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In vitro characterisation of a monovalent and bivalent form of a fully human anti Ep-CAM phage antibody

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Abstract Antibodies to tumour-associated antigens are increasingly being used as targeting vehicles for the visualisation and for therapy of human solid tumours. The epithelial cell adhesion molecule (Ep-CAM) is an antigen that is overexpressed on a variety of human solid tumours and constitutes an attractive target for immunotargeting. We set out to obtain fully human antibodies to this antigen by selecting from a large antibody repertoire displayed on bacteriophages. Two single-chain variable antibody fragments (scFv) were identified that specifically bound recombinant antigen in vitro. One of the selected antibodies (VEL-1) cross-reacted with extracellular matrix components in immunohistochemistry of colon carcinoma, whereas the other scFv (VEL-2) specifically recognised colon cancer cells. The latter antibody was further characterised with respect to epitope specificity and kinetics of antigen-binding. It showed no competition with the well-characterised anti Ep-CAM MOC-31 monoclonal antibody and had an off-rate of 5×10^{-2} s^{-1}. To obtain an antibody format more suitable for in vivo tumour targeting and to increase the apparent affinity through avidity, the genes of scFv VEL-2 were re-formatted by fusion to a human (γ1) hinge region and CH3 domain. This "minibody" was expressed in *Escherichia coli*, specifically bound the Ep-CAM antigen and showed a 20-fold reduced off-rate in surface plasmon resonance analysis. These results show that phage antibody selection, combined with antibody engineering, may result in fully human antibody molecules with promising characteristics for in vivo use in tumour targeting.

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Introduction

During malignant transformation, a number of cellular (self-)antigens are up-regulated or mutated, providing targets for immunotherapy of human cancer. The epithelial cell adhesion molecule (Ep-CAM, also known as EGP-2, EGP40, KSA, CO17-1A antigen or GA733-2 antigen) is a type I transmembrane glycoprotein that is abundantly expressed on a variety of human carcinomas, notably on colorectal carcinoma (CRC). The protein is present on almost all cell types derived from the ectoderm and endoderm germlineages [10] and has been reported to function as an intercellular adhesion molecule [26]. Recently Ep-CAM was shown to modulate cadherin-based intercellular junctions, causing a redistribution of E-cadherin and catenins and a down-regulation of α-catenin in transfected L cells and epithelial cells [27]. This suggests a role for the antigen in tumour metastasis: increased expression of Ep-CAM may lead to a decrease of the number of cadherin-based junctions, thereby possibly destabilising intercellular contacts and promoting metastasis (for review, see [4]). In CRC, adenomas show a low, heterogeneous expression of the antigen, whereas carcinomas are found to be homogeneously positive [33], providing the antigen with an essential characteristic for use as the target in immunotargeting. In addition, it is not shed into the circulation [46], which further increases its usefulness.

Ep-CAM has been widely used as a target for immunotargeting of solid tumours. In most clinical trials, murine monoclonal antibodies have been used either for the visualisation [23, 30] or for treatment [35–37, 40] of tumours. However, the therapeutic use of most murine antibodies in humans is limited by the induction of a human anti-(mouse Ig) antibody (HAMA) [22] response. And although part of this response may consist of an anti-idiotype antibody network which is expected to

augment the therapeutic effect, the induction of this cascade has been reported not to correlate with the response of patients treated with the 17-1A anti-Ep-CAM antibody [12]. On the other hand, therapeutic strategies designed around anti-idiotype antibodies and the induction of the anti-idiotype network have been shown to induce clinical responses [9, 38, 42].

To partially overcome the problems inherent to the use of murine antibodies in humans, chimeric and humanised versions of murine antibodies to Ep-CAM have been developed [7, 50]. To date, only very few fully human antibodies have been described that recognise the native antigen [5, 20].

Therefore, to improve on existing modalities for antibody targeting of Ep-CAM-expressing solid tumours, we have used the power of phage display to select fully human antibodies to the antigen. From a large, non-immune single-chain Fv (scFv) phage antibody repertoire [49], two clones were selected that recognised recombinant antigen in vitro. However, one of these antibodies also reacted with extracellular matrix components in immunohistochemistry of colon carcinoma, which made the antibody unsuited for in vivo use. The second antibody specifically bound the antigen in several in vitro tests, but showed a relatively fast dissociation rate in kinetic analysis. Therefore, to improve the apparent affinity by means of avidity and to obtain a suitable antibody format for in vivo use, the genes of scFv VEL-2 were re-formatted as a minibody. This fully human VEL-2 minibody is shown to be a promising candidate for further evaluation as a tumour-targeting vehicle.

Materials and methods

Escherichia coli strain

The *E. coli* strain TG1: K12,Δ*(lac-pro)*, *supE*, *thi*, *hsdD5*/F′ *traD*36, $proA^{+}B^{+}$, $lacI^{q}$, *lacZ*ΔM15 was used.

