Monoclonal Antibody to Human Esophageal Cancer Endothelium Inhibits Angiogenesis and Tumor Growth

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Abstract. Background: Monoclonal antibodies to tumor endothelial cells (TECs) hold great promise for cancer angiogenesis-targeted therapy. The aim of the present study was to develop such an agent for esophageal cancer treatment. Materials and Methods: BALB/c mice were immunized with human esophageal tumor endothelial cells (ETECs) cultured with tumor homogenate. MAbs were produced, screened by immunofluorescence and immunohistochemistry (IHC) and an IgG1 κ mAb 4B3 was selected. The mAb 4B3 antigen was analyzed by IHC and Western blotting. The antibody’s effects on ETECs were determined by adhesion and tube formation assays, while its therapeutic potential was evaluated with a tumor model established by co-inoculating mice with the human esophageal cancer cell lines KYSE180 and ETECs. Results: MAb 4B3 recognized a 40-kDa surface antigen preferentially expressed on TECs and other stromal cells in human malignant tissues of esophagus, stomach, colon, liver, lung and breast compared with their normal counterparts. The antigen was not detected on cancer cells or normal epithelia in these tissues, nor was it detectable on any cells in the mouse xenografts of KYSE180, including the host endothelia. MAb 4B3 inhibited ETEC adhesion to extracellular matrix proteins and tube formation in vitro. The antibody inhibited angiogenesis and growth of the tumor formed by co-inoculation. Conclusion: These results suggest that mAb 4B3 has therapeutic potential for esophageal cancer.

Esophageal cancer is the eighth most common tumor and the sixth leading cause of cancer death worldwide (1). Despite recent improvements in traditional therapy, overall survival is still very poor, with over 90% of patients succumbing to the disease (2). Therefore, developing new strategies and therapeutic agents for the management of the malignancy is an urgent necessity.

Anti-angiogenic therapy is emerging as a promising strategy because the growth and metastasis of esophageal tumors, like other solid tumors, depend on angiogenesis, the formation of new blood vessels from pre-existing ones (3, 4). The blockade of angiogenesis suppresses tumor development in many preclinical models, while Avastin, a recombinant humanized monoclonal antibody (mAb) against VEGF, has demonstrated therapeutic efficacy in treating human metastatic colorectal cancer (5). Among a number of anti-angiogenic agents that are under active investigation both in the laboratory and the clinic, mAbs against tumor endothelial cell (TEC) surface antigens are a group of molecules crucial for angiogenesis. This type of agent not only easily and preferentially binds to TECs in vivo, but also interferes with the proliferation, adhesion and/or migration of the targeted cells, or simply destroys them, resulting in the inhibition of angiogenesis and tumor development. However, the specificity and curative effects of the existing TEC mAbs are still not sufficient and no such mAb or its derivative is currently in clinical use (6). On the other hand, the genetic and molecular events underlying the structural and functional differences between normal and tumor vasculature are constantly being revealed (7-9), suggesting the possibility of producing more specific and clinically meaningful TEC mAbs.

To generate antibodies valuable for the treatment of esophageal cancer, BALB/c mice were immunized with human esophageal tumor endothelial cells (ETECs) and mAb 4B3, that recognizes a surface antigen preferentially expressed on tumor blood vessel endothelial cells and other stromal cells, was raised. In vitro functional analysis indicated that mAb 4B3 reduced the adhesion of ETECs to extracellular matrix (ECM) proteins and ETEC tube

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Key Words: Monoclonal antibody, esophageal cancer, endothelium, angiogenesis.
formation. When injected in vivo, the antibody inhibited angiogenesis and tumor growth. Our findings suggest that mAb 4B3 has therapeutic potential for esophageal cancer.

