Phage probes for malignant glial cells

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Abstract

Early diagnosis and effective treatment of malignant gliomas, which are heterogeneous brain tumors with variable expression of cell surface markers, are inhibited by the lack of means to characterize and target tumor-selective molecules. To create molecular profiles for RG2 rat glioma cells, we used phage display technology, an approach capable of producing valuable ligands to unknown cell surface targets. The ligands were selected from libraries of peptides displayed as fusion molecules on phage particles. Modifications of the selection conditions resulted in identification of three distinctive families of peptide ligands for malignant glioma cells. The first family with V $^{D}/_{G}$ L P $^{E}/_{T}$ H^{3} binding motif appeared to target a marker that is common for glioma cells, normal brain cells, and cells of non-brain origin. The second group of peptide-presented phage displayed D $^{T}/S/_{L}$ T K consensus sequence and contained peptides with pronounced glioma-selective properties. Phage clones expressing peptides with E $^{L}/V/_{S}$ R G D S motif were found in cell lysates and represented the third family of glioma-specific ligands. All peptides within this family contain the RGD amino acid sequence, which is known to bind to a number of integrins. Phage clones that belong to this family were internalized by RG2 glioma cells about 63-fold more efficiently than by astrocytes. The approach described could be applicable for accurate detection of glioma expression patterns in individual tumors. Such patterns could be beneficial in the design of effective combinations of drugs for anti-glioma treatments. (Mol Cancer Ther. 2003;2:1129–1137)

Introduction

Cancer is a complex heterogeneous disease with molecular, morphological, and clinical differences that exist both between and within tumors (1). While histopathologic features of tumor cell morphology, invasiveness, and metastasis remain the ''gold standard'' for diagnosis and staging of cancers, molecular profiles of neoplastic cells based on DNA, mRNA, and/or protein alterations are rapidly being developed and utilized not only to augment diagnosis, but to provide new therapeutic measures (2). Of these profiles, the pattern of protein expression based on cell surface markers is the most functional, holding the potential to offer direct correlations between cancer cell ''portraits'' and therapeutic responses to anti-cancer drugs (3).

Several strategies have been implemented to date for identification of distinctive protein profiles for a given cancer. For example, separation of proteins on two-dimensional gels has been used widely for protein expression analysis in research laboratories for many years. However, such analyses are unlikely to be used routinely in a clinical setting due to their complexity and the absence of 2-D protein databases of different tumor types. A peptide microarray method was proposed recently by Aina *et al.* (4) for identification of cell surface binding profiles of cancer cells. With this technique, cell-specific peptides isolated by selection from a one-bead one-compound (OBOC) combinatorial library were linked to a polystyrene slide in a microarray format and used to detect a binding profile of human T-lymphoma cells. While the method seems promising for profiling cancer cells derived from individual cancer specimens, the diversity of OBOC libraries is relatively low and could be a limiting factor for their broad applications.

Most of the surface markers described in association with malignant glioma cells have been detected using corresponding antibodies or naturally occurring ligands. These markers include growth factor receptors such as epidermal growth factor receptor (5) and platelet-derived growth factor receptor (6, 7), cytokine receptors such as interleukin-4 receptor (8) and interleukin-13 receptor (9), transferrin receptor (10), urokinase-type plasminogen activator receptor (11), chloride channels (12, 13), membrane-type matrix metalloproteinases (14), and cell adhesion molecules such as integrins (15) and CD44 (16, 17). Variation in marker expression within the same tumor mass, over time with neoplastic progression, or among the same type of tumors from different individuals occurs frequently. For example, the multifunctional adhesion molecule involved in cell-cell and cell-matrix interactions, $CD44_s$, was shown to be expressed differentially, whereby low-grade astrocytomas (9.5%) had far fewer cells with high expression of $CD44_s$ than did glioblastomas (59%), with the positive staining being heterogeneous even within samples (16).

While some of the glioma-specific cell surface markers can be recognized by antibodies, a critical need remains for inexpensive probes that can provide a more complete profile of an individual tumor. Phage provide several

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advantages as probes for such markers: (*a*) phage probes can be developed for unknown, non-antigenic cell surface markers; (*b*) phage display libraries are readily available from commercial and private sources and are very diverse (10^9–10^{10} per library); (*c*) propagation of phage in bacterial cultures is well standardized and inexpensive compared to antibody production in animals or cell cultures; and (*d*) phage preparations are stable without loss of titer for many years when stored at proper conditions. In this study, we have utilized a landscape phage display library (18) to select phage clones that display peptides specific for cell surface markers of RG2 rat glioma cells, a well-studied model of human glioma. Probes designed using this approach could be applied instead of antibodies (that are expensive and can be developed only if the antigen is known and available) to profile individual glioma specimens, such as biopsies and tissue sections. Based on the cancer profile, a combination of anti-cancer drugs can be designed to increase therapeutic effectiveness and reduce toxicity.

Materials and Methods

Cells

Cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The RG2 (CRL-2433) and F98 (CRL-2397) cell lines are undifferentiated malignant rat gliomas. Normal rat cells included fetal skin fibroblasts 27FR (CRL-1213), cardiac myoblasts H9c2(2-1) (CRL-1446), astrocytes CTX TNA2 (CRL-2006), and hepatocytes BRL 3A (CRL-1442). All cells were grown in media as recommended by ATCC and incubated at 37°C, 5% CO_2. For use in phage display selection protocols, all cell types were grown in 25 cm^2 polystyrene flasks for 48 h to reach sub-confluent monolayers. To accomplish this, RG2 cells were plated at 2.5×10^6 cells/flask; myoblasts, hepatocytes, and fibroblasts were plated at 1×10^6 cells/flask; and astrocytes and F98 glioma cells were plated at 2×10^6 cells/flask.

