Human/mouse cross-reactive anti-VEGF receptor 2 recombinant antibodies selected from an immune b9 allotype rabbit antibody library

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Abstract

Vascular endothelial growth factor (VEGF) and its receptors have been implicated in promoting solid tumor growth and metastasis via stimulating tumor-associated angiogenesis. Models of murine tumor angiogenesis and receptor-specific antibodies are required to evaluate roles of VEGF receptors in mouse models of human cancer. Human VEGFR2 (also known as KDR) and murine VEGFR2 (or Flk-1) share 85% amino acid sequence identity in their extracellular domain. We describe here the development of antibodies that cross-react with mouse and human VEGFR2. High-affinity, species cross-reactive, Fabs specific for KDR/Flk-1 were selected from an antibody phage display library generated from an immunized rabbit of b9 allotype. The selected chimeric rabbit/human Fabs were found to bind to purified KDR and Flk-1 with nanomolar affinity. Three of the selected Fabs detected KDR expression on human endothelial cells as well as Flk-1 on murine endothelial cells. The availability of anti-VEGFR2 Fab with species cross-reactivity will help to decipher the functional role of KDR/Flk-1 in tumor biology as well as facilitate the preclinical evaluation of the

Abbreviations: Flk-1, fetal liver kinase receptor 1 (or murine VEGFR2); Flt-1, fms-like tyrosine kinase receptor 1 (or human VEGFR1); HRP, horseradish peroxidase; HMEC-1, human microvasculature endothelial cells; HUVEC, human umbilical vein endothelial cells; KDR, kinase insert domain-containing receptor (or human VEGFR2); $K_a$, equilibrium dissociation constant; $k_{on}$, association rate constant; $k_{off}$, dissociation rate constant; mAb, monoclonal antibody; mFlt-1, fms-like tyrosine kinase receptor 1 (or murine VEGFR1); RT, room temperature; RU, reference units; VEGF, vascular endothelial growth factor; $V_H$ and $V_L$, the variable domain of antibody heavy and light chain, respectively.

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suitability of KDR/Flk-1 for drug targeting. This report underscores our earlier finding that b9 rabbits are excellent sources for high-affinity cross-reactive antibodies with therapeutic potential.

Keywords: Antibody engineering; Phage display; Angiogenesis; VEGF/KDR/Flk-1; Endothelial cells

1. Introduction

Angiogenesis is a highly regulated and complex process that results in formation of new blood vessels from preexisting vasculature. Collective experimental and clinical studies strongly suggest that uncontrolled angiogenesis is a major contributor to chronic diseases such as cancer (tumor growth and metastasis), diabetic retinopathy, rheumatoid arthritis, and psoriasis (Folkman, 1995). Vascular endothelial growth factor receptors and their ligand (VEGF) may promote tumor-associated angiogenesis and lead to solid tumor growth and metastasis (Ferrara et al., 2003). VEGF exerts its effects via two high-affinity tyrosine kinase receptors called VEGFR1 (or \textit{fms}-like tyrosine kinase, Flt-1) and VEGFR2 (also known as kinase insert domain-containing receptor or KDR in humans, and fetal liver kinase receptor 1 or Flk-1 in mice) (Ferrara, 1999). Of these two receptors, VEGFR2 appears to be responsible for transduction of the majority of VEGF signals in endothelial cells that lead to proliferation, migration, differentiation, tube formation and increased vascular permeability, and also for transduction of signals that contribute to vascular integrity (Neufeld et al., 1999; Zhu et al., 2002). Furthermore, inhibition of angiogenesis and the resultant tumor growth has been achieved by using specific agents that interrupt either the VEGF/VEGFR2 interaction or that block the VEGFR2 signal transduction pathway (Zogakis and Libutti, 2001; Margolin, 2002). Such blocking agents include soluble receptor constructs, antisense RNA strategies, RNA aptamers to VEGF, and low molecular weight VEGF receptor inhibitors (Siemeister et al., 1998). Work has also been done with inhibitory antibodies to VEGF (Kim et al., 1993; Yuan et al., 1996; Margolin et al., 2001) or VEGFR2 (Zhu et al., 1998; Prewett et al., 1999; Jendreyko et al., 2003). Inhibition of VEGFR2-mediated signal transduction represents an excellent approach for arresting angiogenesis in cancer.

Monoclonal antibodies (mAbs) are a novel class of potentially therapeutic angiogenesis inhibitors (Glennie and Johnson, 2000; Yancopoulos et al., 2000). With recent progress in molecular engineering of mAbs and the ability to produce human mAbs from transgenic mice and antibody libraries, a variety of chimeric, humanized, and fully human mAbs with desired biological properties can be readily produced (Hoogenboom and Chames, 2000; Rader, 2001). Although tumor-associated blood vessels in patients appear to be an attractive target, the potential for antibodies in antiangiogenic therapy has not been fully explored. In preclinical models, based on human tumors xenografted onto immunodeficient mice, newly formed blood vessels in the transplanted tumor originate from the host, and conventional mouse anti-human mAbs typically do not react with cell surface receptors displayed by mouse endothelial cells. In fact, the lack of cross-reactivity of humanized mouse mAbs directed to human integrin \(\alpha_v\beta_3\), human VEGFR2, and human VEGF with the mouse antigen has been a major obstacle in their preclinical development (Klohs and Hamby, 1999).

