Fully Human Antibodies to MCAM/MUC18 Inhibit Tumor Growth and Metastasis of Human Melanoma

Lisa Mills, Carmen Tellez, Suyun Huang, Cheryl Baker, Marya McCarty, Larry Green, Jean M. Gudas, X. Feng, and Menashe Bar-Eli


ABSTRACT

MCAM/MUC18 expression correlates with tumor thickness and metastatic potential of human melanoma cells in nude mice. Moreover, ectopic expression of MUC18 in primary cutaneous melanoma cells leads to increased tumor growth and metastasis in vivo. Here we tested the effect of a fully human anti-MUC18 antibody, ABX-MA1, on angiogenesis, tumor growth, and metastasis. ABX-MA1 had no effect on melanoma cell proliferation rate in vitro. However, when cells of the metastatic melanoma lines A375SM and WM2664 (which express high levels of MUC18) were injected s.c. into nude mice and treated with ABX-MA1 (100 μg, i.p. for 5 weeks), tumor growth was significantly inhibited compared with control IgG-treated mice. ABX-MA1 treatment also suppressed experimental lung metastasis of these melanoma cells. ABX-MA1 disrupted spheroid formation by melanoma cells expressing MUC18 (homotypic interaction) and the ability of these cells to attach to human vascular endothelial cells [HUVECs (MUC18 positive)] in vitro. ABX-MA1 treatment of melanoma cells in vitro significantly inhibited the promoter and collagenase activity of matrix metalloproteinase 2, resulting in decreased invasion through Matrigel-coated filters. Decreased expression of matrix metalloproteinase 2 was also observed in the implanted tumors in vivo. Moreover, because HUVECs also express MUC18, ABX-MA1 directly disrupted the tube-like formation by HUVECs in an in vitro vessel formation assay. Collectively, these results point to usefulness of ABX-MA1 as a modality to treat melanoma either alone or in combination with conventional chemotherapy or other immunotarget agents.

INTRODUCTION

The ability of tumor cells to detach from the primary site and produce metastases in a distant organ is due to the survival and growth of unique subpopulations of cells with metastatic properties (1, 2). One tumor cell property that is essential for metastasis is the expression of CAMs, which mediate cell-to-cell or cell-to-matrix interactions. For example, expression of adhesion molecules such as the integrin VLA-4 (α4β1) and the vitronectin receptor (αvβ3) correlates with progression of human melanoma (3, 4). Other molecules, the expression of which increases with the advanced stage of disease in melanoma, are particular isoforms of CD44, a cell surface glycoprotein that functions as the major receptor for hyaluronate (4), HLA-DR, and intracellular adhesion molecule 1 (5). CAMs do not, however, simply glue cells together. CAMs are also involved in signal transduction; i.e., upon adhesion to fibronectin, a signal transduction cascade is activated through the phosphorylation of focal adhesion kinase, which in turn activates the RAS pathway of signal transduction (6).

MCAM, previously known as MUC18, Mel-CAM, or CD146, is a newly recognized CAM belonging to the immunoglobulin superfamily (7–9). cDNA cloning and sequencing revealed that MCAM has significant homology to several CAMs of the immunoglobulin superfamily, including BEN (10), N-CAM (11), MAG (12), and DCC (13). Some of these proteins are known to exhibit changes in their expression pattern in several human tumors. MCAM is an integral membrane glycoprotein with an apparent molecular weight of 113,000. It contains five immunoglobulin-like domains, and its cytoplasmic domain contains several protein kinase recognition motifs, suggesting the involvement of MCAM in cell signaling (9). MCAM can mediate heterotypic adhesion between cells, although the counter receptor or ligand for MCAM has yet to be identified (14). The malignant potential of cutaneous melanoma is directly related to the vertical thickness of the lesion (15, 16). Analysis of primary melanomas indicates that although the majority of advanced and metastatic tumors strongly express the MCAM antigen, its expression on thin tumors (<0.75 mm), which have only a low probability of metastasizing, and on benign nevi is weaker and less frequent (7, 17). In addition, we have previously demonstrated a positive correlation between MCAM expression and the ability of human melanoma cell lines to metastasize in nude mice (18). Our studies in murine models suggest that MCAM/MUC18 contributes to tumor growth and metastases formation in vivo (19). Indeed, ectopic expression of MCAM/MUC18 in radial growth phase melanoma cell lines (MCAM negative) resulted in an increase in their tumorigenicity and metastatic potential in nude mice (19). The transfected cells displayed increased homotypic adhesion, increased attachment to human endothelial cells, up-regulation of the metalloproteinase MMP-2, and increased invasiveness through Matrigel-coated filters (19). Moreover, it has recently been shown that the production of tumorigenic variants from a nontumorigenic melanoma cell line is accompanied by MCAM up-regulation (20).

These observations have established MCAM/MUC18 as a candidate mediator of tumor growth, angiogenesis, and metastasis in human melanoma and lend credence to the rationale that blockade of MCAM/MUC18 might be a potential target for immunotherapy against human melanoma.

In the present study we used a fully human anti-MCAM/MUC18 Ab (ABX-MA1; produced by Abgenix) to block the MCAM/MUC18 adhesion molecule on melanoma cells and analyzed its effect on tumor growth, angiogenesis, and metastasis of human melanoma. We show that ABX-MA1 treatment of nude mice bearing melanoma cells suppressed their tumor growth and the formation of metastases. ABX-MA1 disrupted spheroid formation by melanoma cells expressing MCAM/MUC18 (homotypic interaction) and the ability of these cells to adhere to vascular endothelial cells (extravasation). Inhibition of tumor growth and metastasis in vivo correlated with decreased vascularization, attributed in part to a decrease in MMP-2 expression and activity. Moreover, in an in vitro vessel formation assay, ABX-MA1 directly interfered with tube formation by HUVECs. These results

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2 The abbreviations used are: CAM, cell adhesion molecule; FACS, fluorescence-activated cell sorting; HUVEC, human umbilical vein endothelial cell; IHC, immunohistochemistry; MMP, matrix metalloproteinase; CMEM, complete minimal essential medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; Ab, antibody; MVD, microvessel density; EGF, epidermal growth factor.

