Species Cross-Reactivity of Antibodies Used to Treat Ophthalmic Conditions

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Purpose. The species cross-reactivity of the monoclonal antibodies infliximab, bevacizumab, and an anti–VEGF-B antibody, 2H10, in humans and rodents was determined.

Methods. The binding of infliximab to human, mouse, and rat TNF-α, of bevacizumab to human, mouse, and rat VEGF-A, and of the 2H10 antibody to human, mouse, and rat VEGF-B was evaluated by ELISA. The sequence of human, mouse, and rat TNF-α and VEGF-A at the binding sites for infliximab and bevacizumab were compared.

Results. Infliximab bound to human TNF-α, but no binding to mouse or rat TNF-α was detected between 10 pg/mL and 10 μg/ml. Sequence comparison of the binding site revealed four changes in mouse and five in rat TNF-α compared with human. Bevacizumab bound strongly to human VEGF-A, but showed 5-log weaker binding to both mouse and rat VEGF-A. There was a single amino acid substitution in mouse and rat VEGF-A at the bevacizumab binding site. The 2H10 antibody displayed a similar binding profile to human, mouse, and rat VEGF-B.

Conclusions. The species cross-reactivity of monoclonal antibodies should be determined prior to their use in preclinical animal models. The 2H10 antibody binds to human, mouse, and rat VEGF-B making it suitable for testing in rodent models of human disease.

Keywords: biologic, species cross-reactivity, monoclonal antibody, TNF-α, VEGF-A, VEGF-B, human, mouse, rat

The use of "biologics" for the treatment of human disease is becoming increasingly common. A number of murine, chimeric, fully humanized monoclonal antibodies and Fab fragments have been Food and Drug Administration approved, and are used in the treatment of diseases, such as breast cancer,[1] head and neck cancer,[2] rheumatoid arthritis,[3] Crohn's disease,[4] and systemic lupus erythematosus.[5] Biologic antineovascular agents targeting members of the VEGF family of growth factors have revolutionized the treatment of ocular conditions, such as proliferative diabetic retinopathy,[6] diabetic macular oedema,[7] and neovascular AMD.[8,9] Furthermore, infliximab, an antibody to TNF-α, is used for the treatment of some forms of noninfectious uveitis.[10,11] The development of additional novel agents could see widespread use of biologics to treat other ocular conditions.

The high specificity of monoclonal antibodies for their target epitopes can pose problems if the antibody does not bind to cross-species homologues. This is particularly important if the safety and efficacy of a biologic agent is to be evaluated in a preclinical animal model prior to human use.[12] The feasibility of conducting safety and efficacy testing in animal models hinges on the species cross-reactivity of the antibody.[13] The vast majority of monoclonal antibodies in clinical use demonstrate limited species cross-reactivity.[14–18]

Here, we examined the species cross-reactivity of three monoclonal antibodies, with applications in ocular diseases, by ELISA. The binding of bevacizumab (Avastin; Genetech, South San Francisco, CA, USA), a humanized monoclonal antibody, to human, mouse, and rat VEGF-A was assessed. The specificity of infliximab (Remicade; Janssen Biotech, Horsham, PA, USA), a chimeric monoclonal antibody, for human, mouse, and rat TNF-α was also evaluated. Finally, the cross-reactivity of a murine anti–VEGF-B antibody, 2H10, for human, mouse, and rat VEGF-B was examined.

Methods

Sources of Antibodies and Growth Factors

Human, mouse, and rat TNF-α and VEGF-A were sourced from ProspecTany (Rehovot, Israel). Anti-human (AB-210-NA) and anti-rat (AF-510-NA) TNF-α capture antibody, and anti–VEGF-A capture antibody (AF564) were obtained from R&D Systems (Minneapolis, MN, USA). A biotinylated goat–anti-human IgG antibody (109-065-008) was purchased from Jackson ImmunoResearch (West Grove, PA, USA). Infliximab was purchased from Johnson and Johnson Health Care Systems (Piscataway Township, NJ, USA). Bevacizumab was sourced from Genentech (South San Francisco, CA, USA). Antibody-ligand binding was quantified by ELISA. Two independent experiments were performed for each ELISA, and representative data depicting the mean of three technical replicates ± SD are displayed.

