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ABSTRACT

Identification of the genes that are differentially expressed in brain tumor cells but not in normal brain cells is important for understanding the molecular basis of these neurological cancers and for defining possible targets for therapeutic intervention. In an effort to discover potentially antigenic proteins that may be involved in the malignant transformation and progression of human glioblastomas, a novel antibody-based approach was developed to identify and isolate gene products that are expressed in brain tumors versus normal brain tissue. Using this method, whereby tumor-specific antibodies were isolated and used to screen a glioblastoma cDNA expression library, 28 gene products were identified. Nine of these clones had homology to known gene products, and 19 were novel. The expression of these genes in multiple different human gliomas was then evaluated by cDNA microarray hybridization. One of the isolated clones had consistently higher levels of expression (3–30-fold) in brain tumors compared with normal brain. Northern blot analysis and in situ hybridization confirmed this differential overexpression. cDNA sequence analysis revealed that this gene was identical to a relatively new class of growth regulators known as granulins, which have tertiary structures resembling the epidermal growth factor-like proteins. The 2.1-kb granulin mRNA was expressed predominantly in glial tumors, with lower levels in spleen, kidney, and testes, whereas expression was not detected in non-tumor brain tissues. Functional assays using [3H]thymidine incorporation indicated that granulin may be a glial mitogen, as addition of synthetic granulin peptide to primary rat astrocytes and three different early-passage human glioblastoma cultures increased cell proliferation in vitro, whereas increasing concentrations of granulin antibody inhibited cell growth in a dose-dependent manner. The differential expression pattern, tissue distribution, and implication of this glioma-associated molecule in growth regulation suggest a potentially important role for granulin in the pathogenesis and/or malignant progression of primary brain neoplasms.

INTRODUCTION

Cancer is the result of cumulative multiple genetic mutations, which result in the activation of oncogenes and/or the inactivation of tumor suppressor genes. It is the differential expression of these critical genes and their downstream effectors that enables cells to override growth controls and undergo carcinogenesis (1, 2). The pathological changes that arise in cancer, whether caused by a single gene mutation or multiple genetic alterations, are essentially driven by changes in gene expression (1, 2). In the malignant progression of astrocytic cancers, it has been shown that accumulation of multiple genetic lesions underlies the neoplastic process. These lesions include mutations of the genes p53, p16, RB, and PTEN, as well as amplification of CDK4 and EGFR (3, 4). Although these known genetic abnormalities have been well documented in the formation of the most malignant brain tumor, glioblastoma, recent insight into the extent of gene expression differences underlying malignancy reveals that hundreds of gene transcripts may be expressed at significantly different levels between normal and neoplastic cells (5). Therefore, there is considerable room for the identification of novel genes that are differentially expressed in brain tumor cells to further our understanding of the complex molecular basis of these neurological cancers. Furthermore, this endeavor has direct clinical relevance if combined with the development of innovative rational therapies that specifically target these differentially expressed gene products.

A variety of methods are currently used to isolate genes associated with particular differential phenotypes. Subtractive hybridization (6), differential display (7–10), representational difference analysis (11–14), serial analysis of gene expression (5, 15), and suppression subtractive hybridization (16, 17) all allow for the cloning and identification of differentially expressed sequences. Although all these techniques identify tissue-enriched mRNAs, none select for tissuespecific proteins. Because we initially were interested in identifying glioma-associated antigens that may be potential targets for brain tumor immunotherapy, we set out to devise a differential screening technique that provided actual confirmation of the presence of a protein product, not just the capacity to synthesize a protein. Furthermore, we wanted to select for proteins with antigenic determinants that may be potentially recognized by the immune system.

Here we report an approach to identifying differentially expressed gene products that are actually translated from mRNA species, using antibody-based screening of a cDNA expression library. We further show that this method, which we termed DIA,3 can be coupled to cDNA microarray screening and allowed the identification of a putative growth factor gene, granulin, which may play a role in the malignant progression of glioblastomas.

