THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 19, Issue of May 11, pp. 16464–16468, 2001
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Printed in U.S.A.

Systematic Evolution of a DNA Aptamer Binding to Rat Brain Tumor Microvessels

SELECTIVE TARGETING OF ENDOTHELIAL REGULATORY PROTEIN PIGPEN*

Received for publication, January 16, 2001
Published, JBC Papers in Press, February 13, 2001, DOI 10.1074/jbc.M100347200

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Tumor microvessels differ in structure and metabolic function from normal vasculature, and neoangiogenesis is associated with quantitative and qualitative changes in expression of endothelial proteins. Such molecules could serve as molecular addresses differentiating the tumor vasculature from those of the normal brain. We have applied Systematic Evolution of Ligands by EXponential enrichment (SELEX) against transformed endothelial cells as a complex target to select single-stranded DNA-ligands (aptamers) that function as histological markers to detect microvessels of rat experimental glioma, a fatal brain tumor that is highly vascularized. Both the SELEX selection procedure as well as subsequent deconvolution-SELEX were analyzed by fluorescence based methods (flow cytometry and fluorescence microscopy). Of 25 aptamers analyzed, one aptamer was selected that selectively bound microvessels of rat brain glioblastoma but not the vasculature of the normal rat brain including peritumoral areas. The molecular target protein of aptamer III.1 was isolated from endothelial cells by ligand-mediated magnetic DNA affinity purification. This protein was identified by mass spectrometry as rat homologue of mouse pigpen, a not widely known endothelial protein the expression of which parallels the transition from quiescent to angiogenic phenotypes *in vitro*. Because neoangiogenesis, the formation of new blood vessels, is a key feature of tumor development, the presented aptamer can be used as a probe to analyze pathological angiogenesis of glioblastoma. The presented data show that pigpen is highly expressed in tumor microvessels of experimental rat brain glioblastoma and may play an important role in warranting blood supply, thus growth of brain tumors.

Neoangiogenesis, the new formation of blood vessels is associated with endothelial cell (EC)[1] proliferation, migration, and formation of new capillaries as a response to the increased demand of tumor tissue for oxygen and nutrients (1). In normal physiological processes, such as wound-healing, angiogenesis is tightly balanced by positive and negative regulators. In several disease states, such as tumor growth, overactive angiogenesis contributes to advancement of disease (2, 3). Positive regulators of angiogenesis are recruited by the tumor to dominate negative regulators to ensure proliferation and organization of microvessel-forming EC. Thus, neoangiogenic EC, associated with tumor development, differ from preexisting, quiescent EC by qualitative or quantitative changes of molecular addresses, which could serve as potential targets for tumor homing diagnostic and therapeutic agents (4–6). A series of targets and corresponding antibodies to address the activated endothelium of pathological blood vessels are known (2). But it must been supposed that there are other regulators, so far unknown, to be involved in the complex process of angiogenesis. To define novel molecular addresses of the tumor vasculature, combinatorial chemistry approaches, the libraries of which are not biased by natural mechanisms of selection such as immune tolerance (7), have been successfully applied to develop antibody fragments by phage display that bind to tumor blood vessels (8–12). However, peptides require circularization, dimerization, or presentation in the context of a larger protein for binding to a target (13). These structural requirements limit the number of potential ligands that can be sampled in an experiment directed against complex targets. Systematic Evolution of Ligands by EXponential enrichment (SELEX) is a nucleic acid based combinatorial chemistry procedure that has been used to isolate relatively short high affinity ssDNA or RNA ligands, termed aptamers, to a wide variety of protein or low molecular weight compounds (14, 15). Single-stranded nucleic acids, which can fold into very small and complex three-dimensional shapes with a great diversity of binding specificities, are isolated from a large pool of random sequence molecules (10^{14}-10^{15} sequences) by reiterative rounds of selection and amplification (reviewed in 16).

Here we describe a fluorescence-based SELEX procedure. Using transformed EC as a complex target allowed systematic evolution of fluorescence-labeled oligonucleotides and subsequent *in situ* deconvolution-SELEX by flow cytometry and fluorescence microscopy on cryostat tissue sections (Fig. 1).

