MT95-4, a fully humanized antibody raised against aminopeptidase N, reduces tumor progression in a mouse model

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Aminopeptidase N (APN/CD13) is involved in tumor cell invasion and tumor angiogenesis and is considered a promising therapeutic target in the treatment of cancer. To develop a novel monoclonal antibody-based cancer therapy targeting APN/CD13, we established a fully humanized anti-APN/CD13 monoclonal antibody, MT95-4. In vitro, MT95-4 inhibited APN/CD13 enzymatic activity on the tumor cell surface and blocked tumor cell invasion. B16 mouse melanoma cells stably expressing human APN/CD13 were also established and were inoculated s.c. or injected i.v. into nude mice. We found that expression of human APN/CD13 in murine melanoma cells increased the size of subcutaneous tumors, extent of lung metastasis and degree of angiogenesis in the subcutaneous tumors; these tumor-promoting and angiogenesis-promoting characteristics were reduced by the i.p. administration of MT95-4. To further verify the specificity of MT95-4 for neutralization of APN/CD13 activity, MT95-4 was administered into NOD/SCID mice inoculated s.c. with H1299 or PC14 cells, which exhibit high expression of APN/CD13, or with A549 cells, which exhibit weak expression of APN/CD13. MT95-4 reduced tumor growth and angiogenesis in mice bearing H1299-derived and PC14-derived tumors, but not in mice bearing A549-derived tumors. These results suggested that the antitumor and anti-angiogenic effects of MT95-4 were dependent on APN/CD13 expression in tumor cells. Given that MT95-4 is the first fully humanized monoclonal antibody against APN/CD13, MT95-4 should be recognized as a promising candidate for monoclonal antibody therapy against tumors expressing APN/CD13.
fore, to promote the potential clinical application of our work, we aimed to establish a fully humanized monoclonal antibody for inhibition of APN/CD13 activity. In the current study, we raised fully humanized monoclonal antibodies by immunization of KM mice, which produce humanized antibodies, with HT1080 cells and, from the resulting antibodies, we selected a monoclonal antibody (named MT95-4) on the basis of its ability to inhibit APN/CD13 activity in vitro. To evaluate the antitumor effects of MT95-4 in vivo, we established subcutaneous tumor models and tail vein metastasis models using B16-F1 melanoma cells transfected with human APN/CD13 and human lung cancer cells, which exhibited high expression of APN/CD13, in immune-deficient mice. Tumor-bearing mice were then treated with MT95-4, and the tumor progression and angiogenesis were analyzed.

Materials and Methods

Cells and cell culture. HT1080, B16-F1 melanoma, H1299 and A549 cells were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). PC14 cells were obtained from Immuno-Biological Laboratories (Gunma, Japan). HT1080, B16-F1, and A549 cells were cultured in DMEM (Gibco, Grand Island, NY, USA), and H1299 and PC14 cells were cultured in RPMI 1640 Medium (Gibco) supplemented with 10% FBS and 1% penicillin-streptomycin. All cells were incubated at 37°C in an atmosphere containing 5% CO2.

A retroviral vector (pDON-5neoCD13; Takara Bio, Shiga, Japan) containing APN/CD13 cDNA and a control pDON-5neo vector were transfected into B16-F1 melanoma cells according to the manufacturer’s protocol. Transfectants resistant to 1 mg/mL G-418 (Promega, Madison, WI, USA) were selected and maintained in DMEM with 10% FBS and 1 mg/mL G-418. From these B16-F1 transfectants, one cell line that expressed high levels of APN/CD13 (APN-B16 cells) and another cell line that did not express APN/CD13 (control-B16 cells) were selected. The expression levels of APN/CD13 were determined by flow cytometry and real-time PCR.

Reagents and animals. Matrigel was purchased from BD Biosciences (Bedford, MA, USA). KM mice were kindly provided by Kyowa Hakko Kirin (Tokyo, Japan). Male BALB/c nude mice and NOD/SCID mice (6–8-weeks old) were purchased from Charles River Laboratories (Wilmington, MA, USA). Animals were maintained according to guidelines for the ethical use of animals in research at Hiroshima University.