Phage antibody selection on recombinant Ep-CAM

A large phage antibody repertoire in scFv format [49] was used for the selection. The library was rescued with helper phage M13K07 and phages were panned for binding to antigen. Two selection methods were used: panning on antigen coated in immunotubes [28] or panning on biotinylated antigen in solution (100 nM concentration) followed by retrieval on streptavidin-coated magnetic beads, as described [14]. Three selection rounds were performed on purified, recombinant, baculovirus-expressed Ep-CAM [47] (a kind gift of Prof. D. Herlyn, the Wistar Insitute, Philadelphia, Pennsylvania, USA). After the second and third round of selection, individual clones were tested for binding to antigen in enzyme-linked immunosorbent assays (ELISA).

ELISA and competition ELISA

Individual bacterial clones were picked and expression of soluble scFv fragments was induced by activation of the upstream *LacZ* promotor with isopropyl β-D-thiogalactopyroside (IPTG) as described [28]. The assay is further described by Roovers et al. [39]. Recombinant minibody was detected with a polyclonal antiserum to human Fc regions (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA; 0.1% v/v) and peroxidase-conjugated rabbit anti-(goat-Ig) immunoglobulins (Dako, Glostrup, Denmark; 0.1% v/v). Staining was performed as described [39].

For competition experiments, individual clones were rescued with helper phage M13K07 as described [28]. Phages expressing the respective scFv were then mixed with a tenfold molar excess of MOC31 monoclonal antibody [45] or an irrelevant control antibody (the Ki-4 anti-CD30 antibody [18]) and added to antigen-coated wells. Phages were then detected with a polyclonal sheep antiserum to fd phage (Pharmacia, Uppsala, Sweden) and peroxidase-conjugated rabbit anti-(goat-Ig) immunoglobulins (Dako). Staining was performed as described [39].

Analysis of antibody binding to cell-surface-expressed antigen by means of flow cytometry

The colorectal cancer cell line CaCo2 (ATCC number HTB37) was cultured in Dulbecco's modification of Eagle's medium (Dulbecco, Life Technologies) supplemented with 10% (v/v) fetal calf serum and 2 mM glutamine at 37 °C in a humidified atmosphere containing 5% (v/v) CO_2. Cells were harvested by a short treatment with trypsin/EDTA, washed in phosphate-buffered saline (PBS) and aliquots of approximately 500 000 cells were prepared in polystyrene FACSscan tubes (Falcon: Becton & Dickinson, Heidelberg, Germany). All incubations were carried out for 1 h at room temperature in 2% (w/v) skimmed milk powder (Marvel) in PBS, containing 0.05% (w/v) NaN_3 (2%MPBS/N_3). Three washes, each consisting of centrifugation of the cells (400 g, 3 min, room temperature) and resuspension in 1 ml 2%MPBS/N_3, were performed between every incubation step. Cells were stained with recombinant scFv fragments (approximately 100 μg/ml concentration), the 9E10 monoclonal antibody [31] directed to the C-terminal c-*myc*-derived epitope tag (0.1% v/v in 2%MPBS/N_3) and fluorescein isothiocyanate-labeled rabbit anti-(mouse Ig) immunoglobulins (Dako, Glostrup, Denmark; 2% v/v in 2%MPBS/N_3). Finally, cells were washed three times with 2%MPBS/N_3 and once with PBS, resuspended in 500 μl PBS and analysed in a FACSCalibur flow cytometer (Becton & Dickinson, Heidelberg, Germany). Data were analysed by the Cellquest software program (Becton & Dickinson, Heidelberg, Germany).

Cloning of an engineered minibody

The polymerase chain reaction (PCR) was performed using Expand High Fidelity Taq polymerase (Boehringer Mannheim, Mannheim, Germany) together with the buffer supplied by the manufacturer in a 50-μl reaction containing 1.25 mM $MgCl_2$, 250 μM each of the four different deoxyribonucleoside triphosphates (dATP, dTTP, dCTP, dGTP), 500 pM both primers: reverse (BACK) and forward (FOR), 100 pg DNA template and 0.1 U DNA polymerase. The PCR consisted of 25 cycles of 94 °C, 45 s; 55 °C, 45 s and 72 °C, 1 min 30 s, and products were analysed by agarose gel electrophoresis with ethidium bromide staining using standard techniques [41].

The human (IgG1) CH1 gene, present in vector pCES1 [13], was amplified with primers CL-ASS-BACK and Hinge-CH1-FOR (Table 1), which introduces the human IgG1 hinge region at the 3′ end of the CH1 gene. The human (IgG1) CH3 (the third constant domain of the heavy chain) gene was amplified from vector VH-Express [34] with primers Hinge-L-CH3-BACK(1) and His6-CH3-FOR (Table 1). This introduces part of the hinge region and a flexible $(Gly_4Ser)_2$ linker at the 5′ end of the CH3 gene and a region encoding a hexa-histidine (His6) tag, two stop codons and an *Eco*RI restriction site for cloning at the 3′ end of the CH3 gene. Both fragments (CH1-Hinge and Hinge-L-CH3-His6) were purified from the PCR mix with the Wizard PCR prep purification kit (Promega, Madison, USA). Approximately 75 ng of the two DNA fragments were then joined in ten cycles of splice overlap extension

