Overexpression of Oncogene Products Can Cause Tumor Progression without Parenchymal Infiltration in the Rat Brain

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ABSTRACT

Tumor cell progression and parenchymal infiltration play important roles in the pathogenesis of gliomas. Using retrovirally marked rat 9L gliosarcoma cells, which when injected in the CDF rat brain form noninfiltrating tumors which grow only surrounding existing blood vessels, we were able to investigate elements of tumor growth and progression within the substance of the brain. Transfection of 9L by oncogenes associated with tumor progression and expressed in gliomas resulted in alterations in the in vivo phenotype of these cells, with the production of faster growing, well-vascularized, large solid tumors. However, diffuse infiltration was not seen. Moreover, coinfection of 9L or its transfecants together with the infiltrative C6 cell line resulted in a mixed tumor of noninfiltrating 9L cells in an environment of infiltrating C6 cells, suggesting that the infiltrative ability of C6 cells is controlled on the individual cell level.

INTRODUCTION

Neoplastic growth in vivo is a complex phenomenon which requires the activity of a variety of factors. Essential to tumor growth is the ability to interact with and alter its environment. Many tumor cells have been shown to secrete angiogenic substances (1), and in order to form metastases tumor cells must be able to attach to basement membrane, degrade ECM3 components, and move through the ECM (2–8). ras oncogenes are associated with the metastatic phenotype (9), and highly metastatic behavior has been induced in tumor cells with low metastatic potential by ras transfection (10–13). This metastatic behavior has been linked to the production of collagen-degrading proteases (14, 15). Malignant neoplasms provide an environment in which, through genetic instability and progressive mutation, cells may acquire abilities such as these and be selected for further growth (16–19).

Tumor progression plays an important role in the pathogenesis of gliomas, with low-grade lesions often progressing to more malignant glioblastomas (20, 21). Glioblastomas are highly angiogenic (22, 23), and they typically infiltrate surrounding brain parenchyma (24) by mechanisms the details of which probably differ from systemic neoplasms due to dissimilarities between the CNS and systemic ECM (25–27), as well as the facts that primary CNS tumors rarely metastasize systematically (28) and that even the most aggressive metastases to the brain tend to form noninfiltrative masses with well-defined borders (29). However, like systemic tumors, glioblastomas have been shown to produce ECM-degrading enzymes (30–32).

Several oncogenes which are associated with tumor growth and progression have been shown to be inappropriately expressed in human glioblastomas, including the PDGF-A chain and c-sis (PDGF-B) (33, 34), neu (35), and Ha-ras (36). An infiltrative phenotype has been induced in hamster primary glial cells following transfection with activated c-Ha-ras (37). In order to study the ability of these genes to induce various aspects of tumor progression in the CNS, constructs containing these genes were transfected into a rat brain-derived tumor cell line, 9L gliosarcoma, which has lost its infiltrative ability after successive passages and upon cerebral injection invades primarily by growing along preexisting blood vessels in Virchow-Robin spaces without brain parenchymal infiltration and without formation of large, solid masses (38). The cells were tagged by infection with a replication-incompetent β-galactosidase-containing retrovirus (39), allowing straightforward identification at the histological level. This specific method of individual tumor cell identification permitted us also to analyze whether an in vivo infiltrative phenotype could be conferred upon noninfiltrative cells by locally growing infiltrating tumor cells. The relative role of membrane-associated versus diffusible proteolytic enzymes in the ability of glial tumor cells to infiltrate surrounding tissue is not well understood. Cultured glioblastoma cells have been shown to produce secreted (31, 40) as well as membrane-associated (30, 32) proteolytic enzymes. In vitro studies have demonstrated that the infiltrative ability of a rat glioblastoma cell line, C6, is due to the presence of a membrane-bound metalloprotease (32). In order to analyze if the in vivo noninfiltrative growth pattern of 9L tumor cells (or their oncogene-transfected derivatives) could be altered by neighboring infiltrating glial tumor cells, coinjections of retrovirally tagged 9L cells together with infiltrative C6 glial tumor cells were performed, allowing tracing of individual 9L cells in the microenvironment of the infiltrating C6 cells. Thus, we were able to assess if 9L cells could be induced to grow infiltratively secondary to diffuse destruction of the brain ECM by the C6 cells or whether the process of C6 infiltration is highly localized to the individual cell level.