Infliximab ELISA

The binding of infliximab to human, mouse, and rat TNF-α was assayed using an indirect sandwich ELISA. ELISA plates were coated with 100 μL 1 μg/mL anti-human or anti-rat/mouse TNF-

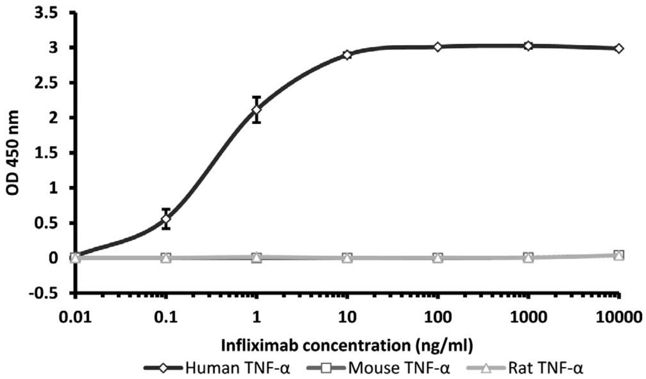

Figure 1. Binding of infliximab to recombinant human, mouse, and rat TNF-α. The binding of infliximab to recombinant human, mouse, and rat TNF-α was assayed by an indirect sandwich ELISA. Infliximab did not bind to mouse or rat TNF-α at the concentrations tested (100 pg/mL–100 ng/mL). Binding to human TNF-α was observed above 1 ng/ml. Representative data from two independent experiments, with mean of three technical replicates ± SD.

α antibody overnight at 4°C. Each plate was washed three times with 0.05% vol/vol Tween20 (Sigma-Aldrich Corp., St Louis, MO, USA) in PBS pH 7.4 (wash buffer) and 300 μL blocking buffer (5% wt/vol skim milk powder [Fonterra, North Adelaide, Australia], 5% wt/vol sucrose [Merck, Kenilworth, NJ, USA]) in PBS was added for 1 hour at room temperature. One hundred microliters of 0.1 μg/mL recombinant human, recombinant mouse, or recombinant rat TNF-α was added for 1 hour at room temperature with shaking at 800 rpm. The plate was washed three times with wash buffer and 100 μL infliximab (100 ng/mL–100 pg/mL) was applied for 1 hour at room temperature with shaking at 800 rpm. The plate was washed three times with wash buffer and bound infliximab was detected with a biotinylated goat–anti-human IgG antibody, applied at a 1:20,000 dilution in 100 μL for 1 hour at room temperature with shaking at 800 rpm. The plate was washed three times with wash buffer and 100 μL 200 ng/mL streptavidin conjugated to horseradish peroxidase (Life Technologies, Carlsbad, CA, USA) was added for 20 minutes. The plate was washed three times with wash buffer and developed with 100 μL TMB solution (BD OptEIA; Becton Dickenson, San Diego, CA, USA) for 20 minutes, protected from light. Fifty microliters of 1 M H_2SO_4 was added and the absorbance at 450 nm was measured using a plate reader (Molecular Devices, Sunnyvale, CA, USA).

Bevacizumab ELISA

The binding of bevacizumab to recombinant human, mouse, and rat VEGF-A was assessed using an indirect sandwich ELISA. ELISA plates (Nunc, Roskilde, Denmark) were coated with 50 μL VEGF-A capture antibody (1 μg/mL) overnight at 4°C. The plate was washed and blocking buffer was applied as described above. The plate was washed three times with wash buffer and 50 μL recombinant human, mouse, or rat VEGF-A (10 μg/mL; R&D Systems, Minneapolis, MN, USA) was added for 1 hour at room temperature with shaking at 800 rpm. The plate was washed three times with wash buffer and 50 μL bevacizumab (10 ng/mL–1 mg/mL) was incubated for 1 hour at room temperature with shaking at 800 rpm. Bound bevacizumab was detected with a biotin-conjugated anti-human IgG antibody (0.13 μg/mL, 50 μL/well), incubated for 1 hour at room temperature with shaking at 800 rpm. The plate was washed three times with wash buffer and 50 μL 200 ng/mL streptavidin conjugated to horseradish peroxidase was added for 20 minutes. The plate was developed and the absorbance read at 450 nm, as described above.

