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In vitro Karyotypic and Immunophenotypic Characterisation of Primitive Neuroectodermal Tumours: Similarities to Malignant Gliomas

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Monoclonal antibody (Mab) mediated immunotherapy of brain tumours requires the identification of tumour-restricted cell surface antigens. We have characterised four primitive neuroectodermal tumours, which included pineoblastoma, medulloblastoma and ependymoblastoma cultures, that demonstrated *in vitro* evidence of malignant behaviour (anchorage-independent growth and nu/nu xenograft tumour formation). The cytogenetic findings ranged from normal G-banded and Q-banded karyotypes through mixed near-diploid/hyperdiploid. These cultures resembled the cell surface immunophenotypic spectrum of malignant gliomas. They were distinguished from normal glia *in vitro* by the expression of restricted fetal mesenchymal, neuronal, myoblastic, melanocytic, epidermal, chondrocytic, lymphoid and epithelial antigens. Certain antigens appeared sufficiently represented among central nervous system (CNS) neoplasms to afford potential targets for Mab-mediated immunotherapy.
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INTRODUCTION

LITTLE is understood of the biological determinants of central nervous system (CNS) neoplasms which are responsible for their poor prognosis. However, tumour-specific traits may be exploited for the development of novel therapies. This preliminary report seeks to correlate the cytogenetic findings, experimental growth characteristics (anchorage-independent growth, nu/nu xenograft tumour formation) and the surface immunophenotype of early passage CNS primitive neuroectodermal tumours (PNET), which include pineoblastoma (PBL), medulloblastoma (MBL) and ependymoblastoma (EPD) cultures. The purpose was to identify qualitative and quantitative differences in cell surface immunophenotype between normal glia with malignant gliomas and PNET, which may be targeted for passive immunotherapy with monoclonal antibodies (Mabs) [1, 2]. The process of inquiry has emphasised the demonstration of tumour-restricted cell surface antigenic expression with correlation to other attributes of the transformed phenotype. The tumour cell surface immunophenotype is relevant for therapeutic purposes as systemically administered Mab will probably be afforded access principally to cell surface and interstitial antigens. Previous studies have demonstrated general correspondence between *in vivo* and *in vitro* expression for many of these antigens [3, 4].

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PATIENTS AND METHODS

Derivation of cell cultures

Solid tumour cell cultures of intrinsic CNS neoplasms were generated and characterised, as previously described [1]. The specimens were obtained through the tumour procurement

services of the participating hospitals; the procedures were reviewed and approved by their respective institutional review boards. The malignant nature of the cultures was confirmed through both cytogenetic analysis and accepted experimental measures of malignant behaviour such as anchorage-independent growth and the ability to form Swiss nu/nu mouse xenografts, as previously described [1]. The presence of glial fibrillary acidic protein (GFAP) *in vitro* was established as previously reported [5]. Only mycoplasma-free specimens were used for analysis [6]. Cultures derived from recurrent tumours were designated -R. All specimens were coded to ensure confidentiality and to avoid bias regarding pathogenesis or embryogenesis.

Culture SK-PBL-1-R was established at autopsy from a PBL arising within the pineal recess of an 11-month-old male, who had been initially diagnosed at 5 months of age. Culture SK-MBL-10 was derived from a primary MBL resected from the vermis cerebelli of a 16-year-old female, who died after 27 months of therapy. The MBL culture, IARC-186, was generated from the cerebrospinal fluid of a 4-month-old infant with a disseminated MBL of the cerebellum.This child died 6 weeks after diagnosis. The EPD culture, SK-EPD-2-R, was derived from a recurrent tumour resected from the lateral ventricle of a 29-month-old male, 9 months into his illness.

Karyotyping procedures

Chromosomal analysis of no less than 30 informative metaphases was performed both among the primary cultures and the earliest possible passage, using the convention of the International System for Human Cytogenetic Nomenclature [7, 8]. Karyotypically delineated stem-lines and side-lines were categorised as normal, near diploid (35–57 chromosomes/metaphase), hyperdiploid (58 to > 100 chromosomes/metaphase) or mixed populations.