Phage Display Library

A landscape f8-1/8-mer phage display library (18) was used to identify specific peptide ligands for RG2 rat glioma cells. In this library, phage express foreign peptides as part of the major coat protein, pVIII. The size of the library was approximately 2×10^9 clones. All general methods of handling phage, including propagation, purification, titering, production of pure phage clone, and isolation of phage DNA were those described in detail in *Phage Display: A Laboratory Manual* (19).

Phage Display Library Selection

The method of Barry *et al.* (20) with our modifications was used to select phage clones which recognize glioma-specific cell surface markers. All selections began with depletion of phage clones binding to plastic (see Fig. 1). An aliquot of the primary library (100 copies of each phage clone in Selections 1 and 2; 10 copies of each in Selection 3) in 2 ml of washing/blocking buffer (0.1% BSA, 0.1% Tween 20 in DMEM) was added to an empty flask (depletion flask) and incubated for 1 h at room temperature. At the same time, cells were incubated for 1 h at 37°C, 5% CO_2 in serum-free medium that was removed immediately before application of the phage. In Selection 1, buffer containing phage that did not bind to plastic was transferred from the depletion flask to the flask containing RG2 cells for 1 h, room temperature. In Selections 2 and 3, phage that did not bind to plastic were transferred from the depletion flask to a flask containing normal rat cells to deplete phage clones that bind to common receptors (see Fig. 1). Following the final incubation with RG2 cells in all selection schemes, phage not binding to tumor cells were washed away with washing/blocking buffer. For retrieval of cell-surface bound phage, RG2 cells in flask 1 (see Fig. 1, Selection 1) were treated with 1 ml elution buffer (0.1 M glycine-HCl, pH 2.2) for 10 min on ice. The eluate was removed from the flask, neutralized with 175 μl 1 M Tris (pH 9.1), and phage were concentrated by centrifugal concentrators (Centricone 100 kDa, Fisher Scientific, Pittsburgh, PA) to an approximate volume of 150 μl. To recover all cell-associated phage in Selection 1, RG2 cells were scraped from flask 2 in 5 ml DMEM and pelleted by centrifugation at $130 \times g$ for 10 min. DMEM was removed and cell pellet was lysed with 200 μl of lysis buffer [2% deoxycholic acid (sodium salt), 10 mM Tris, 2 mM EDTA (pH 8.0)]. For retrieval of phage in Selections 2 and 3 (Fig. 1), the procedure was the same as for Selection 1 with the exception that both phage fractions (eluted and lysis) were obtained sequentially from the same flask. These phage fractions were amplified separately in bacteria and used in subsequent rounds of selection for RG2 tumor cell recognition. The remaining rounds of selection were accomplished according to procedures described above, but without negative selection steps on normal cells. Following the 4th round (Selections 1 and 2) or the 6th round (Selection 3), phage DNAs were isolated, sequenced, and foreign oligonucleotide inserts translated to reveal peptide sequences responsible for binding to tumor cells.

Quantitation of Cell-Associated Phage

Binding specificity of RG2-selected candidate phage clones was confirmed by comparison to a control phage (phage library vector, f8-5, which does not express any foreign peptides) and a second control phage clone (1F20, which expresses a random peptide EAGPRSAP). Briefly, RG2 cells were grown in 25 cm^2 flasks for approximately 48 h to sub-confluence. Before application of phage, media were changed to serum-free for 1 h. Each phage clone ($\sim 10^9$ cfu/flask) was added to the cells in 2 ml of washing/blocking buffer, and incubated for 1 h at room temperature. The media with unbound phage were removed carefully from the flasks, and cells were washed eight times with 4 ml of cold washing/blocking buffer for 5 min/wash. Cell surface-bound phage were eluted with 1 ml of elution buffer, for 10 min on ice. The eluate was removed from flasks and neutralized with 1 M Tris. Cells were washed again two times with washing/blocking buffer and lysed with 1 ml lysis buffer for 30 min at room temperature and processed as above. Phage titers were determined by infection of bacteria and presented as a ratio of output to input phage. All analyses were done in triplicate.

To test phage selectivity, RG2-selected candidate phage clones were incubated with RG2 glioma cells or various control cell lines including F98 glioma, CTX TNA2 astrocytes, 27FR fibroblasts, H9c2(2-1) cardiac myoblasts, and BRL 3A hepatocytes. Bound phage were recovered and titered as in the specificity test described above.

