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Morphological, immunocytochemical and growth characteristics of three human glioblastomas established in vitro

T. Bilzer[1], D. Stavrou[2], E. Dahme[3], E. Keiditsch[4], K.F. Bürrig[5], A.P. Anzil[6], and W. Wechsler[1]

[1] Department of Neuropathology, University of Düsseldorf, Düsseldorf, Federal Republic of Germany
[2] Department of Neuropathology, University of Hamburg, Hamburg, Federal Republic of Germany
[3] Institute of Veterinary Pathology, University of Munich, Munich, Federal Republic of Germany
[4] Institute of Pathology, Clinicum Bogenhausen, Technical University of Munich, Munich, Federal Republic of Germany
[5] Institute of Pathology, University of Düsseldorf, Düsseldorf, Federal Republic of Germany
[6] Department of Pathology, State University of New York Health Science Center, Brooklyn, New York, USA

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Summary. The human glioblastoma-derived cell lines 86HG-39, 87HG-28 and 87HG-31, used for the production of monoclonal antibodies (mAbs) against glioma-associated antigens (GAA), were characterized in terms of morphology, growth behaviour, chromosomes and antigen expression. In the primary tumours, differential expression of glial fibrillary acidic protein, S100 protein, Leu-7 and GAA as defined by mAbs MUC 2–39, MUC 2–63 and MUC 8–22 was demonstrated. Receptors for epidermal growth factor (EGFr) and nerve growth factor (NGFr) were found in many cells in short-term cultures, but the transferrin receptor (Tr) was found in only a few cells of 87HG-28. In permanent cell lines, differentiation antigens and EGFr decreased and Tr increased markedly. NGFr and GAA remained stable. Transplantation tumours of 86HG-39 were partly positive for Tr and GAA. Chromosomal analysis revealed that the 86HG-39 and 87HG-28 cell lines had a hypodiploid or diploid stem line with lines in the hypotetraploid to tetraploid region for 50 in vitro passages. The 87HG-31 cell line had chromosomal patterns in the hypotriploid to triploid region. A gain of chromosomes was seen in the groups C7, C8, C10, D14, F19, F20, G21, G22. The variability of antigens in these tumours and especially during long-term cultivation probably reveals an ability to influence the growth of malignant glioma cells via the respeective effector molecules.

Key words: Glioma antigens – Epidermal growth factor receptor – Nerve growth factor receptor – Transferrin receptor – Chromosomes

Offprint requests to: T. Bilzer, Department of Neuropathology, University of Düsseldorf, Moorenstrasse 5, W-4000 Düsseldorf, Federal Republic of Germany

Introduction

Malignant human gliomas are highly variable with regard to morphology, growth fraction (Deckert et al. 1989) and antigenicity (Wikstrand et al. 1985; Rettig et al. 1986). Thus, heterogeneity is a major problem in glioma diagnosis and therapy. Immunochemical methods are valuable in the analysis of brain tumour cell populations (Kleihues et al. 1987; Perentes and Rubinstein 1987; Reifenberger et al. 1987) and the presence of growth-related molecules such as epidermal growth factor receptor (EGFr) (Libermann et al. 1984), transferrin receptor (Tr) (Prior et al. 1990) and glioma-associated antigen (GAA) (Carrel et al. 1982; Bourdon et al. 1983; Wikstrand et al. 1986; Stavrou et al. 1987; Vrionis et al. 1988) can help to identify neoplastic cells, monitor tumour progression and act as possible targets for antibody-guided therapy (Epenetos et al. 1985; Takahashi et al. 1987; Wikstrand et al. 1987; Lee 1988).

Cell lines and glioma xenograft models offer the opportunity of detailed neuropathological, immunological and pharmacological studies. Such models may represent some characteristics of the primary tumour and indicate some of its developmental potentials.

The objective of the present study was to describe (a) the morphology, antigen expression, in vitro propagation, growth behaviour and cytogenetics of three human gliomas which have been used for the production of monoclonal antibodies (mAbs) against GAA (Stavrou et al. 1987) and (b) to report on the expression of growth factor receptors and GAA in permanent cell lines and transplantation tumours.

Materials and methods

Primary tumours, the age and sex of the patients, tumour location and the histological features of the surgical specimens from which the cell lines were derived are summarized in Table 1. Samples

Table 1. Origin, location, diagnosis and histopathological characteristics of primary brain tumours established as permanent glioma cell lines 86HG-39, 87HG-28 and 87HG-31

Cell line	Patient (age/sex)	Location	Diagnosis	Histopathological characteristics					
				Cell			Necroses	Vascular proliferation	Lymphocytic infiltration
				Density	Polymorphism	GF[a]			
86HG-39	56 years/M	L frontal	Glioblastoma	Moderate	High	24%	Small+large	Moderate	Marked
87HG-28	55 years/F	R temporal	Glioblastoma	Moderate	Moderate	14%	Small+large	Marked	Moderate
87HG-31	61 years/M	L parietal	Glioblastoma	High	Moderate	12%	Small+large	Moderate	Rare

[a] Growth fraction according to nuclear Ki-67 expression

for in vitro propagation obtained from the brain tumours were processed within 1 h. Tumour tissue was kept under sterile conditions, freed from blood and connective tissue and then mechanically dissociated in cell suspensions, or cut into pieces of 1 mm^3. Pieces for cryostat sections and paraffin histology were treated according to conventional methods.

Tumour fragments were placed in 25 cm^2 plastic culture flasks and covered with Dulbeccos modified Eagle medium (DMEM) culture medium containing 10% fetal bovine serum (FBS), and supplemented with non-essential amino acids (Stavrou et al. 1987). Suspensions and explants were incubated at 37° C in humidified air with 5% carbon dioxide. After outgrowth, cells were detached and dispersed by use of 0.025% trypsin/0.04% EDTA in phosphate buffered saline (PBS). Subsequent cultures were fed with supplemented DMEM/10% FBS, changing the medium twice weekly. Confluent cultures were divided in ratios between 1:2 and 1:10, depending on the proliferation rate. Cell culture reagents were purchased from Boehringer (Mannheim, FRG), and Flow (Meckenheim, FRG). For immunocytochemistry, cells were kept under serum-free conditions for 72 h. Serum-free media consisted of a 1:1 mixture of DMEM and nutrient mixture F12 (Ham's) supplemented as above, additionally enriched by 10 µg/ml insulin, 10 µg/ml transferrin, 10 nM sodium selenite and 1 mg/ml bovine serum albumin (Westphal et al. 1988). These products were obtained from Gibco (Paisley, UK) and Sigma (Deisenhofen, FRG). Plating efficiency, doubling time and saturation density were estimated according to conventional methods by counting the cells in a haemocytometer (Coulter Electronics, Harpenden, UK) and under an inverse phase contrast microscope (Zeiss ICM 405), respectively. Viability of cells was tested by trypan blue exclusion. The morphological features of the cell lines were monitored by phase contrast or differential interference contrast microscopy with a Leitz photomicroscope. All cell lines were routinely tested for mycoplasma contamination.

Material for histological investigation was either frozen in liquid nitrogen or fixed in formalin. Paraffin sections were stained with haematoxylin and eosin (H & E), cresyl violet, and according to Masson's trichrome and Tibor PAP methods. Frozen sections were stained with H & E. Immunochemistry was carried out on tissue sections and monolayer cell cultures for glial fibrillary acidic protein (GFAP), vimentin, desmin, cytokeratin, neurofilaments, neuron specific enolase (NSE), S100, Leu-7, Leu-M1, chromogranin, synaptophysin, *Ulex europaeus* agglutinin (UEA 1), EGFr, NGFr, Tr, MUC 2–63, MUC 2–39, and MUC 8–22. Origins of antibodies, dilutions etc. are listed in Table 2.

