

Generation and Characterization of a Panel of Monoclonal Antibodies Against Distinct Epitopes of Human CD146

Ying Zhang,^{1,2*} Chaogu Zheng,^{1,2*} Jinbin Zhang,^{1,2} Dongling Yang,¹ Jing Feng,¹ Di Lu,¹ and Xiyun Yan¹

Abstract

CD146 (MUC18, Mel-CAM/MCAM) is a transmembrane protein, originally identified as a biomarker of melanoma, and plays an important role in cancer invasion and metastasis. Further studies revealed that CD146 as a novel endothelial marker was also involved in angiogenesis. Previous studies reported several anti-CD146 antibodies, such as MUC18, A32, S-endo1, and P1H12, showing different binding patterns to the endothelium of various types of blood vessels. To examine the possibility that antibodies targeting different epitopes on CD146 could have different behaviors, we generated a panel of anti-human CD146 monoclonal antibodies, named AA1–5 and AA7, by immunizing mice with human CD146 protein purified from HUVEC. Their specificity and binding affinity were intensively characterized using Western blotting, flow cytometry, and immunohistochemical assay. On the basis of epitope mapping, we divided the six monoclonal antibodies (MAb) into two groups, groups V1 and C2-2, corresponding to the different extracellular domains harboring these epitopes, the first IgV and the second IgC2 domains, respectively. Furthermore, owing to different epitopes, the two groups of antibodies behaved differentially in cellular and histological levels. Therefore, these anti-CD146 MAbs targeting different domains should be useful tools in studying the expression and function of human CD146.

Introduction

BELONGING TO THE IMMUNOGLOBULIN SUPERFAMILY, CD146, also known as MUC18, Mel-CAM, or MCAM, is a glycosylated cell adhesion molecule. Its extracellular region contains the characteristic V-V-C2-C2-C2 immunoglobulin-like domain structure.⁽¹⁾ CD146 was first cloned and sequenced from a human melanoma cDNA library screened by a monoclonal antibody, named MUC18, which reacts specifically with human melanoma cells.^(2,3) It was therefore identified as the biomarker of melanoma and involved in tumor progression and metastasis.^(4,5) Using amino acid sequence analysis, Shih et al. identified the same molecule as melanoma-associated antigen using another monoclonal antibody, A32.⁽⁶⁾ Although CD146 expression can be detected in a relatively limited spectrum of normal tissues, such as smooth muscle, endothelium, and trophoblast,^(7,8) ectopic expression of CD146 in melanoma mediates increased cell-cell adhesion and contributes to melanoma invasion and tumor cell migration.^(9–11) Pathological study showed a direct correlation

between CD146 expression and metastatic potential of melanoma cells.⁽⁹⁾ Consistently, overexpressing CD146 in CD146-negative primary melanoma cells resulted in remarkably increased tumor growth and metastasis.⁽⁹⁾

Also, CD146 has been proven to be expressed on circulating endothelial cells as a marker of endothelium.^(12–14) Two monoclonal antibodies, S-endo1^(15,16) and P1H12,⁽¹⁷⁾ which are demonstrated to recognize CD146, had been developed to detect circulating endothelial cells in ANCA-associated small-vessel vasculitis. Both of them can bind to the endothelia of various tissue sections and different types of vessels, indicating the widespread expression of CD146 in endothelium. Although the ligand for CD146 have not been identified, engagement of CD146 with anti-CD146 antibodies, such as S-endo1, could induce association of tyrosine kinase p59^{lyn} with the cytoplasmic tail of CD146, trigger tyrosine phosphorylation of p125^{FAK}, and Paxilin and then lead to rearrangement of cytoskeleton.^(18,19) These evidences indicated a role of CD146 in mediating outside-in signaling and the control of cell-cell contact.

¹National Laboratory of Biomacromolecules, Chinese Academy of Sciences, University of Tokyo Joint Laboratory of Structural Virology and Immunology, Institute of Biophysics, Beijing, China.

²Graduate University of the Chinese Academy of Sciences, Beijing, China.

*These authors contributed equally to this work.

In recent years, therapeutic antibodies targeting CD146 have been developed for the treatment of melanoma. Mills et al. reported a fully human anti-CD146 antibody, named ABX-MA1, which inhibited melanoma growth, invasion, and lung metastasis in nude mice.^(20,21) Our previous work demonstrated that the monoclonal anti-CD146 antibody, AA98, could inhibit angiogenesis in CAM assay and significantly suppress tumor growth in animal models.⁽²²⁾ Moreover, immunohistochemical assay showed that AA98 specifically binds to various types of tumor vessels, but not blood vessels in most of normal tissues, indicating a possibly new role of CD146 in tumor angiogenesis.⁽²²⁾ Further studies revealed that the mechanism of AA98-mediated anti-angiogenesis might involve its inhibition of CD146 dimerization,⁽²³⁾ MAPK phosphorylation, and NF κ B activation.⁽²⁴⁾ These evidences bring new insight into the function of CD146 in vascular and cancer biology.

Although CD146 is widely considered a specific endothelial marker, monoclonal anti-CD146 antibodies developed by different groups have displayed different patterns of immunoreactivity to various types of blood vessels. S-endo1 strongly reacted with the majority of human endothelium from different types of vessels, including arteries, arterioles, veins, venules, capillaries, high endothelial venules, and lymphatic vasculature.⁽¹⁶⁾ However, MUC 18 staining was restricted on endothelium to capillaries and high endothelial venules.⁽²⁵⁾ Moreover, S-endo1 strongly reacted with vessel of umbilical cord in frozen cryostat sections whereas MUC 18 did not, regardless of identical antigen of the two monoclonal antibodies (MAb).⁽¹²⁾ Recognitions of P1H12 localized to endothelial cells of all vessels, including microvessels of normal and cancerous tissues,⁽¹⁴⁾ while AA98 specifically bound to angiogenic endothelium in tumor vessels, but limited types of normal vessels.⁽²²⁾ In the biochemical studies, S-endo1 and AA98 only recognized CD146 under non-reducing conditions, indicating their conformational epitopes involving disulfide bond.^(12,22) Collectively, observations above using anti-CD146 antibodies with different epitopes apparently raise the possibility that CD146 might display different expression patterns, structures, or even biological functions in different circumstances.

