Influence of Extracellular Matrix on the Proliferation and Differentiation of Glioma Cells in Culture

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Abstract

The influence of extracellular matrix on the proliferation and differentiation of glioma cells *in vitro* was investigated by culturing glioma cells in collagen gel and on collagen and laminin films. Rat C-6 glioma cells extended thin cytoplasmic processes, proliferated, and differentiated in collagen gel and on collagen film. The cells were stellate with multiple thin processes like the astrocyte *in vivo*. The intracellular content of cyclic adenosine-monophosphate in rat C-6 glioma cells on collagen film increased approximately four fold over the control level. In laminin film culture, rat C-6 glioma cells extended thin cytoplasmic processes with many knotty structures and proliferated. These findings confirm that extracellular matrix induces the differentiation of glioma cells.

Key words: glioma, extracellular matrix, collagen, laminin, cyclic adenosine-monophosphate

Introduction

Extracellular matrix is the basic structural component of tissue. It surrounds the cells *in vivo* and influences cellular proliferation and differentiation. Recent studies indicate that extracellular matrix components, especially collagen, participate in cellular proliferation and differentiation.^{3,8,20} Myoblasts differentiate into muscle fibers in collagen film culture.⁵ Mammary epithelial cells produced milk proteins in collagen gel culture in the presence of related hormones.^{4,19} We found that mature fat cells in collagen gel culture proliferate and maintain cell functions.¹⁵ The extracellular matrix in the central nervous system (CNS) is closely associated with important cellular functions, such as proliferation, differentiation, and migration of neural cells.^{2,18} Rutka *et al.*¹² showed that the collagen component in extracellular matrix significantly influenced the proliferation and differentiation of glial tumor cells *in vitro*.

Astrocytes in brain tissue extend multiple thin cytoplasmic processes to form complex networks around the capillary basement membrane, which is mainly collagen and laminin. The blood-brain barrier is a combination of capillaries and astrocyte foot processes. Glioma cells around blood vessels

demonstrate intense immunohistochemical staining for glial fibrillary acidic protein. This suggests that the extracellular matrix around local blood vessels causes glioma cells to differentiate.¹¹ However, it is not clear how the CNS extracellular matrix influences the proliferation and differentiation of glial or glioma cells. Here, we investigated the proliferation and differentiation of cultured glioma cells in an extracellular matrix.

Materials and Methods

I. Cell cultures

Rat C-6¹¹ and human KC-P14¹⁷⁾ glioma clonal cell lines were maintained in monolayer cultures on 65 mm plastic dishes under standard tissue culture conditions (37°C, under 5% CO₂, 95% air) on Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum and gentamycin. The rat C-6 glioma cells maintained S-100 protein immunoactivity over serial passages. Human KC-P14 glioma cells were derived from a 40-year-old female with a glioblastoma (Prof. A. Nishimoto, Okayama University Medical School, Okayama).

Monolayer culture: The monolayer cell cultures had a concentration of 5×10^3 cells/cm² in a 35 mm plastic dish.

Collagen, laminin, and fibronectin film cultures: Collagen solution (types I, III, and IV; Nitta Gelatin Co., Ltd., Yao, Osaka) was diluted 1:10 with dis-

Fig. 1 A: Isolated glioma cells were plated on extracellular matrix (*upper*). Some rat C-6 glioma cells extended thin cell processes and proliferated 12 hours after plating on collagen or laminin film (*lower*). B: Isolated glioma cells were embedded in collagen gel (*upper*). The glioma cells extended thin processes multidirectionally and proliferated 12 hours after embedding (*lower*).

tilled water. Laminin (Nitta Gelatin Co., Ltd.) and fibronectin (Organon Teknika Corp.-Cappl Products, West Chester, U.S.A.) were diluted 1:10 with phosphate-buffered saline containing no calcium or magnesium [PBS(-)]. The surfaces of culture dishes were coated with these solutions. After drying at 4°C, the dishes were washed five times with PBS(-). Cell suspensions were cultured in these dishes (Fig. 1A).