MATERIALS AND METHODS

DIA. GBM tumor tissue was immediately snap frozen in liquid nitrogen at the time of surgery. Non-tumor brain was obtained from a surgical resection for trauma and similarly frozen. Both tissue specimens were homogenized in PBS (pH 7.0) with a glass mortar and pestle. The soluble material was aspirated, and the insoluble material was re-extracted into a second fraction, using 0.1% SDS. Both fractions were used for affinity purification and immunizations. Affinity chromatography was carried out using an aliquot of extracted material immobilized on CNBr-activated Sepharose 4B columns (Pharmacia) according to the manufacturer’s specifications. The immobilized GBM tumor tissue extract was loaded into a fritted column (Varian), blocked with 1 M glycine, precycled with 0.1 M HCl, and neutralized with 0.1 M PBS (pH 8.4). The extract of non-tumor brain tissue was similarly immobilized.

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The abbreviations used are: DIA, differential immuno-absorption; GBM, glioblastoma multiforme; BBS, borate-buffered saline; PBS, fetal bovine serum; EGFR, epidermal growth factor; EGFR, EGFR receptor.
Antisera were raised against the GBM tumor homogenate by s.c. and i.m. immunization of New Zealand White rabbits, using complete and incomplete Freund’s adjuvants. Several bleedings were collected from two animals, pooled, and diluted 1:2 with 0.1 M BBS (pH 8.4). The diluted antisera was passed over the GBM affinity column, and unbound material was washed off with BBS. Bound material was eluted off using glycine buffers adjusted to pH 3, pH 2, and then pH 1. The eluate and effluent were monitored at 280 nm (LKB), and the antisera was passed repeatedly through the column until depleted. The eluate was then collected into BBS, checked for neutral pH, and cross-absorbed repeatedly (until depleted of cross-reactive antibodies) against the column of non-tumor brain to select out antibodies that may bind normal brain antigens. The unbound material was further cross-absorbed against normal human plasma to select out nonspecific antibodies. The final product was concentrated with a column of Amicon (Amicon) and dialyzed against carbonate buffer (pH 9.5). The antibodies were biotinylated at a molar ratio of 15:1 using NHS long-chain biotin (Sigma) and repurified using a column of G-25 (Pharmacia). These biotinylated antibodies were then used to screen a glioblastoma phagemid cDNA expression library.

Construction and Screening of cDNA Expression Library. For construction of the glioblastoma cDNA expression library, a human GBM tumor was snap frozen in liquid nitrogen at the time of surgery and stored at −80°C. Total RNA was extracted from 500 mg of fresh frozen tumor tissue, using Trizol reagent according to the manufacturer’s protocol (Life Technologies, Inc.). mRNA from a total of 30 μg of RNA was isolated using double chromatography on oligo-dt cellulose columns (Life Technologies, Inc.). Double-stranded cDNA was synthesized from this mRNA, using a Superscript II cDNA synthesis kit (Life Technologies, Inc.), and the cDNAs were ligated into a λ ZipLox phagemid vector (Life Technologies, Inc.). We obtained a library titer estimated at 5.0 × 10^8 plaque-forming units. Approximately 2.0 × 10^10 plaque-forming units were plated and grown in the presence of isopropyl-1-thio-β-D-galactoside, lifted onto nitrocellulose membranes, and incubated with biotinylated anti-GBM antibodies (1:10,000 dilution). The membranes were then incubated with streptavidin-horseradish peroxidase and diazobenzenediazonium tetrachloride (Pierce). Positive clones were isolated, re-screened, and subcloned into the pZL1 plasmid vector (Life Technologies, Inc.) by in vivo excision. Inserts were verified by agarose gel electrophoresis and partially sequenced using a dsDNA cycle sequencing kit (Life Technologies, Inc.) according to the manufacturer’s protocol.

