A Strategy for the Identification of Differentially Expressed Proteins Secreted by Fibroblasts

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Abstract: Monoclonal antibodies, which recognise markers of the modified tumour extracellular matrix, may be valuable tools for the diagnosis and therapy of cancer. The pattern of expression of some good-quality tumour-associated antigens is known to be regulated by intracellular pH. In this article, a strategy is described for the isolation of human antibodies specific for proteins which are differentially secreted by fibroblasts at different pH values. We panned a large synthetic antibody phage library against biotinylated proteins secreted by normal human dermal fibroblasts, cultured at pH 7.5, in the presence or in the absence of a molar excess of unbiotinylated proteins, secreted by fibroblasts cultured at pH 6.7. A panel of monoclonal antibodies was isolated, whose reactivity was tested by enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry.

Keywords: Angiogenesis · Antibody phage technology · Extracellular matrix · Fibroblast · Pharmaceutical chemistry · Tumour targeting

1. Introduction

Tumour targeting, *i.e.* the selective delivery of molecules to the tumour site, typically by intravenous injection, is a promising approach to tumour imaging and therapy [1][2]. This approach relies on the availability of good-quality markers, which are abundantly expressed at the tumour site but absent in normal tissues, and of targeting agents (*e.g.* antibodies), with high affinity for the target and suitable pharmacokinetic properties.

The tumour stroma and its extracellular matrix (ECM) are a rich source of tumour-associated antigens. These antigens are typically more abundant and possibly more stable than tumour-associated antigens located at the surface of tumour cells. During tumour progression, the ECM of the tissues in which a tumour grows is remodelled through proteolytic degradation of the existing ECM components and by neo-synthesis of ECM components (by both neoplastic and stromal cells). It appears that this tumour extracellular matrix generates a more suitable environment for tumour progression.

Some tumour-associated ECM antigens are isoforms of proteins with a wide distribution in normal adult tissues (*e.g.* fibronectin and tenascin-C), generated by a mechanism of alternative splicing of their primary transcript.

The ED-B domain of fibronectin is highly conserved in different species. The corresponding exon can be inserted into the fibronectin-mRNA by a mechanism of alternative splicing, which only occurs in transformed cells, malignancies and under strictly controlled physiological conditions. Thus, with some very rare exceptions, ED-B is undetectable in normal tissues, but exhibits a much greater expression in foetal and tumour tissues as well as during wound healing. Its selective accumulation around neo-vascular structures has been extensively demon-strated in studies on many different tumour types, in particular in invasive breast carcinomas and in brain tumours [3][4], as well as in ocular angiogenesis [5]. Furthermore, recombinant human antibody fragments with high affinity for the ED-B domain have been shown to efficiently target tumour neo-vasculature *in vivo* [5–7]. Anti-ED-B antibodies, fused to a pro-coagulant factor, cause the complete and selective thrombosis of tumour blood vessels and can provide complete remissions in murine tumour models which are not cured by conventional chemotherapy [8].

Like fibronectins, tenascins show a wide distribution in normal adult tissue. Tenascin-C exists in several polymorphic isoforms due to alternative splicing of nine fibronectin-like type III repeats. Large tenascin-C isoforms are present in almost all normal adult tissues but are upregulated in foetal, regenerating and neoplastic tissue to a much greater extent. Changes both in synthesis and accumulation of the different tenascin-C isoforms are induced by mitogenic stimulation [9]. In addition, in cultured normal human fibroblasts, small pH variations of the culture medium (from 7.2 to 6.9) strikingly modify the alternative

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splicing pattern of the tenascin-C primary transcript [10]. Since such extracellular pH variations occur in many normal and pathological conditions, microenvironmental pH may be an important element for the regulation of mRNA alternative splicing *in vivo* [10]. Furthermore, tumour cells often have an elevated intracellular pH, which may result in a different pattern of protein neosynthesis or alternative splicing of ECM components.

The systematic investigation of the differential expression of proteins secreted by fibroblasts at different pH values is an issue of interest both in basic research and for tumour targeting applications. In particular, recent advances in antibody phage technology [11] make it possible to rapidly generate human antibody fragments against virtually any target, without immunisation and by-passing hybridoma technology. In this article, we describe the use of a good-quality human antibody phage library for the discovery of tumour-associated ECM antigens.