Collagen gel culture: We used the Elsdale and Bard procedure³⁾ as modified by Enami *et al.*⁴⁾ Briefly, eight volumes of acid-soluble type I collagen solution were mixed with one volume of 10× concentrated Eagle's MEM and one volume of reconstruction buffer (2.2 gm NaHCO₃, 4.77 gm N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 100 ml 0.05 N NaOH). This mixture cooled in ice was mixed with separated glioma cells. Cell mixture (2 ml) containing 5×10^4 cells/ml was placed in a 35 mm culture dish and immediately warmed to 37°C to form a gel. After 30 minutes, when the gel had sufficient strength, a coating of medium 2 ml/dish was formed (Fig. 1B).

Soft agar gel culture: Soft agar (Agar noble; DIFCO Laboratories, Detroit, Michigan, U.S.A.) was dissolved in distilled water to form a 1% solution. This was autoclaved for 15 minutes, then kept at 60°C. One volume of this solution was mixed with one volume of 2.25× concentrated Eagle's MEM supplemented with 20% fetal calf serum. Two volumes of this mixture were mixed with one volume of the prepared cell suspension (5×10^4 cells/ml) at 37°C.

II. Examination of cell cultures

Immunohistochemistry for S-100 protein: Rat C-6 glioma cells cultured on collagen film were fixed with methanol and ethanol (1:1) at 4°C for 10 minutes, reacted with a 1:200 dilution of mouse anti-S-100 protein (Cosmo Bio., Tokyo) primary antibody for 30 minutes, then rinsed with PBS(-). The resultant cells were used for immunohistochemical testing by the avidin-biotin-peroxidase complex method, and staining with diaminobenzidine (Sigma, St. Louis, Mo., U.S.A.).

Immunohistochemistry for tubulin: Cells cultured on collagen film were fixed with 2% paraformaldehyde at 4°C for 1 minute and acetone (-20°C) for 15 minutes. A 1:500 dilution of mouse anti-tubulin primary antibody (Amersham, Buckinghamshire, England) was applied for 1 hour, followed by staining with fluorescein isothiocyanate conjugated rabbit anti-mouse immunoglobulin G (IgG).

Bromodeoxyuridine (BrdU) labeling: BrdU was added to the cell culture for 2 hours. The cells were then fixed with acidic ethanol for 15 minutes, and reacted with anti-BrdU antibody (Amersham) for 1 hour, then rinsed with PBS(-). The culture was then reacted with anti-mouse IgG by the indirect immunohistochemical method, and stained with diaminobenzidine. Hematoxylin and eosin stains were performed last.

III. Cyclic adenosine-monophosphate (cAMP) assay in cells

The cAMP content in cells was assayed in the normal monolayer and collagen film cultures. Duplicate control and collagen-coated 35 mm dishes were inoculated with 1×10^6 glioma cells. After 24-hour culture, the cAMP content in the cells were measured by the radioimmunoassay method.⁶⁾

IV. Effect of N-2'-dibutyryl adenosine-3'-5'-cyclic monophosphate Na-salt (db-cAMP)

Normal monolayer and collagen film cell cultures were plated on dishes. Six hours later, db-cAMP (Seikagaku-kogyo, Tokyo) was added at final concentrations of 0.1–1 mM to the mediums.

Results

I. Cell cultures

Usual monolayer culture: Monolayer rat C-6 glioma cells on a plastic dish proliferated extensively and became confluent within 4–5 days. The cells were short spindle-shaped and bipolar with short processes (Fig. 2A). Human KC-P14 glioma cells proliferated and became confluent within 3 days. The

Fig. 2 A: Rat C-6 glioma cells on usual plastic dish proliferated extensively in a monolayer. Cells are short spindle-shaped and bipolar with short processes. $\times 200$. B: Human KC-P14 glioma cells on usual plastic dish proliferated in a monolayer. Cells are rectangular without obvious processes. $\times 200$.

cells were rectangular without obvious processes (Fig. 2B).