Finally aptamers were generated that function as a histological marker to selectively stain microvessels of experimental rat glioblastoma, a brain tumor that is highly vascularized (17). We present aptamer III.1, a ssDNA-ligand binding to pigpen, a

* This work was supported by the Wilhelm Sander Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: EC, endothelial cell(s); ssDNA, single-stranded DNA; SELEX, Systematic Evolution of Ligands by EXponential enrichment; nt(s), nucleotide(s); FITC, fluorescein isothiocyanate; 18C, 18-carbon ethylene glycol spacer; trB, triple biotin; PCR, polymerase chain reaction; BSA, bovine serum albumin; DAPI, 4′,6 diamidino-2-phenylindole.

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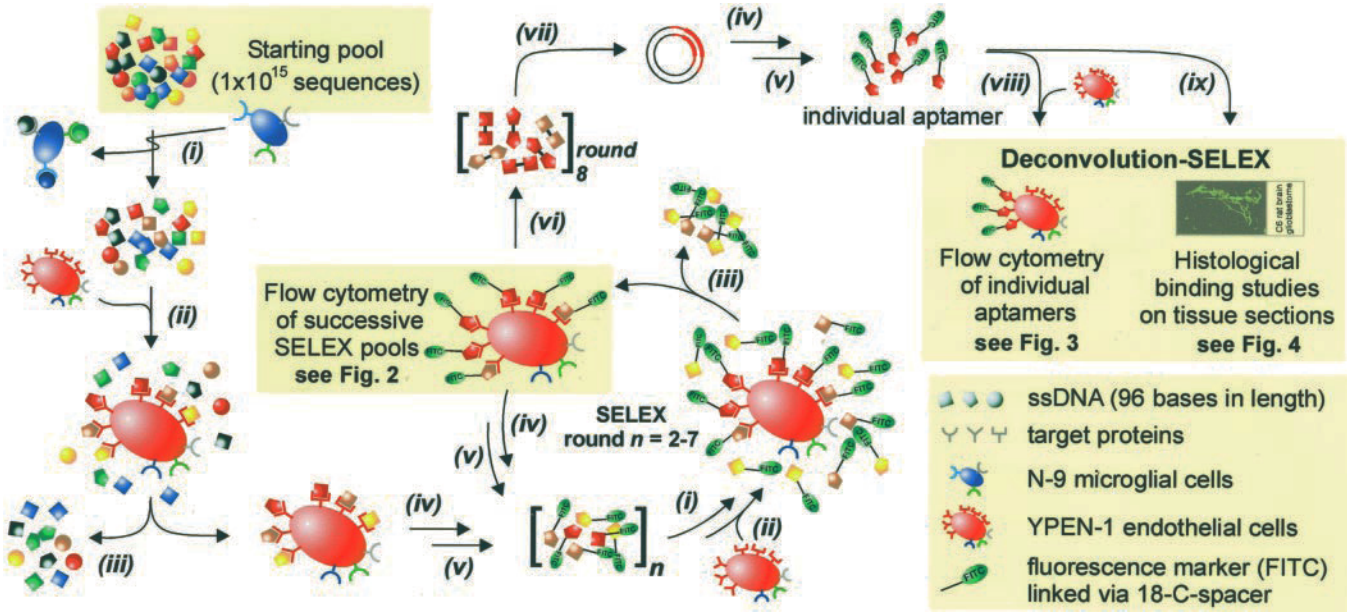

FIG. 1. **Selection procedure for the generation of DNA aptamers binding to angiogenic microvessels of rat experimental glioma.** *(i)*, counterselection against N9 microglial cells (to reduce co-selection of aptamers binding to other cell types). *(ii)*, incubation of successive SELEX pools with YPEN-1 endothelial cells. *(iii)*, removal of unbound sequences. *(iv)*, PCR amplification of cell-bound aptamers using modified primers. *(v)*, FITC-ssDNA generation. *(vi)*, PCR amplification using unmodified primers for *(vii)* sorting round 8 pool aptamers into individual clones. *(viii)*, deconvolution-SELEX (step 1) of cloned aptamers as a preselection to evaluate aptamers binding to endothelial cells. *(ix),* deconvolution-SELEX (step 2) to evaluate preselected aptamers selectivity against tumor microvessels embedded in their natural surrounding on tissue sections of rat brain glioblastoma.

not widely known endothelial protein of the Ewing's sarcoma family that parallels the transition from quiescent to angiogenic phenotypes *in vitro* (18).

