ELSEVIER

Journal of Immunological Methods 287 (2004) 31–47

JIM
Journal of
Immunological Methods

www.elsevier.com/locate/jim

Research Paper

Isolation of human antibodies to tumor-associated endothelial cell markers by in vitro human endothelial cell selection with phage display libraries

Ricardo Mutuberria, Sietske Satijn, Angelique Huijbers, Edith van der Linden, Hera Lichtenbeld, Patrick Chames [1], Jan-Willem Arends [2], Hennie R. Hoogenboom *,[3]

Department of Pathology, Maastricht University, PO Box 5800, 6202 AZ Maastricht, The Netherlands

Received 24 October 2003; received in revised form 7 January 2004; accepted 12 January 2004

Abstract

Human antibodies selectively targeting angiogenic vessels hold great promise for the immunotherapy of human malignancies and can help to elucidate the molecular mechanisms regulating angiogenesis. By selecting a large antibody phage display library on proliferating stimulated HUVEC, we have isolated 15 human antibodies that bind to HUVEC in flow cytometric analysis, 11 of which target the vasculature of colorectal carcinomas as demonstrated by immunohistochemical analysis. The four most specific antibodies, TEM-A, TEM-C, TEM-M and TEM-Q, also stain the vasculature of a series of carcinomas derived from liver, ovary, kidney, bladder, lung and breast, and either react weakly or not all with the vasculature of corresponding normal tissues. All four antibodies react with the vasculature of endometrium, but only TEM-M and TEM-Q react with the vasculature of placenta. As shown by non-reducing western blot analysis, 9/15 of the antibodies recognize either one or two distinct bands on HUVEC cell lysates, with molecular weights of 175 and/or 110–125 kDa. Antibodies identified by this approach may be used for the identification of new markers of angiogenesis and/or tumor vasculature. The selected antibodies may prove useful as new prognostic markers, for in vivo tumor imaging purposes and for further development of targeted therapies.
© 2004 Elsevier B.V. All rights reserved.

Keywords: Tumor vasculature; Angiogenesis markers; Phage display; Human antibodies; Cell selections; HUVEC

Abbreviations: CDR, complementarity determining region; EC, endothelial cells; HUVEC, human umbilical vein endothelial cells; PBL, peripheral blood leucocytes; TEM, tumor-associated endothelial cell marker; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; FACS, fluorescence activated cell sorting.

* Corresponding author. Hertogsingel 46, 6214 AE, Maastricht, The Netherlands. Tel.: +31-62-709-4528; fax: +31-43-358-1863.
E-mail address: hhoogenboom@attglobal.net (H.R. Hoogenboom).
[1] Present address: Cellectis SA, Paris, France.
[2] Present address: Department Pathology, Deventer Hospital, Deventer, The Netherlands.
[3] Present address: Merus BV, Utrecht, The Netherlands.

0022-1759/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jim.2004.01.011

1. Introduction

A hallmark of pathological angiogenesis is the persistent growth of new blood vessels from preexisting ones, sustaining the progression of many neoplastic and non-neoplastic diseases (Folkman, 1995). The proliferation index provides a measure of pathological angiogenesis in human tumors, with endothelial cells (EC) residing in tumors showing proliferation indices up to 100 times higher in tumors (Vermeulen et al., 1995) compared to normal tissues. Tumor vasculature also presents specific morphology, uncontrolled permeability and a high percentage of immature vessels (Eberhard et al., 2000).

The complexity and variability of the angiogenesis process in tumors suggests the existence of specific markers of tumor angiogenesis. EC from different sites of the vascular tree differ in structure and function and are highly adaptable to environmental stimuli. As a result, the antigenic expression of the endothelial cell compartment becomes heterogeneous within the same tumor and among different tumors. Molecular heterogeneity may result from the nature of the angiogenic pathway, the different functions of EC in the sequential events in angiogenesis or the nature of the host tissue in which angiogenesis takes place (as reviewed by Ribatti et al., 2002).

The presence of different endothelial cell phenotypes in tumors has important therapeutic implications. Specific sets of antibodies may be generated to target each of these phenotypes. Such antibodies are promising candidates for quantifying angiogenesis, monitoring the status of tumor progression and estimating the malignant potential of tumors. Furthermore, they may provide the key for identification of new markers of tumor angiogenesis, resulting in the development of novel anti-angiogenic therapies or new strategies in vascular immunotargeting.

The search for vascular addresses has been intensified since the concept of tumor infarction by vascular targeting was shown to be an effective therapy for solid tumors in a mouse model (Huang et al., 1997). In the last decade, a growing number of molecular targets on EC, including tumor endothelium, have been identified (Griffioen and Molema, 2000). However, the use of tumor-associated endothelial gene expression analysis (Carson-Walter et al., 2001; St Croix et al., 2000) has demonstrated that a large number of tumor-associated endothelial cell markers (TEM) have not yet been identified or characterized.

Antibodies capable of selectively targeting angiogenesis markers have proven highly effective in the diagnosis, imaging and therapy of human malignancies and other forms of pathologic or non-pathologic angiogenesis (Birchler et al., 1999; Neri et al., 1997). Such antibodies, or antibody fragments, are readily selected and engineered using phage display technology (Viti et al., 2002). Phage display antibody libraries have proven highly effective in the isolation of high affinity human antibodies against almost any given antigen or antigenic mixtures (as reviewed by Winter et al., 1994). The first phage-display engineered antibody, the TNF-targeting HUMIRA, was recently approved for therapy of rheumatoid arthritis (Piascik, 2003). The versatility of the phage expression system makes it ideal for the selection of ligands directed to novel targets present on specific tissues, cell types or cells in specific differentiation or disease-induced states (Hoogenboom et al., 1998). Phage display is also the method of choice for the identification of peptides homing to specific vascular addresses (Arap et al., 2002).

Under normal growth conditions, cultured proliferating human umbilical vein endothelial cells (HUVEC) already present a proliferative phenotype and express angiogenesis associated antigens present in the vasculature of solid tumors, such as CD44 (Griffioen et al., 1997), VEGFR2 (Soker et al., 1997), Tie-2 receptor (Willam et al., 2000) or $\alpha_v\beta_3$ integrin (Yeh et al., 1999). Immunotherapy of tumors using fixed proliferating HUVEC as a vaccine has resulted in the inhibition of angiogenesis and regression of solid tumors (Wei et al., 2000). Furthermore, the immunization of mice with HUVEC cultured in the presence of tumor-conditioned medium has resulted in the generation of a monoclonal antibody against human endoglin (Wang et al., 1993). These experimental data suggested that selections on proliferating HUVEC further stimulated with angiogenic factors and tumor conditioned media, using magnetic beads for cell immobilization, could result in the selection of a panel of human antibodies directed against different markers of angiogenesis, including antigens present in tumor neovasculature.

We have employed a large, non-immunized Fab phage library to select binders to tumor vasculature.

The library was constructed by combining heavy and light chain antibody variable regions that had been previously PCR amplified from B-lymphocytes from healthy human donors (de Haard et al., 1999). The genes encoding the displayed human antibody fragments are carried in the phage genome, creating a link between phenotype and genotype. While hundreds of phage display antibodies have been successfully isolated by selection on purified antigens, antibody selections on cells, and in particular EC, have to date proven very problematic and to require optimization. We have consequently applied different selection approaches. In vivo selection is the most direct approach for the identification of vascular bed specific phenotypes. Phage libraries are injected directly into the circulation of a living organism for the later recovery of binders. This approach has resulted in the selection of phage-peptides against the vasculature of different organs and tumors (Arap et al., 2002; Pasqualini, 1999; Rajotte et al., 1998) and phage-antibodies against thymic endothelium and the perivascular epithelium (Johns et al., 2000). In our hands, four rounds of selection with a non-immune phage antibody library in mice bearing human tumor xenografts did not result in the enrichment of specific phage antibodies, probably resulting from the low clearance of unbound phage from the poorly perfused tumors (Mutuberria et al., 1999). Similarly, an in vitro selection, in which we have used EC isolated from human tumor biopsies as the target antigen source, did not result in discernible enrichment of endothelium-specific binders (R.M., Mashasi Kanasawa and H.R.H., unpublished data). Two probable causes for the lack of enrichment of specific binders in these selections are the antigenic changes induced by the isolation procedure itself, and the potentially different arrays of antigens presented by different tumor biopsies used in subsequent rounds of selection. Recently, we demonstrated the efficient selection in a model system of a phage antibody directed to a marker on endothelial cells, by gentle in vitro immobilization of endothelial cells using magnetic beads (Mutuberria et al., 1999). Cells were first labeled with an endothelial cell-specific antibody, and incubated with the phage antibodies. The labeled cell population was subsequently isolated using magnetic columns, which simultaneously permitted efficient removal of unbound phage antibodies by simple washing of the column.

In the current study, we describe a simple but efficient procedure for selecting phage antibodies to targets present on tumor-associated endothelial cells, by using in the selection process proliferating HUVECs which were further stimulated with growth factors and tumor-spent medium. Our results demonstrate that, with the appropriate method, ex vivo selections allow the isolation of binders to antigens over-expressed in vivo. With this approach, we have retrieved a large collection of antibodies specific for proliferating EC surface membrane antigens, including antibodies that show high levels of specificity for tumor vasculature. Finally, we demonstrate that a subset of the selected antibodies that stain the vasculature of tumors, recognize on immunoblots proteins of M_r 110–125 and 175 kDa.

