Concise Report

Prophylactic but not therapeutic activity of a monoclonal antibody that neutralizes the binding of VEGF-B to VEGFR-1 in a murine collagen-induced arthritis model

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Objective. To assess the therapeutic potential of a mAb that neutralizes the binding of VEGF-B to VEGFR-1, to inhibit the pathogenesis of CIA in mice.

Methods. CIA was induced in C57BL6/J and DBA-1 mice by intradermal injection of chick collagen type II (CII) in adjuvant. A neutralizing VEGF-B mAb or an isotype control mAb was then administered by intraperitoneal injection twice weekly beginning either post CII booster injection but prior to or immediately following clinical disease diagnosis.

Results. Neutralizing VEGF-B mAb inhibited the development of CIA in C57BL6/J mice in a dose-dependent manner when administered following the CII booster injection, but prior to clinical disease diagnosis. This result was also confirmed in DBA-1 strain mice. In contrast, the neutralizing VEGF-B mAb had no measurable effect on disease severity or progression when treatment commenced from the day of clinical disease diagnosis.

Conclusions. Treatment with an mAb that neutralizes the binding of VEGF-B to VEGFR-1 exhibits prophylactic but not therapeutic actions in a mouse model of RA. These data indicate that while VEGF-B/VEGFR-1 signalling is involved in the early development of arthritis it may not be required for maintenance or progression of established disease.

KEYWORDS: Collagen-induced arthritis, VEGF-B, Prophylactic, Therapeutic, Angiogenesis, Monoclonal antibody, Animal model.

Mounting evidence supports a contribution of VEGFR-1 signalling in arthritis [1–4]. Specifically, over-expression of soluble VEGFR-1 receptor using an adenoviral vector suppressed CIA in mice, thus identifying a requirement for VEGFR-1 ligands in the progressive inflammatory pathology leading to joint destruction [5]. Consequently, interrupting VEGFR-1 signalling by inhibiting the binding of VEGF-1 ligands may be a potential therapy for RA.

VEGF-A, via interaction with VEGFR-1 and not VEGFR-2, has been implicated in the pathogenesis of arthritis [1, 2, 4, 6]. While there is general acceptance that VEGF-A is involved in arthritis there is still some contention regarding its involvement in the maintenance and progression of established disease [1, 2]. Recent studies suggest that other VEGF-1 ligands may also be involved in the pathogenesis of arthritis [4]. VEGF-B is a structural homologue of VEGF-A, which binds to VEGFR-1. We have previously shown that Vegfb-null mice displayed reduced severity of arthritis inflammation in two mouse models of arthritis, antigen and CIA [7]. Although VEGF-B signalling has been implicated in directly promoting both the pathological angiogenesis and the inflammatory response in arthritis [3], the data from our previous studies only identified impaired synovial angiogenesis in the absence of VEGF-B [7].

Vegfb-null mice have provided a useful tool for establishing a role for VEGF-B in the development of arthritis and further studies are now required to establish the potential benefits of blocking VEGF-B signalling as a therapy for RA. To test this, we have used a neutralizing VEGF-B mAb that inhibits the binding of VEGF-B to its receptor [8] and which effectively inhibited VEGF-B-induced angiogenesis ex vivo [9]. We have assessed the ability of this mAb to inhibit CIA in mice as an indicator of the therapeutic potential of blocking VEGF-B binding to VEGFR-1 as a treatment for RA.

Materials and methods

Mice

All mice in this study were treated in accordance with the National Health and Medical Research Council guidelines for the care of experimental animals. Dose effects of neutralizing VEGF-B mAb administration on CIA development and severity were assessed using C57BL6/J strain mice. This strain of mice was chosen based on previous CIA studies using Vegfb-null mice that established a role for VEGF-B in the pathogenesis of arthritis [7]. DBA-1 strain mice were used for confirmation and therapeutic studies. The DBA-1 mice are widely used for CIA studies, including studies involved in assessment of potential arthritis therapies [10]. In all CIA studies, female mice 8–12 weeks of age (20–25 g) were used.