MATERIALS AND METHODS

Cell Cultures and Animals. Cells were grown on tissue culture-treated plates in DMEM supplemented with 10% FBS, L-glutamine and penicillin/streptomycin and were passed (1:50) when they grew to confluence at 5 to 7 days.

Retrovirus Infection. The producer cell line Ebag2 (gift of C. Cepko), releasing the BAG retrovirus particles (39), was maintained in DMEM/10% FBS. The BAG vector contains both the β-gal gene, promoted by the Moloney leukemia virus long terminal repeat, and the Tn5 neo gene under SV40 control conferring resistance to G418. Ebag2-conditioned medium was filtered and placed over a 80% confluent plate of rat 9L cells. These cells were then selected using medium containing G418 (1 mg/ml). Isolated colonies were picked and stained with X-gal (1 mg/ml; from 40 mg/ml in dimethylformamide stock) plus 5 mM K₄Fe(CN)₆ and 5 mM K₃Fe(CN)₆·3H₂O plus 2 mM MgCl₂ in PBS. One strongly blue-staining colony, 9L/β-gal, was chosen for transfection studies.
Transfection. Stably transfected lines of 9L/β-gal were made by transfecting 70% confluent plates of these cells with 10 μg pSV-PDGF-A (human glioma-derived PDGF-A chain in pBR327 under the control of the SV40 enhancer and promoter (41), pSM-1 (human c-sis [PDGF-A] under SV40 control elements (42), pSV2-neu-NT (rat neuro/glioblastoma-derived neu under SV40 control elements (43), pEJ (human bladder carcinoma-derived Ha-ras under its own control elements (44), or pSV2-CAT as a negative control (coding sequence for CAT under SV40 control (45)). Each of these plasmids was cotransfected with 1 μg pSV2-gpt, conferring resistance to mycophenolic acid, using a modified calcium phosphate coprecipitation method (46). Medium was changed 24 h later, and after 72 h cells were split into selective media containing mycophenolic acid (5 mg/ml), xanthine (250 μg/ml), hypoxanthine (15 μg/ml), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid in DMEM/10% FBS (46). Resulting colonies were pooled and expanded. Expression of these oncogenes at the mRNA level was confirmed using Northern blot analysis of total RNA.

Northern Blot Analysis. Total RNA was extracted from the oncogene-transfected 9L/β-gal derivatives and probed to hybrids generated by random hexamer 32P labeling of either the entire transfected plasmid (pSV-PDGF-A, pSM-1, pSV2-neu-NT, pSV2-CAT) or the excised full-length gene (pEJ). Strongly hybridizing bands of the expected sizes for the transfected oncogenes were identified in the corresponding transfected cell lines: 1.8 kilobases for the PDGF-A gene; 4.2 kilobases for c-sis; 6.8 kilobases for neu-NT; and 1.2 kilobases for EJ-ras (41-44) (data not shown). In nontransfected 9L cells, a very low expression level was noted for ras while no expression was detected, in the sensitivity range of the Northern blot analysis, for the other genes tested.

CAT Assay. 9L/β-gal cells transfected with pSV2-CAT or with pSV2-PDGF-A were grown to confluency on 100-mm tissue culture plates and were processed using the Promega (Madison, WI) CAT Enzyme Assay System. Cell extract was prepared using the Tris buffer method. A standard curve of CAT activity was generated using 0.005 to 0.1 unit of CAT with 14C chloramphenicol and n-butyryl-CoA, and enzymatic activity of cell extracts was compared to this curve.

Growth Curves. Using 6-well tissue culture plates, 20,000 viable cells (as determined by trypsin blue exclusion) per well were plated in DMEM/10% FBS. For growth curves in 10% FBS, cells were trypsinized at 72 or 144 h, and 0.1 ml were counted on a hemocytometer. For growth curves in 1% FBS, medium was changed to DMEM/1% FBS after 24 h. Cells were counted after 72 or 144 h from the time medium was changed. All wells were run in duplicate, and all counts were duplicated.

