Gliosarcoma: Its Origin and Morphological Pleomorphism

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Abstract

The histogenesis of gliosarcoma has been concerned in that the sarcomatous component is of vascular origin, or fibroblasts, pluripotent mesenchymal cells of the perivascular adventitia or perivascular spaces, or a suggestive common origin. Recent molecular-biological analysis, however, makes one to consider the possibility of this tumor originating from common precursor cells. To clarify the histogenesis of this tumor, an experimental study using nude mice with transplanted human glioblastoma cells was carried out. The grown tumor demonstrated fibrosarcomatous features, differing from those of originally cultured glioblastoma cells, except for some remaining cells with positive GFAP immunoreactivity and intracytoplasmic intermediate-sized glial filaments on electron microscopy. The presence of \textit{Alu} sequence in the grown tumor proved that the tumors were composed of human originated tissue. In addition, DNA restriction fragment length polymorphism analysis showed the transplanted tumor consisted of human cells. These results indicated that the fibrosarcomatous lesion of glioblastoma exografts in nude mice was derived only from originally cultured human glioblastoma cells, but not from the recipient mouse. It may thus be assumed that human gliosarcoma showing fibrosarcomatous features might have originated from the same totipotential cells.

Key Words

gliosarcoma, glial filament, nude mouse, \textit{Alu} sequence, RFLP,

Introduction

Glioblastoma is a poor prognostic brain tumor, occupying one-third of primary brain tumors. The average life expectancy after diagnosis is about one year. Histopathologically, the tumor shows various characters and features such as heteromorphism, pleomorphism, and sometimes even forming a sarcoma. Gliosarcoma among them is defined as a glioblastoma variant characterized by a biphasic tissue pattern with alternating areas displaying glial and mesenchymal differentiation according to the new World Health Organization (WHO) classification, and corresponding to WHO grade IV. The histogenesis of this tumor has been concerned in that the sarcomatous component is of vascular origin, or fibroblasts, pluripotent mesenchymal cells of the perivascular adventitia or perivascular spaces, or a suggestive common origin. Recent molecular-biological analysis, however, makes one to consider the possibility of this tumor originating from common precursor cells. From this point of view, an experimental study using nude mice transplanted human glioblastoma cells is scheduled to prove the histogenesis of the tumor, and to establish the animal model of human glioblastoma.

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Materials and Methods

Animal model

Male nude mice (Balb/c: nu/nu) at 5 weeks of age were purchased from Japan Clea Laboratory. The mice were fed with autoclaved water and sterilized feed by irradiation (CE-2, Japan Clea Lab. Tokyo, Japan) and kept in box fixed by autoclaved chips of wood in the filter conditioned Isolac (Sanki Kagaku Inc., Kanagawa, Japan). Sixteen mice were xenografted. Additional 6 mice were xenografted for short term effects of transplantation. Mice were sacrificed under ether anesthesia, approved by the institution's guide for the care and use of laboratory animals.

Cell culture of glioblastoma multiform and xenografted glioblastoma

Glioblastoma cell line (U-118 MG) was purchased from American Type Culture Collection. The cells were cultured in MEM (minimum essential medium, Invitrogen Corp., CA, USA) containing 10% fetal calf serum in the CO2 incubator under the condition of 5% concentration. The cells were cultured in 75 cm2 flasks and on slide chambers at the same time. The cells in flasks were collected by rubber scratch and divided for xenograft and for electron microscopic findings. The cultured glioblastoma cells on slide chamber, on the 1th, 3th, 4th, and 7th day were stained immunohistochemically with GFAP. The xenografted lesions were examined on the 3th and 7th day after subcutaneous injection of 1×107 cells in 100μl of PBS into the back. Xenografted human glioblastoma tumors were established in nude mice by subcutaneous injection of 2×106 cells in 100μl of PBS, into the back of 16 individual mice[11]. The grown tumors were measured on the 25th day and following every week up to on the 25th week after transplantation, and measured tumor size at 25 days, 18 weeks, and 25 weeks after transplantation. Grown tumors on the 18th week after transplantation were examined for pathological findings and molecular analysis. The purchased U-118 MG cells (passage number 446) were used at passage 446-450.

