells.

AP-16 cells might be derived from a progenitor that is distinct from O-2A progenitors and type-1 astrocyte progenitors. Recently, Fok-Seang and Miller (1992) have identified a distinct type of astrocyte precursors, which are $A2B5^{+}$ and separate both from O-2A progenitors and type-1 astrocyte progenitors, in neonatal rat spinal cord cultures. AP-16 cells showed unique growth characteristics: EGF dependency and growth inhibition by FCS. Cells with these properties may disappear from glial cultures in conventional serum-supplemented medium. Further analysis is necessary to determine the in vivo cell type from which AP-16 cells originate.

In spite of an exponential increase in research on TGF-β, little is known about its effects on cells in the central nervous system. We demonstrated that TGF-β induced the appearance of GFAP with the disappearance of A2B5 in AP-16 cells. TGF-β also changed the morphology of AP-16 cells. Recently, Sakai et al. (1990) established a cell line (SFME) in which TGF-β1 induces GFAP. Although this report does not describe other glial markers of SFME cells,

the finding of the effect of TGF-β1 on the expression of GFAP is significant. Ciliary neurotrophic factor has been shown to be responsible for the differentiation of O-2A progenitors into type-2 astrocytes (Lillien et al., 1988). TGF-β may play a role in the differentiation of some type(s) of astrocytes in the developing central nervous system.

We found that fibronectin and laminin were expressed by cultured glial cells (Section 4.3). Fibronectin and laminin are expressed during a defined period of the embryonic stage in brain (Stewart and Pearlman, 1987; Liesi, 1985) and are induced in adult brain by injury (Liesi et al., 1984; Egan and Vijayan, 1991). These molecules are considered to play important roles in the development of embryonic neurons and the regeneration of damaged adult nerve fibers (Akers et al., 1981; Manthorpe et al., 1983; Liesi et al., 1984). However, the regulatory mechanism for the biosynthesis of these molecules by glial cells is completely unknown. In the present study, we found that AP-16 cells synthesized both molecules and that the expression of fibronectin was increased by TGF-β1. Recently, TGF-β1 has been shown to be present in developing mouse brain (Flanders et al., 1991) and to be induced in adult brain by injury (Lindholm et al., 1992). TGF-β1 may play a role as an inducer of the fibronectin expression in vivo. TGF-β1 has also been shown to increase the expression of the neural adhesion molecule L1

by modulating production of nerve growth factor in cultured immature astrocytes (Saad et al., 1991). These results suggest that TGF-β1 plays a crucial role in the regulation of glia-neuron interaction.

5.5 Summary

An immortalized clonal cell line (AP-16) has been established from glial cultures obtained from neonatal mouse cerebra by multipassages under serum-free conditions. Immunofluorescent experiments showed that AP-16 cells expressed a marker for glial progenitors (A2B5) and did not express markers for oligodendrocytes (galactocerebroside) or mature astrocytes (glial fibrillary acidic protein; GFAP). Treatment with transforming growth factor-β1 (TGF-β1) or fetal calf serum (FCS) for 2 days induced AP-16 cells to differentiate into A2B5-negative, GFAP-positive, phenotypically mature astrocytes. These results indicate that AP-16 cells retained the properties of astrocyte progenitors. An enzyme-linked immunosorbent assay showed that AP-16 cells synthesized fibronectin and laminin and that the expression of fibronectin was increased by TGF-β1. AP-16 cells should be useful for studying the roles of TGF-β1 in the differentiation of astrocyte progenitors.

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Table IV. Effects of TGF-β1 on the expression of fibronectin and laminin in AP-16 cells

		Amount detected (ng/10^6 cells)		
Molecule	TGF-β1	Intracellular	Matrix	Medium
Fibronectin	-	4.2 ± 0.9	5.6 ± 2.7	23 ± 12
	+	4.8 ± 0.5	8.9 ± 3.5	194 ± 47
Laminin	-	6.7 ± 3.2	3.5 ± 0.2	2.9 ± 1.6
	+	5.6 ± 2.1	4.2 ± 1.6	1.8 ± 0.8

Cells were seeded (5 x 10^5 cells/35-mm dish) and cultured with or without TGF-β1 (10 ng/ml). After 3 days, the amounts of fibronectin and laminin were determined by ELISA. Viable cells were counted by the trypan blue dye exclusion method. Values represent amounts of fibronectin and laminin in the three portions of cultures: intracellular, extracellular matrix (matrix) and culture medium (medium). The values shown are means and standard deviations from three experiments with duplicate samples per experiment.