The rabbit antibody repertoire has been used in diagnostic applications for decades in the form of polyclonal antibodies. Rabbit antibodies are an attractive alternative to the mouse antibody repertoire for the generation of mAbs to both murine and human antigens. Significantly, rabbits are evolutionarily quite distant from mice. Rabbits (\textit{Oryctolagus cuniculus}) belong to the family of Leporidae, in the order Lagomorpha whereas mice are part of the large and diverse order Rodentia. As we have demonstrated recently, rabbit mAbs selected from antibody libraries by phage display can be humanized while retaining both high specificity and strong affinity to the human antigen (Rader et al., 2000; Steinberger et al., 2000). In contrast, humanized and human antibodies that are derived from immune mice (either indirectly through humanization or directly through transgenic mice...
containing human immunoglobulin loci) are negatively selected against epitopes displayed by the mouse antigen and, thus, typically lack cross-reactivity (Rader, 2001). Rabbit mAbs that cross-react with human and mouse antigens are of particular relevance for the preclinical evaluation of therapeutic antibodies in mouse models of human diseases.

The antibody diversity generated by \( \text{V}_{\text{H}}\text{DJ}_{\text{H}} \) rearrangements in rabbits is more limited than in mice and humans, because only 1 out of >50 functional \( \text{V}_{\text{H}} \) gene segments, \( \text{V}_{\text{H}}\text{1} \), is predominantly used. In contrast to the limited \( \text{V}_{\text{H}}\text{DJ}_{\text{H}} \) rearrangements, \( \text{V}_{\text{K}}\text{J}_{\text{K}} \) rearrangements in rabbits are much more diverse and, thus, the resulting rearranged kappa light chain genes may compensate for the limited diversity of rearranged heavy chain genes (Sehgal et al., 1999). In contrast to mice and humans, which have only one kappa light chain isotype, rabbits have two, K1 and K2 (Akimenko et al., 1984; Benammar and Cazenave, 1982), including highly diverse allelic variants of K1 (b4, b5, b6, and b9 allotypes). In normal rabbits, ~70–90% of the expressed antibodies are of the K1 isotype. Most K1 light chains have an unusual disulfide bridge that joins the variable and constant domains, usually through cysteines at positions 80 and 171 (McCartney-Francis et al., 1984). Exceptions are Basilea mutant rabbits that cannot express the K1 isotype and rabbits of the wild-type parental b9 allotype where a cysteine at position 108 in framework region 4 of the variable kappa light chain domain can substitute for cysteine 80. The genes of bas and b9 wild-type rabbits are evolutionarily close and frequently express kappa light chains that do not encode cysteine 80. This is of particular interest for the generation of chimeric antibodies consisting of rabbit variable and human constant domains, which is an established format for the selection of rabbit mAbs and their subsequent humanization by phage display (Rader et al., 2000; Steinberger et al., 2000; Popkov et al., 2003). The fusion of rabbit variable kappa light chain domains containing cysteine 80 to the human kappa light chain constant domain, which does not provide a matching cysteine, results in a free thiol group that is likely to be disadvantageous for the expression of antibody fragments in \( \text{E. coli} \) (Schmiedl et al., 2000). This is also a disadvantage for the selection of antibody fragments from phage display libraries. Indeed, we have recently demonstrated that rabbit immune repertoires with higher expression of kappa light chains that do not encode cysteine 80 are a superior source in terms of selectable diversity (Popkov et al., 2003).

We previously selected a rabbit/human chimeric anti-KDR Fab from immune rabbits and demonstrated that, when expressed as an intrabody in the endoplasmatic reticulum of human endothelial cells, this antibody exhibited strong antiangiogenic activity (Jendreyko et al., 2003). The antibody does not, however, recognize Flk-1, the mouse homologue of human KDR; therefore, it is not a suitable candidate for preclinical evaluation of the anti-VEGFR2 intrabody-based strategy in mouse models. Here we describe the selection of human/mouse cross-reactive anti-VEGFR2 chimeric rabbit/human Fabs derived from a b9 allotype rabbit immune repertoire. The selected Fabs were characterized for binding to both purified recombinant and naturally expressed KDR/Flk-1 receptors.

2. Materials and methods

2.1. Reagents

The lyophilized recombinant fusion proteins KDR/Fc (320 kDa), Flk-1/Fc (320 kDa), Flt-1/Fc (246 kDa), and mFlt-1/Fc (400 kDa), which contain the extracellular domain of human or murine VEGF receptors fused to human IgG1 Fc via a polypeptide linker, and recombinant human VEGF (42 kDa) were purchased from R&D Systems (Minneapolis, MN). Goat anti-KDR and goat anti-Flk-1 polyclonal antibodies and biotinylated goat anti-human VEGF polyclonal antibodies were also purchased from R&D Systems. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, Fc fragment specific polyclonal antibodies, and FITC-conjugated donkey anti-goat, anti-human, and anti-rabbit IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA). Goat anti-human Fab polyclonal antibodies were from Bethyl Laboratories (Montgomery, TX). Goat anti-human kappa light chain polyclonal antibodies were from Pierce (Rockford, IL). HRP-conjugated rat anti-HA mAb 3F10 was from Roche Molecular Biochemicals (Mannheim, Germany). HRP-conjugated goat anti-human kappa light chain polyclonal antibodies
were from Southern Biotechnology Associates (Birmingham, AL). HRP-conjugated strepavidin was from Zymed Laboratories (South San Francisco, CA).

2.2. Cell lines

Primary cultured human umbilical vein-derived endothelial cells (HUVEC) were purchased from BioWhittaker (Walkersville, MD) and maintained in EGM complete medium supplemented with bovine brain extract (BioWhittaker). Immortalized human microvasculature endothelial cells (HMEC-1) derived from foreskin, pulmonary, and hepatic endothelium were developed by Drs. Edwin Ades, Francisco J. Candal, and Thomas Lawley (Ades et al., 1992) and obtained from CDC/Emory University. Cells were maintained in EBM basal medium supplemented with 10 ng/ml epidermal growth factor, 1 μg/ml hydrocortisone, 10% FCS, and antibiotics (BioWhittaker). SV40 transformed, mouse endothelial pancreatic islet cell line MS1 (MILE SVEN1) was purchased from American Type Culture Collection and maintained in DMEM supplemented with 4 mM l-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 1 mM sodium pyruvate, 10% FCS, and antibiotics.

