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De novo protein sequencing, humanization and in vitro effects of an antihuman CD34 mouse monoclonal antibody

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ABSTRACT

QBEND/10 is a mouse immunoglobulin lambda-chain monoclonal antibody with strict specificity against human hematopoietic progenitor cell antigen CD34. Our in vitro study showed that QBEND/10 impairs the tube formation of human umbilical vein endothelial cells (HUVECs), suggesting that the antibody may be of potential benefit in blocking tumor angiogenesis. We provided a de novo protein sequencing method through tandem mass spectrometry to identify the amino acid sequences in the variable heavy and light chains of QBEND/10. To reduce immunogenicity for clinical applications, QBEND/10 was further humanized using the resurfacing approach. We demonstrate that the de novo sequenced and humanized QBEND/10 retains the biological functions of the parental mouse counterpart, including the binding kinetics to CD34 and blockage of the tube formation of the HUVECs.

1. Introduction

The CD34 protein belongs to the family of single-pass transmembrane sialomucin proteins, with an apparent molecular mass (Mr) of approximately 115 kD [1,2]. Cells with the CD34 surface protein could be found in the bone marrow, umbilical cord blood and peripheral blood as hematopoietic progenitor cells and vascular endothelial cells [3–7]. $CD34^{+}$ hematopoietic cells enriched from bone marrows have traditionally been used clinically in patients after radiation therapy or chemotherapy [8,9]. In previous studies, it was revealed that $CD34^{-/-}$ mice start to exhibit an abnormal vessel morphology when they are triggered by disease models, such as autoimmune arthritis [10], tumor angiogenesis [11], and oxygen-induced retinopathy [12]. Furthermore, CD34 expressed on human umbilical vascular endothelial cells (HUVECs) show the angiogenic tip cell phenotype [13]. Therefore, anti-CD34 should be considered when developing antiangiogenic therapy.

Angiogenesis is a physiological process related to the sprouting and growth of new vessels from an existing vasculature. Angiogenesis is the predominant pathway for neovessel growth in malignancy [14]; therefore, the process is called tumor angiogenesis. In 1971, Folkman first proposed the hypothesis that tumor growth is angiogenesis dependent [15], according to which angiogenesis presents unique opportunities for therapeutic intervention in cancer treatment. During vascular network expansion, sprouting angiogenesis requires a subset of highly specialized endothelial cells, namely tip cells. The tip cells start migrating and existing at the leading front of the growing vessels to guide migration toward a source of angiogenic growth factors, such as vascular endothelial growth factor (VEGF) [16–20], platelet-derived growth factor [21], placental growth factor [19,20,22], fibroblast growth factor-2 [18,23], interleukin-8 [24,25], transforming growth factor-beta [26–28], and angiopoietins [29,30].

Most monoclonal antibodies (mAbs) originate from mice; therefore, a human antimouse [31] or antichimeric [32] antibody might be evoked when mouse antibodies are applied in human therapy. To circumvent such an adverse immune response, mouse antibodies must be humanized for clinical applications [33]. Antibody humanization involves maintaining the specificity and affinity of the parental nonhuman antibody and designing an antibody molecule to reduce the immunogenicity to the greatest extent possible.

QBEND/10 is a mouse mAb against CD34; it reacts with the class II epitope of CD34 [34]. The CliniMACS CD34 reagent system (Miltenyi Biotec, Bergisch Gladbach, Germany) is a clinically approved device for selecting hematopoietic stem cells from donor apheresis. CliniMACS uses mouse QBEND/10 directly conjugated to an iron oxide particle. Notably, in our current in vitro study, mouse QBEND/10 impaired the tube formation of HUVECs. To address the therapeutic potential of QBEND/10, we used de novo protein sequencing and antibody

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humanization technology as well as analyzed the effects of humanized QBEND/10 on angiogenesis in vitro by inhibiting the tube formation of HUVECs.

2. Materials and methods

2.1. Materials

Mouse QBEND/10 was purchased from AbD Serotec. Ammonium bicarbonate, dithiolthreitol (DTT), iodoacetamide (IAM), formic acid (FA), thermolysin, and subtilisin were purchased from Sigma–Aldrich. Urea and acetonitrile (ACN) were purchased from J.T Baker. Trypsin and chymotrypsin were purchased from Promega. Endoproteinase Glu-C (Glu-C) and peptide N-glycosidase F (PNGase F) were purchased from New England BioLabs and Roche, respectively. Furthermore, 4–12% and 4–20% NuPAGE Bis-Tris polyacrylamide gels were purchased from Invitrogen. Amicon Ultra centrifugal filters (molecular weight cut-off, 100 kDa) were purchased from Millipore.