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suggest that blocking MCAM/MUC18 with ABX-MA1 could be beneficial in treating melanoma patients either alone or in combination with other chemotherapeutic or antiangiogenic agents.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The A375-P human melanoma cell line was established in culture from a lymph node metastasis of a melanoma patient (21). The highly metastatic A375SM cell line was established from pooled lung metastases produced by A375-P cells injected i.v. into nude mice (22). The SB-2 cell line was isolated from a primary cutaneous lesion and was a gift of Dr. B. Giovanella (St. Joseph’s Hospital Cancer Center, Research Laboratory, Houston, TX). In nude mice, SB-2 cells are poorly tumorigenic and nonmetastatic (18, 23, 24). The melanoma cell line WM2664, purchased from American Type Culture Collection, is highly metastatic in nude mice (25). All cell lines were maintained in cell culture as monolayers in Eagle’s minimal essential medium supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, HEPES buffer, and penicillin-streptomycin and incubated at 37°C with 5% CO₂.

Animals. Male athymic BALB/c nude mice were purchased from the Animal Production Area of the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD). The mice were housed in laminar flow cabinets under specific pathogen-free conditions and used at 8 weeks of age. Animals were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care in accordance with current regulations and standards of the United States Department of Agriculture, Department of Health and Human Services, and NIH.

Fully Human Anti-MUC18 Ab (ABX-MA1). ABX-MA1 is a human IgG2 monoclonal Ab directed against human MUC18/AMCAM that was generated using Abgenix’s proprietary XenoMouse mice. In XenoMouse technology, murine heavy and light chain loci have been inactivated and subsequently replaced with a majority of human heavy and κ light chain immunoglobulin loci. When immunized, these mice produce fully human Abs (26). The mice used for this immunization contained only the human IgG2 heavy chain sequences and human κ light chain. ABX-MA1 detects a single specific M, 113,000 MCAM/MUC18 band in Western blot analysis and binds specifically to MCAM/MUC18 as determined by FACS analysis with metastatic melanoma cells. Chemopure human IgG control Ab was purchased from Jackson Immunoresearch (West Grove, PA) and used at the same concentration as ABX-MA1 in all experiments.

Western Blot Analysis. Melanoma cell lines were seeded at 1 × 10⁶ in 100-mm tissue culture plates in 10 ml of CMEM. After overnight incubation, the plates were washed two times in PBS and scrapped off in 400 μl of Triton lysis buffer, 1 μl of DTT, and 4 μl of protease inhibitor mixture. After a 30-min incubation on ice, the cells were centrifuged at 15,000 rpm for 15 min. The protein concentration was determined (Bio-Rad and BSA standards), and 40 μg of protein were loaded onto a 10% SDS-PAGE gel and electrophoretically transferred to a 0.45-μm nitrocellulose membrane (Millipore, Bedford, MA). The membrane was blocked with 5% milk in tween tris buffered saline for 1 h. Primary incubation of both cell lines was accomplished by cutting the membranes and incubating them in 1 ml of either control IgG (1:500 dilution) or ABX-MA1 overnight. Membranes were probed with secondary Ab peroxidase-conjugated AffiniPure rabbit antihuman IgG (H+L) for 1 h and then washed with tween tris buffered saline. Probed proteins were detected by enhanced chemiluminescence (Amerham) following the manufacturer’s protocol.

Flow Cytometry. Quantitative analysis of MUC18 on cell surfaces was determined by FACS analysis. A375SM and SB-2 cells (1 × 10⁶) were plated in 6-well plates (Fisher Scientific, Houston, TX) and incubated for 24 h. After attachment, wells were scraped with a disposable cell scraper (Sarstedt) and incubated with control IgG or ABX-MA1 for 1 h at 4°C. After several washings with FACS buffer, all samples were incubated with R-phycocerythrin-conjugated AffiniPure F(ab’)2 fragment goat antihuman IgG (H+L) [1:200 dilution (Jackson Immunoresearch)] for 1 h at 4°C in the dark. The cells were fixed in 1% paraformaldehyde in PBS and examined by flow cytometry.

Three-dimensional Spheroid Culture. Multicellular spheroids were generated by the liquid overlay technique (27, 28). Briefly, 24-well tissue culture plates (Costar) were coated with 250 μl of prewarmed 1% Seaplaque agarose (FMC Bioproducts, Rockland, ME) solution in serum-free MEM. After the agarose was allowed to solidify and form a thin layer on the bottom of the dish, a single-cell suspension of A375SM or SB-2 (1 × 10⁴) was diluted in 25 μl of hybridoma medium, plated with 475 μl of control IgG (1:200 dilution) or ABX-MA1, and incubated at 37°C in 5% CO₂/95% air. After 24 h, spheroid formation was determined. Images were captured by bright-field microscopy and photographed in digital format.

Attachment of Melanoma Cells to HUVECs. Attachment of melanoma cells to endothelial cells treated with 12 μg/ml control IgG or ABX-MA1 was measured by plating 4 × 10⁶ HUVECs in 96-well plates and allowing them to attach for 24 h. A thin overlay of 2% BSA was placed in each well and incubated overnight at 37°C. Then 5 × 10⁶ cells with or without treatment were added to each well and incubated overnight at 37°C. Wells were rinsed twice with PBS, and cells in each well were counted. Results are presented as the percentage of cells adhered from the total number of cells seeded.