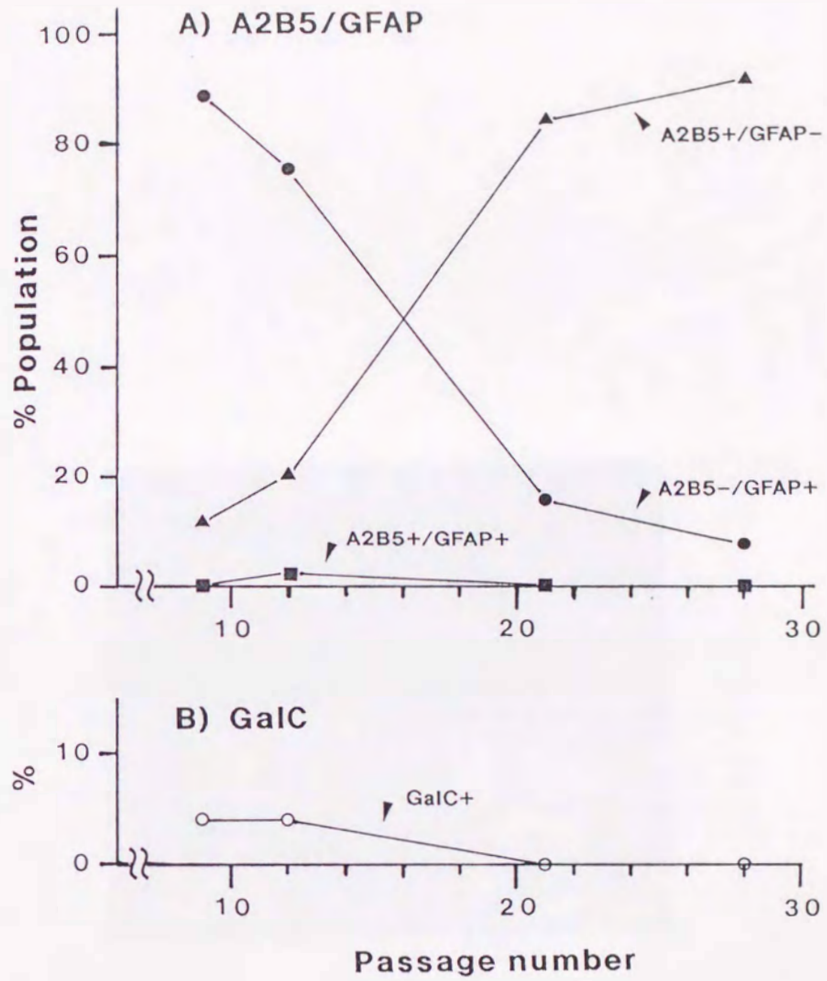

Fig. 10. The shift in cell population during multipassages characterized by immunofluorescent staining. Cells cultured in G5 medium at the indicated passage were double-stained with A2B5 monoclonal antibody and anti-GFAP (A) or stained with anti-GalC (B). Percentages were determined as the ratio of immunocytochemically defined cells to the corresponding total cells identified by phase-contrast optics. The mean percentage was determined for more than 500 cells on three separate coverslips.

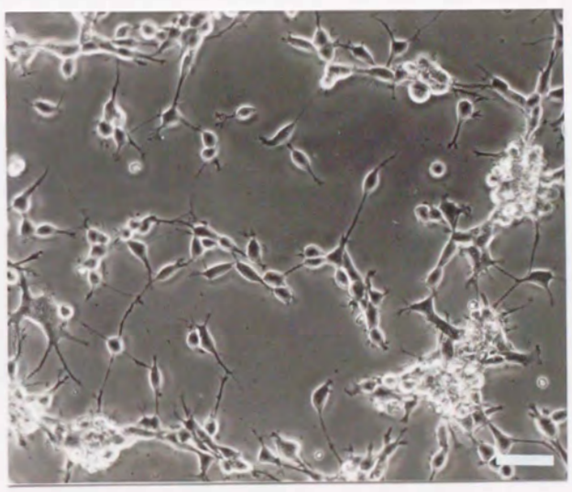

Fig. 11. Morphology of AP-16 cells grown in the standard culture medium. Bar, 50 μm.

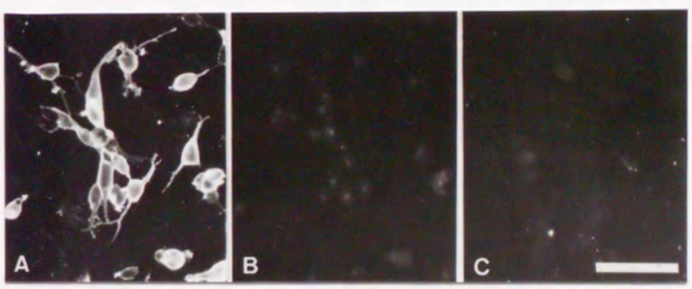

Fig. 12. Antigenic characterization of AP-16 cells. Double immunofluorescence illustrating the same field illuminated by Texas red optics demonstrating A2B5 immunoreactivity (A) and fluorescein optics demonstrating GFAP staining (B). Immunofluorescence of the cells labelled with anti-GalC (C). Bar, 50 μm.

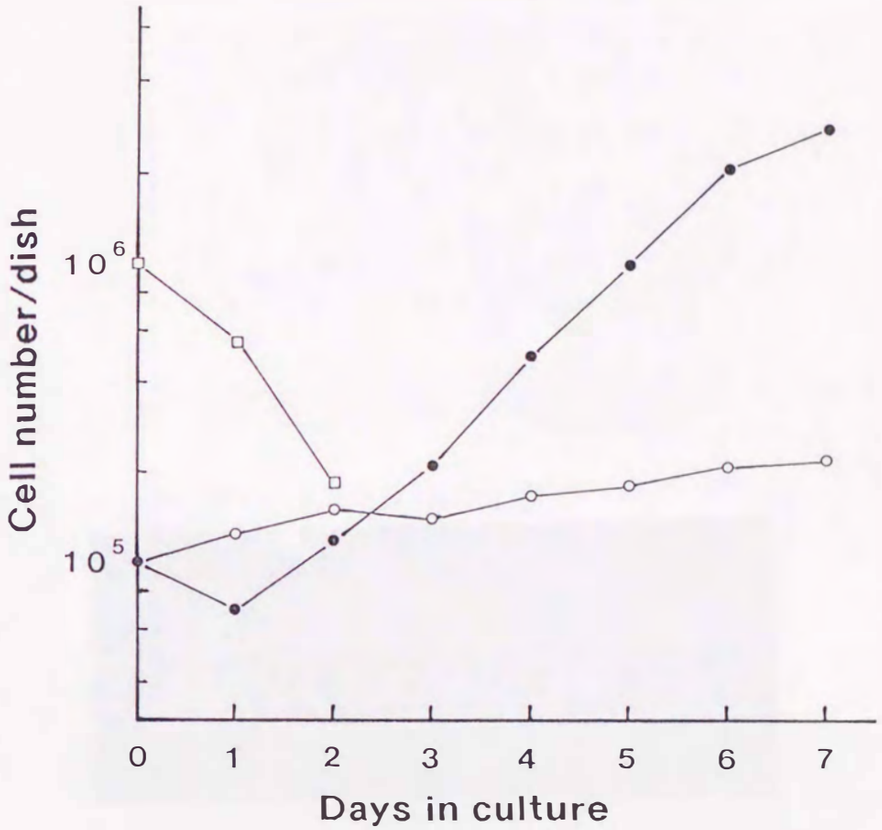

Fig. 13. Growth characteristics of AP-16 cells. Cells (10^5 cells/35-mm dish or 10^6 cells/35-mm dish) were seeded and cultured in the standard culture medium (closed circles), in medium supplemented with 10% FCS (open circles) or in medium lacking EGF (open squares). The medium was replaced every 2 days. Viable cells were counted at the indicated days by the trypan blue dye exclusion method. Values are the means of three dishes. Individual values varied less than 10% from the means.

Fig. 14. Effects of TGF-β1 and FCS on the morphology of AP-16 cells. Cells were cultured for 2 day in the culture medium (A), medium supplemented with 10 ng/ml TGF-β1 (B) or medium supplemented with 10% FCS (C). Bar, 50 μm.

