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Establishment and Characterisation of a Human Glioma Cell Line

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A new cell line, CB109, has been established from a human glioblastoma multiforme. The cytoskeleton was positive for glial fibrillary acidic protein, vimentin and fibronectin. Hyaluronan (HA) and the HA-binding protein hyaluronectin (HN) were expressed in the cell cytoplasm and in the extracellular matrix of spheroids and plated cells. Hyaluronidase did not prevent spheroid formation suggesting that HA was not involved in the cell–cell adhesion. HA precoating prevented cell adherence to the plates and favoured spheroid formation. HA was secreted in relatively large amounts into the culture medium. High performance liquid chromatography demonstrated that HA was in the high molecular weight form. The rate of HN secretion by cells was very low. Basic fibroblast growth factor significantly increased the proliferation *in vitro* and tumour growth after grafting into nude mice. The epidermal growth factor receptor was not expressed on cultivated CB109 cells. Cytogenetic analysis showed polysomy 7, structural rearrangement of chromosome 10 short arm and a translocation 13q13-q14 without detectable alteration of the RB gene.
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INTRODUCTION

MALIGNANT GLIOMAS are relatively easy to establish as continuous cell lines [1, 2]. Glioma-derived cell lines offer the opportunity to analyse different aspects of tumour response *in vitro* and *in vivo*. We have cultivated 120 human primary tumours and isolated 10 glioma-derived cell lines. One of them (CB109), initiated from a human glioblastoma multiforme, exhibits several interesting features which are: cytoplasmic markers, hyaluronic acid secretion, basic fibroblast growth factor (bFGF) sensitivity and karyotype pattern. No RB gene alteration was detected.

MATERIALS AND METHODS

Origin of cell line

The CB109 cell line was derived from a left parietal glioblastoma multiforme of a 58-year-old man, taken at surgery.

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Histology

Tissues from the original tumour were fixed in liquid nitrogen. Tissues from the grafted tumours were fixed in acetic alcohol (1% glacial acetic acid in 95° alcohol) or 4% formalin. Sections were stained with haematoxylin and eosin.

Cell culture

A 3 × 2 × 2 cm fragment with a large necrotic and haemorhagic part was obtained from surgery. After removal of the necrotic tissue the remainder was clearly identified as tumour tissue. The material was rinsed twice with Ca^{2+} and Mg^{2+} free Tris buffer, minced with scalpels and allowed to settle. The supernatant was discarded and the pellet disaggregated in culture medium. Cells were separated by gentle aspiration in a 2 ml pipette about 10 times. Following centrifugation at 80 g for 5 min, the pellet was disaggregated in culture medium and the suspension was distributed in four 75 cm^2 tissue flasks (Costar). Primary culture medium was Ham's F10 with 20% heat decomplemented fetal calf serum (FCS), 1% glutamine, 100 U/ml penicillin. The culture was grown in a 5% CO_2 in air incubator, with 100% humidity at 37°C. At the first passage, the FCS concentration in the medium was reduced to 10%. The medium was changed twice weekly until cell confluence, when the cells were removed by a 0.05% trypsin–0.02% EDTA solution in

Ca^{2+} and Mg^{2+}-free balanced salt solution at a split ratio of 1:2. Cells were subcultured until the 70th passage (P70) when CB109 cell line was considered to fulfil the criteria for an established cell line (Tissue Culture Association Recommendations). Further cells were subcultured as far as passage P101.

All media and additive solutions were obtained from Biochrom, Intermed, Noisy le Grand. Tris solution was from Eurobio (Paris, France). Only mycoplasma-free cells were used. Large scale production of cells was done in 150 cm^2 flasks (CML France); growth experiments were performed in 96-well plates (Falcon), and immunostaining was performed, after acetic alcohol fixation, on cells grown on glass coverslips at various passages.

The cell doubling time was studied by counting cells daily at the P6 and P101 passages. The morphological features of cultivated cells were observed with a Zeiss phase contrast microscope and camera.

