

Malignant Transformation of Cultured Low Grade Glioma Cells: An Immunohistochemical and Ultrastructural Study

Xue-Yun Zhong,^{*} Masachika Senba,^{*} Rui Situ^{**} and Yun-Xian Chen^{***}

^{*}Department of Pathology, Institute of Tropical Medicine, Nagasaki University, Nagasaki 852, Japan

^{**}Department of Pathology, Jinan University School of Medicine, Guang-zhou, China

^{***}Department of Internal Medicine, Atomic Disease Institute, Nagasaki University School of Medicine, Nagasaki 852, Japan

Using cultured glioma cell line, we demonstrated *in vitro* and *in vivo* that when low grade glioma was pass through glioma cell culture at 19th passage, these tumor cells were malignantly transformed. This cell line was established from tissue culture of neurosurgical specimen, pathologically diagnosed as glioma (low grade glioma; grade I-III) with glial fibrillary acidic protein (GFAP) positive but vimentin negative. When glioma cell cultured at 19th passage was injected into xenograft nude mice, histopathological and immunohistochemical analysis demonstrated that low grade glioma cells were transformed to high grade glioma (grade IV). High grade glioma cell can produce vimentin, laminin and fibronectin, but can not produce GFAP. It is suggested that vimentin and GFAP are useful and important marker in differentiating low grade glioma from high grade glioma. The mechanism of malignant transformation of low grade glioma cells is not clear. The possible explanation may be that the low grade glioma gene include malignant potential originally, as a result, low grade glioma cells change gradually to high grade glioma.

Introduction

In humans, gliomas account for 60% of primary intracranial neoplasms. Gliomas arise most commonly from malignant transformation of astrocytes and are classified according to histopathological criteria as astrocytoma (low grade glioma) or glioblastoma (high grade glioma). To investigate this question, neurosurgical pathology has shown that primary brain tumors were characterized by local invasive growth, recurrence, malignant transformation, and unusual metastasis.^{5,11,14} The mechanism to clarify the factors causing malignant transformation in low grade glioma is unknown. Recent studies indicate that invasion of the host tissues is likely enhanced by the strong interaction of malignant cells with extracellular matrix.¹⁻⁴ Extracellular matrix is a large multifunctional molecule that affects the behavior of many cells including the attachment

of cells to a substrate and junction of morphological changes and cell differentiation,⁶ and neurite outgrowth.²⁰ Vimentin is expressed in poorly differentiated astrocytoma, but not in well differentiated astrocytoma. On the other hand, glial fibrillary acidic protein (GFAP) is demonstrated in well differentiated astrocytoma, but not in poorly differentiated astrocytoma.²⁰ Vimentin and GFAP are considered to be important markers of differentiation of astrocytoma.^{11,12,15}

The authors reported here a new finding that demonstrated *in vitro* and *in vivo*, low grade glioma when pass through glioma cells culture at 19th passage and injected into xenograft nude mice, histopathological and immunohistochemical analysis showed that the tumor cells were malignantly transformed.

Materials and Methods

1. Cell cultures:

The human SWO-38 glioma cell line was provided by Dr. Rui (Department of Pathology, Jinan University, Guangzhou, China). This cell line was established from tissue culture of neurosurgical specimens. The patient was a 12-year old Chinese boy. This tumor grew on the cerebellar medium. The tumor was diagnosed as glioma (grade I-III) with glial fibrillary acidic protein (GFAP) positive by immunohistochemistry. The cell line was maintained in PRMI 1640 medium with 10% FBS (fetal calf serum), penicillin (100IU/ml), and streptomycin (100 μ g/ml). They were kept in a standard tissue culture in the incubator with 5% CO₂. After cultivation the cell line was cloned at limiting dilution.

2. Morphology and growth characteristics of the cell line:

A monolayer cultured cell with rapid growth was obtained on the 4th passage. The cell morphology and growth characteristics were examined ultrastructurally, and immu-

All correspondence to: Dr. Masachika Senba
Department of Pathology, Institute of Tropical Medicine,
Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852,
Japan

nohistochemically.