Table 1 Primer sequences

Name	Sequence
CL-ASS-BACK	5′-TAA TAA GGC GCG CCC GGT GGA GGC GGT AAT TCT ATT TCA AGG AGA CAG T-3′
Hinge-CH1-FOR	5′-CCA CCG CAC GGT GGG CAT GTG TGA GTT TTG TCA CAA GAT TTG GGC TCA AC-3′
Hinge-L-CH3-BACK(1)	5′-CAC ACA TGC CCA CCG TGC GGT GGA GGC GGT TCA GGC GGA GGT GGC TCT GGG CAG CCC CGA GAA CCA CAG G-3′
His6-CH3-FOR	5′-TTC TCG ACT GAA TTC TTA TTA GTG ATG GTG ATG ATG ATG TTT ACC CGG AGA CAG GGA GAG-3′
Hinge-L-CH3-BACK(2)	5′-TTC TCG ACT GCG GCC GCA GAC AAA ACT CAC ACA TGC CCA-3′
M13-FOR	5′-CGC CAG GGT TTT CCC AGT CAC GAC-3′

PCR (SOE-PCR [16]) without primers and re-amplified by 20 cycles using outer primers (CL-ASS-BACK and His6-CH3-FOR). The re-amplified PCR product was then purified from the reaction mixture with the Wizard PCR preparation purification kit, cut with the restriction enzymes *Eco*RI and *Bst*EII and ligated into pCES1, thereby replacing the bacteriophage gene III and creating vector pCES1/CH3.

To synthesise a minibody of scFv VEL-2, the human IgG1 hinge region, linker sequence and CH3 domain, present in pCES1/CH3, were amplified with primers Hinge-L-CH3-BACK(2) and M13-FOR (Table 1). This introduces a *Not*I restriction site at the 5′ end of the hinge region and removes the first five residues (EPKSC amino acid sequence) of this domain. The PCR product was cut with restriction enzymes *Not*I and *Eco*RI and cloned into pCANTAB6 [29], creating PC6/CH3. This removes the bacteriophage gene III from the vector. Antibody V genes of VEL-2 in scFv format were excised from plasmid DNA of VEL-2/pCANTAB6 using restriction sites *Sfi*I and *Not*I and cloned into PC6/CH3, resulting in minibody VEL-2. Both vectors pCES1/CH3 and PC6/CH3 were sequenced using the dideoxy-DNA chain-termination method [43] to ensure that no mutations were introduced in the genes by amplification.

Production and purification of soluble antibody fragments

For large-scale purification, the selected scFv antibody fragment VEL-2 was re-cloned in an expression vector lacking the bacteriophage gene III (as a *Sfi*I/*Not*I fragment in pUC119-poly-HIS6MYC, a kind gift of Dr. Andrew Griffiths, MRC Laboratory of Molecular Biology, Cambridge, UK) This method ensures less toxicity to the *E. coli* host through expression of the bacteriophage gene III during induction and thus a higher yield of protein. Production of soluble scFv and minibody fragments and purification from the *E. coli* periplasmic space using immobilised metal ion affinity chromatography (IMAC) and fast protein liquid chromatography (FPLC) were performed as described for scFv by Roovers et al. [39].

Western blotting

After purification of the recombinant minibody fragment, different samples were analysed by means of sodium dodecyl sulfate polyacrylamide gel electroforesis [25]. Samples were either dissolved in reducing loading buffer (containing dithiothreitol) or in non-reducing loading buffer (lacking dithiothreitol) and loaded onto a 10% (w/v) polyacrylamide gel. Proteins were separated according to size, electroblotted to nitrocellulose by standard techniques [41], and blots were dried in air. Minibody was then revealed with a polyclonal antiserum to human Fc regions (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA; 0.1% v/v/) and peroxidase-conjugated rabbit anti-(goat Ig) immunoglobulins (Dako; 0.1% v/v). Staining was performed with diaminobenzidine/H_2O_2.

Kinetic measurement using surface plasmon resonance (SPR) with BIAcore technology

To determine the kinetics of binding of different antibody fragments, recombinant Ep-CAM [47] was covalently coupled to a CM5 sensor chip via amine coupling, resulting in an antigen surface of 900 resonance units. IMAC-purified (and, for scFv, FPLC-purified) antibody fragments were then run over the surface at high (20 µl/min) flow rate to saturation of binding. From the sensorgrams obtained, off-rates were determined by curve-fitting on the first 10 s. of the dissociation phase using the BIAevaluation (version 2) software (Pharmacia, Uppsala, Sweden).

Immunohistochemical analysis of antibody specificity

Cryosections of different tissues (normal colonic epithelium and colon carcinoma) were cut and mounted on 3-aminopropyl-2-ethoxysilane(APTS)-coated glass slides. Sections were subsequently stained with recombinant antibody fragments, an antibody to the hexahistidine tag (SeroTec, Raleigh, USA; 0.1% v/v) and peroxidase-conjugated rabbit anti-(mouse Ig) immunoglobulins essentially as described [39].

