Characterization of initiated cells in *N*-methylnitrosourea–induced carcinogenesis of the CNS in the adult rat¹

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Glial tumors may originate from the malignant transformation of multipotent glial progenitor cells, but tools to study malignant transformation leading to gliomas are limited by the lack of biological systems that represent early stages of this disease in adult animals. In order to characterize the initiated cells that give rise to gliomas, we have employed the *N*-methylnitrosourea (MNU) model

for induction of brain tumors in adult rats (Rushing et al., 1998). Specifically, we have isolated and cultured transformed (pre-malignant) cells from normal-appearing brains of rats exposed to MNU for 10 weeks and from histologically abnormal brains of rats exposed to MNU for 15 weeks. We compared them with cells cultured from control animals under identical conditions. Cultured cells were classified according to their morphology, immunophenotype, karyotype, proliferation capacity, and tumorigenicity in athymic mice. Cultures from untreated normal rat brains grew as monolayers and had normal karyotypes (42 X,Y), epithelioid morphology, and slow proliferative capacity (doubling time >120 h). In contrast, cultured cells from brains of MNU-exposed animals had karyotypes that ranged from normal to highly aneuploid. Aneuploid lines grew rapidly in multilayers (doubling time <24 h), had differentiated astrocytic or oligodendroglial morphology and immunohistochemical staining profile, and yielded tumors in athymic mice. Initiated cells with minor chromosomal aberrations assumed mixed bipolar or tripolar morphologies in high density cultures, proliferated rapidly, but showed contact inhibition and failed to induce tumors when injected s.c. in athymic mice. In general, lines showing no evidence of chromosomal aberrations had the most epithelioid morphology, proliferated slowly (doubling time >72 h), and retained strict contact growth inhibition. The presumed undifferentiated glial progenitor cells in culture from either control or MNU-treated rats variably expressed markers such as vimentin, nestin, and NG2 proteoglycan, and they weakly expressed the mature astrocytic or oligoden-

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⁵Abbreviations used are as follows: bFGF, basic fibroblast growth factor; BrdU, bromodeoxyuridine; db-cAMP, dibutyl-cyclic-AMP; GalC, galactocerebroside; GFAP, glial fibrillary acidic protein; MAP-2, microtubule-associated protein-2; MNU, *N*-methylnitrosourea; PBS, phosphate buffered saline; SWRB, Southwestern rat brain line; TRITC, tetramethylrhodamine isothiocyanate.

droglial markers glial fibrillary acidic protein or galactocerebroside, respectively. These cultures differentiated to bipolar-tripolar morphology with concomitant maturation to a GFAP+ or GalC+ phenotype upon exposure to secondary messengers such as dibutyryl-cyclic-AMP and/or growth factors such as basic fibrillary growth factor. Continuous stimulation with these messengers resulted in terminal differentiation and consequent death upon withdrawal of the stimulus. These results provide information that could lead to detailed characterization of initiated, premalignant cells in the adult brain and to a better understanding of glial carcinogenesis. *Neuro-Oncology* 3, 99–112, 2001 (Posted to *Neuro-Oncology [serial online]*, Doc. 00-041, February 27, 2001. URL <neuro-oncology.mc.duke.edu>)

A prominent hypothesis regarding the induction of primary tumors in the adult CNS is that they arise from proliferating precursor or stem cells (Cushing and Bailey, 1928; Goldman, 2000). This hypothesis is contrasted with the theory that tumors arise from differentiated mature cells that are mutated and subsequently undergo a series of transformations to a less differentiated phenotype (Mabron et al., 1950; Doetsch et al., 1999). The involvement of precursor cells in CNS carcinogenesis is supported by evidence that replicating multipotential stem cells exist in the adult rodent or primate brain (Gage, 1998; Gould et al., 1998, 1999; Goldman, 2000; Johansson et al., 1999; Palmer et al., 1997) and are even demonstrable in humans (Eriksson et al., 1998; Frisen et al., 1998; Gage, 2000). The hypothesis that the rare progenitor stem cell rather than the mature glial cell is the target of carcinogens could explain the common occurrence of histologically “mixed” glial tumors in the CNS, and it is also consistent with the recognized ability of gliomas to infiltrate surrounding brain parenchyma (Silbergeld and Chicoine, 1997). Recent studies describe a normal resident population of multipotent stem cells located on or near the lateral ventricles, usually in the subventricular zone. In rodents, this population replicates and migrates outward through adjacent differentiated brain, developing into mature cell types, including astrocytes, oligodendroglia, and less frequently, neurons. Division of these cells, and thus their susceptibility to mutagens, can be demonstrated in vivo by labeling with ^3H -thymidine, viral infection, or bromodeoxyuridine (Doetsch et al., 1999; Eriksson et al., 1998; Gould et al., 1998, 1999; Johansson et al., 1999; Levison et al., 1999).