Microarraying of Cloned DIA Products. After the subtractive products were cloned into the pZL1 vector, plasmid inserts were PCR-amplified using vector-specific primers. PCR was performed in 50-μl reactions containing 10 mM Tris (pH 9.0), 50 mM KCl, 0.1% gelatin, 2.5 units of Taq DNA polymerase, and 150 μM deoxynucleotide triphosphates. Thermal cycling conditions consisted of an initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 68°C for 1 min, and 72°C for 1.5 min, with a final 72°C extension for 10 min, in a PTC100 thermal cycler (MJ Research). Five microglobules of each PCR amplification product were examined by agarose gel electrophoresis with ethidium bromide staining. A single band was detected in 26 of the 28 PCR reactions performed. Each of the 26 successfully amplified PCR products (1–2 μg) was recovered from the remaining 45 μl of each PCR reaction by ethanol precipitation.

The PCR products were arranged onto glass slides, following a protocol similar to that described previously (18). Briefly, the PCR products were resuspended in 15 μl of 1× SSC. A custom-built arraying robot picked up ~600 nl of DNA solution and deposited 1–4 nl of DNA solution in triplicate onto a silanized glass slide surface (Sigma). After printing, the slide was hydrated for 10 s over a 37°C water bath, snap dried for 2 s on a 100°C heating block, and then UV cross-linked with 4000 mJ short-wave irradiation (Stratagene Stratallinker). The slide was then washed for 2 min sequentially in 0.2% SDS and distilled water. The bound DNA was denatured in distilled water at 100°C, desiccated in an ice-cold bath of 95% ethanol, and air-dried.

Probe labeling, microarray hybridization, and washes were performed as described previously (19). mRNA from a large batch of pooled tumor and non-tumor brain specimens was used to make cDNA labeled with Cy5. The Cy5-labeled cDNA from this collective batch served as the common reference probe. The subtracted products (RNA samples 2 μg from 10 individual tumors and non-tumor brain specimens (e.g., 8 glioma and 2 normal brain tissues) were used to make cDNA labeled with Cy3.

After hybridization with the arrayed subtractive clones, Cy3 and Cy5 intensities were scanned using a custom-built two-color laser scanning fluorometer. The image files were analyzed with custom-written software that performed quantification similar to that published previously (20, 21). The relative abundance of each of our 26 subtractive clones (L1–L26) in tumor versus normal brain was calculated using the equation:

\[
\frac{[(\text{Cy3 signal}) - \text{Cy3 background}]}{[(\text{Cy5 signal}) - \text{Cy5 background}]} = \frac{\text{Tumor}}{\text{Normal}}
\]

Northern Blot Analysis of Granulin mRNA Expression. Tissue total RNA was extracted using Trizol reagent (Life Technologies, Inc.) according to the manufacturer’s instructions, and 10 μg/lane were separated on 1.2% denaturing agarose gels, transferred overnight to Hybond membranes (Amer sham) using 10× SSC, and irreversibly fixed by UV cross-linking. Prehybridization and hybridization were performed at 65°C in ExpressHyb solution (Clontech). 32P-labeled cDNA probes were generated from our plasmid DNA containing granulin cDNA using random primers according to the manufacturer’s protocol (NEB). After hybridization, membranes were washed (2× SSC containing 0.1% SDS at 37°C for 20 min, followed by 0.2× SSC containing 0.1% SDS at 61°C for 20 min), and exposed to X-ray film (Kodak) at 80°C. Blots were then stripped with 0.1% SDS at 100°C for 15 min and reprobed with 32P-labeled ribosomal 18S cDNA to control for gel loading and RNA integrity.