3. Nude mouse inoculation:

The tumorigenesis of culture cells was determined by subcutaneous injection into a 4-week-old male nude mice (BALB/c-nu/nu). The mice were inoculated with 2×10^5 culture cells (19th passage) suspended in 0.5ml of PBS. When subcutaneous tumor achieved the greatest diameter of 1.5cm or more, the tumor was investigated by light microscopy and immunohistochemical stainings.

4. Monolayer cultures:

Monolayer cultures of glioma cell line were propagated on glass coverslips in 10cm petri tissue culture dishes (Nunc, Roskilde, Denmark), using the same culture medium as described above. After 7 days, glioma cells were cultured on glass coverslips and fixed in absolute alcohol for 1 hour at 4 °C and then washed three times in PBS with immunocytochemical staining by the peroxidase-antiperoxidase technique. Antibodies to human laminin, fibronectin, vimentin, GFAP and keratin were used in this study.

5. Histopathological and immunohistochemical analysis:

The specimens were fixed in 10% formalin, and embedded in paraffin for histopathological and immunohistochemical studies, sections were cut at 4 micron and stained with hematoxylin and eosin (H & E) stain for histological examination. These specimens were further stained by the avidin-biotin-peroxidase complex (ABC) methods for GFAP, vimentin, laminin, fibronectin and keratin. The antibodies were used DAKO, Copenhagen, Denmark. We used primary rabbit antibodies (1:40), goat anti-rabbit immunoglobulin G (1:100, Cappel Laboratories, Cochranville, USA) and rabbit peroxidase-antiperoxidase (1:100, Cappel Laboratories) After being washed with phosphate buffered saline pH 7.2, the sections were stained as described here. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol. The reaction product was developed with 3, 3-diaminobenzidine, and the sections were counterstained with hematoxylin.

6. Ultrastructural examination:

For transmission electron microscopy, the glioma cultured cells were fixed with 2.5% glutaraldehyde solution for 1.5 hour, subsequently in 2% osmic acid for 2 hour, and dehydrated and embedded in epoxy-resin. Ultrathin sections were cut with ultramicrotome, and observed in an electron microscope (Hitachi H-600, Japan), after additional staining with uranyl acetate and lead citrate.

For scanning electron microscopic observation, fixed and dehydrated cell layers were treated with amyloacetate,

and dried in a critical-point drier (Hitachi HCP-2, Japan) The specimens were then observed using a scanning electron microscope (Hitachi S-800, Japan).

Results

1. Original tissue (Glioma; grade I-II) for culture:

The microscopic sections showed that the tumor cells were well-differentiated astrocytoma which were diffusely reacted with GFAP (Fig. 1), but were not reacted with vimentin, fibronectin, laminin, and keratin. A monolayer permanent cell line with rapid growth was obtained, and it was named SWO-38. Epithelial-like cell processes ap-

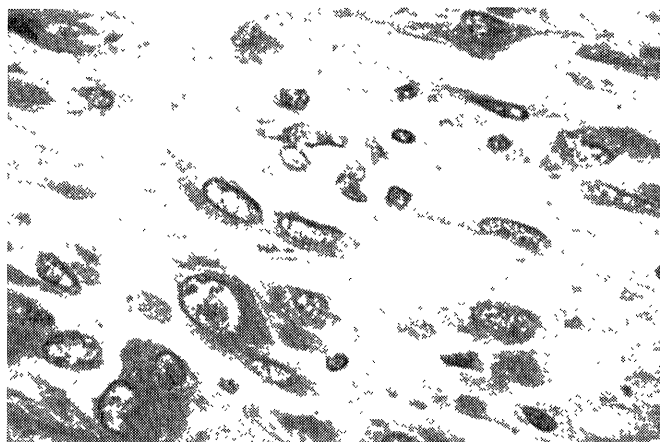


Fig. 1. Immunohistochemical staining with GFAP expression of the low grade glioma. (human original tissue, immunoreaction for GFAP x 400).