Effect of ABX-MA1 on Proliferation. Cells (2000 melanoma cells/well in 96-well plates) were treated with 100 μg/ml ABX-MA1, IgG control Ab, or CMEM for 5 days and then analyzed for viability by MTT assay (which determines relative cell numbers based on the conversion of MTT to formazan in viable cells). MTT (40 μg/ml) was added to each well and incubated for 2 h. The medium was removed, and 100 μl of DMSO were added to lyse cells and solubilize formazan. Absorbance was determined on a microplate reader.

In Vivo Tumor Growth and Metastasis. To prepare tumor cells for inoculation, cells in exponential growth phase were harvested by brief exposure to a 0.25% trypsin/0.02% EDTA solution (w/v). The flask was tapped sharply to dislodge the cells, and supplemented medium was added. The cell suspension was pipetted to produce a single-cell suspension. The cells were washed and resuspended in Ca²⁺/Mg²⁺-free HBSS to the desired cell concentration. Cell viability was determined by trypan blue exclusion, and only single-cell suspensions of >90% viability were used. s.c. tumors were produced by injection of 1–5 × 10⁶ tumor cells in 0.2 ml of HBSS on the right scapular region. Growth of s.c. tumors was monitored by weekly examination of the mice and measurement of tumors with calipers. The mice were killed 5 weeks after injection, and tumors were processed for H&E staining.

For experimental lung metastasis, 1 × 10⁶ tumor cells/0.2 ml HBSS were injected into the lateral tail vein of nude mice. The mice were killed after 60 days, and the lungs were removed, washed in water, and fixed with Bouin’s solution for 24 h to facilitate counting of tumor nodules as described previously (29). The number of surface tumor nodules was counted under a dissecting microscope. Sections of the lungs were stained with H&E to confirm that the nodules were melanoma and to monitor the presence of micrometastases. Both s.c. and i.v. groups were treated once or twice weekly with either 1 mg or 100 μg of ABX-MA1 as indicated or control IgG Ab by i.p. injection.

Zymography. MMP-2 activity was determined on substrate-impregnated gels (30) with minor modifications. We plated 5 × 10⁶ metastatic A375SM cells in 6-well plates and allowed them to attach for 24 h. Cells were treated with 100 μg/ml ABX-MA1, control IgG, or CMEM for 5 days. Treatment for 5 days was found to be optimal for the Ab to affect MMP-2 activity. On day 5, CMEM was removed and replaced with serum-free medium overnight. The supernatant was collected, the volume was adjusted for cell number, and the supernatant was loaded on gelatin-impregnated (1 mg/ml; Difco, Detroit, MI) SDS-8% polyacrylamide gels under nonreducing conditions, followed by 30 min of shaking in 2.5% Triton X-100 (BDH, Poole, United Kingdom). The gels were then incubated for 16 h at 37°C in 50 mM Tris, 0.2 mM NaCl, 5 mM CaCl₂, and 0.02% Brij 35 (w/v) at pH 7.6. At the end of the incubation, the gels were stained with 0.5% Coomassie G 250 (Bio-Rad, Richmond, CA) in methanol/acetic acid/H₂O (30:10:60). The intensity of the various bands was determined on a computerized densitometer (Molecular Dynamics type 300A).

Invasion Assay through Matrigel. Invasion of highly metastatic A375SM and WM2664 cells was measured by plating 2.5 × 10⁵ cells on 6-well plates and allowing them attach for 24 h. After 5 days of treatment with 100 μg/ml ABX-MA1, control IgG, or CMEM, cells were released from the plates by a brief exposure to trypsin-EDTA (Life Technologies, Inc.,) counted, and centrifuged. Biocoat Matrigel invasion chambers (Becton-Dickinson) were primed according to the manufacturer’s directions. A solution of 20% CMEM was placed in the lower well to act as a chemotractant, and 2.5 × 10⁵ cells in 500 μl of serum-free medium with appropriate Ab were placed in the upper chamber of the Matrigel plate and incubated at 37°C for 22 h. Cells on the
lower surface of the filter were stained with Diff-Quick (American Scientific Products, McGraw Park, IL) and quantified with an image analyzer (Optimas 6.2) attached to an Olympus CK2 microscope. The data were expressed as the average number (+SD) of cells from 10 fields that migrated to the lower surface of the filter from each of three experiments performed.

**Transient Transfection and Luciferase Assays: Effect of ABX-MA1 on MMP-2 Promoter.** The MMP-2 promoter construct was generated by digesting the MMP-2 promoter region, −390 to +290 (31), out of p682 basic (chloroamphenicol acetyltransferase-driven MMP-2 promoter; Ref. 30) at the HindIII XhoI sites and ligating into it pGEM-9Zf(−) vector (Promega, Madison, WI) using the same restriction enzyme. The MMP-2 promoter region was then removed via the SpeI/Sall sites and ligated into the pGL3-Enhancer (Promega; Ref. 32). Melanoma cells were treated with 100 μg/ml ABX-MA1, control Ig, or CMEM for 4 h and then transfected with 10 ng of plasmid DNA of either luciferase basic vector, SV40 positive control, or MMP-2 promoter vector, using 10 μl of Lipofectin reagent (Life Technologies, Inc.). After 12 h, the medium was changed, and treatments were added. Cells were lysed and analyzed using dual luciferase assay (Promega) and Ascent Lumiskan plate reader and software (33).