Monoclonal antibodies were applied to paraffin and frozen sections and on cell cultures grown on cover slides with immunochemical methods as usual, in particular by a four step PAP, avidin-biotin-complex, or immunogold method, respectively. For staining of transplantation tumours in the mouse, biotinylated primary antibodies were used to avoid reations of the anti-mouse secondary antibodies with the host immunoglobulins in the tumour. Frozen sections and cells for immunocytochemical stainings were fixed in ice-cold acetone. After immunochemistry (Reifenberger et al. 1989), sections were counterstained with Meyer's haemalum or left unstained and mounted in Depex[R] (Serva, Heidelberg, FRG). Controls were performed by replacing the primary antibodies with either the immunoglobulin fraction from non-immunized rabbits or with non-specific mouse IgG at dilutions identical to those of the primary antibodies.

Cell enzyme linked immunosorbent assay (ELISA) was performed for the detection of mAb reactivity against surface antigens on viable cells as previously described (Stavrou et al. 1983). Glioma cells were seeded into 96-well round-bottomed vinyl plates (Linbro 76.364-05, Flow) and grown to a subconfluent state. Thereafter, DMEM/FBS was removed and replaced by serum-free conditioned medium as described above. After 48 h cells were washed with cold PBS and then covered with the primary antibody during 1 h at 4° C. After washing in PBS cells were fixed; rabbit anti-mouse IgG was added and incubated for 30 min. After the final wash, 100 µl substrate solution containing 1 mg/ml orthophenylendiamine (Sigma) in 0.1 M citrate buffer, pH 4.5, was added to each well. The reaction was stopped by 8 N sulphuric acid and the supernatants measured in a Titertek Multiscan (Flow) at 492 nm.

For scanning electron microscopy (SEM) cell cultures were fixed in 1% phosphate-buffered glutaraldehyde (pH 7.3) for 24 h, dehydrated in graded ethanol and saturated in amylacetate. Samples were then dried with the critical-point method and covered with gold-paladium. SEM micrographs were taken in a Philips SEM 515. Material for transmission electron microscopy (TEM) were fixed in 2.5% paraformaldehyde/1% glutaraldehyde, buffered with Sorensen buffer, pH 7.4, postfixed in osmium and embedded in Epon. Cell cultures were fixed in 0.5% glutaraldehyde. Ultrathin sections were stained with uranyl acetate and lead citrate and examined under a Zeiss EM-10.

The tumorigenicity of the cell lines in athymic mice was determined by subcutaneous injections of 10^6–2×10^7 cells grown in vitro. Cells were harvested mechanically, washed twice in PBS, suspended in 100-µl aliquots and placed in the right chest wall. Cell numbers were determined by counting in a Coulter counter and the percentage of living cells was then evaluated by trypan blue exclusion test. Tumours were removed at a size of more than 1 cm in diameter. They were dissected free of connective tissue aseptically and processed for examination by light and electron microscopy and tissue culture.

For chromosome preparation, subconfluent cell cultures were treated with Colcemid (Ciba, 0.4 µg/ml, 1–3 h), detached with trypsin, centrifuged at 100 *g* and resuspended in 0.5% potassium chloride solution. After swelling sufficiently, cells were pelleted and fixed in acetic acid/methanol 1:3 for 45 min. Metaphases were spread on microscopic slides and air-dried. Staining of chromosomes was performed with 2.5% Giemsa solution after treatment with 0.0125% trypsin in sodium chloride solution. Samples were examined with immersion optic under a Zeiss photomicroscope.

Table 2. Origin of antibodies for immunochemistry of human gliomas and glioma cell lines

Antigen	Species	Antibody	Source	Dilutions	References
GFAP	M	m clone G-A-5	Boehringer, Mannheim, FRG	1:100	Debus et al. Differentiation 25:193–203 (1983)
	R	p	Dakopatts Hamburg, FRG	1:2000	
Vimentin	M	m clone V9	Boehringer	1:100	Osborn et al., Eur J Cell Biol 34:137–143 (1984)
Desmin	M	m clone D33	Dakopatts	1:100	
Neurofilaments	M	m pool NF	Dianova, Hamburg, FRG	1:100	Osborn and Weber, Lab Invest 48:372–394 (1983)
Cytokeratins	M	m clone KL 1	Dianova	1:100	Viac et al., Invest Dermatol 81:351–354 (1983)
		m clone Lu5	Boehringer	1:100	v. Overbeck et al., Virchows Arch [A] 407:1–12 (1985)
S100	R	p	Dakopatts	1:2000	
NSE	R	p	Dakopatts	1:2000	
Chromogranin	M	m clone LK2H10	Hybritech, San Diego, Calif.	1:300	Lloyd and Wilson, Science 222:628 (1983)
Synaptophysin	M	m clone SY 38	Boehringer	1:200	Wiedenmann and Franke, Cell 41:1017–1028 (1985)
Leu-7 (HNK1)	M	m	Becton and	1:100	
Leu-M1(FAL, CD15)	M	m clone MMA	Dickinson, Heidelberg, FRG	1:100	
EGFr	M	m clone EGFR1	Amersham, UK	1:100	Waterfield et al., J Cell Biochem 20:149–161 (1982)
NGFr	M	m clone ME20-4	Amersham	1:100	Ross et al., Ann NY Acad Sci 486:115–123 (1983)
Tr	M	m clone 2EB	Amersham	1:100	Gatter et al., J Clin Pathol 36:539–545 (1983)
GAA	M	m clone MUC 8–22		1:100	Stavrou et al., J Neurol Sci 80:205–220 (1987)
	M	m clone MUC 2–63		1:100	
	M	m m clone MUC 2–39		1:100	
Ki-67	M	m PC	Dakopatts	1:50	Gerdes et al., Int J Cancer 31:13–20 (1983)
UEA 1		biotin. lectin	Vector, Burlingame, Calif.	1:150	
MIgG	R	p	Dakopatts	1:500	
	G	p	Dakopatts	1:500	
	H	p+Biotin	Vector	1:300	
	G	p+Gold	Janssen, Olen, Belgium	1:200	
RIgG	S	p	Dakopatts	1:500	

M, Mouse; R, rabbit; H, horse; G, goat; S, swine; m, monoclonal antibody; p, polyclonal antisera

Results

The three brain tumours described here show many histological characteristics of the glioblastoma group: a moderate to high cell density with small to medium-sized gliogenic cells, small anaplastic cells, mono- or multinucleated giant cells, abundant nuclear polymorphism and many mitotic figures. Most cell nuclei are chromatin-rich, round or oval in polygonal, and carrot-like in spindle cells. All tumours have solid and microcystic areas; regressive changes consist of small necrotic foci with palisading and large necroses. Tumour vascularization shows numerous capillaries but also small and medium-sized vessels with glomerular formation and garland-like endothelial proliferations. Diffuse lymphocytic infiltrations as well as perivascular lymphocytic cuffs containing 2–6 layers are often prominent.