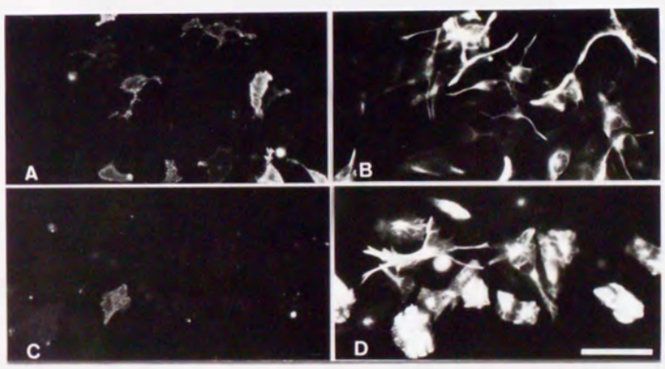

Fig. 15. Effects of TGF-β1 and FCS on the antigenic features of AP-16 cells. Cells were cultured in standard culture medium supplemented with 10 ng/ml TGF-β1 (A, B) or with 10% FCS (C, D) as described in Materials and Methods. After 2 days, the cells were double-labelled with A2B5 monoclonal antibody (A, C: Texas red optics) and anti-GFAP (B, D: fluorescein optics) and examined by fluorescence microscopy. Bar, 50 μm.

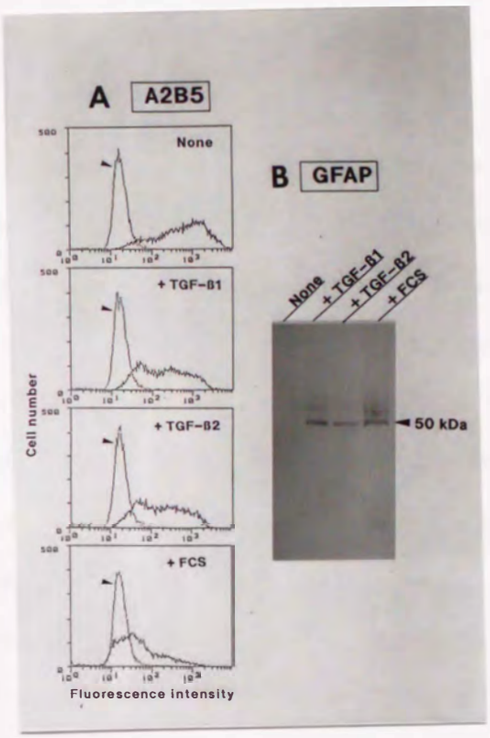

Fig. 16. Expression of A2B5 and GFAP. AP-16 cells were cultured in standard culture medium or medium supplemented with 10 ng/ml TGF-β1, 10 ng/ml TGF-β2 or 10% FCS. After 2 days, the cells were subjected to the following analyses. A) Flow cytometric analysis of A2B5 expression on AP-16 cells. Dotted lines (arrowheads) indicate background staining with the second antibodies alone. The data shown are from a single experiment representative of the three experiments. B) Western blot analysis of GFAP. After electrophoresis (50 μg protein per lane) under reducing conditions and transfer onto nitrocellulose membranes, blots were incubated with anti-GFAP and with goat anti-rabbit IgG conjugated with horseradish peroxidase. Molecular weight (50 kDa) is indicated on the right-hand side.

6. CYTOKINES AFFECTING SURVIVAL AND DIFFERENTIATION OF AN ASTROCYTE PROGENITOR CELL LINE

6.1 Introduction

In vitro studies have provided important clues to the identification of cytokines that may control glial cell development in the central nervous system (Cameron and Rakic, 1991). Significant progress has been made in understanding cytokines affecting survival (Barres et al., 1992), proliferation (Bögler et al., 1990; McKinnon et al., 1990) and differentiation (Hughes et al., 1988) of oligodendrocyte-type-2 astrocyte (O-2A) progenitors (Raff, 1989). However, very little information on other glial progenitors has been obtained due to the lack of enriched cultures or cell lines of these cells.

In our previous study, we established a cell line (AP-16) of astrocyte progenitors from neonatal mouse cerebrum by long-term, serum-free culturing (Chapter 5). Cell line AP-16 is considered to be a suitable model for studying the roles of cytokines in the development of astrocyte progenitors. In fact, AP-16 cells were found to be dependent on epidermal growth factor (EGF) for survival and to differentiate into astrocytes in the presence of transforming growth factor-βs (TGF-β1, TGF-β2). In this study, we found that EGF, transforming growth factor-α (TGF-α) and basic fibro-

blast growth factor (bFGF) acted as survival factors that prevented death of AP-16 cells by apoptosis (Kerr et al., 1972). In addition, we found that leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) induced AP-16 cells to differentiate into astrocytes.

6.2 Materials and Methods

Culture of AP-16 cells

AP-16 cells were maintained in Dulbecco's modified minimum essential medium (D-MEM) containing sodium selenite (30 nM), insulin (5 μg/ml), transferrin (50 μg/ml), biotin (10 ng/ml) and murine EGF (10 ng/ml, Takara Shuzo Ltd).

EGF deprivation experiments

Cells in the culture medium were washed with D-MEM and incubated in the absence or presence of EGF (10 ng/ml) for 24 hours. The cells were then examined for apoptosis by the following experiments. First, nuclear morphology was observed after the cells were fixed and stained with DNA fluorochrome Hoechst 33258. Second, total DNA was extracted and its organization was analyzed by horizontal electrophoresis (5 μg/lane) in a 1% agarose gel for 3 hours at 80 V (Barres et al., 1992). DNA was visualized after electrophoresis by ethidium bromide staining. In addition, the effects of cycloheximide and actinomycin D on AP-16 cells in

the absence of EGF were examined. Cells were seeded at 10^6 cells/35-mm dish in the culture medium. After 4 hours the dishes were changed to medium lacking EGF, and cycloheximide (0.1 μg/ml) or actinomycin D (0.01 μg/ml) was added. Twelve hours later cell viability was determined by trypan blue dye-exclusion method.