2. Materials and methods

2.1. Cell lines and endothelial cell preparation

Peripheral blood leucocytes (PBL) were isolated from buffy coats of healthy donors by density gradient centrifugation over histopaque (Sigma). The colorectal cancer cell line $CaCo_2$ was obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI 1640 medium (Life Technologies, Paisley, Scotland) supplemented with 10% (v/v) fetal calf serum and 2 mM glutamine. HUVEC were harvested from normal human umbilical cords by perfusion with 0.125% trypsin/EDTA as described previously (Groenewegen et al., 1986). Cells were cultured in 0.2% (w/v) gelatin coated culture flasks in culture medium (RPMI 1640, 20% (w/v) heat inactivated human pooled serum, 2 mM glutamine, 0.1 mg/ml streptomycin and 100 U/ml penicillin). Confluent HUVEC were subcultured 1:3 to a maximum of three passages. Stimulation was induced on HUVEC subconfluent monolayers by a 24-h incubation with stimulation media (SM), comprising 0.1 ng/ml recombinant human basic fibroblast growth factor (bFGF; Prepotech, Rocky Hill, NJ), 0.01 ng/ml recombinant human VEGF-165 (Prepotech) and 10% (v/v) supernatant of colorectal carcinoma cell line $CaCo_2$. For fixation, HUVEC were harvested from gelatin-coated flask by incubation with 0.5%

(w/v) EDTA in PBS, and afterwards, detachment culture medium was added to the cells to neutralize EDTA. Cells were spun down at 1500 rpm for 5 min, resuspended in 1 ml 1% (w/v) paraformaldehyde and incubated for 30 min at room temperature. Cells were washed with 9 ml PBS and spun down at 1500 rpm for 5 min. The pellet was resuspended in 2 ml PBS supplemented with 0.1% (w/v) BSA.

2.2. Preparation of phage-displayed and soluble fabs

A large, non-immunized phage antibody repertoire (de Haard et al., 1999) was used for selection. This is a nonimmune phage antibody library, with naturally diversified VH and VL pools derived from healthy human donors, with antibodies cloned into the Fab format and with 3.7×10^{10} clones. The library and specific phage-displayed Fabs were rescued with helper phage M13K07 as described (Marks et al., 1991) and phage particles were purified and concentrated from the bacterial culture supernatant by polyethylene glycol (PEG) precipitation as described (Sambrook et al., 1990). Soluble antibodies were produced from *Escherichia coli* periplasmic fractions as already described (de Haard et al., 1999). The bacterial strain used was *E. coli* TG1 (K12, Δ(*lac-pro*), *sup*E, *thi*, *hsd*D5/F′ *tra*D36, proA + B+, *lac*I^q, *lac*ZΔM15).

2.3. Selections on stimulated HUVEC using a high gradient magnetic separation column

For column selections, cultured stimulated HUVEC were harvested by EDTA treatment, washed twice with PBS/0.1% BSA, fixed with 1% paraformaldehyde and selected as previously described (Mutuberria et al., 1999). Briefly, approximately 5×10^5 cells were incubated for 45 min in 1 ml PBS/0.1% BSA containing a 1:50 dilution of the anti-CD31 mouse monoclonal antibody EN4 (Monosan, Uden, the Netherlands). Cells were washed twice by centrifugation to remove excess EN4, and incubated for 1 h with 1 ml of phage pre-blocked for 1 h in 2% (w/v) semi-skimmed milk powder in PBS (MPBS). Cells were then magnetically labeled by suspension in 100 μl PBS/0.1% BSA containing 20 μl of goat anti-mouse IgG MACS magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 20 min. Unbound beads were removed by two washes followed by centrifugation. Labeled cells were resuspended in 100 ml PBS/0.1% BSA and loaded onto a MiniMACS-MS column, previously equilibrated with 1 ml PBS/0.1% BSA and placed in a magnetic field (Miltenyi Biotec, Bergisch Gladbach, Germany). The column was washed 10 times with 1 ml of PBS/1% BSA to remove unbound phage. The column was removed from the magnet and eluted two times with 500 μl of PBS/0.5% BSA. Bound phage was eluted from HUVEC with 0.5 ml of 100 mM tri-ethylamide (TEA; pH 12) for 10 min at room temperature. The eluted phage was neutralized with 0.5 ml 1 M Tris–HCl (pH = 7.5). Phage were allowed to infect exponentially growing *E. coli* TG1 and titrated as described (Marks et al., 1991).

2.4. DNA fingerprinting and sequencing of antibody clones

Antibody-encoding V-genes of selected antibody fragments were amplified by PCR from a single bacterial colony as described (Roovers et al., 2001a,b). Fingerprint analysis was performed by *Bst*NI fingerprinting of the PCR products obtained by amplification with the oligonucleotide primers, M13-reverse and gene III-forward (Marks et al., 1991). For sequencing, plasmid DNA was prepared from 50 ml cultures grown at 30 °C in LB medium, containing 100 μg/ml ampicillin and 2% glucose, using the Qiagen Midi-kit (Qiagen, Hilden, Germany). Sequencing was performed with the primers CH1FOR (5′-GTC CTT GAC CAG GCA GCC CAG GGC-3′) and M13REV (5′-CAG GAA ACA GCT ATG AC-3′) (Base Clear, Leiden, the Netherlands). Sequences were compared to the human Ig set from the International Immunogenetics Database (IMGT) reference directory (http://imgt.cines.fr).

2.5. Analysis of phage antibody binding by flow cytometric analysis

Approximately 1×10^{10} pre-blocked phage particles bearing antibody were added to about 1×10^5 HUVEC, PBLs or $CaCo_2$ cells in 100 μl PBS/1% BSA. The mixture was incubated for 1 h at room temperature and washed three times. Phage binding was detected by a 1/500 dilution of goat anti-fd polyclonal antibody

(Amersham Pharmacia Biotech, Uppsala, Sweden) followed by a 1/50 dilution of FITC-conjugated rabbit anti-goat antibody for 1 h (Dako, Glostrup, Denmark). Detection of fluorescent cells was performed by flow cytometry using a FACS-Calibur (Becton Dickinson, Oxnard, CA). Data were analyzed using the Cellquest software program (Becton Dickinson). Fluorescence activated cell sorting (FACS) analysis of soluble Fabs was performed as above but detection was done with 10 μl/ml mouse Mab 9E10 (hybridoma supernatant), followed by FITC-conjugated anti-mouse antibody (Dako).

2.6. Immunohistochemistry using phage-displayed antibodies

Tissue samples were obtained from patients after surgical resections. All patients gave written consent for the investigation in accordance with the ethical guidelines of the Maastricht University Hospital. Patient-derived tumors and normal tissues were cut into 5 μm frozen tissue sections. These were mounted on 3-aminopropyltriethoxysilane (APS) coated glass slides, air dried and fixed with 1% (v/v) paraformaldehyde in PBS for 10 min. Slides were washed in PBS and incubated with 1% (v/v) H_2O_2 in PBS for 20 min to quench peroxidase activity. Tissues were washed three times in PBS and blocked with 20% (v/v) FCS for 30 min. The slides were then incubated overnight at 4 °C with 100 μl pre-blocked phage suspension (2% (w/v) skim milk powder, 0.1% Tween 20 in PBS). Slides were washed three times for 10 min in PBS/0.1% Tween 20. The slides were incubated with 1/100 dilution of biotin-conjugated anti-M13 (Research Diagnostics, Flanders, NJ) for 1 h. Slides were washed again three times for 5 min in PBS/0.1% Tween 20, and incubated for 1 h with horseradish peroxidase conjugated StreptABC (Dako). The slides were washed three times for 5 min in PBS/0.1% Tween 20. Staining was performed with diaminobenzidine (DAB) using H_2O_2 as substrate. Slides were washed in demineralized water, counterstained with hematoxylin, dehydrated and mounted with coverslips. Two negative controls were used for staining: phage particles displaying an irrelevant antibody or phage particle containing no antibody insert into the phagemid vector. The monoclonal antibody EN-4 (Monosan) directed against the pan-endothelial cell antigen CD-31 was used as a positive control to stain the vasculature of the various tissues analyzed.

