A Novel Antibody AA98 V_H/L Directed Against CD146 Efficiently Inhibits Angiogenesis

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Abstract. *Background: An anti-CD146 monoclonal antibody, AA98, has been identified as an inhibitor of tumor angiogenesis. To overcome the inherent immunogenicity of murine antibody as well as to facilitate immunotoxin construction, a single chain AA98 V_H/L with three-domain fragments was constructed and expressed in mammalian cells. Materials and Methods: The genes of the AA98 heavy chain variable region and the light chain were linked with a modified 12 amino acid sequence that was derived from the heavy chain C_H1 region, thus constituting the three-domain antibody V_H/L. Soluble AA98 V_H/L was produced by mammalian cells and purified by affinity chromatography. The specificity of AA98 V_H/L for the CD146 molecule was detected by ELISA, immunofluorescence staining and flow cytometry. Results: AA98 V_H/L alone showed anti-angiogenic properties in a chicken chorioallantoic membrane (CAM) assay as the parent mAb AA98 did. Conclusion: This newly generated AA98 V_H/L antibody displays a therapeutic potential for tumor and other angiogenesis disorders, as well as providing a new strategy for antibody engineering for clinical applications.*

Angiogenesis, the growth of new microvessels (1, 2), plays an essential role in many physiological and pathological processes, such as the female menstrual cycle, embryo implantation, ocular disorder, tumor growth and metastasis. This process enrolls a series of angiogenic factors and receptors, including vascular endothelial growth factor (3), E-selectin (4), integrin (5), and adhesion molecules such as CD146 (5). CD146 is a 110 kDa glycoprotein with 5 immunoglobulin (Ig)-like domains (V-V-C2-C2-C2), one transmembrane region and a short cytoplasmic tail. It was initially identified as a progression marker of melanoma (7). CD146 has been considered as a marker of vascular endothelium (8) and a structural component of interendothelial junctions (9). It has been found that CD146 is an angiogenesis-related adhesion molecule which plays an important role in tumor angiogenesis from the observations that anti-CD146 mAb AA98 displays anti-angiogenic properties both *in vitro* and *in vivo*, which renders CD146 an attractive target for angiogenic intervention. An antibody-based approach has proven to be effective in blocking vascularization in chicken chorioallantoic membrane (CAM) assays and human tumor xenografts in mice (6).

The limitations to the use of murine antibodies in clinical applications include the human-antimouse antibody response, poor tumor penetration and effect, as well as economic obstacles regarding the production of antibody in high amounts. To circumvent these problems, we developed a three-domain antibody fragment V_H/L, whose structure has been previously reported to confer it with more desirable properties for immunotherapy. This antibody fragment not only offers advantages such as its small size, facilitating diffusion into tumors, and easy modifications for fusion with other proteins, as does single chain variable fragment (scFv), but is also a more stable antigen-binding fragment (Fab), provides sensitive detection in genetic manipulation and is a promising candidate for use in gene therapy.

Here the cloning, expression and characterization of AA98 V_H/L from the parent monoclonal antibody AA98 are described and its binding specificity to CD146 and anti-angiogenic activity are examined.

Materials and Methods

Antibodies. Anti-CD146 antibody AA98 was purified as described previously (6). Alkaline phosphatase (AP)-conjugated anti-mouse κ was purchased from Southern Biotechnology Associates (Birmingham, AL, USA). Rabbit anti-mouse κ antibody, FITC-conjugated anti-rabbit IgG and isotype-matched mouse IgG were purchased from Biodee Biotechnology Ltd (Beijing, China).

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Cell lines, tissues and animals. Human umbilical vein endothelial cells (HUVEC) and human microvascular endothelial cells (HMVEC) were purchased from the Chinese Type Culture Collection (Wuhan, China). A375 was purchased from the American Type Culture Collection (Rockville, MD, USA). All cell lines were cultured in Dulbecco's modified Eagle's Medium at 37˚C and 5% carbon dioxide in a humidified incubator.

Human tumor tissues were obtained from the tissue bank of the 301 Hospital in Beijing. Fertilized eggs of white Leghorn chicken were supplied by the Chicken Center of the Chinese Agriculture University, Beijing, China.

