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An Agonistic Antibody to EPHA2 Exhibits Antitumor Effects on Human Melanoma Cells

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Abstract. *Background/Aim: EPH receptor A2 (EPHA2) is highly expressed in aggressive types of human cancer, and is expected to be an excellent target molecule for antibody treatments. In this study, we investigated the therapeutic potential of antibody to EPHA2 against melanoma in vitro. Materials and Methods: We generated three monoclonal antibodies (mAbs) to EPHA2 and examined cell-surface expression by flow cytometry. To investigate the ability to inhibit tumor cell migration therapy with mAbs to EPHA2, we performed a wound scratch assay and invasion assay. We investigated the therapeutic effects of immunotoxins consisting of toxin-conjugated EPHA2 mAbs. Results: All human melanoma cell lines studied expressed EPHA2. Like natural ligand ephrin-A1, one of EPHA2 mAbs, SHM16, inhibited metastatic behavior of cells, such as migration and invasion. In addition, drastic growth inhibition and cytotoxicity were found using immunotoxin-conjugated SHM16. Conclusion: These observations indicate a promising role for EPHA2 as a target in antibody treatments for melanoma, and demonstrate the potential therapeutic effects of an agonistic antibody to EPHA2.*

Melanoma is the most aggressive type of skin cancer. While melanoma accounts for fewer than 5% of all skin cancer cases, it is responsible for the large majority of deaths due to skin cancer. The type of treatment will depend not only on stage and location of the melanoma, but also on the patient's overall health. When melanoma has not grown deeper than the epidermis, it is usually treated by surgery (wide excision). When this is not possible, therapeutic options including injections of Bacille Calmette-Guerin (BCG) vaccine, interferon, or interleukin-2 (IL2) directly into the melanoma, radiation therapy, and chemotherapy (1, 2). The treatment of widespread melanoma has changed in recent years as newer forms of immunotherapy and molecularly-targeted drugs have been shown to be more effective than chemotherapy (3-6).

Receptor tyrosine kinases (RTKs) transmit signals that regulate cell proliferation and differentiation, promote cell migration and survival, and modulate cell metabolism. They play critical roles in a wide range of biological processes, including embryonic development, growth of an organism, angiogenesis, synaptic plasticity, and oncogenesis (7-9). EPH receptors, the largest subfamily of RTKs, are involved in many biological processes including angiogenesis, tissue-border formation, cell migration, and axon guidance (10, 11). EPH receptors and their ephrin ligands are important mediators of cell-to-cell communication that regulate cell attachment to the extracellular matrix, cell shape and cell motility. They have been studied extensively for their roles in the developmental process. In recent years, EPH receptors and ephrins have been found to be integral players in cancer formation and progression. EPH receptor A2 (EPHA2) and ephrin-A1 are especially known to be involved in the development and maintenance of many types of solid tumors (12-14).

EPHA2 was originally named epithelial cell kinase, due to its widespread expression in epithelial cells. Subsequent studies revealed that EPHA2 was overexpressed in human cancer, including melanoma, and that its overexpression was correlated with malignancy and poor prognosis. A large number of studies have demonstrated that EPHA2 overexpression and activation promote tumorigenesis, suggesting a potential role as an oncogene (15). EPHA2 activation by its ligand, ephrin-A1, regulates cell behavior in a manner more consistent with it being a tumor suppressor. Specifically, EPHA2 activation leads to induction of apoptosis, inhibition of cell proliferation, and suppression of cell migration (16, 17). Accordingly, EPHA2 has been found to be

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an integral player in cancer formation, and is expected to be a good target for antibody and small-agent therapy (18-20).

Recently, we established several monoclonal antibodies (mAbs) specific for human EPHA2. One of these mAbs, called SHM16, exhibited a unique function as an agonistic antibody to EPHA2. The first aim of the study was to determine if SHM16 could mimic the effects of the natural ligand ephrin-A1 in regulating melanoma cell migration and invasion. The second aim was to assess whether SHM16-mediated targeting of toxin conjugates (immunotoxins) to EPHA2 would have antitumor effects against melanoma cells.

Materials and Methods

Cell lines. The melanoma cell lines A375, A2058, G361 were purchased from JCRB Cell Bank (Osaka, Japan), and SK-MEL28 was purchased from the American Type Culture Collection (Manassas, VA, USA). All cell lines, except fibroblasts and melanocytes (purchased from Lifeline Cell Technology, Frederick, MD, USA), were maintained in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Gibco by Life Technologies, Grand Island, NY, USA), 100 units/ml penicillin G (Gibco), 100 μg/ml streptomycin (Gibco), and 25 mmol/l HEPES (Sigma-Aldrich) at 37˚C in a humidified incubator with a 5% CO_2 atmosphere. Fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) supplemented with 10% FBS, penicillin G and streptomycin, and HEPES. Melanocytes were maintained DermaLife Basal Medium (Lifeline Cell Technology) at 37˚C in a humidified incubator with a 5% CO_2 atmosphere.