To that end, 24-well plates were coated with reconstituted Matrigel (Beckett) by HUVECs, we analyzed the effect of ABX-MA1 on HUVEC tube formation. Cells were briefly trypsinized, and 2 × 10^4 cells were added to each well of and incubated at 37°C in 5% CO_2 for 8 h. Plates were captured with bright-field microscopy using a Sony digital camera equipped with the Optimas 6.2 program.

**Effect of ABX-MA1 on Vessel-like Tube Formation by HUVECs.** The basement membrane-like substrate (Matrigel) induces HUVECs to rapidly form vessel-like tubes in *vitro* (34). Because MUC18/MCAM is also expressed by HUVECs, we analyzed the effect of ABX-MA1 on HUVEC tube formation. To that end, 24-well plates were coated with reconstituted Matrigel (Beckett-Dickenson) following the manufacturer’s directions. HUVECs were pretreated with medium containing 100 μg/ml ABX-MA1, 100 μg/ml IgG, or MCDB (FIND) alone for 4 days. Treatment for 4 days was found to be optimal for the Ab to affect tube formation. Cells were briefly trypsinized, and 2 × 10^4 cells were added to each well of and incubated at 37°C, in 5% CO_2, for 8 h. Plates were captured with bright-field microscopy using a Sony digital camera equipped with the Optimas 6.2 program.

**In Situ TUNEL Assay.** Tissues were fixed in 10% buffered formalin solution and then embedded in paraffin. Thin sections (4 μm) were prepared, and the TUNEL assay was performed using a commercial kit according to the manufacturer’s protocol (Promega). Briefly, tissue sections were deparaffinized and fixed at room temperature for 5 min in 4% paraformaldehyde. Cells were stripped of proteins by incubation for 10 min with 20 μg/ml proteinase. The tissue sections were then permeabilized by incubating them with 0.5% Triton X-100 in PBS for 5 min at room temperature. After being rinsed twice with PBS for 5 min, the slides were incubated with terminal deoxynucleotidyl transferase buffer for 10 min. Terminal deoxynucleotidyl transferase and buffer were then added to the tissue sections, which were incubated in a humid atmosphere at 37°C for 1 h. The slides were washed three times with PBS for 5 min. Prolong solution (Molecular Probes, Eugene, OR) was used to mount the coverslips. Immunofluorescence microscopy was performed using a Sony digital camera equipped with narrow bandpass excitation filters mounted on a filter wheel (Ludl Electronic Products, Hawthorne, NY) to select for green fluorescence. Images were captured using a cooled charge-coupled device camera (Photometrics, Tucson, AZ) and SmartCapture software (Digital Scientific, Cambridge, United Kingdom) on a Macintosh computer. Images were further processed using Adobe Photoshop software (Adobe Systems, Mountain View, CA).

**RESULTS**

**Generation of Specific Fully Human Anti-MUC18 (ABX-MA1).** ABX-MA1 is a human IgG2 monoclonal Ab directed against human MUC18/MCAM that was generated using Abgenix’s propriety XenoMouse mice. XenoMouse IgG2 mice were immunized three times with SK-Mel28 cells. The animals were subsequently boosted with the same melanoma cells admixed with 5 μg of soluble MUC18-human IgG2 Fc fusion protein administered via base of tail/i.p. injection. After selection, the B cells derived from spleen and lymph nodes were fused with the myeloma P3X63Ag8.653. The resulting hybridoma supernatants were cultured in medium containing hypoxanthine, aminopterin, and thymidine (HAT) and subsequently screened for binding to the soluble MUC18 antigen coated on plastic plates using an ELISA followed by FACS analyses.

Clone 3.19.1 was chosen for further in *vitro* and in *vivo* analyses and designated ABX-MA1. ABX-MA1 recognized and detected a single band corresponding to the M, 113,000 MUC18 protein in the metastatic melanoma cell lines A375SM and WM2664 [both expressed high levels of MUC18 (Ref. 19; Fig. 1A, Lanes 1 and 4)]. This Ab failed to detect any MUC18 protein in the nonmetastatic melanoma cell line SB-2 [MUC 18 negative (Ref. 19; Fig. 1A, Lane 2)]. The specificity of the Ab was further confirmed by detection of the M, 113,000 MUC18 band in SB-2 cells after transfection with the MUC18 gene as we have described previously (Ref. 19; Fig. 1A, Lane 3). In addition to metastatic melanoma cells, MUC18 is also expressed...
on HUVECs (7, 8). ABX-MA1 detected the M_{r} 113,000 protein on HUVECs (Fig. 1A, Lane 6), but not on murine nude mouse endothelial cells (NME, Lane 5). The MUC18 adhesion molecule was also detected with ABX-MA1 by FACS analysis on the cell surface of A375SM cells, but not on SB-2 cells (Fig. 1B). Thus, ABX-MA1 specifically bound and detected the M_{r} 113,000 MUC18 receptor on metastatic human melanoma and HUVECs.

**ABX-MA1 Is Functional: Disruption of Spheroid Formation by Metastatic Melanoma Cells.** Clumping or emboli formation of tumor cells is an important step before extravasation and metastasis. We and others have shown that MUC18/MCAM plays a significant role in homotypic aggregation of metastatic melanoma cells (7, 8, 14, 19). To determine the role of ABX-MA1 in homotypic aggregation, we evaluated the ability of MUC18-negative (SB-2), and MUC18-positive (A375SM) cells to grow in a three-dimensional culture system, i.e., as so-called multicellular spheroids (27, 28) in the presence of ABX-MA1. Under such culture conditions, adherence and formation of monolayers by the cells at the bottom of the dish are prevented by the presence of a thin layer of solid agarose, thus forcing the cells to form tumor-like homotypic multicellular aggregates (19, 27, 28). Under these conditions, the MUC18-negative SB-2 cells grew in monolayers, whereas the MUC18-positive A375SM cells formed spheroids (Fig. 2). When we cultured the cells with ABX-MA1 (added at time 0), spheroid formation was disrupted only in A375SM cells and not in SB-2 cells. This effect was not observed when control IgG Ab was added to the cultures (Fig. 2). This assay confirms the ability of ABX-MA1 to disrupt spheroid formation by metastatic MUC18-positive melanoma cells.