2.3. Rabbit immunization

Two rabbits from the New Zealand White (NZW) laboratory strain and one rabbit of b9 allotype (from an NIAD/NIH allotype-defined colony) were immunized. Each NZW rabbit received an initial immunization with a complex of equimolar amounts of KDR and VEGF (7.5 μg KDR and 1 μg VEGF) that had been incubated at 37 °C for 30 min immediately before emulsification with Ribi adjuvant (MPL+TDM+CWS in PBS) according to the manufacturer’s instructions (Ribi Immunochem Research, Hamilton, MT). The b9 allotype rabbit immunization was done with Flk-1 emulsified in RIBI adjuvant as described above (20 μg per immunization and boost). A total of 1 ml was distributed in four subcutaneous sites on the back. After the initial immunization, three identical boosts were given at 3-week intervals. Antisera from immune rabbits were analyzed for binding to the immunogens by ELISA using HRP-conjugated goat anti-rabbit IgG, Fc fragment specific polyclonal antibodies for detection. For antibody detection in the rabbit serum, Costar 3690 96-well ELISA plates (Corning, Acton, MA) were coated with 100 ng of antigen (KDR, Flk-1, human IgG, or BSA) in 25 μl of PBS and incubated overnight at 4 °C. After blocking with 150 μl of 3% BSA in TBS for 1 h at 37 °C, 50 μl of a 1:10,000 dilution of rabbit serum was added into each well and the plates were incubated for 2 h at 37 °C. To eliminate the binding to the human IgG1 Fc part of recombinant Fc fusion proteins, 2.5 mg/ml human IgG (Pierce) was added to the diluted serum. Washing and detection were performed essentially as described (Barbas et al., 2001) using HRP-conjugated goat anti-rabbit IgG, Fc fragment specific polyclonal antibodies (diluted 1:5000 in 1% BSA/TBS).

2.4. Library generation and selection

Five days after the final boost, spleen and bone marrow from both femurs of the immune rabbits were harvested separately and used for total RNA preparation and first strand cDNA synthesis as described (Barbas et al., 2001). Two libraries, one representing immune repertoires derived from combined individual NZW rabbits and one from the b9 allotype rabbit were generated. Detailed protocols for the generation of chimeric rabbit/human Fab libraries in the phagemid vector pComb3X are published elsewhere (Barbas et al., 2001). In brief, rabbit Vκ, Vλ, and VH encoding sequences were amplified from first strand cDNA and fused to human Cκ and CH1 encoding sequences, respectively, followed by assembly of chimeric rabbit/human light chain and Fd fragment encoding sequences and by asymmetric SfiI cloning into phagemid vector pComb3X. The reverse primers that hybridize to the Jκ region for amplification of rabbit Vκ encoding sequences eliminated the b9 wild-type cysteine at position 108. The resulting chimeric rabbit/human Fab libraries were designated NZW (NZW rabbits) and b9 (b9 allotype rabbit). For validation, approximately 20 IPTG-induced clones from each unselected library were analyzed for the expression of chimeric rabbit/human Fab by ELISA using goat anti-human IgG and goat anti-human kappa light chain polyclonal antibodies for capture and a rat anti-HA mAb (an epitope tag from pComb3X) conjugated to HRP for detection. Clones that gave a signal at least four-fold over background were defined as positive and further analyzed by DNA sequencing.
Two libraries were panned in parallel against human and mouse VEGFR2 immobilized on Costar 3690 96-well ELISA plates (Corning). Four rounds of panning (Barbas et al., 2001) were carried out in microtiter wells coated with 500 ng of KDR or Flk-1 in the first round, 250 ng in the second round, and 100 ng in the third and fourth rounds. To eliminate the selection of clones that bind to the human IgG1 Fc part of recombinant Fc fusion proteins, 2.5 mg/ml human IgG (Pierce) was added to the phage preparation during selection. After the final round of panning, several clones selected randomly from each library were IPTG-induced and analyzed for binding to 100 ng immobilized KDR, Flk-1, IgG, or BSA by ELISA using a rat anti-HA mAb conjugated to HRP for detection. Clones that bound receptors were further analyzed by DNA fingerprinting and sequencing.

2.5. DNA fingerprinting and nucleotide sequencing

The diversity of the selected Fab clones after four rounds of panning was analyzed by comparison of restriction enzyme digestion patterns (i.e., DNA fingerprints). For DNA fingerprinting, Fab encoding inserts in pComb3X were amplified by PCR, using the primers GBACK (5′ -GCC CCC TTA TTA GCG TTT GCC ATC-3′ ) and OMPSEQ GTG (5′ -AAG ACA GCT ATC GCG ATT GCA GTG-3′ ) and digested with AluI, a frequently cutting restriction enzyme with recognition sequence AG/CT (Promega, Madison, WI). The restriction fragments were separated in 4% (w/v) agarose gels and stained with ethidium bromide. Primers NEWPELSEQ and OMPSEQ (Rader et al., 2000) were used for DNA sequencing of rabbit V H and V L encoding regions, respectively, from purified phagemid DNA.

2.6. Expression and purification Fabs

Expression of Fabs from gene III fragment-depleted phagemid vector pComb3X in E. coli strain ER2537 and purification of the soluble Fab were carried out as previously described (Barbas et al., 2001).