Induction of CIA, mAb treatment and disease assessment

CIA was induced in the C57BL6/J and DBA-1 strain mice as previously described [7]. Briefly, mice were immunized by intradermal injection at multiple sites at the base of the tail with a total of 100 μl of emulsion consisting of equal parts of complete Freund’s adjuvant (CFA, Sigma-Aldrich, St. Louis, MO, USA) containing 5 mg/ml heat-killed Mycobacterium tuberculosis antigen (H37 RA, Gibco BRL, Gaithersburg, MD, USA) and 2 mg/ml chick type-II collagen (CII, Sigma-Aldrich) in 10 mM acetic acid. Booster injections of 100 μl of emulsion consisting of equal parts of incomplete Freund’s adjuvant (IFA, Sigma-Aldrich) and 2 mg/ml CII in 10 mM acetic acid were administered at the same site 8 or 21 days later (see Table 1 for an experiment summary). In the dose response study with C57BL6/J mice, day 8 booster CIA immunizations were used for comparison with previous studies [7]. In the two prophylactic studies with DBA-1 mice, either a day 8

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or day 21 booster CII immunization was used to determine the effects of strain and immunization protocol. Lastly, in studies designed to assess the effects of anti-VEGF-B antibody on established CIA, the day 21 booster CIA immunization was used as this protocol had been found to be optimal for disease induction in the DBA-1 mouse strain (data not shown).

For studies designed to assess the prophylactic dose effects of a neutralizing VEGF-B mAb [8] on CIA, groups of 9–10 C57BL6/J mice were administered 10, 50 or 200 μg neutralizing VEGF-B mAb 2H10 or IgG2a isotype control mAb C44 (ATCC; CRL-1945) in 200 μl of PBS by intraperitoneal (i.p.) injection, twice weekly from day 8 for the duration of the experiment. An additional group of mice was administered vehicle phosphate buffered saline (PBS) to control for non-specific effects. In confirmation CIA studies, groups of 8–10 DBA-1 mice were administered 200 μg of neutralizing VEGF-B mAb or control mAb in 200 μl of PBS by i.p. injection, twice weekly from day 8 for the duration of the experiment. In studies designed to assess the effects of the neutralizing VEGF-B mAb [8] on the progression of established CIA, groups of 11 DBA-1 mice were administered 200 μg neutralizing VEGF-B mAb or control mAb in 200 μl of PBS by i.p. injection, twice weekly from the day of clinical disease diagnosis for a period of 2 weeks (Table 1).

Arthritic disease in digits and paws was determined twice weekly in prophylactic studies from experimental day 21 and daily in therapeutic studies from the day of disease diagnosis. Disease was determined by clinical assessment based upon the cumulative scoring of the level of inflammation (swelling and erythema) of each digit and paw, as previously described [7]. Clinical scoring of disease severity was performed blind to the treatment regimen. At peak clinical score (Fig. 1) the mice were euthanased and their digits removed for immersion fixation for 48 h in 4% paraformaldehyde in PBS. Joints were decalcified in 10% EDTA for 2–3 weeks and then paraffin embedded. All clinically diseased joints from the anti-VEGF-B mAb-treated mice (n = 22) and an equivalent number from the isotype control mAb-treated mice (prophylactic studies, DBA-1 strain) were analysed by histology and the level of inflammation [haematoxylin and eosin (H&E) sections] and osteoclast-associated levels of bone degradation [tartrate-resistant acid phosphatase (TRAP), leucocyte acid phosphatase staining kit from Sigma-Aldrich) in each joint graded (blinded respective to clinical grade) using a scale of 0–3, with 3 being the most severe.

**Statistical analyses**

Differences in clinical disease severity between groups were analysed using two-factor analysis of variance, with replication function. Linear differences were considered significant if \( P \leq 0.05 \). Differences in histological disease severity (inflammation and osteoclast-associated bone degradation) of the diseased joints from mice in the anti-VEGF-B mAb and isotype control mAb treatment groups were analysed using Student’s t-test. Differences in means were considered significant if \( P \leq 0.05 \). Correlations between clinical disease severity and (i) histological levels of inflammation and (ii) osteoclast-associated levels of bone degradation were analysed using linear regression.

**Results**

Immunization of C57BL6/J mice with CII induced limb and digit inflammation with characteristic oedema and erythema that was first evident 25 days after primary immunization, as previously observed [7]. Disease severity within animals, as well as the number of affected animals, steadily increased until day 69 when the experiment was terminated (Fig. 1A). Disease induction kinetics were slightly delayed in comparison with those observed in other CIA studies using this strain of mice [11], which may reflect variations in the immunization protocol between studies.