2.7. Immunoblotting

HUVEC membrane bound antigens were prepared as described (Ausubel, 1995). Briefly, one volume of TSA buffer containing 10 mM Tris–HCl pH 8, 140 mM NaCl, 0.025% NaN_3 and one volume of lysis buffer (TSA solution with 2% triton X-100) was added to the cells and stirred at 4 °C for 1 h. The lysate was centrifuged 10 min at 4000 × *g* to remove nuclei. The supernatant was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) on 8% gels under non-reducing and reducing (DTT) conditions and electroblotted to nitrocellulose using standard techniques (Sambrook et al., 1990). Blots were blocked overnight in 5% skim milk at 4 °C and incubated for 1 h with phage antibodies previously blocked in 5% (w/v) skim milk, 1% (w/v) gelatin, washed five times with washing buffer (5% skim milk, 0.1% gelatin, 0.1% Tween 20) and incubated with 1/2000 diluted peroxidase-labeled anti-M13 antibody (Amersham Pharmacia Biotech) for 1 h. Finally, membranes were washed four times with washing buffer and one time in PBS and developed using ECL detection (Amersham Pharmacia Biotech) or DAB/H_2O_2. Standard precision blue markers from BioRad (Veenendaal, Netherlands) were employed as molecular standards. The following commercially available antibodies were used for molecular weight comparisons: anti-human VEGFR2 (220 kDa), anti-human VEGFR1 (180 kDa), anti-human Tie-1 (117 kDa), anti-human Tie-2 (140–160 kDa) antibodies (R&D Systems, Minneapolis, MN, USA), anti-CD31 Mab EN-4 (130 kDa) (Monosan), anti-human endoglin antibody (98–180 kDa) (US Biological, Swampscott, MA).

3. Results

3.1. Selection of HUVEC-binding antibodies from the phage display library

To isolate antibodies specific for tumor endothelium, a Fab antibody fragment displaying phage library (de Haard et al., 1999) was subjected to four rounds of

panning and enrichment on proliferating HUVEC. In an effort to further mimic the tumor associated angiogenic phenotype, the proliferating HUVEC were stimulated with the angiogenic factors bFGF and vascular endothelial growth factor (VEGF) and tumor-conditioned medium from the colorectal cancer cell line $CaCo_2$. HUVEC were detached from culture flasks by EDTA alone, to minimize proteolytic cleavage of cell surface antigens. HUVEC were fixed in 1% paraformaldehyde to maintain the integrity of the cell membrane during the selection procedure. Selection resulted in over 600-fold enrichment between rounds 3 and 4, clearly indicating that a subset of antibody clones was being selected and amplified (Table 1).

To determine the number of unique antibody clones, Fab genes from 132 randomly picked colonies (69 from round 3 and 63 from round 4) were PCR amplified and digested with *Bst*N1 restriction enzyme. Seventeen unique fingerprint patterns were identified. Fifteen patterns were present in round 3 (R3) and seven in round 4 (R4). Five of the clones found in R4 were identical to those found in R3, and two were new (antibody clones TEM-M and TEM-N). On the basis of the frequency of the fingerprints, the three most abundant clones were TEM-A, TEM-C and TEM-Q, representing 66% of the R3 population and 78% of the R4 population. In contrast, 7 of the remaining 15 antibody clones only represented each 2% or less of the fingerprinted population (Table 2). The clones representing the 17 fingerprint patterns were sequenced and the nucleotide and amino acid sequences between the FR1 and FR4 of the antibody light and heavy chains compared. All the antibodies were grouped according to their similarities in VH and VL families and the amino acid sequence of their complementarity determining region (CDR) 3 (Table 3). The sequences of several antibody clones are homologous, with identical CDR3 in the VH or VL chains, but with differences in the amino acid sequence of other CDRs (i.e., TEM-H and TEM-P). The two antibodies found only in the R4 population, TEM-M and TEM-N, are highly homologous, with identical CDR regions and differing only in the framework region.

Table 1
Results of phage antibody selections on stimulated HUVECs

Selection round	Input titer[a]	Output titer[a]	Ratio o/i	Enrichment factor[b]
1	9.5×10^{11}	1.7×10^{5}	1.7×10^{-7}	not relevant
2	2.9×10^{13}	9.3×10^{5}	3.2×10^{-8}	none
3	6.2×10^{13}	1.4×10^{7}	2.2×10^{-7}	7×
4	5.3×10^{13}	7.2×10^{9}	1.3×10^{-4}	617×

[a] Input (i) and output (o) titers are given in total amounts of colony forming units (CFU).

[b] Enrichment factor is defined as the ratio o/i (selection round $n+1$)/ratio o/i (selection round n).

Table 2
Frequency and characteristics of the selected antibodies

Antibody	R3[a] (%)	R4[b] (%)	FACS[c]	IHC[c]	M_r (kDa)
TEM-A	31	6	++	+++	175
TEM-C	15	58	++	++	110/175
TEM-D	1	0	+	+	–
TEM-E	2	0	++	–	110
TEM-F	8	2	++	–/+	110
TEM-G	1	0	++	++	110
TEM-H	3	0	++	+	110
TEM-I	6	12	++	–/+	–
TEM-J	4	0	+	–	–
TEM-L	3	0	++	–	175
TEM-M	0	3	++	+++	110
TEM-N	0	2	+++	+++	110
TEM-O	2	0	++	–/+	–
TEM-P	2	0	++	–	–
TEM-Q	20	14	+	+++	–
TEM-R	1	3	–	–	–
TEM-T	1	0	–	–	–

[a] Percentage of the total fingerprints in round 3.

[b] Percentage of the total fingerprints in round 4.

[c] Results of FACS (on HUVEC) and IHC (on colorectal carcinoma) are presented as: – negative, –/+ weak positive, + moderate positive, ++ strong positive, +++ very intense positive.

3.2. Binding of selected phage clones to HUVEC

Flow cytometric analysis was employed to determine the binding specificity of the selected antibodies. Fifteen clones were found to bind to proliferating stimulated HUVEC at least two times above the background signal (relative mean fluorescence intensity values >2), set by the mean fluorescence intensity observed with irrelevant antibody displaying phage (phage Z) (Table 4). All antibodies except TEM-R and TEM-T bound to all proliferating HUVEC, with and without stimulation. For most antibodies, the FACS values on both types of proliferating HUVEC were similar, with the exception of antibodies TEM-O and TEM-P, which produced relative mean fluorescent

Table 3
Classification of the selected antibody clones according to their amino acid sequences

Clone	Closest VL family[a]	CDR3 (VL)	Closest VH family[a]	CDR3 (VH)
TEM-A	Z73673 IGLV6-57*01	QSYDSSP	X92274 IGHV4-30-4*03	GGHMDPFFDY
TEM-C	X12686 IGKV3-20*01	QQYGSSR	X92283 IGHV3-30-3*01	AYYYDRARL
TEM-G	X12686 IGKV3-20*01	QQYGSSR	X92283 IGHV3-30-3*01	LITMIGRDY
TEM-M	X12686 IGKV3-20*01	QQYGSSR	X92283 IGHV3-30-3*01	LITMIGRDY
TEM-N	X12686 IGKV3-20*01	QQYGSSR	X92283 IGHV3-30-3*01	LITMIGRDY
TEM-Q	M94115 IGLV3-21*03	QVWDSSSDH	X62112 IGHV4-4*07	ENFGAFDH
TEM-E	X12691 IGKV2D-28*01	MQALQTP	X62112 IGHV4-4*07	HYPQQLPPNYYYYYYMDV
TEM-D	X12691 IGKV2D-28*01	MQALQTP	X92224/J IGHV6-1*01	LPSVYYYYYGMDV
TEM-F	X12691 IGKV2D-28*01	MQALQTP	X92283 IGHV3-30-3*01	ASGYYYYYGMDV
TEM-H	Z73671 IGLV5-45*02	LIWDNAAW	L06615 IGHV3-30*04	AVIAAAHDY
TEM-P	Z73671 IGLV5-45*02	LIWDNAAW	L06615 IGHV3-30*04	AVIAAAHDY
TEM I	N.D[b]	N.D	M99660 IGHV3-23*01	GAPAGSYFDY
TEM-J	X01668 IGKV3-11*01	QQRSNWP	M99675 IGHV3-48*01	EFRRFGDRAYGMDV
TEM-T	X17264 IGKV3D-11*01	QQRSNWP	X92274 IGHV4-30-4*03	GDHYDSSSYRP
TEM-L	X01668 IGKV3-11*01	QQRTMWP	X92283 IGHV3-30-3*01	MGLMSSSPGGV
TEM-O	Z73671 IGLV5-45*02	MIWHSSAW	M99660 IGHV3-23*01	GGGMDV
TEM-R	X12686 IGKV3-20*01	QHYGSSI	M83132 IGHV1-69*04	VTAAGPIDY

[a] Sequences are compared to the human Ig set from the International Immunogenetics Database (IMGT) reference directory (http://imgt.cines.fr).
[b] N.D. = not determined.