Important information regarding the progression of CNS neoplasms has been derived mainly from examining human surgical specimens, but complexities of the biologic behavior and biochemical and genetic characteristics of brain tumors require the development of animal models and in vitro systems. The use of transgenic mice with mutant *EGFR* (epidermal growth factor receptor; Holland et al., 1998), activated *RAS* (Ding et al., 1999; Holland et al., 2000), aberrant *v-erbB*,⁴ or homozygously deleted *P53* (Donehower et al., 1992; Jacks, 1996) may provide interesting test systems for the involvement of these genes. However, these mice express oncogenes in an aberrant manner in all glial cells, so these systems may be less applicable in determining the

pathways relevant to the sporadic development of CNS tumors in humans in which the tumor might originate from a single cell. Historically, alkylating agents such as ethylnitrosourea have often been used for induction of brain tumors in rats (Kleihues et al., 1968; Koestner et al., 1971; Padhy et al., 1982). Transplacental mutagenesis of rats with ethylnitrosourea induces a variety of CNS tumors (Schubert et al., 1974), whereas early postnatal exposure induces peripheral Schwann cell and glial-like tumors (Kindler-Rohrborn et al., 1999; Perantoni et al., 1987). The model has been successful in identifying the significance of the *neu* oncogene in the induction of schwannomas (Nikitin et al., 1991) and holds promise for investigating genetic events leading to induction and propagation of gliomas. The problem with both the transplacental or the early postnatal exposure to ethylnitrosourea is that the target cell for the carcinogen may not be the same as the one leading to CNS tumors in adults. Certainly, development of various types of tumors in rats indicates that ethylnitrosourea has more than one cellular target when given during development, and a less complex scenario is expected in adult animals in which the number of potential targets is limited. The induction of malignant gliomas by exposure of adult animals to carcinogens is technically challenging, but it has been achieved by continuous exposure of Fisher rats to MNU⁵ (Shibutani et al., 1993). We have also induced a high incidence of glial lineage tumors (>80%), including low-grade astrocytomas, oligodendrogliomas, anaplastic astrocytomas, and glioblastomas multiformes in Sprague-Dawley rats, using MNU (Rushing et al., 1998). The large percentage of mixed glial neoplasms of various grades suggests that the model is appropriate for the study of progressive pathways (Louis and Gusella, 1995) in which tumors may arise from multipotent progenitor cells via intermediate formation of both astrocytic and oligodendroglial tumors. In this work, we seek to characterize the target cell that is altered by MNU during the carcinogenic process in the adult brain.

Materials and Methods

Animals

Thirty-six adult male Sprague-Dawley rats weighing 220 ± 10 g were obtained from Charles River (Wilmington, Del.) and were fed a commercial, complete diet (Prolab Feed RMH 3000; Agway, Waverley, N.Y.) until they reached 240 g. The animals were then divided into 3 groups of 12 each. Two groups were given slightly acidified water (pH 6.0) containing 100 ppm MNU for 10 and 15 weeks, respectively. The third group was given regular water and served as control (Fig. 1). MNU is relatively stable in water at pH 6.0 with a half-life of about 30 h, compared with 2.3 h at pH 7.0 (Lijinsky, 1992). At the end of each period (10 or 15 weeks), the respective MNU-exposed group and 6 controls were killed, and their brains were removed and cut sagittally under sterile conditions at 300- μm section thickness with a vibratome. Sections were alternatively fixed in 10% alcoholic formalin (PenFix; Richard Allen Medical, Richland, Mich.) for histologic examination, or placed into tissue culture (Fig. 1). Six sections from each

Fig. 1. Presentation of the in vivo/in vitro systems for the initiation of brain carcinogenesis in rats and subsequent culture of mutated mobile cells. In vivo: Mature Sprague-Dawley rats are exposed to MNU (100 ppm) in the drinking water continuously for 10 or 15 weeks. Controls are not exposed to MNU. In vitro: At the end of treatment, brains are sectioned at 300 μ m and placed on filters wetted with growth medium. Cells that are migrating to the filters are cultured to yield normal progenitor cells of flat epithelioid morphology (group A), mutated progenitor-flat epithelioid cells (group B), and glioma cells at various stages of maturation (group C).

brain were placed into tissue culture. Tumorigenicity experiments were performed on 4-week-old Balb/C *nu/nu* athymic mice purchased from Simonson (Gilroy, Calif.). Mice were maintained under germ-free barrier conditions and were given sterilized food (Teklad Laboratory Diet; Harlan, Indianapolis, Ind.) and water.

Tissue and Cell Culture

Tissues and cells were cultured in Eagle's minimum essential medium supplemented with lysine, valine, methionine, and leucine (100 μ M each) nonessential amino acids (1:100 dilution of stock), 1 mM sodium pyruvate, 1 μ M cyanocobalamin, 10 μ M folic acid, 0.2 mg/ml gentamicin, and 10% fetal bovine serum (GIBCO-BRL, Gaithersburg, Md.). Sections of brain tissue were placed on 40- μ m pore size Millicell-CM (Millipore, Bedford, Mass.) filters and incubated in 100-mm diameter plastic dishes containing 7 ml of medium, replaced at 3-day intervals. Under these conditions, cells with the ability to migrate moved from the tissue section to colonize the filter, both at the tissue-filter interface and also peripherally to the tissue. These cultures were maintained for 3 to 4 weeks, at which time the tissue was removed. The cultures were washed with PBS, and the cell colonies attached on the filter were trypsinized and cultured in the same medium.

Proliferation Assays

Cell growth was evaluated by daily assessment of the cell number (growth curves) or by the determination of cells

synthesizing DNA. Growth curves were constructed from the colorimetric absorbance resulting from cells fixed at daily intervals when stained with crystal violet. The labeling index was determined from immunostaining cells for BrdU incorporated into DNA after 24-h pulse treatment. After the cells were fixed, BrdU incorporated into DNA was identified using tetramethyl-benzidine-coupled antibodies (Boehringer Mannheim, Indianapolis, Ind.).