In particular, N39/86 (Fig. 1A, B) is a recurrent glioblastoma. It was removed from the left frontal lobe of a 56-year-old male patient, 4 months after the first surgery. Immunoreactivity of the proliferating cell marker Ki-67 demonstrates a growth fraction of approximately 24% (Table 1). Some areas are extremely rich in reticulin fibres and show a sarcomatous pattern (Fig. 1B). The tumour N28/87 was from the right temporal lobe of a 55-year-old woman. The tumour has a proliferation index of approximately 14% (Table 1), is variable, containing several angiomatous areas with

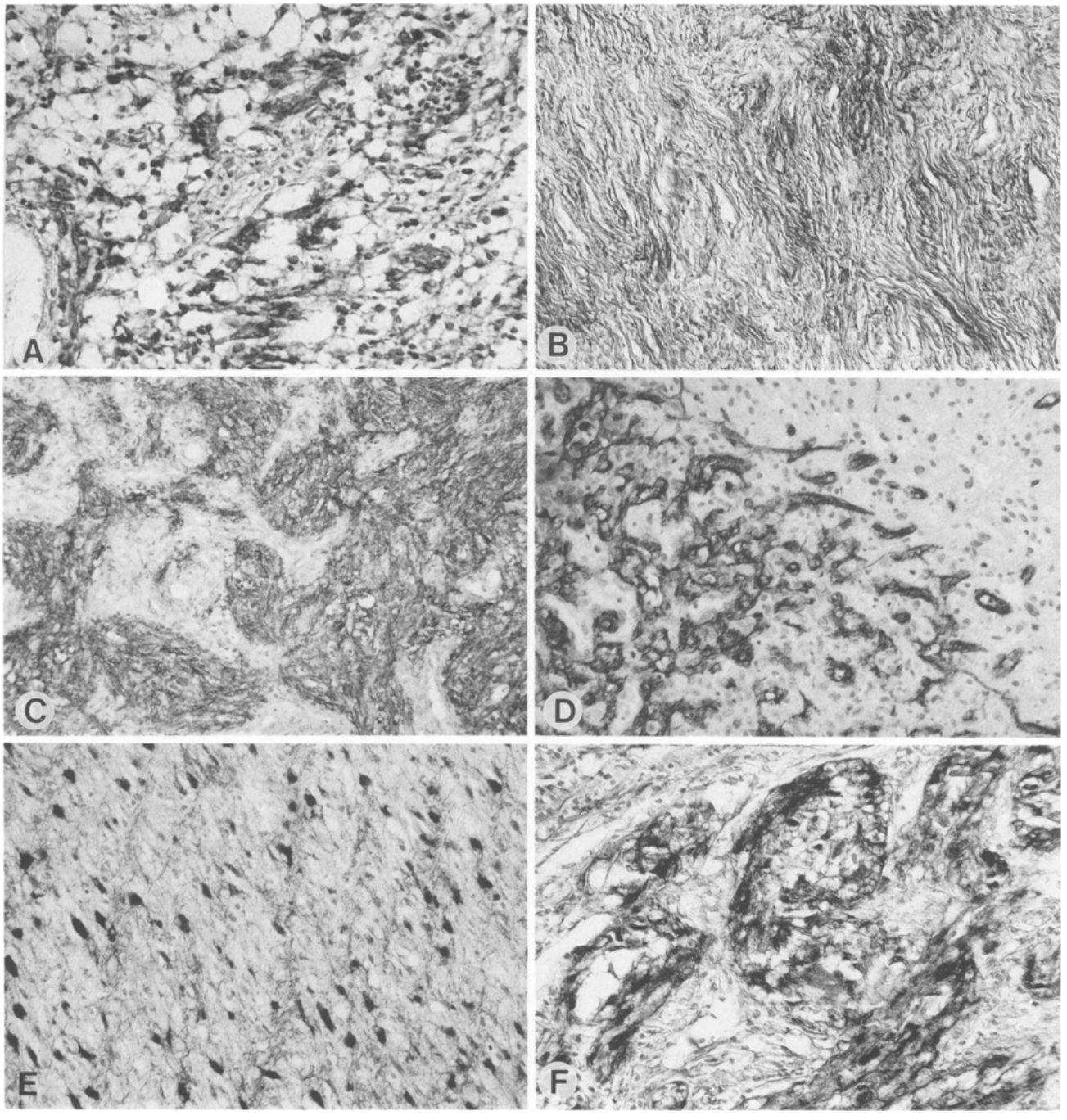


Fig. 1 A–F. Human glioblastomas N39/86, N28/87, N31/87. **A, B** Recurrent glioblastoma N39/86 in a 56-year-old man. Gliomatous tissue with microcystic parts, large necroses, endothelial proliferations and nuclear polymorphism. Immunohistochemistry of glial fibrillary acidic protein (GFAP) in gliomatous cell groups (**A**) and sarcomatous areas (**B**). **C, D** Glioblastoma N28/87 of a 55-year-old woman. GFAP-positive tumour parts between marked vascular proliferation (**C**), partly resembling an angioma, as demonstrated by binding of *ulex europaeus agglutinin* (UEA 1; **D**). **E, F** Glioblastoma N31/87 of a 61-year-old man, composed mostly of gemistocytic areas with strong expression of GFAP (**E**) and Leu-7-positive gliogenic areas between vascular proliferation. **A, B, D** PAP method; **C, E, F** ABC method. **A, F** ×320; **B–D** ×160; **E** ×200

blood vessels of various sizes (Fig. 1 C, D). Reticulin fibres are also prominent in large areas of the tumour tissue. The tumour N31/87 was removed from the left parieto-occipital region of a 61-year-old man. It is of medium to high cell-density containing large polymorphic protoplasmatic astroglial cells (Fig. 1 E) and anaplastic areas with marked vascular proliferation (Fig. 1 F). The proliferation index is 12% (Table 1).

Immunohistochemical data are given in Table 3 and Fig. 2. In N39/86 and N28/87, most of the GFAP-posi-

Table 3. Antigen expression in human gliomas and glioma cell lines

Antigen	Primary tumours			Short-term cultures			Long-term cultures						Transplantation tumours
				Passages 5–15			Passages 30–50			Passages 80–100			
	N39/86	N28/87	N31/87	86HG-39	87HG-28	87HG-31	86HG-39	87HG-28	87HG-31	86HG-39	87HG-28	87HG-31	TT 86HG-39
GFAP	2	2	3	2	2	2	1	1	2	1	0	0	0
Vimentin	2	3	2	4	4	4	4	3	4	4	4	4	1
S100	3	2	2	2	2	2	2	3	3	1	2	1	1
Leu-7	3	2	2	2	2	2	2	1	1	1	1	0	0
EGFr	–	–	–	2	2	1	1	0	0	0	0	0	0
NGFr	–	–	–	3	2	0	3	2	0	3	2	0	0
Tr	–	–	–	0	1	0	1	1	1	3	2	3	2
Ki-67	2	1	1	2	1	1	–	–	–	–	–	–	2
MUC 2–39	3	0	0	4	0	0	4	0	0	4	0	0	3
MUC 8–22	2	3	2	3	3	2	2	3	2	3	3	2	3
MUC 2–63	2	2	2	3	3	1	3	3	2	3	3	1	2

Values: 1, −25%; 2, 25%–50%; 3, 50%–75%; 4, >75%; −, not tested

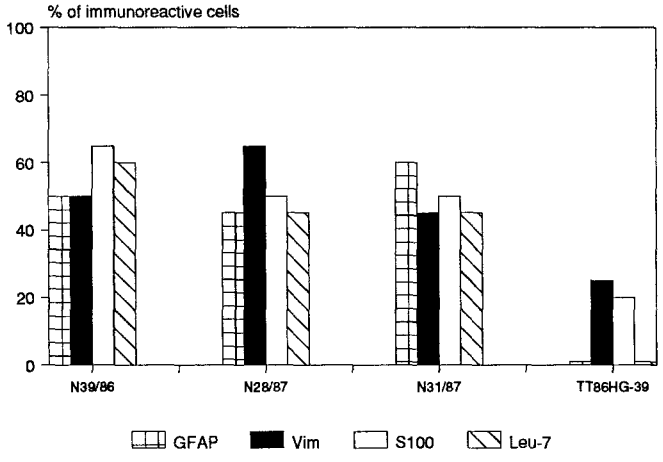


Fig. 2. Immunohistochemistry of glial antigens GFAP, vimentin, S100 and Leu-7 in primary brain tumors N39/86, N28/87 and N31/87 as well as in xenotransplantation tumours of the 87HG-39 cell line in nude mice, taken from the 50th in vitro passage

tive cells are diffusely distributed (Fig. 1A) or arranged in gliomatous cell clusters. Moreover, strong immunoreactivity is found in sarcomatous areas of N39/86 (Fig. 1B). In N28/87 and N31/87, GFAP is intensely expressed around blood vessels. In N31/87 areas resembling gemistocytic astrocytoma are very rich in GFAP, whereas small anaplastic cells are negative (Fig. 1E). In all tumours, S100 and vimentin reactivities are similar to GFAP, with vimentin somewhat more pronounced in the anaplastic fields. Leu-7 is mostly restricted to gliogenic cell groups (Fig. 1F). Desmin, cytokeratin, neurofilaments, NSE, chromogranin and synaptophysin are not found. Leu-M1 is mostly expressed in reactive astrocytes in the tumour periphery. MUC 2–39 stains cells only in N39/86. MUC 8–22 and MUC 2–63 stain cells in N39/86, N28/87 and N31/87. In transplantation tumours of 86HG-39 GFAP, S100 and Leu-7 are weak or negative. Expression of growth factor receptors was investigated in cryostat sections of secondary tumours of the 86HG-39 cell line. Immunoreactivity can be shown for Tr in most and for NGFr and EGFr in single cells. Reactivity patterns of MUC 2–39, MUC 8–22 and MUC 2–63 in the transplantation tumours are comparable with those found in the primary tumours.