Table 4
Characterization of specificity of selected monoclonal phage antibodies by FACS analysis

	$CaCo_2$	HUVEC, stimulated with						
		(A) Mix[a]	(B) None[a]	A/B	bFGF[b]	VEGF[b]	TS-med[b]	None[b]
TEM-A	0.7	74.9	70.4	1.1	0.85	1.0	1.2	1.0
TEM-C	1.9	50.6	41.4	1.2	0.67	1.0	1.0	1.0
TEM-D	0.6	11.9	18.8	0.6	–	–	–	–
TEM-E	0.5	29.7	29.2	1	–	–	–	–
TEM-F	0.9	38.8	37.5	1	–	–	–	–
TEM-G	1.0	41.1	50.6	0.8	–	–	–	–
TEM-H	0.7	35	40.5	0.9	–	–	–	–
TEM-I	1.0	39.8	23.7	1.7	0.61	0.9	1.1	1.0
TEM-J	1.9	25.0	16.0	1.6	–	–	–	–
TEM-L	0.6	50.9	62.4	0.8	–	–	–	–
TEM-M	1.3	56.7	65.7	0.8	–	–	–	–
TEM-N	1.5	133.1	157.9	0.8	0.72	1.0	1.1	1.0
TEM-O	0.6	44.5	9.6	4.6	–	–	–	–
TEM-P	0.8	36.4	6.8	5.3	–	–	–	–
TEM-Q	1.2	5.4	3.2	1.7	0.49	0.8	0.9	1.0
TEM-R	1.2	0.8	2.1	0.4	–	–	–	–
TEM-T	1.3	1.3	1.1	1.1	–	–	–	–
CD-31	1.8	12	12.8	0.9	0.65	0.6	1.0	1.0
Phage Z[c]	1	1	1	1	1.0	1.0	1.0	1.0

[a] Relative mean fluorescence intensity values; calculated by (mean fluorescence of phage antibody/mean fluorescence of control phage).
[b] Relative stimulation values: calculated by: (relative mean fluorescence/relative mean fluorescence of unstimulated HUVEC); TS-med = tumor spent medium.
[c] Phage carrying irrelevant antibody.

values 4.6- and 5.3-fold higher for proliferating HUVEC after tumor-stimulation (data not shown). These data suggest that the selected antibodies bind to antigens associated with EC proliferation, and that the stimulation by tumor spent medium and angiogenic factors contributed largely to the upregulation of the target antigens for antibodies TEM-O and TEM-P. For the 15 HUVEC binding clones, mean fluorescent values for binding to stimulated proliferating HUVEC ranged between 5.4 (TEM-Q) and 133 times (TEM-N) (Table 4). Non-fixed HUVEC were also tested to confirm that these 15 phage antibodies reacted with the target antigen in the native form (data not shown). To identify the antibodies binding to endothelial cell specific antigens, the phage antibodies were tested for binding to CaCo2 cells and peripheral blood leukocytes (PBL). $CaCo_2$ cells were tested since conditioned medium from this cell line was used to stimulate HUVEC. PBL were tested to determine whether the antibodies bound any of the antigens shared by both cell populations, and to evaluate whether these antibodies had potential application in vascular targeting. Interestingly, none of the selected antibodies bound to either $CaCo_2$ cells or PBL (Table 4 and Fig. 1).

Five antibodies, TEM-A, TEM-C, TEM-I, TEM-N and TEM-Q, were chosen for further testing for binding to HUVEC under different culture conditions involving the various stimulatory agents, bFGF, VEGF and tumor spent medium (Table 4). All five antibodies bound strongly to cultured HUVEC without further stimulation, indicating that the targeted antigens are overexpressed by cultured proliferating HUVEC. Exposure of HUVEC to CaCo2 tumor spent media increased the FACS values of three of the five antibodies tested (TEM-A, TEM-N and TEM-I). On the other hand, the values obtained after bFGF or VEGF stimulation were in most cases lower than the values obtained with proliferating non stimulated HUVEC. However, of the five antibodies, only TEM-N resulted in higher FACS values for non-stimulated compared to

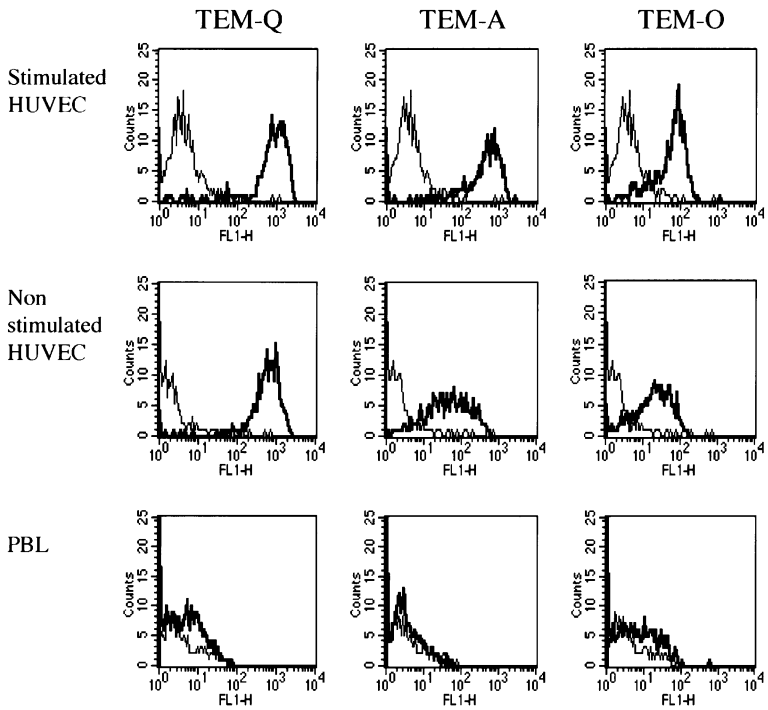

Fig. 1. Characterization of specificity of selected phage antibodies by FACS analysis on HUVEC and PBL. Three representative selected phage antibodies were tested for binding to: stimulated, non-stimulated HUVEC and PBL. Bound antibody displaying phage was detected by sequential incubations with polyclonal goat anti-fd antibodies and rabbit anti-goat Ig antibodies coupled to fluorescein. Binding of TEM-Q, -A and -O (thick line); negative control is irrelevant antibody displaying phage, phage Z (thin line).

stimulated HUVEC. Therefore, these angiogenic factors seem to have a stimulatory effect only in combination with tumor spent medium.

3.3. Characterization of HUVEC antigens by immunoblot analysis

We tested the polyclonal phage from selection rounds 3 and 4, as well as the individual phage antibodies that bound in FACS to HUVEC for their ability to bind proliferating HUVEC cell lysate preparations via immunoblotting. An immunoblot with the polyclonal phage revealed that the phage antibody populations from R3 and from R4 recognized, on a non-reducing SDS-PAGE gel, one band of approximately M_r 175 kDa, and a much wider band, of approximately M_r 110–125 kDa (Fig. 2). Nine of the individual phage antibodies produced either one or both of these bands by this immunoblot analysis. The antibodies TEM-A and TEM-L recognize a 175-kDa antigen, while TEM-E, TEM-F, TEM-G, TEM-H, TEM-N and TEM-M recognized predominantly an antigen of 110–125 kDa. TEM-C recognized both antigens (Fig. 2a and data not shown). The other antibodies, TEM-D, TEM-I, TEM-J, TEM-O, TEM-P and TEM-Q did not react in this assay (summarized in Table 2). We have used commercially available antibodies to compare these antigens with the molecular weights of possible target antigens such as CD31 (PECAM), Tie-1, Tie-2, VEGFR1 and VEGFR2. None of those commercial antibodies detected the exact same molecular weights as the selected phage antibodies (Fig. 2b and data not

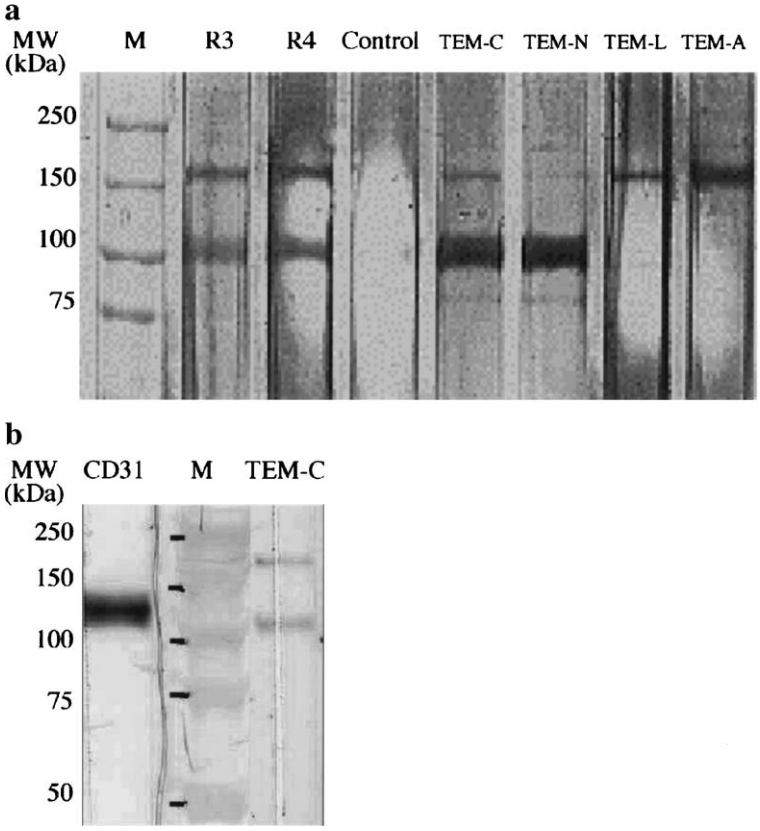

Fig. 2. Immunoblot analysis with selected phage antibodies. HUVEC membrane fractions separated by SDS-PAGE and blotted on nitrocellulose were analyzed with: (a) M, Mr markers; R3 and R4, rounds 3 and 4 selected phage antibody polyclonal mixtures, respectively; Control, phage Z; individual phage antibodies TEM-C, TEM-N, TEM-L and TEM-A; (b) CD31, anti CD31 monoclonal antibody; M, Mr markers; TEM-C phage antibody. Bound phage particles were detected by a peroxidase conjugated anti-M13 antibody; anti-CD31 antibody was detected by peroxidase conjugated anti mouse antibody. The blot was developed using DAB/H_2O_2.

shown). The relatively broad band detected by the pan endothelial cell marker CD31 (PECAM) (of 130–140 kDa) was only slightly higher in M_r than the 110–125-kDa protein band detected by phage antibodies such as TEM-C (Fig. 2b). However, immunohistochemistry and FACS data ruled out CD31 as a target antigen. Under reducing conditions, the immunoblot analysis was negative, both for polyclonal and monoclonal phage antibodies, indicating that these antibodies bound epitopes that are altered due to the breaking of disulfide bonds, a phenomenon also noted for phage display selected antibodies to other antigens such as epithelial cell adhesion molecule (EpCAM) (Roovers et al., 2001a,b).