Fig. 1. Strategy for the recovery of proteins secreted by fibroblasts, cultured at different pH values. For further details, see **4. Experimental Procedures**.

Fig. 2. 2D-PAGE analysis of proteins secreted by fibroblasts. A, unconcentrated; B, 10-fold concentrated; C, 80-fold concentrated culture supernatants.

2. Results

2.1. *Proteins Secreted by Fibroblasts Grown at Different pH Values*

The differential expression of tenascin-C isoforms upon culture of fibroblast at different pH values has been described by Borsi *et al.* [10]. We followed the same experimental strategy for the culture of fibroblasts, under the assumption that environmental pH may be an important element for the regulation of alternative splicing or neo-synthesis of other proteins. Fig. 1 schematically depicts the experimental approach that we followed. Normal human dermal fibroblasts were grown to confluence in serum-rich medium and then incubated for four days in serum-free medium of different pH values (6.7 or 7.5). The supernatant was then dialysed, concentrated and biotinylated. Secreted medium was concentrated using ultrafiltration devices, and the quality of the samples monitored during concentration by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Fig. 2 shows 2D gels of medium collected from NHDF cultures before concentration (Fig. 2A) and at different steps of the concentration procedure (Figs. 2B and 2C). No apparent selective loss of protein spots was detected. We further used 80-fold concentrated medium for the following biopanning steps, using an antibody phage library (see below).
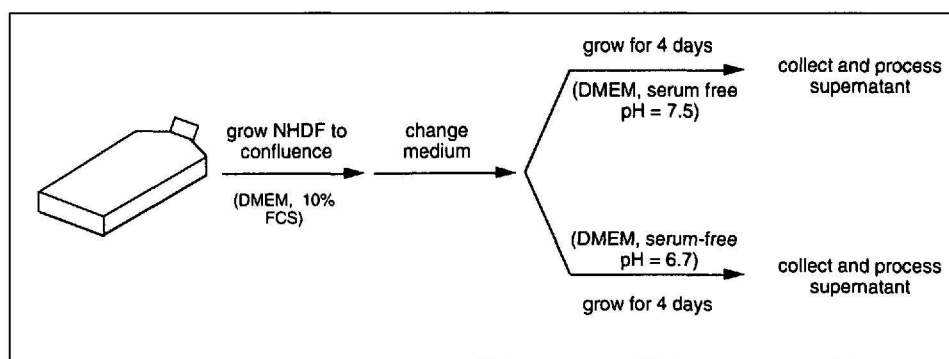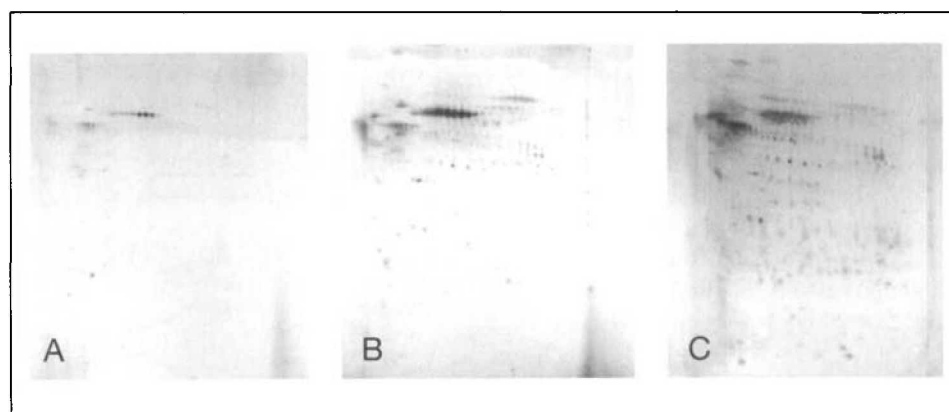
The effect of extracellular pH on the differential expression of protein isoforms was investigated by Dot Blot, analysing the expression pattern of the large and small tenascin-C isoforms (data not shown). The larger isoform of tenascin-C is present at higher levels in medium of fibroblasts incubated at pH 7.5, compared to pH 6.7 (data not shown).