Gene cloning of AA98 V_H/L. AA98 hybridoma cells secreting in AA98 were homogenized with Trizol reagent (Gibco BRL, Carlsbad, USA). Total RNA was isolated and cDNA was synthesized using the Superscript preamplification system (Gibco BRL, Carlsbad, USA). With cDNA as the template, Fd and L chains were amplified separately by PCR using specific primers. For the Fd gene, the forward primer 5'ag gtc cag ctg ctc gag tct gg3', and reverse primer 5'gat atc act agt ggg ccc gct ggg ctc3' were used. For the L chain gene, the forward primer 5'gat att gag ctc gtg atg ac(c/a) ca(g/a) (t/a)ct cc3' and reverse primer 5'gc tct aga aag ctt att aac act cat tcc tgt tga a3' were used. The PCR products of the Fd and the L chains genes were cloned into pGEM-T (Promega, Madison, USA). Sequence analysis was performed in both directions using universal T_7 or T_3 primers.

The AA98 V_H/L gene was constructed by connecting V_H and L chains through a linker derived from C_H1 of AA98. V_H together with a modified 12 amino acid sequence of the C_H1 elbow region was amplified by PCR from recombinant pGEM-T-Fd. The amino acid sequence of the original C_H1 elbow region was Ala Lys Thr Thr Ala Pro Ser Val Tyr Pro Leu Ala, while in the modified linker, the second amino acid Lys was replaced by Ser. The L chain was obtained by PCR from pGEM-T-L and ligated with V_H-linker into a eukaryotic expression plasmid pCEP-Pu/AC7 (a gift from Dr. T Sasaki, Max-Planck Institute of Biochemistry, Germany).

Expression and purification of AA98 V_H/L. The recombinant plasmid with AA98 V_H/L gene pCEP-Pu/AC7 was transfected into 293T cells (CTCC, Wuhan, China) using a Fugene 6 transfection kit (F. Hoffmann-La Roche Ltd, Basel, Switzerland). The stable transfectants secreting AA98 V_H/L were screened by an enzyme-linked immunosorbent assay (ELISA). The AA98 V_H/L antibodies were purified from the culture supernatant by affinity chromatograpy with anti-mouse κ-protein A-Sepharose column. The bound proteins were eluted with 100 mM glycine, pH 2.8, followed by neutralization with 1M Tris-HCl, pH 8.0.

Immunoblotting. Purified protein was separated using 12% SDS-PAGE and electrically transferred to nitrocellulose filters (Invitrogen, Carlsbad, USA). The blots were blocked with 5% milk in phosphate-buffered saline (PBS) for 1 h at room temperature and detected by alkaline phosphatase-conjugated goat-anti-mouse κ for 1 h at room temperature, followed by three washes and alkaline phosphatase detection by 4-Nitro blue tetrazolium chloride / 5-Brom-4-chlor-3-indoxylphosphate (Sigma, Deisenhofen, Germany).

Purification of human CD146. The cellular lining of human umbilical veins was collected by scraping and homogenized in lysis buffer (150 mM NaCl, 1 mM EDTA, 50 mM HEPES, 10 mM sodium phosphate, pH 7.6, 10% glycerol, 1% TritonX-100, 1 mM phenylmethylsulfonyl fluoride, 25 μg/mL aprotinin, 2 μg/mL leupeptin and 2 μg/mL pepstatin). The supernatant of the prepared cell extract was applied to a mAb AA98-protein A-Sepharose column (Amersham, Piscataway, USA). Bound proteins were eluted with 100 mM glycine, pH 3.0, followed by neutralization with 1M Tris, pH 8.0.

Enzyme-linked immunosorbent assay (ELISA). Briefly, immobilized antigen CD146 (100 μg/ml) was coated onto 96-well plates and blocked for 1 h at 37˚C with 5% milk in PBS. Culture supernatants of each clone were added and incubated for 2 h at 37˚C. After 3 washes with PBST (PBS with 0.05% Tween-20), the bound antibodies were detected using AP-conjugated anti-mouse κ antibody for 1 h at 37˚C. Finally 4-nitrophenyl phosphate (Sigma) was added as a substrate and the color reaction was measured at 405 nm with a Bio-Rad ELISA reader (Richmond, CA, USA). Positive clones were selected and maintained.