Establishment of mAbs to human EPHA2. SHM16, SHM17, and SHM20 mAbs against human EPHA2 were generated by immunizing BALB/c mice with the human melanoma cell line A375. The immunized splenocytes were then fused to P3U1 mouse myeloma cells. The supernatants were harvested at 14 days post-fusion, and screened in a bioassay for activity against A375. This protocol for producing and screening targeted mAbs has been described in detail elsewhere (21, 22). After cloning by limiting dilution, we selected hybridoma cell colonies named SHM16, SHM17, and SHM20 (mouse IgG1, κ). EPHA2 was identified as the antigen for all three antibodies by immunoprecipitation and biochemical analysis. To confirm the reactivity of SHM16, SHM17, and SHM20 with EPHA2, the A375 cell line was transfected with short interfering RNA (siRNA) targeting EPHA2 and incubated at 37˚C for 72 h. After this treatment, the reactivity of EPHA2 with A375 was examined by flow cytometry.

Reagents. Anti-EPHA2 mAbs were dissolved in sterilized phosphate-buffered saline (PBS) and kept as 1 mg/ml stock solutions. The mouse immunoglobulin G1 (IgG1) isotype control was purchased from BioLegend (San Diego, CA, USA). An ephrin-A1 fusion with the human IgG fragment crystallizable (Fc) domain (ephrin-A1-Fc) was purchased from R&D systems (Minneapolis, MN, USA). Saporin-conjugated goat anti-mouse IgG (Mab-ZAP) was purchased from Advanced Targeting Systems (San Diego, CA, USA).

Identification of antigen recognized by SHM16. The molecules recognized by the mAb, SHM16 were identified by a two-step method as previously described in detail (21, 22). First, the cell-surface proteins of A375 cells were biotinylated and immunoprecipitated with the mAbs. The precipitated proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 5-20% gradient polyacrylamide gels (Bio-Rad, Hercules, CA, USA) and transferred onto nitrocellulose membranes (Millipore, MA, USA), and molecular weights of the immunoprecipitated proteins were then determined. In the second step, the immunocomplexes of A375 cells generated by the mAb separated by polyacrylamide gel was stained by using a Silver Stain kit (Wako Pure Chemical Industries, Ltd, Osaka, Japan) according to the company's recommendations. The specifically stained protein bands were extracted from the gel, digested by trypsin, and analyzed by oMALDI-Qq-TOF MS/MS QSTAR Pulsari (Applied Biosystems Japan Ltd, Tokyo, Japan).

Flow cytometric analysis. Cell-surface expression of EPHA2 was determined by flow cytometry. Approximately 1×10^6 cells were harvested from a monolayer culture, washed, and incubated with a saturating amount (10 μg/ml) of primary antibody in PBS (pH 7.4) with 2% FBS (staining buffer) for 30 min on ice. The cells were then washed and stained with Alexa Fluor 488-conjugated goat anti-mouse IgG1 secondary antibody (diluted 1:100; Invitrogen) for 15 min on ice. Cells were then incubated with fluorescein-conjugated mAb for 30 min on ice. The cell suspension was washed three times with PBS, and then analyzed using a FACSCalibur flow cytometer (BD Immunocytometry Systems, Franklin Lakes, NJ, USA).

Cell proliferation assay. A375 cells were seeded and incubated at 37˚C for 4 days with ephrin-A1-Fc or one of SHM16, SHM17, or SHM20 EPHA2 mAbs. After 4 days, AlamarBlue® reagent (AbD Serotec, Oxford, UK) was added to a final concentration of 10%, and incubated with cells at 37˚C for 3 h. The fluorescence intensity was measured at a wavelength of 590 nm following excitation with a wavelength of 570 nm.

Competitive inhibition assay of ephrin-A1-binding to EPHA2 by mAbs. Approximately 1×10^6 cells (500 μl per tube) harvested from a monolayer culture were incubated with one of the anti-EPHA2 mAbs (1 μg/ml SHM16, or SHM17) for 1 h on ice, and then incubated subsequently with 3 μl per tube ephrin-A1-Fc (10 μg/ml) for 1 h at room temperature. Cells were washed, and then stained with goat anti-human IgG (Fc-specific) conjugated with fluorescein 5-isothiocyanate (FITC) (Caltag Laboratories, Carlsbad, CA, USA) for 30 min on ice. The cell suspension was then washed three times with PBS, and analyzed using a FACSCalibur flow cytometer.

Scratch assay. Confluent A375 cells in a six-well plate were scratched with a pipette tip, and medium that also contained either 20 ng/ml of ephrin-A1-Fc, 10 μg/ml of SHM16 or SHM17 was added to induce migration. Cells were subsequently incubated at 37˚C for 24 h before being photographed. The area of the scratch gap that remained open after this time was measured using Olympus DP2-BSW software (Tokyo, Japan).

Invasion assay. Biocoat Matrigel invasion chambers were purchased from BD Biosciences (Bedford, MA, USA). A375 cells (2.5×10^4 cells per well) were seeded into the upper chamber of trans-well inserts, and were maintained at 37˚C throughout the experiment. Cells were allowed to migrate toward the lower chamber in media containing either 20 ng/ml of ephrin-A1-Fc, or 10 μg/ml of SHM16, for 24 h. Cell migration was evaluated after crystal violet staining by counting cells in six randomly chosen fields.