2.7. Antigen binding of purified Fabs

Costar 3690 96-well ELISA plates (Corning) plates were coated with 100 ng of antigen (KDR, Flk-1, Flt-1, goat anti-human kappa light chain antibody, or BSA) in 25 μl of PBS and incubated overnight at 4 °C. After blocking with 150 μl of 3% BSA in TBS for 1 h at 37 °C, 50 μl of 2 μg/ml Fab solution was added into each well and the plates were incubated for 2 h at 37 °C. Washing and detection were performed essentially as described (Barbas et al., 2001) using HRP-conjugated goat anti-human kappa light chain antibody (diluted 1:5000 in 1% BSA in TBS).

To analyze the interference of the purified Fabs with VEGF binding to VEGFR2, plates were coated with 50 ng of antigen (KDR, Flk-1, or BSA) in 25 μl of PBS and incubated overnight at 4 °C. After blocking with 150 μl of 3% BSA in TBS for 1 h at 37 °C, 50 μl of 0.5 nM (20 ng/ml) of human VEGF in PBS was added into each well in the presence or absence of 50 nM (2.5 μg/ml) of control or tested Fabs and the plates were incubated for 2 h at 37 °C. After washing, the wells were incubated with 0.5 μg/ml of biotinylated goat anti-human VEGF antibody for 2 h at 37 °C. Detection was performed essentially as described (Barbas et al., 2001) using HRP-labeled strepavidin diluted 1:2500 in 1% BSA/TBS.

2.8. Flow cytometry

HUVEC, HMEC-1, and MS1 cells were washed with HEPES-buffered saline solution (HBSS; BioWhittaker), detached by mild trypsinization with 0.025% trypsin, 0.01% EDTA in HBSS (BioWhittaker), washed with PBS, and resuspended at a concentration of 10^6 cells/ml in flow cytometry buffer [1% (w/v) BSA, 0.03% (w/v) NaN3, 25 mM HEPES in PBS, pH 7.4]. Aliquots of 100 μl containing 10^5 cells were distributed into the wells of a V-bottom 96-well plate (Corning) for indirect immunofluorescence staining. Cells were stained with 5 μg/ml purified rabbit/human Fab as a primary antibody and a 1:100 dilution of FITC-conjugated goat anti-human IgG polyclonal antibodies in flow cytometry buffer as a secondary antibody. Incubation with primary and secondary antibodies was for 40 min at RT.

For detection of antibodies in the rabbit sera by indirect immunofluorescence, aliquots of 100 μl containing 10^5 cells were distributed into the wells of a V-bottom 96-well plate (Corning) and incubated
first with a 1:200 dilution of serum followed by a 1:100 dilution of FITC-conjugated donkey anti-rabbit IgG polyclonal antibodies in flow cytometry buffer. Incubation with primary and secondary antibodies was for 40 min at RT. Flow cytometry was performed using a FACScan instrument from Becton-Dickinson (Franklin Lakes, NJ).

2.9. Binding kinetics analysis of the Fab fragments

Surface plasmon resonance for the determination of association ($k_{\text{on}}$) and dissociation ($k_{\text{off}}$) rate constants for the interaction of chimeric rabbit/human Fab with VEGFR2 was performed on a BIAcore 1000 instrument (Biacore, Uppsala, Sweden). A CM5 sensor chip (Biacore) was activated for immobilization with N-hydroxysuccinimide and N-ethyl-N’-(3-dimethylaminopropyl)carbodiimide according to the methods outlined by the supplier. Recombinant KDR/Fc or Flk-1/Fc fusion protein was coupled at a low density [800–1000 resonance units (RU)] to the surface by injection of 10 to 20 µl of a 1 ng/µl sample in 20 mM sodium acetate (pH 3.5). Subsequently, the sensor chip was deactivated with 1 M ethanolamine hydrochloride (pH 8.5). Binding of chimeric rabbit/human Fab to immobilized VEGFR2 was studied by injection of Fab at five different concentrations ranging from 50 to 150 nM. PBS was used as the running buffer. The sensor chip was regenerated with 20 mM HCl and remained active for more than 30 measurements. The $k_{\text{on}}$ and $k_{\text{off}}$ values were calculated using BIA Evaluation software 3.0 (Biacore). The equilibrium dissociation constant $K_d$ was calculated from $k_{\text{off}}/k_{\text{on}}$. Data obtained from different sensor chips were highly consistent.

Pairwise epitope mapping was performed as described by Lu et al. (2003). Recombinant KDR/Fc or Flk-1/Fc fusion protein was coupled at a low density (500–750 RU). A chimeric rabbit/human Fab was first injected at high concentration (250 nM) to saturate all receptor epitopes immobilized on the chip, which was followed by injection of a second Fab. The antigen surface was regenerated over 10 min using 25 mM HCl between each new mapping. The value of the RU signal from the second Fab over the RU signal from the first Fab was used to draw an epitope map.

3. Results

3.1. Immunization and library generation

Two rabbits from the New Zealand White (NZW) laboratory strain were immunized and boosted with a complex of equimolar amounts of KDR and VEGF. One b9 allotype rabbit (from an NIAD/NIH allotype-defined colony) was immunized and boosted with Flk-1 alone. Analyses of the sera from the three immunized rabbits by ELISA and flow cytometry showed that all rabbits produced a strong cross-reactive immune response against both the KDR and Flk-1 proteins (recombinant and native) regardless of the immunogen used (Fig. 1). Chimeric rabbit/human Fab libraries in phagemid vector pComb3X were generated from cDNA derived from spleen and bone marrow RNA of the immune rabbits as described (Rader et al., 2000; Barbas et al., 2001). Tissues from two combined NZW and one b9 allotype rabbits were handled separately, resulting in two libraries. The immune repertoires were represented by a complexity of approximately $3.8 \times 10^8$ and $1.9 \times 10^8$ independent transformants, respectively (Table 1). Randomly picked independent transformants from each library were analyzed for protein expression and DNA sequence. The expression of chimeric rabbit/human Fab was analyzed by ELISA using goat anti-human Fab and goat anti-human kappa light chain polyclonal antibodies for capture and a rat anti-HA mAb conjugated to HRP for detection. DNA fingerprinting and nucleotide sequencing of several randomly picked colonies revealed no identical patterns and nucleotide sequences between different clones, indicating an excellent diversity of both libraries (data not shown).