2.2. *Antibody Phage Library Selections*

Antibody phage technology was used in an attempt to isolate human antibodies specific for proteins or protein isoforms expressed by fibroblasts at basic pH (pH 7.5) and not at more acidic pH (pH 6.7). The large synthetic ETH-2 antibody phage library [12] was used in a biopanning strategy, depicted in Fig. 3, which relies on the pre-binding of the library with an excess of unbiotinylated pH 6.7 proteins, before exposing the library to biotinylated pH 7.5 proteins. The unbiotinylated proteins and irrelevant phage were discarded, whereas using streptavidin-coated magnetic beads, biotinylated pH 7.5 proteins were recovered together with the specific phage bound to them. Recovered phage were

eluted and could be used for further rounds of biopanning. In total, we performed four rounds of panning. As negative control, a selection was performed in parallel, without pre-incubation of the ETH-2 library with the pH 6.7 supernatant. The phage titres rescued at the end of the various rounds of panning are shown in Fig. 4. As expected, phage titres increased after the second round of panning, indicating that a selection and amplification of binding specificities had occurred. Also, the higher titres observed in the negative control selection were expected, since the absence of competitor proteins would result in the isolation of more binders.

Five hundred selected clones, isolated after four rounds of competitive biopanning, were screened by ELISA on plates coated either with biotinylated pH 7.5 proteins, or with biotinylated pH 6.7 proteins. Clones which preferentially bound to proteins present in the pH 7.5 medium, and not in the pH 6.7 medium (approx. 70/500 clones), were further characterised. In particular, we focused our further work on ten antibody clones which did not bind to ED-B or serum, and which were able to bind to fibroblasts, as detect-

ed by fluorescence microscopy (data not shown).

2.3. *Characterisation of Selected Antibody Clones*

The ten antibody clones were sequenced. This analysis revealed three distinct protein sequences (clones MS-1, MS-2 and MS-3; Fig. 5), represented at a 4/10, 4/10 and 2/10 frequency, respectively.

The three antibodies were produced by bacterial fermentation and were purified on a Protein A column as described [12]. They were further investigated by immunohistochemistry on tumoral and normal tissues: glioblastoma multiforme (an aggressive tumour of the brain), breast carcinoma, lung carcinoma, and their normal counterparts.

The staining results obtained with the three antibodies were similar. For example, antibody MS-1 showed a strong staining around tumour blood vessels in sections of glioblastoma multiforme, whereas vessels in the normal brain tissue are only weakly stained (Fig. 6). However, the usefulness of MS-1 is limited by the fact that this antibody stains strongly and homogeneously the ECM in both normal and tumour breast and lung.

3. Discussion

We have devised a biopanning strategy, featuring the use of a synthetic human antibody phage library and proteins secreted by human fibroblasts, for the discovery of antigens which are differentially expressed in slightly basic or acidic media. This research had been stimulated by the observation that the pattern of alternative splicing of a relevant tumour marker, such as tenascin-C, is regulated by intracellular pH.