Immunohistochemistry

The neuropathologic diagnosis of these PNET was based upon WHO criteria and the presence or absence of GFAP, S-100 protein (S-100), "neuron specific" enolase (NSE), neurofilament, chromogranin, desmin, Leu-7 and leucocyte common antigen (LCA) [9]. Immunohistochemical analysis was performed using formalin fixed, paraffin embedded tumour specimens. Immunoreactivity with monoclonal antibodies to GFAP (Boehringer Mannheim), neurofilament (Biognex), chromogranin (Hybritech), Leu-7 (Becton Dickinson), LCA (Dako) was detected using the three step indirect method with peroxidase conjugated rabbit antimouse and swine antirabbit antibodies (Dako, 1:40 dilution). Diaminobenzidine (DAB) was used as the peroxidase substrate. Polyclonal antisera recognition of S-100 (Dako, 1:250), NSE (Dako, 1:150) and desmin (1:50 dilution, Euro-Diagnostic BV) was detected using the peroxidase–antiperoxidase (PAP) technique (Dako) [10].

Serological assays

The immunophenotype was determined using a panel of antigenic systems selected for their utility in identifying lineage-consistent, lineage-independent and putative tumour-restricted surface antigenic expression [11]. The serological mixed haemadsorption assay (MHA), protein A assay and quantitative absorption assays for the identification of the surface immunophenotype of cultured cells have been described previously [3]. High-titred murine Mab-enriched serum or ascites were sequentially diluted for the determination of these reactivity curves, with the exception of supernatant which was used for S6. Titration end-points were presented using a semi-log scale, as previously reported [1, 2].

RESULTS

Histological and immunohistochemical description of tumour specimens

SK-PBL-1-R. On histological examination, this appeared to be an extremely cellular neoplasm composed of primitive, often carrot-shaped, cells with extreme nuclear hyperchromasia and limited, ill-defined cytoplasm. Numerous mitoses and extensive necrosis were present throughout the tumour. Classic rosette formation was not seen in the areas sectioned. There was no evidence of astrocytic, oligodendroglial, ependymal or rhabdomyoblastic differentiation (Fig. 1a). Immunohistochemical analysis of SK-PBL-1-R demonstrated extensive NSE and Leu-7 immunostaining, and scattered neurofilament immunoreactivity. No GFAP, S-100, chromogranin or LCA immunoreactivity was detected.

SK-MBL-10. Histologically, this was a highly cellular neoplasm of primitive relatively uniform cells with ovoid hyperchromatic nuclei and ill-defined cytoplasmic boundaries. Poorly defined Homer Wright rosettes were presented throughout the neoplasm accompanied by numerous mitoses and focal necrosis. No ganglionic, astrocytic, oligodendroglial, ependymal or rhabdomyoblastic differentiation was identified (Fig. 1b). Immunohistochemical studies revealed extensive NSE and Leu-7 immunostaining, rare neurofilament immunoreactivity without evident GFAP, S-100, chromogranin or LCA immunostaining. Scattered GFAP and S-100 immunoreactivity was seen in reactive astrocytes in the surrounding cerebellum.

IARC-186. On light microscopy, the original tumour biopsy was hypercellular with irregularly shaped basophilic nuclei and scanty eosinophilic cytoplasm. A small number of cells showed eccentric nuclei containing several nucleoli and more abundant cytoplasm. A well-developed stroma was observed. There was no ependymal, neuronal or myoblastic differentiation evident on Mallory and Masson's staining. Electron microscopy of the tumour exhibited irregular tumour cells with indented nuclei and dark and light cytoplasm. Numerous intermediate filaments filled the cytoplasm of the tumour cells and a few lipid droplets were encountered. True synapses and neurotubules were not seen but small intermediate junctions were consistent with primitive synaptic contact. Immunochemistry demonstrated the filaments to be GFAP positive and desmin negative [12]. A more complete description of this cell line is in preparation.

SK-EPD-2-R. Histologically, this was an extremely cellular neoplasm with focal necrosis and infiltration of brain parenchyma. The relatively uniform primitive tumour cells formed numerous ependymal rosettes in the tumour proper and infiltrating edges. Tumour cells displayed severe nuclear hyperchromasia and a very large number of mitoses. However, giant cell formation, astrocytic, oligodendroglial, ganglionic and rhabdomyoblastic differentiation were not evident. There was no papillary formation (Fig. 1c). Immunohistochemical analysis revealed moderate Leu-7 immunostaining but no GFAP, S-100, neurofilament, chromogranin or LCA reactivity. Scattered GFAP immunoreactivity was seen in reactive astrocytes near points of parenchymal invasion.