EXPERIMENTAL PROCEDURES

Cell Lines—Endothelial cell line YPEN-1 (CRL-222) immortalized by an Adenovirus-12 SV40 hybrid virus (19), microglial cell line N9 (20), and rat glioblastoma cell line C6 (21) were obtained from ATCC (American Type Culture Collection). YPEN-1 cells and N9 microglial cells were cultured in RPMI 1640 (Life Technologies, Inc.), supplemented by 10% fetal calf serum (Seromed) to subconfluence, and harvested by carefully dislodging the cells from tissue culture flasks.

SELEX Library and Primers—The high pressure liquid chromatography-purified library contained a central randomized sequence of 60 nucleotides (nt) flanked by 18-nt primer hybridization sites (5′-ATA CCA GCT TAT TCA ATT- 60-nt -AGA TAG TAA GTG CAA TCT-3′; MWG-Biotech AG) (22). A fluorescein isothiocyanate (FITC)-labeled 5′-primer (5′-FITC-18C-ATA CCA GCT TAT TCA ATT-3′; 18C denotes an 18-carbon ethylene glycol spacer) and a triple biotinylated (trB) 3′-primer (5′-trB-AGA TTG CAC TTA CTA TCT-3′) were used in the PCR reactions for the synthesis of double-labeled, double-stranded DNA molecules. After alkaline melting, the biotinylated ssDNA strand was separated from the FITC-conjugated ssDNA aptamer by streptavidin-coated magnetic beads (M-280 streptavidin dynabeads were obtained from Dynal) according to the supplier's instructions (23). FITC- and biotin-labeled primers were synthesized by Operon Technologies, Inc. Unmodified primers were purchased from Amersham Pharmacia Biotech. All steps with FITC-labeled DNA were performed in light-protecting (ambra) reaction tubes (Eppendorf GmbH, Hamburg, Germany). Incubation of cells and tissue sections with the FITC-aptamers was performed in the dark.

SELEX—Selection of ssDNA against YPEN-1 endothelial cells was carried out essentially as described for pure protein targets (14, 15, 24, 25). Briefly, ssDNA pools were denatured by heating at 80 °C for 10 min in selection buffer (initial round: 10^{15} sequences in 1 ml; subsequent rounds: 200 pmol in 200 μl) containing 50 mM Tris·HCl (pH 7.4), 5 mM KCl, 100 mM NaCl, 1 mM $MgCl_2$, and 0.1% NaN_3 and then renatured at 0 °C for 10 min. To reduce background binding, a 5-fold molar excess of yeast tRNA (Life Technologies, Inc.) and bovine serum albumin (BSA; Merck) were added. To reduce co-selection of ssDNA binding to other cell types, in particular macrophages, counterselection was performed with 3×10^7 N9 microglial cells, a population of brain monocytes, by incubation at 37 °C for 30 min in selection buffer. Partitioning of bound and unbound ssDNA sequences was done by centrifugation. To ensure elimination of aptamers against the polypropylene-partitioning matrix of the reaction tubes, tubes were replaced against polyethylene ones after the third round of selection. The counterselected SELEX library was incubated with endothelial cells (10^6 in the initial round, 10^5 in subsequent rounds) at 37 °C for 30 min. After centrifugation and three washes with 1 ml selection buffer (with 0.2% BSA), cell-bound aptamers were amplified by PCR (*Taq*-polymerase and dNTP's were obtained from Promega). FITC- and biotin-labeled primers were used in PCR amplification (28 cycles of 1 min at 94 °C, 1 min at 46 °C, and 1 min at 72 °C, followed by 10 min at 72 °C). For cytofluorometric analysis FITC-labeled ssDNA was prepared as described above.

Sorting into Individual Clones—Aptamers obtained from the eighth round of selection were PCR-amplified using unmodified primers and cloned into *Escherichia coli* using the TA cloning kit (Invitrogen). Plasmids of individual clones were isolated by alkaline lysis (26), and inserts were amplified by PCR. For binding studies of individual aptamers by flow cytometry and fluorescence microscopy, FITC-ssDNA was prepared as described above.

Flow Cytometric Analysis of Aptamers Bound to YPEN-1 EC in Successive SELEX Rounds—Specific EC-binding, FITC-labeled aptamers were monitored in the second and subsequent rounds. After incubation of 10^5 EC with 200 pmol aptamer in 200 μl of selection buffer (with a 5-fold molar excess of tRNA and BSA), cells were washed twice in 1 ml of selection buffer (with 0.2% BSA), and FITC fluorescence was monitored with a FACScan cytometer (BD Pharmingen) by counting 5000 events. Nonspecific control ssDNA (FITC-18C-96-nt) was prepared from the starting pool by PCR as described above.