Cell lines were designated as 86HG-39, 87HG-28 and 87HG-31, whereby the first digits stand for the year, the latter for the number of the biopsy and the letters for human glioma. All cell lines grew as monolayers; 86HG-39 cells could also be grown as spheroids. Cell proliferation increased continously up to the 30th in vitro passage. Cell lines were regarded as permanent from the 30th in vitro passage onward. Growth characteristics of the three glioma cell lines are summarized in Fig. 3. Cell growth with elongation of processes was observed after 24 h. The major cell type present initially was small to medium-sized, bi-or multipolar with long thin processes in 86HG-39 and 87HG-31 and short plump processes in 87HG-28, respectively. Parallel growing spindle cells also occurred. Mono- or multinuclear giant cells were numerous. Between the short-term cultures and the 20th in vitro passage, cultures developed into a more uniform cell population dominated by bi-or tripolar cells with occasional polygonal and rare giant cells (Fig. 4). However, during a cultivation period of over 90 in vitro passages, the cell line 87HG-28 remained stable as a mixed monolayer of the above-mentioned type. The continuously growing cell line 87HG-31 represented a mixed monolayer culture, whereby bi-or tripolar cells were dominant, but single pleomorphic giant cells were a consistent finding.

Immunocytochemistry was carried out in the cell lines at different passage levels, in particular between passages 5 and 15, 30 and 50, 80 and 100 (Table 3, Figs. 5–7). In short-term passages, glial antigens are comparable to those of the original tumours. Receptors of EGF and NGF are stained in variable amounts in most of cells in 86HG-39 and 87HG-28, EGF in single cells of 87HG-31. Tr is found in single cells or is negative. MUC 2–39 is present in most of the cells in 86HG-39 but negative in the two other cell lines. MUC 8–22 and MUC 2–63 react with cells in all cell lines. In long-term passages,

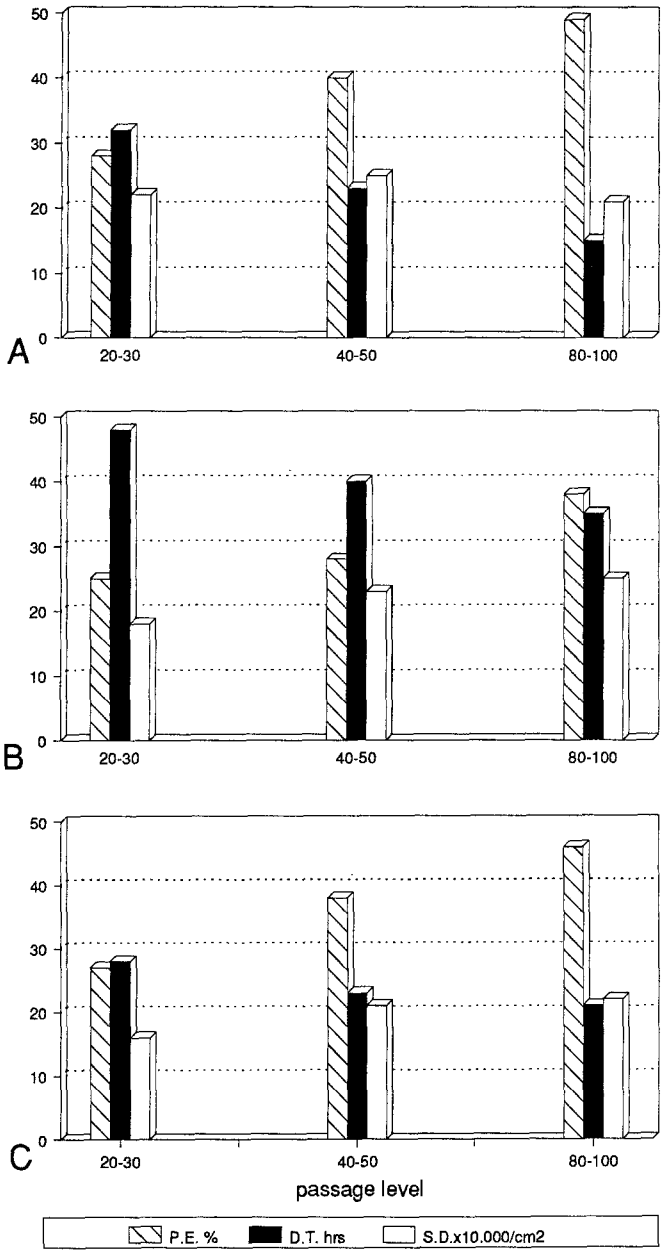


Fig. 3. In vitro growth of human glioma cell lines 86HG-39 (**A**), 87HG-28 (**B**) and 87HG-31 (**C**). Plating efficiency (*P.E.*), doubling time (*D.T.*) and saturation density (*S.D.*) at different passage levels that illustrate a marked increase of the in vitro proliferation

expressions of GFAP, S100 and Leu-7 are markedly reduced, whereas vimentin gives identical results as in the early passages (Figs. 5–7, 8A, E). EGFr is still present in a few cells of 86HG-39, but negative in 87HG-28 and 87HG-31. Surprisingly, Tr is increased in all cell lines (Table 3; Figs. 5–7, 8D). NGFr (Fig. 8B), MUC 2–39, MUC 8–22 (Fig. 8C) and MUC 2–63 (Fig. 8F) remain unchanged.

Micro-ELISA was performed concerning the expression of EGFr, Tr, MUC 2–39 and MUC 2–63 on 86HG-39 cells. In passages 25–35, extinctions concerning EGFr were at a mean value of 0.48, Tr 0.25, MUC 2–39, 0.73, MUC 2–63 0.68. Controls were at 0.1–0.2. In passages 80–90, EGFr was at 0.18, Tr 0.65, MUC 2–39 0.78, MUC 2–63 0.7.

After subcutaneous injection of 10^6–10^7 cells, growing tumours developed only from the 86HG-39 cells; other lines showed short-term nodules only. Treatment of the recipients with dexamethasone and cyclosporin A did not improve the tumour take. Secondary tumours were obtained in 19 of 20 animals with the 86HG-39 cell line. These were similar to the original tumour but were more homogeneous with less necrosis and vascular proliferation. In tumours larger than 1.5 cm in diameter, large central necroses with lymphocytic and macrophage infiltration occur. Cells are small to intermediate in size, round to polygonal with some giant cells. The mitotic index ranges from 32% to 38%. Tumours are sharply demarcated from the surrounding tissue, with some infiltration of muscle and skin and a delicate collagen network reaching from the periphery into the tumour.

SEM was carried out on cell cultures and revealed similar results for the three glioma cell lines. At higher magnifications, flattened spindle shaped and multipolar cells are observed. Processes reach a length of approximately 100 μm. There is no contact inhibition, and cells are often double-layered (Fig. 4C, D). At higher magnifications bubbles, folds and short microvilli are observable at the cell surface. Filopodia of different length occur.