Materials and Methods

Cell culture. Human ETEC were isolated from fresh samples of squamous cell carcinoma and identified as described previously (10). The ETECs were cultured on 2% gelatin-coated (Sigma Chemical, St. Louis, MO, USA) flasks or plates (Corning, Cambridge, MA, USA) in Medium 199 (HyClone Laboratories, Logan, UT, USA) supplemented with 20% fetal calf serum (FCS; Beijing Yuanheng Biotechnology, Beijing, China). 50 µg/ml endothelial cell growth supplement (ECGS; Sigma), 16 µg/ml human esophageal tumor homogenate, 100 µg/ml heparin (Sigma), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mmol/l L-glutamine in an atmosphere of 5% CO2 and 95% air at 37°C. The human esophageal squamous cell carcinoma cell line KYSE180 (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) was cultured in PRMI 1640Medium (HyClone) supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mmol/l L-glutamine. Esophageal tumor homogenate was prepared as follows: pooled tumor tissues were minced and homogenized in DMEM supplemented with 10% FCS on ice. After centrifuging, the supernatant was collected, dialyzed against phosphate-buffered saline (PBS), sterilized by passing through filters (0.45 µm; Millipore, Billerica, MA, USA) and frozen at -80°C until use. The protein concentration was determined by a spectrophotometer at 280 nm.

Generation of mAbs. ETECs cultured to passages 8 to 10 were detached with a rubber policeman. The cells were fixed with 4% paraformaldehyde for 24 h at 4°C, washed three times with PBS and used as an immunogen (11). Female 6-week-old BALB/c mice (Experimental Animal Center of Peking University Medical College, Beijing, China) were injected subcutaneously with 1x108 fixed cells weekly until the serum titer reached 1:40000, determined by a spectrophotometer at 280 nm.

Immunohistochemistry (IHC). The tissues were fixed in 10% neutral buffered formalin overnight at 4°C, embedded in paraffin and sectioned (6 µm thick). The tissue sections were deparaffinized in xylene and rehydrated in graded alcohol. Antigen retrieval was performed with heat treatment. Immunoperoxidase staining was carried out using an UltraVision Anti-Polyvalent kit (for human tissues) or an UltraVision Mouse-on-Mouse kit (for mouse tissues, Lab Vision, Fremont, CA, USA), according to the manufacturer’s instructions. Briefly, the tissue sections were incubated with various blocking solutions. The sections were then incubated for 16 h at 4°C with hybridoma supernatants at varying dilutions, polyclonal rabbit anti-vWF (A0082, Dako, Glostrup, Denmark) diluted 1:500 or monoclonal mouse anti-human vWF (F8/86, Dako) diluted 1:200. After washing, the slides were incubated with biotinylated goat anti-mouse/rabbit followed by streptavidin-peroxidase. DAB substrate was applied for 5 min to develop the stain. The sections were washed and counterstained with Mayer’s hematoxylin, dehydrated and mounted with neutral resin. For assessment of vessel density, vWF-positive vessels in ten areas with relatively high vascular density (hot spots) were counted in three separate x400 fields (14).

Western blotting. ETEC membrane proteins were prepared as described with slight modification (15). Briefly, one volume of TSA buffer [10 mM Tris-HCl pH 8.0, 140 mM NaCl, 0.025% NaN3], one volume of lysis buffer (TSA solution with 2% TritonX-100) and proteinase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 5 µg/ml aprotinin, and 5 µg/ml leupeptin) were added to the cells, followed by stirring at 4°C for 1 h. The lysate was centrifuged for 10 min at 4000 xg to remove nuclei. The supernatant was separated by 8% SDS-PAGE under non-reducing and reducing conditions and electrophoretically transferred to the nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The membrane was blocked in 5% non-fat milk for 1 h, incubated with hybridoma supernatant for 2 h and then incubated with 1:1000 diluted peroxidase-conjugated anti-mouse for 1 h, all at room temperature. The proteins were visualized using a chemiluminescence detection system (Pierce, Rockford, IL, USA).

Adhesion assay. A 96-well plate was coated with 10 µg/ml Matrigel (BD Biosciences, San Jose, CA, USA) overnight at 4°C. The remaining protein binding sites were blocked with 1% BSA in PBS for 1 h at 37°C. The ETECs were grown to confluence and labeled with 5 µM Calcein-AM fluorophore (Molecular Probes, Eugene, OR, USA) as described (16). The cells were trypsinized, washed and resuspended at 5x104 cells/ml in serum-free medium containing 1% BSA. The cells were then mixed with varying amounts of mAb 4B3 or isotype-matched antibody (mIgG, Sigma) for 1 h at 37°C. After incubation, 5x104 cells were added to each well and the plates were incubated for 30 min at 37°C in 5% CO2. Non-adherent cells were removed by gentle rotation washing with serum-free medium,
calculated as \( \frac{\pi}{6} \times \text{length} \times \text{width}^2 \). The tumors were weighed at the termination of the experiment.