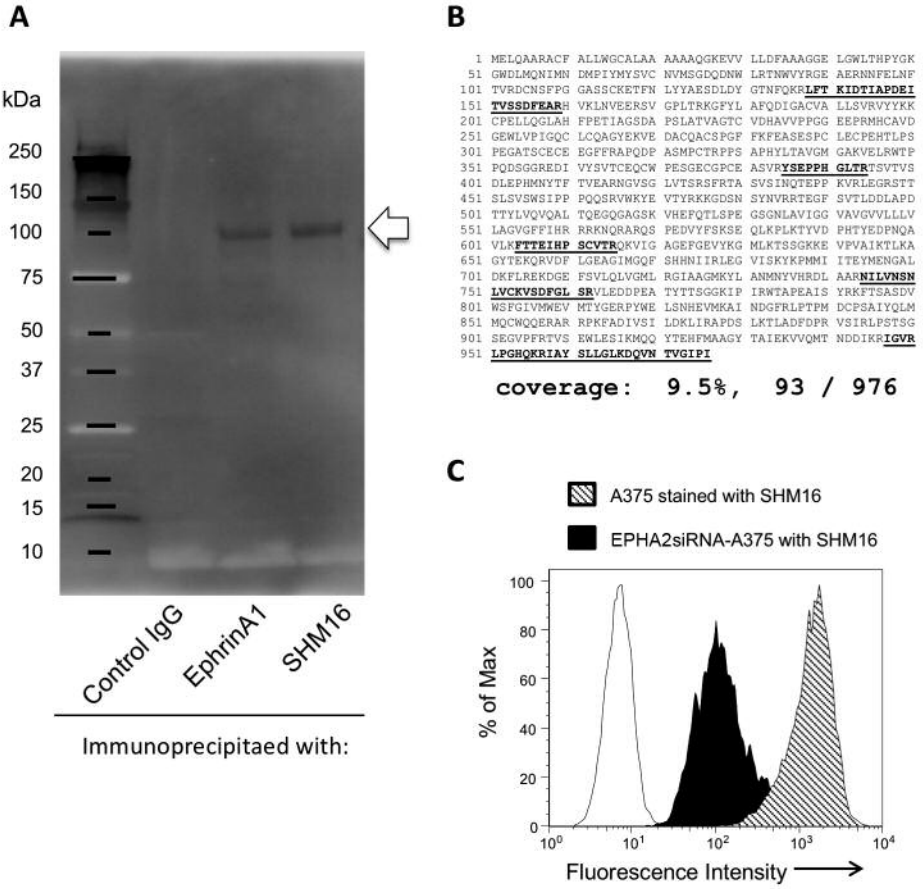

Figure 1. *Characterization of the SHM16 antibody to EPH receptor A2 (EPHA2) by immunoprecipitation, mass spectrometric analysis and siRNA-mediated knockdown of EPHA2 expression in A375 melanoma cells. A: The biochemical nature of the antigen recognized by SHM16 was examined by immunoprecipitation. The precipitates were first subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions, and then to fluorography. A band of 100 kDa (indicated by an arrow) was precipitated by ephrin-A1 (lane 2) and the SHM16 antibody (lane 3). No band was precipitated with the IgG1 isotype control monoclonal antibody (lane 1). B: High-intensity spectra indicate the peptide, the sequence of which corresponded to the amino acid sequence of human EPHA2. Bold typeface indicates the sequence of the detected peptides by mass spectrometry. C: To knock-down EPHA2 protein levels, the A375 cell line was transfected with short interfering RNA (siRNA) targeting EPHA2 mRNA. After 3 days, the surface expression of EPHA2 on A375 cells was examined by flow cytometry.*

Internalization of the SHM16, SHM17, and SHM20 EPHA2 mAbs. A375 cells (1.0×10^4 cells per well) were seeded into each well of four-well imaging chambers in media that included 2 μg/ml EPHA2 mAbs (SHM16, SHM17, or SHM20) and 2 μg/ml Alexa Fluor 488 goat anti-mouse IgG1 secondary antibody. After 24 h of incubation at 37˚C, photographs were taken using a confocal laser scanning microscope (FV10; OLYMPUS).

Targeted delivery of immunotoxin into melanoma cells. A375 cells were plated into a flat-bottom, 96-well plate (2,000 cells per well) and incubated for 4 days at 37˚C in a humidified incubator. This cell suspension with RPMI1640 medium included different concentrations of Mab-ZAP, along with either an anti-EPHA2 mAbs (SHM16, SHM17, or SHM20 at 2 μg/ml final concentration), or a control IgG1 mAb (2 μg/ml final concentration). After 24 h, AlamarBlue reagent was added and plates were incubated at 37˚C for a further 48 h. The fluorescence intensity was then measured at an emission wavelength of 590 nm.