Results

Cell surface molecular expression patterns may include receptors which are common to multiple cell lineages, restricted to one or a few cell lineages, and those which are unique to individual cell types. Additionally, among various types of cells, common receptors may be expressed similarly or at different densities. To identify ligands for common as well as unique glioma cell markers, we developed three phage display selection strategies (Fig. 1). The first selection protocol was designed to identify phage clones which bind to high-affinity/density RG2 glioma cell surface receptors and might or might not recognize receptors on other cell types, since the scheme did not include pre-selection on other cells. Steps of negative selection on fibroblasts, myoblasts, and hepatocytes were included in the second protocol (Fig. 1) to remove phage clones that bind to receptors on these cells which represent major cell types in the body. Introduction of these steps might lead to identification of phage clones with brain specificity. In the third protocol, we significantly reduced the number of copies of each phage clone in the library to remove excess phage capable of binding to normal cells, including astrocytes (see Selection protocol 3, below). Additionally, blocking phage (same as control phage) were added to the library in incubation medium to minimize non-specific binding (Fig. 1, Selection 3). This protocol was developed to find phage clones that might be selective for tumor cells of glial origin. To exclude phage that bind to plastic, all three protocols started with incubation of the library in an empty (cell-free) flask made of the same plastic as those used to grow cells.

Selection Protocol 1

Since we began our study in search of dominant glioma-specific phage clones, no depletion on normal cells was done in these experiments. After incubation with RG2 cells, phage not bound to the cells were removed in multiple washing steps. To determine cell-specific phage clones that bound to and/or were internalized by glioma cells, the following two approaches were utilized.

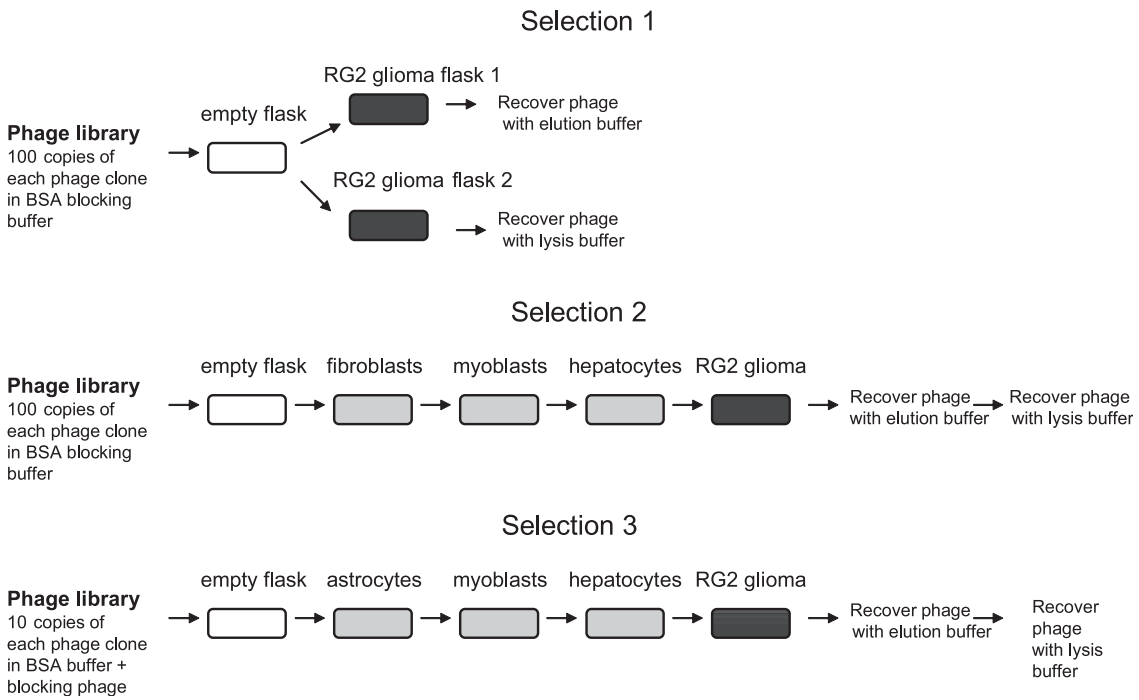

Figure 1. Selection schemes for identification of phage clones that recognize molecular markers on RG2 glioma cells. *Selection 1*, RG2 cells were grown to a sub-confluent monolayer. First, an aliquot of the primary library in washing/blocking buffer was added to an empty flask (depletion flask) to remove plastic-binding phage clones. Buffer containing phage that did not bind to plastic was transferred to two flasks with RG2 glioma cells for incubation. After that, phage not associated with tumor cells were washed away. Cell-surface bound phage were recovered with low pH elution buffer (*RG2 glioma flask 1*). In a parallel experiment (*RG2 glioma flask 2*), all phage clones including cell surface bound and internalized phage were recovered using lysis buffer. Eluted phage and those obtained after cell lysis were amplified in bacteria and used in subsequent rounds of selection for tumor cell binding. *Selection 2*, before incubation with RG2 cells, preselection steps with fibroblasts, myoblasts, and hepatocytes were included in the protocol to remove phage binding to these normal cells. Phage associated with RG2 cells were recovered from a single flask in two sequential steps, first with elution buffer and then with lysis buffer. *Selection 3*, here, compared to the previous protocols, 10-fold less of the primary library was used and astrocytes were included in pre-selection steps. For each protocol, only the first round of selection is shown in the figure. The consecutive rounds were performed in the same manner in all three schemes (see ''Materials and Methods'').

Cell Surface-Bound Phage Clones. Cell surface-bound phage were recovered with low pH elution buffer (Fig. 1, RG2 glioma flask 1). Eluted phage were used for amplification in bacteria and in subsequent rounds of selection for tumor cell binding. Four rounds of screening on RG2 cells were performed. In each round, the enrichment in phage binding to the cells was determined via titering of input and output phage. The ratio of output to input phage increased from one round to another (280-fold increase after four rounds), indicating successful selection for phage clones that bind to RG2 glioma cells.