Results

Selection and in vitro characterisation of human anti Ep-CAM scFv antibodies

We set out to select fully human antibodies to the tumour antigen Ep-CAM for the purpose of immunotargeting of human cancer. To this end, we first performed phage antibody selections on recombinant antigen coated in immunotubes, using a large, non-immunised single-chain Fv (scFv) phage antibody library [49]. After three and four rounds of selection, individual clones were picked and DNA-fingerprinted as described [28]. One predominant clone was found (data not shown) and selected for further analysis. Soluble scFv was then expressed as described [17], and shown to specifically recognise recombinant antigen in ELISA (Fig. 1). However, when the antibody was used to stain cryosections of primary colorectal carcinoma, staining of fibroblasts and extracellular matrix components was also observed, apart from the (expected) epithelial cell reactivity (Fig. 2). When the epitope specificity of the scFv fragment was compared to that of the well-characterised MOC-31 antibody [45] in a competition ELISA, clone VEL-1 did not compete with this antibody (data not shown). Measurement of the kinetics of antigen binding of scFv VEL-1 was performed by surface plasmon resonance (SPR). The off-rate of the monomeric

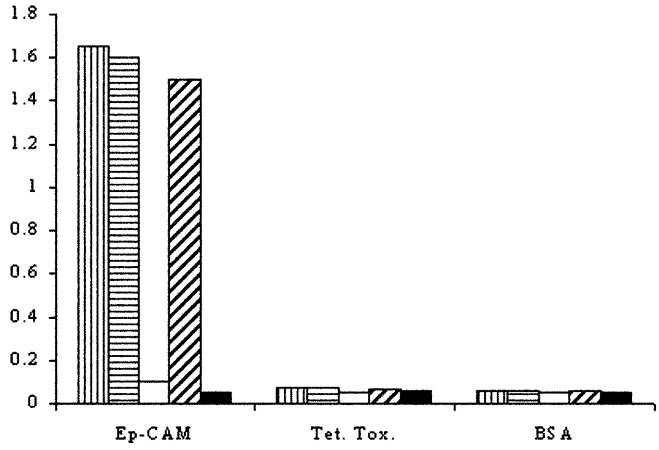

Fig. 1 Enzyme-linked immunosorbent assay showing specific binding of recombinant antibody fragments. ScFv VEL-1 (*vertically striped bars*), scFv VEL-2 (*horizontally striped bars*) and minibody VEL-2 (*diagonally striped bars*) were tested on the antigens epithelial cell adhesion molecule (*Ep-CAM*), tetanus toxoid (*Tet.Tox.*) and bovine serum albumin (*BSA*). Negative controls for scFv (*open bars*) and minibody (*solid black bars*) are also indicated

scFv was measured to be 8.4×10^{-3} s^{-1} under the conditions used (Table 2). To further characterise the epitope recognised by the VEL-1 antibody, the Ep-CAM antigen was treated with $NaIO_4$ (as described by [53]), which cleaves carbohydrate-vicinal hydroxyl groups under mild acidic pH [6]. The reactivity of scFv VEL-1 with the antigen in ELISA was slightly inhibited by $NaIO_4$ treatment (data not shown), revealing that the epitope recognised by this selected antibody fragment could be partially composed of carbohydrate. This may explain (part of) the cross-reactivity with different cell types and extracellular stroma, observed in immunohistochemical analysis (Fig. 2).

In an attempt to overcome the problem of selecting antibodies to cross-reactive epitopes of the Ep-CAM antigen, which may be caused by the partial denaturation of the antigen during coating of the immunotubes, a different selection procedure was chosen. Selections were performed on biotinylated antigen in solution with retrieval on streptavidin-coated magnetic beads [14]. Again, one predominant clone was found after three rounds of selection, as determined by DNA fingerprint analysis (data not shown). This scFv (VEL-2) was also shown to specifically recognise the Ep-CAM antigen in ELISA (Fig. 1). In addition, it specifically stained

Table 2 Characteristics of the binding kinetics of selected anti-epithelial cell adhesion molecule (anti-Ep-CAM) antibodies. $t_{1/2}$ was calculated as $t_{1/2} = \ln 2/k_{off}$

Antibody:	$10^3 \times k_{off} \pm$ SE (s^{-1})	$t_{1/2}$ (s)
VEL-1 scFv	8.4 ± 0.145	83
VEL-2 scFv	50 ± 4	14
Minibody (VEL-2)	2.2 ± 0.3[a]	315
MOC-31 scFv	0.34 ± 0.05	2,039