In Situ Hybridization of Granulin mRNA Expression. In situ hybridization was performed using 35S-labeled riboprobes following previously published protocols (22). Briefly, surgically resected human brain tissues (tumor and non-tumor) were rapidly frozen in isopentane directly from the operating room. Frozen tissues were sectioned on a cryostat at 20-μm thickness, post-fixed in 4% paraformaldehyde, washed, and stored at −75°C. Sections were washed, acetylated, defatted, and incubated with 35S-labeled sense or antisense granulin cRNA probe (10^7 cpm/ml) at 60°C overnight (18–24 h). Following RNase A (20 μg/ml) treatment at 45°C, sections were washed in descending concentrations of SSC, air dried, and dipped for emulsion autoradiography in Kodak NTB2 (1:1 dilution). Following exposure to emulsion for 5 weeks, the slides were developed and counterstained with H&E.

Hybridization densities were measured from the in situ slides by counting silver grains within representative cells, using an image analysis computer (Olympus microscope and MCID imaging software; Imaging Research, Inc.; Ref. 23). Sections through several different tumor and non-tumor human brain specimens that had been hybridized with granulin cRNA were chosen for counts. Briefly, two independent observers outlined labeled regions within each slide, and the computer determined the absorbance and quantity of silver grains within each outlined area. Ten measurements were performed for each slide and averaged into single values per mm² per specimen. These values were then divided by the estimated number of cells per mm² for each specimen to get the average units of silver grains per cell. The average quantity of silver grains per cell for each tumor was compared to that of non-tumor brain specimens using the Student’s t-test.

Cell Cultures. Primary cultures of rat astrocytes from the brains of adult Fischer 344 rats were isolated following a protocol described previously (24). Cultures were maintained in DMEM (Life Technologies, Inc.) supplemented with 10% FBS, l-glutamine, and antibiotic drugs (100 units/ml penicillin and 100 μg/ml streptomycin) at 37°C in 5% CO 2.

Primary human glioblastoma cell cultures were established in our laboratory using a protocol similar to that published previously (25). Tumors were taken directly from the operating room at the time of surgery. Tissues were finely minced using sterile scissors, rinsed with PBS, and dispersed with trypsin-EDTA. Monolayer cells were plated in T75 flasks (Costar) and cultured in DMEM/Ham’s F12 (Irvine Scientific) supplemented with 10% FBS (Life Technologies, Inc.), l-glutamine, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin).

Measurement of Cell Proliferation. The effect of granulin D peptide and granulin antibody on the proliferation of primary rat astrocytes and three early-passage human glioblastoma cell lines were examined. Synthetic peptide, consisting of the 55-amino acid sequence of granulin D (26), was provided by Research Genetics. For the antibody studies, a polyclonal antibody was raised against this 55-amino acid synthetic peptide conjugated to keyhole limpet hemocyanin. The IgG fraction was isolated from sera, using protein A-Sepharose (Zymed), concentrated using a Centri-cell concentrator (Amicon), and partially sequenced using a dsDNA cycle sequencing kit (Life Technologies, Inc.).
RESULTS

Isolation of Glioblastoma-associated Gene Products by DIA. We hypothesized that tumor-specific antibodies could be generated and used to screen a glioblastoma cDNA expression library to identify gene products specifically expressed in brain tumors. To test this possibility, we used human GBM tumor homogenate to immunize rabbits to obtain anti-GBM antiserum. This antiserum was passed over a GBM affinity column, and anti-GBM antibodies were eluted off. Antibodies that were not specific for tumor antigens were absorbed out by subsequent passage through a normal brain affinity column and cross-absorption against normal human plasma. The tumor-associated antibodies were then biotinylated and used to screen a glioblastoma plasmid cDNA expression library (Fig. 1). Using this approach, which we term DIA, positive reactions were found in 28 plaques. Positive clones were isolated, subcloned into the pZL1 plasmid vector by in vivo excision, and partially sequenced.