**In vivo tumor therapy.** Twenty-four female BALB/c nude mice aged 5 to 6 weeks (Weitonglihua Biotechnology, Beijing, China) were inoculated s.c. with a mixture of \( 4 \times 10^6 \) ETECs and \( 1.5 \times 10^6 \) KYSE180. The tumors were allowed to reach a diameter of 3 to 5 mm and were then randomly divided into three groups of eight animals. These groups received i.p. injections of 200 \( \mu \)g/mouse of mAb 4B3, mIgG or PBS, respectively, twice a week for 3 weeks. Tumor volumes were measured with a caliper twice weekly and calculated as \( \pi/6 \times \text{length} \times \text{width}^2 \). The tumors were weighed at the termination of the experiment.

**Statistical analysis.** The values are expressed as mean±SD and compared by the Student’s 2-tailed \( t \)-test. Differences with \( p<0.05 \) were considered significant.

**Results**

**Producing and screening of mAbs binding to ETEC surface antigens.** Two hundred and eighty-five mAbs which react with ETECs, as determined by ICC, were produced. Since antibodies that affect cell function are probably those which recognize antigens on the cell surface, these mAbs were then screened using live cell immunofluorescence. Seventy-three mAbs were found to bind to the ETEC surface, among which was an IgG1κ mAb 4B3 (Figure 1).

**Screening of mAbs which preferentially react with tumor vessel endothelia.** To obtain therapeutically significant antibodies, five human esophageal carcinoma and adjacent normal tissues were subjected to IHC analysis with all the 73 surface antigen-recognizing mAbs. Eleven antibodies preferentially reacted with tumor vessel endothelial cells (ECs), as opposed to normal ECs. Further IHC analysis of esophagus, stomach, colon, liver, lung and breast carcinomas (44, 30, 29, 27, 29 and 30 cases, respectively), as well as the adjacent normal tissues, demonstrated that mAb 4B3 reacted with EC and other stromal cells such as fibroblast in esophageal carcinoma tissues at a rate of 84% (41/49), while it only reacted slightly with the normal counterparts at a rate of 12% (6/49). The staining with tumor stromal cells was weaker than with TECs. This antibody exhibited a similar staining pattern in other cancer and normal tissues examined. The antibody did not react with cancer cells or normal epithelial cells (Figure 2).

Due to the expression specificity and subcellular location of the antigen recognized by mAb 4B3, this antibody was chosen for further study.

**Characterization of antigen recognized by mAb 4B3.** Immunoblotting revealed that mAb 4B3 recognized a protein of approximately 40 kDa under both reducing and non-reducing conditions (Figure 3).
AR 5, mAb 4B3 inhibited ETEC tube formation significantly. A dose-dependent inhibition was observed in the range of 25 to 100 μg/ml.

MAb 4B3 inhibits tumor angiogenesis and growth. MAb 4B3 did not recognize any host cells, including blood vessel ECs in the human esophageal carcinoma cell line KYSE180 xenograft in mouse, nor did it recognize KYSE180 either in vitro or in vivo (data not shown). Preliminary experiments showed that ETECs co-inoculated with KYSE180 in nude mice survived, assembled to many humanized blood vessels (wholly human or human-murine chimeric vessels) and profoundly promoted tumor growth, while ETECs alone did not. To evaluate the effects of mAb 4B3 on tumor angiogenesis and growth, mice were s.c. injected with a mixture of 4x10⁶ ETECs and 1.5x10⁶ KYSE180. The tumors were allowed to reach a diameter of 3 to 5 mm and 200 μg/mouse of mAb 4B3, mIgG or PBS were given twice a week for 3 weeks. The tumor volume and