[a] Apparent (bivalent) dissociation rate constant

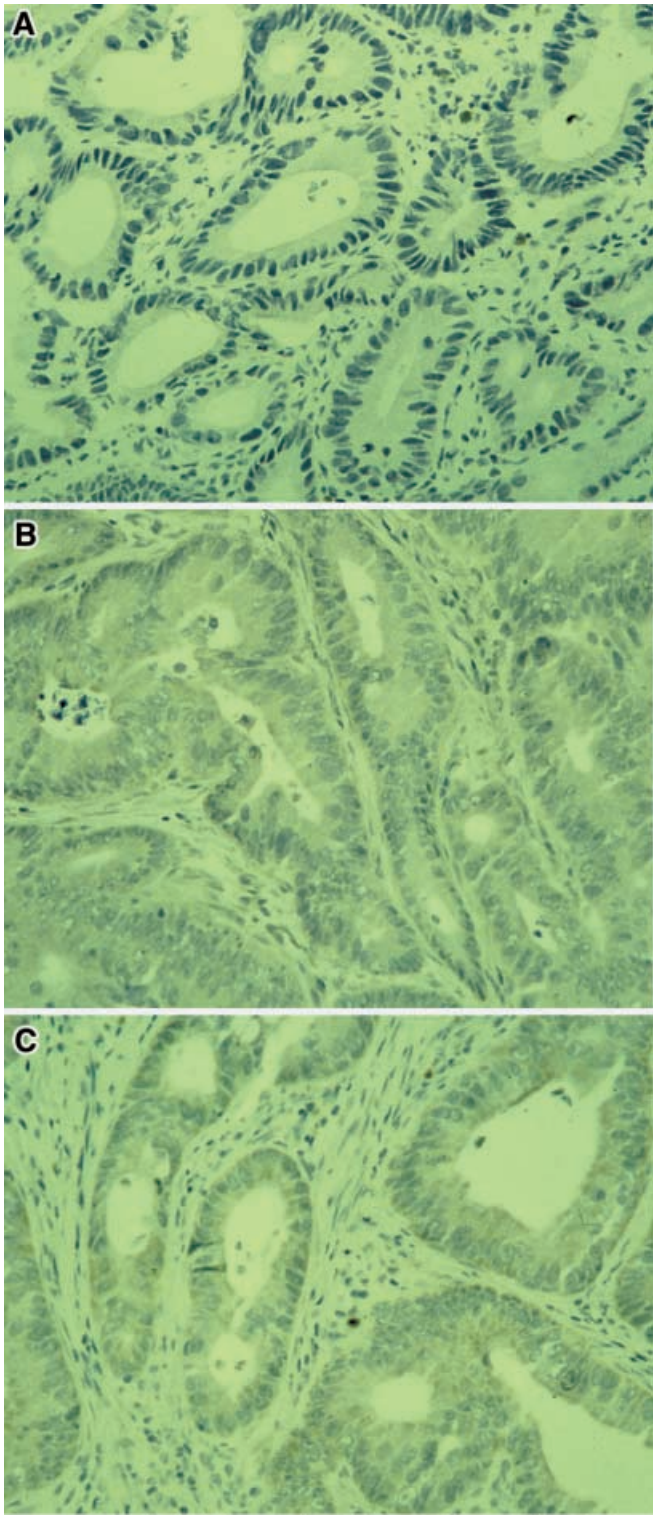

Fig. 2A–C Immunohistochemical analysis of selected human antibody fragments. Staining of primary colorectal carcinoma with (**A**) an irrelevant scFv as negative control, (**B**) clone scFv VEL-1 and (**C**) clone scFv VEL-2. Bound antibody fragments were detected with the 9E10 antibody and peroxidase-conjugated rabbit anti-(mouse Ig) immunoglobulins

Ep-CAM-positive epithelial tumour cells in immunohistochemical analysis of primary colorectal carcinoma; it did not show the cross-reactivity observed with scFv VEL-1 (Fig. 2). However, the off-rate of the monovalent scFv was higher than that of the VEL-1 antibody: $5.0 \times 10^{-2}\ s^{-1}$ (Table 2). For comparison: the off-rate of the high-affinity monovalent MOC31 scFv has been reported to be $3.4 \times 10^{-4}\ s^{-1}$ [39]. As was determined for the VEL-1 antibody, the VEL-2 scFv did not cross-react with the MOC-31 antibody in competition ELISA (data not shown). In addition, the reactivity of the VEL-2 antibody was not affected by $NaIO_4$ treatment of the Ep-CAM antigen (data not shown), showing that the epitope recognised is most probably composed of a protein sequence. The amount of scFv purified from *E. coli* was measured to be between 100 μg/l and 200 μg/l bacterial culture.

Sequencing of the V genes of both VEL-1 and VEL-2 and alignment of the genes to their closest germile V segment, using V base (http://www.mrc-cpe.cam.ac.uk/imt-doc/restricted/DNAPLOT.html) revealed that they originated from commonly used variable heavy-chain domian (VH) and variable light-chain domain (VL) gene families; the VH and VL genes of scFv VEL-1 are both derived from the largest VH (VH3) and Vλ (Vλ3) V gene families (Table 3). Only a few mutations were found in the non-primer-encoded VH gene segment and the Vλ gene showed no mutations at all (Table 4). Mutations in the VH gene were largely confined to the CDR1, except for two mutations in FR3 (E-(85) → G and K-(94) → R). The observed mutations in Vλ-CDR3 are probably due to the J segment usage and junctional diversity (Tables 3, 4). The V genes of scFv VEL-2 belonged to the VH1 and Vλ3 V gene families (Table 3). Strikingly, the VH gene of scFv VEL-2 is different from that of scFv VEL-1, but the VL gene shows a marked homology to that of scFv VEL-1. Both VL genes are derived from the same germline Vλ segment and show no mutations compared with this segment, but have a different CDR3 (Table 4). In addition, both VL genes also use the same J segment (Jλ2/Jλ3a: Table 3). The VH gene of scFv VEL-2 also shows no mutations compared with the germline V segment (Table 4).