3.4. Immunohistochemical analysis of the HUVEC binding antibodies with normal and tumor tissues

Since the goal of the selection was to obtain antibodies amenable for targeting the vasculature of human tumors, we continued with the characterization of the identified antibodies by performing a series of immunohistochemical analyses on normal and neoplastic tissue sections of human origin (Table 5). Since the HUVEC used for selection were stimulated with tumor-conditioned media from $CaCo_2$, a colorectal carcinoma derived cell line, the assay was first performed on colorectal carcinomas and the corresponding normal colorectal tissue from the same biopsies.

Antibodies TEM-A, TEM-C, TEM-D, TEM-F, TEM-G, TEM-H, TEM-I, TEM-M, TEM-N, TEM-O and TEM-Q reacted with colorectal carcinoma associated neovasculature. While antibodies TEM-H and TEM-Q cross-reacted sporadically with the vasculature of normal colonic epithelia, the rest of the antibodies were completely unreactive on normal colonic vasculature. Antibodies TEM-F, TEM-I and TEM-O reacted weakly and only with a subset of all the colorectal carcinomas tested. On the other hand, antibodies TEM-A, TEM-C, TEM-M, TEM-N and TEM-Q reacted strongly with the vasculature of all colorectal carcinomas. These antibodies, except TEM-N (whose sequence differs to TEM-M in one amino acid), were selected for a more extensive immunohistochemical analysis.

All four antibodies, TEM-A, TEM-C, TEM-M and TEM-Q, stained the vasculature of all carcinomas tested, for all three patient-derived samples used

Table 5
Immunohistochemistry results on endothelial cells of tumors and corresponding normal tissues[a,b]

Tissue	Tumor				Normal			
	TEM-A	TEM-C	TEM-M	TEM-Q	TEM-A	TEM-C	TEM-M	TEM-Q
Colon[c]	+++/D	++/D	+++/D	+++/D	–	–	–	– +/S
Kidney	++/D	++/F	++/D	++/D	+/F[d]	– +/S	– +/S	– +/S[e]
Liver	+++/D	+++/D	+++/D	++/D	– /F[f]	– +/S	– +/F[g]	– +/Fg
Ovary	+++/D[h]	++/D[h]	+++/D	+++/D	–	– +/F	– +/F	–
Lung	+/D	++/F	+/F	+/F	– +/S[i]	– +/S[i]	–	– +/S[i]
Pancreas	+/F	– +/S	+/F	+/F	–	– +/S	– +/S	–
Bladder	+/F	++/D	+/F	++/D	–	– +/S	– +/S	– +/S
Breast	+++/D	+/D	++/D	+/D	N.D.[j]	N.D.	N.D.	N.D.
Endometrium[c]					++/D	++/D	++/D	++/D
Placenta					–	–	+/S	++/D

[a] –, no endothelial cell staining, – + weak staining of endothelial cells, + moderate staining of endothelial cells, ++ strong staining of endothelial cells, +++ very intense staining of endothelial cells.

[b] S, sporadic staining, 1–5% of vessels; F, focal staining, 5–80% of vessels; D, diffuse staining, 80–100% of vessels.

[c] For all tissues $n = 3$ except for colon and endometrium where $n = 6$.

[d] Glomeruli and large vessels positive.

[e] Luminal tubuli positive.

[f] Sinusoidal lining positive.

[g] Portal vessels positive.

[h] Also matrix surrounding vessels positive.

[i] Large vessels positive.

[j] N.D. = not determined.

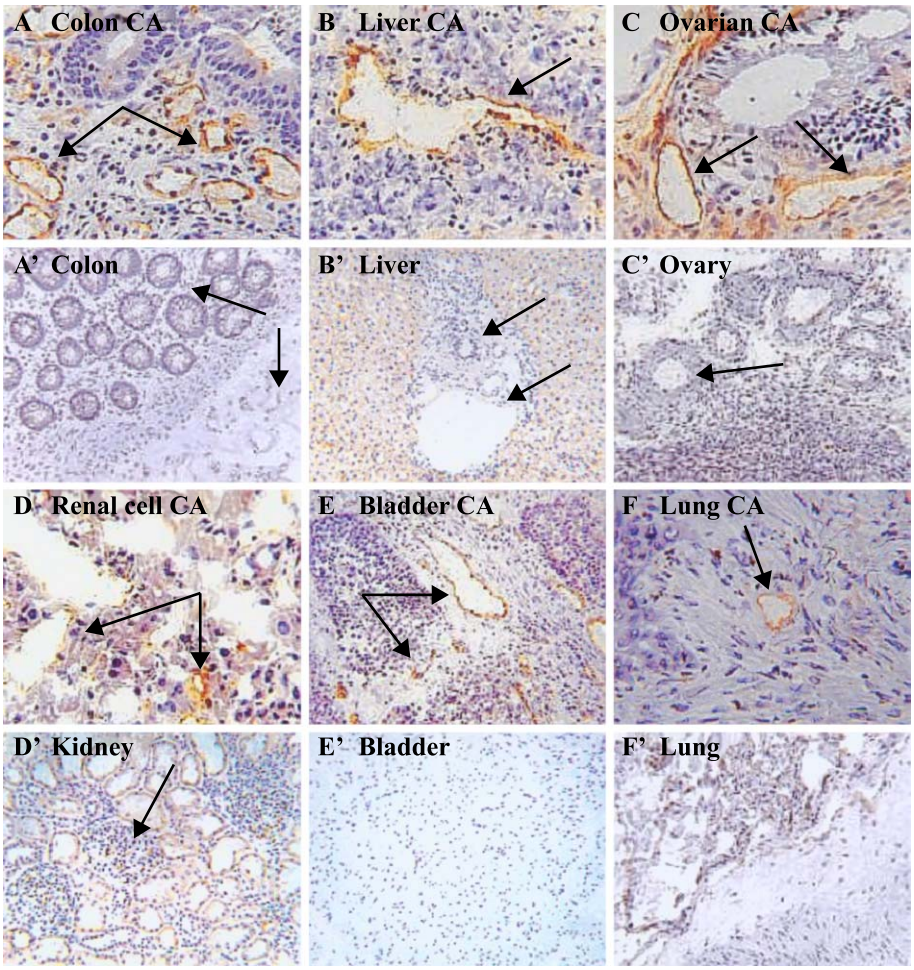

Fig. 3. Immunohistochemical staining with phage antibody TEM-A on a series of human tumors and corresponding normal tissues. Strong staining of tumor vasculature was found in (A) colorectal carcinoma, (B) hepatocellular carcinoma, (C) ovarian carcinoma, (D) renal cell carcinoma, (E) bladder carcinoma, (F) lung carcinoma (original magnification × 400; A, B, C, D, E, F). Staining was negative (A′ , C′ , E′) or focally positive (B′ , D′ , F′) in the corresponding normal tissues (original magnification × 200; A′ , C′ , E′ , B′ , D′ , F′). Vessels indicated by arrows.

for the staining: renal cell, hepatocellular, breast, ovarian, lung and bladder carcinoma (Table 5). The antibodies revealed the most striking staining on ovarian, colorectal and hepatocarcinomas (Fig. 3 and Table 5). All four antibodies bound these tissues with a high level of specificity, showing homogeneous staining of the malignant tissue and localized or negative reactivity on the vasculature of corresponding normal tissues. In these four tissues and in breast tumors the intensity of the staining of tumor vasculature with the selected antibodies was almost identical to the staining produced by the anti-pan-endothelial cell marker CD31. Diffuse or focal staining of tumor vasculature was mainly observed in lung, pancreas and bladder carcinoma.