Tumor Injection. For cerebral injection, 70 to 80% confluent cells were trypsinized, spun down, resuspended in normal saline at 4000 cells/ml, and kept on ice prior to use. Male 126- to 150-g CD Fischer rats were anesthetized with Nembutal and placed in a stereotactic frame. Injections were made with a 10-μl Hamilton syringe bilaterally 3.5 mm lateral to bregma and 4.5 mm deep to dura. Five μl were injected at each site. Coinjections were made with C6 cells suspended together with 9L/β-gal, 9L/β-gal/neu, or 9L/β-gal/ras at 1:3 and 3:1 ratios.

Animals were sacrificed when they became ill or after more than 4 weeks. They were etherized and underwent intracardiac perfusion first with PBS and then with Pipes buffer-2% paraformaldehyde-0.1 M Pipes buffer-2 mM MgCl2, 1.25 mM ethyleneglycol-bis(2-aminoethyl ether)-N,N'-tetraacetic acid). After 30 min brains were carefully removed, cut into 4-mm sections, and frozen on liquid nitrogen or dry ice in OCT embedding material (Miles). Embedded tissue was stored at −70°C until processed.

Tissue Preparation and Staining. 20 μm sections were cut at −17°C at 50-100-μm intervals and mounted on poly-δ-lysine-coated slides. Slides were fixed in cold Pipes buffer-10 min, rinsed in 2 mM MgCl2 in PBS for 10 min, permeabilized in 0.5% Triton X-100 in PBS for 10 min, and stained overnight in 37°C with X-gal (1 mg/ml; from 40 mg/ml in dimethylformamide stock) plus 5 mM K3Fe(CN)6 plus 5 mM K4Fe(CN)6·3H2O plus 2 mM MgCl2 plus 0.01% sodium deoxycholate plus 0.02% Nonidet P-40 in PBS for 10 min, stained overnight in 37°C with X-gal (1 mg/ml; from 40 mg/ml in dimethylformamide stock) plus 5 mM K3Fe(CN)6 plus 5 mM K4Fe(CN)6·3H2O plus 2 mM MgCl2 plus 0.01% sodium deoxycholate plus 0.02% Nonidet P-40 in PBS, counterstained lightly with eosin Y or hematoxylin and eosin, and coverslipped (40, 47). Endothelial cells were selectively stained using a Vectastain ABC kit with biotinylated Bandeiraea (Griffonia) simplicifolia lectin I isoelectric B4 (Vector Laboratories, Burlingame, CA).

Table 1  Latency between intracerebral injection of 9L-derived tumor cells and morbidity

<table>
<thead>
<tr>
<th>Four animals/group.</th>
<th>Illness latency (days)</th>
<th>Means separation*</th>
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<tbody>
<tr>
<td>Cell line</td>
<td>Range</td>
<td>Mean</td>
</tr>
<tr>
<td>9L/β-gal/neu</td>
<td>17-18</td>
<td>17.50</td>
</tr>
<tr>
<td>9L/β-gal/ras</td>
<td>19-23</td>
<td>21.25</td>
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<tr>
<td>9L/β-gal/PDGF-A</td>
<td>26-28</td>
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<tr>
<td>9L/β-gal/PDGF-B</td>
<td>29-53</td>
<td>40.50</td>
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<tr>
<td>9L/β-gal/CAT</td>
<td>29-56</td>
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<tr>
<td>9L/β-gal/PDGF-A</td>
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* Student-Newman-Keuls test showing significant differences between the first and last three rows (P < 0.05).
RESULTS

CAT Assay. Production of biologically active CAT enzyme in CAT-transfected cells was confirmed by CAT assay. CAT activity of one confluent plate of 9L/β-gal transfected with pSV2-CAT was increased approximately 800-fold over 9L/β-gal PDGF-A gene transfectants, which served as a negative control (data not shown).

Growth Curves. These are presented in Fig. 1. There were no significant differences in growth rates among the entire group of cell lines. Growth was maintained through and beyond the transition to confluence.