Finally, both scFv antibodies were tested for their ability to bind to cell-surface-expressed antigen by means of flow cytometry, and only scFv VEL-2 was shown to recognise the Ep-CAM-positive CRC cell line CaCo2 (Fig. 4). This made VEL-2 the only antibody suitable for development as vehicle to target Ep-CAM-positive malignancies.

Table 3 Germline segment usage of selected anti Ep-CAM antibodies. NA not applicable

Antibody	V gene	V gene family	V segment used	D segment used	J segment used
VEL-1	VH	VH3	DP-47/V3-23	D6-25	JH3a
VEL-1	VL	Vλ3	DPL16/VL3.1	NA	Jλ2/Jλ3a
VEL-2	VH	VH1	DP-7/21-2	D6-19	JH3b
VEL-2	VL	Vλ3	DPL16/VL3.1	NA	Jλ2/Jλ3a

Synthesis and characterisation of a bivalent minibody of the anti-Ep-CAM scFv VEL-2

To increase the apparent affinity of the specific anti-Ep-CAM scFv VEL-2) through avidity and to obtain a suitable antibody format for use in in vivo targeting of tumours, the minibody format [19] was chosen. In this format, the scFv cassette is genetically linked to a human hinge region, a flexible peptide linker and a human CH3 domain that causes dimerisation of the monovalent scFv moiety. In addition, it causes an increase of the molecular mass to approximately 90 kDa,

Table 4 V gene-segment alignments of selected V -genes and their closest germline match. Primer-encoded sequences are indicated with lower-case letters

1. VH sequences:

```
                        FR1                         CDR1       FR2                 CDR2                          FR3                            CDR3           FR4
            ------------------------------ ------- -------------- ------------------- -------------------------------- -------------- ----------
                     1         2         3           4         5          6          7         8           9
            123456789012345678901234567890 1ab2345 67890123456789 012abc3456789012345 67890123456789012abc345678901234

DP-47/V3-23 evqllesgGGLVQPGGSLRLSCAASGFTFS S--YAMS WVRQAPGKGLEWVS AISG--SGGSTYYADSVKG RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK
VEL-1 VH    q...v......................... .--FG.H .............. ...--.............. ......................G........R DWRAVATRGGYGMDV WGQgttvtvs

DP-7/21-2   qvqlvqsgAEVKKPGASVKVSCKASGYTFT S--YYMH WVRQAPGQGLEWMG IINP--SGGSTSYAQKFQG RVTMTRDTSTSTVYMELSSLRSEDTAVYYCAR
VEL-2 VH    .............................. .--.... .............. ...--.............. ................................ GYNSAFDI        .....l....
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2. Vλ sequences

```
                     FR1                 CDR1           FR2          CDR2                 FR3                       CDR3      FR4
             ---------------------- -------------- --------------- ------------ ---------------------------------- ----------- -----------
                                 2      3             4          5            6         7         8
             1234567891234567890123 45678901abc234 567890123456789 01abcde23456 789012345678ab9012345678901234567 8 9012345ab

DPL16/VL3.1  sseltqdPAVSVALGQTVRITC QG-DS-LRSY-YAS WYQQKPGQAPVLVIY GK-----NNRPS GIPDRFSGSSSG--NTASLTITGAQAEDEADYYC NSRDSSGNH
VEL-1 VL     ...................... ..-..-....-... ............... ..-----..... ............--.................... .....T.HPRVL FGGgtkltvlg
VEL-2 VL     ...................... ..-..-....-... ............... ..-----..... ............--.................... ........VV  ...........
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Fig. 3 Features of minibody VEL-2. Nucleotide sequence and amino acid translation of the engineered scFv-Hinge-L-CH3 region and C-terminal region of the CH3 domain. The construct contains a C-terminal hexahistidine tag for immobilised metal ion affinity chromatography (IMAC)

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                                        NotI
--VL gene-- ACC GTC CTA GGT GCG GCC GCA GAC AAA ACT CAC ACA TGC CCA CCG TGC
              T   V   L   G   A   A   A   D   K   T   H   T   C   P   P   C
             --VL gene--                                --IgG1 Hinge--

GGT GGA GGC GGT TCA GGC GGA GGT GGC TCT GGG CAG CCC CGA --IgG1 CH3 gene--
 G   G   G   G   S   G   G   G   G   S   G   Q   P   R
            --Linker--                --CH3 gene--