Phage Clones from Whole Cell Lysate. In parallel experiments, all phage clones including cell surface-bound and internalized phage were recovered using lysis buffer (Fig. 1, RG2 glioma flask 2). Phage were used for amplification and further selection for tumor specificity as above. The enrichment in phage binding to the tumor cells was determined via titering of input and output phage for each round. After the 4th round, the ratio of output to input phage isolated after cell lysis was approximately 320-fold higher than for the original library. This increase demonstrated effective selection for phage clones that bind to and are internalized by RG2 glioma cells.

Peptide Sequences from Phage Display Selection on RG2 Glioma Cells. In both experiments following the 4th round, phage DNAs were isolated and sequenced. Translation of foreign oligonucleotide inserts in phage DNA revealed the peptide sequences of candidate ligands for tumor cell-specific receptors (Fig. 2). For cell surface-binding phage, a consistent motif of six amino acids, V $^{D}/_{G}$ L P $^{E}/_{T}$ H, was identified.

For phage recovered from whole cell lysate, several different types of sequences were found (Fig. 2). Three of the sequences had a similar motif as those from the cell surface fraction. Additionally, dominant sequences were recovered which contained the RGD motif known to bind to integrins. Two kinds of RGD sequences were obtained, differing from each other by the amino acid in the second position. The first group contained leucine (L), the second group contained valine (V). These amino acids have non-polar side chains differing only in length and, thus, are likely to perform the same role in the recognition of cell receptor(s). Having two similar, but not identical, sequences is indirect proof of the specificity of the selection process.

Specificity and Selectivity of Identified Phage Clones. Phage clones shown in Fig. 2 were tested further in a binding assay to determine the strongest binder among them and to confirm the specificity of these clones to glioma cells. As seen in Fig. 3, two cell surface-binding clones (#3 and #19) appeared to be the best binders since they demonstrated the highest ratio of output to input phage. Binding of cell surface clones selected for glioma was compared to two control phage clones. One of the controls was the phage library vector f8-5 which does not express any foreign peptides, and the second control was a phage clone 1F20 that expresses a random peptide. Binding of both controls to glioma cells was negligible, approximately 1000-fold less than that of clones selected for these cells.

Elution buffer

sequence	*frequency*
V D L P E H G K	9
V G L P E H T Q	5
V G L P E H S A	4
V D L P T H S S	7
V D L P E H R Q	1
V D L P T H Q S	1
V D L P T H N Q	1
V D L P Q **H** G Q	1
D T T K N G S G	1
random	1

Lysis buffer

sequence	*frequency*
E L R G D S L P	10
E V R G D S L P	2
V D L P S H P E	1
V N L P E H P E	2
V D L P R S D T	1
H **T T K** E Q M A	1
random	12

Figure 2. Selection protocol 1. Peptide sequences from phage display selection on RG2 glioma cells.

Additionally, phage clone VGLPEHTQ binding to RG2 cells with the highest affinity was used to test its selectivity. Binding of this phage clone to RG2 glioma cells was compared to that of other cell types (Fig. 4A). Binding to another glioma cell line, F98, appeared to be 41% of that to RG2 cells. Binding to astrocytes was 64% of that occurring with RG2 cells. Approximately 5-fold less binding occurred with unrelated normal control cells (hepatocytes, myoblasts, and fibroblasts were used to represent other major cell lineages). Thus, overall selectivity of the phage clone was found to be in the range of 2- to 5-fold, depending on the type of control cells used.

Similar selectivity studies have been performed for ELRGDSLP phage clone recovered after lysis of the cells (Fig. 4B). While this phage clone specifically recognized glioma, it also recognized other cells such as hepatocytes, myoblasts, and fibroblasts, which are non-brain in origin. This may be due to the presence of different RGD-binding receptors on those cells. The phage clone was, however, selective for glioma when compared to brain cells (astrocytes) by approximately 63-fold.

Selection Protocol 2

To find glioma-specific ligands which do not cross-react with other cell types, the previous selection scheme was modified. The starting library was initially depleted in three additional steps of negative selection on normal cells that represent major cell types in the body (fibroblasts, myoblasts, and hepatocytes). Normal brain cells were not included in this protocol. Phage clones that remained following depletion were screened against RG2 glioma cells to select for tumor-specific binding molecules.

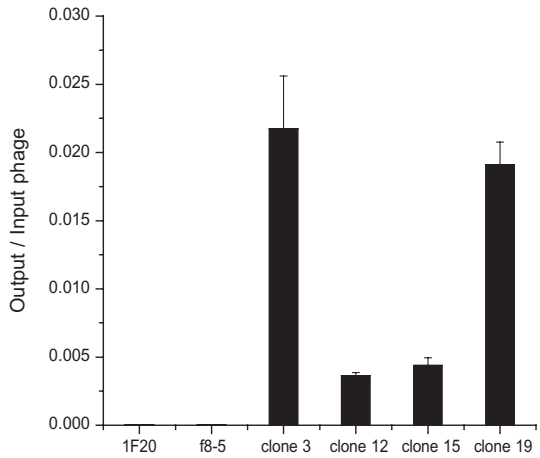

Figure 3. Selection protocol 1. Binding of selected and control phage clones to RG2 glioma cells. RG2 cells were grown to sub-confluent monolayer and incubated with different pure phage clones selected for RG2 cells (*clones 3, 12, 15,* and *19*) or control phage clones (*1F20* and *f8-5*). After that, unbound phage were washed away and attached phage were eluted from cell surfaces with a low-pH buffer. Phage titers for output and input phage were determined by infection of bacteria. *Charts,* ratio of output to input phage for each phage clone. The numbers for control phage were too low to be seen in the chart. The sequences of the clones used in these experiments were: clone 3 is V G L P E H T Q; clone 12 is V D L P T H S S; clone 15 is V D L P E H G K; clone 19 is V D L P Q H G Q.