We have isolated monoclonal antibodies which stain vascular structures in glioblastoma multiforme, but not in normal brain. The corresponding antigen has not been identified yet, but can be observed in a 2D immunoblotting analysis of 2D gels of fibroblast culture supernatants (data not shown). The applicability of these antibodies is limited by the fact that they also stain normal tissues such as breast and lung. However, the
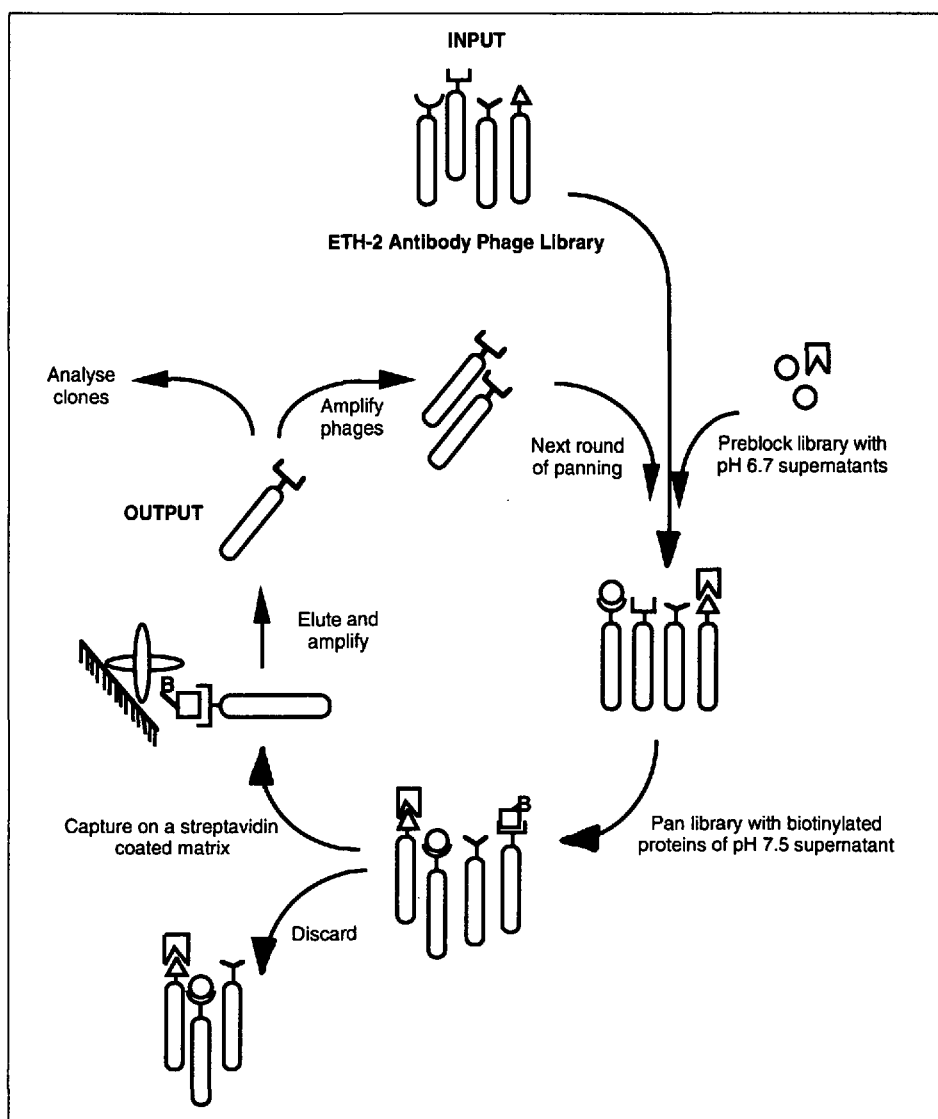

Fig. 3. Schematic representation of the strategy for the selection of human antibodies from the ETH-2 library, which preferentially react with proteins (or epitopes) secreted by fibroblasts at pH 7.5, but not at pH 6.7. A round of panning, which can be repeated several times, is illustrated. Antigens are depicted as geometric objects; biotinylated antigens are marked by the symbol B.
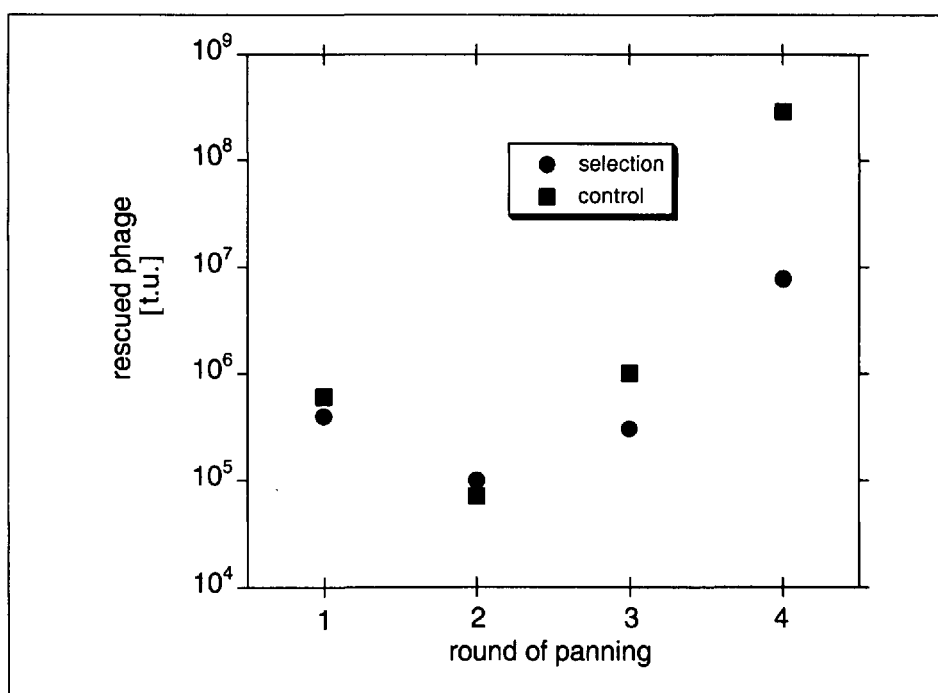
Fig. 4. Phage titres (transforming units) rescued after each of the four rounds of panning with biotinylated pH 7.5 protein supernatants in the presence ('selection') or in the absence ('control') of an excess of unbiotinylated pH 6.7 supernatants.