Figure 2. Immunohistochemical staining with mAb 4B3 on human malignant and normal tissues. Formalin-fixed, paraffin-embedded sections were deparaffinized. Immunoperoxidase staining was performed using UltraVision Anti-Polyvalent kit, with mAb 4B3 as the primary agent, followed by biotinylated goat anti-mouse antibody and streptavidin-peroxidase as subsequent agents. Stains were developed with DAB. Strong staining of tumor vessel EC and other stromal cells was found in carcinomas of (A) esophagus, (B) stomach, (C) colon, (D) liver, (E) lung and (F) breast. Staining was negative or weak in adjacent normal tissues (a, b, c, d, e and f). Bar, 50 μm.

Figure 3. Western blotting analysis of 4B3 antigen. ETEC membrane proteins were prepared, electrophoresed on 8% SDS-PAGE under both reducing (A) and non-reducing (B) conditions, and transferred to nitrocellulose membrane. The membrane was incubated with mAb 4B3 followed by peroxidase-conjugated anti-mouse antibody. Proteins were visualized with a chemiluminescence detection system. MAb 4B3 identified a membrane protein of about 40 kDa.
Figure 4. Effect of mAb 4B3 on ETEC adhesion to Matrigel. ETECs labelled with Calcein AM and incubated with control mIgG or mAb 4B3 were allowed to adhere to Matrigel-coated 96-well plate. After incubation and washing, bound cells were photographed and counted. (A) Representative pictures of adhesion of ETECs incubated with 100 µg/ml mIgG or mAb 4B3. (B) Percentage of adherent cells treated with mAb 4B3 to control. The inhibitory effect of mAb 4B3 was dose-dependent with maximal inhibition at 50 to 200 µg/ml. This experiment was performed in triplicate and repeated three times with similar results. *p<0.05.

Figure 5. Effect of mAb 4B3 on ETEC in vitro angiogenesis. MAb 4B3 or control mIgG-treated ETECs were seeded on Matrigel. Tube formation was assessed 6 h later. (A) Representative photographs of the tube formation of ETECs incubated with 100 µg/ml mIgG or mAb 4B3. (B) Quantification of branching points of the tubular network in x100 field. MAb 4B3 inhibited tube formation in a dose-dependent manner. This experiment was performed in triplicate and repeated three times with similar results. *p<0.05.

Figure 6. Effect of mAb 4B3 on tumor angiogenesis and growth. A mixture of 4x10⁶ ETECs and 1.5x10⁶ KYSE180 was inoculated in nude mice (n=8), which were then treated with mAb 4B3, mIgG or PBS. The tumor volumes were monitored (A) and weights were measured on termination of the experiment (B). Serial sections of mIgG- and 4B3-treated tumors were stained with the indicated antibodies (C), total and humanized blood vessel densities were counted and the difference (mouse vessel density) was calculated (D). MAb 4B3 inhibited tumor angiogenesis and growth. Bar, 50 µm. *p<0.05; **p<0.01.
ETECs cultured with esophageal tumor tissue homogenate identified in this way. In the present study, we used human simulations, relatively few TEC markers have been quiescent ECs (15, 22). Partially due to the imprecise hybridomas which react differentially with stimulated and raise antibodies in this approach, followed by screening of tumor-conditioned medium are utilized as immunogens to umbilical vein endothelial cells (HUVEC) stimulated with has been used for decades and a few such molecules were to cell adhesion.

The antibody-based approach to identify TEC markers has been used for decades and a few such molecules were validated (20, 21). Usually, surrogate TECs such as human umbilical vein endothelial cells (HUVEC) stimulated with tumor-conditioned medium are utilized as immunogens to raise antibodies in this approach, followed by screening of hybridomas which react differentially with stimulated and quiescent ECs (15, 22). Partially due to the imprecise simulations, relatively few TEC markers have been identified in this way. In the present study, we used human ETECs cultured with esophageal tumor tissue homogenate to immunize BALB/c mice and screened the differentially-reacting hybridomas directly with malignant and normal tissues of the esophagus. To select those mAbs that possibly affected cell behavior, and to decrease the workload, we first isolated membrane epitope-binding mAbs using live cell immunofluorescence. As a result, mAb 4B3 and another ten antibodies preferentially reacting with TECs were identified. Extensive IHC analysis indicated that mAb 4B3 also preferentially reacts with TECs in five other common tumors examined. The antibody also reacted with tumor stromal cells such as fibroblast, but the staining was weaker. MAb 4B3 did not react with any parenchyma cells. These findings suggest that the 4B3 antigen is a possible molecular target for anti-angiogenic therapy, and that the immunizing and screening strategy reported here is efficient to generate mAbs which preferentially react with TECs.