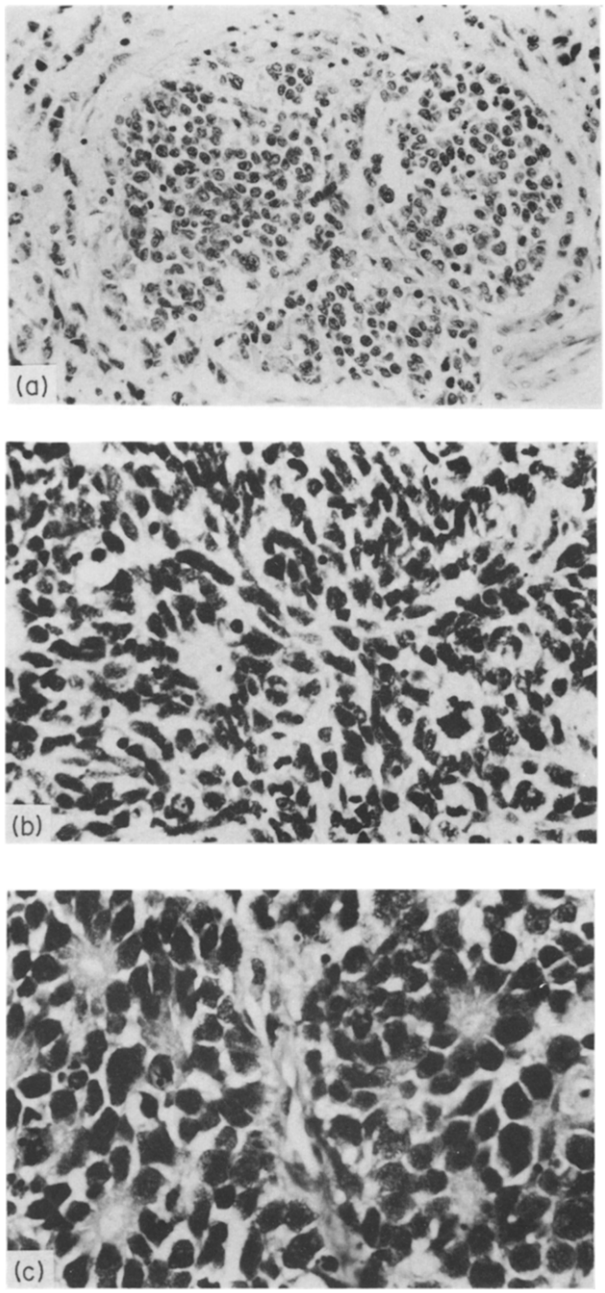

Fig. 1. Histological appearance of original PNET from which the cell cultures were derived. (a) Pineoblastoma (SK-PBL-1-R). Rare nest of malignant cells reminiscent of pineal architecture were encountered among sheets of undifferentiated small cells comprising this neoplasm (hematoxylin and eosin, magnification = 320 ×). (b) I11-defined Homer Wright rosettes are present throughout the medulloblastoma (SK-MBL-10) depicted in (b) (hematoxylin and eosin, magnification = 280 ×). (c) Ependymal rosettes of ependymoblastoma (SK-EPD-2-R) with central lumina formed by primitive neuroectodermal cells with markedly hyperchromatic nuclei (hematoxylin and eosin, magnification = 360 ×).

In vitro *characterisation of the PNET cultures*

To minimise the possibility of *in vitro* artefact, the four PNET cultures were characterised earlier. Each demonstrated functional evidence of malignant behaviour *in vitro*, as indicated by anchorage-independent growth and nu/nu xenograft tumour formation (Table 1). In contrast to the immunohistochemical characterisation, cultures SK-PBL-1-R, SK-MBL-10, and SK-EPD-2-R expressed GFAP (data not shown). The IARC-186 culture expressed GFAP, as did the original tumour, and grew indefinitely *in vitro*. Serial passage of IARC-186 in Swiss nu/nu mice was successful. The light microscopic examination of the nu/nu xenograft tumour was notable for deep basophilia and hypercellularity, with a well-developed stroma. There was no ependymal or myoblastic differentiation, which was similar to the original biopsy [12].