Establishment of a fully humanized monoclonal antibody that inhibited APN/CD13 activity. Because the establishment of a murine monoclonal antibody against APN/CD13 had been successful by immunizing Balb/c mice with HT1080 human fibrosarcoma cells,(13) KM mice, which lack endogenous genes encoding the immunoglobulin (Ig) H and L κ chains and instead carry human chromosome 14 fragments containing the entire human Ig H chain loci and the human κ L chain segments,(20) were injected i.p. with HT1080 cells three times. Three days after the last injection, spleen cells from the mice were fused to SP2/O-AG14 myeloma cells (ATCC) to generate hybridomas. A clone that had an inhibitory effect on APN/CD13 activity assessed by aminopeptidase assay was selected (MT95-4).

Aminopeptidase assay. Cell-associated aminopeptidase activity was assessed by measuring the amount of 7-amino-4-methylcoumarin (AMC) liberated from L-alanine-4-methylcoumarin-7-amide (Ala-MCA; Peptide Institute, Osaka, Japan), as previously described.(8,9) A mixture containing 0.1 mM Ala-MCA, 5 × 10^5 HT1080 cells, and a raised antibody or human control IgG (Sigma, St. Louis, MO, USA) suspended in 200 μL of...
PBS was seeded into each well of a 96-well plate and incubated for 2 h at 37°C in an atmosphere containing 5% CO2. After incubation, 10 μL of 0.2 M EDTA was added to each well to terminate the reaction, and the level of AMC was then analyzed with a TriStar LB 941 Multimode microplate reader (excitation, 355 nm; emission, 460 nm; Berthold Technologies, Bad Wildbad, Germany).

Matrigel invasion assay. The invasive properties of tumor cells were assessed using Matrigel invasion chambers (24-well inserts, pore size: 8 μm [BD Biosciences]). Tumor cells were resuspended in medium containing 0.1% FBS. Cell suspensions with MT95-4 or control IgG were added to the upper chamber at a density of 2.5 × 10⁴ cells/500 μL medium/well and incubated for 22 h at 37°C in an atmosphere containing 5% CO2. The cells invading to the lower surface of the membrane were stained with Diff-Quik stain after removing noninvading cells with cotton swabs. Images were captured using a microscope at a magnification of 200 × (model BZ-9000; Keyence, Osaka, Japan), and the number of invading cells was counted in five random microscopic fields per well.

Flow cytometric analysis. FACS was performed to assess the expression level of APN/CD13 on the cell surface. The cells were incubated for 30 min at 4°C with murine monoclonal anti-APN/CD13 antibodies (WM15; BD Biosciences) and isotype control. Cells were then washed with PBS and stained with Alexa-Fluor-488-conjugated goat anti-mouse secondary antibodies (Invitrogen, Carlsbad, CA, USA). The samples were analyzed on a FACS Calibur flow cytometer (BD Biosciences), and the data were analyzed using CELL Quest software (BD Biosciences).

Quantitative real-time PCR. Total RNA was isolated using an RNeasy Mini kit (Qiagen, Valencia, CA, USA). The isolated total RNA was reverse transcribed into cDNA using a High Capacity RNA-to-cDNA kit (Applied Biosystems, Framingham, MA, USA) following the manufacturer’s instructions. Quantitative real-time PCR was performed on an ABI Prism 7700 (Applied Biosystems) for detection and quantification of human APN/CD13 using beta-actin as a control housekeeping gene.