3.2. Library selection and initial analysis of selected clones

The chimeric rabbit/human Fab libraries were selected by panning against immobilized recombinant KDR and Flk-1 proteins. After four rounds of panning, several phage clones were picked randomly and analyzed for binding to KDR and Flk-1 by ELISA using a rat anti-HA mAb conjugated to HRP for detection. The highest percentage of positive clones was obtained from the b9 library selected on KDR (95%), followed by selection on Flk-1 (90%).
Fig. 1. Immune rabbit serum binding to VEGF receptors. (A) Direct binding of a 1:10,000 dilution of pre-immune and immune serum from three rabbits to immobilized KDR and Flk-1 was measured by ELISA as described in Materials and methods. (B) Flow cytometry histograms showing the binding of a 1:200 dilution of pre-immune (dotted line) and immune rabbit sera (bold line) to human (HMEC-1 and HUVEC) and mouse (MS1) endothelial cell lines. For indirect immunofluorescence staining, cells were incubated with corresponding serum except for the control (fine line) followed by FITC-conjugated donkey anti-rabbit IgG secondary antibodies.
The percentage of anti-KDR-positive clones in the NZW library was 79%. No positive clones were obtained from the NZW library after selection on Flk-1 (Table 1). Positive clones were further analyzed by DNA fingerprinting using the restriction enzyme AluI. Among the anti-KDR positive clones from the NZW rabbit antibody library, 11 distinct fingerprints were identified (Table 2). Although all clones selected from the NZW library bound to KDR, none of them was cross-reactive with Flk-1 (Table 2). By contrast, the b9 library yielded five distinct fingerprints among anti-KDR positive and eight distinct fingerprints among anti-Flk-1 positive clones (Table 3). All clones that were selected from the b9 library on KDR bound to both KDR and Flk-1, that is, to both human and mouse VEGFR2, whereas five of the eight distinctly fingerprinted clones from the selection on Flk-1 recognized both human and mouse VEGFR2 (Table 3). The three others were found to exclusively recognize Flk-1 protein.

Table 1

<table>
<thead>
<tr>
<th>Library</th>
<th>Size</th>
<th>Number of pannings on</th>
<th>Total clones positive/analyzed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>KDR Flk-1 KDR Flk-1</td>
</tr>
<tr>
<td>NZW</td>
<td>$3.8 \times 10^8$</td>
<td>4*</td>
<td>58/73 (79) 0/40 (0)</td>
</tr>
<tr>
<td>b9</td>
<td>$1.9 \times 10^8$</td>
<td>1</td>
<td>21/22 (95) 20/22 (90)</td>
</tr>
</tbody>
</table>

* Signifies four independent selections.

Table 2

<table>
<thead>
<tr>
<th>Clone identity</th>
<th>Selected (Sequenced)</th>
<th>Binding properties</th>
<th>Flk-1</th>
<th>KDR</th>
<th>IgG</th>
<th>BSA</th>
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<tbody>
<tr>
<td>KDR selection</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1V02*</td>
<td>14 (1)</td>
<td></td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>1V09*</td>
<td>16 (2)</td>
<td></td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2V05</td>
<td>3 (1)</td>
<td></td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>1V03*</td>
<td>4 (1)</td>
<td></td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2V01*</td>
<td>1 (1)</td>
<td></td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2V02*</td>
<td>1 (1)</td>
<td></td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2V03*</td>
<td>4 (1)</td>
<td></td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2V08</td>
<td>1 (1)</td>
<td></td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
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<tr>
<td>2V07*</td>
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<td>−</td>
<td>−</td>
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<tr>
<td>2V10*</td>
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<td>−</td>
<td>+</td>
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</tr>
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<td>VC06</td>
<td>5 (2)</td>
<td></td>
<td>−</td>
<td>+</td>
<td>−</td>
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</tbody>
</table>

Flk-1 selection none

*a,b,c Clonally related chimeric rabbit/human Fabs that share an amino acid sequence identity of >95% in their variable Ig domains are assembled in three groups.

* Unsequenced clones were considered identical by DNA fingerprinting.

* Binding properties of chimeric rabbit/human Fab in supernatants of IPTG-induced clones selected on KDR (top) and Flk-1 (bottom) recombinant receptors as detected by ELISA; +, binding; −, no binding.

3.3. Cross-reactivity with human and mouse antigen

For further analysis, eight clones were chosen for Fab purification: four cross-reactive (VR01, VR05,
Fig. 2. Deduced amino acid sequence alignment of rabbit variable domains of selected clones derived from the NZW (A) and b9 (B) Fab libraries. Shown are frameworks (FRs) and CDRs of VL and VH sequences, which are marked according to Kabat et al.'s (1991) definition. GenBank accession numbers for nucleotide sequences selected from NZW and b9 libraries are AY596399–AY596420 and AY596421–AY596446, respectively.
VR08, and VR12), three KDR specific (1V09, 2V05, and VC06), and one Flk-1 specific (VR16). Fabs were produced as soluble Fab in *E. coli* and purified by affinity chromatography using goat anti-human F(ab′)2 coupled to NHS resin columns. The KDR and/or Flk-1 antigen-binding selectivities of the purified rabbit/human Fabs were determined by ELISA. As shown in Fig. 3A, all clones were found to specifically bind to VEGFR2 and not to VEGFR1 (Flt-1). Moreover, b9-derived clonally related Fabs VR01 and VR08, as well as b9 Fabs VR05 and VR12 were confirmed to bind both
human and mouse VEGFR2 (Fig. 3A). Fabs 1V09, 2V05, and VC06 bound only to KDR, and Fab VR16 bound only to Flk-1 (Fig. 3A).