Karyotypic analysis of 30 or more metaphases was completed for each culture. The SK-PBL-1-R culture was composed of cells which were karyotypically normal 46, XY, by G-banding and Q-banding. SK-MBL-10 revealed mixed near-diploid and hyperdiploid populations. A representative stemline karyotype is exhibited (Fig. 2). The IARC-186 culture demonstrated a 46, XY karyotype with normal G-banding and Q-banding, with a minor sideline of tetraploid cells which appeared to be endoreduplicated. The EPD culture, SK-EPD-2-R, was composed of several populations of normal, near-diploid and tetraploid cells (Table 1).

Immunophenotypic profile

A panel of Mabs has defined qualitative and quantitative differences in cell surface antigenic expression between normal and malignant glial cells *in vitro*. The immunophenotypes (Tables 1–4) are arranged for ease of comparison with those previously reported for normal glia and malignant gliomas [1, 11].

DISCUSSION

Immunohistochemical characterisation, with antisera against S-100, NSE, synaptophysin, neurofilament proteins, intermediate filaments as well as other antigens, has been proposed to clarify differentiation and the diversity of lineages among PNET [13–17]. However, the diagnostic specificity of individual immunohistochemical reagents for such purposes has been questioned by few investigators [14, 18, 19]. Application of large panels of reagents reactive to neuroectodermal markers, astrocytic and neuronal cytoskeletal proteins and hematopoietic-lymphoid antigens has revealed intra- and intertumoural heterogeneity among PNET [20–25]. However, no correlation between

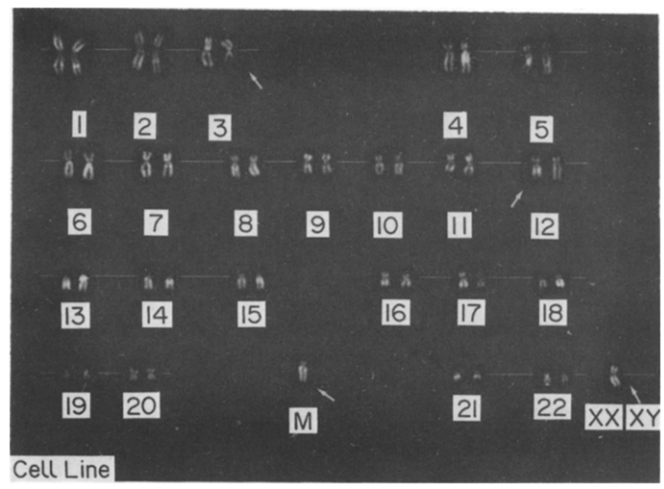

Fig. 2. Representative stemline karyotype from SK-MBL-10 cell line, passage (3). Karyotype 46,X-X,del(3)(q21)/96,XX, del(3)(q21). The arrows indicate the abnormal chromosomes found in this (2n) karyotype. The del(3)(q21) and the absence of a single sex chromosome (X) were observed in all cells analysed. Other markers indicated (a marker 12, and an unidentifiable marker) were unique to this karyotype.

Table 1. Karyotypic analysis (modal chromosome number and range, and stem-line karyotype), anchorage-independent growth (AI), nu/nu xenograft tumour formation (NU), as well as group I immunophenotype of the MBL, PNET and EPD cultures

	Cytogenetic characterisation					Group I phenotype			
	Modal	Range	Stem-line karyotype	AI	NU	CNT/11	AJ8	A010	HLA-DR
SK-PBL-1-R	46	(42–46)	46,XY	+	+	4	—	5	—
SK-MBL-10	46	(39–90)	46,X,−X del(3) (q21)/ 90,XX, del(3) (q21)	+	+	5	—	—	—
IARC-186	46	(38–92)	46,XY/ 92,XXYY*	+	+	—	—	5	5
SK-EPD-2-R	46	(37–92)	46,XY/ 45,X,−Y*/ 92,XXYY*	+	+	5	—	5	—

* Side-lines consisting of a fraction (5–30%) of the studied metaphases.
Cultures were ranked in terms of progressive cytogenetic divergence. The relative karyotypic distribution among mixed cultures is depicted by the order of presentation, with the most common first.

expression of cellular markers and the biological behaviour of the putatively embryonal CNS neoplasms has been identified as yet [26].