Western blot analysis. HT1080 cells were lysed with lysis buffer containing 1% 3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonate. The soluble fraction of the cell lysate was electrophoresed on 10% sodium dodecyl sulfate SDS-PAGE and transferred electrophoretically to membranes. The lower part of the membrane around 43 kDa was cut and

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**Fig. 2.** Effects of human APN/CD13 expression in murine B16-F1 melanoma cells. (a) Expression levels of human APN/CD13 mRNA in control-B16 and APN-B16 cells were evaluated by quantitative real-time PCR. (b) Expression levels of APN/CD13 on the surface of control-B16 and APN-B16 cells were assessed by flow cytometry. Data (a) represent the mean (±SEM) of triplicate samples. ***P < 0.001. (c) Comparison of the numbers of lung surface nodules in the tail vein metastasis model between control-B16 and APN-B16 cells. The numbers of lung surface nodules in mice were counted 21 days after injection of control-B16 and APN-B16 cells. Each bar represents the value of the mean number of nodules, for eight mice per group. *P < 0.05. (d) Comparison of tumor volumes in a subcutaneous tumor model for tumors derived from control-B16 and APN-B16 cells. The sizes of subcutaneous tumors were measured twice a week for 2 weeks after inoculation of control-B16 and APN-B16 cells. The data represent the mean (±SEM) for eight mice per group. *P < 0.05. (e) Evaluation of angiogenesis in subcutaneous tumors derived from control-B16 and APN-B16 cells. The area containing CD31-positive vessels was measured. Data represent the means (±SEM) from eight mice per group. **P < 0.01. (f) Immunohistochemical staining for CD31 in a subcutaneous tumor. Scale bar, 100 μm.
incubated overnight with rabbit anti-β-actin antibodies (4976S; Cell Signaling Technology, Danvers, MA, USA). The remaining section of each membrane was evenly divided into two parts and incubated with rabbit anti-APN/CD13 antibodies (2972-1; Abcam, Cambridge, UK) or MT95-4. After washing, the membranes were incubated with HRP-conjugated anti-rabbit or anti-human antibodies (NA934 or NA933, respectively; GE Healthcare, Buckinghamshire, UK). The signals were detected using enhanced chemiluminescence reagent (GE Healthcare) followed by exposure to X-ray film.

Cell proliferation assays. Control-B16 and APN-B16 cells (5 × 10⁴ cells/well) were incubated in 96-well plates, with 100 μL medium per well. After culturing for 1–4 days, 10 μL of Cell Counting Kit-8 reagent (Dojindo, Kumamoto, Japan) was added to each well according to the manufacturer’s protocol. The absorbance was measured at 450 nm using a microplate reader.

Subcutaneous tumor model. Control-B16 or APN-B16 cells (1 × 10⁵) were inoculated s.c. into the right flanks of nude mice. H1299, PC14 or A549 cells (1 × 10⁵) were inoculated into the right flanks of NOD/SCID mice. Tumor-bearing mice were injected i.p. with 1 mg/kg MT95-4 or control human IgG (Sigma) twice per week. The length and width of the tumors were measured using calipers, and the tumor volume was calculated using the formula: \( \text{tumor volume} = \text{length} \times \text{width}^2 \times 0.5 \) (21).

Tail vein metastasis model. Control-B16 or APN-B16 cells (2 × 10⁶) were injected into nude mice through the tail vein. H1299 cells (1 × 10⁶) were injected into NOD/SCID mice. Tumor-bearing mice were injected i.p. with 1 mg/kg MT95-4 or control human IgG (Sigma) twice per week.

Evaluation of microvessel density in subcutaneous tumors. Frozen sections of subcutaneous tumors were incubated with rat polyclonal antibodies against mouse CD31 (550274; BD Biosciences) and then reacted for 30 min with a biotinylated rabbit anti-rat IgG antibody (Vector Laboratories, Burlingame, CA, USA). The immunoreaction was amplified with a Vectastain ABC Kit (Vector Laboratories) and visualized by incubation with a 3, 3-diaminobenzidine solution acting as a chromogen. The sections were then counterstained with hematoxylin and dehydrated. Images were captured using a microscope at a magnification of 200× (model BZ-9000; Keyence), and the area of CD31-positive vessel-like structures was measured in five random microscopic fields per section using Dynamic Cell Count software (BZ-HIC; Keyence).

Statistical analysis. Statistical analyses were performed using Prism 5 (GraphPad software, San Diego, CA, USA). All the results are expressed as means ± SEM. Differences between the groups were evaluated using Student’s t-tests or Mann—Whitney U-tests. Differences with P-values of < 0.05 were considered statistically significant.