Spheroid formation

Exponential phase cells were removed by trypsin and seeded in hyaluronic acid-coated (100 mg/l) enzyme linked immunosorbant assay plates. Cells (10^5 and 10^4) were grown in Hams' F10 medium with 2% Ultroser (IBF, France) which contains a very low and constant HA level. In order to study the role of hyaluronidase in cell adhesion and spheroid formation we also incubated the cells in the presence of 0.1 and 1 U/ml streptomyces hyaluronidase (Sigma). Spheroids were fixed with acetic alcohol, paraffin embedded and 5 μm sections were cut.

Hyaluronan detection in tissues

Using the reciprocal affinity of HA and hyaluronectin (HN), HA was demonstrated with biotinylated ovine HN [3]. A biotinylated probe was prepared from sheep brain hyaluronectin [4]. The hyaluronectin probe was incubated at 1 μg/ml for 30 min on sections or on glass coverslips at room temperature, washed 3 times with phosphate-buffered saline (PBS) and detected with streptavidin-peroxidase (Biogenex Laboratories), incubated for 30 min, following the manufacturer's recommendations, at room temperature. This was followed by 5 min 3-3'diamino-benzidin tetrahydrochloride (DAB) staining (Fluka, Switzerland), 1 mg/ml in Tris–HCl buffer, pH 7.4, with 0.003% H_2O_2. Controls were performed using biotinylated HN which had previously been supplemented with 20% HA.

Immunochemistry

Immunolocalisation was performed on cryostat sections of the primary tumour, on paraffin-embedded sections of nude mouse xenografts, on paraffin sections of spheroids and on cells grown on glass coverslips. All procedures were conducted at room temperature.

Non specific staining was suppressed by H_2O_2 pretreatment: 5 μm thick sections were deparaffinised, treated with H_2O_2 3% in methanol for 5 min, 1% normal horse serum in distilled water for 30 min. Cells on glass cover slides were incubated in 0.0015% H_2O_2 in PBS. Primary antibody incubations were overnight for the anti-HN monoclonal antibodies and 30 min for all other antibodies. For the other steps the ABC technique was used according to the manufacturers' recommendations (Vector Laboratories, Burlingame, California). Each step was followed by three washings with PBS. After DAB staining the preparations were embedded in Eukitt mounting medium (Gassalem Paris).

Polyclonal anti-glial fibrillary acidic protein (GFAP) antibodies were raised in rabbits.

Monoclonal antibody to GFAP, dilution 1:20, was from a mouse hybridoma supernatant [5]. Antibodies to HN were either polyclonal antibodies (1:100) raised in rabbit [6] or undiluted monoclonal mouse hybridoma supernatant [7]. Monoclonal antibodies to vimentin (1:5), epidermal growth factor (EGFr)(1:10), desmin (1:5) were from Amersham; antibodies to neurofilaments (1:5) from Immunotech, antibodies to laminin (1:20) from Heyl and antibodies to human fibronectin (FN) from Sigma (1:600). Controls were SP2/0 myeloma supernatant for monoclonal antibodies and rabbit anti-HN antibodies absorbed out on insolubilised HN for polyclonal antibodies.

Chromosome analysis

CB109 cells at P80 passage were incubated for 1 h at 37°C in the presence of colchicine, 0.04 μg/ml (Sigma), trypsinised, and resuspended in 0.075 mol/l KCl for 35 min at 37°C, fixed with three changes of methanol:acetic acid (3:1), spread on a clean, dry slide and R banding was performed [8]. Karyotype was described according to the International System for Human Cytogenic Nomenclature.

Retinoblastoma (RB) gene study

DNA from CB109 cells was digested with restriction endonuclase *Hind* III and analysed by DNA blotting with the cDNA probe RB 3.8 from the RB gene. RB 3.8 is the 3' portion of RB cDNA and was a generous gift from Dr Tyffa (Boston, USA). Filters were washed twice in 2 × SSC (sodium saline citrate buffer) plus 1% SDS (sodium dodecyl sulphate) at room temperature for 15 min and twice in 0.1 × SSC with 0.1 × SDS at 50°C for 30 min.

Determination of HA and HN in culture supernatants

The CB109 cells were grown in culture medium with 10% FCS for 3 days, rinsed and cultivated in serum free culture medium for one day.