In this study, we immunized mice with human CD146 protein purified from human umbilical vein endothelium and then generated a panel of anti-CD146 antibodies, named AA1, AA2, AA3, AA4, AA5, and AA7. The specificity and binding affinity of these antibodies were characterized using ELISA, flow cytometry, Western blotting, immunoprecipitation, and immunohistochemistry. Further epitope mapping identified different epitopes recognized by two groups of antibodies, showing different behaviors in the cellular and histological levels. Together these anti-CD146 MAbs binding different epitopes should be useful tools in studying the expression and functions of CD146, as well as in cancer diagnosis and therapy targeting CD146.

Materials and Methods

Cell lines, tissues, and animals

Cell lines A375, T24, and SP2/0-Ag14 were obtained from American Type Culture Collection (Rockville, MD). Human microvascular endothelial cells (HMEC) were kindly provided by Dr. Luo Yongzhang (Tsinghua University, Beijing,

China), and U937 cells were kindly provided by Dr. Tang Hong (Institute of Biophysics, Chinese Academy of Sciences, Beijing). A375 cells were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Invitrogen, Carlsbad, CA) and grown at 37°C in humidified atmosphere of 10% CO₂. Other cells were all maintained in RPMI 1640 supplemented with 10% fetal bovine serum and grown at 37°C in humidified atmosphere of 5% CO₂. Human umbilical vein endothelial cells (HUVEC) were prepared from human umbilical cords as previously described⁽²⁶⁾ and cultured in DMEM with 10% FCS. Human umbilical cord was obtained from Anzheng Hospital in Beijing. Female inbred BALB/c mice (6–8 weeks old) were obtained from the Animal Center of the Chinese Academy of Medical Science, Beijing.

Purification of CD146 from human umbilical cord

HUVECs were peeled from umbilical cord and lysed in extract buffer (3[G/V] PEG4000, 0.01 M Tris-HCl [pH 8.0], 10 mM EDTA, 2 mM PMSF, freshly added). Protein extracts were passed through Sephacryl S-200, collected for 97 kDa protein, and followed by murine anti-human CD146 monoclonal antibody AA98 coupled covalently to cyanogen bromide-Sepharose 4B (Amersham, GE Healthcare, Uppsala, Sweden). The eluate was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.

Generation and production of monoclonal antibodies

BALB/c mice were immunized by intraperitoneal (i.p.) injection with CD146 purified from HUVEC (100 μ g/mouse) in complete Freund's adjuvant. In the following month, mice were immunized again with purified CD146 (100 μ g/mouse) in non-complete Freund's adjuvant every 2 weeks. Before the fusion process, mice were boosted with purified CD146 (100 μ g/mouse) in non-complete Freund's adjuvant, and then mice were sacrificed and spleen cells were fused with mouse SP2/0-Ag14 myeloma cells, as described previously. Positive hybridoma cell clones were identified by rapid screening using an enzyme-linked immunosorbent assay (ELISA) with the recombinant extracellular domain of CD146 as antigen.

Expression and purification of recombinant CD146 domains

The different fragment of extracellular domain of CD146 was cloned into pET28a, pET30a, and pET32a to generate the expression constructs His6-S tag-D1 (residues 24-145), Thioredoxin (Trx)-His6-S tag-D2 (residues 128-248), Trx-His6-S tag-D3 (residues 232-335), Trx-His6-S tag-D4 (residues 294-442), Trx-His6-S tag-D5 (residues 381-561), and His6-sCD146 (residues 24-552). All the constructs were expressed in *Escherichia coli* BL21(DE3).

For soluble expressed protein, supernatant after sonication was incubated with Ni-NTA Sepharose 6 Fast Flow (GE Healthcare) to get purified fusion protein. For inclusion body, the pellet was washed by solutions 1 (2.5 M NaCl), 2 (0.5% Triton X-100 and 10 mM EDTA [pH 8.0]) and 3 (2 M Urea, 50 mM Tris, 1 mM EDTA [pH 8.0]) in turn. Each step was followed by centrifugation at 12000 g for 30 min at 4°C to harvest a pellet for the next step. After washing, the pellet was solubilized in solution 4 (8 M Urea, 25 mM Tris, 150

mM NaCl, 25 mM DTT [pH 8.0]) to 1 mg/mL for refolding process. Refolding buffers included 25 mM Tris, 150 mM NaCl, 1 mM glutathione reduced, 2 mM glutathione oxidized, and gradually decreased urea and arginine. Protein solution was dialyzed against refolding buffers to remove urea and finally stayed in 25 mM Tris and 150 mM NaCl.

sCD146-AP, (residue24-559 of CD146 fused with alkaline phosphatase) was secreted by 293T cells transiently transfected with eukaryotic expression vector with CMV promoter. Then the supernatant was collected and purified to obtain the recombinant fusion proteins.

Enzyme-linked immunoabsorbent assay

Plates were coated with different proteins (1 μ g/mL) at 4°C overnight and then blocked with 2% bovine serum albumin for 2 h at room temperature. Next, the primary antibodies were added and incubated for another 2 h at room temperature. After washing with PBS, the bound antibodies were detected by incubation with HRP-conjugated anti-mouse IgG at room temperature for another hour. After carefully washing, 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma, Deisenhofen, Germany) was added as substrate for color development. Sample absorption at the wavelength of 450 nm was measured by a Bio-Rad ELISA reader (Richmond, CA).

Immunofluorescence

A375 were plated on coverslips and cultured in a 6-well plate until confluence. Cells were washed with PBS, fixed with acetone-methanol (1:1) for 1 min. After washing with PBS, cells were blocked with 5% normal goat serum for 30 min at 37°C, and then incubated with a panel of anti-CD146 MAbs overnight at 4°C, followed by incubation with FITC conjugated anti-mouse IgG for 30 min at 37°C. Finally, the coverslips were examined with a confocal laser scanning microscope (Olympus, Tokyo, Japan).