Pathology and Immunohistochemistry

The xenografted lesion on the 3th day and the grown tumor on the 18th week after transplantation were fixed in 10% buffered formalin (pH 7.0) and embedded in paraffin. The paraffin-embedded sections were stained with hematoxylin-eosin (HE) and reticulin silver impregnation. Immunohistochemistry on para section was performed with a streptoavidin-biotin staining kit (DAKO, Glostrup, Denmark) using diaminobenzidin (DAB) as the chromogen. The sections were treated with anti-GFAP antibody (DAKO, Glostrup, Denmark) at 1:4000 working dilution, and visualized by DAB. Samples for transmission electron microscopy (H-7500, Hitachi Ltd., Tokyo, Japan) were fixed with 2.5% glutaraldehyde and osmium acid, embedded in Epon 812 (Nakarai, Kyoto, Japan), and ultrathin sections were made.

Molecular analysis and in situ hybridization

The tumor was frozen under liquid nitrogen, and BIOFOOD identification kit (BIOTOOLS B&M Labs, S.A., Spain) was applied to distinguish human DNA from mouse DNA. The process was done following the manufacture's protocol. Human control DNA was freshly isolated from leukocytes of a healthy donor and mouse DNA from liver of an experimental animal. DNAs of the transplanted tumors were obtained from formalin-fixed and paraffin-embedded sections through needle dissection (Laser Microdissection CRI 337, Cell Robotics, Inc., NM, USA). A 359-bp DNA product was amplified by PCR program using special restriction enzyme (Enzyme 3), resulted in the different gel pattern between human DNA and mouse DNA in a species-specific manner. The formalin-fixed, paraffin embedded sections were used for in situ hybridization for detecting Alu sequence in the nuclei of transplanted tumor cells. The slides were micro-waved in citrate buffer for 5 min, prehbiridzied with salmon sperm DNA, and add probe (Alu DNA, ISH probe, PR-1001-01-2601, Inno Genex, CA, USA). Heat the slide to 85°C for 10 min and incubate in a moist chamber at 37°C for overnight. Post-hybridization wash and detection according to the manufacture's manual (Inno Genex, CA, USA) were done and a kit (DAKO, Glostrup, Denmark) was used with minor changes.

Results

Tumor growth and size

The transplanted glioblastoma cells proliferated to form a solitary tumor in the subcutaneous region on the back of 14 in 16 mice, measuring 2×2 mm up to 5×5 mm (2.9±0.3×3.4±0.9 mm; n=14), 4×4 mm up to 18×22 mm (12.5±4.0×16.5±
Pathologic features of the tumor

The cultured glioblastoma cells (U118 MG) showed positive GFAP immunostaining on whole experimental day (1st to 7th cultured day), but the cultured control human fibroblasts were negative (Fig. 1). Also, there were intracytoplasmic intermediate-sized filaments (glial filament) on electron microscopy (Fig. 2).

The xenografted lesion was hard to notice as a tumor on the 3rd and 7th day. However, histopathologically, the region transplanted after 3 days preserved the features of glioblastoma keeping GFAP positive staining and with focally appearing reticulin fibers in the stroma.

All established tumors were whitish pink and relatively firm with homogeneous fibrous tissue like sarcomatous appearance from 4 to 25 weeks after transplantation. They showed smooth and circumscribed cut-surface, grossly, without massive adhesion and invasion to the host tissues, except for a few adhesive lesions. Histologically, the tumor demonstrated fibrosarcomatous features, losing positive GFAP immuno-reactivity resulted in hard to find and demonstrated increased reticulin fibers at 18 weeks after transplantation (Fig. 3). However, a few positive GFAP immuno-reactive cells were found in the muscle layer, which indicated that the invasive character of glioblastoma multiform was preserved although its morphological feature was fibrosarcomatous. In addition, electron microscopically, there were a few cells with a bundle of intracytoplasmic intermediate-sized glial filaments, which was direct proof of glial origin, or partially remaining elements of glial filament among the cells with hardly any remnant of glial filament (Fig. 4) at 18 weeks after transplantation, which indicated the remnant of the original character of oligoblastoma even in fibrosarcomatous cell appearance.