HA molecular weight was estimated by high performance liquid chromatography (HPLC) through a Superose 12 column (Pharmacia) driven by Beckman equipment. HA concentration was determined with the indirect enzymoimmunological assay on HA coated microtest plates using sheep brain hyaluronectin [3]. HN was assayed with a sandwich enzymoimmunological assay on anti-HN antibody coated plates after hyaluronidase digestion of samples, in the presence of antiproteases, for 18 h. Samples for assay were diluted 10^{-1} and 10^{-2} and incubated for 4 h on plates.

Preparation of bFGF

Brain was extracted in 0.2 mol/l glycine HCl at pH 2.2 (1/2 w:w) and centrifuged at 32 000 *g* for 5–10 min. The supernatant was adjusted to pH 7 (1) [mean (S.D.)] with sodium hydroxide, centrifuged as above and passed through a 4 ml Heparin Ultrogel (Industrie Biologique Française, Villeneuve la Garenne, France) column at 50 ml/h flow rate [9]. The unbound solution was used for HN preparation and Heparin Ultrogel was washed with 1 l 1 mol/l NaCl. Basic FGF was eluted with 2 mol/l NaCl and the elution was recorded at 220 nm. Fractions whose absorbance was higher than 0.05 were pooled, filtered through a 0.22 μm sterile membrane, aliquoted and stored in liquid nitrogen. The bFGF activity was tested on human fibroblasts using the method described below for growth experiments.

Tumorigenicity

Nu/nu males Swiss-nu mice aged at least 6 weeks (Iffa-Credo, Les Oncins, France) were used. They were SSCup (Statut

Sanitaire Contrôlé/usage particulier: Specific pathogen free and free from potentially pathogenic micro-organisms). CB109 at passages P9 and P97 were suspended in serum free Ham's F10 medium or PBS and $5 \times 10^6 - 10^7$ cells were injected subcutaneously into each mouse of the five mouse groups. Mice were barrier-maintained and appeared to be in good health at the end of the experiment except for the tumour site.

Growth experiments were conducted in vitro and in vivo

In vitro, cells were seeded in 96-well plates. Outer wells were filled with Hank's balanced salt solution (HBSS) to avoid edge effects. On each plate a calibration series was established with 5×10^3, 10^4, 2×10^4, 4×10^4 cells per well, in triplicate. Other wells were filled with 250 μl of culture medium containing 10^4 cells. 24 h after seeding, cells of the calibration series were fixed with 1% glutaraldehyde in HBSS. In the other wells, the culture medium was replaced by serum free medium for controls or by serum free medium containing bFGF in the case of treated cells. The bFGF optimum concentration used (0.2 ng/ml) was determined using CB109 cell cultures (not shown). 48 h later, media were removed and stored at −20°C before assays. Cells were fixed by 1% glutaraldehyde and cell number was evaluated according to the crystal violet staining method [10]. Briefly, the 1% crystal violet solution was deposited on wells for 30 min. Wells were rinsed with distilled water and air dried. The stain adsorbed on the cells was then solubilised with 10% acetic acid and the absorbance was measured at 620 nm on a Flow Titertek multiskan spectrophotometer coupled to a Victor VPC II Computer.

In vivo, 5×10^6 CB109 cells were grafted into nude mice at passage P88 and P97 in 0.25 ml of serum free medium for controls, and in 0.25 ml of serum free medium containing 0.25 ng of bFGF for treated groups (5 or 6 mice per group). Tumour diameters were measured twice per week. At the end of the experiments (4 and 9 weeks) tumours were weighed and tissue samples were taken for histology.

RESULTS

The primary tumour showed polymorphic proliferation with marked nuclear and cellular variation: bipolar cells, protoplasmic astrocytes, numerous very small cells and multinucleated giant cells were present (Fig. 1a). Extensive necrotic areas and abnormal vascular proliferations were observed. All characteristics were that of a glioblastoma multiforme.

Suspensions of 5×10^6 cultured cells injected subcutaneously to nude mice gave tumours within 2 months at the two passages studied (P9 and P97). Tumours were well vascularised, with a central core of necrosis and developed desmoplasia. The peripheral part was composed of viable cells and necrotic tissue in alternating layers of irregular thickness. Haematoxylin and eosin staining showed cellular pleomorphism with very large nuclei, prominent nucleoli and atypical mitoses (Fig. 1b).