The reactivity of antibodies TEM-Q and TEM-M was limited to the endothelial cells, whereas antibodies TEM-A and TEM-C occasionally reacted with other vascular elements (perivascular staining). Perivascular staining was more manifest on ovarian carcinomas, with antibody TEM-C. Antibodies TEM-A and TEM-M showed the strongest immunoreactivity

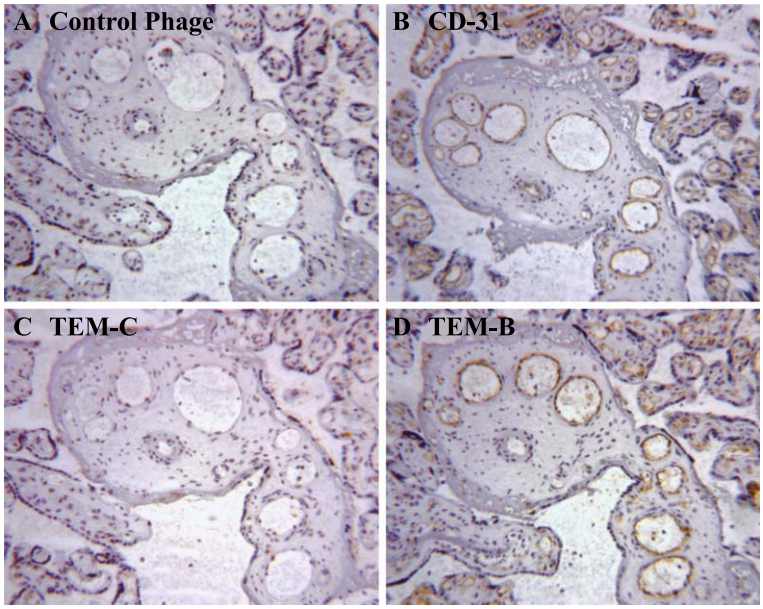

Fig. 4. Example of patterns of immunohistochemical reactivity on frozen sections of human term placenta. (A) Negative control phage Z; (B) anti-CD31 antibody staining of blood vessels; (C) lack of reactivity of phage-displayed antibody TEM-C with placental vasculature; (D) phage antibody TEM-B (original magnification × 400).

with the vasculature of all tested tumors, and resulted in sporadic, focal or absent reactivity with the endothelium of the corresponding normal tissues (Fig. 3 and Table 5). Normal non-angiogenic tissues predominantly demonstrated focal or negative staining of vessels. However, differences in the staining pattern of the selected antibodies emerged in several normal tissues. On normal liver only antibody TEM-A reacted with the sinusoidal lining, and antibodies TEM-M and TEM-Q reacted with portal vessels of liver tissue. In normal kidney antibody TEM-A reacted with the vasculature of glomeruli and large vessels (Fig. 3), whereas the staining produced by TEM-Q was localized in luminal tubuli. On normal lung, antibody TEM-A showed no reactivity, and the rest of the antibodies showed weak reactivity on some of the larger lung vessels.

Once we had established the presence of the target antigens on the tumor vasculature, we further determined tumor specificity of the four antibodies by testing their reactivity with the angiogenic vasculature of placenta and endometrium. All four antibodies reacted positively on the vasculature of endometrium. Antibody TEM-Q also stained the placental neovasculature homogeneously. Antibody TEM-M stained less than 5% of placental vasculature, and antibodies TEM-A and TEM-C did not show reactivity on placental vasculature (Fig. 4 and Table 5).

4. Discussion

The search for new targets in angiogenesis has recently resulted in the generation of new selection and screening methods for the rapid isolation of binding specificities from peptide or human antibody fragment repertoires (Arap et al., 2002; Giordano et al., 2001; Giovannoni et al., 2001). In our hands, a simple and efficient selection method has resulted in a population of antibodies against endothelial proliferation associated antigens, with diverse specificities for tumor vasculature and the vasculature of other angiogenic tissues. By selection of a large non-immune Fab phage library on stimulated proliferating HUVEC, we have successfully identified a series of human antibodies that recognize as yet unidentified antigens associated with tumor endothelium and/or other forms of angiogenic vasculature.

Peptide or antibody phage library selections on whole cells presents a substantial technical challenge when searching for binders specific for that cell type, in particular when searching for binders to cells specific for a unique differentiation or disease-induced state. Nearly all of the selected HUVEC-binding antibodies reacted with antigens present on endothelial cells only, and there was no necessity to reduce the frequency of pan-cell reactive antibodies by employing subtractive or depletive methods as previously reported (De Kruif et al., 1995). Furthermore, without taking any specific precaution during the selection, such as alternation of selections between HUVEC and isolated tumor EC or tumor tissue sections, the majority of the HUVEC binding antibodies (15 out of 17 identified clones) bound almost exclusively to the endothelium of the carcinomas analyzed.

In the literature there are several reports on how cell-binding antibodies are isolated from phage libraries (Mutuberria et al., 1999; Ridgway et al., 1999; Roovers et al., 2001a,b). For EC limited data have been published on using phage-displayed peptide libraries (Giordano et al., 2001; Pasqualini, 1999; Pasqualini and Ruoslahti, 1996), or in vivo selections with a phage-displayed antibody library (Johns et al., 2000). For cell panning selections, the procedure and nature of the cells employed have a profound effect on the quality of retrieved phage antibodies. In our hands the use of in vitro cultured proliferating HUVEC in combination with MiniMacs magnetic cell retrieval (Mutuberria et al., 1999) has given the best results. These cells are easily isolated and can be readily cultured under reproducible conditions. It is known that cultured HUVEC maintain important cell–cell and cell–matrix interactions, and like EC in tumors, they have a high proliferation rate (Grant et al., 1989). In addition, culture conditions can be further modulated to mimic the microenvironment of the cell population in vivo, e.g., by the addition of tumor spent media and/or angiogenic factors to mimic the microenvironment of tumors. We found that magnetic cell retrieval resulted in the best selection technique. During this study we compared two different types of magnetic beads with comparable cell-retrieval efficiency, Dynabeads and Minimacs beads. The use of Dynabeads yielded antibodies predominantly to the cell-capturing antibody, and only very few binding to HUVEC (P.C., R.M. and H.R.H., data not shown). Dynabeads are large magnetic particles covered at high density with the cell-capturing antibody, and are normally present in excess during the selection procedure. As a result, the library is exposed to a large proportion of irrelevant antigen, and despite subtraction on beads before the selection, a large number of selected clones are directed against the cell-capturing antibody (data not shown). On the contrary, with MiniMACS selection, the excess beads bearing the capturing antibody are removed by mild centrifugation prior to loading the cells onto the magnetic column. As a result, the irrelevant epitopes (capturing antibody-coupled beads) are negligible compared to the antigenic mix present on the cell surface, thus, resulting in the sole retrieval of phage antibodies directed to antigens present on HUVEC surface membranes. This underlines the importance of defining a proper cell selection procedure before embarking on this approach (Mutuberria et al., 1999). This is the first report of a successful selection of tumor vasculature specific antibodies by panning a phage antibody library on endothelial cells.

Our data suggest that proliferating HUVEC dominantly express a limited set of cell-surface markers that are also present on tumor EC. Indeed, endothelium is a highly specialized organ system, with a distinct inherent phenotype. When the angiogenic switch occurs, environmental stimuli induce genetically programmed determinants that contribute to major changes in endothelial cell behavior and function, resulting also in significant changes in antigenic expression. The antigenic expression of stimulated HUVEC may well be very different compared to other types of cells, thus preventing selection of pan-reactive cell binding antibodies, and highly similar to the EC that are subjected to the same stimuli in vivo, leading to the selection of a high proportion of antibodies binding to tumor endothelium.

Half of the antibodies isolated in this study detect either one or two bands of M_r 110–125 and 175 kDa, respectively, under non-reducing conditions in immunoblotting of a proliferating HUVEC cell extract. Indeed, also in the selected polyclonal antibody mixture, these are the dominant bands recognized. The dual reactivity of TEM-C with both suggests that this antibody detects either two splice variants of the same antigen, or both a full length and a fragment of the identical antigen, but we cannot exclude the

possibility that the antibodies like TEM-C, or even those antibodies recognizing the same Mr band, react with unrelated antigens. The wide variation of the staining pattern on tumor and normal tissues suggests that the selected antibodies bind either unrelated antigens or variants of the same antigen with different tissue expression patterns. The other antibodies did not show any activity on immunoblotting, either due to lack of sensitivity of the assay, or lack of reactivity of the antibody with the antigen in the HUVEC cell extracts. Further investigations are warranted for those antibodies that show reactivity to EC in flow cytometry or in immunohistochemistry. The limited number of antigens that the selected phage antibodies appear to recognize may be due to the presence on the proliferating HUVEC of selection-dominant epitopes (a preferential selection of phage antibodies to one particular epitope or antigen despite the presence of many others in the selection procedure), as has been reported for other systems (Hoogenboom et al., 1999), which in this case happens to be present on tumor EC. Further deviations from the selection procedure (i.e. reduced washing on the column), the use of a different EC source (i.e. microvascular EC, lymphatic EC), and variations in the culture conditions (i.e. hypoxia, coculture with pericytes or tumor cells) or stimulation protocol (i.e. different angiogenic factors or condition media from different tumors) combined with a more extensive phage screening, such as with automated systems, would result in a wider spectrum of antigens recognized by the selected phage antibodies.