**Suppression of Tumorigenicity and Metastasis of Human Melanoma Cells by ABX-MA1.** Because expression of MUC18/MCAM by melanoma cells contributes to tumor growth and metastasis (18, 35), we next determined the effect of ABX-MA1 on tumorigenicity and metastasis of human melanoma cells in nude mice. To that end, MUC18-positive A375SM (1 × 10^5) and WM2664 (5 × 10^5) cells were injected s.c. into nude mice (n = 10). Three days later, animals injected with A375SM were injected with 1 mg of ABX-MA1 i.p. once weekly; animals injected with the less aggressive WM2664 cells were injected with 100 μg of ABX-MA1 twice weekly for a period of 4–5 weeks. Control animals were injected with normal IgG Ab. Tumor cells in the animals treated with control IgG grew progressively and produced large tumors reaching a volume of 1200 mm^3 for WM2664 and 500 mm^3 for A375SM (Fig. 3, A and B). In contrast, treatment with ABX-MA1 reduced tumor growth to approximately 400 mm^3 for WM2664 and 180 mm^3 for A375SM during the same periods of time (Fig. 3).

To determine the effect of ABX-MA1 on metastasis of human melanoma cells, 1 × 10^5 A375SM and WM2664 cells were injected i.v. into nude mice to produce experimental lung metastasis. Three days later, animals that had received injection of tumor cells subsequently received injection of ABX-MA1 or control IgG i.p., once or twice weekly as indicated, for a period of 4 weeks for A375SM cells and 8 weeks for WM2664 cells. Two different doses of ABX-MA1 (100 μg or 1 mg) were used to treat the injected animals. As shown in Table 1, both the incidence and number of lung metastases of both melanoma cell lines were lower in ABX-MA1-treated mice than in the control IgG-treated group. Both doses of ABX-MA1 were equally effective in inhibiting the formation of metastases. In IgG-treated mice, A375SM cells produced numerous lung metastases (median, 17; range, 5–34), whereas the WM2664 cells produced a median of 11 lung colonies (range, 4–21). In contrast, treatment with ABX-MA1 significantly inhibited the ability of both A375SM and WM2664 cells to form metastases in nude mice (median, 1–2; range, 0–8; P < 0.001). Collectively, these data demonstrate that treatment of mice with ABX-MA1 suppressed melanoma tumor growth and metastasis.

The decrease in tumor growth and in the ability to produce lung metastases attributed to ABX-MA1 was not due to differences in cell division time because no significant differences in cell doubling time were found when A375SM and WM2664 were cultured in the presence of ABX-MA1 (Fig. 4, A and B). These data suggest that ABX-MA1 did not affect melanoma cell proliferation in vitro. ABX-MA1 also did not alter the proliferation rate of HUVECs in vitro (Fig. 4C).

**Down-Regulation of MMP-2 Activity in Human Melanoma Cells by ABX-MA1 in Vitro.** The metastatic potential of tumor cells depends on vascularization and their ability to degrade type IV col-

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**Fig. 2. Disruption of spheroid formation by ABX-MA1.** ABX-MA1 but not control IgG blocked spheroid formation in MUC18-expressing A375SM cells but not in MUC18-negative SB-2 cells.

**Fig. 3.** Effect of ABX-MA1 on tumor growth of human melanoma cells in nude mice. A375SM (1 × 10^5) or WM2664 (5 × 10^5) cells were injected s.c. into nude mice (n = 10). Three days later, A375SM-bearing mice were treated with ABX-MA1 (1 mg, weekly, i.p.), whereas mice injected with WM2664 cells were treated with ABX-MA1 (100 μg, twice weekly, i.p.) or control IgG. The volume of the s.c. tumors was determined every 3–5 days. Treatment with ABX-MA1 significantly inhibited the growth of A375SM and WM2664 in nude mice.

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**FULLY HUMAN ANTI-MUC18 INHIBITS GROWTH AND METASTASIS OF HUMAN MELANOMA CELLS**

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**Table 1.** Incidence and Number of Lung Metastases of Both Melanoma Cell Lines in Nude Mice Treated with ABX-MA1 or Control IgG. Both doses of ABX-MA1 were equally effective in inhibiting the formation of metastases. In IgG-treated mice, A375SM cells produced numerous lung metastases (median, 17; range, 5–34), whereas the WM2664 cells produced a median of 11 lung colonies (range, 4–21). In contrast, treatment with ABX-MA1 significantly inhibited the ability of both A375SM and WM2664 cells to form metastases in nude mice (median, 1–2; range, 0–8; P < 0.001). Collectively, these data demonstrate that treatment of mice with ABX-MA1 suppressed melanoma tumor growth and metastasis.