Deconvolution-SELEX (Step 1): Flow Cytometric Analysis of Individual Aptamer—5×10^4 YPEN-1 endothelial cells and N9 microglial cells, respectively, were each preincubated with selection buffer containing 1 μg/μl tRNA for 20 min on ice and incubated with 200 μl of denatured and renatured FITC-aptamer solution (0.25 pmol/μl with 1 μg/μl tRNA) for 30 min at 37 °C. Cells were washed twice in selection buffer (with 0.2% BSA), and fluorescence was determined by flow cytometry.

Deconvolution-SELEX (Step 2): Preparation of Rat Brain Glioblastoma Tissue Sections and Histological Analysis—2–2.5 $\times$ 10^4 tumor cells (C6 glioblastoma cell line) were transplanted intracranially into Harlan Sprague-Dawley rats as described (21). Rats were perfused 23 days after transplantation with phosphate-buffered saline, and brains were removed for cryostat sectioning. Binding of individual FITC-aptamers was analyzed on rat cryostat sections of normal and glioblastoma brain. Tissue sections (10 μm) were preincubated with selection buffer (with 1 μg/μl tRNA) at 4 °C for 20 min and then incubated with FITC-aptamer solution (0.25 pmol/μl with 1 μg/μl yeast tRNA) at room

temperature for 40 min. Tissue sections were washed twice in selection buffer (with 0.2% BSA), and fluorescence patterns were analyzed microscopically. For double labeling, cryostat tissue sections of rat brain glioblastoma were stained with aptamer III.1 as described above and subsequently stained with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) (300 nM, 2–3 min; Molecular Probes) or mouse CD-31 (1 h at a dilution of 1/1000; Dako) with Cy™3 anti-mouse secondary IgG (30 min at a dilution of 1/200; Amersham Pharmacia Biotech), respectively. After two washes in selection buffer (with 0.2% BSA) fluorescence patterns were analyzed microscopically.

Wounding experiments were performed in Petri dishes. The confluent monolayer of YPEN-1 EC was wounded by carefully dislodging cells with a sterile pipette tip. The cells in Petri dishes were cultured for an additional 4 h, washed twice with selection buffer, and subsequently fixated with methanol (10 min, −20 °C; Merck). After incubation of the cell layer with FITC-18C-aptamer III.1 (0.25 pmol/μl with 1 μg/μl tRNA) in selection buffer (30 min, 37 °C), staining patterns were analyzed by fluorescence microscopy.

Ligand Mediated Protein Purification—1 mg (100 μl) of magnetic streptavidin beads were coated with 200 pmol of trB aptamer III.1 (MWG-Biotech AG) by incubation in 1 ml selection buffer (30 min, room temperature). As a control, 100 μl of magnetic beads were coated with 200 pmol of unselected FITC-ssDNA (trB-96-nt). 1.5×10^8 Ypen-1 endothelial cells were lysed in the presence of protease inhibitors as described (27). After centrifugation, the protein pellet was resuspended in 400 μl of selection buffer, treated by ultra-sonication (0 °C, 20 s), and incubated with aptamer III.1-coated magnetic beads in the presence of a 100-fold excess of tRNA (20 nmol) as an unspecific competitor in selection buffer (total volume 1.5 ml, 0 °C, 15 min). The protein-aptamer-magnetic bead-complex was removed in a magnet stand and washed four times (first wash: 1 ml of selection buffer with 150 mM NaCl; second through fifth wash: 200 μl of selection buffer with 100 mM NaCl with 2 nmol tRNA). Protein is removed from aptamer-coated beads by incubation in 30 μl of 1 M NaCl (0 °C, 30 min) and analyzed by polyacrylamide gel electrophoresis after staining with Coomassie Blue.