Recovery of Cell-Associated Phage from RG2 Rat Glioma Cells. In each round of selection, phage associated with glioma cells were recovered in two sequential steps: first, with elution buffer and then with lysis buffer. For both phage fractions, elution and lysis, the selection process was monitored by titering of input and output phage. As in Selection 1, the selection was stopped after the 4th round when the significant increase in phage associated with the cells was observed. Fifty-three random individual phage clones from the output of the last round were propagated and the DNA inserts encoding foreign peptide were sequenced.

Peptide Sequences from Phage Display Selection on RG2 Glioma Cells. Among clones in the elution fraction, a family of phage bearing peptides with V $^{D}/_{G}$ L P $^{E}/_{T}$ H consensus sequence similar to those identified from Selection 1 was found (Fig. 5). While represented by nearly 100% in Selection 1 (see Fig. 2, elution fraction), they appeared only as one third of all the sequences under changed conditions (see Fig. 5, elution fraction). In addition, a new dominant family of peptides with D $^{T}/_{S}$ T K consensus motif was found. In the lysis fraction, most of the sequences revealed the similar motif of three amino acids, D $^{T}/_{S}$ T. Demonstrating a great diversity, all the sequences in this family were represented only once, each with differing flanking amino acids.

Selectivity of Phage Clones. Two phage clones, one from the elution and one from the lysis fraction, were tested for selectivity to rat glioma by quantitation of cell-associated phage after phage-cell incubation. The binding of DSTKSGNM phage to RG2 and F98 gliomas, and normal rat cells including astrocytes, is shown in Fig. 6A. This clone demonstrated highly selective binding to RG2 glioma cells when compared to normal astrocytes (25-fold increase) or to other normal cells, including fibroblasts, myoblasts, and hepatocytes (32- to 86-fold increase). Moreover, DSTKSGNM clone showed even better binding to another rat glioma, F98, than to the target RG2 cells. The second phage clone tested in these experiments, DYDMTKNT, was selected for glioma after cell lysis. Unlike the other phage clones, it contains DMTK motif in the middle of the sequence and methionine is in the second position of the motif. This phage clone appeared to be very selective for both types of glioma cells as well (Fig. 6B).

Selection Protocol 3

Analysis of the data obtained in the two selection protocols described above dictated the rationale for

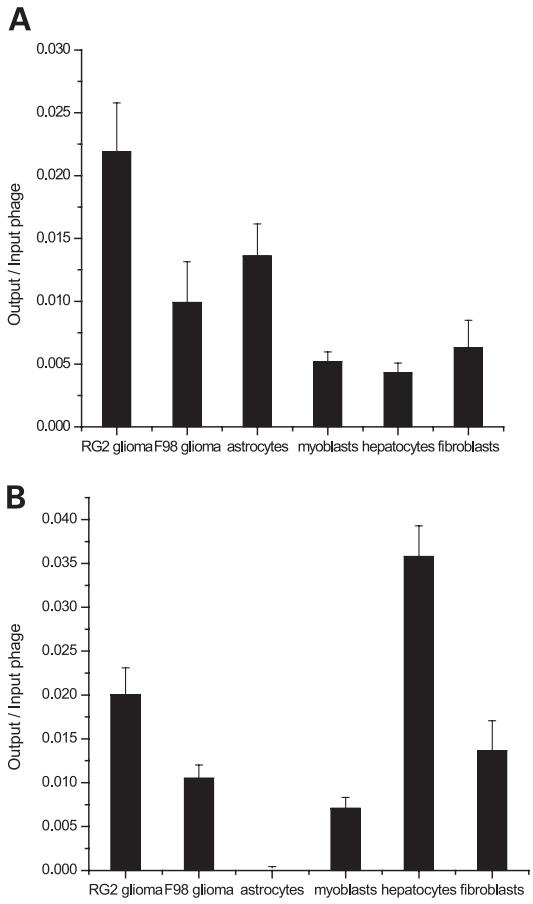

Figure 4. Selection protocol 1. Selectivity of glioma-specific phage clones for RG2 and control cells. **A,** RG2 glioma and control (*F98 glioma*, *astrocytes*, *myoblasts*, *fibroblasts*, and *hepatocytes*) cells were incubated with RG2-specific phage clone VGLPEHTQ. After the incubation, unbound phage were removed and phage attached to the cells were eluted with a low-pH buffer. Phage titers in the eluates were determined, and plotted as output to input ratios (*Y* axis) against different cell types (*X* axis). **B,** ELRGDSLP phage clone selected for RG2 glioma was recovered by lysis after the incubation with cells of all types, including RG2 and control cells shown in the figure. Phage associated with each cell type are presented as the ratios of output to input phage.