Flow cytometry

Detached cells were processed to obtain single-cell suspensions followed by staining with anti-CD146 antibodies on ice for 40 min. After three washings with PBS containing 0.3% bovine serum albumin (BSA), cells were incubated with corresponding fluorescein isothiocyanat (FITC) conjugated anti-mouse IgG (Sigma) on ice for 30 min, then washed and analyzed using a FACS Calibur flow cytometry system (Becton Dickinson, San Jose, CA).

SDS-PAGE and Western blot analysis

For preparation of cell extracts, cells were scraped off, washed with ice-cold PBS, and lysed in lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 1% NP-40, 10% glycerol, 100 μ g/mL PMSF) on ice for 20 min. After centrifuging at 12,000 g for 5 min at 4°C, the supernatants were subjected to electrophoresis on a 8% SDS-PAGE and then electrophoretically transferred to nitrocellulose membrane (Invitrogen). After blocking with 5% non-fat milk at room temperature for 2 h, the membranes were incubated with primary anti-CD146 antibodies and then reacted with corresponding HRP-conjugated secondary antibody. The specific immunoreactive proteins were visualized with enhanced chemiluminescence reagent (Pierce, Rockford, IL).

Immunoprecipitation

1×10^7 cells of A375 were lysed in a culture dish by adding 0.6 mL ice-cold lysis buffer. The supernatant was collected by centrifugation at 12,000 g at 4°C for 10 min and then pre-cleaned with protein A-Sepharose (Santa Cruz Biotechnology, Santa Cruz, CA) to remove the protein A-bound proteins. The total amount of proteins in the pre-cleaned supernatants was measured by Bradford kit (Bio-Rad). Each sample containing 3.2 mg total protein was immunoprecipitated with either anti-CD146 MAbs or control mIgG at 4°C for 2 h, followed by incubation with protein A-Sepharose for 1 h. Immunoprecipitates were washed twice with the lysis buffer and then boiled for 5 min in 1x loading buffer. Samples were analyzed by Western blotting.

Immunohistochemistry

For immunohistochemistry, specimens from human umbilical cord were prepared in frozen or paraffin-embedded tissue sections, according to standard procedures. Sections were incubated with 0.3% H₂O₂ in methanol for 30 min to quench endogenous peroxidases. For paraffin-embedded sections, slides were boiled in Tris-EDTA (pH 9.0) buffer for 30 min to retrieve antigen. After every incubation step, the sections were washed with PBS three times. Sequentially, the sections were blocked with 5% horse serum in PBS at 37°C for 30 min and then incubated with primary antibodies in PBS overnight at 4°C. Appropriate biotin-conjugated secondary antibodies were applied followed by HRP-conjugated streptavidin (Dianova, Rodeo, CA). After every incubation step, the sections were washed with PBS three times. Freshly prepared 3,3'-Diaminobenzidine (DAB) was added to the sections as substrate. The sections were finally counterstained with hematoxylin.

Results

CD146 antigen preparation

In order to obtain monoclonal antibodies binding to the epitopes harbored in the extracellular region of CD146, two kinds of protein antigens were prepared. The full-length purified CD146 protein maintaining the native conformation was used to immunize mice, while recombinant CD146 only containing extracellular domains was employed in screening positive hybridoma clones producing anti-CD146 MAbs. Therefore monoclonal antibodies directly against natural epitopes in the extracellular region of CD146 could be generated and screened out.

Natural human CD146 protein was purified from the lysate of HUVECs using an MAb AA98 protein A-Sepharose column.⁽²³⁾ The purified proteins, which were used as immunogen, were evaluated by SDS-PAGE and found to be >90% pure as estimated by Coomassie blue staining (Fig. 1A [lane 2]). Recombinant CD146 proteins, rhCD146 (aa24-552), containing the extracellular V-V-C2-C2-C2 immunoglobulin domains was expressed in *E. coli*, renatured, and purified as indicated in the Materials and Methods section. The recombinant soluble CD146 proteins were analyzed by protein electrophoresis (Fig. 1A [lane 1]) and applied as antigen in screening of antibodies. Both natural and recombinant CD146 proteins were recognized by MAb AA98 under non-reducing conditions (Fig. 1A [lanes 3 and 4]).

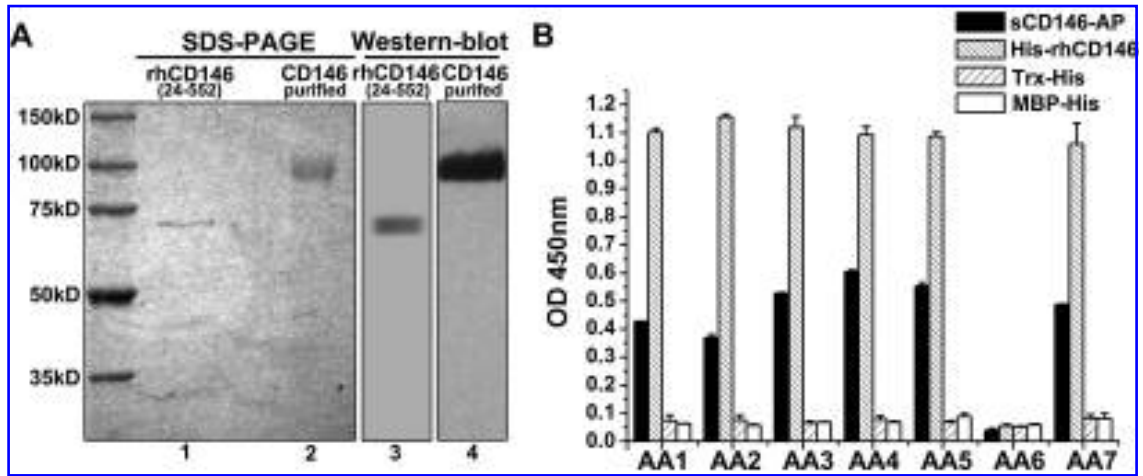


FIG. 1. Anti-CD146 MAbs strongly bound to recombinant human CD146 protein. (A) The purified natural CD146 from HUVEC and recombinant rhCD146 (aa24-552) were examined by SDS-PAGE (lanes 1 and 2) and recognized by MAb AA98 (lanes 3 and 4). (B) The binding activity of anti-CD146 MAbs, AA1-7, to CD146 antigens, including His-rhCD146, sCD146-AP, Trx-His, and MBP-His, respectively. The data represented at least three instances of independent experiments and all samples were tested in triplicate in each experiment.

