vectors for gene therapy and animal transgenesis, Gene ther. 18 (2011) 384–393.
[9] Y. Kuroiwa, K. Tomizuka, T. Shinohara, Y. Kazuki, H. Yoshida, A. Ohguma, T. Yamamoto, S. Tanaka, M. Oshimura, I. Ishida, Manipulation of human minichromosomes to carry greater than megabase-sized chromosome inserts, Nat. Biotechnol. 18 (2000) 1086–1090.
[10] M. Katoh, F. Ayabe, S. Norikane, T. Okada, H. Masumoto, S. Horike, Y. Shirayoshi, M. Oshimura, Construction of a novel human artificial chromosome vector for gene delivery, Biochem. Biophys. Res. Commun. 321 (2004) 280–290.
[11] M. Oshimura, M. Katoh, Transfer of human artificial chromosome vectors into stem cells, Reprod. Biomed. Online 16 (2008) 57–69.
[12] K. Okita, T. Ichisaka, S. Yamanaka, Generation of germline-competent induced pluripotent stem cells, Nature 448 (2007) 313–317.
[13] N. Fusaki, H. Ban, A. Nishiyama, K. Saeki, M. Hasegawa, Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome, Proc. Jpn. Acad. Ser. B Phys. Biol. Sci. 85 (2009) 348–362.
[14] Y. Okada, E. Matsuura, Z. Tozuka, R. Nagai, A. Watanabe, K. Matsumoto, K. Yasui, R.W. Jackman, T. Nakano, T. Doi, Upstream stimulatory factors stimulate transcription through E-box motifs in the PF4 gene in megakaryocytes, Blood 104 (2004) 2027–2034.
[15] H. Kurosaki, M. Hiratsuka, N. Imaoka, Y. Iida, N. Uno, Y. Kazuki, C. Ishihara, Y. Yakura, J. Mimuro, Y. Sakata, H. Takeya, M. Oshimura, Integration-free and stable expression of FVIII using a human artificial chromosome, J. Hum. Genet. 56 (2011) 727–733.
[16] N. Komatsu, K. Kirito, R. Shimizu, M. Kunitama, M. Yamada, M. Uchida, M. Takatoku, M. Eguchi, Y. Miura, *In vitro* development of erythroid and megakaryocytic cells from a UT-7 subline, UT-7/GM, Blood 89 (1997) 4021–4033.
[17] K. Tomizuka, H. Yoshida, H. Uejima, H. Kugoh, K. Sato, A. Ohguma, M. Hayasaka, K. Hanaoka, M. Oshimura, I. Ishida, Functional expression and germline transmission of a human chromosome fragment in chimeric mice, Nat. Genet. 16 (1997) 133–143.
[18] K. Eto, R. Murphy, S.W. Kerrigan, A. Bertoni, H. Stuhlmann, T. Nakano, A.D. Leavitt, S.J. Shattil, Megakaryocytes derived from embryonic stem cells implicate CalDAG-GEFI in integrin signaling, Proc. Natl. Acad. Sci. USA 99 (2002) 12819–12824.
[19] W.M. McCormack, M.P. Seiler, T.K. Bertin, K. Ubhayakar, D.J. Palmer, P. Ng, T.C. Nichols, B. Lee, Helper-dependent adenoviral gene therapy mediates long-term correction of the clotting defect in the canine hemophilia A model, J. Thromb. Haemost. 4 (2006) 1218–1225.
[20] D.A. Roth, N.E. Tawa, J.M. O'Brien, D.A. Treco, R.F. Selden, F.V.T.T.S. Group, nonviral transfer of the gene encoding coagulation factor VIII in patients with severe hemophilia A., N. Engl. J. Med. 344 (2001) 1735–1742.
[21] C.B. Doering, G. Denning, K. Dooriss, B. Gangadharan, J.M. Johnston, K.W. Kerstann, D.A. McCarty, H.T. Spencer, Directed engineering of a high-expression chimeric transgene as a strategy for gene therapy of hemophilia A, Mol. Ther. 17 (2009) 1145–1154.
[22] F.S. Tedesco, H. Hoshiya, G. D'Antona, M.F. Gerli, G. Messina, S. Antonini, R. Tonlorenzi, S. Benedetti, L. Berghella, Y. Torrente, Y. Kazuki, R. Bottinelli, M. Oshimura, G. Cossu, Stem cell-mediated transfer of a human artificial chromosome ameliorates muscular dystrophy, Sci Transl Med 3 (2011). 96ra78.
[23] M. Stadtfeld, M. Nagaya, J. Utikal, G. Weir, K. Hochedlinger, Induced pluripotent stem cells generated without viral integration, Science 322 (2008) 945–949.
[24] J. Yu, K. Hu, K. Smuga-Otto, S. Tian, R. Stewart, I.I. Slukvin, J.A. Thomson, Human induced pluripotent stem cells free of vector and transgene sequences, Science 324 (2009) 797–801.
[25] K. Takahashi, K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, S. Yamanaka, Induction of pluripotent stem cells from adult human fibroblasts by defined factors, Cell 131 (2007) 861–872.
[26] D. Xu, Z. Alipio, L.M. Fink, D.M. Adcock, J. Yang, D.C. Ward, Y. Ma, Phenotypic correction of murine hemophilia A using an iPS cell-based therapy, Proc. Natl. Acad. Sci. USA 106 (2009) 808–813.
[27] T. Ohmori, J. Mimuro, K. Takano, S. Madoiwa, Y. Kashiwakura, A. Ishiwata, M. Niimura, K. Mitomo, T. Tabata, M. Hasegawa, K. Ozawa, Y. Sakata, Efficient expression of a transgene in platelets using simian immunodeficiency virus-based vector harboring glycoprotein Ibalpha promoter: *in vivo* model for platelet-targeting gene therapy, FASEB J. 20 (2006) 1522–1524.
[28] Q. Shi, D.A. Wilcox, S.A. Fahs, H. Weiler, C.W. Wells, B.C. Cooley, D. Desai, P.A. Morateck, J. Gorski, R.R. Montgomery, Factor VIII ectopically targeted to platelets is therapeutic in hemophilia A with high-titer inhibitory antibodies, J. Clin. Invest. 116 (2006) 1974–1982.