Anti–VEGF-B mAb ELISA

The binding of the anti–VEGF-B antibody 2H10 to recombinant human, mouse, and rat VEGF-B was assessed using a direct sandwich ELISA. An ELISA plate (Nunc, Roskilde, Denmark) was coated with 50 μL VEGF-B (1 μg/mL in PBS) overnight at 4°C. The plate was washed with PBS and 50 μL blocking buffer (2% BSA in PBS) was applied for 2 hours. The plate was washed with TPBS (0.05% Tween-20, PBS) and a serial dilution of 2H10 antibody, 100 μL, in antibody buffer (0.5% BSA, TPBS) was applied for 1 hour. The plate was washed twice with TPBS and 50 μL detection antibody, anti-mouse IgG-HRP (Merck Millipore, Billerica, MA, USA) at 1 μg/mL in antibody buffer was applied for 30 minutes. The plate was washed three times with TPBS and developed with 50 μL TMB/E Substrate (Merck Millipore, Billerica, MA, USA) for 3 minutes. The reaction was stopped with 25 μL 1M phosphoric acid and the absorbance read at 450 nm.

Sequence Comparison at Antibody Binding Sites

The sequences of human (ADV31546), mouse (BAF02298.1), and rat (ADV31545.1) TNF-α, human (NM003376), mouse (Q00731), and rat (NM031836) VEGF-A, and of human (NP_001230662.1), mouse (NP_001172093.1), and rat (NP_446001.1) VEGF-B were obtained from NCBI Protein Database (in the public domain, http://www.ncbi.nlm.nih.gov/protein). Sequences were aligned using the AlignX program (VectorNTI 9.0.0; Thermo Fisher Scientific, Waltham, MA, USA).

Results

Binding of Infliximab to Human, Mouse, and Rat TNF-α

Binding of infliximab to human TNF-α was observed above 1 ng/mL antibody concentration (Fig. 1), but the antibody did not bind to mouse or rat TNF-α at any of the concentrations tested (10 pg/mL–10 μg/mL). Similar results were observed when binding was assayed using a direct ELISA (data not shown).

Binding of Bevacizumab to Human, Mouse, and Rat VEGF-A

Strong binding of bevacizumab to human VEGF-A was observed between 1 mg/mL and 10 ng/mL antibody concentration (Fig. 2). Binding to mouse and rat VEGF-A was not observed below 10 μg/mL. Bevacizumab bound to human VEGF-A at a 5-log lower concentration when compared with either mouse or rat VEGF-A. The binding profile of bevacizumab to human, mouse, and rat VEGF-A was similar when tested by direct ELISA (data not shown).

Binding of 2H10 to Human, Mouse, and Rat VEGF-B

Binding of the 2H10 antibody to human, mouse, and rat VEGF-B was assessed by direct ELISA. The 2H10 antibody bound to human, mouse, and rat VEGF-B above 0.01 μg/mL antibody concentration (Fig. 3). The binding profile of the 2H10 antibody to human, mouse, and rat VEGF-B was similar over the range of concentrations tested.