The potency of the Fab to interfere with the KDR/VEGF and/or the Flk-1/VEGF interaction was determined by analysis of binding of VEGF to immobilized KDR and Flk-1. Only cross-reactive Fab VR05 blocked VEGF binding to both immobilized KDR and Flk-1 (Fig. 3B). In the presence of a 100-fold molar excess of VR05 over VEGF, binding of VEGF to KDR and Flk-1 was reduced by 43% and 58%, respectively. Fab VR16 demonstrated inhibition (41%) of VEGF binding to Flk-1, but not to KDR. None of the other six clones efficiently inhibited VEGF binding to either VEGF receptor (Fig. 3B).

3.4. Binding kinetics

The binding kinetics of various anti-KDR/Flk-1 Fabs were determined by surface plasmon resonance on a BIAcore instrument. The cross-reactive Fabs from the b9 library, VR01, VR05, VR08, and VR12, revealed a monovalent affinity to KDR of 1.2, 3, 5.5, and 72 nM, respectively (Table 4). Their affinity to Flk-1 was 0.9, 1.0, 2.4, and 1.6 nM, respectively, which arose from lower dissociation rate constants (Table 4).

3.5. Pairwise epitope mapping

BIAcore analysis was also employed to examine the topographical relationship of the epitopes defined by the Fabs. In this assay, the KDR-specific Fab was first injected onto a KDR-coated chip at a high concentration to saturate all the receptor immobilized on the chip; this was followed by injection of a second Fab. An increase in binding density (as measured by reference units, or RU) to the KDR-coated chip upon the injection of the second antibody indicated no

---

### Table 4

<table>
<thead>
<tr>
<th>Fab</th>
<th>Antigen</th>
<th>$k_{on}/10^4$ (M$^{-1}$ s$^{-1}$)</th>
<th>$k_{off}/10^{-4}$ (s$^{-1}$)</th>
<th>$K_d$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1V09 KDR</td>
<td>13.06±1.10</td>
<td>1.30±0.08</td>
<td>1.00±0.11</td>
<td></td>
</tr>
<tr>
<td>1V09 Flk-1</td>
<td>NDB</td>
<td>NDB</td>
<td>NDB</td>
<td></td>
</tr>
<tr>
<td>2V05 KDR</td>
<td>7.60±0.78</td>
<td>1.80±0.20</td>
<td>2.37±0.32</td>
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<tr>
<td>2V05 Flk-1</td>
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<td>NDB</td>
<td>NDB</td>
<td></td>
</tr>
<tr>
<td>VC06 KDR</td>
<td>4.30±0.65</td>
<td>0.63±0.08</td>
<td>1.55±0.36</td>
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</tr>
<tr>
<td>VC06 Flk-1</td>
<td>NDB</td>
<td>NDB</td>
<td>NDB</td>
<td></td>
</tr>
<tr>
<td>VR01 KDR</td>
<td>3.43±0.80</td>
<td>3.96±0.12</td>
<td>11.55±3.18</td>
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</tr>
<tr>
<td>VR01 Flk-1</td>
<td>2.24±0.57</td>
<td>8.71±0.77</td>
<td>40.35±7.62</td>
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<tr>
<td>VR05 KDR</td>
<td>9.73±1.19</td>
<td>2.96±0.32</td>
<td>3.06±0.34</td>
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</tr>
<tr>
<td>VR05 Flk-1</td>
<td>8.13±2.61</td>
<td>2.93±0.14</td>
<td>3.00±1.08</td>
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<tr>
<td>VR08 KDR</td>
<td>6.70±0.91</td>
<td>3.66±0.25</td>
<td>5.52±0.55</td>
<td></td>
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<tr>
<td>VR08 Flk-1</td>
<td>6.88±0.59</td>
<td>12.93±0.24</td>
<td>18.90±1.58</td>
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<tr>
<td>VR12 KDR</td>
<td>0.37±0.01</td>
<td>2.71±0.20</td>
<td>72.35±5.36</td>
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<tr>
<td>VR12 Flk-1</td>
<td>16.58±1.91</td>
<td>4.91±0.29</td>
<td>3.01±0.48</td>
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<tr>
<td>VR16 KDR</td>
<td>NDB</td>
<td>NDB</td>
<td>NDB</td>
<td></td>
</tr>
<tr>
<td>VR16 Flk-1</td>
<td>2.22±1.28</td>
<td>1.08±0.09</td>
<td>0.89±0.15</td>
<td></td>
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</tbody>
</table>

Association ($k_{on}$) and dissociation ($k_{off}$) rate constants were determined using surface plasmon resonance. KDR or Flk-1 was immobilized on the sensor chip. The dissociation constant ($K_d$) was calculated from $k_{off}/k_{on}$. All numbers represent the mean±S.D. of five measurements.

* NDB, no detectable binding.