Pleomorphism was also observed in the culture. In early passages cells grew rapidly and displayed mixed cell types (Fig. 1c): numerous large, flat, more or less stellate cells, less numerous smaller, near polygonal cells, bipolar cells, and some rare rounded cells which frequently detached from the flask bottom. Some multinucleated giant cells were also present. All cells presented a large nucleus showing two or more nucleoli and intracytoplasmic vesicles.

After serial passages (Fig. 1d) large flat cells were rare, partially polygonal cells were more numerous, and bipolar and rounded cells were abundant. Some multinucleated giant cells

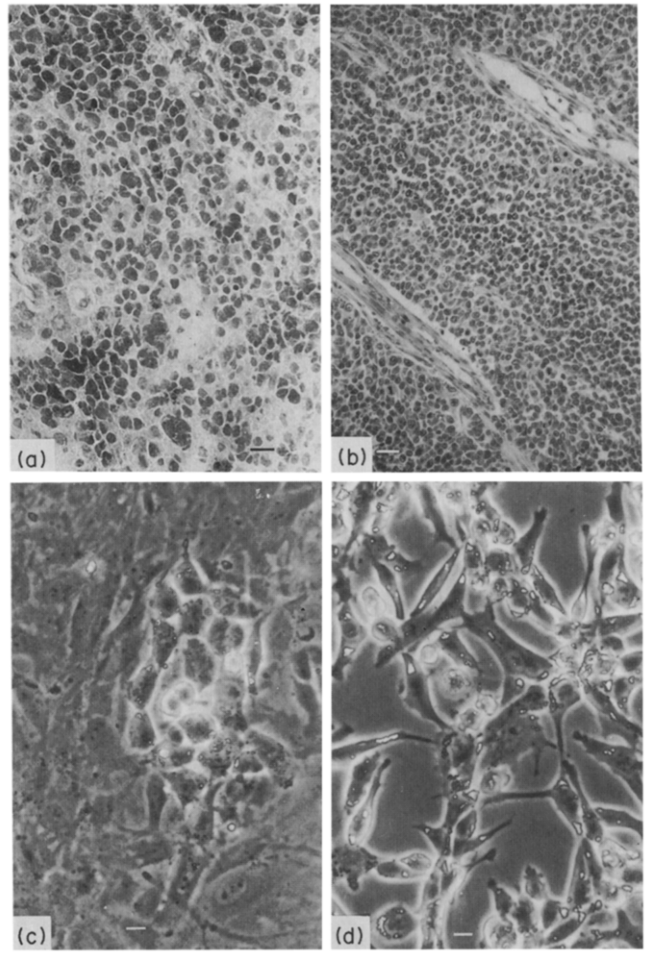

Fig. 1. Morphological features of CB109 tumors and cultures. (a) Original tumour (cryostat section): high cellular density of small cells next to necrotic areas and some giant cells. Haemotoxylin and eosin stain. Bar = 20 μm. (b) Heterotransplanted tumour (paraffin section): a central area of necrotic cells is surrounded by small viable cells among which giant cells and abnormal mitosis can be observed. Haematoxylin and eosin stain. Bar = 40 μm. (c and d) Phase contrast photomicrographs of cultivated CB109 cells: Similar polymorphism is seen at the 1st (c) and 60th (d) passages: near polygonal, bipolar and refringent free cells surrounded by flat stellate cells in (c). Bar = 20 μm.

were still present. All cells formed a network becoming thicker when the cell numbers approached confluence. On this network, spheroid-like aggregated cells were observed when cultivated at hyperconfluence. Most cells were firmly attached to the flask during the first 2 or 3 days, then numerous cells began to detach giving loosely attached or non-adherent cells. The non-adherent cells were viable since they formed new monolayers when plated again on plastic dishes.

Cell proliferation kinetics were studied by establishing growth curves. Cell doubling time was 50 h at the 6th passage with a 3 day lag phase and 30 h at 101st passage with no lag phase.

Multicellular spheroids formed easily when CB109 cells were cultivated on HA coated plates. In all studied conditions spheroids were observed from 1 to 15 days, then cells turned slowly necrotic and disaggregated. HA and HN were detected in the extracellular matrix (ECM) of spheroids. The spheroid formation was not affected by the presence of hyaluronidase which destroyed 99% of HA in the culture medium.