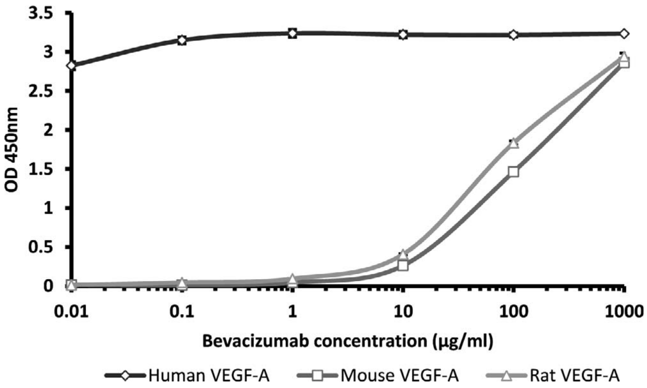

Figure 2. Binding of bevacizumab to recombinant human, mouse, and rat VEGF-A. An indirect sandwich ELISA was used to assess the binding of bevacizumab to recombinant human, mouse, and rat VEGF-A. Bevacizumab demonstrated strong binding to human VEGF-A at the concentrations tested (10 ng/mL–1 mg/mL). Binding to mouse and rat VEGF-A was observed above 10 μg/mL antibody concentration. Binding to mouse and rat VEGF-A was similar to human VEGF-A at five orders of magnitude higher antibody concentration. These data demonstrate that bevacizumab at best binds poorly to rodent VEGF-A. Representative data from two independent experiments, with mean of three technical replicates ± SD.

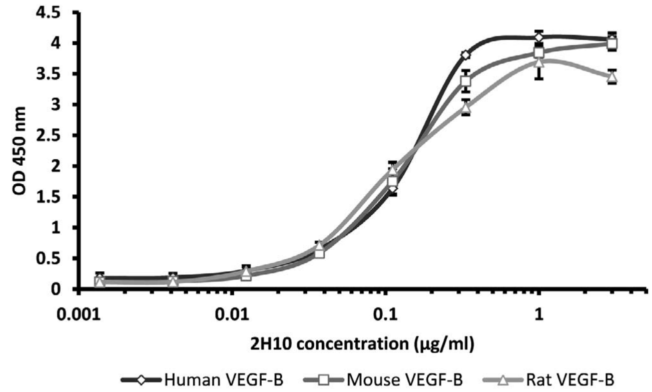

Figure 3. Binding of the 2H10 antibody to recombinant mouse, human, and rat VEGF-B. The binding of the anti–VEGF-B antibody to human, mouse, and rat VEGF-B was assayed by direct ELISA. The antibody had a similar binding profile to human, mouse, and rat VEGF-B. Representative data from two independent experiments, with mean of three technical replicates ± SD.

Disparities at the Binding Sites of Infliximab, Bevacizumab, and 2H10 mAb for Human, Rat, and Mouse TNF-α, VEGF-A, and VEGF-B, Respectively

Infliximab interacts with 12 amino acids in TNF-α.[19] Mouse TNF-α differs from human at four of these residues, including a deletion of a serine at position 71 (Table 1). Rat TNF-α is identical to mouse TNF-α except for an additional substitution of an arginine for a glutamine at position 67 (Table 1).

The binding site of bevacizumab on VEGF-A consists of 21 residues.[20] There is a single amino acid change in the bevacizumab binding site in mouse and rat VEGF-A compared with human VEGF-A (Table 2): A serine is substituted for glutamine at position 88 in rat and mouse VEGF-A.

The 2H10 monoclonal antibody interacts with the VEGF-B homodimer at 18 residues.[21] Human and rat VEGF-B have identical sequences at the 2H10 binding site, while a proline is substituted for a serine residue at position 16 in mouse VEGF-B (Table 3).

Table 1. Sequence Variation in TNF-α at the Infliximab Binding Site

	Infliximab Interaction With TNF-α		
TNF-α Residue	**Human**	**Mouse**	**Rat**
67	Q	Q	**R**
70	P	P	P
71	S	-	-
73	H	**Y**	**Y**
105	T	T	T
107	E	E	E
109	A	A	A
110	E	E	E
137	N	N	N
138	R	**L**	**L**
140	D	**K**	**K**
141	Y	Y	Y

Amino acids in TNF-α interacting with infliximab have been compared in human, mouse, and rat. Rodent TNF-α differs from human at a number of residues. Residues that are different in mouse or rat compared with human have been bolded, - indicates a deletion.