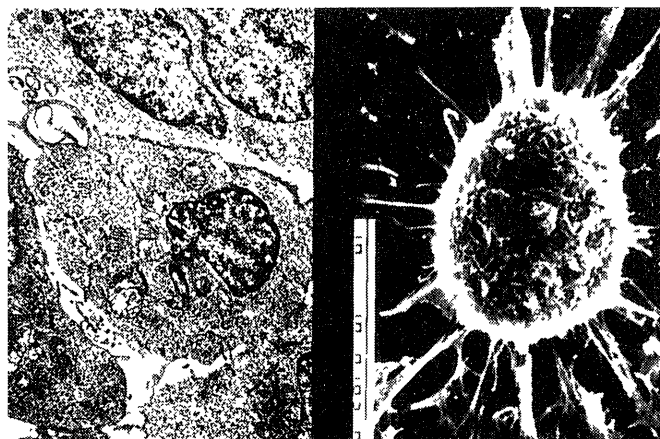


Fig. 2. Electron micrograph of glioma cultured tumor cells (4th passage). a: showing the tumor cells were characterized by relatively round cells with prominent nucleoli and abundant mitochondria. (transmitted electron microscopy x 6,000). b: glioma cell surface include rich microvillus and filopodium (scanning electron microscopy x 2,000).

peared on the 4th passage in culture cells. Ultrastructurally, the cells were characterized by round cells with prominent nucleoli and with abundant mitochondria (Fig. 2). The cell culture at the 19th passage was used as culture cell xenograft to nude mice. The nude mice were inoculated subcutaneously with 2×10^5 cells, and a visible tumor developed at the site of inoculation after 1 month. Histopathological analysis demonstrated that tumor cells were malignant, showing high density, cellular polymorphism, mitosis, and necrosis. The tumor cells were not reacted with GFAP and keratin, however, these were reacted with vimentin (Fig. 3), fibronectin, and laminin by immunohistochemical procedure. Specifically, laminin (Fig. 4) was demonstrated around the cytoplasm of the tumor cells.

2. Monolayer of glioma cells:

The glioma cell culture passed through 58th passage was propagated on glass coverslips in 10cm petri tissue culture dishes. After 7 days, glioma cells cultured on glass coverslips were investigated for the cell characteristics using immunohistochemistry. The glioma cells were negative with GFAP, but strongly positive with laminin, fibronectin, and vimentin. The laminin and fibronectin were strongly expressed in glioma cell line *in vitro* and *in vivo* (Table 1).

Discussion

The clinical status of primary brain tumors was characterized by local invasive growth, recurrence, malignant transformation, and metastasis uncommonly. Clinical and pathological studies in humans showed that glioma account for 60% of primary intracranial neoplasms. Glioma arises most commonly from malignant transformation of astrocytes.^{5 11 14)} Our present study demonstrated *in vitro* and *in vivo*, low grade glioma after passing through glioma cell culture at 19th passage and injected into xenograft nude mice. Histopathological and immunohistochemical analysis demonstrated that these tumor cells were malignantly transformed. Recent studies indicate that invasion of the host tissues is likely enhanced by the strong interaction of malignant cells with extracellular matrix.^{1-4 10, 19)} Extracellular matrix proteins are known to influence cell proliferation, including cell attachment, growth, cell migration and dedifferentiation.^{7 10, 18)}

Our results showed that both established glioma cell

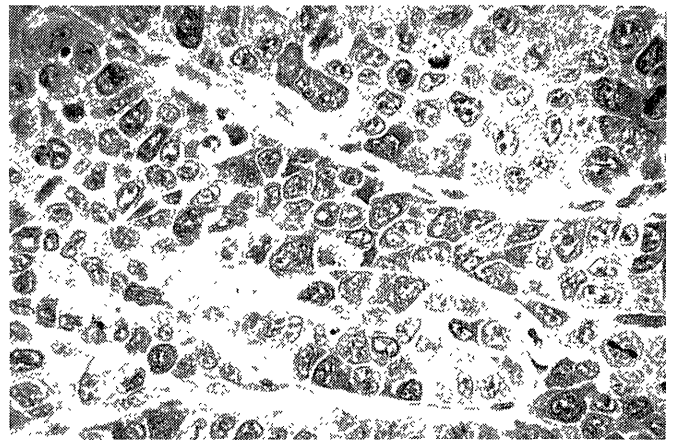


Fig. 3. The glioma cell cultured 19th passage, the culture cell injected xenograft nude mice, the tumor cells showed high density, cellular polymorphism, mitosis, and necrosis with laminin positive. (xenograft immunoreaction for laminin x 200).