2.2. Enzymatic digestion and deglycosylation of QBEND/10

Mouse QBEND/10 was first processed for detergent removal and buffer exchange into 50 mM ammonium bicarbonate buffer solution by using the centrifugal filters. QBEND/10 was subsequently denatured using 6 M urea, reduced with 10 mM DTT at 37 °C for 1 h and alkylated using 50 mM IAM for 30 min in the dark at room temperature (RT). The resulting protein was individually digested with trypsin, Glu-C, thermolysin, chymotrypsin, and subtilisin at 37 °C for 18 h (protein:enzyme=20:1). One aliquot of the trypsin digest was added to Glu-C for 20-h digestion at 37 °C. Thereafter, PNGase F was added for the deglycosylation reaction. The samples were subsequently diluted and acidified to 0.1% FA for liquid chromatography (LC)–mass spectrometry (MS) analysis.

2.3. In-gel tryptic digestion

In a parallel experiment, a mini gel (8 cm×8 cm, 4–20% NuPAGE Bis-Tris polyacrylamide gel) was used for separation through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie Brilliant Blue R-250 staining. Two bands containing proteins with an apparent Mr of approximately 25 and 50 kDa were excised from the gel, washed, in-gel reduced, alkylated, and digested overnight with trypsin.

2.4. Liquid chromatography–tandem mass spectrometry analysis

The samples were analyzed with a Q Exactive mass spectrometer (Thermo Scientific) coupled with an Ultimate 3000 RSLC system (Dionex). LC was performed using the C18 column (Acclaim PepMap RSLC; 75 μm×150 mm, 2 μm, 100 Å) with a linear gradient of 1–25% of mobile phase B (mobile phase A: 5% ACN/0.1% FA; mobile phase B: 95% ACN/0.1% FA) for 40 min, 25–60% of mobile phase B for 3 min, and 60–80% of mobile B for 2 min for a total separation time of 70 min. A full MS scan was obtained in the range of m/z 350–2000, and the 10 most intense ions from the scan were subjected to fragmentation for MS/MS spectra. Raw data were processed into peak lists by using Proteome Discoverer 1.3 for a Mascot database search.

2.5. Database search and de novo sequencing

A customized database was prepared by collecting the sequences of immunoglobulins (IgGs) from the National Center for Biotechnology Information (NCBI) database. The database was searched using Mascot version 2.4.0. Carbamidomethylation was selected as the fixed modification, and deamidation (NQ), oxidation (M), and pyroglutamate (N-term Q) were included as variable modifications. Up to five missed cleavages were allowed for each enzyme digestion and ± 5 ppm and ± 0.02 Da were used as the mass tolerance window for parent and fragment ions, respectively. Furthermore, an error-tolerant search was performed, in which all modifications and sequence variations were considered. The MS/MS spectra with high intensities were manually sequenced if they had not been identified using Mascot. A customized computational algorithm was constructed to categorize the observed peptides as a heavy or light chain and then align the peptides into a complete sequence. The results were specified in Mascot as a new database for protein identification and an error-tolerant search. The process was repeated iteratively until the protein sequence with the highest possible score was obtained. All MS/MS spectra in this study were manually validated to assure their quality.

2.6. Molecular modeling

Molecular modeling of the variable fragment (Fv) of mouse QBEND/10 was performed using the Prediction of Immunoglobulin Structure (PIGS; http://www.biocomputing.it/pigs) [35,36] Web server through single sequence submission. The structural model of the mouse QBEND/10 Fv region was generated from the corresponding amino acid sequence by using PIGS with default settings. The most suitable heavy and light chain templates were selected from the 20 templates displayed. The Protein Data Bank (PDB) codes 2GKI_H [37] and 2QHR_L [38], exhibiting 86.67% and 94.92% sequence similarity with mouse QBEND/10 VH and VL, respectively, were used to model the three-dimensional (3D) structure of mouse QBEND/10. For the automated construction of the 3D structure of the Fv region of mouse QBEND/10, a canonical loop grafting approach was used for CDRs L1–L3 and H1–H3. The position of the conserved amino acid side chains was maintained, whereas the nonconserved amino acid side chains were modeled using SCWRL 4.0 [39]. Energy minimization was performed using the Swiss-PdbViewer application [40].

2.7. QBEND/10 humanizaiton

Mouse QBEND/10 was humanized using the resurfacing approach [41]. The variable heavy and light (VH and VL, respectively) chains and CDRs were numbered and identified according to the method proposed by Kabat [42]. First, the generated Fv model of mouse QBEND/10 was used to identify surface accessible residues by using Swiss-PdbViewer [40], with the threshold set at 30% [43]. Second, the sequence of mouse QBEND/10 VH and VL chains was searched using NCBI IgBLAST against the human IgG germline database (http://www.ncbi.nlm.nih.gov/igblast/). Human germline V sequences with the highest identity to mouse VH and VL regions were used. The J region for the heavy and light chains was selected from the most identical human consensus sequence. Third, these crucial surface residues of the framework regions were manually exchanged with those from the selected human IgG germline sequence. These side chains were rotated manually to evaluate stable side chain conformation and were subsequently subjected to energy minimization using Swiss-PdbViewer. Finally, the sequence composition for the Fv region of the resurfaced QBEND/10 was assembled. Two resulting models, mouse and humanized QBEND/10, were analyzed, visualized, and superimposed with Swiss-PdbViewer [40]. On the basis of the superimposition result, structural changes in the CDRs were determined.

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