Molecular aspects of the tumor

The results from the existence of Alu sequence in the formalin-fixed, paraffin-embedded tissue specimen of grown tumor proved that the tumors were composed of human originated tissue (Fig. 5a). In order to identify whether the components originated from only human glioblastoma or not, the result of restriction fragment length polymorphism (RFLP) helped to identify human and mouse tissue using a 359-bp DNA product amplified by PCR and digested to specify the species-specific restriction length pattern. The RFLP analysis showed different pattern of digested DNA length from human and mouse (Fig. 5b). The restriction length patterns of the transplanted tumors were identical with human DNA without mixture of any mouse restricted DNA fragment. Therefore, there might be no mixture or less detectable sensitivity of mouse tissue in the transplanted and formed tumor.
Fig. 3. The histological feature of xenografted lesion in the back of nude mouse at 3 days (a, b, c, x100) and grown tumor at 18 weeks (d, e, f, x100) after transplantation. The fibrosarcomatous features are shown by HE staining (a, d). The preserved feature of glioblastoma with positive GFAP staining was noticed (b: arrows denote typical positive cells), and also showed scattered reticulin fibers (c) on the 3rd day. However, minimal remaining original character of glioblastoma with positive GFAP immunoreactivity (e) is shown among the fibrosarcomatous pattern. On the contrary, characteristic fibrosarcomatous features were clearly demonstrated by silver stained reticulin fibers at 18 weeks after transplantation (f).

Fig. 4. Inserted arrow area on low magnification (a) was magnified to identify the glial filament at high magnification (b).

Electron microscopically, a few fibroblastic cells showed bundles of intra-cytoplasmic intermediate-sized filaments: glial filament (b), which indicated that the cell originated from glial cell at 18 weeks after transplantation. Coincidently, among numerous tumors cells (e), partial glial components (arrow; magnified inset at the right) were hardly noticed.
Discussion

Glioblastoma, the most frequent malignant brain tumor shows various characters and features such as anaplasia and dedifferentiation and sometimes even form a sarcoma. Recently, glioblastoma has been classified into two types, primary glioblastoma and secondary glioblastoma, lead by the different genetic pathways with little overlapping\(^1\). Gliosarcoma is comprised in approximately 2 to 8\(^{1, 10, 12, 13}\) of total glioblastomas\(^1\). It is morphologically defined as a glioblastoma variant, corresponding to WHO grade IV\(^1\). It was described originally in 1895 by Strobe\(^{et\ al.}\) and defined as a glioblastoma subtype by Feigin\(^{et\ al.}\)\(^10, 14\). Gliosarcomas are morphologically characterized by a biphasic tissue pattern, showing a glial differentiation pattern and a mesenchymal one\(^1\). It has been hitherto widely accepted that the histogenesis of this tumor has been concerned in that the sarcomatous component is of vascular endothelial cell and pericytic origin, or of the potential cell origin from the mesenchymal tissue component of the meninx close to the glioblastoma\(^2, 9, 15\). The former hypothesis was supported by experimental work of transplantation of glioblastoma multiform fragments obtaining the formation of endothelial sarcoma\(^16\). The latter hypothesis was supported by the study of the expression of \(\alpha\)-smooth muscle actin in the sarcomatous region\(^17\). Both hypotheses for the sarcomatous components have been concerned with the mixture of neuro-originated ectodermal glioblast and mesodermal sarcoma. However, recent molecular and biological analysis, such as common mutation of p53 in both components and resemblance to chromosomal abnormality, makes one to reinforce the consideration for the possibility of this tumor to originate from common precursor cells\(^5, 9\). Further genetic analysis of both primary gliosarcoma and a secondary one strongly supports the concept of a monoclonal origin of gliosarcomas by identical genetic alterations in both gliomatous and sarcomatous components\(^10\). From this point of view, the previous cases in humans can be classified morphologically into three different phenotypes, such as anaplastic sarcomatous cells proliferating around vessels mixed with gliomatous cells, existence of a fibro-sarcomatous tumor between glioblastoma and meninx, and mingled and mixed fibro-sarcomatous components of glioblastoma and sarcoma\(^17-19\).

Our experimental study using nude mice transplanted with human glioblastoma cells may give a direct proof to solve the histogenesis of the gliosarcoma. The experimentally established tumor, originating from human glioblastoma multiform, showed the third type feature of gliosarcoma; fibrosarcoma, which could not be discriminated from a glioblastoma component and a fibrosarcoma one.