Results

Specificity of SHM16 mAbs to human EPHA2. We established several mAbs specific for EPHA2 by immunizing BALB/c mice with the previously described A375 human melanoma cell line (23). In order to examine the specificity of the mAbs to EPHA2, we performed immunoprecipitation and siRNA transfection. The lysate of the surface biotinylated A375 was subjected to immunoprecipitation with SHM16-coupled or ephrin-A1-Fc-coupled Sepharose beads, and SDS-PAGE analysis was performed under reducing conditions. As shown

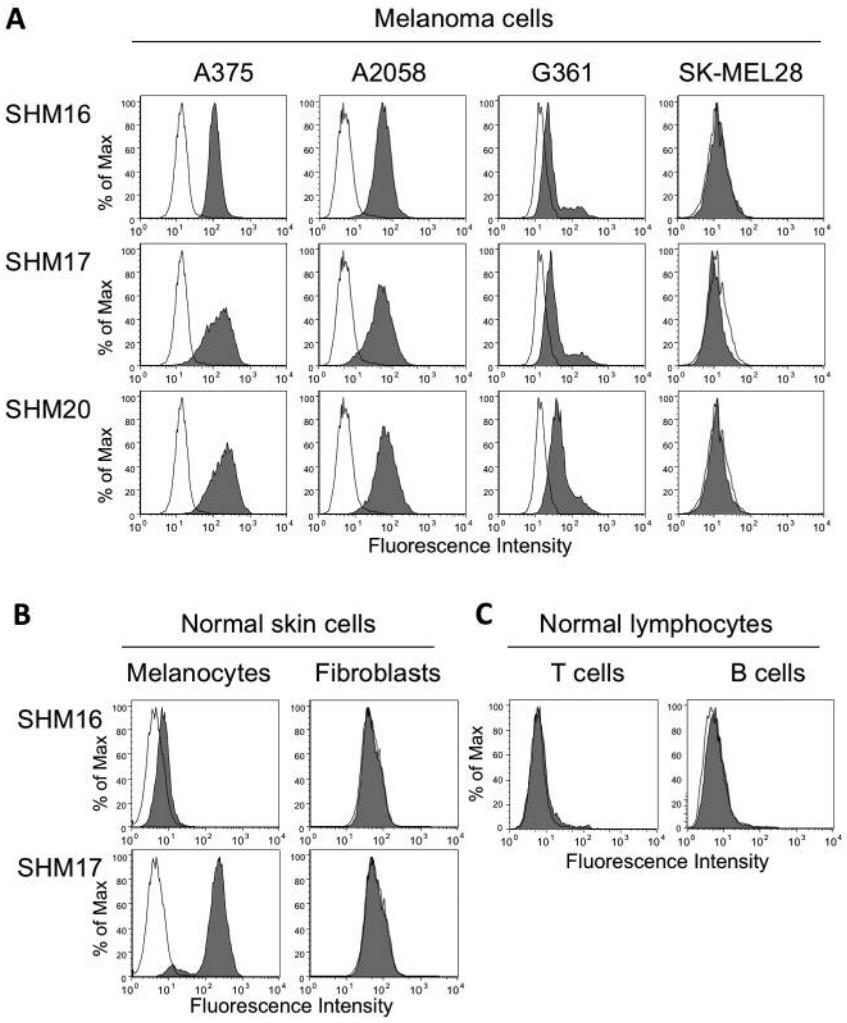

Figure 2. *Cell surface expression of EPH receptor A2 (EPHA2) on melanoma cells versus non-transformed cells. A: The reactivity of the EPHA2 antibodies to human melanoma cell lines was tested. Histograms show the results of flow cytometric analysis of (from left to right) A375, A2058, G361, and SK-MEL28 cell lines treated with (from top to bottom) SHM16, SHM17, and SHM20 antibodies. On each plot, the histogram from an isotype mouse IgG1 antibody control treatment is shown (clear histogram) along with the histogram from the respective antibody treatment (shaded histogram). B: The reactivity of the SHM16 (top) and SHM17 (bottom) antibodies with non-transformed human melanocytes (left) and fibroblasts (right) was examined by flow cytometry. Each plot shows the histogram from a control treatment with an isotype mouse IgG1 antibody (open histogram) and the histogram from an experimental treatment with the indicated antibody (shaded histogram). C: Expression of EPHA2 on human lymphocytes. Mononuclear cells were isolated from human peripheral blood and stained with fluorescence isothiocianate (FITC) conjugated anti-CD3 or -CD19 antibody and SHM16 followed by phycoerythrin-conjugated anti-mouse IgG.*

in Figure 1A, the antigens immunoprecipitated by SHM16 and ephrin-A1-Fc had similar apparent molecular masses of about 100 kDa. We then performed affinity purification andliquid chromatograph/tandem mass spectrometrywith the SHM16 antibody, and this analysis clearly identified human EPHA2 as the antigen (Figure 1B). To confirm the specificity of SHM16 for human EPHA2, we examined the reactivity of SHM16 to A375 cells that were transfected with EPHA2-specific siRNA or a control siRNA (scrambled). Transfection with EPHA2 siRNA resulted in a significant decrease in the reactivity of A375 with not only SHM16 mAb (Figure 1C), but also with SHM17 and SHM20 mAbs (data not shown). We, therefore, conclude that the antigen recognized by SHM16, SHM17, and SHM20 is EPHA2.