Results

A fully humanized monoclonal antibody (MT95-4) recognized APN/CD13 and inhibited invasion and aminopeptidase activity in HT1080 cells in vitro. By immunizing KM mice with HT1080 fibrosarcoma cells abundantly expressing APN/CD13, we established a fully humanized monoclonal antibody, MT95-4, which had inhibitory effects against aminopeptidase activity. To verify the specificity of MT95-4, western blotting analysis was performed using HT1080 cell lysates. As shown in Figure 1a, MT95-4 detected a band at 150 kDa corresponding to the predicted size of APN/CD13, which coincided with the band from a commercially available anti-APN/CD13 antibody.

To confirm the selection of MT95-4, we examined the inhibitory effects of MT95-4 on the aminopeptidase activity of HT1080 cells by measuring the level of AMC liberated from alanine-MCA. As shown in Figure 1b, MT95-4 neutralized the
aminopeptidase activity of HT1080 cells. We then evaluated whether MT95-4 affected the invasive properties of HT1080 cells using Matrigel invasion chambers. Compared to control IgG, MT95-4 inhibited the invasion of HT1080 cells (Fig. 1c).

**APN/CD13 promoted tumor growth and angiogenesis in vivo.** We established two B16-F1-derived cell lines that differed in the expression levels of human APN/CD13 (control-B16 and APN-B16 cells). Quantitative real-time PCR and flow cytometric analysis revealed that APN-B16 cells expressed high levels of human APN/CD13, while control-B16 cells did not (Fig. 2a,b). When the in vitro cell proliferation rates of the two cell lines were compared, no differences were observed (data not shown).

Next, to determine whether the expression of human APN/CD13 in murine B16-F1 melanoma cells affected tumor progression in vivo, control-B16 cells or APN-B16 cells were injected into nude mice through the tail vein or inoculated s.c. into right flanks of nude mice. As shown in Figure 2c and d, mice bearing APN-B16 cells exhibited significantly increased numbers of lung surface nodules and larger subcutaneous tumors than mice bearing control-B16 cells. In addition, to confirm the role of APN/CD13 in tumor angiogenesis, the degrees of angiogenesis in these subcutaneous tumors were assessed by immunohistochemical staining with anti-CD31 monoclonal antibodies. The CD31-positive areas in subcutaneous tumors derived from APN-B16 cells were larger than those of tumors derived from control-B16 cells (Fig. 2e,f).

**MT95-4 reduced tumor progression and angiogenesis promoted by the expression of human APN/CD13 in murine B16-F1 melanoma cells.** To confirm the neutralizing effect of MT95-4 against APN/CD13 in vivo, we established a tail vein metastasis model and a subcutaneous xenograft model using APN-B16 cells.
cells and administered MT95-4 (1 mg/kg) i.p. twice per week in tumor-bearing mice. As shown in Figure 3a and b, administration of MT95-4 reduced the number of lung surface nodules and the size of subcutaneous tumors in mice bearing APN-B16 cells. In addition, the sections of subcutaneous tumors consisting of APN-B16 cells were immunohistochemically stained with anti-CD31 antibodies, and the areas of CD31-positive vessels were significantly smaller in the MT95-4-treated group than in the control group (Fig. 3c,d).

Antitumor and anti-angiogenic effects of MT95-4 were dependent on the expression of APN/CD13 in tumor cells. To further verify whether the antitumor effects of MT95-4 were associated with the expression of APN/CD13 in cancer cells, we administered MT95-4 to mice bearing human lung cancer cells abundantly or weakly expressing APN/CD13. As shown in Figure 4a and b, real-time PCR analysis and flow cytometric analysis revealed that APN/CD13 was abundantly expressed in H1299 and PC14 cells but weakly expressed in A549 cells. Thus, we established subcutaneous tumor xenografts using H1299, PC14 and A549 cells in NOD/SCID mice, and MT95-4 or control IgG was administered i.p. The administration of MT95-4 reduced tumor volumes in mice bearing H1299 and PC14 cells (Fig. 4c,f) but not in mice bearing A549 cells (Fig. 4i). Moreover, the degree of angiogenesis in subcutaneous tumors derived from H1299 and PC14 cells was lower in the MT95-4-treated group than in the control group (Fig. 4d,g); however, there were no differences in the degree of angiogenesis between MT95-4-treated and control IgG-treated mice bearing tumors derived from A549 cells (Fig. 4j).