Table 1. Immunocytochemical characterisation of CB109 line

	Original tumour		Cultures		Grafted tumours	
Markers	Cells	ECM	Cells	ECM	Cells	ECM
GFAP	++	−	+ to +++	−	− or +	−
Human HN	− or +	− or ++	++	+	− or + to +++	− or + to +++
HA	−	+	+ to ++	+	+	++
Fibronectin	ND	ND	+ to ++	−	ND	ND
Vimentin	ND	ND	+ to ++	−	ND	ND
Desmin	ND	ND	−	−	ND	ND
Laminin	ND	ND	−	−	ND	ND
EGFr	ND	ND	−	−	ND	ND
Neurofilaments	ND	ND	−	−	ND	ND

Intensity of staining, scale established comparatively to control staining −: negative, +: weak, ++: moderate, +++: strong, ND: not done.

Cell markers studied by immunostaining are shown on Table 1.

In the primary tumour many cells were stained by the anti-GFAP antibody. Hyaluronectin distribution in the primary tumour was irregular: some areas were rich in extracellular strongly HN stained deposits whereas others were negative. Hyaluronic acid was found in the ECM of the tumour.

In cell culture no change was observed from early to later passages. The staining revealed 95% GFAP positive cells on the 94th passage with some cells demonstrating fibrillar staining and others with a more granular pattern. About 50% of cells were HN positive, showing spots or fibrillar formation in the cell cytoplasm or processes. About 50% of cultivated cells were positive for HA in the cytoplasm and cell processes. All cells were vimentin positive at passage P82, 1% of them being strongly labeled. All cells were FN positive in the cytoplasm. The ECM which was deposited on the glass coverslips by cultivated cells was also positive for several components: HA, HN, whereas fibronectin and vimentin were absent.

No staining was seen for desmin, laminin, EGF receptor and neurofilament antigens.

In grafted tumours, some cells were positively stained for GFAP and positive fibrillar processes were also observed. In the case of the grafted tumours two types of HN were detected: mouse HN produced by mouse tissues and human HN produced by CB109 cells which could be characterised by its specific human antigenicity. The location of human HN in the grafted tumours was irregular with positive and negative areas. Also, positivity within the cytoplasm was seen occasionally: in some areas, cell cytoplasm was strongly HN positive whereas the ECM was slightly stained; in other areas cells were negative or weakly stained, and ECM was very strongly HN positive. Some areas were completely HN negative. HA was present mainly in the ECM and was also seen in some CB109 cells.

HA and HN were also found in the culture medium but whereas CB109 cells secreted 0.55 (0.07) 10^{-13}g of HA/cell/h, HN was found in minimal amounts 1/100 to 1/300 that of HA. HA was essentially found in the void volume of HPLC through Superose 6 columns, indicating a molecular weight higher than 10^6 Da. In extracts made in neutral buffers HN was linked to HA as shown by HPLC and hyaluronidase digestion which reduced the apparent molecular weight of HN.

Chromosome analysis monitored at passage P80 showed that cells were near tetraploid, with 83 to 92 chromosomes (mode 88) and minor cell to cell variation (Fig. 2). Common clonal structural abnormalities were: der(1)t(1;?)(q11;?), der(3)t(3;?)(q11;?), der(5)t(5;6)(q14;q15), i(8q),der(10)t(dup(2)(q11q21;10)(q11;p15), der(10)t(10;12)(p11;p11), der(12)t(12;13)(q14;q13)