Discussion

In this study, we examined the species cross-reactivity of three monoclonal antibodies. In summary, we found that infliximab bound strongly to human TNF-α but did not bind to either mouse or rat TNF-α at the concentrations tested (10 pg/mL–10 μg/mL). Bevacizumab bound to human VEGF-A at a 5-log lower

Table 2. Sequence Variation in VEGF-A at the Bevacizumab Binding Site

	Bevacizumab Interaction With VEGF-A		
VEGF-A Residue	**Human**	**Mouse**	**Rat**
17	F	F	F
21	Y	Y	Y
45	Y	Y	Y
47	F	F	F
48	K	K	K
79	Q	Q	Q
80	I	I	I
81	M	M	M
82	R	R	R
83	I	I	I
84	K	K	K
85	P	P	P
86	H	H	H
87	Q	Q	Q
88	G	**S**	**S**
89	Q	Q	Q
90	H	H	H
91	I	I	I
92	G	G	G
93	E	E	E
94	M	M	M

Amino acids in VEGF-A interacting with bevacizumab have been compared in human, mouse, and rat. The bevacizumab binding site on rodent VEGF-A differs from human at a single residue: Glycine 88 is replaced by serine. Residues that are different in mouse or rat compared with human have been bolded and underlined.

Table 3. Sequence Variation in VEGF-B at the 2H10 mAb Binding Site

	2H10 mAb Interaction With VEGF-B		
VEGF-B Residue	**Human**	**Mouse**	**Rat**
16	S	**P**	S
17	W	W	W
18	I	I	I
21	Y	Y	Y
25	T	T	T
26	C	C	C
27	Q	Q	Q
48	V	V	V
49	P	P	P
62	P	P	P
79	Q	Q	Q
81	L	L	L
83	I	I	I
87	S	S	S
88	S	S	S
90	L	L	L
101	C	C	C
102	E	E	E

Amino acids in VEGF-B interacting with the anti–VEGF-B mAb 2H10 have been compared in human, mouse, and rat. Rat VEGF-B is identical to human at the binding site for the 2H10 mAb. A proline is substituted for a serine at residue 16 in mouse VEGF-B (bold).

concentration than mouse or rat VEGF-A. The 2H10 antibody displayed a very similar binding profile to human, mouse, and rat VEGF-B at the range of concentrations tested.

The observed differences in cross-species reactivity of infliximab and bevacizumab might be explained by sequence differences at the binding site of their target molecules. Infliximab interacts with a 12 amino acid sequence on the human TNF-α molecule.[19] Mouse TNF-α differs at four of these residues and rat TNF-α at five (Table 1). These differences include the deletion of a serine residue at position 71, which in combination with the amino acid substitutions may be responsible for the loss of binding activity to rodent TNF-α.

Bevacizumab interacts with human VEGF-A at 21 residues.[20] There is a single amino acid substitution in both mouse and rat VEGF-A (Table 2). The glycine at position 88 in human VEGF-A is replaced with a serine in rodent VEGF-A. This small change at the binding site might explain why bevacizumab binding to rodent VEGF-A is much weaker, but not completely abolished. Our results were in keeping with the literature on binding of bevacizumab to murine VEGF-A.[22] Yu et al.[23] demonstrated that bevacizumab bound weakly to murine VEGF-A by Western blot, but no binding was observed by surface plasmon resonance. Furthermore, the authors showed that bevacizumab could not neutralize the biological activity of murine VEGF-A.[23]

The binding site of the 2H10 antibody on the VEGF-B homodimer consists of 18 amino acid residues.[21] Rat and human VEGF-B are identical at these residues, whereas proline is substituted for serine at position 16 in mouse VEGF-B (Table 3). 2H10 exhibited very similar binding profiles to human, mouse, and rat VEGF-B. Sequence identity at the binding site explains these findings for human and rat VEGF-B. The single amino acid substitution in mouse VEGF-B was clearly insufficient to alter its binding characteristics, given that serine 16 lies outside the direct interaction site and has only two van der Waals' contacts with tyrosine at position 49 of the 2H10 mAb.[21]