Discussion

In the present study, we established a fully humanized monoclonal antibody, MT95-4, that had the potential to neutralize human APN/CD13 activity. We also established two murine melanoma B16-F1-derived cell lines; one exhibited abundant expression of human APN/CD13, while the other did not exhibit expression of human APN/CD13. By inoculating these cells into nude mice, the expression of human APN/CD13 in murine melanoma cells was shown to promote tumor growth.
and angiogenesis in vivo. Interestingly, MT95-4 was able to reduce tumor growth and angiogenesis promoted by the expression of human APN/CD13 in vivo. In addition, we demonstrated that the ability of MT95-4 to inhibit tumor growth and angiogenesis was dependent on the expression of APN/CD13 in tumor cells. In vitro, MT95-4 dose-dependently inhibited the invasive properties of malignant tumor cells.

The most notable result from the current study was that we were able to establish a fully humanized monoclonal antibody, named MT95-4, that could neutralize human APN/CD13 activity. Given that MT95-4 recognized cell surface APN/CD13, we can speculate that the neutralization of APN/CD13 activity on the tumor cell surface by MT95-4 reduced tumor progression by inhibiting tumor cell invasion and angiogenic drive. Interestingly, MT95-4 could not recognize murine APN/CD13 (data not shown) and, thus, did not inhibit APN/CD13 activity in mouse endothelial cells. Therefore, the results of the present study suggested that the APN/CD13 activity of tumor cells promoted angiogenesis regardless of the APN/CD13 activity of endothelial cells, and this angiogenic drive from tumor cells was inhibited by MT95-4.

In the tumor microenvironment, APN/CD13 is expressed in stromal cells and endothelial cells, and the expression of APN/CD13 in these nonmalignant cells is involved in tumor growth and angiogenesis. In the present study, however, we could not affect the APN/CD13 activity of host murine cells in the tumor microenvironment because MT95-4 could not react with mouse APN/CD13. Interestingly, we confirmed that MT95-4 had the ability to inhibit in vitro tube formation by human umbilical vein endothelial cells (data not shown). Therefore, we can speculate that MT95-4 may inhibit APN/CD13 activity not only on tumor cells but also on endothelial cells in the human tumor microenvironment and may exert stronger antitumor effects in humans than in our mouse xenograft tumor models. In addition, we were not able to assess whether MT95-4 has antitumor effects related to antibody-dependent cellular cytotoxicity and/or complement-dependent cytotoxicity in the present study. Further experiments are required to evaluate these points.

APN/CD13 is considered a potential therapeutic target for the treatment of cancer. Currently, bestatin is clinically used as an APN/CD13 inhibitor. To the best of our knowledge, MT95-4 is the first fully humanized monoclonal antibody against APN/CD13 and has been shown to possess antitumor effects. Given that monoclonal antibodies against vascular endothelial growth factor or epidermal growth factor receptor are currently used in the clinical setting, we believe that MT95-4 may be a good candidate for monoclonal antibody-based anticancer therapy. Preparation for the clinical application of this antibody will begin in the near future.

In conclusion, we established a fully humanized monoclonal antibody against APN/CD13 (MT95-4) that inhibited the invasive properties of malignant tumor cells in vivo and prevented tumor progression and angiogenesis in vivo. These antitumor effects of MT95-4 were thought to be mediated by the neutralization of APN/CD13 activity at the tumor cell surface. Given that MT95-4 is a fully humanized antibody and may inhibit APN/CD13 activity in nonmalignant cells in the tumor microenvironment, which promote tumor growth, MT95-4 may be a promising candidate for monoclonal antibody-based anticancer therapy.

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Disclosure Statement

The authors have no conflict of interest to declare.

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