Immunofluorescence staining. HUVEC cells were grown in chamber slides, washed with PBS and incubated with AA98 V_H/L culture supernatant (5 μg/ml); mAb AA98 (50 μg/ml) was used as positive control and 293T cell culture medium as negative control. The specificity of mAb AA98 binding to the CD146 positive HUVEC cells has been demonstrated previously (6, 9). Bound protein was detected by incubation with rabbit anti-mouse κ followed by FITC-conjugated anti-rabbit IgG. Each incubation was carried out in PBS for 1 h at 37˚C and the cells were analyzed under a fluorescence microscope (Leica, Wetzlar, Germany).

Flow cytometry. Cells were detached with 0.03% EDTA and incubated with purified antibodies for 1 h, followed by incubation with anti-IgG κ chain antibody and FITC-conjugated antibody. Cells were washed three times with PBS/1% bovine serum albumin before the next incubation. After the final incubation, cells were washed, resuspended in PBS and immediately analyzed on a FACSCalibur flow cytometry system (Becton & Dickinson, San Jose, CA, USA). A total of 10,000 cells were analyzed per sample.

CAM angiogenesis assay. Fertilized eggs of white Leghorn chicken were incubated at 37˚C with 65% to 70% humidity. The 6-day-old embryos with intact yolks were placed in a bowl and incubated at 37˚C with 3% CO_2. Small filter disks carrying either 1, 5, or 15 μg purified AA98 V_H/L, or mAb AA98 or control mIgG, respectively, were applied to the CAM of individual embryos. After 24 hours of incubation, neovascularization in CAMs was observed under a stereomicroscope.

Results

Construction and expression of AA98 V_H/L antibody. To obtain the AA98 V_H/L gene, the V_H with a modified linker and L chain genes were separately amplified by RT-PCR from the total RNA of hybridoma secreting mAb AA98. Notably, the linker with the 12 amino acid peptide which comes directly from the Fd region next to the V_H gene was modified at the Lys by substitution with Ser through mutated primers to prevent possible interference with antigen-binding sites. Two restriction endonuclease sites

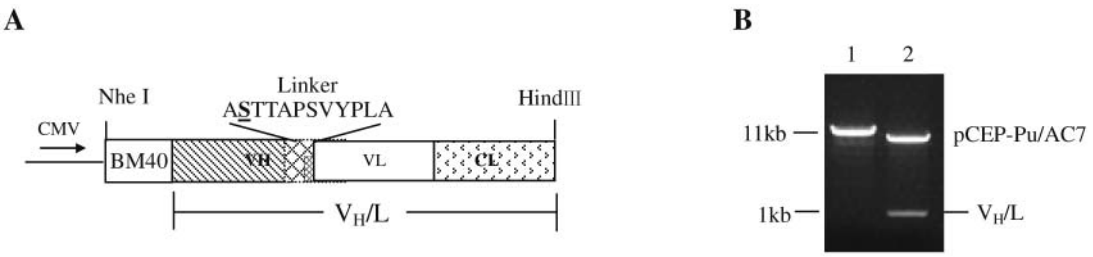

Figure 1. *Construction of three-domain antibody AA98 V_H/L. (A) Schematic representation of the recombinant mammalian expression vector pCEP-Pu/AC7 containing the AA98 V_H/L gene. The AA98 V_H/L expression is regulated by CMV promoter and directed by the BM40 secretion signal from the 293T cells to culture medium. The linker sequence derived from the elbow region of CH1 contains a Lys to Ser mutation at the second position (underlined). (B) Characterization of recombinant vector pCEP-Pu/AC7 containing AA98 V_H/L by restriction endonuclease digestion. The 1kb AA98 V_H/L gene (Lane 2) was cut with Nhe I and Hind III digestion from the recombinant vetor (Lane 1).*

were created on both sides of the linker, which make the modified linker a useful module for designing other antibodies.