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**Figs. 3A and B.** Tumor volume (mm^3) in nude mice treated with ABX-MA1 or control IgG. The volume of the s.c. tumors was determined every 3–5 days. Treatment with ABX-MA1 significantly inhibited the growth of A375SM and WM2664 in nude mice.
A, the natants for cell numbers. Treatment with the Ab for 5 days was found analyzed by zymography after normalizing the volume of the super-

regardless of treatment with ABX-MA1 (data not shown).

observed a faint band of MMP-9 activity in all melanoma cells tested,

metalloproteinase expressed and secreted by melanoma cells, we treated (grown in CMEM) control cells. Because MMP-2 is the major
cells was significantly lower than that of either IgG-treated or un-
72,000 MMP-2 collagenase activity of ABX-MA1-treated A375SM

To examine the effect of ABX-MA1 on MMP-2 transcription, we next analyzed the promoter activity of MMP-2 in ABX-MA1-treated and control melanoma cells. A375SM cells were transfected with the MMP-2 promoter-driven luciferase reporter gene and treated with ABX-MA1 (100 μg/ml) or control IgG Ab for 48 h. Consistent with a decreased MMP-2 collagenase activity, MMP-2 promoter activity in ABX-MA1-treated A375SM cells was decreased by 3-fold when compared with IgG-treated and untreated cells (Fig. 5f). These results suggest that MUC18 may directly regulate MMP-2 expression at the transcriptional level and that blocking of MUC18 by ABX-MA1 suppressed MMP-2 expression and activity in melanoma cells.

Suppression of Human Melanoma Cell Invasion by ABX-MA1. We next analyzed whether the decreased expression of MMP-2 in ABX-MA1-treated cells correlated with their ability to invade through the basement membrane, an important component in the process of tumor invasion and metastasis. To that end, 2.5 × 10⁵ A375SM or WM2664 melanoma cells that had been treated with 100 μg/ml ABX-MA1 or control IgG Ab for 5 days were placed in the upper compartment of an invasion chamber in the presence of 100 μg/ml ABX-MA1 or control IgG. After 22 h of incubation, the cells on the lower surface of the filters were counted. A375SM and WM2664 cells treated with ABX-MA1 exhibited significantly less invasion through Matrigel-coated filters than IgG-treated or untreated cells [2068 ± 129 versus 57 ± 8 (number of migrated cells ± SD) for A375SM (P < 0.001) and 1866 ± 130 versus 56 ± 7 (P < 0.001) for WM2664; Table 2]. These results indicate that blockade of MUC18 in melanoma cells by ABX-MA1 inhibited the ability of cells to penetrate through the basement membrane. Collectively, our data indicate that inactivation of MMP-2 by ABX-MA1 in melanoma cell may account for the decrease in their metastatic potential.

Decreased Expression of MMP-2 in Tumors Treated with ABX-MA1. To determine whether ABX-MA1 suppressed the expression of MMP-2 in vivo, we performed immunohistochemical analysis on the melanoma specimens derived from the implanted A375SM and WM2664 tumors. As shown in Fig. 6, MMP-2 staining was strong in

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<td>ABX-MA1 (1 mg/ml)</td>
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<td>0–8</td>
<td>2/5</td>
</tr>
</tbody>
</table>

*P < 0.01 as determined by Mann-Whitney U test.

Fig. 4. Effect of ABX-MA1 on proliferation of melanoma and HUVECs in vitro. A375SM, WM2664, and HUVECs were treated with 100 μg/ml ABX-MA1 or control IgG for 1–4 days. Cell proliferation was determined by MTT assay. This is one of three representative experiments.

lager. We previously demonstrated that MUC18/MCAM contributes to the metastatic phenotype of melanoma cells through the induction of metalloproteinase MMP-2 (19). We therefore tested whether blocking MUC18 with ABX-MA1 had any effect on MMP-2 activity in melanoma cells. To that end, A375SM cells were treated with ABX-MA1 (100 μg/ml) or control IgG for 5 days, and MMP-2 activity was analyzed by zymography after normalizing the volume of the supernatants for cell numbers. Treatment with the Ab for 5 days was found to be optimal to affect MMP-2 activity. As shown in Fig. 5a, the M₅₀ of 72,000 MMP-2 collagenase activity of ABX-MA1-treated A375SM cells was significantly lower than that of either IgG-treated or untreated (grown in CMEM) control cells. Because MMP-2 is the major metalloproteinase expressed and secreted by melanoma cells, we observed a faint band of MMP-9 activity in all melanoma cells tested, regardless of treatment with ABX-MA1 (data not shown).

To examine the effect of ABX-MA1 on MMP-2 transcription, we next analyzed the promoter activity of MMP-2 in ABX-MA1-treated and control melanoma cells. A375SM cells were transfected with the MMP-2 promoter-driven luciferase reporter gene and treated with ABX-MA1 (100 μg/ml) or control IgG Ab for 48 h. Consistent with a decreased MMP-2 collagenase activity, MMP-2 promoter activity in ABX-MA1-treated A375SM cells was decreased by 3-fold when compared with IgG-treated and untreated cells (Fig. 5f). These results suggest that MUC18 may directly regulate MMP-2 expression at the transcriptional level and that blocking of MUC18 by ABX-MA1 suppressed MMP-2 expression and activity in melanoma cells.

Suppression of Human Melanoma Cell Invasion by ABX-MA1. We next analyzed whether the decreased expression of MMP-2 in ABX-MA1-treated cells correlated with their ability to invade through the basement membrane, an important component in the process of tumor invasion and metastasis. To that end, 2.5 × 10⁵ A375SM or WM2664 melanoma cells that had been treated with 100 μg/ml ABX-MA1 or control IgG Ab for 5 days were placed in the upper compartment of an invasion chamber in the presence of 100 μg/ml ABX-MA1 or control IgG. After 22 h of incubation, the cells on the lower surface of the filters were counted. A375SM and WM2664 cells treated with ABX-MA1 exhibited significantly less invasion through Matrigel-coated filters than IgG-treated or untreated cells [2068 ± 129 versus 57 ± 8 (number of migrated cells ± SD) for A375SM (P < 0.001) and 1866 ± 130 versus 56 ± 7 (P < 0.001) for WM2664; Table 2]. These results indicate that blockade of MUC18 in melanoma cells by ABX-MA1 inhibited the ability of cells to penetrate through the basement membrane. Collectively, our data indicate that inactivation of MMP-2 by ABX-MA1 in melanoma cell may account for the decrease in their metastatic potential.