Generation of monoclonal antibodies against CD146

To establish a panel of monoclonal antibodies to CD146, we immunized mice with natural CD146 protein purified from HUVECs and obtained hundreds of hybridomas, from which 30 hybridomas were identified as anti-CD146 producers. After three rounds of screening using ELISA with rhCD146 (aa24-552) as antigen, six anti-CD146 MAbs with stable and high affinity were selected. These MAbs were referred to as AA1, AA2, AA3, AA4, AA5, and AA7. All of the MAbs were purified from mice ascites and their isotypes were IgG1 κ , as determined by a mouse monoclonal antibody isotyping kit (BD Pharmingen, San Diego, CA). AA6 was also a MAb produced by another hybridoma clone generated along with AA1-5 and AA7. Because of the weak binding activity in screening ELISA assay, AA6 served as a negative control in further study.

Two CD146 fusion proteins, His-rhCD146 and sCD146-AP (Fig. 2A), were used for testing the binding activity of these

MAbs. The His- rhCD146 is a fusion of CD146 (aa24-552) with 6 \times His-tag, expressed in *E. coli* and purified using Ni-NTA column. The sCD146-AP is CD146 (aa24-559) fused with alkaline phosphatase and produced in mammalian cells. As shown in Figure 1B, all MAbs, but not AA6, strongly recognized His-rhCD146, whereas they did not bind to His-Trx and His-Mannose binding protein (His-MBP), indicating their binding was specific to CD146. Recombinant sCD146-AP proteins expressed in eukaryotic cells also reacted with AA1-5 and AA7, but not AA6, further proving the identity of these anti-CD146 MAbs.

Epitope mapping of anti-CD146 monoclonal antibodies

Based on the high immunoreactivity to recombinant CD146 protein, refined epitope mapping was performed to reveal the specific binding sites of these MAbs on CD146 protein. The five extracellular domains, including two IgV and

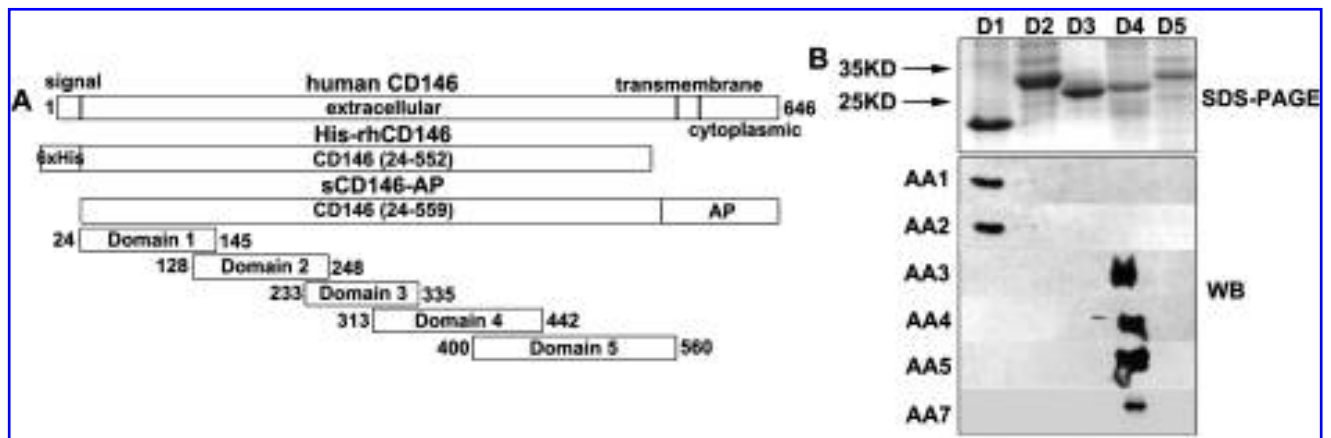


FIG. 2. Epitope mapping of these anti-CD146 antibodies. (A) The schematic presentation of CD146 constructs, including the sketch map of full-length CD146 molecule, two fusion proteins containing the intact extracellular domains, and five fractions in CD146 extracellular region (domains 1-5). (B) Recombinant CD146 fractions were analyzed by SDS-PAGE (the upper panel) and recognized by six anti-CD146 MAbs, respectively (the lower panels).

three IgC2, were expressed in *E. coli*, respectively, and designated D1, D2, D3, D4, and D5 as the sequence of V-V-C2-C2-C2 from extracellular N-terminus to the transmembrane part (Fig. 2A). As indicated in the Materials and Methods section, all of the domains except for D1 were fused with Thioredoxin. The overlapping regions between two neighboring domains were designed to eliminate the possibility of missing linear epitopes. Interestingly, D1, D2, and D4 turned out to be severely insoluble, whereas D3 and D5 solved well, implying different biochemical characteristics of these five domains.

As determined in the Western blotting assay using these recombinant fractions of CD146, AA1 and AA2 only recognized D1 (aa24-145) and the other four MAbs, AA3-5 and AA7, only bound to D4 fraction (Fig. 2B). Since D2 (aa128-248) could not be recognized by AA1 and AA2, epitopes for AA1 and AA2 would be harbored in the region from aa24 to aa128. However, the group of MAbs, including AA3-5 and AA7, recognized the different epitopes from AA1 and AA2, which were located in the non-overlapped D4 region (aa335-381). Based on the epitopes located in the different domains, we divided these six MAbs into two groups: group V1, containing AA1 and AA2, suggests the epitopes in the first IgV domain; group C2-2, including AA3, AA4, AA5, and AA7, means the recognition sites are in the second IgC2 domain. The different epitopes recognized by the two groups of anti-CD146 MAbs implicated possibly different behaviors and binding patterns in biochemical, cell biological, or histological levels.