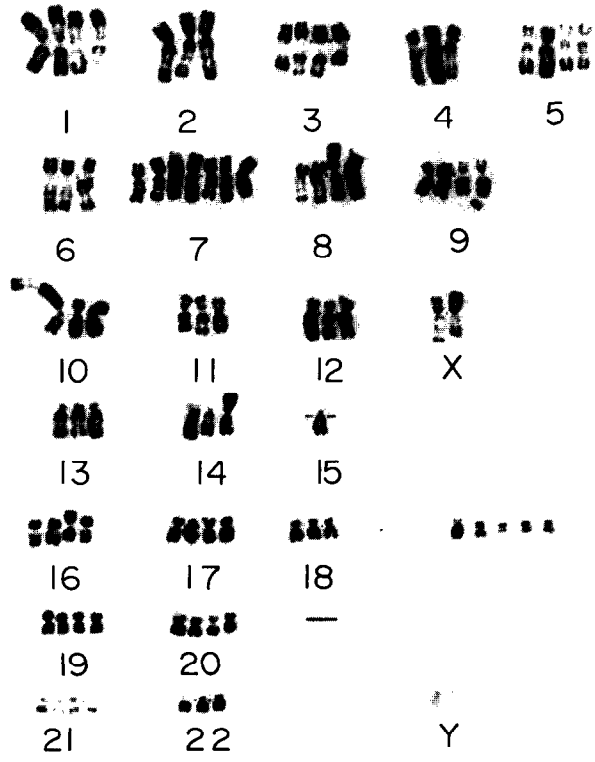

Fig. 2. G-banded karyotype representative of CB109 cells in P80 passage.

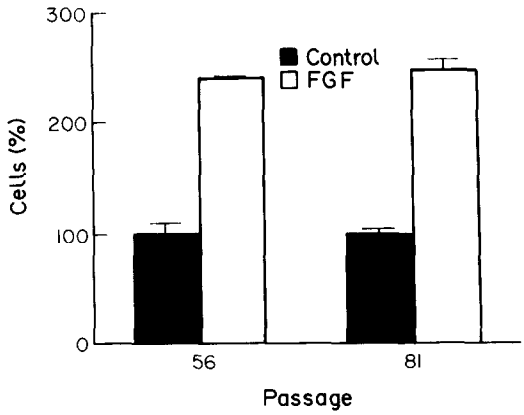

Fig. 3. Effect of bFGF on the growth of CB109 *in vitro*. Each value is mean (S.D.) (bars) of six replicate cultures ($P < 0.0001$). Growth is measured by the ratio: number of cells in experiment × 100: number of cells in control.

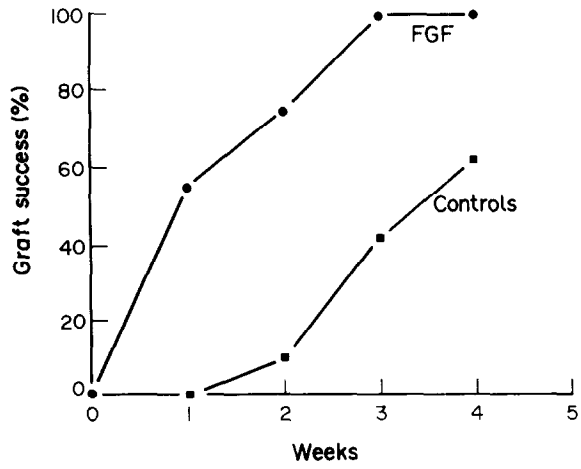

Fig. 5. Kinetics of growth of CB109 cells grafted to nude mice in the presence of bFGF. Each value is the ratio *n*:11 (number of graft sucesses:number of mice).

and i(16q), and marker chromosomes looking like centromeric regions of acrocentric chromosomes. Heptasomy of chromosome 7 was notable in all analysed metaphases.

The CB109 DNA pattern did not reveal any gross genomic rearrangement of the RB gene in CB109 cells when compared with DNA of normal lymphocytes from blood donors.

The addition of bFGF to cells produced significant cell proliferation (240% of control) in passage 56 (Fig. 3) and a similar response (250% of control) was obtained on passage 81 (Fig. 3).

In vivo, 0.25 ng of bFGF added to 5×10^6 cells enhanced tumour weights (Fig. 4) by a factor of up to 3 ($P < 0.0001$) and the latency period (Fig. 5) was reduced to 1 week (compared with 2 weeks in controls).

DISCUSSION

We have established a new human glioma cell line CB109 which possesses several interesting features, the most important are: GFAP, vimentin, fibronectin, hyaluronan and hyaluronectin synthesis, sensitivity to bFGF, multicellular spheroid formation and typical chromosomal abnormalities. CB109 morphology was clearly that of gliomas with large cells, numerous cellular and nuclear abnormalities, and a tendency for cells to become non-adherent, which is a classical feature of gliomas [11].