Collagen, laminin, and fibronectin film cultures:

In type I collagen film culture, 20–30% of rat C-6 glioma cells extended thin cell processes 12 hours after plating. The cells were stellate and extended multiple thin processes like the astrocyte *in vivo* (Fig. 3A). The processes stained positively for S-100 protein by the immunohistochemical technique (Fig. 3B). Tubulin immunostaining revealed an intracytoplasmic network (Fig. 3C). The cells were apparently morphologically differentiated, but cell divisions were also observed. Rat C-6 glioma cells were positive to BrdU labeling in the presence and absence of differentiation, with labeling indices of 2.5 and 3.1%, respectively. There was no significant difference. In type III or IV collagen film culture, similar morphological changes were observed, but to lesser extents (Table 1). Human KC-P14 glioma cells extended thin processes multidirectionally, but had shorter processes than rat C-6 glioma cells. The cells proliferated.

Fig. 3 A–C: Rat C-6 glioma cells in collagen film culture. Cells extend thin cell processes and are stellate in form like the astrocyte *in vivo* (A). Cell processes are positively immunostained for S-100 protein (B). A network of intracytoplasmic filaments of microtubules is seen (C). $\times 300$. D: Rat C-6 glioma cells in laminin film culture. Cells extend long processes, which are bipolar with many knotty structures. $\times 430$.

Table 1 Effect of different environments on the differentiation and proliferation of rat C-6 glioma cells

	Usual monolayer culture	Collagen film culture			Collagen gel culture	Laminin film culture	Fibronectin film culture	Addition of db-cAMP
		Type I	III	IV				
Thin process	0	++	+	+	++	+	0	+++
Proliferation	0	0	+	+	0	0	0	-

-: inhibition, 0: no effect, +: mild effect, ++: moderate effect, +++: severe effect.

Fig. 4 A-C: Rat C-6 glioma cells in collagen gel culture. Background, collagen gel. Cells extend thin processes multidirectionally (A). Some processes are branched (B). Cell divisions and proliferations are observed (C). A, $\times 200$; B and C, $\times 300$. D: Human KC-P14 glioma cells in collagen gel culture. Cells extend similar cell processes and proliferate. $\times 300$.

In laminin film culture, 30–40% of rat C-6 glioma cells extended long processes. The processes were bipolar and had many knotty structures (Fig. 3D). No inhibition of proliferation was observed. Human KC-P14 glioma cells underwent no morphological change and proliferation was not inhibited.

In fibronectin film culture, neither rat C-6 nor human KC-P14 glioma cells showed morphological changes and neither was inhibited from proliferation.

Collagen gel culture: In the collagen gel culture, more than 90% of rat C-6 glioma cells extended thin processes 12 hours after embedding. The processes were five to ten times longer than the cell bodies. Three to four processes were extended multidirec-

tionally from each cell body (Fig. 4A) and grew branches (Fig. 4B). Morphologically, they were as differentiated as those of astrocytes. However, there was distinct evidence of cell divisions and no inhibited proliferation (Figs. 4C and 5). Human KC-P14 glioma cells extended similar cell processes, but these were shorter (Fig. 4D). The human glioma cells also proliferated.

Soft agar gel culture: In soft agar gel culture, rat C-6 glioma cells were round, proliferated, and formed colonies, but did not extend cell processes.

II. cAMP assay in cells

The content of cAMP in rat C-6 glioma cells in collagen film culture was approximately four times that

Fig. 5 Growth curves for rat C-6 glioma cells. Cells in normal monolayer (○) and collagen film cultures (△) proliferate similarly to in collagen gel culture (■).

in control cells in the normal monolayer culture 24 hours after plating.