Meanwhile, previous studies showed that both AA98 and S-endo1 could only bind to CD146 protein in non-reducing form, implicating disulfide bond-based conformational epitopes.^(12,22) Therefore, this epitope mapping study under reducing conditions identified recognition sites of the six newly generated anti-CD146 MAbs, which are demonstrated to be distinguished from epitopes of AA98 and widely used S-endo1. It indicated these MAbs might have new applications for detecting CD146.

Characterization of anti-CD146 monoclonal antibodies

Human melanoma cell line A375 had been reported to express a great amount of CD146 proteins⁽⁹⁾ and was used in characterizing the six anti-CD146 monoclonal antibodies in this study. Western blotting assay showed that all of these anti-CD146 antibodies, unlike MAb AA98, recognized both reduced and non-reduced CD146 protein in A375 cell lysate (Fig. 3A). There was a slight shift from 110 kDa (unreduced) to 130 kDa (reduced), consistent with the previous observation.⁽²²⁾ These results also proved that these MAbs recognized linear epitopes of CD146. It is interesting that used in the same dilution fold AA3-5 and AA7 exhibited greater binding affinities to the reduced CD146, but not the non-reduced form, than did AA1 and AA2 (Fig. 3A; compare lanes 7 and 8 to lanes 9-12), possibly caused by different epitopes.

Flow cytometry analysis displayed that AA1 and AA2 could significantly bind to CD146 molecule expressed on A375 cell surface, compared with the undetectable reactivity of normal IgG (Fig. 3B). Unexpectedly, AA3-5 and AA7 could hardly recognize CD146 protein in the living A375 cells (Fig. 3B). This data hinted that probably epitopes of group V1 MAbs naturally maintained in the living cells; however, the different epitopes recognized by group C2-2 MAbs, including AA3-5 and AA7, could not be exposed on the surface of CD146 protein in its native conformation. Moreover, HUVEC and HMEC could also be stained by AA1 and AA2, but not the other group of MAbs, AA3-5 and AA7 (data not shown). In contrast, U937, a human histiocytic lymphoma cell line, which expresses no CD146,⁽¹⁰⁾ was not recognized by the panel of anti-CD146 antibodies (data not shown). It supported the specificity of these MAbs directly against CD146.

The binding capabilities of these anti-CD146 MAbs to cell membrane-uncoupled CD146 were further characterized by immunoprecipitation assay. The A375 cell lysates were first immunoprecipitated by the panel of anti-CD146 MAbs respectively and then captured immunocomplexes were analyzed in Western blotting using previously proved rabbit

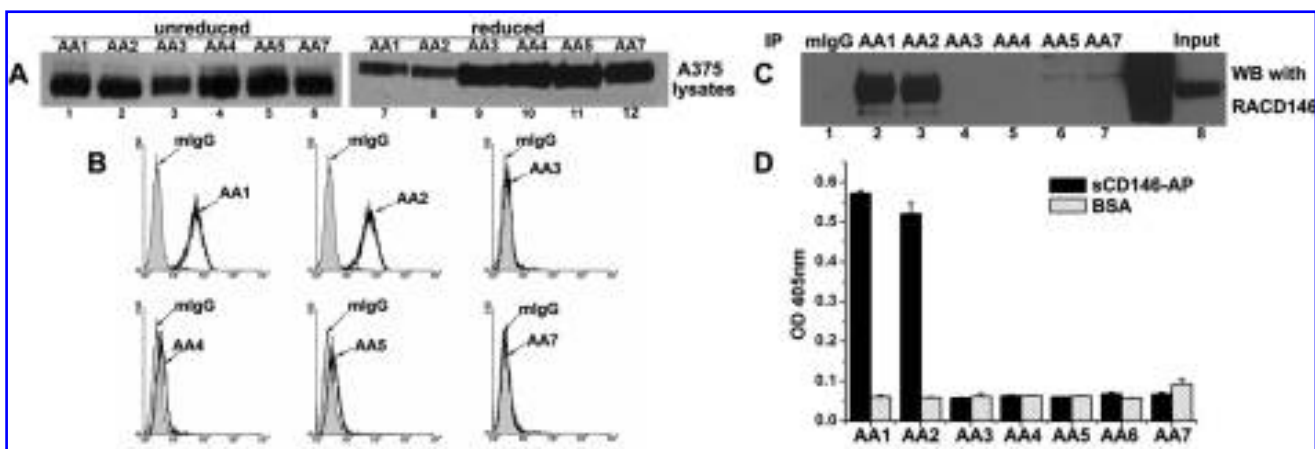


FIG. 3. Characterization of anti-CD146 monoclonal antibodies. (A) All anti-CD146 monoclonal antibodies bound to human CD146 from A375 cell lysates under both unreduced (lanes 1-6) and reduced (lanes 7-12) conditions. (B) Flow cytometry assay showed only AA1 and AA2, rather than AA3, AA4, AA5, AA7, and mIgG control, bound to CD146 protein on A375 cells. (C) CD146 was immunoprecipitated by AA1 and AA2 from A375 cell lysates, but not by AA3, AA4, AA5, AA7, and mIgG control. The CD146 either from precipitates or A375 cell lysates (input) was recognized by rabbit-anti-human CD146 polyclonal antibody (RACD146). (D) Seven anti-CD146 MAbs showed their different abilities for capturing sCD146-AP fusion proteins, which was detected by PNPP, a kind of specific substrate for alkaline phosphatase.

polyclonal anti-CD146 antibody RACD146. Figure 3C shows that proteins pulled down by either AA1 or AA2, but not normal IgG, reacted with RACD146 (lanes 1 and 2), demonstrating the authenticity of the two MAbs targeting CD146. The presence and molecular weight of CD146 protein in cell lysates was also tested (lane 8). Again, AA3–5 and AA7 were unable to bind to the natural CD146 protein released from the cell membrane (lanes 4–7). Interestingly, unlike the results in Figure 1B, when the seven anti-CD146 antibodies were coated on the ELISA plate respectively, only AA1 and AA2, but not AA3–5 and AA7, captured the sCD146-AP protein in the solution, of which the AP (alkaline phosphatase) activity was then detected by PNPP, a kind of specific substrate for alkaline phosphatase (Fig. 3D).