Differentiation experiments

Experiments on the differentiation of AP-16 cells were performed by culturing the cells for 2 days with one of the following cytokines: recombinant murine LIF (10 ng/ml, Pepro Tech), recombinant rat CNTF (10 ng/ml, Pepro Tech), recombinant human TGF-α (10 ng/ml, GIBCO-BRL), recombinant human interleukin-2 (IL-2, 10 ng/ml, Wako Pure Chemicals), recombinant murine interleukin-6 (IL-6, 10 ng/ml, GIBCO-BRL) and murine 2.5S nerve growth factor (NGF, 10 ng/ml, Boehringer-Mannheim). After 2 days of treatment with the various cytokines, the expression of A2B5 (a marker for glial progenitors) was analyzed on FACStar (Becton Dickinson), and the expression of GFAP (a marker for astrocytes) was examined by Western blotting as described in Section 5.2. Antibodies used were A2B5 murine monoclonal antibody (IgM), rabbit anti-GFAP (Dako Co.), FITC-conjugated goat anti-mouse IgM (TAGO) and horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad).

6.3 Results

Characterization of cell death in AP-16 cells by EGF deprivation

When AP-16 cells were deprived of EGF, they not only ceased proliferating but their viability was also severely impaired (Fig. 13). Two days after deprivation of EGF, most cells detached from the dish and floated in the medium. The floating cells could be recovered with the medium, and neither attached onto a new dish nor proliferated even in the presence of EGF. Thus, we concluded that the cells that floated into the medium were dead.

Morphological, biochemical and physiological responses of AP-16 cells to EGF deprivation were analyzed to define the mechanisms of cell death in these cells. Upon fluorescent microscopy after Hoechst 33258 staining, the nuclei of the AP-16 cells incubated in the absence of EGF were usually seen to be shrunken, highly condensed and fragmented (Fig. 17). Control cells in the presence of EGF showed a regular diffuse chromatin staining pattern characteristic of viable cells. DNA from AP-16 cells incubated in the presence or absence of EGF was subjected to agarose gel electrophoresis (Fig. 18). DNA obtained from cells incubated in the absence of EGF was not randomly cleaved but, rather, showed a regularly spaced ladder pattern of DNA fragmentation. DNA from cells grown in the presence of EGF did not show any sort of fragmentation. These features of nuclear morphology and DNA

organization are characteristics of a form of cell death termed apoptosis (Wyllie et al., 1980).

One of the most striking features of apoptosis is that it is dependent on protein and RNA synthesis in the dying cells (Ellis et al., 1991). We therefore monitored cell death after EGF deprivation in the presence of cycloheximide (an inhibitor of protein synthesis) or actinomycin D (an inhibitor of RNA synthesis) (Fig. 19). The inhibition of cell death by these inhibitors was significant, providing further evidence for apoptosis.

Survival factors for AP-16 cells

We have showed that the death of AP-16 cells after EGF deprivation was due to apoptosis, indicating that EGF promotes survival by suppressing the process of apoptosis. This action of EGF was mimicked by TGF-α (10 ng/ml) and bFGF (10 ng/ml, Boehringer-Mannheim) (Fig. 20) but not by TGF-β, platelet-derived growth factor-AA (PDGF-AA), NGF, LIF, CNTF, IL-2 or IL-6. AP-16 cells proliferated in the presence of EGF, TGF-α or bFGF.

Differentiation factors for AP-16 cells

We have shown that treatment of AP-16 cells with TGF-βs led to the appearance of GFAP and a decrease in A2B5 expression (Section 5.3). When AP-16 cells were treated with LIF or CNTF for 2 days, similar antigenic changes, the

appearance of GFAP and decrease in A2B5 expression, were observed by Western blotting and flow cytometry, respectively (Fig. 21). Treatment of AP-16 cells with other cytokines, TGF-α, IL-2, NGF or IL-6, had no effect on the expression of GFAP or A2B5. Neither LIF nor CNTF altered the morphology or growth of AP-16 cells. These results show that LIF and CNTF induced AP-16 cells to differentiate into astrocytes.

6.4 Discussion

AP-16 cells were strictly dependent on EGF not only for growth but also for maintaining cell viability. After removal of EGF, AP-16 cells progressively died. The cell death appeared to fulfill the criteria used to define the apoptotic process (Ellis et al., 1991): cells showed a typical morphology (condensed and fragmented nuclei); DNA electrophoresis analysis demonstrated the characteristic pattern of fragmentation in multiples of nucleosome-sized subunit (185 bp); and the cell death was suppressed by the inhibition of RNA or protein synthesis. Some glial cells die during the development of the central nervous system, but the types of glial cells that die and mechanisms responsible for their death are unclear (Knapp et al., 1986). Recently, Barres et al. (1992) showed that immature oligodendrocytes and O-2A progenitors require signals (PDGF and insulin-like

growth factors) from other cells to survive in culture and that these cells die by apoptosis in large numbers during normal development. Our in vitro studies using AP-16 cells suggest that EGF, an EGF-like molecule (e.g., TGF-α) or bFGF promotes survival and proliferation of astrocyte progenitors in vivo.

LIF and CNTF have been reported to exert multiple and partially overlapping effects on neuronal survival and differentiation in vitro (Hall and Rao, 1992; Manthorpe et al., 1993; Patterson, 1993). To our knowledge, there are no reports describing the biological effects of LIF on glial cells. CNTF has been reported to induce the transient expression of GFAP in O-2A progenitors from optic nerve (Hughes et al., 1988). These findings, together with our results, suggest that LIF and CNTF play roles in the control of both neuronal and glial development.

The results obtained with AP-16 cells suggest that various cytokines are involved in astrocyte development. This cell line should provide useful model systems for examining the relationships between cytokines and their receptors, intracellular signalling and the roles of cytokines in regulating the growth and differentiation of astrocyte progenitors.

6.5 Summary

The effects of various cytokines on survival and differentiation of an astrocyte progenitor cell line (AP-16) were examined. Epidermal growth factor (EGF) deprivation caused death of AP-16 cells by apoptosis. Transforming growth factor-α (TGF-α) and basic fibroblast growth factor (bFGF) prevented the apoptosis occurring in the absence of EGF. Leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) induced glial fibrillary acidic protein (GFAP) and decreased A2B5 antigen in AP-16 cells, indicating that these cytokines induced AP-16 cells to differentiate into astrocytes. The results obtained with AP-16 cells suggest that these cytokines are involved in the control of astrocyte development in vivo.