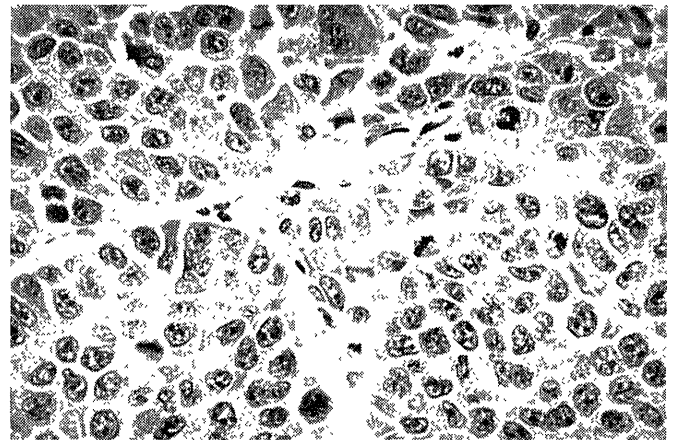


Fig. 4. Vimentin staining demonstrated around the cytoplasm of the glioma cells. (xenograft nude mice, immunoreaction for vimentin x 200).

lines and glioma in primary culture, even in early passage, simultaneously expressed laminin and fibronectin. First, a representative culture glioma cell line can secrete laminin and fibronectin to the culture medium which may help in promotion and dedifferentiation of tumor cells.^{1 3, 4)} Second, surgical specimens showed expression of GFAP in low grade glioma by immunohistochemical examination. The established glioma cell line was found negative for GFAP, whereas, it was positive for fibronectin and laminin. Spe-

Table 1. Immunohistochemical results for low and high grade of glioma

	morphology	GFAP	vimentin	laminin	fibronectin	keratin
Original tumor	Glioma (I-II)	+	-	-	-	-
Cell Line		-	+	+	++	-
Xenograft	Glioma (III-IV)	-	+	++	++	-

GFAP: glial fibrillary acidic protein, +: positive, ++: strongly positive, -: negative.

cifically, laminin was not only found in the basement membrane of the tumor but also was demonstrated clearly around the cytoplasm of the tumor cells. This can be explained by the fact that glioma cells produce laminin. Third, glioma cell culture at the 19th passage after injection into xenograft nude mice, histopathological analysis demonstrated that the tumor cells were malignantly transformed.

Low grade glioma was malignantly transformed. This tumor cells showed high cell density, cellular polymorphism, mitosis, necrosis with palisading cells, and prominent vascularization with endothelial cell proliferation. A recent study pointed out that glioblastoma was distinguished from astrocytoma by the presence of necrosis and vascular proliferations.¹⁴⁾ Clinical and experimental studies suggest that angiogenesis factor is an early event in tumorigenesis and may facilitate tumor progression and metastasis. Using a rabbit brain tumor model, it was found out that inhibition of angiogenesis may be an approach to destroy tumors which are highly vascularized and not treatable by conventional methods.²⁾

The authors propose that vimentin and GFAP are useful as a tumor marker in distinguishing low from high grade glioma. Vimentin and GFAP coexist in glioma cells *in vivo*, similarly to what has been previously observed in cultured glioma cells, but GFAP is expressed in more differentiated and specific cytoskeletal protein in glial cells, and astrocytes stained for vimentin was lower than for GFAP.^{12, 17, 18, 20)} Our present study demonstrated *in vitro* and *in vivo* that when low grade glioma pass through glioma cells culture at 19th passage, they were malignantly transformed. The mechanism of malignant transformation of low grade glioma cells is unknown. It is suggested that low grade glioma gene may contain malignant potential, resulting in dedifferentiation of low to high grade malignancy.