TEM on the three cell lines and in transplantation tumours of the 86HG-39 cell line showed that 86HG-39 cell cultures consist of medium-sized cells with long processes and large hypodense nuclei. Nuclei contain up to four nucleoli and chromatine-dense clumps at the nuclear membrane. Sometimes nuclei have multiple finger-like invaginations (Fig. 9A). The cytoplasm is rich in filaments and polysomes (Fig. 9B). Numerous mitochondria occur in the perikaryon. Autophagolysosomes with straight or concentric membranes are numerous (Fig. 9C). The Golgi apparatus appears normal (Fig. 9D). Gap junctions and single tight junctions can be found in vitro as well as in the secondary tumours. In the tumours, the cells are loosely arranged with abundant extracellular space containing collagen fibres. 87HG-28 cells are characterized by very large and often bizarre hypodense nuclei with multiple long and branched invaginations. They contain one to three nucleoli and varying numbers of chromatin clumps. In the cytoplasm, only a few small mitochondria and large numbers of autophagolysosomes filled with myelin-like structures can be found (Fig. 9E). There are a great number of filaments, mostly associated with the cell surface (Fig. 9F). Rough endoplasmatic reticulum (RER) is rare, but many free ribosomes are disseminated in the cytoplasm. Single cell contacts occur. 87HG-31 cells have small or medium-sized normally configured hypochromatic nuclei and one or two nucleoli. Mitochondria and Golgi apparatus are normal. RER is rare, but numerous free ribosomes can be found. Many filaments are located near to the nucleus and the cell membrane, which occasionally showed single junctions.

The karyotype of 86HG-39 was analysed at the 27th

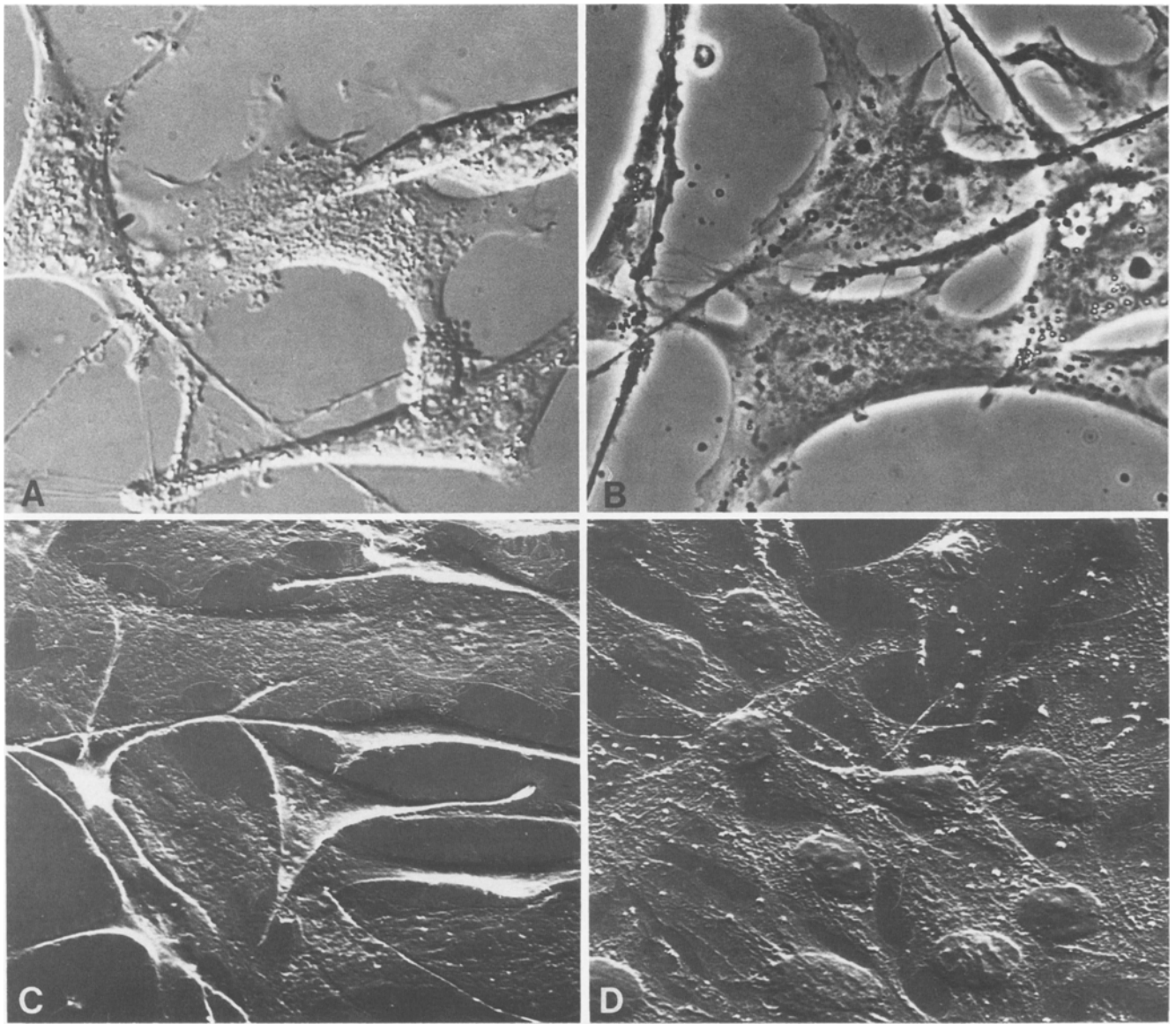
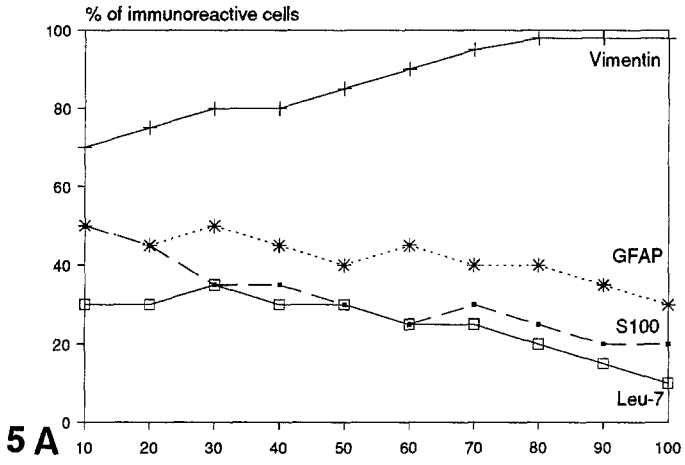


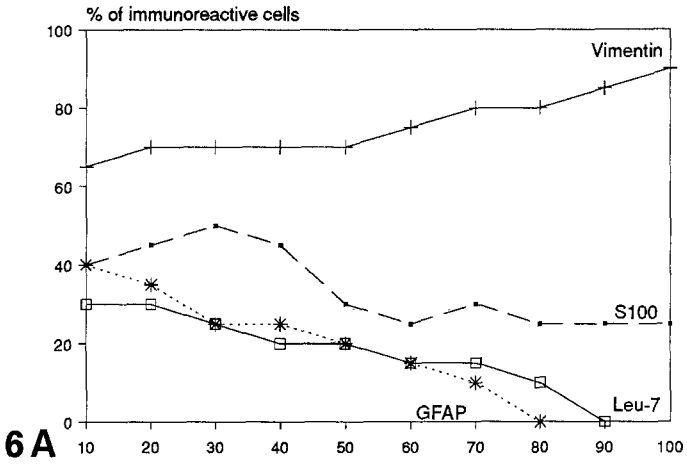
Fig. 4. Human glioma cell line 86HG-39 in the 30th (**A, B**), and in the 90th in vitro passage (**C, D**). Cell morphology is more uniform in late passages and cultures consist mostly of flattened spindle-shaped cells but still contain single multipolar cells. Cells are often double-layered without contact inhibition. At the cell surface, bubbles, folds and microvilli can be seen (**C, D**). **A** DIK; **B** phase contrast; **C, D** SEM, × 500