Cell-surface expression of EPHA2 on human melanoma cells. Flow cytometry was used to examine the expression of EPHA2 on the surface of the human melanoma cell lines

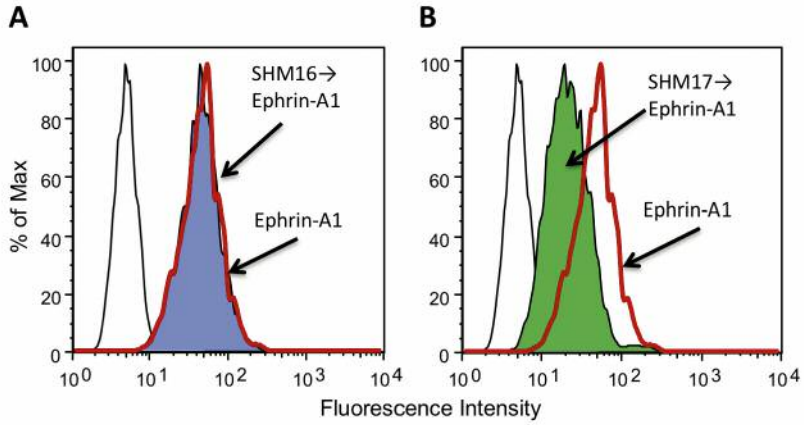

Figure 3. *Assays for competitive inhibition of ephrin-A1 binding to EPH receptor A2 (EPHA2) by SHM16 and SHM17. Approximately 1×10^6 A375 cells (500 μl per tube) harvested from a monolayer culture were incubated with 3 μl anti-EPHA2 antibody per tube (SHM16 or SHM17, 1 μg/ml) for 1 h on ice, and subsequently incubated with 3 μl ephrin-A1-Fc per tube (10 μg/ml) for 1 h at room temperature. Cells were then washed and stained with goat anti-human IgG (Fc specific), conjugated with fluorescence isothiocianate (FITC) for 30 min on ice.*

A375, A2058, G361, and SK-MEL28, as well as on normal skin melanocytes and fibroblasts. Of the four cell lines examined, EPHA2 was expressed on A375, and A2058 and faintly expressed on G361 cells (Figure 2A). Conversely, human dermal fibroblasts were found to be negative for EPHA2 using SHM16 and SHM17 (Figure 2B). The expression of EPHA2 was found in normal melanocytes using SHM17, however the reactivity of SHM16 with melanocytes was far weaker. In addition, human T- and B-lymphocytes were also negative for EPHA2 (Figure 2C). These data indicated that EPHA2 would be a suitable target for antibody treatment of melanoma using the SHM16 mAb.

SHM17, but not SHM16, interferes with binding of ephrin-A1 to EPHA2. In order to determine the difference between the epitopes recognized by SHM16 and SHM17, we next tested their ability to competitively inhibit ephrin-A1 binding to EPHA2 on the surface of A375 cells, using flow cytometry to detect binding. We found that SHM17 significantly interfered with binding of ephrin-A1 to EPHA2 (Figure 3). In contrast, SHM16 did not affect ephrin-A1 binding to EPHA2 on the surface of A375 cells. These data suggested that SHM17 would act as an antagonistic mAb, while SHM16 recognizes an alternative epitope of EPHA2 (Figure 3).

Inhibition of tumor cell migration by anti-EPHA2. It has been reported that EPH receptor ligands influence cell migration and invasion (24, 25). To investigate whether EPHA2 mAbs have the ability to inhibit tumor cell migration, we performed a wound scratch assay and a Matrigel invasion assay using A375 melanoma cells. Similarly to the natural ligand ephrin-A1, SHM16 suppressed migration and invasion by A375 human melanoma cells (Figures 4). However, the antagonistic antibody SHM17 was unable to inhibit tumor cell migration and invasion (Figure 4A). Overall, SHM16 stimulated signaling by EPHA2 that reduced A375 cell motility, indicating that it may be an agonistic EPHA2 mAb.

Therapeutic effect of a combination of immunotoxin and anti-EPHA2. We finally investigated the therapeutic effects of immunotoxins consisting of combinations of EPHA2 mAbs with a saporin-conjugated secondary antibody. Firstly, the internalization of SHM16 and SHM17 by A375 melanoma cells was analyzed using confocal laser scanning microscopy. As shown in Figure 5A, fluorophore- labeled SHM16 was clearly internalized by A375 cells, whereas SHM17 remained on the cell surface. Next, we examined the effect of anti-EPHA2 immunotoxins on cell growth and survival. Growth inhibition and cytotoxicity were found following targeted delivery of saporin by SHM16 and SHM20 (Figure 5B). As shown in Figure 5C, the therapeutic potential of antibody-mediated cytotoxin delivery by SHM16 and SHM20 was investigated using A375, A2058, G361, and SK-MEL28 human melanoma cell lines. Drastic growth inhibition and cytotoxicity were observed following targeted delivery of saporin by SHM16, and occurred in a dose-dependent manner.