The treatment of mice with the control mAb at any of the tested doses was without effect when compared with mice that were treated with control vehicle, PBS (Fig. 1A and Table 1). In contrast, mice treated prophylactically with anti-VEGF-B mAb at doses of 50 and 200 μg per injection beginning on day 8, which was prior to disease diagnosis, displayed significant reductions in average clinical disease severity when compared with control groups (Fig. 1A and Table 1). The treatment of mice with 10 μg of anti-VEGF-B mAb per injection was without effect on average clinical disease severity when compared with mice treated with the same concentration of control mAb or control vehicle, PBS.

There was a significant dose effect of anti-VEGF-B mAb treatment on disease severity, as demonstrated by the sequential reductions in disease at increasing doses. There were significant differences in average clinical disease severity between mice treated with 10 and 50 μg per injection (\( P < 0.001 \)) and between mice treated with 50 and 200 μg per injection (\( P < 0.001 \)). These data illustrate a dose-dependent inhibition of CIA by this mAb. The anti-VEGF-B mAb also exhibited inhibitory activity on CIA in DBA-1 mice (using either the day 8 or day 21 booster CIA immunization protocol) when administered prophylactically at 200 μg per injection, indicating that the action of the mAb was not strain specific (Fig. 1B and C and Table 1). Interestingly, the control mAb group in the day 21 booster CIA immunization study (Fig. 1C) had a higher final clinical score than the equivalent control group in the day 8 booster CIA immunization study (Fig. 1B), suggesting that the day 21 immunization protocol was more effective at stimulating disease than the day 8 immunization protocol in DBA-1 strain mice.

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**Table 1. CIA incidence and statistical comparison between treatment groups**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Booster immunization</th>
<th>Treatment regime</th>
<th>Treatment dose (μg)</th>
<th>Disease incidence</th>
<th>Statistical comparison (μg)</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL6/J</td>
<td>Day 8</td>
<td>Prophylactic</td>
<td>PBS</td>
<td>8/9</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C44 10</td>
<td>10/10</td>
<td>PBS</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C44 g</td>
<td>10/10</td>
<td>PBS</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C44 200</td>
<td>9/9</td>
<td>PBS</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2H10 10</td>
<td>9/10</td>
<td>C44 10</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2H10 50</td>
<td>9/10</td>
<td>C44 50</td>
<td>0.0004*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2H10 200</td>
<td>6/10</td>
<td>C44 200</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>DBA-1</td>
<td>Day 8</td>
<td>Prophylactic</td>
<td>C44 200</td>
<td>16/19</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2H10 200</td>
<td>4/19</td>
<td>C44 200</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2H10 200</td>
<td>10/10</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2H10 200</td>
<td>6/10</td>
<td>C44 200</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>DBA-1</td>
<td>Day 21</td>
<td>Prophylactic</td>
<td>C44 200</td>
<td>11/11</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2H10 200</td>
<td>11/11</td>
<td>C44 200</td>
<td>0.98</td>
</tr>
</tbody>
</table>

*Statistically significant, \( P < 0.05 \). NA, not applicable.
In contrast to prophylactic studies, the treatment of mice with anti-VEGF-B mAb from the day of disease diagnosis at 200 μg per injection neither decreased the clinical severity of pre-existing CIA in affected digits (data not shown) nor inhibited the progression of CIA to other digits or paws in comparison with mice treated with isotype control mAb (Fig. 1D and Table 1).