Our previous investigations established qualitative differences between the surface immunophenotype of normal and malignant glia *in vitro*. The low-grade astrocytomas were distinguished from normal glia by their mixed near-diploid karyotype and anchorage-independent growth *in vitro*. In contrast, malignant gliomas formed nu/nu xenograft tumours and demonstrated a progression of cytogenetic abnormalities which ranged from intermixed normal and near-diploid stem-lines, through mixed near-diploid/hyperdiploid to predominantly hyperdiploid cultures. This spectrum of worsening cytogenetic complexity correlated with qualitative and quantitative immunophenotypic divergence between normal glia and malignant gliomas. 17 neuroectodermal and non-neuroectodermal antigens (the "group II" antigens) were expressed predominantly among the hyperdiploid malignant gliomas but were not detected on normal glia *in vitro*. Anaplastic astrocytomas and glioblastomas exhibited restricted fetal mesenchymal, neuronal, myoblastic, melanocytic, epidermal, chondrocytic, trophoblastic, lymphoid and epithelial antigens. Statistical correlations between immunophenotype with the histopathological grade of glioma, natural history and duration of clinical survival suggested the hypothesis that certain antigens may confer a Darwinian advantage to the expressing neoplasm [1, 2].

Within this same study, these PNET tumour cultures were characterised and proven to demonstrate malignant behaviour *in vitro*. The spectrum of cytogenetic findings ranged from normal G-banded and Q-banded karyotypes through mixed near-diploid/hyperdiploid (Table 1). No specific chromosomal anomaly was noted, nor was there an obvious karyotypic–histopathological association. However, these malignant neuroepithelial cultures were distinguishable from normal glia by the differential expression of the group I neuroectodermal tumour antigens and qualitative immunophenotypic differences for certain of the group II antigens *in vitro*. Notably, coded analysis did not diagnostically segregate the PNET from the malignant gliomas [1]. These newly established tumour cultures variably expressed restricted fetal mesenchymal (CNT/11), neuronal (A010), melanocytic (M111/M231, C350), myoid (5.1H11, 24.1D5), chondrocytic (OST7), lymphoid (HLA-DR, LEU13) and epithelial (M111/M231) surface antigens (Tables 1–3). In contrast, a putative marker of MBL and PNET, UJ181.4, was not found to be expressed as a cell surface antigen [27]. The well-known difficulties in establishing PNET and MBL cultures have prevented accession of sufficient numbers to establish a statistical correlation between surface immunophenotype and the natural history of these diseases. Functionally important antigens would afford more attractive targets for Mab-mediated immunotherapy. Attention to shared immunophenotypic aberrations among different CNS neoplasms may identify prognostically relevant antigens.

Table 2. Cell surface expression of the group II antigens among the tumour cultures

	Group II phenotype								
	CNT/2	UJ181.4	2G9	OST7	M111	M231	C350	B2.6	C437
SK-PBL-1-R	—	—	—	3	—	—	—	—	—
SK-MBL-10	—	—	—	—	—	—	—	—	—
IARC-186	—	—	—	3	5	5	—	—	—
SK-EPD-2-R	—	—	—	—	—	—	—	—	—

Table 3. Cell surface expression of the group II antigens among the tumour cultures

	Group II phenotype					
	MC25	CAT301	BT-9	LK26	LEU13	5.1H11
SK-PBL-1-R	—	—	—	—	—	—
SK-MBL-10	—	—	—	—	3	5
IARC-186	—	—	5	—	—	3
SK-EPD-2-R	—	—	—	—	—	—

Table 4. Cell surface expression of the group III antigens among the tumour cultures

	Group III phenotype										
	3F8	R24	S4	24.1D5	G171	S6	F24	V1	AJ225	I12	MC139
SK-PBL-1-R	5	—	2	—	4	3	5	—	3	4	5
SK-MBL-10	2	—	†	5	3*	2	—	—	4*	3	4
IARC-186	4	—	†	4	5	—	—	5	4	4	5
SK-EPD-2-R	4	—	3	—	3	1	5	—	—	—	—

* Side-lines consisting of a fraction (5–30%) of the studied metaphases.
† Result not available/not done.

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