Discussion

EPHA2 is a member of the EPH family of RTKs and is highly expressed in aggressive human cancer, including melanoma (26). By immunization with the A375 human melanoma cell line, we have established several mouse mAbs specific for human EPHA2. In this study, we examined whether EPHA2

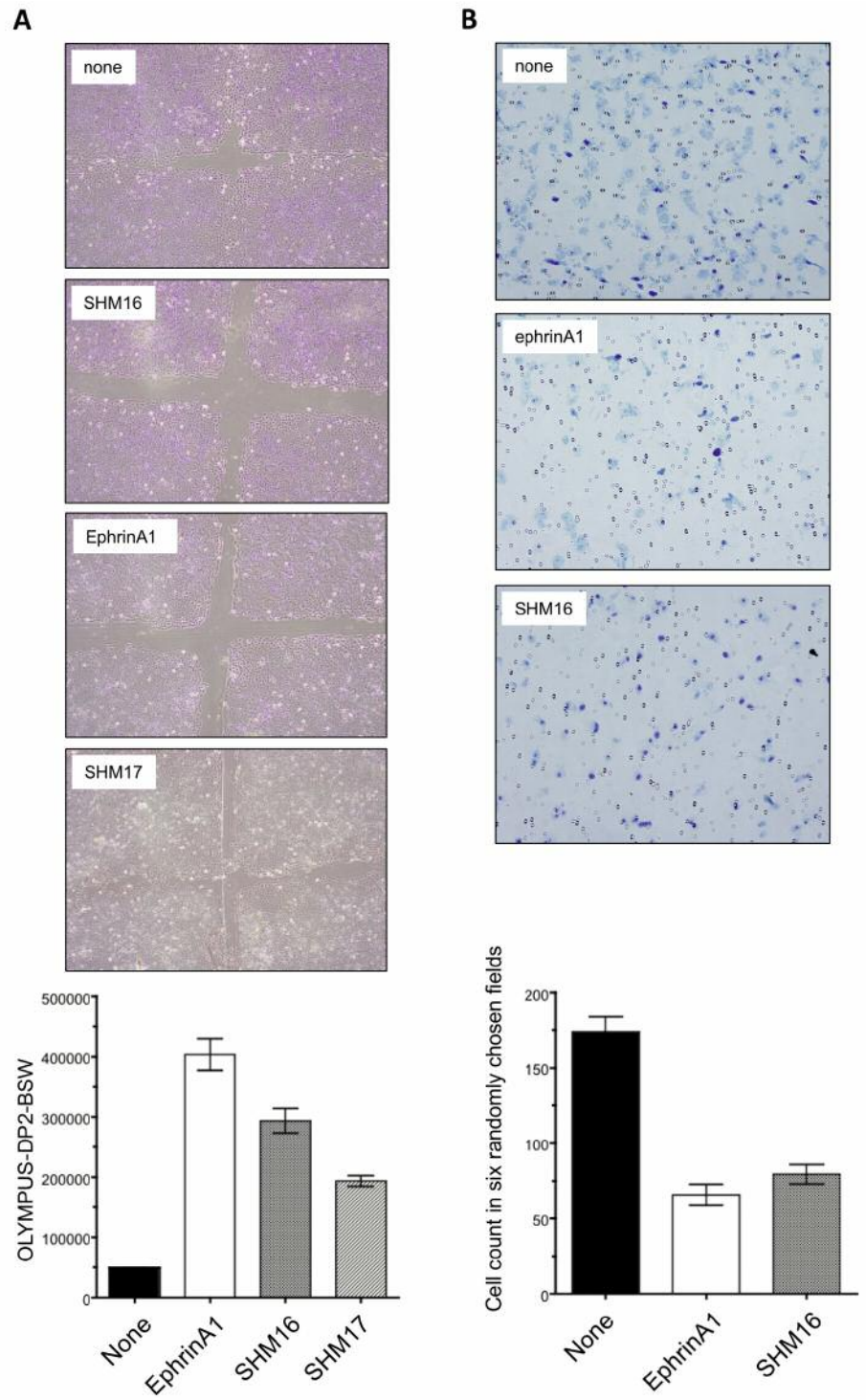

Figure 4. *Impact of SHM16 and SHM17 treatment on migration by A375 melanoma cells in vitro. A: Scratch assay. A confluent A375 cell culture was scratched with a pipette tip, and medium was added to induce migration of cells in the wound. The medium was either supplemented with ephrin-A1-Fc (20 ng/ml), SHM16 (10 μg/ml), SHM17 (10 μg/ml), or not at all (none). After a 24-h incubation, photographs were taken of each condition (left). The area of the open gap that remained was measured using the Olympus DP2-BSW software and is indicated on the bar graph (right) for each condition. *Significantly different at p<0.01 compared to the non-supplemented condition. B: Invasion assay. A375 cells were seeded into the upper chambers of trans-well inserts. Cells were allowed to migrate for 24 h toward the lower chamber in medium containing ephrin-A1-Fc (20 ng/ml) or SHM16 (10 μg/ml), or without anything added (none). Cell migration was evaluated after crystal violet staining by counting cells in six randomly chosen fields. *Significantly different at p<0.01 compared to control.*