Taken together, biochemical studies implicated that the CD146 protein in native conformation cannot be detected by AA3–5 and AA7. However, denaturing in either SDS-PAGE loading buffer or alkaline ELISA coating buffer (pH 9.6) leads to exposure of their epitopes. Protein sequence analysis found many hydrophobic amino acids in the region (aa335–381) containing epitopes of group C2-2 MAbs, which indicated they might be the potentially structural components of the hydrophobic core of the second IgC2 domain, namely D4, and be buried inside this domain. It might explain the weak binding activity of antibodies in this group to the natural CD146. In contrast, both AA1 and AA2

strongly bind to CD146 molecules in both natural and denatured forms, indicating linear epitopes on the surface of the protein.

Subcellular localization of CD146

Immunofluorescent staining using anti-CD146 MAbs confirmed the subcellular localization of CD146 in A375 cells. As a transmembrane cell adhesion molecule, CD146 expression had been observed to be restrained on the cell membrane when AA1 or AA2 was employed to stain the fixed cells (Fig. 4A). However, consistent with the test of flow cytometry, AA3–5 and AA7 failed to recognize CD146 in this assay, as well as the negative control using only normal murine IgG. It indicated epitopes of group C2-2 MAbs might also not be exposed in the membrane-linked form of human CD146 protein.

Differential application of anti-CD146 monoclonal antibodies in immunohistochemistry

Besides being overexpressed in melanoma, CD146 is also reported to express in some normal tissues, such as endothelium and smooth muscle.⁽⁷⁾ To further characterize the panel of anti-CD146 MAbs, frozen and paraffin-embedded sections of human umbilical cord were involved in studying the histological distribution of CD146 expression using the

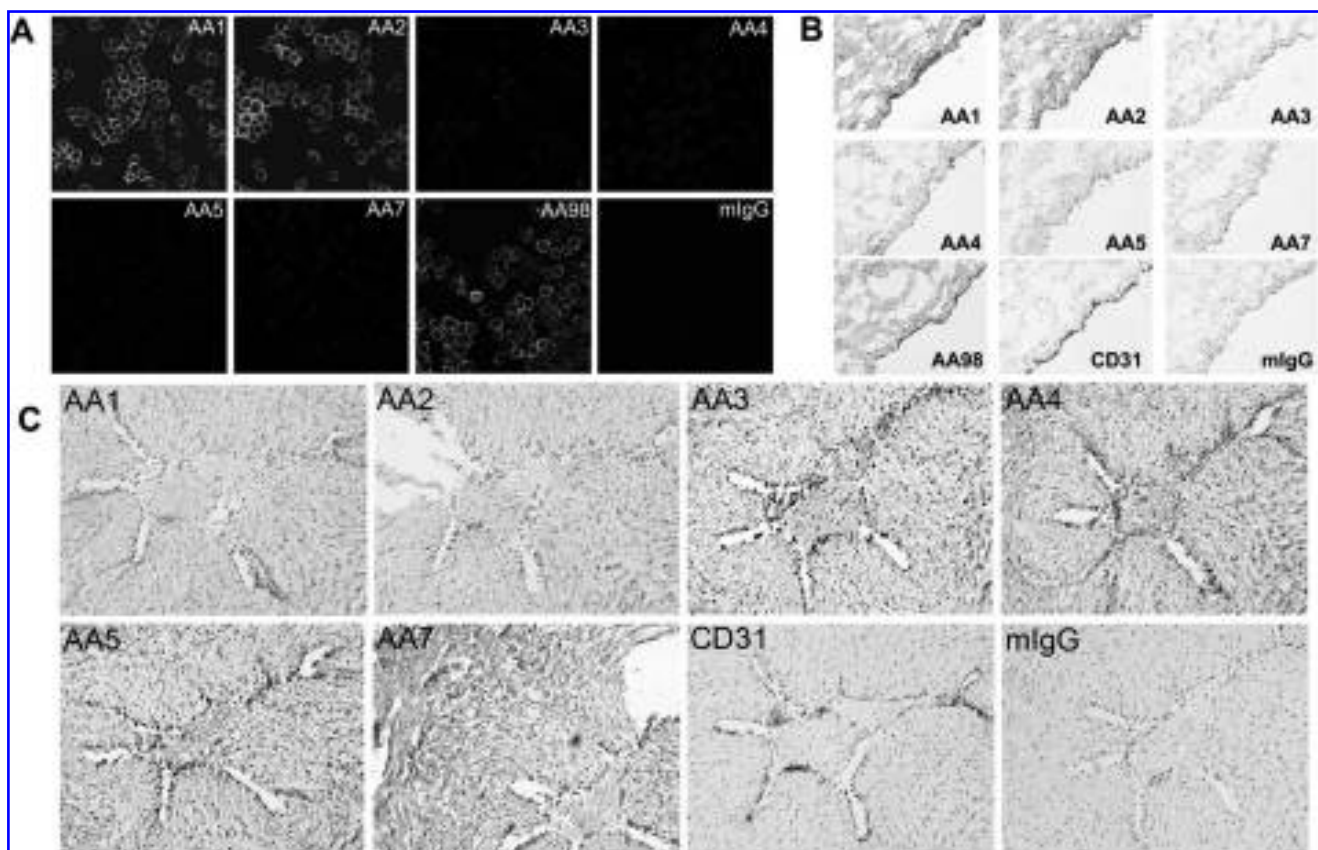


FIG. 4. Subcellular and histological distribution of CD146. Binding of anti-CD146 MAbs AA1–AA5 and AA7 to A375 cells and human umbilical cord vein and artery were determined by cellular immunofluorescent (A) and immunohistochemical assay (B and C). (B) Frozen sections of umbilical cord vein and (C) the paraffin-embedded artery tissue sections. MAbs AA9B and anti-CD31 served as positive control and mIgG as negative control.