In view of this bias towards a few selection-dominant epitopes, which sometimes translates into an immunodominance of the epitope (Pelsers et al., 1999), it remains intriguing to identify the molecular nature of the antigens recognized by most of our selected phage antibodies. The M_r of the targeted antigens does not correspond with the M_r of known markers such as VERGFR1, VEGFR2, Tie-1 or Tie-2 when empirically compared by immunoblot analysis. Significant differences are also observed between mean fluorescent values obtained with the selected antibodies and the antibodies to these known markers by FACS analysis (data not shown). According to their M_r similarity, the putative targets for antibodies TEM-A and TEM-C are CD105, CD106, CD107a and b, CD109, αv integrin, platelet-derived growth factor receptors α and β, angiotensin converting enzyme or the leptin receptor; antibody TEM-M could be targeting integrins β1 or β3, CDw93, Ephrin A2 receptor, FGF receptor, C1qPR, VE cadherin, CD98, CD107 or CD133; antibody TEM-C could bind angiotensin converting enzyme or integrin αvβ3. Taking into consideration the FACS and IHC results produced by the selected antibodies, we have reduced the candidate targets to antigens that are highly specific for endothelial cells, and are only present on the endothelial cell compartment of tumors with an expression pattern similar to that of CD31. This rules out most of the known endothelial cell targets. Furthermore, we know that the 100- and 175-kDa antigens are both expressed on endometrium, the 110–175- and 175-kDa antigen is not expressed in placenta and the 110-kDa antigen shows a sporadic expression on placental vasculature. Endoglin, a candidate target, is highly specific for tumor endothelium and is also expressed in placenta (Dye et al., 2001) corresponding only with the IHC results obtained with antibody TEM-M and TEM-Q. However, unlike the staining pattern of the selected antibodies this antigen is also expressed on tumor cells of ovarian neoplasm and epithelial cells of normal ovary (Henriksen et al., 1995; Jindal et al., 1995) and localizes to the proliferating endothelium at the edge of tumors (Miller et al., 1999). We also discard PDGF receptors as target antigens since they are expressed in a number of non-endothelial cell types such as monocytes, macrophages, smooth muscle cells or fibroblasts, to which the selected antibodies show no binding (Kim et al., 2003). Antibodies to integrin αvβ3 preferentially stain the blood vessels of small calibre and localize in angiogenic hot spots within the vasclature of breast tumors (Gasparini et al., 1998). In contrast, the selected antibodies produce a diffuse staining of the vasculature of all breast carcinomas analysed and the molecular target is nearly as abundant as CD31 in the analysed breast tumors (data not shown).

The available data on the expression patterns of known tumor-associated endothelial cell markers appear insufficient to identify the antigens targeted by the selected phage antibodies. Immunohistochemical analysis demonstrates that 11 of the selected antibodies bind to antigens present in tumor vasculature. The level of reactivity for tumor vasculature is variable,

which can be caused by the differences of antigenic expression or related to the affinity of the antibody. For example, the staining intensity on lung, pancreas and bladder tumors is lower and in some cases focal or sporadic. This can be related to the expression of the target antigens in these tissues, which could be associated to proliferating endothelial cells present on angiogenic hot spots. However, we can conclude from the IHC staining produced by antibodies such as TEM-A or TEM-M, that the antigens of M_r 110–125 and 175 kDa are good quality targets for both tumoral and non-tumoral neovasculature. Most importantly these targets are highly abundant in the vasculature of tumors such as breast, liver, ovarian and colorectal carcinomas. The targeted antigens have been detected in proliferating HUVEC membrane fractions and, due to the nature of the selection procedure, it is highly possible that the targets of these antibodies are angiogenesis related EC surface membrane receptors. Further biochemical analysis of the antigens, for example by N-terminal sequencing of protein immunoprecipitated by the TEM-antibodies, will be needed to identify the antigens.

The variability found in the reactivity of the selected antibodies on normal tissues and non-neoplastic angiogenic tissues clearly indicates that different epitopes or antigens are being targeted, and that the expression of these differs between tissues. The targeted molecules could be splice variants or isoforms of known tumor endothelium markers, or even receptor–ligand complexes. However, it is also possible that novel markers of angiogenesis and tumor vasculature are being targeted.

The recombinant anti-HUVEC antibodies described here have potential applications as selective markers of tumor vasculature. Antibodies such TEM-A, TEM-C, TEM-M and TEM-Q are highly reactive with tumor EC from various carcinomas of all patient samples tested, yet mostly unreactive with quiescent endothelium. It will be interesting to use these antibodies to compare angiogenic intratumoral microvessel density versus the total microvessel density using panendothelial markers, and to determine their relevance as independent prognostic markers, as reported for endoglin (Kumar et al., 1999). Isolation and characterization of the targeted antigens will be needed for further development and applicability of the selected antibodies.

In conclusion, the pattern in IHC suggests a unique tumor-endothelial cell specificity, which would warrant in vivo targeting studies with these antibodies. The TEM-A, TEM-C, TEM-M and TEM-Q antibodies show in principle the appropriate targeting abundancy and specificity to be suitable as labeled agents for in vivo imaging of human solid malignancies. Further immunohistochemical analysis of normal tissues, with the phage antibodies or possibly reformatted versions of these human Fab's will be necessary to establish the full expected specificity. Unfortunately, none of the four antibodies that we extensively tested shows any cross-reactivity with murine endothelial cells (data not shown), a feature which would allow relatively rapid in vivo analysis of this targeting potential of these antibodies. By exploiting the power of this phage antibody display approach, we anticipate that many novel vascular antigens/epitopes will be identified and that the antibodies themselves will rapidly unlock the potential of these targets for cancer immunotherapy.

Acknowledgements

We would like to thank Daisy van der Schaft for technical assistance and Arjan Griffioen and Adriaan de Bruïne for discussion and Maggie Merchant for critical reading and editing of the manuscript. This work was supported by The Basque Country research grant BF195.155 Mod. AK (to R.M.).

References

Arap, W., Kolonin, M.G., Trepel, M., Lahdenranta, J., Cardo-Vila, M., Giordano, R.J., Mintz, P.J., Ardelt, P.U., Yao, V.J., Vidal, C.I., Chen, L., Flamm, A., Valtanen, H., Weavind, L.M., Hicks, M.E., Pollock, R.E., Botz, G.H., Bucana, C.D., Koivunen, E., Cahill, D., Troncoso, P., Baggerly, K.A., Pentz, R.D., Do, K.A., Logothetis, C.J., Pasqualini, R., 2002. Steps toward mapping the human vasculature by phage display. Nat. Med. 8, 121.

Ausubel, F.M., 1995. Current protocols in molecular biology. In: Frederick, R.B., Ausubel, M., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K., Albright, L.M., Coen, D.M., Varki, A. (Eds.), Current Protocols in Molecular Biology. Willey, Amsterdam, p. 10.11.1.

Birchler, M., Viti, F., Zardi, L., Spiess, B., Neri, D., 1999. Selective targeting and photocoagulation of ocular angiogenesis mediated

by a phage-derived human antibody fragment. Nat. Biotechnol. 17, 984.

Carson-Walter, E.B., Watkins, D.N., Nanda, A., Vogelstein, B., Kinzler, K.W., St Croix, B., 2001. Cell surface tumor endothelial markers are conserved in mice and humans. Cancer Res. 61, 6649.

de Haard, H.J., van Neer, N., Reurs, A., Hufton, S.E., Roovers, R.C., Henderikx, P., de Bruine, A.P., Arends, J.W., Hoogenboom, H.R., 1999. A large non-immunized human Fab fragment phage library that permits rapid isolation and kinetic analysis of high affinity antibodies. J. Biol. Chem. 274, 18218.

De Kruif, J., Terstappen, L., Boel, E., Logtenberg, T., 1995. Rapid selection of cell subpopulation-specific human monoclonal antibodies from a synthetic phage antibody library. Proc. Natl. Acad. Sci. U. S. A. 92, 3938.

Dye, J.F., Jablenska, R., Donnelly, J.L., Lawrence, L., Leach, L., Clark, P., Firth, J.A., 2001. Phenotype of the endothelium in the human term placenta. Placenta 22, 32.

Eberhard, A., Kahlert, S., Goede, V., Hemmerlein, B., Plate, K.H., Augustin, H.G., 2000. Heterogeneity of angiogenesis and blood vessel maturation in human tumors: implications for antiangiogenic tumor therapies. Cancer Res. 60, 1388.

Folkman, J., 1995. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat. Med. 1, 27.

Gasparini, G., Brooks, P.C., Biganzoli, E., Vermeulen, P.B., Bonoldi, E., Dirix, L.Y., Ranieri, G., Miceli, R., Cheresh, D.A., 1998. Vascular integrin alpha(v)beta3: a new prognostic indicator in breast cancer. Clin. Cancer Res. 4, 2625.

Giordano, R.J., Cardo-Vila, M., Lahdenranta, J., Pasqualini, R., Arap, W., 2001. Biopanning and rapid analysis of selective interactive ligands. Nat. Med. 7, 1249.

Giovannoni, L., Viti, F., Zardi, L., Neri, D., 2001. Isolation of anti-angiogenesis antibodies from a large combinatorial repertoire by colony filter screening. Nucleic Acids Res. 29, E27.