VH

EVQLLESGGGLVQPGGSLRLSCAASGFTFSSFSMSWVR
QAPGKGLEWVSSISGSSGTTYYADSVKGRFTISRDNSKN
TLYLQMNSLRAEDTAVYYCAK**KRGGW**FDYWGQGTLVTV
SS

VL

SSELTQDPAVSVALGQTVRITCQGDSLRSYYASWYQQK
PGQAPVLVIYGKNNRPSGIPDRFSGSSSGNTASLTITGA
QAEDEADYYCNSS**QPNRNP**VVFGGGTK

Fig. 5. Amino acid sequence of the variable heavy and light chains of recombinant antibody fragment MS-1. Note that residues which have been combinatorially mutated in the ETH-2 phage display library are indicated in bold.
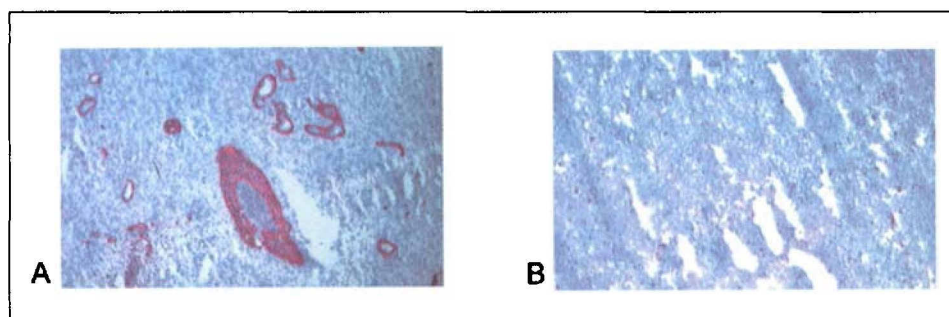

Fig. 6. Immunohistochemical findings with sections of glioblastoma multiforme (A) or normal brain (B), stained in blue with hematoxilin and in red with the MS-1 antibody.

procedure illustrated in this article shows that it is possible to rapidly generate binding specificities against interesting biological specimens, which recognise antigens in their native conformation and which can conveniently be tested by immunohistochemical analysis.

The strategy relies on a pre-culture of fibroblasts in serum-containing medium at basic pH, followed by a change into serum-free culture medium (pH 6.7 or pH 7.5). It is conceivable that, during the pre-culture step, a number of mRNA molecules are being generated, which will continue to express the corresponding protein even after the change of culture medium. We anticipate that a better discrimination between protein production at pH 6.7 or pH 7.5 could be achieved by performing several changes of culture medium and by collecting supernatants only at later time points.

4. Experimental Procedures

4.1. *Cell Culture and Processing of Supernatants*
Cell Cultures
Normal Human Dermal Fibroblasts (NHDF) were purchased from Clonetics

(BioWhittaker, Verviers, Belgium). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Paisley, UK) supplemented with 10% foetal bovine serum (FBS, Life Technologies) in a 5% CO_2 atmosphere at 37 °C.

After reaching confluence, NHDF were incubated in FBS-free medium at different pH values (6.7 and 7.5) for 4 d in a 5% CO_2 atmosphere at 37 °C [10]. The different pH values of the medium were obtained by adding different concentrations (4 to 20 mM) of sodium bicarbonate (Life Technologies) to bicarbonate-free DMEM (Life Technologies).

A daily measurement of the pH of the medium was performed to monitor that the pH remained constant.