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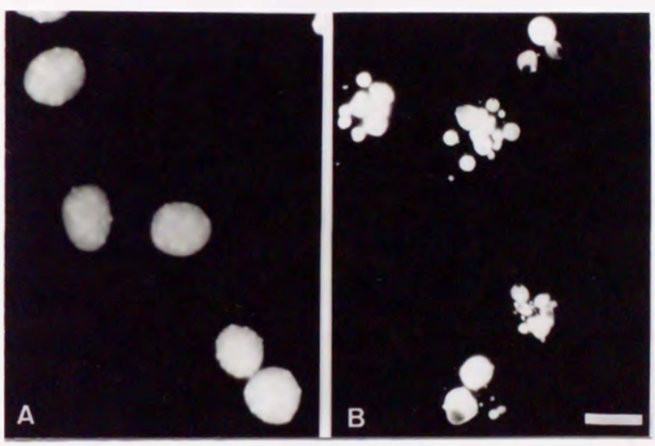

Fig. 17. Apoptotic features of nuclear morphology in AP-16 cells caused by EGF deprivation. Cells in culture medium were washed with D-MEM and incubated in the presence (A) or absence (B) of EGF for 24 hours. The cells were fixed and stained with Hoechst 33258 dye and then nuclear morphology was observed by fluorescence microscopy. Bar, 20 μm.

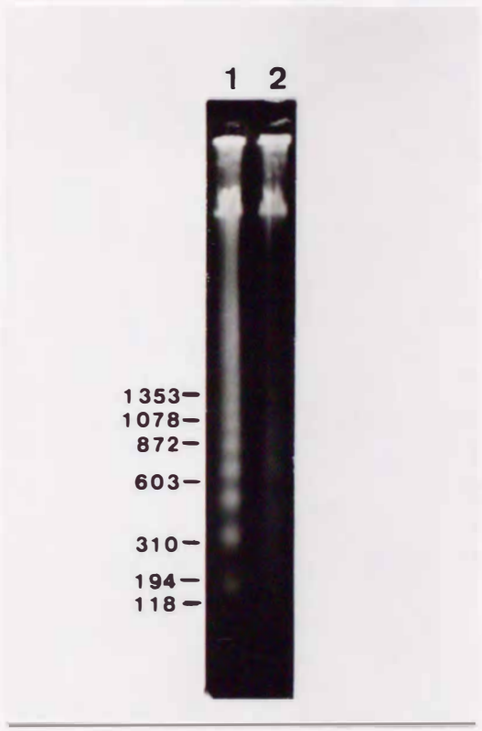

Fig. 18. Gel electrophoresis analysis of DNA fragmentation. Total DNA was extracted from cells cultured in the absence (lane 1) or presence (lane 2) of EGF for 24 hours. Each DNA sample (5 μg) was electrophoresed through a 1% agarose gel and visualized by ethidium bromide staining. Molecular sizes in base pairs are indicated on the left-hand side.

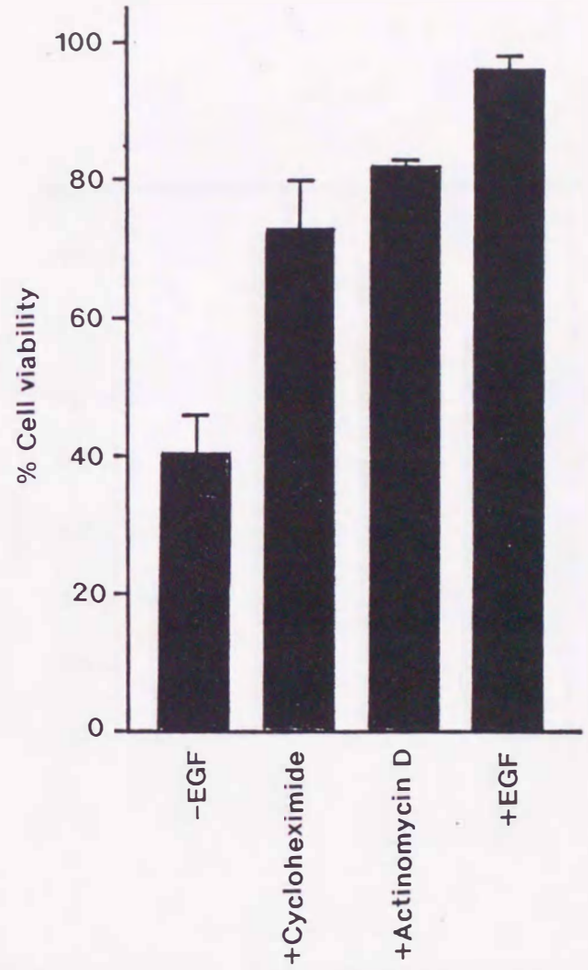

Fig. 19. Increased viability of AP-16 cells with cycloheximide or actinomycin D in the absence of EGF. Cells were incubated without EGF, and with cycloheximide (0.1 μg/ml) or actinomycin D (0.01 μg/ml). Positive control was incubated in the standard medium with EGF. Cell viability was determined 12 hours after the incubation by trypan blue dye-exclusion method. The values shown are means and standard deviations for three experiments.

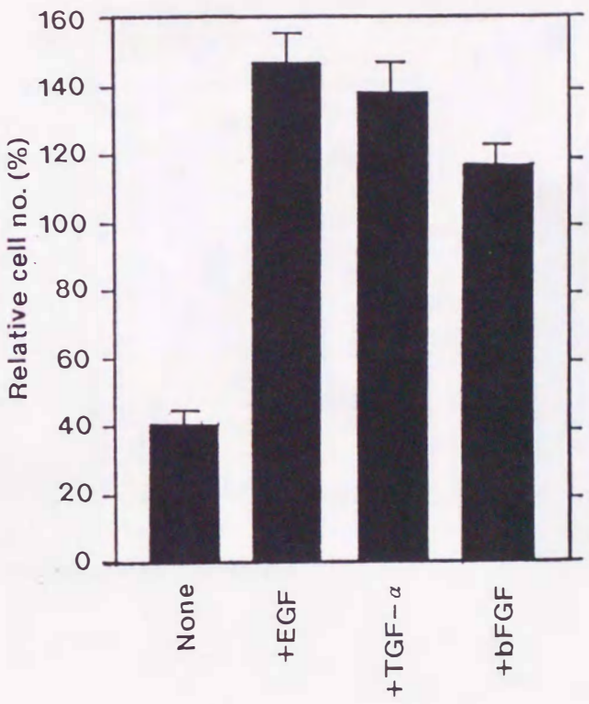

Fig. 20 Prevention of EGF deprivation-induced death of AP-16 cells with TGF-α or bFGF. Cells were incubated without EGF and with EGF (10 ng/ml), TGF-α (10 ng/ml) or bFGF (10 ng/ml). The number of viable cells was determined after 24 hours of incubation by the trypan blue dye-exclusion method. The means of triplicate wells in each experiment were calculated and expressed as a percent of the mean value of the number of viable cells at 0 hour. The values shown are means and standard deviations for three experiments.