Decreased Expression of MMP-2 in Tumors Treated with ABX-MA1. To determine whether ABX-MA1 suppressed the expression of MMP-2 in vivo, we performed immunohistochemical analysis on the melanoma specimens derived from the implanted A375SM and WM2664 tumors. As shown in Fig. 6, MMP-2 staining was strong in
IgG-treated A375SM and WM2664 tumors but was considerably decreased in ABX-MA1-treated tumors. Thus, blocking of MUC18 by ABX-MA1 Ab in melanoma cells inhibited expression of the MMP-2 gene in vivo as well as in vitro.

**Inhibition of Tumor Angiogenesis in ABX-MA1-treated Cells.**
Because both MUC18 and MMP-2 are involved in invasion, metastasis, and angiogenesis, we determined whether blockade of MUC18 by ABX-MA1 could result in suppression of tumor angiogenesis. Tumor-associated neovascularization as indicated by MVD was examined by IHC using anti-CD31 Ab. As shown in Fig. 7, we found a significant reduction in tumor MVD/field after treatment with ABX-MA1 as compared with control IgG-treated tumors. The mean MVD was 8.1 ± 5.1 in ABX-MA1-treated A375SM tumors and 20.1 ± 3.3 in ABX-MA1-treated WM2664 tumors. In contrast, the mean MVD was 22.2 ± 3.1 and 36.1 ± 5.8 for control IgG-treated A375SM and WM2664 tumors, respectively. Moreover, the number of TUNEL-positive tumor cells was inversely correlated with MVD in the studied tumors. The number of tumor cells undergoing apoptosis was higher in the ABX-MA1-treated animals than in tumors derived from control IgG-treated mice (Fig. 7). Quantitative analysis of the TUNEL assay has revealed that the percentage of apoptotic cells was 20 ± 4.8% in ABX-MA1-treated A375SM and 18.6 ± 5.3% in ABX-MA1-treated WM2664 tumors. In contrast, the percentage of apoptotic cells was 1 ± 0.2% and 1.2 ± 0.3% for control IgG-treated A375SM and WM2664 tumors, respectively. These data indicate that ABX-MA1 treatment significantly decreased tumor-associated neovascularization and subsequently increased apoptosis of tumor cells.

**ABX-MA1 Inhibits Attachment of Metastatic Melanoma Cells to HUVECs.**
Because both metastatic melanoma cells and HUVECs express the MUC18 receptor (see Fig. 1A) and because melanoma cells interact with vascular endothelial cells through MUC18-MUC18

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**Table 2. Invasion of ABX-MA1-treated melanoma cells through Matrigel-coated filters**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No. of migrated cells ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A375SM MEM</td>
<td>2575 ± 94</td>
</tr>
<tr>
<td>A375SM IgG</td>
<td>2068 ± 129</td>
</tr>
<tr>
<td>A375SM ABX-MA1</td>
<td>57 ± 8 (P &lt; 0.001)</td>
</tr>
<tr>
<td>WM2664 MEM</td>
<td>1857 ± 57</td>
</tr>
<tr>
<td>WM2664 IgG</td>
<td>1866 ± 130</td>
</tr>
<tr>
<td>WM2664 ABX-MA1</td>
<td>56 ± 7 (P &lt; 0.001)</td>
</tr>
</tbody>
</table>

Fig. 6. Effect of ABX-MA1 on MMP-2 expression in vivo. MMP-2 expression was determined by IHC in specimens derived from A375SM and WM2664 implanted tumors. MMP-2 expression was reduced in tumors taken from ABX-MA1-treated mice.

Fig. 7. Tumor MVD and apoptosis (TUNEL) in s.c. melanoma xenografts. A375SM and WM2664 cells were injected s.c. into nude mice and treated with ABX-MA1 or control IgG. Thirty to 60 days later, the resulting s.c. tumors with similar size were resected and processed for immunohistochemical analysis (CD31 and TUNEL staining).
interactions, we next examined the ability of ABX-MA1 to block the attachment of melanoma cells to HUVECs. To that end, the attachment of SB-2 (MUC18-negative) and A375SM (MUC18-positive) cells to HUVECs was analyzed in the presence of ABX-MA1 or IgG control Ab. Fig. 8 shows that very few SB-2 melanoma cells attached to HUVECs and that the addition of control IgG or ABX-MA1 Ab to the cultures did not alter SB-2-HUVEC interactions (Fig. 8A). In contrast, A375SM cells attached to HUVECs, and treatment with ABX-MA1, but not with control IgG Ab, inhibited A375SM-HUVEC interaction (Fig. 8A). A quantitative summary of three experiments performed on the attachment of A375SM to HUVECs (Fig. 8B) demonstrates that ABX-MA1 reduced the interaction of A375SM with HUVECs by 80%. These data provide a further potential mechanism by which ABX-MA1 may inhibit extravasation and hence metastasis.