Fig. 5. Alu sequence is found as small dots (arrow) in the nucleus of transplanted tumor cells by in situ hybridization (a, x200). RFLPs analysis of transplanted tumors. Enzyme 3 in a BIOFOOD kit digested a 359-bp PCR product in a species-specific manner; 198/161 and 315/44-bp fragments in human (arrows) and mouse (arrow head), respectively. Two representative tumors (T1, T2) obviously showed human specific RFLP profile (b).
In the experimentally established gliosarcoma, however, it was necessary to solve the question whether the fibrosarcoma component originating from human only or penetrated and mingled with fibroblasts originating from mouse to form a sarcoma. From the previous report on human tumor xenograft, it was uncertain whether the tumor was only originating from human or mixture with fibroblasts of mice. They were established by direct transplantation from human tumor itself, or once cultured cells transplanted into nude mice and its produced tumor were cut into small pieces (2×2–3×3 mm) and inoculated in nude mice again. Also, when the grafted tumor in nude mice was introduced into the culture system, it was known that human tumor cells were overwhelmed by the fibroblasts originated from mice.

Our results indicated that the fibrosarcomatous component did not originate from the recipient mouse, but from only human. The molecular study for the discrimination from human and mouse tissue gave the solution. Before the differentiation to form a fibrosarcoma in the nude mouse, the cultured glioblastoma cells maintained the character of glial cells, proven by positive GFAP staining and keeping glial filaments electron microscopically. However, after transplantation the proliferating tumor cells lost almost all characters of glial cells. Only a few cells kept their original character, determined by a few positive GFAP cells and characteristic electron microscopical finding of glial cell. Most cells lost the characteristic glial filaments or partially retained them losing their original character. From our results, however, xenografted grown tumors were of human origin. And the result proved to establish the animal model of human glioblastoma for further investigation. In addition, it may be assumed that gliosarcoma showing fibrosarcomatous features in human might have originated from the same totipotential cells. At present, there are three hypotheses; vascular pericyte origin, mesenchymal cell origin, and precursor cell origin. The possibility of several growth factors surrounding the glioblastoma cells might affect these three different phenotypic expression, in that when close to the blood vessels they might be affected to lead to endothelial proliferation and when close to the meninx they might be affected to lead to mesenchymal proliferation between the glioblastoma and meninx, and fibroblastic differentiation. It is still unclear whether these hypotheses exist individually or not. Various cytokines are expressed in brain tumor, particularly in gliomas. They may be involved in tumor proliferation, angiogenesis, and immune surveillance evasion. Further investigation including various cytokines and growth factors, using this established animal model of human glioblastoma, may clarify the mechanism to form fibrosarcoma among the tumor lesion of glioblastoma.

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References


神経膠肉腫の由来と多形性に関する研究

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神経膠肉腫は膠芽腫の亜型で、病理組織学的に多形性を示す、予後不良な脳腫瘍である。組織学的には膠芽腫と肉腫の成分が混在し、一般的に肉腫は線維肉腫の形態をとる。この肉腫の由来に関しては、血管壁の間葉系細胞説と腺腫の肉腫化説が挙げられてきたが、最近の解析から、神経膠肉腫の膠芽腫と肉腫の成分が共通の前駆細胞に由来するのではないかと考えられつつある。そこで、膠芽腫の細胞をヌードマウスに移植し、この仏説の正否について病理組織学的および分子生物学的に検討した。その結果、移植した腫瘍の組織は線維肉腫像を呈しており、殆どの細胞は膠芽腫の性状を保持していなかった。しかし、一部の細胞では、神経膠のマーカーであるGFAP免疫反応が陽性を示していた。また電子顕微鏡像でも殆どの腫瘍が線維芽細胞様の構造を示していたが、一部の細胞は神経膠由来の証拠であるグリアフィラメントを有していた。これらの所見は、移植によって得られた腫瘍細胞の一部には移植細胞由来の性状が保持されていることを強く示している。さらに、In situ hybridizationによって、ヒトのAlu遺伝子が腫瘍組織内で検出され、DNA解析からもこの腫瘍にはマウスの成分が含まれず、ヒトの成分のみが構成されていることが明らかになった。以上の結果から、実験的に作成された神経膀胱肉腫の膠芽腫と肉腫の両腫瘍成分は、共通のヒト膠芽腫の細胞に由来する可能性が強く示唆された。

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