Tumorigenicity

Tumorigenicity of cultured cells was assessed by injecting 10^6 cells in 100 μ l of medium s.c. into the flank of athymic mice. Mice were observed for 90 days. When tumors were detected, they were measured in 2 perpendicular dimensions, and their volumes were estimated using the ellipsoid formula ($a^2 \times b/2$), where a is the shorter and b the longer of the two dimensions. Animals having a tumor burden greater than 1200 mm³ were killed and tumors were excised. Tumors were processed for histology or tissue culture or were frozen in liquid nitrogen either by snap-freezing or after shredding in 20% dimethyl sulfoxide in fetal bovine serum.

Immunocytochemistry

Cell monolayer cultures grown on glass coverslips or 35-mm plastic dishes were fixed in PBS-buffered 4% paraformaldehyde (pH 7.4) for 30 min at 4°C. Cultures were permeabilized with PBS and 1% Triton X-100 (PBST), washed with PBS, and placed in blocking solution consisting of either 1% bovine serum albumin in

PBS or of 2% horse serum, 1% bovine serum albumin, and 0.1% Triton X-100. Diluted primary antibodies were applied in blocking solution. Primary antibodies, provenance, and dilutions were as follows: galactocerebroside (Sigma-Aldrich, St. Louis, Mo.; G9152 rabbit anti-GC, diluted 1:50), GFAP (DAKO, Carpinteria, Calif.; rabbit anticow, 1:1000; or Sigma-Aldrich; G-A-5 mouse antipig, 1:100), neurofilament (Developmental Studies Hybridoma Database, DSHD, Iowa City, Iowa; clone RT97, mouse antirat, hybridoma supernatant diluted 1:5), nestin (DSHD; mouse antirat, clone rat-401, 1:5), and NG2 (Chemicon, Temecula, Calif.; rabbit antirat, 1:300). In some experiments, immunoreactivity was detected by fluorescence with appropriate fluorescein isothiocyanate- or TRITC-labeled secondary antibodies diluted 1:200 and nuclear counterstaining with 4 μ g/ml Hoechst 33258 in VectaShield mounting media (Vector Labs, Burlingame, Calif.). In other cases, detection was via biotinylated secondary antibody and the avidin-biotin complex method (Vector Elite kit; Vector Labs) with DAB as chromogen and Crystallmount mountant. Images were captured using an Axiophot epifluorescent microscope (C. Zeiss, Inc., Thornwood, N.Y.) equipped with a digital camera (SenSys, Singapore, Singapore). Multicolor images were combined with the Quips PathVisionTM software (Vysis Inc., Downers Grove, Ill.).

Results

Growth and Morphology

Primary cultures of carcinogen-modified cells were established by histoculturing 300- μ m-thick sections derived from brains of animals exposed to MNU (Fig. 1). A 10-week exposure resulted in histologically normal brains that had no identifiable lesions or tumors. Exposures of 15 weeks resulted in histologic alterations, most often gliosis, focal hemorrhage, increased apoptosis, mitosis, and focal cellularity. Twenty-eight of the 60 histocultured brain sections derived from control animals yielded epithelioid cells with low proliferative activity. Similar cultures were obtained from 41 sections derived from 9 histologically

normal brains of animals that were exposed to MNU for 10 weeks. These cells were defined as stage I according to a previous designation of the first stage between normal and fully malignant glial cells (Laerum and Rajewsky, 1975). In contrast, 11 of 12 brains from animals treated for 15 weeks yielded a variety of morphologically distinct cells that ranged from epithelioid (stage I) to cells with bipolar and tripolar morphologies (stage II), which frequently progressed to acquire morphologies and growth patterns that were reminiscent of glial tumor cell lines (stages III and IV). Overall, 28, 41, and 55 primary cultures were obtained from control animals and those treated for 10 weeks and 15 weeks, respectively.

Slow-dividing cells from control and 10-week exposure brains did not express fibronectin or *PECAM-1* (platelet endothelial cell adhesion molecule-1) and were not fibroblasts, meningeal cells, or endothelial cells. An average of 60 days was required for these cells to become confluent (Fig. 2). When cells derived from brains that had been exposed to MNU for 10 weeks were reseeded before they reached confluency, they retained their epithelioid morphology and marginal proliferative activity. However, when cells were cultured under confluent conditions, they were transformed into "pockets" of round flat cells with dense nuclei (Fig. 3A) and consequently assumed astrocytic morphology (Fig. 3B). These cells differentiated upon continued culturing into monolayers of dense bipolar and tripolar astrocyte-like cells (Fig. 3C) (stage II). Transformation to an astrocytic morphology in dense cultures was observed only in cells isolated from animals exposed to MNU, and this was the first evidence of a biologic distinction between control and MNU-exposed cells.

Stage II cells isolated from brains of rats exposed to MNU for 10 weeks did not show uniform behavior upon continued culturing. Three distinct types of growth and differentiation were observed: some astrocyte-like cells reverted back to flat undifferentiated morphology and continued to proliferate slowly until reaching high densities again; some stage II-type cells did not revert back to epithelioid morphology upon reseeding at low densities, but rather continued to grow indefinitely as mixed populations of bipolar, tripolar, and epithelioid

Fig. 2. Flow diagram of the stages in mutated glial progenitor cell maturation and differentiation. Cells derived from brains of animals treated for 10 weeks with MNU yield cultures of epithelioid-like, slow-proliferating cells (stage I). Continuous culture of these cells results in mixed cultures composed of bipolar, tripolar, or epithelioid cells (stage II). Stimulation of stage II cultures either leads to terminal differentiation (mature normal or mutated astrocytes) or, occasionally, to rapid contact uninhibited growth leading to densely populated cultures (stage III). Complete independence from growth factors and spheroid formation (stage IV) is usually associated with tumorigenicity.