Processing of the Medium
NHDF supernatant was collected after 4 d incubation in medium at pH 6.7 or 7.5.

In order to use it as a source of antigens for the selection of binding recombinant antibody fragments, medium was dialysed to remove small peptides and phenol red which could interfere with the biotinylation reaction. Dialysis was

performed in PBS at 4 °C for 2 d using a Spectra/Por molecular porous membrane tubing (Spectrum Medical Industries, Laguna Hills CA, USA) with a 12–14 kD cut-off.

Samples were then concentrated 80-fold with a Ultrafree-15 centrifugal filter device (Millipore, Bedford MA, USA) with a 10 kD cut-off according to the manufacturer's instructions. Concentration of the sample was monitored by measuring the absorbance at 280 nm in a spectrophotometer. Concentration of the samples is necessary both for the biotinylation reaction and for the success of the selection of binders from the phage display library.

The biotinylation of the proteins present in the pH 7.5 supernatant is crucial for the success of the strategy. Experience of our laboratory has shown that selections can be conveniently performed with biotinylated antigen in the 10 nM concentration range.

Proteins were biotinylated with NHS-SS-Biotin (Biotin disulfide N-hydroxy succinimide ester, Pierce, Rockford IL, USA). 500 μl protein solution were mixed with 50 μl NHS-SS-Biotin (1 mg/ml in DMSO) and incubated at r.t. for 1 h. A PD-10 (Sephadex G25M, Pharmacia, Uppsala, Sweden) gel filtration was then performed according to the manufacturer's instructions to remove free biotin.

Quality Controls of the Processed Medium
Culture supernatants were analysed by 2D-PAGE using the methodology of Görg *et al.* [13].

To evaluate the level of biotinylation of the proteins present in the concentrated medium, a Western Blot was performed: biotinylated proteins could easily be detected with the avidin-HRP complex (data not shown). A monodimensional reducing SDS-PAGE was performed in a Novex system using a 10% acrylamide gel (Novex, San Diego CA, USA), followed by protein transfer onto nitrocellulose membrane and immunodetection, according to the protocol described in Viti *et al.* [12]. Avidin-HRP (Sigma, Buchs, Switzerland) diluted 1:500 in 4% MilkPBS was used for the experiment.

To further analyse the biotinylation of the proteins isolated from culture supernatants, we performed an ELISA on streptavidin-coated plates, onto which the biotinylated supernatants had been applied. The large isoform of tenascin-C and ED-B containing fibronectin were

successfully detected, using the BC-2 monoclonal antibody [14] and the scFv (L19) antibody fragment [15], respectively.

4.2. Antibody Phage Library Selections

Selections of antibody fragments from the ETH-2 library [12] were performed on biotinylated material, following the protocol described in Viti et al. [12]. In short, approx. 10^{12} phages were pre-incubated for 20 min with an excess of unbiotinylated pH 6.7 proteins (150 μl of 80-fold concentrated medium, $O.D._{280nm} = 0.8$). Biotinylated pH 7.5 proteins (30 μl, $O.D._{280nm} = 0.15$) were then added and allowed to bind the phages for 10 min. A control selection was also performed, in which phage were not pre-incubated with the unbiotinylated material. After 30 min incubation at r.t., binding phage were captured on streptavidin-coated paramagnetic beads (Dynabeads M-280 streptavidin, Dynal, Oslo, Norway), washed and eluted with 20 mM dithiothreitol.

4.3. Screening of Selected Antibodies

After four rounds of panning, the eluted phage was used to infect exponentially growing TG1 bacteria. Soluble ELISA of bacterial supernatants obtained from single colonies was performed as described [12][16] on streptavidin coated plates. The clones were in this way tested for their different reactivity towards the proteins present in the pH 6.7 and pH 7.5 biotinylated medium. The clones showing a reactivity against pH 7.5 proteins, but not against pH 6.7 proteins, were further characterised.

4.4. Characterisation of Selected Clones

Relevant clones were sequenced using an ABI PRISM 310 Genetic Analyzer, as described in Viti et al. [12].

Recombinant scFv fragments were expressed in bacteria and purified on Protein-A-Sepharose as described [12].

Immunohistochemical studies with cryostat sections of human glioblastoma multiforme, breast carcinoma, lung carcinoma and normal brain, breast and lung were performed as described previously [17].

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