BLAST analysis of these sequences revealed 19 novel clones and 9 genes contained in the GenBank database. Of the nine known clones that were identified by our DIA technique, two are known to regulate growth, one codes for a chemotherapy resistance protein, and two regulate gene expression (Table 1). Of the 19 novel sequences that were identified, Northern blot analyses were performed on 9 of these cDNAs and differential expression was confirmed in tumor tissues versus normal brain in all nine of the clones tested (data not shown).

cDNA Microarray Analysis of DIA Products. To perform high-capacity screening of our DIA clones in multiple tumor and non-tumor brain tissues, we coupled our novel DIA approach with cDNA microarray analysis (18, 20, 21). Twenty-six of the 28 plasmid inserts isolated were successfully PCR-amplified using vector-specific primers that amplify the inserted fragment from the pZL1 vector. Each PCR product was then arrayed onto glass slides and hybridized with two-color fluorophore-labeled probes in a manner similar to that already published (19). The cDNA made from each sample of tumor or normal brain mRNA was labeled with the fluorescent dye Cy3 (green) and mixed with a common reference probe labeled with a second fluorescent dye, Cy5 (red). Using this high throughput arrayer, we determined the relative abundance of each of our 26 DIA clones in eight different brain tumor samples compared with non-tumor brain tissue (Fig. 2). As seen in Fig. 2, for the majority of the 26 genes tested, the three glioblastoma samples analyzed appeared to have higher levels of differential expression than the other tumor types. Furthermore, analysis of the expression of these candidate tumor-specific genes revealed that one of our isolated clones, L5, consistently had much higher levels of expression in gliomas compared with normal brain (3–30-fold).

This differentially expressed clone, L5, was therefore chosen for further characterization. The 590-bp cDNA of clone L5 was manually

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Table 1 Brain tumor-associated gene products identified by DIA

<table>
<thead>
<tr>
<th>Clone</th>
<th>GenBank ID</th>
<th>Function</th>
</tr>
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<tbody>
<tr>
<td>L2, L6</td>
<td>Human GFAP</td>
<td>Glial fibrillary acidic protein; expressed in astrocytes; used as marker for gliomas</td>
</tr>
<tr>
<td>L5</td>
<td>Human granulin</td>
<td>Peptide isolated from human granulocytes; autocrine growth factor for teratoma-derived PC cells; mitogen for 3T3 fibroblasts</td>
</tr>
<tr>
<td>L9</td>
<td>GAPDH</td>
<td>Glyceroldehyde-3-phosphate dehydrogenase; glycolytic enzyme; increased in lung cancer; putative role in apoptosis and neurodegenerative diseases</td>
</tr>
<tr>
<td>L10</td>
<td>Carboxyl reductase</td>
<td>Enzyme involved in chemotherapy resistance and free radical modulation</td>
</tr>
<tr>
<td>L19</td>
<td>DNA-dependent ATPase (putative) &amp; X-linked nuclear protein</td>
<td>Helicase involved in transcription initiation and brain differentiation (putative)</td>
</tr>
<tr>
<td>L20</td>
<td>Homologous with tyrosine kinases</td>
<td></td>
</tr>
<tr>
<td>L24</td>
<td>Hox2, mitochondrion cytochrome oxidase</td>
<td></td>
</tr>
<tr>
<td>L25</td>
<td>Osteopontin</td>
<td>Ligand for integrin; involved in adhesion, migration, and osteoclastogenesis</td>
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*Function not known.*
sequenced and found to be identical to the human granulin/epithelin precursor. Granulins (also known as epithelins) are cysteine-rich polypeptides that have growth factor-like activity. They represent a relatively new class of growth regulators, first described in 1990, with possible roles in inflammation and tumorigenesis (27, 28). The granulin gene exists as a single copy in the human genome on chromosome 17 (29). It is widely expressed in epithelial and tumorigenic cell lines in vitro, many of which respond to the gene product by enhanced cell proliferation, suggesting an autocrine or paracrine role for these factors (30–32). Several of the known structural and biological properties of the granulins resemble those of the EGF-like proteins (33–35). It is unclear whether this is physiologically significant because the in vivo expression and function of granulin have not yet been well defined.