Fig. 3. Appearance of oligodendrocytic- (A) and astrocytic-like (B) cells in pockets within confluent mutated glial progenitor cells in stage I cultures. Continuous culture under confluent conditions yields stage II cultures, a mixture of bipolar and tripolar cells that continue to divide (C). All cultures of cells derived from brains of 15-week MNU-treated animals and 1 of 5 of those derived from 10-week MNU-treated animals propagated to contact uninhibited growing cultures, stage III (D). Original magnification $\times 75$.

cultures; and some stage II-type cells progressed to stage III cells, a state in which growth was not limited by cell contact and grew in multilayer of astrocytic or oligodendroglial cultures (Fig. 3D). Acquisition of the ability to form spheroids (stage IV) occurred in 1 cultured line derived from a 10-week exposure brain (SWBR6) between the 10th and 15th passage, and it was tumorigenic in athymic mice.

Unlike cells isolated from brains of animals exposed to MNU for 10 weeks, which grew as monolayers and in most cases failed to progress beyond stage II, those isolated from animals exposed to MNU for 15 weeks had the capacity to grow in multilayers at an early passage (3-4 passages), and most entered stage IV spontaneously.

Differentiation to astrocytic morphology was observed with all MNU-initiated, premalignant cells in stage I and II after stimulation with a combination of growth factors and db-cAMP (Fig. 4). Stage I cells (Figure 4A) readily differentiated in the presence of db-cAMP (Fig. 4B) or bFGF (Fig. 4C), but more notably, in the presence of both (Fig. 4D). As shown in Table 1, stage I cells did not respond to low concentrations of cAMP or bFGF. At higher concentrations of db-cAMP and bFGF, rates of proliferation increased to levels that were markedly greater than those of controls. Proliferation that could be sustained for more than 9 days was observed with the synergistic combination of 200 μ M db-cAMP and 20 ng/ml bFGF, which yielded fully differentiated cultures with fine processes (Fig. 5). Although higher concentrations of db-cAMP/bFGF stimulated growth for 3 to 4 days, such concentrations eventually resulted in inhibition of DNA synthesis, as determined by the reduction in the number of cells that incorporated BrdU (Table 1). Low rates of proliferation in continuously stimulated cultures suggest that differentiation limits the proliferation rate even in these low stages of transformation. Prolonged exposure to growth factors caused cultures to senesce, although senes-

cence was not observed in undifferentiated cultures. Withdrawal of bFGF in fully differentiated cultures resulted in acute cell death. Terminal differentiation and cell death could be avoided by the use of low concentrations of bFGF and db-cAMP for a limited time (6 days). Withdrawal of growth factors was required to avoid terminal differentiation. In 1 case (SWRB6), growth factor withdrawal yielded differentiated and viable cells in culture (stage III). However, in most cases, early withdrawal of the stimulating factors resulted in de-differentiation of the cells into epithelioid cells (stage I-II).

Evidence for the transformation of epithelioid cells into astrocytic morphology, as opposed to selection of a certain population of cells upon stimulation with growth factors, was obtained by focally stimulating cultures at stage I with bFGF. Within 6 h from the addition of the growth factor focally at the edge of a colony of epithelioid cells, a 2-stage transformation was noted to occur. Cells developed delicate cytoplasmic processes, followed by reduction of the cytoplasmic volume (Fig. 6). This demonstrated that continuous culture of cells in stage I does not serve in "selecting" a certain minority of cells, but in propagation of a general population of cells that have the ability to transform only under certain conditions. An initial increase in proliferative rates upon stimulation of stage I cells was demonstrated by incorporation of BrdU (Fig. 7). Twenty-four hours after stimulation, the number of astrocyte-like cells in the culture markedly increased, indicating differentiation of the flat cell into astrocytic morphology. Both types of cells incorporated BrdU, indicating that bFGF may stimulate DNA synthesis before epithelioid cells achieve a differentiated state.

Tumorigenicity in Athymic Mice

Sixteen cell lines (2 from control, 6 from 10-week MNU-exposed rats, and 7 from 15-week MNU-exposed rats)

Fig. 4. Stimulation of morphologic transformations of stage I cells with db-cAMP and bFGF. Stage I cultures from brains of animals exposed to MNU for 10 weeks (A) were supplemented with 200 μ M db-cAMP (B), 20 ng/ml bFGF (C), or both (D) for 48 h. Factor-supplemented medium was replaced every 24 h. Original magnification $\times 75$.

were tested for tumorigenicity by flank injection in athymic mice (Table 2). Two of the 10-week exposure lines were from passages 10 and 15 of SWBR6. Lines 22a and 22b were derived from different sections of the same brain, as were lines 26a and 26b. Cultures derived from brains of animals that were exposed to MNU for 15 weeks were tumorigenic when injected into athymic animals with only a single exception (SWRB22b). On the other hand, cells derived from brains of animals exposed to MNU for only 10 weeks were not tumorigenic with the exception of 1 line (SWRB6/p15), which became tumorigenic after being cultured for more than 90 days. The morphology of the induced tumors was typically spindled epithelial cells, characteristic of glial-derived tumor cell lines.