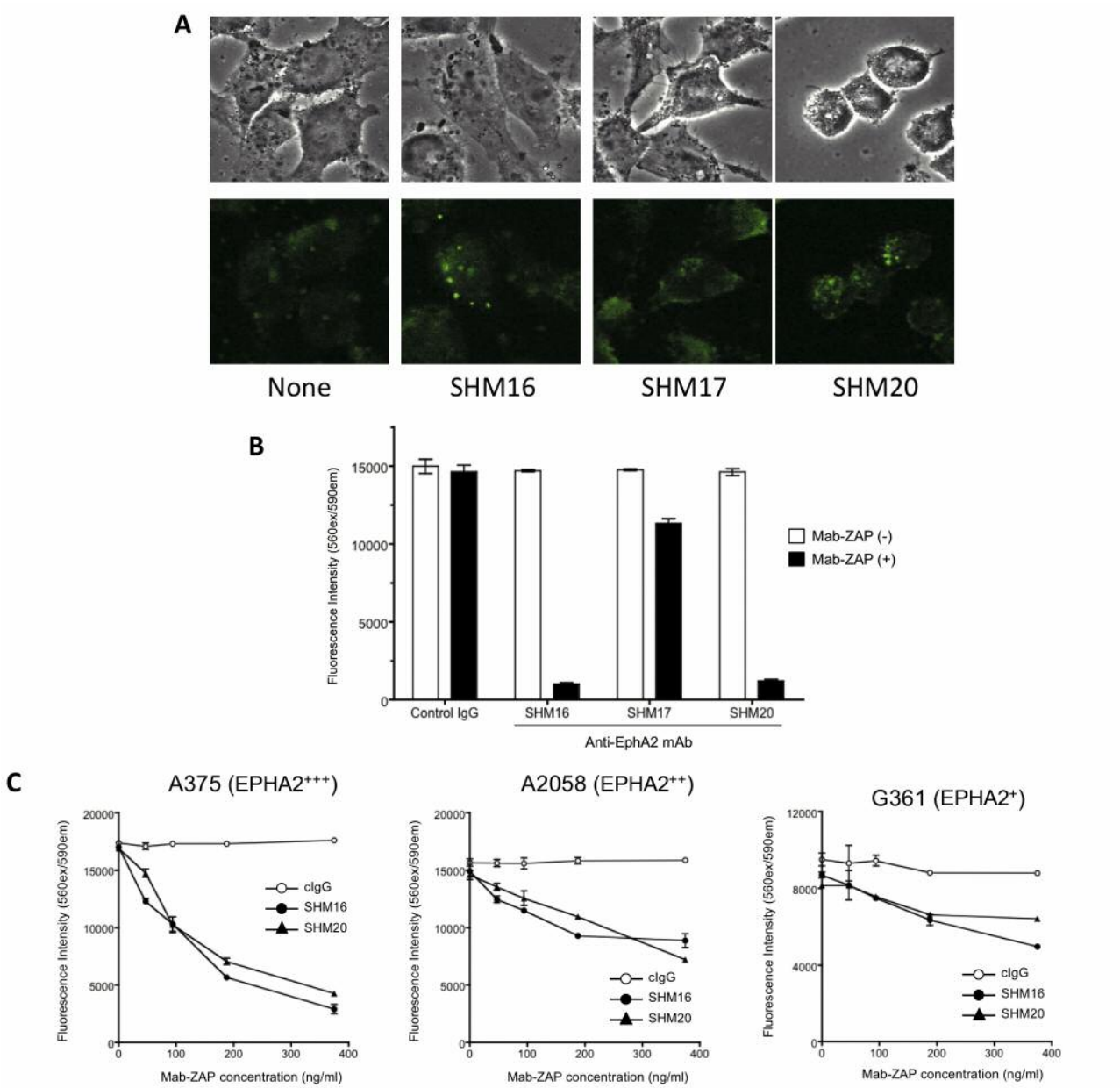

Figure 5. *Therapeutic effects of antibodies to EPH receptor A2 (EPHA2) when combined with an immunotoxin. A: Internalization of EPHA2 antibodies SHM16, SHM17, and SHM20 by A375 cells. Cells were seeded into each well of a four-well tissue plate in medium with one of the EPHA2 antibodies (2 μg/ml), and Alexa Fluor 488-conjugated goat anti-mouse IgG1 secondary antibody (2 μg/ml). A control well (None) had no primary antibody added but contained Alexa Fluor 488-conjugated goat anti-mouse IgG1 secondary antibody (2 μg/ml). After 24 h of incubation, photographs were taken under confocal laser scanning microscopy. Phase-contrast images (top) and fluorescent images (bottom) are shown for each condition. B: The cytotoxic effects of treatment with anti-EPHA2 in combination with an immunotoxin. A375 cells were plated into a flat-bottom, 96-well plate (2,000 cells per well) and incubated for 4 days at 37˚C. Wells contained 2 μg/ml of SHM16, SHM17, SHM20, or a control IgG1 monoclonal antibody, with or without saporin-conjugated goat anti-mouse IgG (Mab-ZAP, 750 ng/ml). After 24 h, AlamarBlue reagent was added to the medium, and cells were incubated at 37˚C for 48 h. The fluorescence intensity was measured at 590 nm following excitation at 570 nm. *Significantly different at p<0.01 compared to the control. C: The therapeutic effects of SHM16 and SHM20 against the human melanoma cell lines A375, A2058, G361, and SK-MEL28 were investigated using an antibody-mediated cytotoxicity assay.*

could be an integral player in cancer formation, and whether it could be a viable target for antibody therapy.