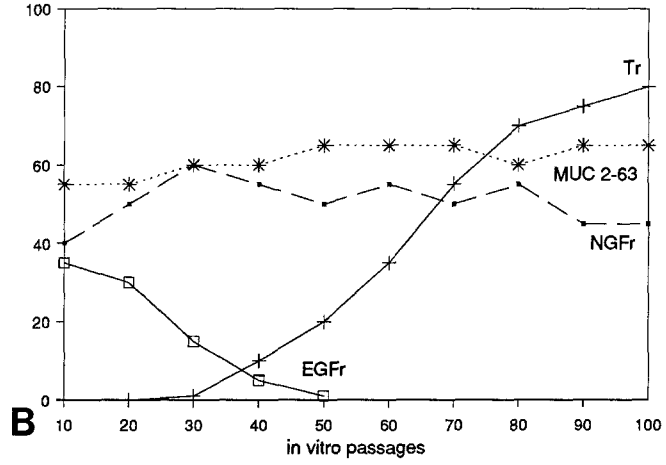
and the 42th in vitro passage. A stemline was found in a hypodiploid to diploid region with sidelines in the hypotetraploid to tetraploid region. The stemline had the following karyotype: 38–46, XY, C9p+, t(+9p− ter::?), −C 13, mar1, mar2, mar3. In this line, only one marker chromosome, i.e. 9p+, could be found. Additionally, there was a nullisomy of chromosome 13. 87HG-28 chromosome numbers were determined at the 50th in vitro passage. The stemline was within the hypodiploid region, with hypotetraploid to tetraploid sidelines. The marker chromosomes in these two cell lines showed a rather uniform pattern, most commonly the markers 6p+, 7q−, 8q−, 10q− and 14q+. 87HG-31 chromosome numbers were determined at the 24th and 64th in vitro passages. The stemline was in the hypertriploid region.

Discussion

Immunohistochemistry of the primary tumours revealed a variety of cell markers, often without obvious morphological correlations. In N39/86, which was a recurrent tumour of a primary glioblastoma, large areas rich in reticulin fibres occur. These may be due to postoperative reaction or radiation-dependent injury. Some, but not all of these structures contain GFAP, vimentin, S100 and Leu-7. In N28/87, angiomatous components can be demonstrated by reticulin staining and by binding of *Ulex europaeus* lectins. However, glial antigens GFAP, S100 and Leu-7 are heterogeneously distributed in all tumour compartments. In N31/87 two different histological patterns can be distinguished immunohistochemically: one representing an anaplastic astrocytoma with







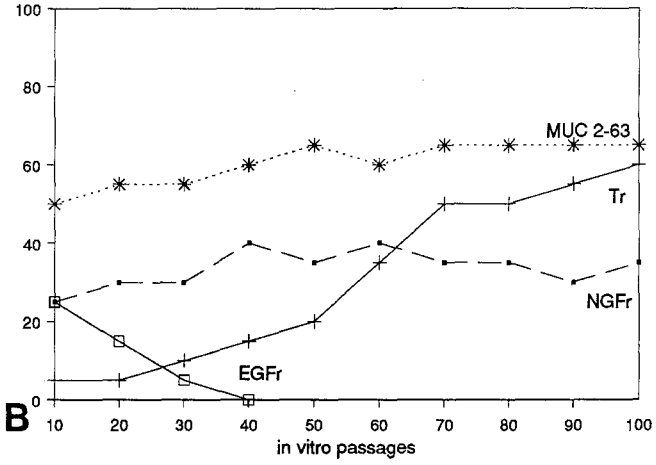


Fig. 5–7. Human glioma cell lines 86HG-39 (**Fig. 5**), 87HG-28 (**Fig. 6**) and 87HG-31 (**Fig. 7**). Differential expression of GFAP, vimentin, protein S100 and Leu-7 (**A**), as well as receptors for epidermal growth factor (*EGFr*) transferrin (*Tr*), nerve growth factor (*NGFr*) and glioma associated antigen (*GAA*) MUC 2–63 (**B**) during in vitro cultivation

gemistocytic elements reacts strongly with GFAP, S100 and Leu-7; the other, containing mostly spindle-shaped cells, shows little staining for glial markers and reticulin, but is positive for vimentin.

Gliomas can exhibit a wide variety of phenotypes (Kepes 1987), including sarcomatous formations (Morantz et al. 1976; Schiffer et al. 1984; Slowik et al. 1985; Kochi and Budka 1987). As outlined recently (Meis et al. 1990) gliosarcoma is widely accepted as a mixed tumour with the strong staining of glial antigens in gliomatous areas and the negative results in the sarcomatous areas which contain many reticulin fibres. In contrast, glioblastomas stain diffusely for GFAP and S100 protein. From this point of view, the classification of N39/86 is difficult, since both appearances are found within the same tumour.

GFAP is widely accepted as a glial cell marker (Osborn et al. 1981; Eng and Smith 1985). GFAP and vimentin have been found in most malignant glial neoplasms (Herpers et al. 1986; Schiffer et al. 1986; Cosgrove et al. 1989; Kleihues et al. 1987; Perentes and Rubinstein 1987; Reifenberger et al. 1987) and in cultures of human glioma (Pateau 1988). Co-polymers of these two intermediate filaments can form heteropolymers in neoplastic astrocytes (Wang et al. 1984). The discussion of whether expression of GFAP and vimentin is inversely correlated to the degree of anaplasia is controversial (De Armond et al. 1980; Reifenberger et al. 1987). In agreement with others (Ponten and Westermark 1978; Pateau 1988) we have observed a decrease and even loss of glial differentiation markers during permanent glioma cultivation. Nevertheless, GFAP and S100 were still present in single cells (87HG-28) or up to 20% of cells (86HG-39, 87HG-31). Vimentin was consistently expressed in nearly all of the tumour cells and was partially co-expressed with GFAP and S100. Positive reactions for GFAP, S100 and Leu-7 in non-gliomatous cells, negative results in gliomatous cells and the vimentin positivity in both seem to us more an indication of variable cellular differentiation and a differentiation-dependent antigen expression than a sign of different histogenesis of the cells involved. Intermediate filament heteropolymers