Karyotype

Giemsa banding of metaphase chromosomes derived from late passage cells from several lines showed a relationship between biologic behavior and karyotypic abnormalities (Fig. 8). Karyotypes from each end of the tumorigenic spectrum are shown in Fig. 8. Cell line SWRB7, with a karyotype showing only one dominant abnormality, the loss of rat chromosome 18, was not tumorigenic in athymic mice (Table 2). Thus SWRB7 behaved both cytogenetically and biologically as expected for a low-grade or even premalignant cell population. In contrast, metaphases from the tumorigenic cell line SWRB6/p15 (the only tumorigenic 10-week

Table 1. Effect of db-cAMP and bFGF on growth and BrdU uptake on stage I rat glial progenitor (SWRB7) cell culture

db-cAMP (μ M)	bFGF (ng/ml) ^a	Cell numbers at day (% of control)				BrdU uptake (% positive) ^b
		1	3	6	9	
0	0	—	—	—	—	89 ^c
0	20	100 ^d	97	99	95	87
0	60	120	163	258	320	89
200	0	98	96	89	97	92
400	0	120	177	253	312	85
200	20	150	245	356	415	97
400	20	120	160	154	165	56
400	60	190	320	280	189	21
400	80	230	423	322	231	16

^aMedium and factors were replaced every second day.

^bPercent of cells taking up BrdU at day 9. BrdU was added to the culture 24 h before termination of experiment.

^{c, d}Mean of 6 determinations.

Fig. 5. Viable, differentiated premalignant glial cells derived from stimulation of stage I cultures of cells extracted from MNU-exposed brains (10 weeks). The culture was stimulated 3 times every 42 h for 6 h with 200 μ M db-cAMP plus 20 ng/ml bFGF and washed with regular culture medium after each stimulation. Original magnification $\times 200$.

exposure line) showed a markedly aneuploid karyotype with mean chromosomal number of 87 (normal male rat = 42XY). Hyperdiploidy was seen for virtually all identifiable autosomes, with many unassignable translocations. Likewise, the tumorigenic line SWRB22a, which was derived from a 15-week exposure brain, revealed an aneuploid karyotype with a mean of 91 chromosomes and many complex translocations. The nontumorigenic MNU-initiated cell lines SWRB11 and SWRB21 revealed a normal 42XY complement of chromosomes, as did the 2 control lines SWRB5 and SWRB10. These results demonstrate that premalignant cells can be propagated *in vitro* with maintenance of a stable normal karyotype and that aneuploidy is associated with tumorigenicity.

Immunohistochemistry

Indirect immunofluorescence on representative cell lines with antibodies that recognize antigens found on mature and progenitor astrocytes and oligodendroglia demonstrated the probable progenitor nature of the cell lines derived from both the MNU-exposed and the control animals. Epithelioid cells from the control cell line SWRB5 were consistently positive for the neuroglial precursor marker NG2, a cell-surface proteoglycan. Positive cells stained uniformly in a plasma membrane pattern, consistent with the typical subcellular location for this protein (Fig. 9A). Nestin, an intermediate filament protein that has been described as an early marker for neuroglial progenitors, was also positive in line SWRB5, with flat cells that stained in a cytoskeletal pattern (Fig. 9B). In SWRB6, a line derived from the 10-week MNU exposure group that subsequently became tumorigenic after exhaustive culturing, most flat cells were nestin-negative, whereas occasional bipolar cells were positive (Fig. 9C). The most undifferentiated SWRB6 cells were consistently negative for all progenitor markers. However, NG2 staining was clearly demonstrated for occasional intermediate-stage SWRB6 cells (Fig. 9D). As SWRB6 cells differentiated to

Fig. 6. Focal stimulation of stage I cultures with the addition of 100 μ l medium containing 300 μ M db-cAMP and 40 ng/ml bFGF. The factors were placed at the edge of a colony after 80% of the medium was removed to allow rapid diffusion of the factors toward the targeted cells. Morphologic changes induced within 6 to 8 h from treatment include rounding of cells (a), formation of fine processes within the cytoplasm of the cell with concomitant condensation of the cytoplasmic mass (b), and stimulation of cell division (c). Original magnification $\times 320$.

a more bipolar and tripolar morphology, these cells became intensely nestin positive and remained NG2 negative. In line SWRB8, both epithelioid and differentiated cells were nestin positive (Fig. 9E). SWRB22 cultures contained mostly flat undifferentiated cells that were positive for either nestin or NG2 but did not coexpress both (Fig.

Fig. 7. BrdU incorporation in cultures of mutated glial progenitor cells derived from the brain of an animal exposed to MNU for 10 weeks. Cells were either stimulated with 200 μ M db-cAMP plus 20 ng/ml bFGF for 24 h (lower), or they were unstimulated (upper). Positive cells stained intensely in the nucleus with anti-BrdU coupled with tetramethyl-benzidine. Original magnification $\times 320$.

Table 2. Tumorigenicity of cell lines generated from culture of cells isolated from the brain of MNU-treated rats

Cell line	Passage	Brain lesions	Tumorigenicity
Exposure to MNU: None			
SWRB5	9,16,20	None	Negative
SWRB10	9,15	None	Negative
Exposure to MNU: 10 wks			
SWRB6	10	None	Negative
SWRB6	15	None	Positive
SWRB7	9,15	None	Negative
SWRB8	9,15	None	Negative
SWRB11	9,15	None	Negative
SWRB21	5,15	None	Negative
Exposure to MNU: 15 wks			
SWRB22a	3,5	Focal gliosis	Positive
SWRB22b	3,5	Focal gliosis	Negative
SWRB26a	3,5	Focal gliosis	Positive
SWRB26b	3,5	Focal gliosis	Positive
SWRB40	3,5	Focal hemorrhage	Positive
SWRB41	4	Focal hemorrhage	Positive
SWRB29	4	Focal hemorrhage	Positive

10⁶ cells were injected s.c. in the flank of Balb/C athymic mice, and animals were kept for 90 days after inoculation.