Granulin mRNA Expression in Human Gliomas. The differential expression of granulin in human gliomas was confirmed by Northern blot analysis, which showed a transcript of 2.1-kb expressed in 86% (18 of 21) of human gliomas and 0% (0 of 3) of the non-tumor brain tissues analyzed (Fig. 3A). Interestingly, of the three gliomas that had absence of any granulin signal, one was from a patient who had received previous radiation therapy and one was from a low-grade oligodendroglioma. These data would suggest that granulin expression may be mitigated by radiation and/or may be related to higher malignancy and tumor progression. Further studies with greater numbers of irradiated or low-grade glioma samples would be necessary to confirm this hypothesis.

To better appreciate the potential role of this gene product, it would be essential to know the extent to which it is expressed within other human tissues. To determine this, radiolabeled granulin cDNA was used to probe Northern blots of a variety of peripheral organs. The probe hybridized predominantly to a 2.1-kb transcript in human testes, spleen, and kidney after 2 weeks of exposure, but not to all other human tissues tested, including normal brain, lung, heart, skeletal muscle, pancreas, liver, and adrenal gland (Fig. 3B).

Differential expression of granulin mRNA was also seen in tumor versus non-tumor tissues, using in situ hybridization. Granulin antisense riboprobe hybridized predominantly to hypercellular areas of tumor tissue (Fig. 4, A and B). The identity of these cells labeled by in situ hybridization was suggested by counterstaining the tissue sections with H&E, which revealed that the majority of the RNA was...
with tumor cells and not in the tissue stroma (Fig. 4, C and D). Sense strand riboprobe cDNA was used as a control and showed no specific labeling (data not shown), indicating that the cellular hybridization obtained with the antisense probe was specific for the granulin mRNA. Quantitation of granulin hybridization densities was measured from the in situ slides, using image analysis software. This analysis revealed significantly greater numbers of silver grains within cells of the most malignant brain tumors (e.g., anaplastic astrocytomas and GBM) compared with non-tumor glial cells (P = 0.006), confirming that elevated levels of granulin mRNA are expressed in high-grade primary brain tumors (Table 2).

**In Vitro Growth Regulation of Glial Cells by Granulin D.** Four granulins, A, B, C, and D, previously have been isolated from human inflammatory cell exudates (28, 33, 36, 37). Each is a small protein of ~6 kDa that is derived from a larger precursor of 593 amino acids, known as acrogranin (38–40). The acrogranin cDNA (clone L5) that we isolated from human glioblastomas contained the entire sequence for granulin D (bp 1254–2099; Ref. 26).

The implication of granulin molecules in growth regulation with a tertiary structure reminiscent of that of EGF suggested a potentially important role for our L5/granulin D clone as a putative growth factor. To determine whether granulin D may modulate glial cell proliferation, we synthesized a 55-amino acid peptide corresponding to the known sequence of granulin D (26). We then studied the effect of this synthetic peptide on proliferation rates of rat astrocytes in culture, using a standard [3H]thymidine incorporation assay. As shown in Fig. 5, addition of synthetic granulin D peptide stimulated DNA synthesis of rat astrocytes in vitro up to 300% in a dose-dependent manner (Fig. 5, A and B). Statistically significant increases in cell proliferation (up to 150% of controls) were seen with the addition of as little as 1 ng/ml (169 pm) of granulin D to cell culture (P = 0.025). Interestingly, this synthetic peptide had a much more modest effect on the proliferation of primary human glioblastoma cells in culture, showing only a 120–150% increase (P = 0.068) in growth with the addition of >1000 ng/ml (169 nm) granulin D (Fig. 5C). This may be explained by the fact that these human cells were tumorous and already expressed high levels of granulin (as shown by Northern blot and in situ hybridization). Thus, the putative receptors of this potential autocrine growth factor may be saturated by endogenous granulin and thereby preclude further growth stimulation by the addition of exogenous peptide. To further evaluate the growth regulatory role of granulin D on human tumor cells in vitro, a polyclonal antibody was raised against the 55-amino acid granulin D peptide and assayed for its ability to inhibit cell proliferation in three primary human glioblastoma cultures. As shown in Fig. 5D, the addition of increasing concentrations of purified granulin D antibody to early-passage human brain tumor cell cultures significantly inhibited cell growth in vitro. [3H]Thymidine incorporation was suppressed down to only 18.6% of controls with the highest concentration of antibody tested (1:100 dilution; P = 0.035).