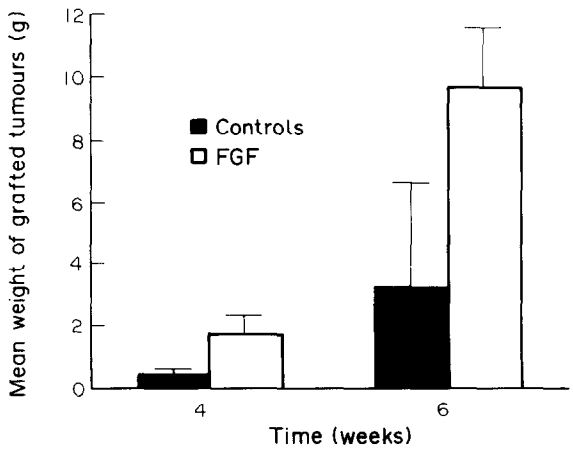

Fig. 4. Effect of bFGF on the growth of grafted CB109 cells in nude mice. Each value is mean (S.D.) (bars) of 5 mice ($P < 0.0001$).

The presence of GFAP is particularly important in assessing the astrocytic phenotype of cultivated cells. The positivity for GFAP frequently disappears rapidly from cultivated glioma cells as shown earlier [12, 13]. A few glioma-derived cell lines have however been described, which are positive for GFAP [2, 11, 14].

Fibronectin was positive as in the majority of glial cell lines. Vimentin has been shown to be the major cytoskeletal component of immature glia [15] and the positivity of CB109 cells for vimentin is consistent with a glial origin as shown already on tumour sections [16].

HA production by cultivated glioma cells is a common process [17, 18] which has a protective effect for glioma cells against attack by activated killer cells [19]. In our study 10 out of 10 glioma-derived cell lines tested were found to produce high molecular weight hyaluronan in amounts similar to that produced by fibroblasts (unpublished). The finding of HA on cells is in accord with the description of hyaluronate synthetase on the cell surface [20]. The production of HA by the cancer cells may explain their failure to adhere to HA-rich ECM and may favour their detachment from ECM. Conversely the production of HN was observed only in two out of these 10 cell lines. In all other cell lines HN was detected neither in cells nor in the culture medium. HN positivity of CB109 cells suggests that HN which is generally found in glioma matrix [6, 7] could originate, at least in part and in some tumours, from the glioma cells. The accumulation of HN in the cytoplasm is however surprising since in the primary brain tumour, as in other human primary tumours HN was found in the extracellular location and generally was not seen as a cellular component. This may suggest that the CB109 cancer cells lack component(s) involved in glycoprotein secretion.

The CB109 cell line is sensitive to bFGF both *in vitro* and *in vivo*. This point seems important since only two out of 5 cell lines tested were shown to be sensitive to bFGF, which suggests that the sensitivity to bFGF which was found with other cancer cells: gliosarcoma [21], breast carcinoma derived cells [22] is not present in all glioma cell lines. In contrast to other observations on glioma cell lines [23] CB109 was negative for EGFr. The promoting effect of bFGF on CB109 growth in the mouse is likely to be related only to the effect of bFGF on cancer cells. Indeed, the grafting of cell lines insensitive to bFGF *in vitro* to nude mice in the presence of bFGF did not enhance tumour

growth, which suggests that the action of bFGF on stroma cells does not have an indirect effect on tumour growth and that bFGF exerts its activity only and directly on the mitogenic activity of CB109 cells.

Polysomy 7 has been described previously [24], and might be correlated with overexpression of EGFr [25] which was not observed in the CB109 cell line. The loss of chromosome 10 was described as a common non-random event in malignant astrocytomas [26]. We did not find loss of chromosome 10 in the CB109 cell line, but we did observe two different structural rearrangements involving the short arm of chromosome 10. That kind of rearrangement may participate in the loss of heterozygoty described recently [27]. A translocation involving the 13q13-q14 region was also present in the CB109 cell line and might conceivably involve the gene related to retinoblastoma. However the RB oncosuppressor gene which has been shown to be involved in different tumours: sarcoma [28], lung [29] and breast carcinoma [30] was expressed normally in CB109.

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