Grant, D.S., Tashiro, K., Segui-Real, B., Yamada, Y., Martin, G.R., Kleinman, H.K., 1989. Two different laminin domains mediate the differentiation of human endothelial cells into capillary-like structures in vitro. Cell 58, 933.

Griffioen, A.W., Molema, G., 2000. Angiogenesis: potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation. Pharmacol. Rev. 52, 237.

Griffioen, A.W., Coenen, M.J., Damen, C.A., Hellwig, S.M., van Weering, D.H., Vooys, W., Blijham, G.H., Groenewegen, G., 1997. CD44 is involved in tumor angiogenesis; an activation antigen on human endothelial cells. Blood 90, 1150.

Groenewegen, G., de Ley, M., Jeunhomme, G.M., Buurman, W.A., 1986. Supernatants of human leukocytes contain mediator, different from interferon gamma, which induces expression of MHC class II antigens. J. Exp. Med. 164, 131.

Henriksen, R., Gobl, A., Wilander, E., Oberg, K., Miyazono, K., Funa, K., 1995. Expression and prognostic significance of TGF-beta isotypes, latent TGF-beta 1 binding protein, TGF-beta type I and type II receptors, and endoglin in normal ovary and ovarian neoplasms. Lab. Invest. 73, 213.

Hoogenboom, H.R., de Bruine, A.P., Hufton, S.E., Hoet, R.M., Arends, J.W., Roovers, R.C., 1998. Antibody phage display technology and its applications. Immunotechnology 4, 1.

Hoogenboom, H.R., Lutgerink, J.T., Pelsers, M.M., Rousch, M.J., Coote, J., Van Neer, N., De Bruine, A., Van Nieuwenhoven, F.A., Glatz, J.F., Arends, J.W., 1999. Selection-dominant and nonaccessible epitopes on cell-surface receptors revealed by cell-panning with a large phage antibody library. Eur. J. Biochem. 260, 774.

Huang, X., Molema, G., King, S., Watkins, L., Edgington, T.S., Thorpe, P.E., 1997. Tumor infarction in mice by antibody-directed targeting of tissue factor to tumor vasculature. Science 275, 547.

Jindal, S.K., Ishii, E., Letarte, M., Vera, S., Teerds, K.J., Dorrington, J.H., 1995. Regulation of transforming growth factor alpha gene expression in an ovarian surface epithelial cell line derived from a human carcinoma. Biol. Reprod. 52, 1027.

Johns, M., George, A.J., Ritter, M.A., 2000. In vivo selection of sFv from phage display libraries. J. Immunol. Methods 239, 137.

Kim, H.R., Yu, J., Ustach, C., 2003. Platelet-derived growth factor signaling and human cancer. J. Biochem. Mol. Biol. 36, 49.

Kumar, S., Ghellal, A., Li, C., Byrne, G., Haboubi, N., Wang, J.M., Bundred, N., 1999. Breast carcinoma: vascular density determined using CD105 antibody correlates with tumor prognosis. Cancer Res. 59, 856.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680.

Marks, J.D., Hoogenboom, H.R., Bonnert, T.P., McCafferty, J., Griffiths, A.D., Winter, G., 1991. By-passing immunization: human antibodies from V-gene libraries displayed on phage. J. Mol. Biol. 222, 581.

Miller, D.W., Graulich, W., Karges, B., Stahl, S., Ernst, M., Ramaswamy, A., Sedlacek, H.H., Muller, R., Adamkiewicz, J., 1999. Elevated expression of endoglin, a component of the TGF-beta-receptor complex, correlates with proliferation of tumor endothelial cells. Int. J. Cancer 81, 568.

Mutuberria, R., Hoogenboom, H.R., van der Linden, E., de Bruine, A.P., Roovers, R.C., 1999. Model systems to study the parameters determining the success of phage antibody selections on complex antigens. J. Immunol. Methods 231, 65.

Neri, D., Carnemolla, B., Nissim, A., Leprini, A., Querze, G., Balza, E., Pini, A., Tarli, L., Halin, C., Neri, P., Zardi, L., Winter, G., 1997. Targeting by affinity-matured recombinant antibody fragments of an angiogenesis associated fibronectin isoform. Nat. Biotechnol. 15, 1271.

Pasqualini, R., 1999. Vascular targeting with phage peptide libraries. Q. J. Nucl. Med. 43, 159.

Pasqualini, R., Ruoslahti, E., 1996. Organ targeting in vivo using phage display peptide libraries. Nature 380, 364.

Pelsers, M.M., Lutgerink, J.T., Nieuwenhoven, F.A., Tandon, N.N., van der Vusse, G.J., Arends, J.W., Hoogenboom, H.R., Glatz, J.F., 1999. A sensitive immunoassay for rat fatty acid translocase (CD36) using phage antibodies selected on cell transfectants: abundant presence of fatty acid translocase/CD36 in cardiac and red skeletal muscle and up-regulation in diabetes. Biochem. J. 337, 407.

Piascik, P., 2003. New antibody approved for treatment of rheumatoid arthritis. J. Am. Pharm. Assoc. 43, 327.

Rajotte, D., Arap, W., Hagedorn, M., Koivunen, E., Pasqualini, R., Ruoslahti, E., 1998. Molecular heterogeneity of the vascular endothelium revealed by in vivo phage display. J. Clin. Invest. 102, 430.

Ribatti, D., Nico, B., Vacca, A., Roncali, L., Dammacco, F., 2002. Endothelial cell heterogeneity and organ specificity. J. Hematother. Stem Cell Res. 11, 81.

Ridgway, J.B., Ng, E., Kern, J.A., Lee, J., Brush, J., Goddard, A., Carter, P., 1999. Identification of a human anti-CD55 single-chain Fv by subtractive panning of a phage library using tumor and nontumor cell lines. Cancer Res. 59, 2718.

Roovers, R.C., van der Linden, E., de Bruine, A.P., Arends, J.W., Hoogenboom, H.R., 2001a. Identification of colon tumour-associated antigens by phage antibody selections on primary colorectal carcinoma. Eur. J. Cancer 37, 542.

Roovers, R.C., van der Linden, E., de Bruine, A.P., Arends, J.W., Hoogenboom, H.R., 2001b. In vitro characterisation of a monovalent and bivalent form of a fully human anti Ep-CAM phage antibody. Cancer Immunol. Immunother. 50, 51.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1990. Molecular Cloning—A Laboratory Manual Cold Spring Harbor Laboratory, New York.

Soker, S., Gollamudi-Payne, S., Fidder, H., Charmahelli, H., Klagsbrun, M., 1997. Inhibition of vascular endothelial growth factor (VEGF)-induced endothelial cell proliferation by a peptide corresponding to the exon 7-encoded domain of VEGF165. J. Biol. Chem. 272, 31582.

St Croix, B., Rago, C., Velculescu, V., Traverso, G., Romans, K.E., Montgomery, E., Lal, A., Riggins, G.J., Lengauer, C., Vogelstein, B., Kinzler, K.W., 2000. Genes expressed in human tumor endothelium. Science 289, 1197.

Vermeulen, P.B., Verhoeven, D., Hubens, G., Van Marck, E., Goovaerts, G., Huyghe, M., De Bruijn, E.A., Van Oosterom, A.T., Dirix, L.Y., 1995. Microvessel density, endothelial cell proliferation and tumour cell proliferation in human colorectal adenocarcinomas. Ann. Oncol. 6, 59.

Viti, F., Giovannoni, L., Neri, D., 2002. Recombinant antibodies for the selective targeting of tumor neovasculature. Curr. Opin. Drug Discov. Dev. 5, 204.

Wang, J.M., Kumar, S., Pye, D., van Agthoven, A.J., Krupinski, J., Hunter, R.D., 1993. A monoclonal antibody detects heterogeneity in vascular endothelium of tumours and normal tissues. Int J. Cancer 54, 363.

Wei, Y.Q., Wang, Q.R., Zhao, X., Yang, L., Tian, L., Lu, Y., Kang, B., Lu, C.J., Huang, M.J., Lou, Y.Y., Xiao, F., He, Q.M., Shu, J.M., Xie, X.J., Mao, Y.Q., Lei, S., Luo, F., Zhou, L.Q., Liu, C.E., Zhou, H., Jiang, Y., Peng, F., Yuan, L.P., Li, Q., Wu, Y., Liu, J.Y., 2000. Immunotherapy of tumors with xenogeneic endothelial cells as a vaccine. Nat. Med. 6, 1160.

Willam, C., Koehne, P., Jurgensen, J.S., Grafe, M., Wagner, K.D., Bachmann, S., Frei, U., Eckardt, K.U., 2000. Tie2 receptor expression is stimulated by hypoxia and proinflammatory cytokines in human endothelial cells. Circ. Res. 87, 370.

Winter, G., Griffiths, A.D., Hawkins, R.E., Hoogenboom, H.R., 1994. Making antibody by phage display technology. Annu. Rev. Immunol. 12, 433.

Yeh, C.H., Peng, H.C., Huang, T.F., 1999. Cytokines modulate integrin alpha(v)beta(3)-mediated human endothelial cell adhesion and calcium signaling. Exp. Cell Res. 251, 57.