9F). In general, all the cells that were tested stained for at least 1 immature glial progenitor marker to various degrees, depending on morphology. Nevertheless, flat, weakly staining cells were still distinctly positive relative to negative fibroblast controls.

Expression of antigens in MNU-initiated glial progenitors depended strongly on the morphology and state of differentiation of the cells. In general, cells at stage I

stained for vimentin but expressed GalC or GFAP only weakly (Fig. 10A and B) and were negative for synaptophysin. GalC and GFAP were expressed in stage II cells and were most prominent in cell lines that later entered stage III. GalC and GFAP were also induced in epithelioid cells upon exposure to bFGF whenever their morphology changed from epithelioid to astrocytic (Fig. 10C and d). Morphologic changes from flat to astrocytic morphology, either spontaneously or by growth factor induction, resulted in the coexpression of GFAP and GalC (Fig. 11). Induction of GalC was further demonstrated in dividing flat cells when they were induced by bFGF and db-cAMP to differentiate to an astrocytic morphology (Fig. 12). The GFAP and GalC phenotypes were not induced during unstimulated division of flat cells in which flat cell morphology was maintained. GalC and GFAP expression was maintained in cultures during malignant transformation from stage II to stage III. None of the cell cultures stained for the neuronal marker synaptophysin at any stage of differentiation or transformation. The immunohistological characteristics of the cell lines tested both in the stimulated and unstimulated stages are listed on Table 3.

Discussion

The evidence presented in these experiments indicates that MNU, when administered for a period of 10 or more weeks, results in the mutation of a certain population(s) of cells in adult rat brain. We isolated these mutated cells based on their ability to migrate in vitro from brain slices. Cells incapable of migrating were not selected since they could not colonize the filter. Vigorous culture conditions ensured selection of nonsenescent, most rapidly dividing cells. We believe these conditions favor cells that have undergone tumorigenic transformation, or are at least at the initiated stages and have retained the property of chemotactic migration. Labeling with ³H-thymidine has

Fig. 8. G-banded karyotypes of rat progenitor cell lines. A. Karyotype of the F344 male cell line SWRB7. The 20 rat autosomes and sex chromosome pair are aligned in order from top to bottom, left to right based on chromosomal assignment. The sex chromosome pair is in row 2, far right. Monosomy 18 is demonstrated (arrow). B. Karyotype of line SWRB6. Note severe aneuploidy and numerous chromosomal translocations, resulting in derivative chromosomes of unknown construction.

Fig. 9. Immunofluorescent studies of rat cell lines. Immunohistochemical analysis was performed on lines representing control animals and on lines from 10- and 15-week MNU-treated animals. A. Control cell line SWRB5 stained with NG2-fluorescein isothiocyanate, which demonstrates typical membrane staining pattern. Hoechst nuclear counterstain. B. The same line stained with nestin-TRITC (antirat-401) showing immunopositive cells suggesting they are glial progenitors. Hoechst nuclear counterstain. C. View of NG2-fluorescein isothiocyanate revealing membrane positivity in a specific population of cells that is intermediate between the flat cell population and the bipolar nestin-positive population. D. SWRB6 showing the intense cytoplasmic nestin-TRITC staining in differentiated cells. Hoechst nuclear counterstain. E. Rare cells from SWRB8, which are nestin-TRITC positive in a coarse cytoskeletal pattern. Hoechst nuclear counterstain. Adjacent flat cells are nestin-negative. F. SWRB22A double-stained with nestin-TRITC and NG2-fluorescein isothiocyanate. The culture of tumorigenic cells contains 2 populations of cells that react only with either nestin or NG2. All positive cells have a flat undifferentiated morphology under phase contrast. Hoechst nuclear counterstain. Original magnification $\times 400$.

shown that, in the brain of rats, immature cells divide most vigorously followed by astrocytes and oligodendroglial cells (McCarthy and Leblond, 1988). In contrast to mature and terminally differentiated cells, immature progenitors not only migrate, but also show evidence of lack of senescence and death (Gage et al., 1995; Laerum and Rajewsky, 1975; Satoh et al., 1994). Thus, under our isolation and culturing techniques, selection of progenitor cells over mature cell types is most likely to occur.

As we previously have shown, exposure of adult rats to MNU for 10 weeks leads to a high incidence of glial tumors after prolonged latency (Rushing et al., 1998). Because the brains of animals were histologically normal after 10 weeks of exposure to MNU in these experiments, it seems reasonable to infer that the biologically altered cells we isolated are the initiated cells that subsequently transform into frank malignancy. It would be of great interest to know how these initiated cells

Fig. 10. Induction of GFAP and GalC expression in stage II cultures. Stage I cultures were stimulated with 200 μ M db-cAMP and 40 ng/ml bFGF for 12 h. A and B show phase-contrast micrographs of 2 cultures stained for GFAP (C) and GalC (D), respectively, by using indirect detection via biotinylated secondary antibodies, ABC kit, and DAB chromogen. Original magnification $\times 100$.

change in situ after the period of exposure but before overt malignancy.