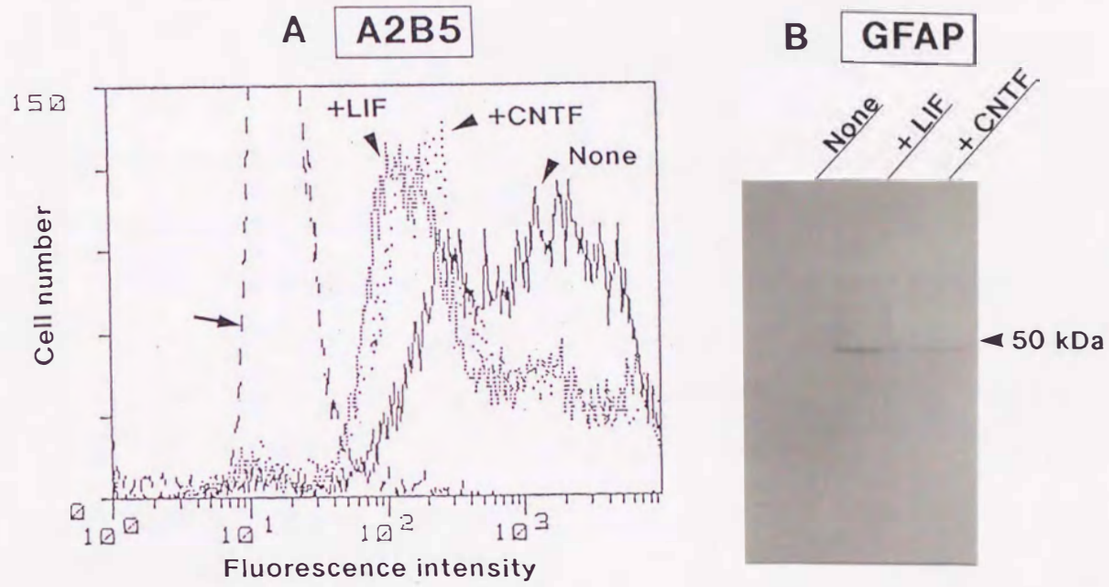

Fig. 21. Effects of LIF and CNTF on the antigenic features of AP-16 cells. AP-16 cells were incubated in the presence of LIF (10 ng/ml) or CNTF (10 ng/ml). After 2 days, the cells were subjected to the following analyses. A) Flow cytometric analysis of A2B5 expression by AP-16 cells. The broken line (arrow) shows background staining with second antibodies alone. B) Western blot analysis of GFAP. After electrophoresis (50 μg protein/lane) under reducing conditions and transfer onto nitrocellulose membranes, blots were incubated with anti-GFAP and horseradish peroxidase-conjugated goat anti-rabbit IgG. Molecular weight (50 kDa) is indicated on the right-hand side.

7. DISCUSSION

Serum-free cultures are efficient tools for characterization of glial cells

The serum-free culture technique provides new approaches in nearly all fields of cell biology. In this study we examined the usefulness of this culture technique in analyzing the properties of glial cells. Since the blood-brain barrier prevents entry of serum proteins into the brain, the properties of glial cells in a serum-free medium are more likely to resemble the properties of their in vivo counterparts than those in medium containing serum (Quarless and Lam, 1989). Using G5 medium, we were able to obtain three types of glial cultures: the primary culture of astrocytes, the mixed glial culture and the cell line of astrocyte progenitors.

These serum-free culture systems provided the following advantages. First, primary culture in G5 medium caused the enrichment of astrocytes. Second, further culturing in G5 medium generated the mixed glial cultures, which can be used for analysis of the properties of each glial subtype. Since serum itself can induce O-2A progenitors to differentiate into type-2 astrocytes, its presence complicates the analysis of the properties of O-2A progenitors. Third, long-term culture in G5 medium allowed culturing of cell types whose growth is inhibited by serum. Such cell types, like AP-16

cells, could not be cultured in serum-supplemented medium on a long-term basis.

From what cell type in vivo does cell line AP-16 originate?

As shown schematically in Figure 22, two glial cell lineages have been identified in the developing rat optic nerve (for review, Raff, 1989). AP-16 cells share some characteristics with O-2A progenitors: a process-bearing morphology and strong A2B5 immunoreactivity. However, unlike O-2A progenitors, AP-16 cells do not differentiate into oligodendrocytes. When grown under conditions that result in the differentiation of O-2A progenitors into oligodendrocytes, AP-16 cells retain a progenitor-like phenotype. AP-16 cells also share some similarities with type-1 astrocyte progenitors. Both cells appear to give rise only to astrocytes and proliferate in response to EGF. These two astrocyte progenitors, however, differ in important ways: type-1 astrocyte progenitors lack A2B5 immunoreactivity and a process-bearing morphology, while AP-16 cells are $A2B5^{+}$ and process-bearing.

Due to these complicated characteristics of AP-16 cells, the relationship between AP-16 cells and O-2A progenitors or type-1 astrocyte progenitors is unclear. One possibility is that AP-16 cells represent O-2A progenitors at a stage of development after commitment to the type-2 astrocyte differentiation pathway but before GFAP expres-

sion. The differences in the responses of O-2A progenitors and AP-16 cells to mitogenic signals (i.e., PDGF, EGF), however, make this unlikely. Furthermore, the differentiated AP-16 cells lack both A2B5 immunoreativity and a process-bearing morphology. AP-16 cells may represent type-1 astrocyte progenitors at an early stage of development, since type-1 astrocytes are thought to be derived from radial glial cells, and $A2B5^+$ radial glial cells have been found in the spinal cord (Frederiksen and McKay, 1988). Another possibility is that AP-16 cells are derived from progenitors that are distinct from O-2A progenitors and type-1 astrocyte progenitors. There are possibly more complicated glial cell lineages in the cerebrum rather than the simple two lineage scheme reported for the optic nerve. To identify in vivo counterparts of AP-16 cells and to clarify their lineage, cell markers specific for AP-16 cells and their counterparts must be developed. In future studies it will be important to determine whether the counterparts of AP-16 cells are present also in the adult brain. The answer to this question will aid in our understanding of the physiology of glial cells.