Elution buffer

sequence	*frequency*
V D L P Q H G G	4
V D L P T H T S	1
V N L P E H A Q	2
V G L P E H Q P	1
D T T K T S A G	1
D S T K I G T S	1
D S T K A S D A	1
D T T Q S M H T	1
D S T K S T N S	1
D S T K A V A L	1
D S T K S G N M	1
D T T K G P G T	1
D G T K M A G G	1
random	4

Lysis buffer

sequence	*frequency*
D T T K G G N P	1
D D T K H S L P	1
D T T R T H M P	1
D S T R G S P A	1
D S T R T T S A	1
D T T R L S D Q	1
D N T R V A A P	1
D D T R Y S S A	1
D E T L Y G I S	1
D Y D M T K N T	1
random	22

Figure 5. Selection protocol 2. Peptide sequences from phage display selection on RG2 glioma cells.

additional modifications to the selection conditions. The continued presence of the V $^{D}/_{G}$ L P $^{E}/_{T}$ H phage clones (found to bind to all cell types tested) after several depletion steps on different cell lines (Selection 2) indicated that depletion of clones recognizing normal cells was not complete. To address this problem, 10-fold less of the initial library than in the previous two protocols was used in the 3rd protocol. Additionally, the presence of many random sequences after screening demonstrated nonspecific binding of the library phage to RG2 cells under the conditions of Selection 2. Therefore, to better block nonspecific interactions which can occur due to the contribution of phage minor coat proteins, the preparation of irrelevant blocking phage was added to the regular blocking BSA buffer. These blocking phage were nonspecific to the target cells, but due to the contribution of phage minor coat proteins, could compete for binding sites with other nonspecific phage clones in the library. To further enhance the selection of brain tumor-specific phage, astrocytes were used for negative library screening.

Recovery of Cell-Associated Phage from RG2 Glioma Cells. Unlike the two previous protocols, it took six rounds of selection to achieve significant enrichment in cell-associated phage. In the first round, phage were collected in two sequential steps from the same flask with RG2 cells. In each following round, the selection was performed using two separate flasks (one for elution fraction and the other for lysis fraction) and the corresponding phage fraction was saved, amplified, and used for the next round. Phage DNAs were sequenced when maximum enrichment in cell-associated phage was observed: after the 6th round for phage eluted from the cells, and after the 5th round for phage in the lysis fraction.

Peptide Sequences Selected for RG2 Cells. Randomly, 21 phage DNAs were isolated and sequenced (Fig. 7). Within the phage population recovered by elution, the diversity of phage clones decreased sharply. Nine out of 11 peptides were identical. Despite this fact, a family with D $^{T}/_{L}$ T K motif was found which differs from similar

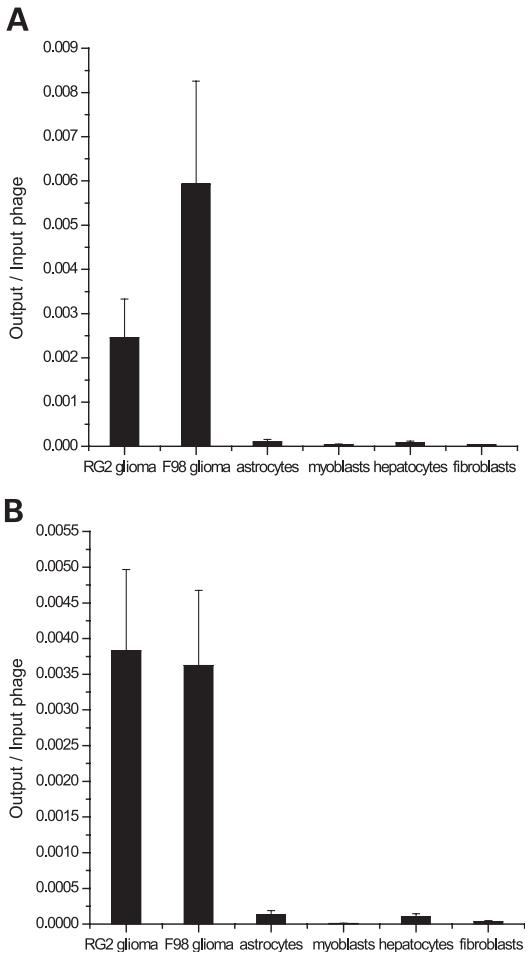

Figure 6. Selection protocol 2. Selectivity of glioma-specific phage clones for RG2 and control cells. **A,** RG2 glioma and control cells were incubated with DSTKSGNM phage clone. Phage attached to the cells were recovered by elution. Phage titers in the eluates were determined by infection of bacteria and plotted as output to input ratios (*Y* axis) against different cell types, including RG2 and F98 gliomas, astrocytes, myoblasts, hepatocytes, and fibroblasts (*X* axis). **B,** after the incubation with DYDMTKNT phage, cells were lysed and cell-associated phage were recovered (see ''Materials and Methods''). Titer of phage in cell cultures of all cell types were determined as above and shown in the figure as ratios of output to input phage.