Protein Identification—In gel tryptic digestion was performed as described (28) and modified as outlined below. Briefly, the protein band was excised from the gel, fully destained, and digested for 3 h with porcine trypsin (sequencing grade, modified; Promega) at a concentration of 67 ng/μl in 25 mM ammonium bicarbonate, pH 8.1, at 37 °C. Prior to peptide mass mapping and sequencing of tryptic fragments by tandem mass spectrometry, the peptide mixture was extracted from the gel by two changes of 50% trifluoroacetic acid/50% water followed by two changes of 50% trifluoroacetic acid/50% acetonitrile. The combined extracts were vacuum-dried. The dried peptides were redissolved in 0.1% trifluoroacetic acid and purified using a ready-to-go pipette tip filled with C18 spherical silica reverse phase material (ZipTip$_{C18}$™, Millipore). Peptides were eluted with 10 μl of 50% methanol/1% formic acid, and sequencing was performed by nanoelectrospray tandem mass spectrometry on a hybrid quadrupole orthogonal acceleration time of flight tandem mass spectrometer (Q-Tof, Micromass, Manchester, England) equipped with a nanoflow electrospray ionization source. Gold-coated glass capillary nanoflow needles were obtained from Protana (Type Medium NanoES spray capillaries for the Micromass Q-Tof, Odense, Denmark). The needle was filled with 3 μl of the sample and subsequently opened by breaking the tapered end of the tip under a microscope. A stable spray was observed applying a needle voltage of 1200–1400 V, a back pressure of 2 p.s.i., and a source temperature of 40 °C. The estimated flow rate was 20–50 nl/min. For nanoflow electrospray ionization tandem mass spectrometry experiments, fragmentation was achieved by collision with argon atoms. Q1 was set to the mass of interest, and an optimized collision energy was applied. The integration time for the time of flight analyzer was 1 s with an interscan delay of 0.1 s. Database searches (NCBInr, non-redundant protein database) were done using the MASCOT software from Matrix Science (29).

RESULTS

Selection of ssDNA Aptamers that Bind Transformed Endothelial Cells—SELEX was used essentially as described (30) to generate ssDNA aptamers against Adenovirus-12 SV40-transformed YPEN-1 rat endothelial cells. Commonly used radioactive labeling of nucleic acids (to monitor the enrichment of aptamers in successive SELEX rounds) was replaced by attaching FITC as a fluorescence reporter molecule. The selection was started with a SELEX library of $\sim 1 \times 10^{15}$ sequences, each containing a central randomized region of 60 nt flanked by two primer hybridization sites (22). To reduce co-selection of aptamers binding to other cell types, counterselection against N9 microglial cells (20), a population of brain monocytes, was performed prior to each selection round. To monitor the enrichment of specific cell-binding aptamers during selection, SELEX pools of the second and following rounds were analyzed by flow cytometry after incubation with YPEN-1 endothelial cells (Fig. 2). Analysis of fluorescence-labeled pools in successive cycles of selection showed a pronounced shift of second round histogram toward higher fluorescence intensity. The histograms of SELEX pools 3–7 showed slow but steady increases in fluorescence intensity and thus enrichment of endothelial cell-binding aptamers.

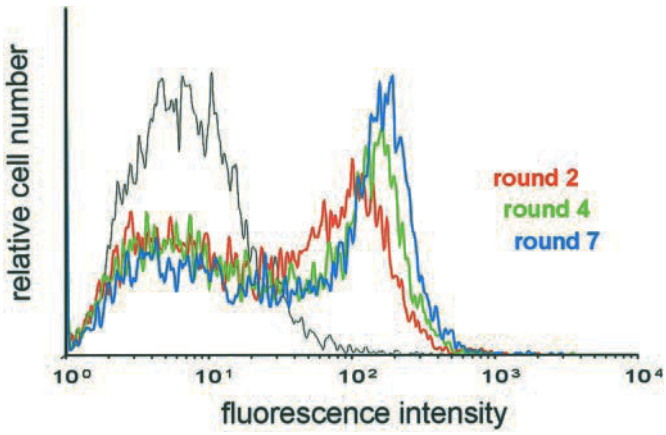

FIG. 2. **Aptamers binding to transformed rat endothelial cells.** Flow cytometry with FITC-conjugated aptamer pools after successive rounds of selection resulted in a progressive increase in fluorescence intensity of the ssDNA population. *Black curve*, unselected SELEX genome (FITC-18C-96-nt). *Colored curves*, increase in fluorescence intensity of aptamers to YPEN-1 endothelial cells after second (*red*), fourth (*green*), and seventh (*blue*) round of selection.