Exposure of the adult rat brain to MNU does not yield a single type of mutant, but rather a series of phenotypes ranging from normal to malignant. Because the latency for inducing a fully malignant phenotype is dependent on the length of exposure to the carcinogen (Rushing et al., 1998), it is not surprising that cells with the ability to induce tumors in athymic mice are found predominantly in the brains of animals exposed to MNU for 15 weeks and seldom in those exposed to the carcinogen for only 10 weeks. These findings are in agreement with previously published results that a 15-week exposure results in a tumor incidence $>80\%$ within 2 months after termination of exposure, whereas animals treated for only 10 weeks were tumor free for approximately 1 year before they developed brain tumors (Rushing et al., 1998). This also suggests that, in the intact animal, target cells that undergo initiation by MNU are able to progress to a malignant phenotype under the influence of factors unrelated to the presence of an exogenous carcinogen. A spontaneous transition from initiated, premalignant to fully malignant phenotype is not observed with the majority of nontumorigenic cell lines in vitro because they do not acquire new chromosomal abnormalities or transform to become tumorigenic in athymic mice even after continuous culturing for more than 18 months.

The most notable difference between control cells and those initiated from 10-week MNU-exposed animals was the ability of initiated cells to undergo spontaneous changes from a flat, epithelioid morphology to a bipolar or tripolar astrocytic morphology. On the contrary, con-

Fig. 11. Double-label immunocytochemistry demonstrating cellular coexpression of GFAP and GC. Cell cultures (SWRB 6) were fixed and stained after 12-h exposure to db-cAMP and bFGF (A and B), or no such exposure (C and D). Panels A/B and C/D represent the 2 fields of cells stained with antibodies against GalC (red) and GFAP (green). GalC and GFAP were detected using Cy3-labeled goat antirabbit (red, A and C), and Alexa 488-labeled goat antimouse (green, B and D), respectively. It is evident that there is minimal staining for GalC or GFAP without stimulation by db-cAMP/bFGF (C and D), and that after stimulation, cells coexpress GalC (A) and GFAP (B). Original magnification $\times 120$.

Fig. 12. Temporal course of induction of GalC in stimulated cells. Cells in stage I cultures (A) were stimulated with db-cAMP/bFGF for 6 h (B) and 96 h (C). GalC staining was detected using biotinylated secondary antibody, ABC kit, and DAB chromogen. Original magnification $\times 100$.

trol glial progenitor cells never assumed fully differentiated morphology without stimulation. Control cells retained their epithelioid morphology even when cultured under confluent conditions for prolonged times.

In this regard, both the *in vivo* and *in vitro* models are representative of human brain tumorigenesis in which progression is considered to occur in stages and genomic instability is rare (Wooster et al., 1994). In fact, genomic stability is required for making this model useful in predicting the genetic abnormalities that are required for the initiation and propagation of brain tumors. The cytogenetic results for the cell lines tested here mirror the typical cytogenetic findings for cell lines generated from high-grade and low-grade human gliomas. For example, glioblastoma-derived cell lines, including U-118 MG, with a near pentaploid complement of chromosomes (range, 110-115 chromosomes) and DBTRG-05MG, which typically shows aneuploid and near tetraploid karyotype (range, 87-91 chromosomes), have been demonstrated to have a high degree of aneuploidy and tumorigenicity (Kruse et al., 1992; Ponten and Macintyre, 1968). Numerical abnormalities are in addition to identified translocations and deletions that involve chromosomal regions, such as the long arm of human chromosome 10, and these can occur concurrently with aneuploidy. The line SWRB6/p15, which forms vigorously growing tumors in nude mice and also has a highly aneuploid karyotype with numerous translocations that generate fusion chromosome products that are complex, is analo-

gous to cell lines derived from high-grade tumors. Fluorescence technologies such as SKY (spectral karyotyping) will enable further definition of the precise molecular events involved in tumor progression in our model.

In comparison, SWRB7, a nontumorigenic line, displays metaphases that are normal except for a single identified abnormality. The sole abnormality is a monosomy for rat Chr 18, which is stereotypical and consistent. This finding is consistent with studies done to date that characterize the karyotypes of human low-grade CNS malignancies (Bigner and Schrock, 1997). Due to technical limitations, human grade I and grade II gliomas are understudied with respect to their karyotypic characteristics, but most studies demonstrate either normal karyotypes, in approximately 50% of cases, or single chromosome losses, frequently in one of the sex chromosomes, leading to monosomy. Thus the nontumorigenic line SWRB7 has karyotypic changes that mimic those observed in human low-grade gliomas. G-banding, of course, has limitations because it detects only large chromosomal abnormalities on the order of 5-10 megabases. Our *in vitro* experiments with lines SWRB8 and SWRB21 show that these lines must have genetic changes that allow transformation even though they are karyotypically normal (Table 3). Clearly, much work remains to be done by complementary techniques such as specific mutation scanning in brain tumor candidate genes and fluorescence *in situ* hybridization of critical genomic loci.

Table 3. Immunohistochemistry of adult rat glial progenitor control and carcinogen-initiated cells before and after stimulation to differentiate with db-cAMP and bFGF

Cell line	bFGF/db-cAMP	GFAP	GC	Nestin	NG2	Morphology
SWRB5 (control)	No	±	–	+	+	Epithelioid
SWRB10 (control)	No	±	±	±	±	Epithelioid
SWRB10 (control)	Yes	±	±	+	±	Spindle
SWRB6 (MNU)	No	±	±	–	–	Epithelioid
SWRB6 (MNU)	Yes	+	+	+	±	Bipolar
SWRB7 (MNU)	No	±	+	+	+	Epithelioid
SWRB7 (MNU)	Yes	+	+	+	+	Spindle
SWRB8 (MNU)	No	±	±	±	±	Epithelioid
SWRB8 (MNU)	Yes	+	+	+	+	Bipolar

When indicated, cells were incubated for 24 h in 20 ng/ml bFGF and 200 μ M db-cAMP in medium containing 5% serum. Staining is designated as absent/negative (–), weakly positive (±), or positive (+).