Apoptosis caused by cytokine deprivation in astrocyte progenitors

We showed that the death of AP-16 cells on withdrawal of EGF is due to apoptosis, indicating that EGF promotes

cell survival by suppression of the process of apoptosis. In the central nervous system, apoptosis caused by trophic factor deprivation seems to be the major developmental strategy for controlling the number of neurons that are innervating a specific target (for review, Oppenheim, 1991). Compared with the intensive search for neurotrophic factors, little effort has been made to define factors that might be required for glial cell survival. To our knowledge, there are no reports describing apoptosis in astrocytes and their progenitors. Our in vitro results suggest that EGF, an EGF-like molecule (TGF-α) or bFGF may be signaling molecules, which promote the cell survival and proliferation of astrocyte progenitors, resulting in an increase in the number of progenitors and the production of astrocytes. Apoptosis is probably involved in restricting the sizes of astrocyte progenitor pools in the absence of relevant growth-promoting stimuli.

Possible roles of TGF-βs in the central nervous system

It has been shown recently that the central nervous system possesses the molecular and cellular machinery to synthesize, release and respond to a wide variety of cytokines (for review, Hefti et al., 1993). These cytokines regulate the differentiation and functions of both neurons and glial cells either directly or via production of trophic factors or adhesion molecules by glial cells, and form an

unique cytokine network in the central nervous system. However, to date, cytokines that regulate the differentiation of astrocyte progenitors are unknown.

The widespread occurrence of isoforms of TGF-βs (TGF-β1-β3) in a variety of cells and tissues and the implication that they may regulate many processes, including embryonic development and maintenance of adult tissues (for review, Sporn et al., 1987), suggest that this multifunctional molecule could also be involved in the development and function of the central nervous system.

In this study, we found that TGF-β1 and TGF-β2 induce the expression of GFAP and fibronectin in AP-16 cells. The number of $GFAP^+$ astrocytes increases in the developing brain and adult brain after injury (for review, Vernadakis, 1986). These stages correspond with the period of the induction of fibronectin expression in the brain (Stewart and Pearlman, 1987; Egan and Vijayan, 1991). Interestingly, TGF-βs are detected in the brain at stages that coincide with the appearance GFAP and fibronectin (Flanders et al., 1991; Lindholm et al., 1992). TGF-βs may act as a regulatory factor of the expression of GFAP and fibronectin in vivo.

Recently, there have been some reports on the roles of TGF-βs in the central nervous system. TGF-βs induce astrocytes to express nerve growth factor (Lindholm et al., 1992), neural adhesion molecule L1 (Saad et al., 1991) or MHC class-II antigen (Johns et al., 1992). These results

suggest that TGF-βs play crucial roles in regulation of both development and repair processes of the central nervous system.

CNTF and LIF act on neurons and glial cells.

The cell line AP-16 has enabled us to investigate and experimantally manupulate in vitro the differentiation of astrocyte progenitors. Using this cell line, we found that CNTF and LIF can act as differentiation factors for astrocyte progenitors. CNTF was originally isolated as a survival factor of ciliary ganglion neurons (Barbin et al., 1984), and it is now also known to promote survival and differentiation of various neurons (for review, Manthorope et al., 1993). While some of the actions of LIF on neurons resemble those of CNTF, LIF also has broad actions outside of the nervous system, such as the regulation of hemopoietic cell maturation (Yamamori et al., 1989) and bone metabolism (Gough and Williams, 1989). CNTF and LIF are indistinguishable in their ability to induce the differentiation of AP-16 cells. Because of the structural similarities between CNTF and LIF, and between their receptors (Hall and Rao, 1992), the potential for functional crossover between these molecules is high.

Variant forms of fibronectin and laminin are produced by glial cells

We analyzed the molecular profiles of fibronectin and laminin synthesized by glial cells. Western blot analysis showed that glial fibronectin has a slightly higher molecular weight than mouse plasma fibronectin. In the case of laminin, glial cells produce the 220 kDa B chain(s) but not the 400 kDa A chain. This confirms the previous finding that glial cells synthesize only the 220 kDa B chain(s) in culture (Liesi and Risteli, 1989). Recently, Ehrig et al. (1990) identified a novel laminin-like protein which they called merosin. Merosin possesses laminin B1 and B2 chains identical to those of EHS tumor laminin, but the third chain (M chain) has a molecular weight of 300 kDa. Glial cells may produce merosin instead of EHS tumor laminin. Merosin has been shown to be effective as a neurite-promoting substrate for embryonic neurons in vitro (Cohen and Johnson, 1991).

There is currently great interest in identifying the sites on fibronectin and laminin that mediate neurite extension to obtain specific probes for evaluation of the functional role of these molecules in neuronal development (Humphries et al., 1988; Sephel et al., 1989). Plasma fibronectin and EHS laminin have been used for these analyses, since fibronectin and laminin have not been purified from brain cells. AP-16 cells cultured in serum-free medium can be used for a cell source to obtain these molecules in a pure form.

Future perspective

Further studies on glial differentiation require a better understanding of the progenitor cells of the central nervous system. Our ability to analyze the characteristics of glial progenitors would be greatly enhanced by the availability of cell lines of different progenitor populations. The availability of large homogeneous populations of such cells would facilitate the identification of marker antibodies for different progenitors. Such cell lines can also be used as genetic assay systems for functions of cloned genes and as assays for external factors involved in glial differentiation.