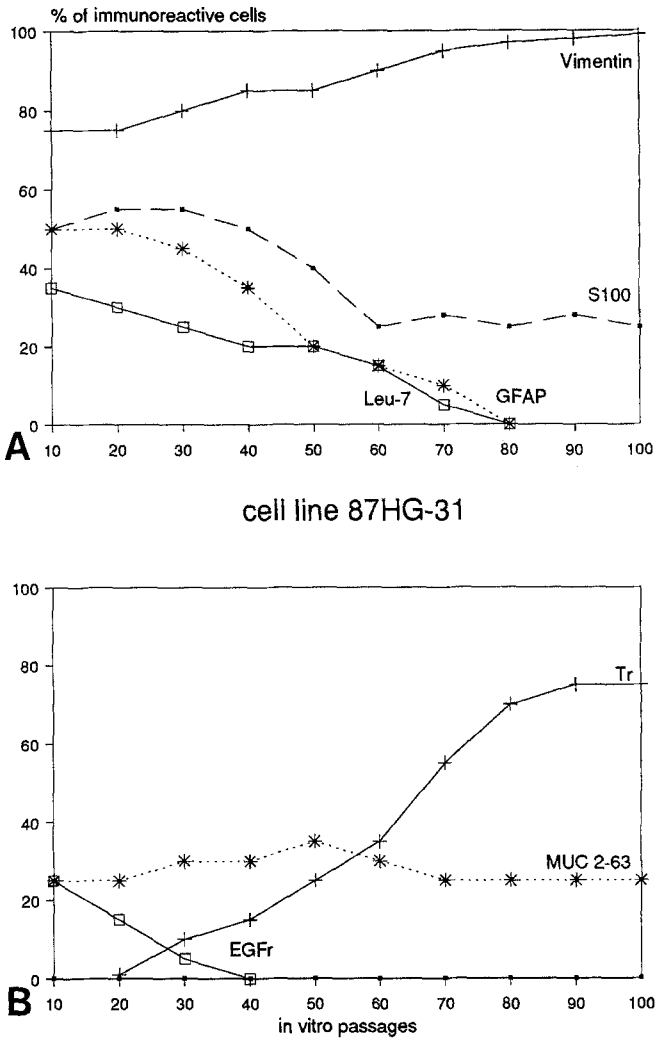


Fig. 7

with varying proportions of GFAP and vimentin as a result of cell differentiation and function have been discussed in detail (Pateau 1988). The characterization of malignant gliomas and the cell lines derived from them generally revealed no simple correlations between different phenotypic and genotypic variables (Shapiro et al. 1981; Bigner et al. 1981; Wikstrand et al. 1985; Rutka et al. 1987; Kennedy et al. 1987; Westphal et al. 1988). Cultures of 86HG-39, 87HG-31 and 87HG-28 exhibited considerable morphological, antigenic and proliferation variability. Although cell lines became more uniform with increasing in vitro passages, cellular heterogeneity never disappeared fully. Interestingly, transplantation tumours of late passages (greater than 100) of 86HG-39 in nude mice were of the same histological type as those from earlier passages (30–40). Long-term in vitro growth and cryoconservation are selective processes which give rise only to those cells which have the highest adaption potentials. Cells which are highly adaptive to culture conditions may represent poorly differentiated forms, and have developmental potential latent in the primary tumour. Such cells could represent the most resistant population to radio- or immunotherapy and if this is so, establishment of in vitro and in vivo model systems of human malignant gliomas would be useful in the development of new diagnostic tools and therapeutic concepts. Different chromosomal aberrations could be demonstrated associated with glioma growth, including loss of sex chromosomes and decrease or loss of chromosomes 4, 9p13, 10, 12, 17p and 22 and gains of nos. 7, 14 and 19 (Bigner et al. 1988; Bigner and Vogelstein 1990). The most prevalent changes are numerical alterations of whole chromosomes, e.g. gains of 7 and loss of 10 (Bigner et al. 1984). Allelic deletions especially on chromosome 10 have been described in glioblastomas (James et al. 1988). In a group of 32 of 38 malignant human gliomas with near diploid stem lines statistically significant gains of 7 were found (Bigner et al. 1988). Nullisomy 13 has also been observed in another glioblastoma cell line (Zang et al. 1988). In the 86HG-39 cell line gains of chromosomes 7 and 16, a decrease of 10, 15 and 17 and a loss of 13 was seen during the 27th to 42th in vitro passage. Those chromosomes which are involved in aberrations harbour important oncogenes responsible for division, growth and differentiation. In gliomas the gene which is most often amplified is the EGFr gene (Bigner and Vogelstein 1990). Although polysomy 7 in gliomas is common, it does not seem to be an essential prerequisite for EGFr amplification (Bigner 1987). To our knowledge, only one human glioma cell line has been reported to retain over-expression of the EGFr in culture (Filmus et al. 1985). Growth factor sensitivity of a human glioma cell line which initially responded to EGF with increased proliferation was reduced in higher passages (Westphal et al. 1988). A possible explanation is that the selective conditions to maintain EGFr amplification are not present in vitro (Humphrey et al. 1988), whereas in anaplastic gliomas, increased expression of EGFr (Libermann et al. 1985; Reifenberger et al. 1989) has been described.

One of the most interesting findings of the present study was a change of growth factor immunoreactivity during permanent glioma cultivation. Binding of transferrin to its receptor induces endocytosis of the receptor/ligand complex and subsequent iron uptake, which is essential for DNA synthesis and cell replication (Trowbridge et al. 1984). Little is known about the expression of Tr in human gliomas (Zovickian et al. 1987; Colombatti et al. 1988), especially during in vitro cultivation. Immunohistochemistry of Tr in human nervous system tumours revealed an increased expression of Tr in high-grade gliomas (Prior et al. 1990). It is quite unclear by which mechanisms increase of Tr in the glioma lines occurred. Since transferrin is a constituent of the defined medium applied 3–4 days before immunocytochemistry, one should not expect Tr increase. Speculatively, it is possible that (a) EGFr has been lost, structurally modified or down-regulated, (b) EGFr-negative/Tr-positive cell sub-populations have been selected during long-term in vitro growth or (c) a switch of growth factor receptors has occurred by epigenetic changes. NGFr was more or less constant during the whole cultivation period. NGFr is found in neural crest tumours such as neurinomas, neurofibromas and ganglioneuromas (Ross et al.