References

- 1) Bjerkvig R, Laerum OD, and Rucklidge GJ: Immunocytochemical characterization of extracellular matrix proteins expressed by cultured glioma cells. *Cancer Res.*, 49:5424-5428, 1989.
- 2) Brem SS, Zagzag D, Tsanaclis AMC, Gately S, Elkouby MP, and Brien SE: Inhibition of angiogenesis and tumor growth in the brain. *Am. J. Pathol.*, 137:1121-1142, 1990.
- 3) Castronovo V, Bracke ME, Mareel MM, Reznik M, and Foidart JM: Absence of laminin deposition in breast cancer and metastases except to the brain. *Pathol. Res. Pract.*, 187:201-208, 1991.
- 4) Hand PH, Thor A, Schlom J, Rao CN, and Liotta: Expression of laminin receptor in normal and carcinomatous human tissues as defined by a monoclonal antibody. *Cancer Res.*, 45:2713-2719, 1985.
- 5) Higuchi M, Ohnishi T, Arita N, Hiraga S, Iwasaki H, Mori S, and Hayakawa T: Immunohistochemical localization of fibronectin, laminin and fibronectin-receptor in human malignant gliomas-in relation to tumor invasion. *Nosinkei*, 43:17-23, 1991.
- 6) Iwasaki K, Kikuchi H, Miyatake S, Kondo S, and Oda Y: Establishment of a new cell line derived from a human gliosarcoma. *Neurosurgery*, 30:228-235, 1992.
- 7) Kleinman HK, Ogle RC, Cannon FB, Little CD, Sweeney TM, and Luckenbill-Edds L: Laminin receptors for neurite formation. *Pro. Natl. Acad. Sci. USA*, 85:1282-1286, 1988.
- 8) Liotta LA: Tumor invasion and metastases-role of the extracellular matrix: Rhoads memorial award lecture. *Cancer Res.*, 46:1-7, 1986.
- 9) McKeever PE, Fligiel SEG, Varani J, Hudson JL, Smith D, Castle RL, and McCoy JP: Products of cells cultured from gliomas. IV. Extracellular matrix proteins of gliomas. *Int. J. Cancer*, 37:867-874, 1986.
- 10) McKeever PE, Fligiel SEG, Varani J, Castle RL, and Hood TW: Products of cells cultured from gliomas VII. Extracellular matrix proteins of gliomas which contain glial fibrillary acidic protein. *Lab. Invest.*, 60:286-295, 1989.
- 11) Ogashiwa M, Asoh Y, Maeda T, Yokoyama H, Hara M, and Takeuchi K: Clinicopathological study on low grade glioma-in relation to malignant transformation. *Neurol. Med. Chir. (Tokyo)*, 30:820-826, 1990.
- 12) Paetau A: Glial fibrillary acidic protein, vimentin and fibronectin in primary cultures of human glioma and fetal brain. *Acta Neuropathol.*, 75:448-455, 1988.
- 13) Panayotou G, End P, Aumailley M, Timpl R, and Engel J: Domains of laminin with growth-factor activity. *Cell*, 56:93-101, 1989.
- 14) Plate KH, Breier G, Weich HA, and Risau W: Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas *in vivo*. *Nature*, 359:845-848, 1992.
- 15) Reifenberger G, Szymas J, and Wechsler W: Differential expression of glial-and neuronal-associated antigens in human tumors of the central and peripheral nervous system. *Acta Neuropathol.*, 74:105-123, 1987.
- 16) Rucklidge GJ, Dean V, Robins SP, Mellao, and Bjerkvig R: Immunolocalization of extracellular matrix proteins during brain tumor invasion in BD IX rats. *Cancer Res.*, 49:5419-5423, 1989.
- 17) Schiffer D, Giordana MT, Mauro A, Migheli A, Germano I, and Giaccone G: Immunohistochemical demonstration of vimentin in human cerebral tumors. *Acta Neuropathol.*, 70:209-219, 1986.
- 18) Tabuchi K: The cell biological characteristics of brain tumors. *Noushinkeigeka*, 16:919-931, 1988.
- 19) Vlodavsky I, and Gospodarowicz D: Respective roles of laminin and fibronectin in adhesion of human carcinoma and sarcoma cells. *Nature*, 289:304-306, 1981.
- 20) Yung WKA, Luna M, and Borit A: Vimentin and glial fibrillary acidic protein in human brain tumors. *J. Neuro-Oncol.*, 3:35-38, 1985.