The differentiated state of these cells can be analyzed by immunochemical staining for proteins specific for individual differentiated cell types, in contrast to antigens indicative of a primitive precursor state, e.g. nestin. More differentiated populations include standard astrocytes, which are positive for GFAP (Bignami et al., 1972) and type 2 astrocytes, also positive for antibody A2B5 (Eisenbarth et al., 1979). The progression of oligodendroglial maturation involves sequential expression of markers including O4, O1, cyclic nucleotide phosphodiesterase, glycoprotein NG2, and mature differentiation markers GalC (Ranscht et al., 1982) and myelin basic protein. In contrast, mature neurons typically express specific cytoskeletal proteins including neurofilament, tau, and microtubule-associated protein-2; cytoplasmic enzymes such as neuron specific enolase; neuronal-specific nuclear proteins such as NeuN; and various neurotransmitter-related enzymes or receptors. Characterization of brain cells and their precursors can thus be accomplished to a certain degree by employing immunohistochemical markers. Evidence suggesting that the target cell for MNU is the glial progenitor comes from the immunohistological properties of the cell lines. These initiated cells do express nestin and NG2, but they do not express the differentiated glial cell markers GFAP and GalC unless they are stimulated to differentiate to astrocytic morphology. Previous data support the concept that glial multipotent progenitor cells coexpress mature glial markers when stimulated. For example, GFAP and GalC are coexpressed in O-2A cells (Yim et al., 1994). Addition of db-cAMP to primary cultures of glial cells from adult rat olfactory bulb results in the coexpression of GalC and GFAP (Bakardjiev, 1997). C6 rat glioma cell cultures contain young oligodendrocytes that are GFAP negative-GalC positive and mixed astrocyte-oligodendrocyte cells positive for both GFAP and GalC, a GFAP plus GalC expressing phenotype that remained after adaptation to serum-free conditions (Coyle, 1995). In all these cases, cells express one of the markers, whereas the other marker is induced by further manipulation of cultures. Cells isolated and cultured under our method expressed both markers either under stimulation with growth factors and/or db-cyclic AMP to induce transition from a flat-cell morphology to an astrocyte-like cell or, in some cases, by spontaneous transformation after repeated passing. However, these *in vitro* experiments cannot allow firm inferences as to the nature of the tumor precursor cells *in vivo*, which remains to be defined.

Stimulation of nonconfluent cultures of both controls and initiated cell lines resulted in rapid differentiation that was sustained for several days as long as the differentiating factors were present in the medium. Differentiated cells returned to epithelioid morphology when the factors were withdrawn, but this depended on the extent of exposure to db-cAMP/bFGF and the culture itself. Cells with mild chromosomal abnormalities maintained some of their differentiated morphology even after the withdrawal of the differentiating factors, thus forming mixed cultures. Cultures of SWRB8 (which had no evident chromosomal abnormalities) lost the ability to return to a nondifferentiated state after prolonged stimu-

lation with db-cAMP and bFGF and senesced when the differentiating factors were removed. We conclude that the process of initiation and transformation is a dynamic one and accounts for the varied behavior among the initiated lines. Thus, the ability to differentiate from a flat to an astrocytic or oligodendroglial morphology is coupled with the degree of chromosomal alterations or malignant transformation of the progenitor cells.

In this regard, stage I cultures derived from the brain of MNU-treated animals respond to bFGF in a manner similar to the behavior of PC12 neuronal cells when they respond to nerve growth factor and other growth factors (Pittman and DiBenedetto, 1996). In PC12 cells, growth factors promote differentiation while inhibiting proliferation of differentiated cells. Following adaptation of PC12 to exogenous growth factors, removal of these factors may result in cell death if the cells are committed to differentiation and can no longer de-differentiate. A trivial mechanism for this phenomenon could be that the growth factor down regulates receptors for serum growth factors that induce proliferation or generate second messengers that normally block the endogenous apoptotic pathways. In carcinogen-initiated glial progenitor cells, the removal of FGF may block cellular responses to survival signals present in serum, thus causing death. This could be overcome by autocrine production of growth factors or increase of endogenous basal levels of cAMP, which will allow survival in the absence of exogenous growth factors or even serum. Such adaptation has been observed with line SWRB6, which underwent a transition from stage II (growth factor dependent) to stage III (independent).

At present it is not clear whether cells that cease to proliferate activate an apoptotic pathway that was not present in the undifferentiated precursor, or if they undergo senescence having completed a definite number of cell divisions (the "Hayflick limit"). Most peculiar is the response of initiated glial precursors to stimulation of the cAMP/PKA signal transduction pathway by the cAMP analog db-cAMP in the presence of bFGF. Stimulation of the cAMP/PKA pathway results in decreased proliferation, increased GFAP expression, differentiation, and death of glioma cells (Chen et al., 1998). However, such response is not as pronounced in premalignant cells (MNU-initiated) without supplementing with exogenous growth factors. It is possible that glioma cell cultures may respond to only cAMP analogs, without the need of growth factors, because they already have decoupled growth factors from mitogenesis, or have their own autocrine production of growth factors.

We believe that we have developed a complex and interesting system that will allow us to further characterize the phenomenon of biological progression from biologically altered, histologically normal cells to frank malignant glioma. Additional experiments should help define the precise genetic chain of events that leads to brain cancer.

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