**DISCUSSION**

The detection and characterization of a putative growth factor differentially expressed in brain tumors versus non-tumor brain tissue demonstrates the usefulness of our novel DIA technique for identification of subtractive tissue-specific gene products that may have significant biological activity. The DIA method described here is an alternative to currently established methods for the purposes of identifying differences in gene expression. It has the advantage of selecting for gene products that are actually translated from mRNA species, which can be readily cloned and synthesized for use in functional assays as we have described here. Furthermore, because this technique is based on the generation of subtractive antibodies used to screen cDNA expression libraries, antibodies to clones of interest can be generated for antibody-based studies. Because it is still speculative whether the tumor-associated antigens we identified using this approach can actually induce effective humoral or cytotoxic antitumor immune responses, further studies are needed before such conclusions can be made. Nevertheless, the results reported in this study demonstrate the potential of this technique to identify candidate glioma-associated peptides that readily allow study of expression patterns and biological function.

With current microarray technology, it is feasible to screen relatively large numbers of tumor samples for the expression of subtractive products. This allows easy discrimination of redundant clones and rapid confirmation of truly differentially expressed genes. Although we isolated and screened only 26 clones from our DIA method, it is
conceivable that thousands of differentially expressed gene products could be identified among the \( \sim 15,000 \) individual mRNA species in a pair of human cell populations (i.e., tumor versus normal). This is based on the assumption that perhaps 15% of the estimated 100,000 genes in the human genome are expressed in any individual cell type at a particular time (41). Even if thousands of differential clones are generated from our subtractive approach, current robotic microarray technology allows for the fabrication of arrays containing up to 20,000 distinct cDNA targets (18). The expression of these thousands of targets can be monitored in multiple tissue samples, just as we measured the relative expression of our 26 clones in various brain tumor tissues. Thus, microarrays in concert with subtractive gene hunting methods could serve as useful tools for the identification of biologically intriguing and clinically relevant human gene sequences.

Using the combination of DIA and microarray hybridization, we have identified a potentially interesting candidate oncogene, granulin \( D \), with a likely function in glial cell proliferation. This granulin peptide belongs to a family of putative growth factors that previously have been characterized by a unique structural motif and implicated in growth regulation (28, 30, 31, 33–35). Structurally, granulins consist of 12 cysteines with four cysteine pairs flanked by two single cysteines at both the NH\(_2\) and the COOH termini (26, 42). The predicted protein architecture consists of four stacked \( b \)-hairpins, each connected to the next with two parallel disulfide bridges, and a peptide backbone arranged as two ladders in a left-handed superhelix (34). Interestingly, this tertiary structure is partially homologous to that of

Table 2 Relative quantitation of in situ hybridization of granulin mRNA in normal brain and primary brain tumors