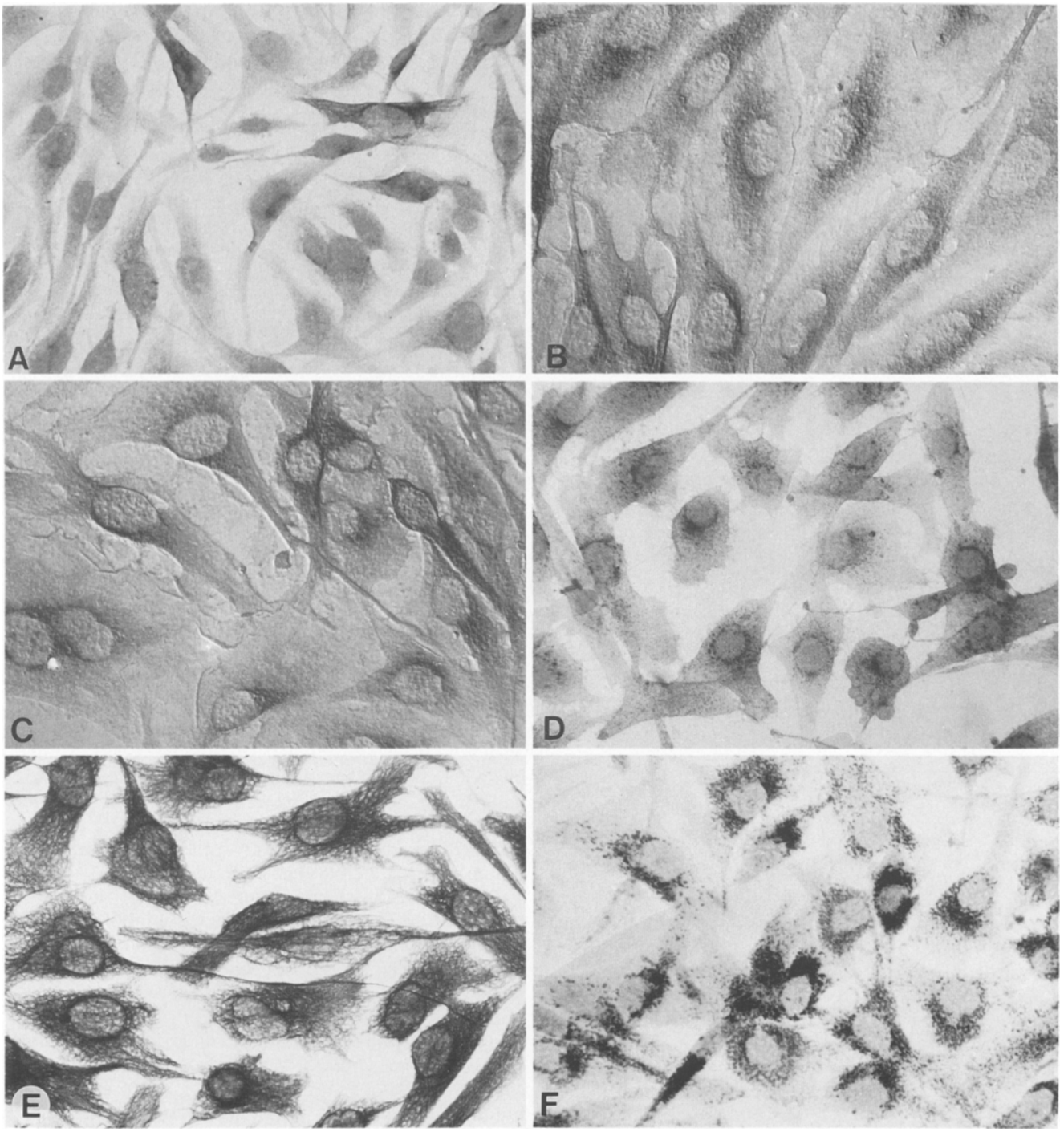


Fig. 8A–F. Human glioblastoma cell lines 86HG-39, 87HG-28, 87HG-31. Cell line 86HG-39 in the 80th in vitro passage with differential expression of GFAP (**A**) and NGFr (**B**). Cell line 87HG-28 with expression of the GAA MUC 8–22 (**C**) and the transferrin receptor (**D**) in the 70th in vitro passage. Cell line 87HG-31 with strong expression of vimentin (**E**) and the GAA MUC 2–63 (**F**) in the 50th in vitro passage. **A–E** ABC method; **F** immunogold with silver enhancement; **A, D** ×350; **B, C, E, F** ×450

1984), whereas gliomas were either negative or only slightly positive (Prior et al. 1989). There is some evidence that NGF (via its receptor) is involved in the differentiation and function of glioma cells. Morphological changes persisted even in the absence of NGF (Marushige et al. 1987). In our studies, cells were kept under serum-free conditions during 3 days before immunochemistry. The content of growth factors in the serum charges cannot be destinated under conventional conditions. If effects of growth factors on cells persist longer than 72 h, it seems likely to suggest that cell characteristics are still under the influence of serum-derived factors.

GAA were defined by mAbs MUC 8–22, MUC 2–63 and MUC 2–39, which preferentially react with neuroectodermal tumours including gliomas, neuroblastomas and melanomas as well as with embryonic and fetal

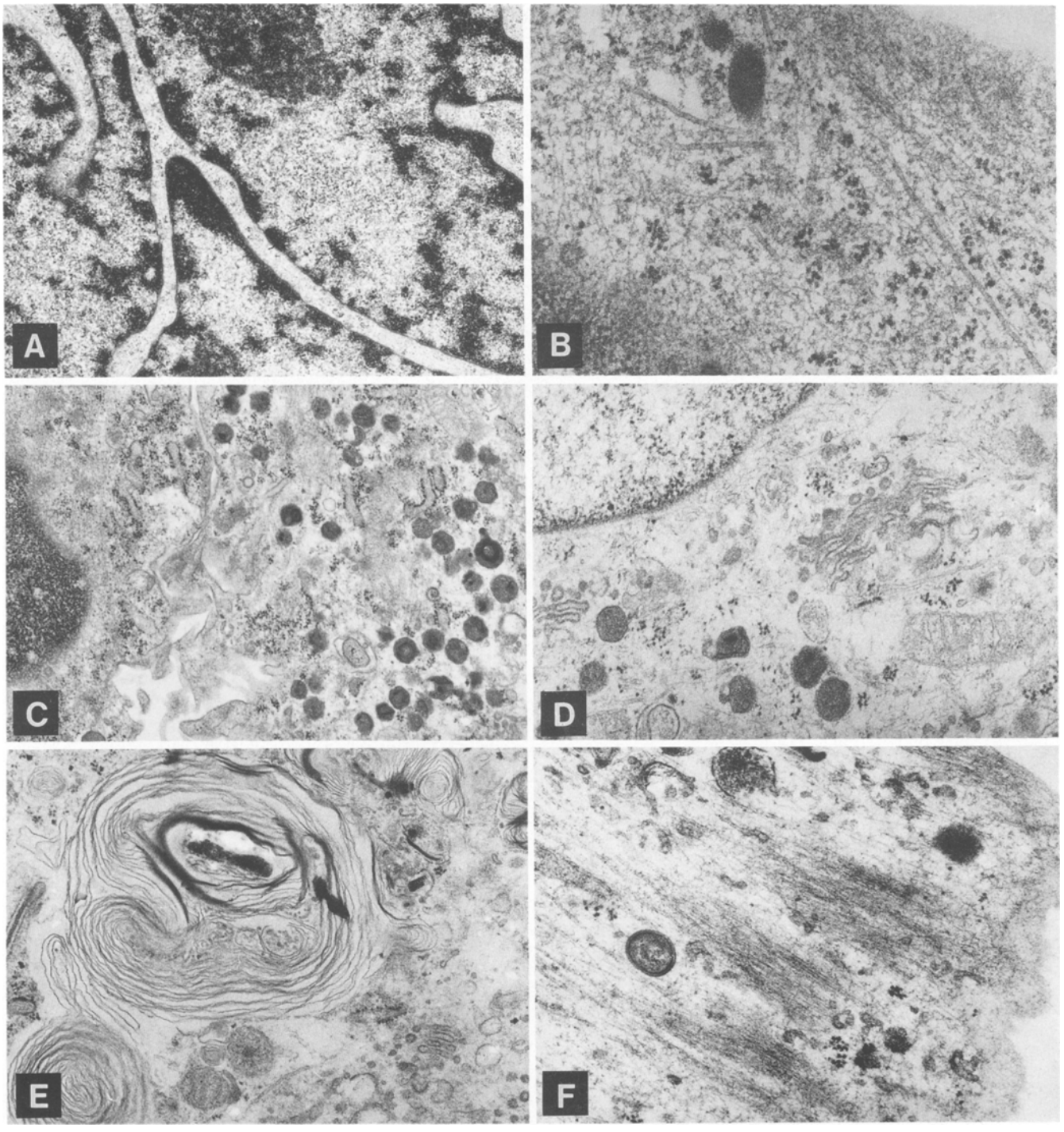


Fig. 9 A–F. TEM of human glioblastoma cell lines 86HG-39 and 87HG-28. Cell line 86HG-39 in the 45th in vitro passage (**A–D**). Cell nuclei are hypodense, contain several nucleoli and chromatin clumps and show multiple invaginations (**A**). The cytoplasm is rich in filaments and polysomes (**B**), autophagolysosomes and a normal Golgi apparatus (**C, D**). Cell line 87Hg-28 in the 50th in vitro passage (**E, F**). Ultrastructure is similar to 86HG-39. Additionally, myelin-like structures can be found in the cytoplasm (**E**). It is rich in filaments, RER is rare but free ribosomes are multiple (**F**). **A**: ×20000; **B, E**: ×25000; **C, D, F**: ×30000

brain, but which do not recognize normal adult brain (Stavrou et al. 1987). The most interesting result concerning the expression of GAA was its constant reactivity during the whole cultivation period and even on paraffin sections of the tumours. Chromosomal analyses of the cell lines 86HG-39, 87HG-28 and 87HG-31 are still in a preliminary stage. Although it could be shown that the profiles found in cells used for immunization were typical for human malignant gliomas, it cannot be concluded that mAbs constructed from these cell lines define antigens representative for gliomas.

The presence or absence of antigens depends on changes in gene expression rather than changes in the tumour cell genome. The loss of immunoreactivity dur-

ing in vitro growth of gliomas is probably due to a failure of cells to express these genes under culture conditions. The capacity of glioma cells to change the expression of genes depending on environmental conditions makes the pathogenesis and biological behaviour of these neoplasms difficult to understand, but offers the opportunity to influence tumour growth by targeting glioma cells with factors which can induce their differentiation. Although the respective reaction potentials of mAbs against GAA are restricted by several cross-reactivities these mAbs seem to be useful molecules for glioma cell targeting.

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