                                                    EcoRI
--- CCG GGT AAA CAT CAT CAT CAC CAT CAC TAA TAA GAA TTC --------------
     P   G   K   H   H   H   H   H   H   *   *
  --CH3 gene--   --Hexahistidine Tag--    Stop
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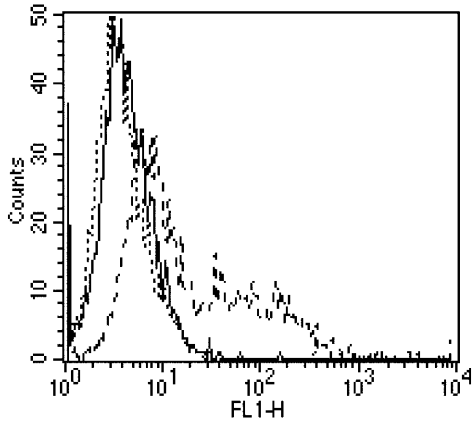

Fig. 4 Flow-cytometric analysis of antibody binding to the colorectal carcinoma cell line CaCo2. Number of events (*counts*) as a function of the fluorescence intensity of the cells (*FL1-H*). —— Negative control, ··· scFv VEL-1, - - - - - scFv VEL-2

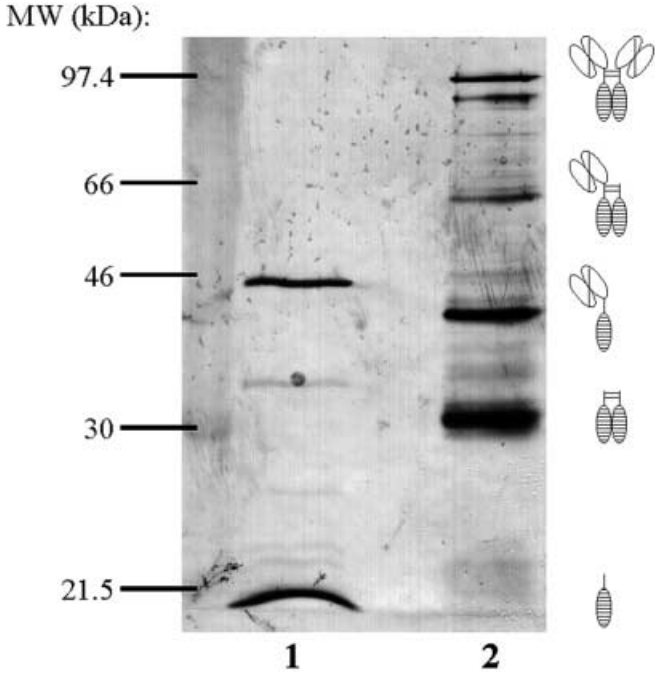

Fig. 5 Analysis of minibody expression by means of Western blotting. IMAC-purified VEL-2 minibody was loaded onto a 10% (w/v) polyacrylamide gel in either reducing (*1*) or non-reducing (*2*) loading buffer. Proteins were separated according to size, blotted to nitrocellulose and revealed by means of a polyclonal goat antiserum to human Fc regions, peroxidase-conjugated rabbit anti-(goat Ig) immunoglobulins and staining with diaminobenzidine/H_2O_2. Molecular mass markers are indicated on the *left*. Antibody formats corresponding to the respective bands are depicted on the *right*. *Open ovals*: variable heavy and variable light domains of scFv moiety, *horizontally striped ovals* CH3 domains

which is a suitable size for radio-immunoimaging with the commonly used ^{99m}Tc radionuclide [51]. The gene for VEL-2 scFv was linked to an engineered human IgG1 hinge region gene that was truncated at the 5′ end, as described by Alt et al. [3]. In contrast to the sequence originally described by Hu et al. [19], the sequence encoding the first five amino acid residues, EPKSC, were omitted to avoid the presence of an unpaired cysteine in the final construct (Fig. 3). In addition, three extra alanine residues were introduced between the C terminus of the VL domain and the N terminus of the hinge region, to encode the *Not*I restriction site. Expression of this cassette in *E. coli* resulted in dimeric molecules of the expected molecular mass (±90 kDa), as determined by Western blotting using a polyclonal anti-human-Fc(γ)-specific serum (Fig. 4). Under reducing conditions, a 45-kDa band was found, corresponding to the scFv-hinge-linker-CH3 cassette and a 15-kDa band, corresponding to free CH3 domains. This indicated that proteolytic cleavage occurred in the linker between the scFv moiety and the CH3 domain. Under non-reducing conditions, several bands were found that corresponded to various degradation products of the minibody (Fig. 5). Most probably because of its increased size, the expression level of minibody VEL-2 was only between 50 μg/l and 100 μg/l bacterial culture. Minibody VEL-2 bound avidly to antigen, as demonstrated by the off-rate of this antibody format, which was reduced by a factor of approximately 20 compared with the parental scFv (2.2×10^{-3} s^{-1} compared with 50×10^{-3} s^{-1}; Table 2) in SPR analysis. As expected, it bound specifically to the Ep-CAM antigen in ELISA (Fig. 1) and immunohistochemistry, showing a staining pattern comparable to that of the VEL-2 scFv (Fig. 2). Furthermore, by using a human Fc-specific antiserum as detecting antibody in ELISA, the physical link between the scFv moiety and the Fc tail (CH3 domains) could be demonstrated (Fig. 1). As was shown for the scFv, minibody VEL-2 also recognised cell-surface-expressed Ep-CAM antigen, as demonstrated by flow-cytometric analysis using the CaCo2 cell line (data not shown).