For expression of soluble AA98 V_H/L, the gene was subcloned into a eukaryotic expression vector pCEP-Pu/AC7, in which the AA98 V_H/L gene was fused to a secretion signal BM40 under the control of CMV promoter (Figure 1A and B). The AA98 V_H/L construction was introduced into 293T cells, and the transfected cells secreted AA98 V_H/L into the culture supernatant. Affinity chromatography was applied for the purification of AA98 V_H/L using an anti-mouse κ-protein A column.

The purified AA98 V_H/L was then analyzed by SDS-PAGE and showed a molecular mass of approximately 72 kDa under nonreducing conditions and of 38 kDa under reducing conditions. Western blot indicated that only the 38 kDa but not 72 kDa molecule was recognized by anti-κ antibody (Figure 2).

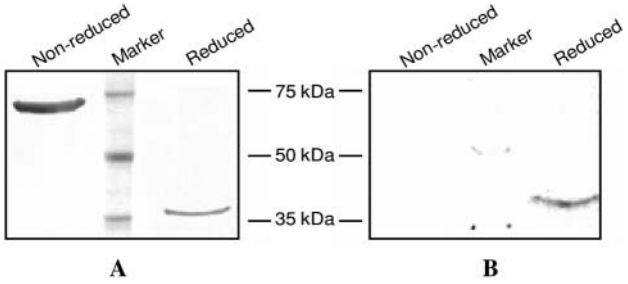

Figure 2. *Characterization of the purified AA98 V_H/L from the culture supernatant. (A) Purified AA98 V_H/L was analyzed by 12% SDS-PAGE under reducing and nonreducing conditions. (B) Western blot analysis of AA98 V_H/L. Proteins were detected by AP conjugated anti-mouse κ antibody after being immunoblotted onto a nitrocellulose membrane.*

Antigen-binding specificity of AA98 V_H/L. We further examined the reactivity of AA98 V_H/L with its antigen CD146 either in purified form or in natural form on the cell surface. ELISA was used to evaluate the binding of AA98 V_H/L to soluble CD146 antigen. Purified human CD146 were immobilized on plate and incubated with AA98 V_H/L, mAb AA98 or culture medium as controls. As shown in Figure 3A, AA98 V_H/L displayed comparable reactivity with its parent mAb AA98, whereas the control medium did not bind to CD146 antigen.

Immunofluorescence staining was adopted to further analyze the recognition of AA98 V_H/L to its antigen on HUVECs, which have been demonstrated CD146 positive on the cell surface (6, 10). The results showed that AA98 V_H/L and mAb AA98, whose binding specificity to HUVECs was elaborated by Yan *et al.* (6), intensively bound to HUVEC cells (Figure 3B). However, the control culture supernatant did not recognize the HUVEC cells, neither did the control mouse IgG (data not shown).

With further flow cytometry, a similar shift profile in both V_H/κ and mAb AA98 stained HMVECs (Figure 3C) was observed. These results further confirmed that mAb AA98 V_H/κ recognizes CD146, both the soluble and membrane-bound form.

Inhibition of angiogenesis in CAM assay by AA98 V_H/L. As it has been proved that mAb AA98 significantly inhibited angiogenesis (6), A CAM assay was carried out to investigate the effect of AA98 V_H/L on angiogenesis *in vivo*. Purified AA98 V_H/L, mAb AA98 or control mIgG absorbed on small filter disks were placed on CAMs of 6-day old embryos. We used 10 embryos for each group. After 24 hours of incubation, AA98 V_H/L and mAb AA98 both induced avascular zones around the antibody-applied areas in which the blood vessels became slim and pale. In contrast, the control mIgG did not show any effect on the CAM angiogenesis. We also found that the avascular zones caused by 15 μg V_H/L were obviously larger than those by 1 μg and 5 μg of V_H/L. These results demonstrated that the inhibitory angiogenic effect of mAb AA98 was induced by AA98 V_H/L