Deconvolution-SELEX—As the utility of a selected pool of nucleic acids to dissect complex systems is contingent upon the ability to quickly identify which sequences are ligands for components of interest within the targeted mixture (30), individual aptamers cloned from the eighth round of selection were evaluated by a two step deconvolution-SELEX procedure: (*i*) each sequence affinity against the transformed YPEN-1 EC cell line was preevaluated by flow cytometry, and (*ii*) the selective binding of aptamers to microvessels of C6 brain tumor was analyzed by fluorescence microscopy on cryostat tissue sections of rat brain glioblastoma. Of 25 sequences (FITC-18C-96-nt) tested by flow cytometry, by comparison of endothelial cells stained with unselected ssDNA (FITC-18C-96-nt), 23 showed binding to YPEN-1 endothelial cells. Of these, 16 bound to the pathological microvasculature of C6 glioblastoma, and 7 showed no binding. Aptamer III.1 displayed the most intensive staining of the vasculature exclusively in areas of solid tumor growth and was therefore chosen as the candidate to be further characterized as outlined below.

Increased fluorescence intensity of YPEN-1 EC stained with fluorescence-labeled aptamer III.1 (sequence and proposed secondary structure is illustrated in Fig. 3*C*) by comparison with unselected ssDNA (FITC-18C-96-nt) as a negative control is shown in fluorescence-activated cell sorter histograms of Fig. 3*A*. To demonstrate also EC-specific binding by flow cytometry, binding analyzes against N9 microglial cells was performed (Fig. 3*B*).

Histological analyzes of aptamer III.1. is illustrated in Fig. 4. FITC-labeled aptamer stained the complex architecture of the pathological microvasculature (Fig. 4*A*). Counterstaining of cellular nuclei with DAPI (*blue*) demonstrated selective targeting

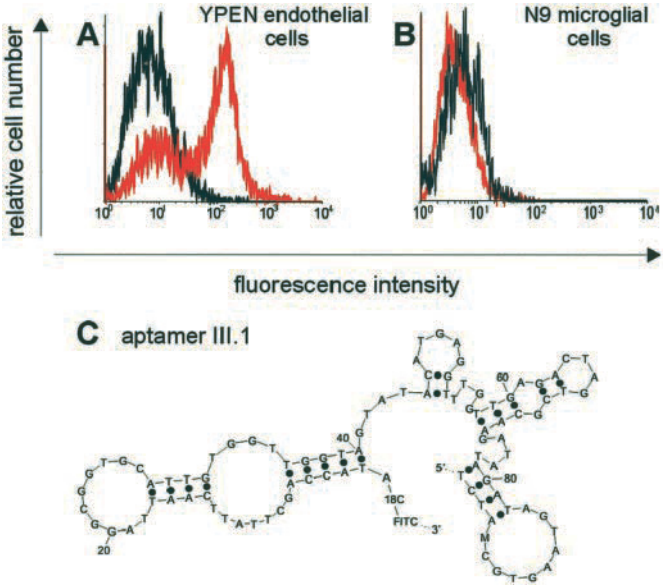

FIG. 3. **Cell binding of FITC-18C modified aptamers.** *Red curves*, aptamer III.1. *Black curves,* unselected ssDNA (FITC-18C-96-nt). *A*, aptamer III.1 shows specific binding to transformed rat endothelial cells but not to *B*, N-9 microglial cells. *C*, proposed secondary structure of aptamer III.1 (FITC-18C-ATACCAGCTTATTCAATTAGGCGGTGCATTGTGGTTGGTAGTATACATGAGGTTTGGTTGAGACTAGTCGCAAGATATAGATAGTAAGTGCAATCT; primer hybridization sites are underlined).