Tumor Growth and Progression. Bilateral intracerebral injections of transfectants were performed stereotactically in CDF rats. Histological sections were prepared 3 to 6 weeks later in order to identify alterations in tumor growth patterns such as mass formation, parenchymal infiltration, and neovascularization. Tracing of individual tumor cells was aided by infecting 9L cells with the replication-incompetent BAG retrovirus which contains the β-galactosidase gene. Tumor cells then stained bright blue using the X-gal histochemical technique. Animals were sacrificed upon signs of illness or after more than 4 weeks postinjection. Illness was manifested by the previously

Fig. 2. Frozen sections (20 μm) stained using the X-gal technique and counterstained lightly with H & E. X-gal selectively stains the BAG retrovirally marked 9L-derived cells. Top, tumors of 9L/β-gal (left) and 9L/β-gal/CAT (right) grow primarily perivascularly in Virchow-Robin spaces; bottom, tumors of 9L/β-gal/neu (left) and 9L/β-gal/ras (right) grow in large, solid masses; middle, tumors of 9L/β-gal/PDGF-B (left) and 9L/β-gal/PDGF-A (right) show an intermediate growth pattern with growth both in masses and with invasion along blood vessels.
TUMOR CELL PROGRESSION AND PARENCHYMAL INFILTRATION

described syndrome of quadriparesis with pigmentation surrounding the eyes in all subjects (38). The latency period between tumor injection and subject morbidity in the various groups is presented in Table 1. Analysis of variance showed significant differences among the groups (p < 0.0001). A Student-Newman-Keuls test revealed means separation between 9L/β-gal/neu, 9L/β-gal/ras, and 9L/β-gal/PDGF-A and the other three groups (P < 0.05).

For each tumor, sections of greatest diameter in the coronal plane were examined in detail. Inspection of sections prepared from the brains of 9L/β-gal-injected animals and stained using the X-gal technique revealed tumors invading along the cerebral vasculature in Virchow-Robin spaces without infiltration of brain parenchyma and without a significant solid component (Fig. 2). Fig. 2 also shows the results of the oncogene transfection experiments. There was a marked alteration of the growth pattern of tumors produced by transfection with the activated Ha-ras or neu. Tumors grown from these transfectant cell lines formed solid masses with little tendency to invade perivascularly. Transfectants of 9L/β-gal with PDGF-A or PDGF-B produced tumors of intermediate appearance, with a central solid component but still considerable perivascular growth in surrounding areas. Solid tumors of 9L/β-gal/neu and 9L/β-gal/ras were shown to be highly vascularized by staining of sections with an endothelia-specific lectin. The resulting slides clearly show proliferation of an extensive vascular system traversing the tumor (Fig. 3).

Coinjection with C6. As in the case of the 9L/β-gal/ras or 9L/β-gal/neu injected alone (see above), all animals in which these cells were injected in combination with C6 showed signs of illness after 17 to 24 days. No animal given an injection of C6 alone or in combination with 9L/β-gal cells became ill prior to 4 weeks after injection. Tumors derived from the coinjection experiments had two distinct components in all cases (Fig. 4a). Although in the presence of C6 cells there was an increased heterogeneity of the growth patterns of 9L cells (9L/β-gal as well as their oncogene-transfected derivatives) with regions of more or less perivascular growth versus mass formation, the counterstained C6 cells displayed an infiltrative growth pattern while blue 9L/β-gal cells or their oncogene-transfected derivatives grew only through Virchow-Robin spaces or as well-defined masses. In no case was there any evidence of brain parenchymal infiltration by blue-stained 9L cells. Some (especially in 9L:C6 at 1:3 ratio) isolated blue 9L cells could be identified, but again they were always tightly associated with blood vessels as shown by staining selectively for endothelial cells (Fig. 4b).