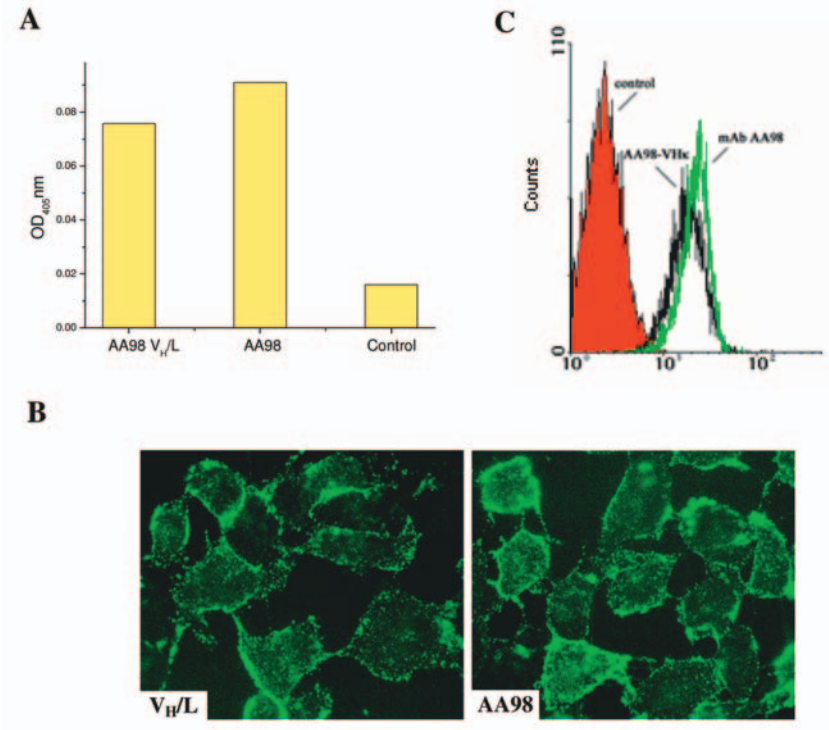

Figure 3. *Binding specificity of AA98 V_H/L to CD146 antigen. (A) ELISA shows the binding activity of AA98 V_H/L to immobilized CD146 purified from HUVEC, similar to that of mAb AA98. (B) Immunofluorescence assay indicates that HUVEC cells were stained by AA98 V_H/L and mAb AA98 (x200). (C) Flow cytometry analysis of mAb AA98-V_H/L binding to CD146 on cell surfaces. HMVECs were incubated with either mAb AA98-V_H/L or mAb AA98, followed by an anti-IgG κ chain antibody, and then a FITC-conjugated antibody.*

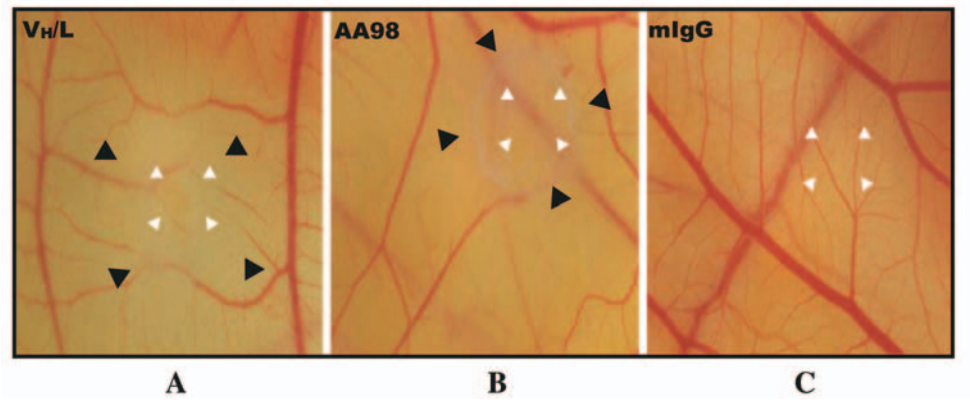

Figure 4. *CAM assays showing that the AA98 V_H/L inhibited angiogenesis during the chicken embryo development. Avascular areas on the CAMs were observed after 24-hour treatment with either 15 μg AA98 V_H/L (A) or mAb AA98 (B), whereas normal angiogenesis was found in the area treated with the same amount of mIgG (C). White arrowheads indicate the area of implanted disks, the black arrowheads indicate avascular area.*

but not its Fc fragment, and the anti-angiogenesis was antibody dose-dependent.