method of immunohistochemistry. Anti-CD31 antibody was applied to identify the umbilical vein endothelium. As shown in Figure 4B, on these frozen sections, either AA1 or AA2 could recognize CD146 protein expressed in both the endothelium labeled by anti-CD31 antibody and the smooth muscle encompassing the vessel endothelial layer. This result is consistent with previous studies concerning the CD146 expression spectrum in normal tissues. Interestingly, when applying these antibodies in paraffin-embedded sections, only MAbs in group C2-2, AA3-5 and AA7, recognized CD146 expressed in the vascular smooth muscle cells (VSMC) and the artery endothelium in umbilical artery tissues (Fig. 4C). Both AA1 and AA2 could hardly stain the paraffin-embedded sections. These data may suggest that the natural epitopes of group V1 MAbs were lost or rearranged during the section preparations; however, epitopes of group C2-2 MAbs, originally buried inside, were somehow exposed onto the surface when denaturing the antigen with ethanol fixation and heated paraffin embedding. Therefore, two groups of anti-CD146 antibodies would differentially apply in histological detection of CD146, which are mainly caused by different recognition epitopes.

Discussion

CD146 is a newly discovered cell adhesion molecule, which is significantly overexpressed in melanoma cells and plays a critical role in tumor growth and metastasis. Although its expression can be detected in a limited spectrum of normal tissues, anti-CD146 antibodies have been involved in oncological diagnosis and cancer therapy for melanoma. Both ABX-MA1, a fully human anti-CD146 antibody generated by Mills and colleagues,⁽²⁰⁾ and AA98, monoclonal anti-CD146 antibody reported in our previous works,⁽²²⁾ can dramatically inhibit melanoma cell growth, migration, and invasion in animal model. Particularly, our works showed AA98 remarkably suppresses tumor angiogenesis through blocking NF κ B activation in endothelial cells, indicating a possible new role for CD146 in vascular and cancer biology.

In the present study, we generated a panel of anti-CD146 antibodies through immunizing mice with purified natural CD146 protein. ELISA using recombinant extracellular region of CD146 and Western blotting assay with A375 cell lysate showed all of the six anti-CD146 antibodies could significantly bind to CD146 protein. Furthermore, by the method of Western blotting, all these MAbs could not recognize the murine CD146 molecule (also called gicerin) in B16 cell lysate,⁽²¹⁾ while rabbit polyclonal anti-human CD146 antibody, as the positive control, got strong signals (data not shown). This result demonstrates the species specificity of these MAbs to human CD146 and rules out species cross-reactivity.

Epitope mapping employing five recombinant extracellular fractions of CD146 found that AA1 and AA2 recognized epitopes located in the region from aa24 to aa127, while the recognition sites of AA3-5 and AA7 were all restrained in the fragment from aa336 to aa380. Based on the different epitopes, these six MAbs are divided into two groups designated by the name of Ig-like domains harboring these epitopes (group V1: AA1 and AA2; group C2-2: AA3, AA4, AA5, and AA7). Interestingly, only the MAbs in group V1 could recognize CD146 proteins in the levels of living cells and frozen tissue sections, whereas

only group C2-2 antibodies stained the paraffin-embedded sections in which antigens were denaturalized. Further analysis of the protein sequences in the region (aa336-380: QELLVNYVSDVRVSPAAPERQEGSSLTLTCE AESSQDLE-FQWLRE) discovered that two-fifths (18/45) of these amino acids are hydrophobic, implying a possible component for the hydrophobic core of the second IgC2 domain. Thus, epitopes of group C2-2 MAbs might not be exposed on the protein surface, resulting in no reactivity for these antibodies in flow cytometry assay, immunofluorescence, and immunohistochemical assays of frozen sections. However, the capability of group C2-2 antibodies to stain the paraffin-embedded tissue sections offered them broad application in histological tests, because most of currently used anti-CD146 MAbs can only stain frozen sections.

Moreover the pI of sCD146-AP fusion protein has been theoretically calculated to be around 5.5, which explains the alkaline environment (pH 9.7)-caused denaturalization and epitope exposure when coating recombinant CD146 proteins on the plate and detecting with group C2-2 MAbs (Fig. 1B). Conversely, sCD146-AP could not be captured by group C2-2 MAbs coated on the solid phase (Fig. 3D) because the neutral buffer (pH 7.2) maintained the native conformation and kept these epitopes covered.

To sum up, this study generated and characterized a panel of monoclonal antibodies against distinct epitopes of human CD146. Based on their different binding sites, the combination of the two groups of MAbs, or with AA98, recognizing another conformational epitope, could generate new sandwich ELISA methods to detect CD146 in solution. These MAbs provide new tools for studying expression and function of CD146.

Acknowledgment

This work was partly supported by grants from the Chinese Ministry of Sciences 863 Project (2006AA02A245), NSFC foundation (nos. 90406020 and 30672436), the Knowledge of Innovation Program of the Chinese Academy of Sciences (KSCX2-YW-R-121, KJCX2.YW.nano02), the Chinese Ministry of Sciences 973 Project (2006CB933204), and MEXT of Japan.

References

1. Holness CL, and Simmons DL: Structural motifs for recognition and adhesion in members of the immunoglobulin superfamily. *J Cell Sci* 1994;107(Pt8):2065-2070.
2. Lehmann JM, Riethmuller G, and Johnson JP: MUC18, a marker of tumor progression in human melanoma, shows sequence similarity to the neural cell adhesion molecules of the immunoglobulin superfamily. *Proc Natl Acad Sci USA* 1989;86(24): 9891-9895.
3. Sers C, Kirsch K, Rothbacher U, Riethmuller G, and Johnson JP: Genomic organization of the melanoma-associated glycoprotein MUC18: implications for the evolution of the immunoglobulin domains. *Proc Natl Acad Sci USA* 1993; 90(18):8514-8518.
4. Lehmann JM, Holzmann B, Breitbart EW, Schmiegelow P, Riethmuller G, and Johnson JP: Discrimination between benign and malignant cells of melanocytic lineage by two novel antigens, a glycoprotein with a molecular weight of 113,000 and a protein with a molecular weight of 76,000. *Cancer Res* 1987;47(3):841-845.