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### Table 5

Epitope mapping of the various anti-KDR and anti-Flk-1 Fabs using BIAcore analysis

(A) Bottom Top Fab

<table>
<thead>
<tr>
<th></th>
<th>1V09</th>
<th>2V05</th>
<th>VC06</th>
<th>VR01</th>
<th>VR05</th>
<th>VR08</th>
<th>VR12</th>
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<tbody>
<tr>
<td>1V09</td>
<td>26</td>
<td>112</td>
<td>357</td>
<td>15</td>
<td>259</td>
<td>97</td>
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<tr>
<td>2V05</td>
<td>143</td>
<td>14</td>
<td>492</td>
<td>24</td>
<td>258</td>
<td>99</td>
<td>6</td>
</tr>
<tr>
<td>VC06</td>
<td>139</td>
<td>204</td>
<td>32</td>
<td>156</td>
<td>162</td>
<td>155</td>
<td>4</td>
</tr>
<tr>
<td>VR01</td>
<td>116</td>
<td>119</td>
<td>477</td>
<td>29</td>
<td>117</td>
<td>25</td>
<td>24</td>
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<tr>
<td>VR05</td>
<td>461</td>
<td>290</td>
<td>571</td>
<td>24</td>
<td>257</td>
<td>−25</td>
<td>−24</td>
</tr>
<tr>
<td>VR08</td>
<td>160</td>
<td>122</td>
<td>481</td>
<td>20</td>
<td>116</td>
<td>24</td>
<td>25</td>
</tr>
<tr>
<td>VR12</td>
<td>237</td>
<td>93</td>
<td>589</td>
<td>174</td>
<td>279</td>
<td>102</td>
<td>22</td>
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</tbody>
</table>

(B) Bottom Top Fab

<table>
<thead>
<tr>
<th></th>
<th>VR01</th>
<th>VR05</th>
<th>VR08</th>
<th>VR12</th>
<th>VR16</th>
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<tbody>
<tr>
<td>VR01</td>
<td>31</td>
<td>5</td>
<td>7</td>
<td>74</td>
<td>65</td>
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<tr>
<td>VR05</td>
<td>250</td>
<td>12</td>
<td>222</td>
<td>170</td>
<td>136</td>
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<tr>
<td>VR08</td>
<td>24</td>
<td>−31</td>
<td>10</td>
<td>80</td>
<td>84</td>
</tr>
<tr>
<td>VR12</td>
<td>210</td>
<td>43</td>
<td>198</td>
<td>4</td>
<td>−6</td>
</tr>
<tr>
<td>VR16</td>
<td>227</td>
<td>63</td>
<td>221</td>
<td>−4</td>
<td>10</td>
</tr>
</tbody>
</table>

Fabs (250 nM) were injected sequentially onto immobilized KDR (A) or Flk-1 (B), without a buffer wash between samples. The table shows RU values for each step in each cycle of two sequentially injected Fabs. The numbers represent the RU signal obtained from the second Fab (top) over the RU signal obtained from the first Fab (bottom).

12, 3, 5.5, and 72 nM, respectively (Table 4). Their affinity to Flk-1 was 40, 3, 19, and 3 nM, respectively (Table 4). Flk-1 monospecific Fab VR16 representing the b9 allotype rabbit immune repertoire and the three KDR monospecific Fabs 1V09, 2V05, and VC06, representing the NZW rabbit immune repertoire, revealed the strongest affinity of 0.9, 1.0, 2.4, and 1.6 nM, respectively, which arose from lower dissociation rate constants (Table 4).
competition for binding between the two antibodies, suggesting non-overlapping epitopes on KDR. This was observed for Fabs 1V09 and VR05, and several other antibody pairs (Table 5A). Binding of Fab VR01 to the KDR-coated chip blocked further binding by Fab VR08, and vice versa, suggesting the two antibodies interacted with either the same or overlapping epitopes on KDR (Table 5A). Both Fabs 1V09 and 2V05 blocked subsequent binding by VR01, but did not block binding by VR08. This observation suggests that VR01 may share an overlapping epitope with both 1V09 and 2V05 (Table 5A). It is noteworthy that the overall KDR-binding RU increased when VR12 was followed by any other Fab, but there was no signal increase when any Fab was followed by VR12. This phenomenon most likely reflected the fact that the strong signal from tight KDR-binders at high concentration mask the signal generated by weak Fab VR12 binding. Overall, six different epitopes were recognized by the seven Fabs mapped in the first matrix (Fig. 4): five separate epitopes (1V09, 2V05, VC06, VR05, and VR12) and one epitope (VR01/VR08) that partially overlapped the 1V09 and 2V05 epitopes. This is seen as asymmetry in the matrix when VR01 is either the top or the bottom Fab (Table 5A).

In the second assay, a pairwise epitope mapping of the Flk-1-specific Fabs was performed on a Flk-1-coated chip. Binding of Fab VR01 to the Flk-1-coated chip blocked further binding by VR08, and vice versa, suggesting the two antibodies interacted with either the same or overlapping epitopes displayed on Flk-1 (Table 5B). The same was found for VR12 and VR16 (Table 5B). The Flk-1 section of the epitope map (Fig. 4) shows a cluster of two separate epitopes (VR01/VR08 and VR12/VR16) and one epitope (VR05) that partially overlaps the VR01/VR08 epitope, seen as asymmetry in the matrix when VR05 is either the top or the bottom Fab (Table 5B).