Discussion

In this study, we constructed a novel AA98 V_H/L antibody targeting the adhesion molecule CD146. This three domain structured antibody contains two variable regions from a heavy and light chain, and a constant region from a light chain. The AA98 V_H/L retains the same specificity for CD146 and activity in angiogenesis as its parent mAb AA98. However, its molecular size is about 1/5 that of the mAb AA98, which endows it with less immunogenecity and better penetration in tumor therapy.

For the AA98 V_H/L construction, the heavy chain variable region and the light chain κ were connected by a 12 amino acid linker derived from the elbow region, this assembles into a three-domain antibody fragment had a tendency to form bivalent structures through the CysSH at the *C*-terminus (10). Because the second amino acid of the linker sequence was positively charged Lys, which could potentially interact with side chains of negative charge in proximity to the surface of the antibody domain (11), we substituted the Lys with Ser. As demonstrated by antigen-binding assays, the specificity of AA98 V_H/L was the same with that of AA98, indicating that the designed linker barely perturbed the antigen-binding site. This uncharged linker is currently adopted in other single chain antibodies under study and has proven to be applicable for antibody engineering.

Bacteria are usually the favorite choice for small antibody fragment expression such as Fab and scFvs, while mammalian cells are occasionally used. We tried both expression systems and found that the V_H/L produced by mammalian cells expressed higher antigen-binding activity than that yielded by *Escherichia coli* (data not shown), which might result from efficient production of fully processed antibody fragment (12). While expressed in mammalian cells, AA98 V_H/L was found to have a molecular mass of 72 kDa, suggesting a bivalent structure of AA98 V_H/L existed in cell culture. This dimerized form has been observed in a prokaryotic expression system (10). Interestingly, only the 38 kDα monomer of AA98 V_H/L can be detected by anti-κ antibody, which was also confirmed in the *E. coli* expression systems (13). This phenomenon was probably caused by a steric hindrance effect. The bivalent antibody fragment represents fairly good specificity for its antigen on cell surfaces and neovasculatures in carcinoma tissues, and proved to be an angiogenesis inhibitor in the *in vivo* CAM assay.

Compared to the whole antibodies, small antibody fragments exhibit desirable properties in tissue penetration and full binding specificity. However, monovalent Fab and scFv are cleared fast in the blood, which leads to a low quantitative retention time on the target (14, 15). To improve this, various methods have been designed to genetically encode dimeric, trimeric or tetrameric scFvs. Clinical applications require a balance between long target retention time and rapid blood clearance, thus intermediate-sized multivalent antibodies are highly desirably for efficient tumor targeting. Diabodies (60 kDa) (16) and Fab dimers (110 kDa) (17) have shown preclinical effectiveness. Given the optimal size and structure, we suggested bivalent V_H/L as an appropriate choice for clinical immune therapy. V_H/L automatically forms dimers in solution without additional molecular manipulations, which differs from Fab and scFv, but still maintains the potential for easy fusion with stabilizing or toxic protein sequences. Moreover, the intact L chain offers sensitive detection in Western blot or ELISA assays and may be carried forward as a promising reagent for diagnostic biosensors.

Blocking angiogenesis to prevent the establishment, growth and metastasis of tumors is becoming a significant strategy (18). CD146 is a newly reported target for anti-angiogenic agents and a potent biomarker for a variety of pathological processes (19, 20, 21). Anti-CD146 mAb AA98 can efficiently inhibit angiogenesis in CAM assays and tumors, as well as tumor growth in mice. The novel AA98 derivative V_H/L has anti-angiogenic potential with its retained binding specificity to CD146 and inhibitory effect in the CAM assay. The introduction of this newly developed antibody fragment will expand the repertoire of reagents for anti-angiogenic immunotherapy and open a new way for antibody engineering aiming at clinical applications.

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