Elution buffer

sequence	*frequency*
D L T K S T A P	9
D T T K S T T T	1
E P V Q P H S T	1

Lysis buffer

sequence	*frequency*
E S R G D S Y A	2
D L T K S S A P	1
D T T K L T N Q	1
D N A I Y T Y Q	2
A S N H V M Y Q	4

Figure 7. Selection protocol 3. Peptide sequences from phage display selection on RG2 glioma cells.

peptides obtained in experiments 1 and 2 by L amino acid in the second position. Phage collected by cell lysis were more diverse than those from the elution fraction. This fraction was comprised of several groups including those with D $^{T}/_{L}$ T K and ESRGDS motifs and a group of identical sequences ASNHVMYQ.

Selectivity of Phage Clones. Selectivity studies analogous to previous experiments (shown in Figs. 4, A and B and 6, A and B) were performed with two phage clones recognized in Selection 3. One of them DLTKSTAP appeared to be very selective for RG2 cells when compared to hepatocytes, myoblasts, and fibroblasts (Fig. 8A). The selectivity was higher than for any previously tested phage clone, and was in the range of 185- to 615-fold. When binding to RG2 glioma cells was measured against binding to astrocytes, it was elevated by approximately 14-fold. The second phage clone tested, ESRGDSTA (Fig. 8B), was similar to that identified in Selection 1, with five identical amino acids at the NH_2 terminus (positions 1, 3, 4, 5, and 6), but different amino acids in the remaining three positions. Interestingly, both phage clones showed similar selectivity patterns; however, the output/input phage ratio was significantly lower for phage clone found in Selection 3 (compare Figs. 4B and 8B). This perhaps reflects the reduced number of copies of each phage clone used for the 3rd protocol.

Discussion

The RG2 rat glioma cell line chosen for this study is an undifferentiated malignant glioma from the brain of a Fisher 344 rat pup following injection of *N*-ethyl-*N*-nitrosourea into the pregnant dam (21). Inoculation of these cells into Fischer rats is a well-established, reproducible animal model of glioma mimicking human brain tumor growth and spread (22). The cell line has been utilized extensively for understanding tumor biology and developing and testing diagnostic and therapeutic techniques for gliomas (reviewed by Barth (22)).

To obtain phage probes for molecular profiling of RG2 glioma cells, three independent phage display selection experiments were performed in this study. In all experiments, a single landscape phage display library was used, while the conditions were modified. This library was constructed by the insertion of foreign oligonucleotides in phage DNA that resulted in expression of random eight amino acids at the NH_2 terminus of each copy of major phage coat protein, pVIII (18). Therefore, each virion displays 4000 copies of the peptide that varies from one phage clone to the next (2×10^9 phage clones total). Depending on peptide copy numbers, all phage display libraries are classified as mono-/oligo- or multivalent. One (rarely two or more) family of binding peptides with the highest affinity to cell surface markers is likely to be selected from monovalent libraries. Due to multiple

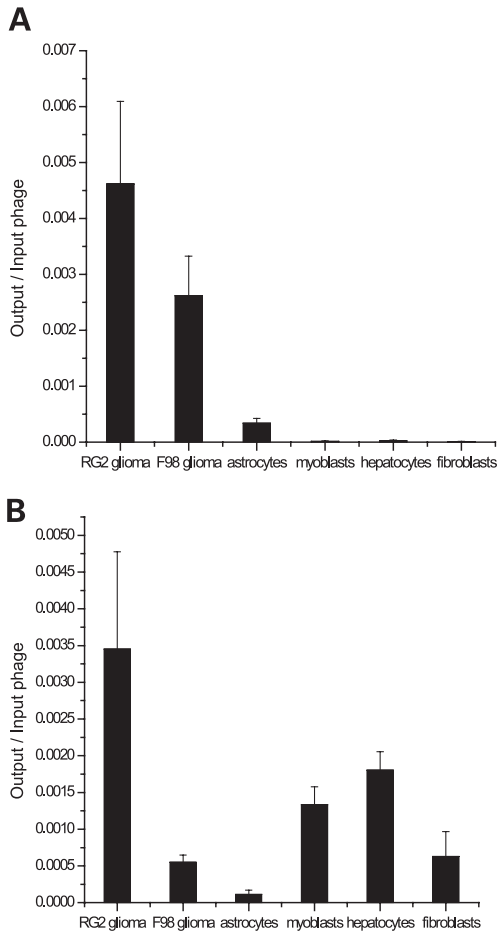

Figure 8. Selection protocol 3. Selectivity of glioma-specific phage clones for RG2 and control cells. **A,** RG2 glioma and control cells (*F98 glioma*, *astrocytes*, *myoblasts*, *hepatocytes*, and *fibroblasts*) were incubated with DLTKSTAP phage clone. Phage attached to the cells were recovered by elution and phage titers determined. **B,** after the incubation with ESRGDSYA phage, cells were lysed and cell-associated phage were retrieved as described in ''Materials and Methods.'' Titer of phage in cell cultures of all cell types were determined. All data are expressed as output to input ratios (*Y* axis) against different cell types (*X* axis).

peptide-cell marker bindings, selection from multivalent libraries (including the landscape library used in this study) is based on the avidity effect and results in identification of a number of cell-binding peptide families (23). Here, we have identified at least three peptide families and found two more groups of identical peptide sequences (Selection 3) that require further examination.