MAbs that disturb the functions of TECs are especially valuable for tumor angiogenesis-targeted therapy. This class of antibodies can be used, not only as therapeutic agents after humanizing, but also as effective tools to identify the corresponding antigens which may serve as therapeutic molecular targets. To verify whether mAb 4B3 had this property, we examined the in vitro effects of the antibody on ETECs. While no inhibitory effect on proliferation and migration was observed, the antibody inhibited adhesion and angiogenesis of the cell. These results prompted us to investigate the in vivo effects of the antibody on tumor angiogenesis and growth. However, preliminary experiments indicated that mAb 4B3 did not cross-react with mouse blood vessel ECs in transplanted human tumor, a common problem confronted by others (23). Several methods have been developed to solve this problem, such as injecting human adult organ-derived ECs into an established tumor (24), or inoculating cancer cells into human skin xenograft (25). Notably, Tei et al. introduced a model in which murine ECs were co-transplanted with human cancer cells into nude rats (26). The murine ECs survived and profoundly promoted the growth of tumor. We co-transplanted ETECs and KYSE180 into nude mice to establish tumors with humanized blood vessels. Our observations were consistent with those for the murine EC model. Using the humanized blood vessel tumor model, we confirmed the in vitro anti-angiogenic effect of mAb 4B3. Importantly, the antibody significantly inhibited tumor growth. Naked antibodies have a complex mechanism of action to block tumor growth. They can activate antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity, induce cell apoptosis and cell cycle arrest, or inhibit cell proliferation as well as angiogenesis (27). IHC analysis showed that treatment with mAb 4B3 induced a 21% reduction in total blood vessel density and a 48% reduction in humanized blood vessel density without any apparent inflammation, obstruction or necrosis in the tissues. The antibody did not recognize
KYSE180. These findings and those of the in vitro functional analysis collectively indicate: (a) that mAb 4B3 inhibited tumor growth by means of inhibiting tumor angiogenesis, and (b) that the anti-angiogenic effect may partially be attributable to the inhibition of adhesion events in the process of angiogenesis. Considering the low percentage (about 34%, as determined by preliminary experiments; a similar percentage can be estimated from data presented in Figure 6 D, mIgG group) of humanized blood vessels in our tumor model and the significant inhibition of tumor growth (51% in tumor weight), the application of mAb 4B3 derivatives in the treatment of human esophageal cancer might hold promise.

Expression specificity, molecular weight and functional characteristics suggest that the 4B3 antigen differs from the known TEC markers such as fibronectin extra-domain B, annexin A1, large tenascin-C isoforms, integrin α5β3, VEGF receptors, CD105, CD44H, prostate-specific membrane antigen, roundabout-4, endothelial-specific protein disulphide isomerase, TEM1, TEM5 and TEM8 (28). It also differs from the known tumor stroma markers such as procollagen I, tenasin, fibroblast activation protein, versican, hyaluronic acid, MMP2 and MMP9 (29). We postulate that the antigen might be a new angiogenesis marker. Future work will focus on the identification and in-depth functional analysis of the protein.

In summary, we raised a monoclonal antibody that preferentially reacted with tumor endothelial cells and other stromal cells. The mAb inhibited the adhesion and tube formation of human esophageal tumor endothelia in vitro. It also inhibited tumor angiogenesis and growth. This mAb might be a useful agent for tumor angiogenesis-targeted therapy.

Acknowledgements

We thank Yangfu Jiang (Sichuan University, China) for critically reading this manuscript. This work was supported by the National Natural Science Foundation of China (Key Program, No. 30230150) and the National Basic Research Program of China (No. 2002CB513100).

References