<table>
<thead>
<tr>
<th>Tissue</th>
<th>No. of silver grains/mm(^2)</th>
<th>Average no. of silver grains/cell</th>
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<tbody>
<tr>
<td>Normal brain (( n = 2 ))</td>
<td>50</td>
<td>1.6</td>
</tr>
<tr>
<td>Pilocytic astrocytoma (( n = 3 ))</td>
<td>3,800</td>
<td>9.6</td>
</tr>
<tr>
<td>Oligodendroglioma (( n = 3 ))</td>
<td>9,500</td>
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<tr>
<td>Anaplastic astrocytoma (( n = 2 ))</td>
<td>17,000</td>
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</tr>
<tr>
<td>Glioblastoma multiforme (( n = 3 ))</td>
<td>20,000</td>
<td>25.9</td>
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Fig. 5. Growth regulatory effects of granulin D in rat and human glioblastoma cell lines. A, photomicrographs of cell cultures of primary rat astrocytes without (left; 0 ng/well) and with (right; 500 ng/well) addition of synthetic granulin D peptide. Original magnification, ×100. B, dose-response graph of granulin D peptide on the proliferation of rat astrocytes. Addition of purified synthetic granulin D to culture media stimulated DNA synthesis of primary rat astrocyte cells up to 300% of controls as measured by standard [[3H]thymidine incorporation assays, with E_{50} = 6 ng/well. C, dose-response graph of granulin D peptide on the proliferation of human glioblastoma cells. D, growth suppressive effect of granulin D antibody in human glioblastoma cell cultures. Results were normalized in terms of percentage of control proliferation, with the control cells receiving equal volumes of heat-inactivated antibody or borate buffer (without antibody). For the human cell culture experiments (C and D), three different human glioblastoma cell lines were studied. For all studies, the controls were set to 100%, and all other counts in each experiment were normalized to this value. The results shown are combined from six separate experiments, using triplicate wells each. Columns represent mean values; bars, SD.

EGF. Functionally, several independent investigators have found granulin peptides to be regulators of cellular proliferation, with biological activities reminiscent of the actions of other polypeptide growth factors such as EGF. Granulin proteins have been shown to have mitogenic activity in murine embryonic 3T3 cells, in the tumorigenic teratoma-derived PC cell line, in human epithelial and fibroblastic cells, and in murine keratinocytes (27, 30, 33, 35). We now report similar growth regulatory effects of this peptide in primary rat astrocytes and in early-passage human glioblastoma cell lines.

The surprising parallels between the granulin and EGF systems are of interest. Given that amplification of the EGFR gene is one of the most common findings in glioblastomas and malignant astrocytomas (1, 43), it is intriguing that one of the glioblastoma-associated clones identified via our novel DIA technique may be related to the EGF/EGFR system. Nevertheless, there are many molecules that have EGF-like domains, and other investigators have found that granulin does not bind to wild-type EGFR (also called erbB-1; Ref. 44). Furthermore, Western blot analysis of EGFR expression in the human glioblastomas used in our bioassay revealed EGFR overexpression in only one of the three tumors tested, with no direct correlation between EGFR overexpression and granulin-induced growth regulation. Interestingly, however, all three of the tumors we studied had overexpression of the closely related EGFR-like transmembrane receptor tyrosine kinase erbB-2 (also called HER-2 or neu; data not shown). The significance of this coincident overexpression of granulin and erbB-2 is unknown at present.

Also intriguing in this context is the fact that both granulin and erbB-2 are genes located on chromosome 17 (29, 45). Previous reports in the literature have found that high-grade gliomas have overrepresentation of chromosome 7 and gain of chromosome 17q at the cytogenetic level (46, 47), which presumably relates to amplification of EGFR and erbB-2 at the gene expression level (48–50). Although overexpression of erbB-2 has been found in a subset of primary brain tumors, its putative ligand in brain cancers is not yet known. It would be interesting to determine whether tumors with amplification of chromosome 17q have coordinate overexpression of erbB-2 and granulin, which may support the idea of granulin being a ligand for the erbB-2 proto-oncogene autocrine/paracrine loop. Additional studies of granulin binding data and signal transduction pathways are warranted and may aid in our understanding of the oncogenesis of brain neoplasms. Given the tissue-specificity of granulin for tumor versus normal brain, its EGFR-like domains, its location on chromosome 17, and its implicated role in glioblastoma cell regulation, it is conceivable that this gene product may be a useful target for the development of new therapeutics for malignant brain tumors.

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