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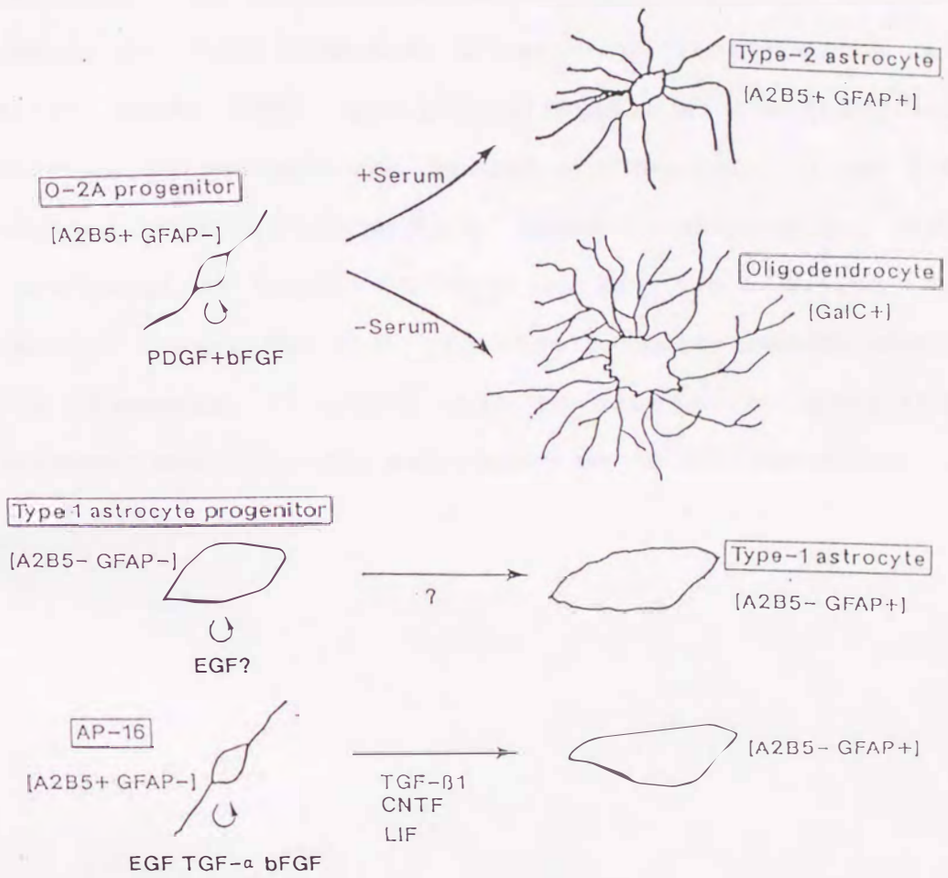

Fig. 22. Schematic diagram of the two glial cell lineages and AP-16 cells. Explanation is provided in the text.

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(Ref. 1): Yoshida T., Takeuchi M. (1991): Primary culture and cryopreservation of mouse astrocytes under serum-free conditions. Cytotechnology 5:99-106.

(Ref. 2): Yoshida T., Takeuchi M. (1991): Expression of fibronectin and laminin by different types of mouse glial cells cultured in a serum-free medium. Cytotechnology 7:187-196.

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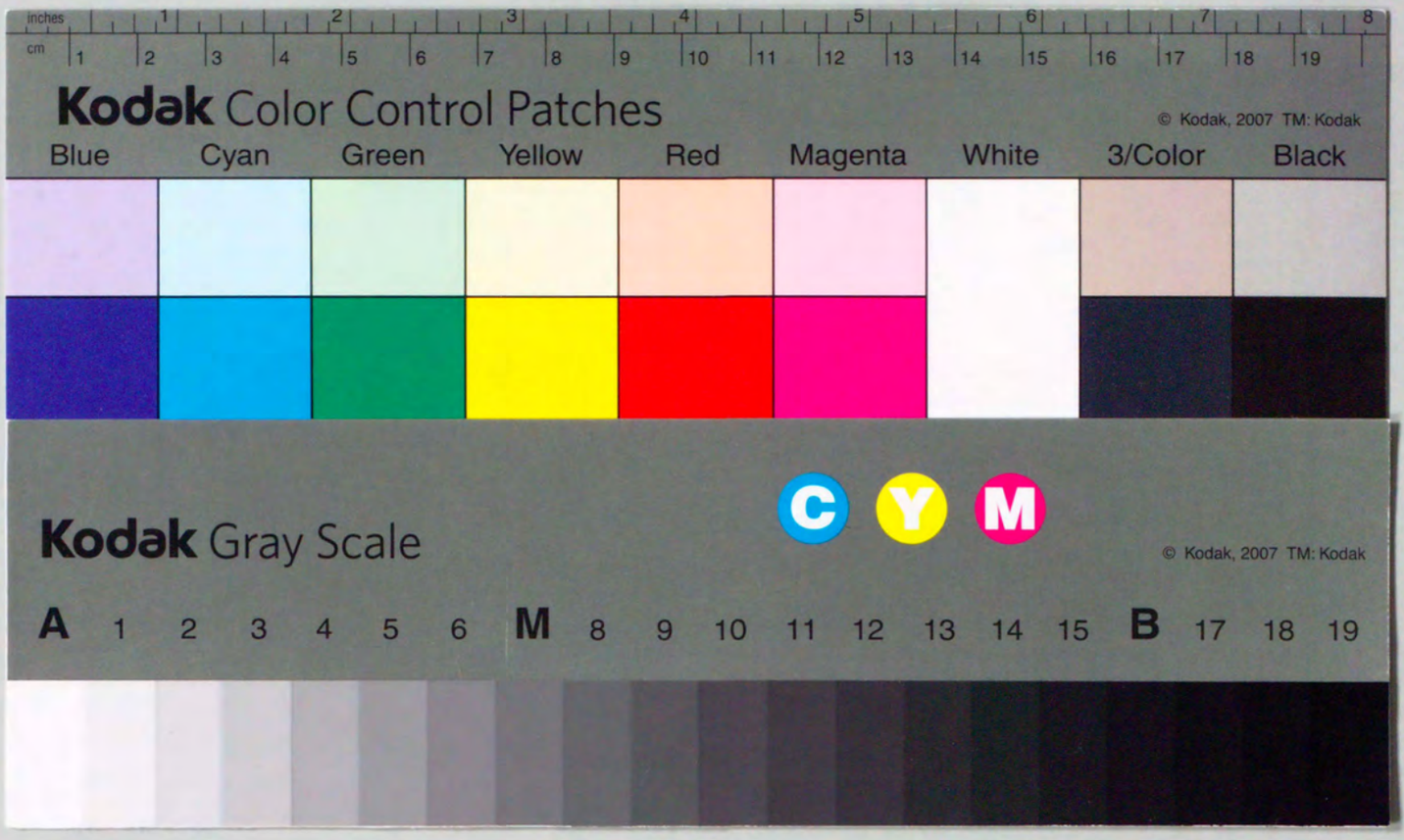
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