DISCUSSION

Tumor progression has been described as a multifactorial process which includes such elements as accelerating division, independence from growth factors, secretion of angiogenic or desmoplastic factors, and invasion and metastases, as well as resistance to treatment modalities such as radiation and chemotherapy (16, 48–55). The natural history of human glial tumors suggests that tumors indeed undergo changes leading from less to more malignant. Biopsies carried out over time commonly show degenerative changes so that low grade lesions may recur in the form of anaplastic astrocytomas or glioblastomas (20, 21). Furthermore, one hallmark of more anaplastic lesions is endothelial hyperplasia (56), and experimental evidences suggest that glioblastomas are among the most actively angiogenic neoplasms (53). An aspect of progression which may be unique to glial tumors is their infiltrative pattern. Glial tumors of all grades typically grow through the surrounding brain parenchyma, intermingling with normal and reactive cells, while even the systematically most invasive metastases to the brain generally grow with well-defined borders (29). This growth pattern has been attributed to differences between primary and secondary brain tumors in expression of ECM-degrading and remodeling enzymes: except around the mesenchymally derived vasculature, the ECM of the CNS is composed of vastly different elements than that of the rest of the body (25, 27).

The rat 9L gliosarcoma cell line, in conjunction with X-gal staining of histological specimens after its labeling with a

![Fig. 3. Frozen section of tumor (20 μm) from 9L/β-gal/neu-stained peroxidase stained via a biotinylated lectin which specifically binds endothelial glycoproteins. The tumor is noted to be highly vascularized.](image-url)
TUMOR CELL PROGRESSION AND PARENCHYMAL INFILTRATION

Fig. 4. Effects of coinjection on growth pattern of 9L gliosarcoma cells. a. 9L/β-gal + C6 (1:3). Blue 9L-derived cells display growth around a blood vessel while infiltrative C6 cells form a clearly identifiable decreasing gradient of hypercellularity moving toward the tumor periphery. × 100. Coinjections of C6 cells together with 9L/β-gal/ras or 9L/β-gal/neu had identical growth patterns. In b, in the same tumors isolated cells were demonstrated to be adjacent to vessels in Virchow-Robin spaces by staining with endothelia-specific lectin. 9L/β-gal/neu plus C6 (1:3), ts 400.

As a cell line which has lost its diffusely infiltrative growth pattern over successive passages (38) and is able to grow only around pre-existing blood vessels, the ability of transfected genes to alter this phenotype can be tested. The failure of 9L cells to grow in large, solid masses can be a result of either the failure of the tumor to induce neovascularization, which would allow it to grow independently of existing blood vessels, or as the inability of the tumor to produce its own supporting solid extracellular matrix, thus being capable of growing only by expansion within existing Virchow-Robin spaces. In this study we have shown that the introduction of activated ras or neu oncogenes into rat 9L gliosarcoma cells results in a change in the in vivo growth pattern of these cells when injected into the rat brain. While 9L/β-gal cells typically grow in a perivascular pattern through Virchow-Robin spaces of preexisting blood vessels without forming solid masses or diffusely infiltrating brain parenchyma, 9L/β-gal/ras and 9L/β-gal/neu cells form more rapidly growing large, solid, highly vascularized, well-defined masses. Tumors produced by transfection of 9L/β-gal with the genes for PDGF-A or PDGF-B (c-sis) produce tumors the growth patterns of which are intermediate between those of the original 9L/β-gal cells and those of 9L/β-gal/ras or 9L/β-gal/neu. Transfection with the bacterial CAT gene as a control produced no change in tumor growth pattern. In vitro growth rates of all cell lines were identical, suggesting that the in vivo changes cannot be explained simply as a result of accelerated cell metabolism or autocrine stimulation. Thus, the alteration in the in vivo tumor growth pattern can be construed as the result of interaction between tumor cells and surrounding brain constituents via one or a number of mechanisms including increased angiogenesis (either directly by the tumor cells or through secondary stimulation by endogenous cells), alterations in desmoplastic functions, or increased responsiveness of the oncogene-transfected 9L tumor cells to the particular growth factor environment of the rat brain. (One point of caution should be noted, and that is that three of the genes introduced into the 9L cells were human in origin (PDGF-A, PDGF-B, and ras), and thus the phenotypic changes caused by them in the 9L cells may not fully replicate those of their rat counterparts; however, the introduction of the rat-derived neu-NT gene into 9L cells caused identical phenotypic changes to that caused by the other, human-derived genes).