Discussion

To improve on existing modalities for immunotargeting of solid tumours expressing the Ep-CAM antigen, we have used phage display to select fully human antibody fragments specific for the antigen. Two different selection procedures were used to obtain anti Ep-CAM antibodies; whereas panning on coated antigen in immunotubes only resulted in an antibody that cross-reacted with extracellular matrix components, selection on biotinylated antigen in solution did result in a specific anti Ep-CAM scFv. Since this scFv antibody showed a relatively fast rate of dissociation from antigen and since the scFv antibody format is not optimal for use in in vivo tumour targeting, the genes of scFv VEL-2 were reformatted into the minibody structure. This engineered minibody was expressed in *E. coli*, showed specific binding to antigen in several in vitro tests and had a markedly lower off-rate than that of the parental scFv. Therefore, this fully human minibody is a promising candidate for further evaluation as a tumour-targeting vehicle.

In agreement with a recent report [20], we were unable to select an antibody that was truly specific for the Ep-CAM antigen by panning a large phage antibody library [49] on coated recombinant antigen in immunotubes. This selection procedure may have the disadvantage of selecting antibodies directed to otherwise cryptic epitopes of an antigen that may become exposed when the immunotubes are coated with the protein. In this respect it is noteworthy that the VEL-1 and VEL-2 scFv antibodies differ mostly in their VH domains and use very similar VL domains (Tables 3, 4). This emphasises the importance of the VH domain for the exact epitope specificity, a phenomenon that has also been noted by others [5].

It is surprising that neither the VEL-1 nor the VEL-2 antibody compete with the MOC-31 murine anti Ep-CAM antibody. Most of the well-characterised anti Ep-CAM antibodies made via the hybridoma technology (e.g. 17-1A [15]; 323/A3 [8] and MOC-31 [45]) all recognise an overlapping epitope on the antigen that is apparently immunodominant in vivo, but is not targeted during in vitro phage antibody selection. However, this possibility of selecting antibodies directed to non-immunogenic epitopes of an antigen is a known feature of the phage display technology [11, 52] and is not uncommon. Whether the epitope specificity of the anti Ep-CAM antibody VEL-2 will have an effect on its in vivo targeting properties remains to be determined.

The off-rates of the scFv fragments we measured are typical for phage antibodies selected from large, non-immunised repertoires [13, 44, 49], except for the VEL-2 scFv (Table 2). It may seem surprising that this scFv was selected, despite of its relatively fast off-rate. This may be explained by multivalent expression on phage, even in the phagemid system used, which compensates the fast off-rate through rebinding (avidity).

Since there is a clear correlation between the off-rate of an antibody and its in vivo tumour targeting [1] performance and since differences in off-rate measured by SPR can be correlated with different retention times on the surface of tumour cells [1], we decided to improve the apparent affinity of scFv VEL-2 for the Ep-CAM antigen by increasing the valency of the molecule. Indeed, the off-rate of the VEL-2 minibody was approximately 20-fold lower than that of the VEL-2 scFv (Table 2). This effect of bivalency is in good agreement with what has been reported for the C6.5 scFv antibody directed to the c-erB-2 antigen [2], but is larger than the avidity effect reported for triabodies [21, 24] (for review, see [48]). Although this effect of multivalency heavily depends on the particular antibody used and on the density of the target antigen on the cell surface, the flexibility of both antigen-binding sites in a minibody will increase the likelihood of one arm rebinding when the other has dissociated from antigen, which may partially explain these contradictory data.

As judged by Western blotting using non-reducing conditions, not all of the expressed minibody was disulfide-bridged (Fig. 4), but the majority (approximately 60%–70%) of the product contained one or two S-S bridges between the two chains. Proteolysis of the minibody was evident and was mostly confined to the flexible linker between the scFv moiety and CH3 domain (Fig. 4). This constitutes a problem for the synthesis of protein to be used in in vivo tumour-targeting studies. However, this may be solved by choosing a protease-deficient *E. coli* strain for expression, or by expressing the genes in a suitable eukaryotic expression system [19].

The different epitope specificity of the VEL-2 antibody compared with that of most of the murine anti Ep-CAM antibodies offers possibilities for the design and engineering of targeting molecules directed to the Ep-CAM antigen. We have recently reported the successful cloning and humanisation of the murine MOC-31 antibody [5, 39]. The combination of both of these specificities into one molecule to develop a chelating antibody or "CRAb" [32] would significantly enlarge the targeting potential of these molecules.

We conclude that minibody VEL-2 is a promising candidate for further evaluation as a tumour-targeting vehicle. It is an engineered form of one of the first fully human anti Ep-CAM antibodies described to date and it combines an antibody format suitable for in vivo tumour targeting with greater affinity than the parental scFv.

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