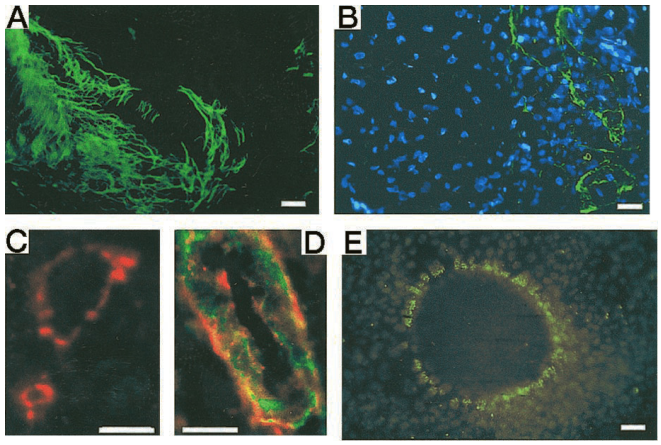

FIG. 4. **Staining of cryostat sections of rat brain glioblastoma.** *A*, FITC-18C-conjugated aptamer III.1 shows selective staining of microvessels embedded in their complex surrounding of glioblastoma. *B*, counterstaining of cellular nuclei with DAPI (*blue*) visualizes selective labeling of microvessels with aptamer III.1 (*green*) in the cell-rich tumor region but not of vessels in peritumoral areas. *C*, staining with CD-31 (*red*) and unselected SELEX genome (FITC-18C-96-nt) shows no double labeling of endothelial cells or vessel-associated structures. *D*, double staining with endothelial CD-31 mAb (Cy™3, *red*) III.1 countersigns aptamer III.1 positive cells (FITC, *green*) as endothelial cells. *E*, staining of Ypen-1 endothelial cell culture after wounding a confluent cell layer with a pipette tip. Aptamer III.1 (*green*) shows significantly increased binding to endothelial cells in the subconfluent area. (*scale bars,* 50 μm)

of microvessels within the cell-rich tumor region but not of vasculature in peritumoral areas (Fig. 4*B*). Fluorescence microscopy of rat normal brain after incubation with aptamer III.1 did not show any staining of EC or vessel-associated structures (data not shown). Staining of tumor tissue sections with mouse IgG directed against EC (CD-31, visualized with anti-mouse IgG-Cy™3, *red*) and subsequent labeling with aptamer III.1 (FITC, *green*) countersigned the aptamers target as EC (Fig. 4*D*). Rat brain tumor tissue sections were analyzed after incubation with unselected FITC-18C-ssDNA and endothelial CD-31 (Cy™3, *red*) as a control. Fig. 4*C* demonstrates negative binding.

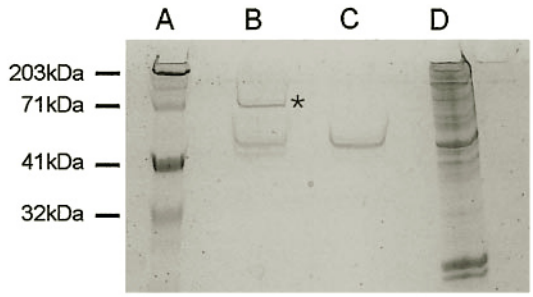

FIG. 5. **Coomassie Blue-stained polyacrylamide gel used to analyze the ligand-mediated target purification.** *Lane A*, molecular marker. *Lane B*, purification with aptamer III.1. *Lane C*, purification with unselected ssDNA (trB-96-nt). *Lane D*, untreated endothelial cells. The target band isolated in the reaction containing the aptamer III.1 is indicated by an *asterisk* and was identified as the 67-kDa protein pigpen.

To get further evidence for aptamer III.1 pathological association, cultured EC were analyzed after wounding the confluent cell layer with a pipette tip. Subsequent staining with aptamer III.1 and fluorescence microscopy showed exclusive up-regulation of the molecular target of aptamer III.1 in endothelial cells next to the lesion, a region that is characterized by increased proliferation compared with areas of adjacent contact-inhibited cells of the confluent monolayer (Fig. 4*E*).

Thus, our histological data gave strong indication that aptamer III.1 binds an endothelial molecular target that is involved in the process of EC proliferation, a key step of angiogenesis.

Aptamer III.1-Mediated Target Identification—The molecular target has been isolated by magnetic DNA affinity purification. Solubilized EC proteins were incubated with trB aptamer III.1 beforehand coupled to magnetic streptavidin beads. After thorough washing and subsequent release of aptamer-bound protein in high salt solution, proteins were analyzed by SDS/polyacrylamide gel electrophoresis (Fig. 5).

Polyacrylamide gel analyses revealed a distinct band migrating at ~70 kDa in the lane of aptamer III.1, which is absent in the lane containing a product of the control reaction (performed with unselected ssDNA) which revealed one single band of ~50 kDa. Peptide mass fingerprinting and sequencing of three of the tryptic peptide fragments of the protein by mass spectrometry and tandem mass spectrometry, respectively, were used to identify the aptamer-specific protein as the rat homologue of mouse pigpen protein (67 kDa), a so far, widely unknown endothelial protein that has been considered to parallel the transition from quiescent to angiogenic phenotypes *in vitro* (31).