To confirm that inhibition of clinical disease in the mice treated prophylactically with anti-VEGF-B mAb was associated with reductions in synovial inflammation and joint destruction, the joints from these studies were histologically evaluated. H&E-stained paraffin sections of clinically diseased joints revealed the presence of substantial synovial inflammation (see Supplementary Fig. 1, available as supplementary data at Rheumatology Online). In contrast, no synovial inflammation was observed in joints without clinical disease from any of the treatment groups (see Supplementary Fig. 1, available as supplementary data at Rheumatology Online). The level of synovial inflammation correlated with the level of clinical pathology (the square of the correlation coefficient for the linear regression, $R^2 = 0.7631$ for the control mAb-treated group and $R^2 = 0.7216$ for the anti-VEGF-B mAb-treated group; see Supplementary Fig. 1, available as supplementary data at Rheumatology Online). Other manifestations of arthritic histopathology including pannus formation, and bone and cartilage erosion were also observed in diseased joints, particularly in joints that displayed severe clinical disease. Increased levels of osteoclasts (identified by TRAP staining) associated with bone degradation were observed in joints of moderate to severe clinical disease (see Supplementary Fig. 1, available as supplementary data at Rheumatology Online), with $R^2 = 0.7631$ and 0.7216 for comparison of the level of clinical disease and the level of osteoclasts in the isotype control and anti-VEGF-B mAb-treated groups, respectively. Few osteoclasts were seen in joints displaying either mild or no clinical disease (see Supplementary Fig. 1, available as supplementary data at Rheumatology Online). Similar levels of histopathology, including osteoclast-associated bone degradation, were observed between treatment groups irrespective of the timing of the booster immunization when comparing joints of similar levels of clinical disease. The level of histological inflammation and osteoclast-associated bone degradation in clinically diseased joints from the anti-VEGF-B mAb-treated mice was significantly reduced ($P = 0.017$ and $P = 0.033$, respectively) in comparison with diseased joints from isotype control mAb-treated mice (prophylactic studies). Comparing the anti-VEGF-B mAb and the isotype control mAb-treated mice, respectively, the level of inflammation was 1.64 ± 0.21 and 2.25 ± 0.21, and osteoclast-associated bone degradation was
Discussion

Vegfb-null mice have a relatively normal phenotype [12], although they may have as yet unidentified physiological changes that underlie our previous findings regarding resistance to CIA [7]. To confirm that VEGF-B is actively required for the inflammatory process in arthritis it was important to undertake the studies using wild-type mice where VEGF-B signalling was antagonized, rather than using mice lacking VEGF-B. In the present study, we demonstrate a dose-dependent prophylactic inhibition of clinical disease development, synovial inflammation and joint destruction in the CIA model in mice with an mAb that blocks the binding of VEGF-B to VEGFR-1. Although mouse strain has been attributed to some variation in the contribution of signalling pathways in the CIA model [13], the inhibitory effect of prophylactic treatment with an anti-VEGF-B mAb on the pathogenesis was observed in both C56BL/J and DBA-1 mouse strains in this study. The level of disease inhibition in the mice treated with anti-VEGF-B mAb in the present study was similar to that previously observed in mice treated with VEGF-A antisera in the same model [1, 2]. These data confirm our previous observations of reduced pathogenesis of CIA in Vegfb-null mice and support our earlier hypothesis that VEGF-B plays a significant role in arthritis. The anti-VEGF-B mAb was not as effective in suppressing disease in the prophylactic day 21 booster CII immunization study in DBA-1 mice when compared with the equivalent study with the day 8 booster CII immunization protocol.

Prophylactic studies can offer valuable insight into the molecular mechanisms underlying disease yet care must be taken in translating such results into a potential therapeutic application. Despite significant inhibition of disease by anti-VEGF-B mAb using a prophylactic treatment regime, the treatment of mice from the day of disease onset using the same mAb at the same dose did not alleviate pre-existing CIA or inhibit disease progression. The effective prophylactic and ineffective treatment regimes with anti-VEGF-B mAb therapy are consistent with the observation of anti-VEGF-A antibodies having a prophylactic but not treatment effect on RA [1]. Although it appears that the angiogenic role of VEGF-A becomes less critical once the vascular networks are organized in the chronic disease, the role of VEGF-B during establishment of disease remains to be identified.

In conclusion, the prophylactic treatment of mice with an mAb that antagonizes the binding of VEGF-B to VEGFR-1 protects mice from development of synovial inflammation and joint destruction and identifies VEGF-B/VEGFR-1 signalling as a major component of the pathogenesis of arthritis. As treatment of mice with the same mAb at the same dose from the day of disease diagnosis neither reduced existing disease severity nor inhibited the progression of CIA, we conclude that blocking VEGF-B/VEGFR-1 signalling alone may not be appropriate for the treatment of established RA.

References


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Supplementary data

Supplementary data are available at Rheumatology Online.