In each protocol, modification of the selection conditions led to identification of unique as well as similar peptides specific for glioma cells. Three major families of peptides with the following consensus motifs have been recognized. The motif in the first family, V $^{D}/_{G}$ L P $^{E}/_{T}$ H, consists of six amino acid residues, four of which were identical for all family members (positions 1, 3, 4, and 6). Variable amino acids were found in the two other positions. Position 2 was occupied by either aspartic acid or glycine with acidic side chain or uncharged polar side chain, respectively. Position 5 was occupied by either glutamic acid (acidic side chain) or threonine (uncharged polar side chain). Both amino acids have similar side chains to those in the second position, demonstrating that their presence within the sequence is not random. It is intriguing that the above-mentioned motif, V $^{D}/_{G}$ L P $^{E}/_{T}$ H, contains a VDL sequence which is the reverse of LDV sequence, a known α4β1 integrin binding motif in fibronectin (reviewed in Ruoslahti (24)).

High amino acid similarity was shown within the second peptide family which included such consensus sequences as DTTK (appeared only once in Selection 1), D $^{T}/_{S}$ T K (was dominant in Selection 2), and D $^{T}/_{L}$ T K (was moderate in Selection 3). Variable amino acids in the second position have nonpolar (leucine) or uncharged polar side chains of different length (threonine or serine) and likely perform the same function (are interchangeable) within the sequence. These data demonstrate a remarkable specificity of cell recognition process, resulting in the isolation of very similar peptide sequences in three independent experiments from approximately 2×10^9 variants. Database searches (via BLAST) for proteins matching the consensus motifs present in the family did not reveal a protein or a peptide that could be considered a natural ligand for glioma cells. Therefore, they might be either novel ligands for glioma cell surface markers or mimotopes of previously characterized ligands. In general, sequences of binding peptides identified via phage display are too short (three to four, rarely five to six amino acids) to make convincing conclusions about their nature, even if a homology to known proteins is found.

Phage clones expressing peptides with E $^{L}/_{V}$ R G D S and ESRGDS motifs were found in the fractions of cell lysates in Selections 1 and 3, respectively, and represent the third family of identified peptides. All peptide sequences within this family contain a well-recognized RGD motif, which is known to bind to a number of integrins which function in cell adhesion (reviewed in Ruoslahti (24)). It is interesting to note that these sequences contain not three, but four amino acids, RGDS, which are present in fibronectin (PIR-PSD protein database), a well-described ligand for a number of integrins expressed on brain cells. In addition, synthetic RGDS peptide is known to block integrin-mediated adhesion to vitronectin (25), an extracellular matrix ligand for αvβ3 and αvβ5 integrins that are overexpressed on brain tumors (26).

By titering cell-associated phage, glioma-selected phage clones described above were evaluated further for cell specificity and selectivity. The specificity was confirmed by counting cell-associated phage following the incubation of glioma cells with individual phage clones, both test and control. It was demonstrated that glioma-selected phage are highly specific and bind to two different glioma cell lines (RG2 and F98) by several orders of magnitude greater than phage clones bearing irrelevant peptides (library vector f8-5, or clone 1F20). In contrast, the study by Spear *et al.* (27) resulted in identification of phage clones with low specificity and selectivity to glioma cells. In our opinion, these results most likely were obtained due to the small size of their library (2×10^7 phage clones) as well as the methodological reasons discussed by the authors.

Similar studies were performed to test peptide selectivity. In this test, binding properties of phage clones selected for glioma to various other cell types were compared. The less selective phage clone with V $^{D}/_{G}$ L P $^{E}/_{T}$ H binding motif was identified in Selection 1, when no preselection on other cell types was conducted. It showed insignificant preferential binding to RG2 glioma cells compared to normal cells, including astrocytes and several other cell types of non-brain origin. In contrast, ELRGDSLP phage clone isolated under the same conditions from whole cell lysate was internalized by RG2 glioma cells about 63-fold more efficiently than by astrocytes. This clone might be used to construct a selective gene therapy vector for intratumoral delivery of therapeutic genes. However, the clone showed no selectivity in comparison to hepatocytes, myoblasts, and fibroblasts. This could be explained by the fact that different RGD binding receptors (*e.g.*, different integrins) which are commonly expressed in many tissues contributed to accumulation of this phage clone within all cell types tested.

The most selective phage clones were identified within the D $^{T}/s/_{L}$ T K family, when normal cell types were used for negative library screening before selection on RG2 glioma cells (Selections 2 and 3). Additionally, phage not specific for glioma was added to the blocking buffer in Selection 3 as an extra blocking agent. Two phage clones examined for cell type selectivity from this family had the consensus motif at the amino-terminal end of the sequence, while the third one contained this motif in the middle (DYDMTKNT). Since each phage clone described above has an individual binding pattern, it is unclear if all members of this family recognize the same cell surface marker. The observed variability is likely due to the different flanking sequences surrounding the consensus sequence. Regardless, the ability of these phage to distinguish glioma from normal cells of brain or other tissues can be readily used for construction of targeting vectors for glioma cells.

In summary, markers on the surfaces of RG2 rat malignant glial cells were portrayed by identification of their ligands

selected from a single phage display library. Usage of different phage display libraries will permit the identification of additional cell-specific ligands for cell surface markers. Data developed from human glioma cell lines and surgical specimens will provide ''portraits'' of individual gliomas which could lead to multiple clinical applications, including improved diagnosis, development of targeting anti-cancer agents, and prediction of responses to existing therapies.

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