EPHA2 pathways are now viewed as intricate signaling networks containing modules of multi-protein complexes that assemble in various intracellular compartments to process, integrate, and transmit information that will ultimately specify a particular biological response (27). The interaction of natural ligands such as ephrin-A1 with EPHA2 receptor induces phosphorylation of a tyrosine motif in the cytoplasmic domain of EPHA2. Downstream of EPHA2 activation, an essential effector cascade required for most of the EPH receptor functions is the mitogen-activated protein kinase (MAPK) cascade. The MAPK cascade is comprised of the MAP kinase kinase (RAF), MAPK kinase (MEK), and extracellular signal-regulated kinase (ERK) kinases. The MAPK pathway includes several key signaling components, and phosphorylation events, that play roles in tumorigenesis. These activated kinases transmit extracellular signals that regulate cell growth, differentiation, proliferation, and migration, as well as apoptosis. Alteration of the RAS (oncogene of rat sarcoma)-RAF-MEK-ERK-MAPK (RAS-MAPK) pathway has been reported in human cancer as a result of abnormal activation of RTKs, or gain-of-function mutations mainly in the RAS or RAF genes (28-30).

Recently, targeted therapies have emerged as promising modality for cancer treatment. Various cancer-associated antigens have been targeted by immunotherapies such as cancer vaccine peptides, small interfering RNAs, and recombinant antibodies (18-20, 31). It is possible that the abundant expression of EPHA2 on the surface of malignant cells would make it a suitable target molecule for various cancer therapies. We found that human melanoma cell lines expressed EPHA2 on their surface, whereas normal dermal fibroblasts did not. We also examined the expression of EPHA2 by normal hematopoietic cells including lymphocytes, neutrophil, erythrocytes, and platelets (as shown in Figure 2C). Our data indicate that normal cells were generally not affected due to lack of EPHA2 expression. Interestingly, significant reactivity of our SHM17 anti-EPHA2 mAb with normal cultured melanocytes was observed, however, only faint reactivity of SHM16 with melanocytes was observed. In order to identify the epitope recognized by these anti-EPHA2 mAbs, we performed a competitive inhibition assay with these antibodies competing against ephrin-A1 to bind to EPHA2. As shown in Figure 3, binding of ephrin-A1 to EPHA2 on melanoma cells was competitively interfered with by treatment with SHM17, but not SHM16. In addition, we found that SHM16, but not SHM17, was internalized by A375 human melanoma cells. Considered together, these results strongly suggest that the epitopes recognized by SHM16 and SHM17 are distinct.

The functions of the EPHA2/ephrin-A1 system in tumorigenesis and angiogenesis make it a very attractive therapeutic target. Several studies showed that artificial ligands to EPHA2 inhibited tumor invasion and metastasis. Petty *et al*. showed that a small-molecule agonist of the EPHA2 RTK inhibited tumor cell migration in prostate cancer (20). They found that the agonistic small molecule inhibited AKT/ERK kinase activities. Synthetic peptides designed to selectively bind EPHA2 were reported to induce tyrosine phosphorylation of EPHA2 in cultured cells (32). Based on these findings, similarly to the natural ligand ephrin-A1, SHM16 also appears to be able to inhibit melanoma metastatic potential by affecting cell migration and invasion.

A number of these therapeutic approaches, including antibody-drug conjugates, immunotoxins, and targeted nucleic acid delivery, require antibodies that not only bind a receptor but also undergo internalization into the cell (33, 34). We previously reported the ability to generate cancer cell-specific internalized antibodies directly from antibody libraries selected for internalization into cancer cell lines (21, 35). Using an Adv-FZ33 screening system, we were able to selectively obtain different mAbs that had high affinity, and recognized antigens of high structure. As shown in Figure 5, specific and efficient immunotoxin delivery into melanoma cells was achieved following treatment with the SHM16 EPHA2 mAb and a saporin-conjugated secondary antibody. Drastic dose-dependent growth inhibition and cytotoxicity were observed using this approach, indicating that SHM16 immunotoxins targeting EPHA2 exhibit antitumor effects. Overall, we have succeeded in generating an agonistic antibody to EPHA2 that might be useful as a targeted therapy against melanoma. Similar results have been shown in colorectal and breast cancer (data not shown). Considering the differences in the cell lines, further studies are needed to clarify the relative contributions of ephrin-A1-EPHA2 signaling activities to tumor growth.

Disclosure Statement

The Authors have no conflicts of interest in regard to this study.

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