DISCUSSION

Selection by Intact Biological Entities—To select a brain tumor-homing ssDNA ligand we applied SELEX against Adenovirus-12 SV40-transformed YPEN-1 rat endothelial cell line, thus against a pathologic endothelial target for several reasons (32): (*i*) it can be supposed that in actively growing endothelial cells the balance between positive and negative angiogenic regulators has been shifted toward the side of regulators required for EC proliferation and thus the transformed endothelial cells bear a profile of molecular addresses predominantly expressed by angiogenic tumor microvessels; (*ii*) the selection by intact biological entities does not require a full understanding of the complex mechanism of EC proliferation, whereas the selection by an isolated protein is limited to cases in which the proteins have been identified and isolated; (*iii*) because of structural differences in protein conformation, the aptamers

selected to bind the protein in its purified form will not automatically bind the protein embedded in its natural surrounding; (*iv*) the selection using intact, activated EC give promises in the identification of components that have not been known for their critical role in angiogenesis, (*v*) moreover, the cellular target allowed fluorescence-based selection thus to monitor the enrichment of cell-binding, fluorescence-labeled ssDNA-ligands in successive pools of selection and amplification by flow cytometry.

Deconvolution-SELEX—The eighth round pool was deconvoluted, and single binding of individual aptamers was quantified by flow cytometry (33–36). Further, screening of these individual aptamers on cryostat brain tissue sections allowed deconvolution for their discriminatory binding to neoangiogenic tumor microvessels but not to vessels of the normal rat brain. Thus histological staining allowed the selection of those aptamer candidates recognizing the differentiation of endothelial cells under *in vivo* conditions of angiogenesis.

These two steps of library deconvolution result in aptamer libraries selectively addressing molecular structures, differentiating neoangiogenic endothelium of the brain. The alignment of the 16 aptamer sequences after eight cycles of systematic evolution and the two subsequent steps of deconvolution did not reveal any obvious binding motifs. This appears to be due to the complexity of the endothelial cell with its high number of potential molecular targets. However, the combination of fluorescence-based SELEX and deconvolution-SELEX allowed identification of aptamer candidates, which perform the desired task to act as a histological marker to selectively stain tumor microvessels.

Aptamers as Histological Markers—Aptamers were used as histological markers in combination with chromosomal dyes and conventional antibodies for immunostaining. This demonstrates that aptamers are versatile tools that rival antibodies in diagnostic applications (reviewed in 7). Unlike antibodies, synthetic aptamers can easily be produced with a high degree of accuracy, reproducibility ,and purity. Therefore, little or no batch to batch variation is expected in aptamer production. They are not sensitive to temperature and undergo reversible denaturation, thus having a much longer self-life (7). Further, aptamers can easily be conjugated to a variety of reporter molecules, chemotherapeutics, or photosensibilisators at precise locations. Thus aptamers selectively binding to tumor endothelium might be of value not only in the development of tumor-homing diagnostics but also in therapeutics.

Complex-SELEX as a Tool to Dissect EC Differentiation—The pronounced and selective staining of rat brain tumor vessels by aptamer III.1 suggested a selective addressing of a molecule involved in neoangiogenesis. We therefore used this aptamer to purify and identify its binding partner, the rat homologous of mouse endothelial pigpen protein. Previous cell culture experiments (18, 31) demonstrate pigpen to be synthesized at barely detectable levels in non-proliferating EC, whereas its expression is up-regulated under conditions where EC are actively migrating and dividing (*i.e.* in subconfluent or wounded cultures) (18). Our observation that the pigpen molecule is highly up-regulated in angiogenic microvessels suggests pigpen as a target for diagnostic imaging or therapeutic strategies *in vivo*.

The combinatorial chemistry procedure SELEX, applied to select ssDNA ligands against transformed EC as intact biological entities followed by deconvolution-SELEX and aptamer-mediated target identification, has demonstrated its potential to identify compounds that are unknown or have not been supposed in probably playing a key role in complex mechanisms like angiogenesis (30, 32). In general, generation of fluoresceinated, cell-binding aptamers by SELEX opens new avenues in the study of cellular differentiation and tumor diagnostics.

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