III. Effect of db-cAMP

In the normal monolayer and collagen film cultures, more than 30% of rat C-6 glioma cells extended extremely slender processes multidirectionally 12 hours after addition of 1 mM db-cAMP. The processes were longer than in the collagen film culture. Moreover, they were richly long-branched. There was no obvious morphological difference between the glioma cells treated with db-cAMP in the normal monolayer and the collagen film cultures. In the normal monolayer culture, proliferation was inhibited by db-cAMP. The morphological changes and the inhibition of proliferation were reversible by removal of db-cAMP from the medium. No morphological changes were caused by 0.1 mM db-cAMP. Human KC-P14 glioma cells extended similar processes and proliferation was inhibited by 1 mM db-cAMP.

Discussion

In the CNS, extracellular matrix is present as the basement membrane between the astrocyte foot processes and the capillary endothelial cells.^{2,14} The collagen component of extracellular matrix inhibits the proliferation and induces the differentiation of

glioma cells.¹² Laminin formation by immature rat astrocytes in primary culture suggests that laminin is important in CNS development.⁹ However, the precise function of the extracellular matrix in the CNS is not yet fully understood.

We found that collagen induced rat C-6 and human KC-P14 glioma cells to develop thin cytoplasmic processes multidirectionally. There was no morphological difference of glioma cells in either collagen film or gel cultures except for the development of cytoplasmic processes. These cells were morphologically differentiated. In a soft agar gel culture, rat glioma cells showed no morphological change. These findings confirm that collagen induces morphological differentiation in glioma cells. The network of thin intracytoplasmic filaments positively stained for S-100 protein by immunohistochemical techniques.

The cAMP content of rat C-6 glioma cells in collagen film culture increased approximately four fold over the control and was associated with morphological changes. It is well known that cAMP is closely associated with cellular differentiation and functions. Previous work suggests that the intracellular cAMP content of undifferentiated glial cells is lower than that of differentiated cells.¹⁰ Our result confirms this.

In this study, db-cAMP induced both rat C-6 and human KC-P14 glioma cells to differentiate morphologically and also inhibited proliferation. Cultured glioma cells differentiate morphologically in the presence of cAMP, and proliferation is inhibited.¹⁶ However, both proliferation and morphological differentiation in human medulloblastoma cell lines are dependent on the db-cAMP concentration.¹³ We found that in collagen matrix and film cultures, glioma cells proliferated and morphologically differentiated. We cannot definitively explain this phenomenon. However, the stimulation mechanism of collagen is different from that of db-cAMP, so in collagen film or gel glioma may both proliferate and differentiate.

We found that rat C-6 glioma showed morphological differentiation but proliferation was not inhibited in type I, III, or IV collagen film culture. In contrast, Rutka *et al.*¹² reported that on type I or IV collagen-coated flasks, glioma cells showed similar morphological changes but were inhibited from proliferation. We also found that glioma cells developed many knotty structures on cytoplasmic processes in laminin film culture, a previously unreported result.

It is not known how collagen induces morphological differentiation in glioma cells. Recently, receptors specific to the extracellular matrix in

various normal or tumor cells have been proposed.⁷⁾ Our result supports this hypothesis of a specific collagen receptor on the glioma cell membrane. We infer that increased intracellular cAMP caused by stimulation of collagen receptors induces cellular differentiation.

Astrocytes form the blood-brain barrier *in vivo* with multiple thin cytoplasmic processes extending to the capillary basement membrane. In contrast, glioma cells *in vivo* have only two or three short processes. These processes do not adhere to the basement membrane, so glioma cells cannot form the blood-brain barrier. We found that glioma cells extended multiple thin processes and demonstrated branching in the presence of collagen. These structures resemble the multiple thin cytoplasmic processes of astrocytes *in vivo*. We suggest that a co-culture of glioma cells and endothelial cells will help elucidate the development of the blood-brain barrier.

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