Infliximab and bevacizumab unquestionably bind strongly to human TNF-α and human VEGF-A, respectively. Furthermore, several reports demonstrate the efficacy of these biologics in rodent models of ocular disease (Table 4). Thus, TNF-α inhibition using infliximab has been shown to inhibit laser-induced choroidal neovascularization in the rat[24] as well as the mouse.[25] Infliximab has also been shown to reduce corneal hemangiogenesis ($P < 0.05$) and lymphangiogenesis ($P < 0.01$) in a murine alkali burn model.[26] A study comparing the antineovascular effect of a number of anti–VEGF-A agents in a rat model of corneal neovascularisation found that bevacizumab was the most effective, although all agents tested significantly inhibited neovascularisation.[27] Further work demonstrated that combination therapy of bevacizumab with etanercept (Enbrel; Amgen, Thousand Oaks, CA, USA) had a greater antineovascular effect than monotherapy with either agent alone.[28] Of particular note, both infliximab and bevacizumab have been shown to be efficacious in models of corneal neovascularization when applied topically.[26,29–31] These results were unexpected, as whole antibodies penetrate poorly through the human, pig, cat, and rabbit cornea.[32] Although rodent corneas are thinner than those of the pig or human, which may have influenced penetration, the major barrier to the penetration of large molecules through the cornea are the corneal epithelial tight junctions.[33] Nevertheless, the data taken together suggest that both infliximab and bevacizumab demonstrate functional species cross-reactivity in rodent models.

The large number of reported studies that have documented a biological effect of human-reactive antibodies in rodent models, in the absence of specific binding, might point to off-

Table 4. Antibody Cross-Reactivity in Animal Models

Condition	Route of Administration	Species	Reference
Infliximab			
Choroidal neovascularization	Intravitreal/intraperitoneal	Rat/mouse	24/25
Corneal neovascularization	Topical	Mouse	26
Asthma	Intraperitoneal	Rat	34
Ischemia reperfusion injury	Intravenous/intraperitoneal	Rat	35, 36/37
Otitis media	Intravenous	Rat	38
Spinal cord injury	Subcutaneous	Rat	39
Bevacizumab			
Corneal neovascularization	Subconjunctival injection/topical	Rat	27, 28/29–31
Tuberculosis granulomas	Intravascular infusion	Rabbit	40
Diabetic neuropathy	Intraperitoneal	Rat	41

Infliximab has been shown to ameliorate a number of inflammatory diseases in rodent models. The antineovascular effect of bevacizumab has been demonstrated in a number of rodent models of human disease.

target effects. Such an effect might be mediated through the Fc portion of the antibody. It is known that intravenous Ig (pooled polyclonal immunoglobulin) has anti-inflammatory and immunomodulatory effects.[42] Of the reports summarized in Table 4, a single study used normal serum as a negative control.[27] The control in the remainder of the studies was saline, which in itself is not ideal.

Off-target effects have also been reported with other agents such as siRNAs. Nonspecific siRNAs showed a comparable antineovascular effect to targeted siRNAs (against VEGF-A or VEGF-R1) in a mouse model of choroidal neovascularization.[43] Another issue is that inherent variations in animal models may lead to a false positive result, especially if a small number of animals are used.

Bevacizumab is a competitive antagonist of VEGF-A and can bind rodent VEGF-A locally, albeit weakly. The biological effect of bevacizumab in rodent models might be attributed to the fact that sufficient bevacizumab was administered to overcome the deficit in binding rodent VEGF-A. Bevacizumab has been shown to reduce the serum levels of VEGF-A at very low concentrations.[44] Bevacizumab activity at low concentrations might explain the antineovascular effects observed in rodent models, even when binding to rodent VEGF-A is limited.

Finally, we established that the 2H10 antibody displayed a similar binding profile to human, mouse, and rat VEGF-B by ELISA and we hypothesize that this antibody is suitable for testing in preclinical rodent models of human diseases. We suggest that the species cross-reactivity of monoclonal antibodies should be tested before being used in animal models of human disease. We caution that the observation of a biological effect in animal models, in the absence of specific binding, may be due to inherent variance in the model, or caused by an off-target effect of the drug in question.

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