5. Johnson JP, Rothbacher U, and Sers C: The progression associated antigen MUC18: a unique member of the immunoglobulin supergene family. *Melanoma Res* 1993;3(5):337–340.
6. Shih IM, Elder DE, Speicher D, Johnson JP, and Herlyn M: Isolation and functional characterization of the A32 melanoma-associated antigen. *Cancer Res* 1994;54(9):2514–2520.
7. Shih IM, Nesbit M, Herlyn M, and Kurman RJ: A new Mel-CAM (CD146)-specific monoclonal antibody, MN-4, on paraffin-embedded tissue. *Mod Pathol* 1998;11(11):1098–1106.
8. Shih IM: The role of CD146 (Mel-CAM) in biology and pathology. *J Pathol* 1999;189(1): 4–11.
9. Xie S, Luca M, Huang S, Gutman M, Reich R, Johnson JP, and Bar-Eli M: Expression of MCAM/MUC18 by human melanoma cells leads to increased tumor growth and metastasis. *Cancer Res* 1997;57(11): 2295–2303.
10. Shih IM, Speicher D, Hsu MY, Levine E, and Herlyn M: Melanoma cell-cell interactions are mediated through heterophilic Mel-CAM/ligand adhesion. *Cancer Res* 1997;57(17):3835–3840.
11. Bani MR, Rak J, Adachi D, Wiltshire R, Trent JM, Kerbel RS, and Ben-David Y: Multiple features of advanced melanoma recapitulated in tumorigenic variants of early stage (radial growth phase) human melanoma cell lines: evidence for a dominant phenotype. *Cancer Res* 1996;56(13):3075–3086.
12. Bardin N, Frances V, Lesaule G, Horschowski N, George F, and Sampol J: Identification of the S-Endo 1 endothelial-associated antigen. *Biochem Biophys Res Commun* 1996;218(1):210–216.
13. Bardin N, Anfosso F, Masse JM, Cramer E, Sabatier F, Le Bivic A, Sampol J, and Dignat-George F: Identification of CD146 as a component of the endothelial junction involved in the control of cell-cell cohesion. *Blood* 2001;98(13):3677–3684.
14. St Croix B, Rago C, Velculescu V, Traverso G, Romans KE, Montgomery E, Lal A, Riggins GJ, Lengauer C, Vogelstein B, and Kinzler KW: Genes expressed in human tumor endothelium. *Science* 2000;289(5482):1197–1202.
15. Bardin N, George F, Mutin M, Brisson C, Horschowski N, Frances V, Lesaule G, and Sampol J: S-Endo 1, a pan-endothelial monoclonal antibody recognizing a novel human endothelial antigen. *Tissue Antigens* 1996;48(5):531–539.
16. George F, Poncelet P, Laurent JC, Massot O, Arnoux D, Lequeux N, Ambrosi P, Chicheportiche C, and Sampol J: Cytofluorometric detection of human endothelial cells in whole blood using S-Endo 1 monoclonal antibody. *J Immunol Methods* 1991;139(1):65–75.
17. Solovey AN, Gui L, Chang L, Enestein J, Browne PV, and Hebbel RP: Identification and functional assessment of endothelial P1H12. *J Lab Clin Med* 2001;138(5):322–331.
18. Anfosso F, Bardin N, Frances V, Vivier E, Camoin-Jau L, Sampol J, and Dignat-George F: Activation of human endothelial cells via S-endo-1 antigen (CD146) stimulates the tyrosine phosphorylation of focal adhesion kinase p125(FAK). *J Biol Chem* 1998;273(41):26852–26856.
19. Anfosso F, Bardin N, Vivier E, Sabatier F, Sampol J, and Dignat-George F: Outside-in signaling pathway linked to CD146 engagement in human endothelial cells. *J Biol Chem* 2001;276(2):1564–1569.
20. Mills L, Tellez C, Huang S, Baker C, McCarty M, Green L, Gudas JM, Feng X, and Bar-Eli M: Fully human antibodies to MCAM/MUC18 inhibit tumor growth and metastasis of human melanoma. *Cancer Res* 2002;62(17):5106–5114.
21. Okumura S, Muraoka O, Tsukamoto Y, Tanaka H, Kohama K, Miki N, and Taira E: Involvement of gicerin in the extension of microvilli. *Exp Cell Res* 2001;271(2):269–276.
22. Yan X, Lin Y, Yang D, Shen Y, Yuan M, Zhang Z, Li P, Xia H, Li L, Luo D, Liu Q, Mann K, and Bader BL: A novel anti-CD146 monoclonal antibody, AA98, inhibits angiogenesis and tumor growth. *Blood* 2003;102(1):184–191.
23. Bu P, Zhuang J, Feng J, Yang D, Shen X, and Yan X: Visualization of CD146 dimerization and its regulation in living cells. *Biochim Biophys Acta* 2007;1773(4):513–520.
24. Bu P, Gao L, Zhuang J, Feng J, Yang D, and Yan X: Anti-CD146 monoclonal antibody AA98 inhibits angiogenesis via suppression of nuclear factor-kappaB activation. *Mol Cancer Ther* 2006;5(11):2872–2878.
25. Kuzu I, Bicknell R, Fletcher CD, and Gatter KC: Expression of adhesion molecules on the endothelium of normal tissue vessels and vascular tumors. *Lab Invest* 1993;69(3):322–328.
26. Jaffe EA, Nachman RL, Becker CG, and Minick CR: Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest* 1973;52(11):2745–2756.

Address reprint requests to:

Xiyun Yan

National Laboratory of Biomacromolecules

University of Tokyo Joint Laboratory of Structural Virology

and Immunology

Chinese Academy of Sciences

15 Datun Road

Beijing 100101

China

E-mail: yanxy@sun5.ibp.ac.cn

Received: April 29, 2008

Accepted: June 9, 2008