ABX-MA1 Interferes with Vessel-like Tube Formation by HUVECs. HUVECs express the MUC18 CAM, suggesting that MUC18 may play a role in the maturation process of vascular endothelial cells. To test this hypothesis, we next analyzed the ability of ABX-MA1 to disrupt the Matrigel-induced formation of vessel-like tubes by HUVECs. To that end, HUVECs were pretreated with 100 µg/ml ABX-MA1, 100 µg/ml control IgG, or CMEM alone for 4 days and then plated on Matrigel to induce vessel-like tube formation. As shown in Fig. 9, CMEM-pretreated HUVECs formed lumen-like structures and anastomosing tubes with multicentric junctions (Fig. 9A). Similar endothelial cell structural morphogenesis occurred in IgG-pretreated HUVECs (Fig. 9B). In contrast, vessel-like tube formation was dramatically disrupted in HUVECs pretreated with ABX-MA1 (Fig. 9C). When ABX-MA1 was added to the cultures after tube formation by HUVECs had occurred, the morphogenesis of preexisting vessel-like tubes was not altered (Fig. 9D). Thus, ABX-MA1 directly inhibited de novo formation of capillary-like networks but did not inhibit preexisting tubule networks.

DISCUSSION

MUC18/MCAM is an adhesion molecule that plays a role in melanoma development and progression. MUC18/MCAM is expressed most strongly on metastatic lesions and advanced primary tumors and is only rarely detected in benign lesions (8, 17). We have shown previously that melanoma cells expressing MUC18/MCAM display increased homotypic interaction, increased attachment to human endothelial cells, decreased ability to adhere to laminin, and increased expression and activity of MMP-2 that resulted in increased invasiveness through Matrigel-coated filters (19). The above-mentioned changes in function attributed to the expression of MUC18/MCAM may underlie the contribution of MUC18/MCAM to the malignant phenotype by human melanomas. In this study, we demonstrated that blockade of MUC/MCAM by neutralizing anti-MUC18 Ab (ABX-MA1) significantly inhibited the growth and metastasis of human melanoma cells in nude mice by suppressing homotypic and heterotypic interactions, angiogenesis, and invasion.

Whereas ABX-MA1 did not alter the proliferation rate of melanoma cells in vitro, it had a profound effect on their tumorigenicity and metastasis potential in vivo. These data indicate that the role of MUC18/MCAM in the progression of human melanoma is more complex than simple autocrine growth stimulation or homotypic interactions.

Indeed, we showed that MUC18/MCAM had a significant effect on angiogenesis and invasion, although the ligand for MUC18/MCAM has yet to be identified. Growth and metastasis of human melanoma cells depend on their ability to develop an adequate vasculature. In fact, the progression of neoplasms from the benign to malignant state is often associated with a switch to an “angiogenic phenotype” representing an increase in proangiogenic molecules produced by the tumor cells and organ-specific environments (36, 37). Melanoma cells secrete a variety of proangiogenic molecules, including basic fibroblast growth factor (38, 39), vascular endothelial growth factor (40),
and interleukin 8 (24, 30). MUC18/MCAM can indirectly affect angiogenesis and invasion of human melanoma cells through up-regulation of MMP-2 and cell interaction with the extracellular matrix and vascular endothelial cells. We found that blockade of MUC18/MCAM by ABX-MA1 suppressed angiogenesis of human melanoma cells grown in nude mice. All of the control tumors were highly vascularized and produced large tumors, whereas the ABX-MA1-treated mice produced small tumors. The retarded tumor growth was directly correlated with decreased blood vessel formation and an increased number of apoptotic tumor cells (TUNEL positive). The inhibition of angiogenesis could be attributed to down-regulation of MMP-2. The proteolytic effect of MMPs facilitates the migration of endothelial cells through the altered extracellular matrix toward the source of the angiogenic stimulus; in this manner, MMP-2 is an integral component of the angiogenesis pathway (41). However, we cannot rule out the possibility that ABX-MA1 can activate murine complement or mediate antibody dependent cell cytotoxicity with murine monocytes through the Fc portion of the Ab. These possibilities are currently under investigation in our laboratory.

In addition to the tumor cells themselves, ABX-MA1 may also affect the host vascular endothelial cells, integrity, formation of new blood vessels, and their interaction with melanoma cells. Because HUVECs also express MUC18/MCAM, ABX-MA1 was found to inhibit their interaction with MUC18-positive metastatic melanoma cells. The inhibition of this interaction could affect extravasation, an important step in the process of metastasis.

Moreover, in an in vitro endothelial cell morphogenesis assay (vessel-like tube formation assay), we found that ABX-MA1 directly inhibited the formation of a capillary-like network by HUVECs. Treatment with ABX-MA1 did not affect existing vessel-like tubes in vitro, disrupting only the formation of the newly formed blood vessels. This observation is of great importance for clinical implications.

The potential of Ab therapy represented by ABX-MA1 fits recent discoveries. Ab immunotherapy provides a novel approach for the treatment of a broad spectrum of diseases including cancer (42–46). Cetuximab (IMC-C225), a mouse-human chimeric anti-EGF receptor monoclonal Ab, and ABX-EGF have been shown to inhibit the proliferation of a variety of cultured human tumor cell lines that overexpress EGF receptor and to inhibit tumor growth in several xenograft models (45, 46).

Currently, both of these Abs are being evaluated in clinical trials. The therapeutic modalities to control tumor growth and metastasis of human melanoma are very limited. The idea of using fully humanized Abs to block MUC18 is especially appealing because multiple dose regimens of the Ab could be administered to the patients with little risk of mounting an immune reaction. Our studies should promote serious consideration for initiating a Phase III clinical trial with ABX-MA1 in patients with metastatic melanoma. ABX-MA1 can be used either alone or in combination with chemotheraphy or other anticancer agents to increase the efficacy of the treatment. In addition, ABX-MA1 should be considered as a treatment modality for other solid tumors in which MUC18/MCAM may play an angiogenic role, including prostate cancer (47).

REFERENCES

Fully Human Antibodies to MCAM/MUC18 Inhibit Tumor Growth and Metastasis of Human Melanoma

Lisa Mills, Carmen Tellez, Suyun Huang, et al.