The neu, PDGF-A, PDGF-B, and ras genes encode proteins which serve different roles in the regulation of cell growth and development. The neu gene encodes the epidermal growth factor receptor (EGFR), which plays a key role in regulating cell proliferation, survival, and differentiation. The PDGF-A and PDGF-B genes encode two distinct subunits of the PDGF dimer, which is a potent mitogen for many cell types. The ras gene encodes a small GTPase protein which regulates cellular proliferation by activating the mitogen-activated protein kinase (MAPK) pathway. The introduction of these genes into tumor cells can lead to increased cell proliferation and altered growth patterns, as observed in the 9L gliosarcoma cells in this study.
metabolism (57–62). An activated new gene product, originally isolated from a series of rat neuro- and glioblastomas (43), has been shown to be expressed in a variety of tumors, including human glioblastomas (35). Both PDGF-A and PDGF-B have been proposed to exhibit angiogenic activity (55) and both have been shown to be expressed in human glioblastomas (33), although in situ hybridization studies have suggested that while PDGF-A is predominantly produced by tumor cells, PDGF-B is predominantly produced by local hyperplastic endothelial cells (55). It is hypothesized that neovascularization in this setting results from paracrine effects inducing autocrine stimulation by PDGF-B in receptor-bearing endothelial cells. In our study introduction of the PDGF-A gene into 9L cells results in a more profound phenotypic change than that of the PDGF-B gene, a result which may be due to unequal levels of PDGF-A and PDGF-B expression in the transfected 9L/β-gal cells or decreased biological activity of the PDGF-B product in the 9L/β-gal/PDGF-B transfectants as compared to the PDGF-A transfectants.

Activation or overexpression of the ras oncogene has been shown to be correlated with several aspects of tumor progression, including an association with increased metastatic potential (9, 10–13) which has been attributed to the production of ECM-degrading enzymes (14, 15). The role of ras in infiltration by glial cells has also been studied by transfection of an activated human Ha-ras into SV40-transformed and primary hamster glial cells, showing its ability to induce diffusely infiltrative behavior by these cells in the hamster cerebral cortex (37). The ability of these ras-transfected cells to infiltrate the brain parenchyma, as opposed to 9L/β-gal/β-gal cells used in our study, is likely due to the enhancement by ras of an initial ability of the hamster glial cells to minimally infiltrate the brain parenchyma, an initial ability that 9L cells lack. Thus, while offering a model for studies of tumor progression, the potential use of the retrovirally tagged 9L cells for studies of brain parenchymal infiltration should be viewed cautiously.

The in vivo phenotypic changes generated in oncogene-transfected rat 9L/β-gal gliosarcoma cells, including the decrease in latency from tumor implantation to subject morbidity, were induced by the transfection of several different oncogenes, each of which acts via a separate mechanism. These results suggest that tumor progression may result from altered function of different regulatory systems which converge from the sites of action of several gene products to a common effector pathway.

The ability to trace individual retrovirally marked 9L cells within histological section enabled us also to investigate whether brain parenchymal infiltration by these cells can be induced by neighboring infiltrating glial tumor cells. Cultured glioma cells have been shown to secrete enzymatically active proteases into the culture medium (31, 40), suggesting that secreted proteases play an important role in the infiltrative behavior of glial tumors. However, in vitro studies have demonstrated that the infiltrative ability of the rat glioblastoma C6 cell line is due to the presence of membrane-bound metalloprotease (32). By coinjecting C6 cells together with 9L/β-gal cells or their oncogene-transfected derivatives (which are able to form large solid masses) and tracing the blue-staining 9L cells within the histological sections, we were able to assess the growth pattern of the 9L cells within the environment of the infiltrating C6 cells. The noninfiltrative growth pattern of 9L cells was maintained, suggesting that although a possible role for secreted products (secreted by the tumor cells themselves or by neighboring cells) in the infiltrative phenotype displayed by C6 glial tumor cells cannot be ruled out, they alone do not account for this phenotype. It is likely that other factors, such as membrane-associated proteases (30, 32) and cell motility (29), are more directly involved in the process leading to brain parenchymal infiltration by the C6 glial tumor cells in vivo.

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Marc S. Schwartz, James Morris and Jacob Sarid


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