3.6. Further analysis of selected clones

Finally, the reactivity of the purified Fabs with native KDR and Flk-1 expressed on the cell surface of human HMEC-1 and HUVEC, as well as on mouse MS1 endothelial cells was tested by flow cytometry. All eight Fabs recognized their corresponding native antigen. Cross-reactive Fabs, VR01, VR05, VR08, and VR12, reacted with both human and mouse endothelial cells (Fig. 5). In contrast, Fabs 1V09, 2V05, and VC06 were able to detect human endothelial cells HMEC-1 and HUVEC, whereas mouse MS1 endothelial cells were uniformly negative (Fig. 5). Fab VR16 bound to Flk-1 on MS1 cells and did not show any significant binding to human endothelial cells (Fig. 5). Thus, the various VEGFR2 epitopes recognized by the selected chimeric rabbit/human

![Fig. 4. Epitope map of human and mouse VEGFR2. Topographical relationship of the epitopes defined by the Fabs against human (KDR) and mouse (Flk-1) VEGFR2. Overlapping circles denote Fabs that cannot bind concurrently.](image-url)
Fab are expressed on the endothelial cell surface and are accessible targets for antiangiogenic therapy.

4. Discussion

Here we describe the efficient production of high-affinity anti-VEGFR2 human/mouse cross-reactive antibodies from a b9 allotype rabbit immunized with a soluble form of mouse VEGFR receptor Flk-1. We have previously demonstrated the advantages of the Basilea mutant and b9 wild-type rabbit immune repertoires over the NZW immune repertoire that correlated inversely with the frequency of kappa light chains containing an unpaired cysteine in the unselected libraries (Popkov et al., 2003). Whereas a
majority of antibodies in immune NZW rabbits contain kappa light chains with a disulfide bridge between cysteine 80 and cysteine 171, a much lower percentage of chimeric rabbit/human Fab contain a kappa light chain with cysteine 80. In addition, the fact that the HCDR3 length distribution in rabbit antibodies is more similar to human than mouse antibodies is highly relevant for the generation of therapeutic mAbs from rabbit immune repertoires since this region is generally conserved in the process of antibody humanization. As a consequence, humanized rabbit antibodies may be more closely related to human antibodies than humanized mouse antibodies.

A key motivation for the generation of therapeutic mAbs from rabbits is their potential cross-reactivity with human and nonhuman primates, as well as mouse antigens: Cross-reactivity facilitates preclinical evaluation (Rader, 2001). Supporting this claim, we here describe for the first time rabbit mAbs that recognize both human and mouse VEGFR2; one mAb, VR05 binds both targets with identical affinity. Several observations from our study are noteworthy. First, based on 85% amino acid sequence identity between domains of human and mouse VEGFR2, the likelihood of obtaining cross-reacting rabbit mAbs was high. However, despite this high sequence similarity between mouse Flk-1 and human KDR, none of the anti-KDR Fabs selected from the NZW library cross-reacted with Flk-1 (Tables 1, 2 and 3). The immune sera from both NZW rabbits were cross-reactive, but no cross-reactive Fabs were recovered. This lends further support to our previous suggestion that yields of desired specificities from NZW are low because an unpaired cysteine in the Vk sequence leading to a free thiol group often interferes with Fab expression.

Second, our BIAcore analysis revealed that among the selected Fabs, the four monospecific Fabs have overall stronger affinity than the four cross-reactive Fabs (Table 4). Interestingly, the dissociation constants of clonally related cross-reactive Fabs, VR01 and VR08, were tighter for KDR than Flk-1, classifying them as heteroclitic antibodies, which bind the cross-reacting antigen (KDR) better than the immunogen (Flk-1).

Third, our epitope-mapping studies revealed that, although Fabs 1V09 and 2V05 compete with the cross-reactive VR01 and VR08 for binding to certain overlapping epitope(s) on KDR, they do not bind to Flk-1. Additionally, cross-reactive Fab VR05 competes with VR01 and VR08 for binding on Flk-1, but does not compete with these antibodies for binding to KDR. Pairwise epitope mapping gave the location of the epitopes recognized by the various Fabs (Fig. 4). The epitope map should not be interpreted, however, as defining the physical locations of the epitopes on the surface of the antigen, since allosteric conformational changes in the antigens might distort the pattern (Fagerstam et al., 1990). Rather it is a functional map, which offers a basis for Fab optimizations.

Although antiangiogenic therapy and vascular targeting therapy of cancer are extremely attractive conceptually, only a few good immunotargets shared by human and mouse vasculature are known. Zhu et al. (1998) described a panel of neutralizing scFv fragments to KDR, derived from mice immunized with a recombinant form of the KDR receptor. One scFv bound to human KDR with high affinity ($K_d=2.1$ nM), blocked the KDR/VEGF interaction, and inhibited VEGF-stimulated receptor activation and mitogenesis of human endothelial cells, but it did not cross-react with the Flk-1 receptor. More recently, the same team has identified several high-affinity human neutralizing Fab fragments to KDR directly from a large naive human phage display library (Lu et al., 2002). The one Fab from this study that cross-reacted with Flk-1 showed much weaker KDR binding ($K_d=49.2$ nM) compared with our selected Fab VR12 ($K_d=72.4$ nM). In contrast to previous observations that none of the anti-KDR antibodies cross-reacted with the same epitope on Flk-1, Fab VR05 generated in this study demonstrated identical binding to human and mouse VEGFR2, thus locating their binding epitope within the conserved domain of the receptor. The binding affinity of the neutralizing monovalent VR05 Fab was 3 nM, which is equal to the best KDR-specific Fabs generated from a naive human library (Lu et al., 2002). Non-neutralizing Fabs generated in this study, especially those with subnanomolar affinity, like Flk-1 specific Fab VR16, can be used as intrabodies to further examine the angiogenic potential of VEGFR2 surface depletion in vivo.

The present study shows that antibodies that bind with strong affinity to both human and mouse
VEGFR2 can be obtained from immunized rabbits, further underscoring the relevance of the rabbit immune repertoire for the generation of therapeutic mAbs that allow mouse animal modeling and should facilitate a faster transition from preclinical models to clinical trials.

Acknowledgements

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