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Robo4 is an effective tumor endothelial marker for antibody-drug conjugates based on the rapid isolation of the anti-Robo4 cell-internalizing antibody

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Full title: Robo4 is an effective tumor endothelial marker for antibody-drug conjugates based on the rapid isolation of the anti-Robo4 cell-internalizing antibody.

Short title: Anti-Robo4 cell-internalizing antibody

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Key Points

- First therapeutic application which targets Robo4 on the tumor blood vasculature
- High-throughput screening system to isolate cell-internalizing monoclonal antibodies useful to develop effective antibody-drug conjugates

Abstract

Monoclonal antibodies (mAbs) that are internalized into cells are a current focus in the development of antibody-drug conjugates (ADCs). We describe a phage display-based high-throughput screening system to rapidly isolate cell-internalizing mAbs. We simultaneously examined the cell-internalizing activities of several hundred independent mAbs, and successfully isolated cell-internalizing mAbs against the tumor endothelial markers Roundabout homolog 4 (Robo4) and vascular endothelial growth factor receptor 2 (VEGFR2). Tumor accumulation of mAbs with high cell-internalizing activity was significantly higher than that of mAbs with low cell-internalizing activity. Furthermore, the anti-tumor effects of ADCs of mAbs with high cell-internalizing activity were significantly stronger than those of mAbs with low cell-internalizing activity. While anti-VEGFR2 therapy caused a significant loss of body weight, anti-Robo4 therapy did not. These findings indicate that cell-internalizing activity has an important role in the biodistribution and therapeutic effects of ADCs. Further, Robo4 can be an effective marker for tumor vascular targeting.

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Introduction

Antibody drug conjugates (ADCs), i.e., monoclonal antibodies (mAbs) labeled with certain anti-cancer agents, are currently the focus of antibody-based drug discovery. ADCs have mAb-derived specificity and allow for targeted delivery of cytotoxic drugs to a tumor, which is expected to significantly enhance the anti-tumor activity of mAbs[1]. Trastuzumab ematansine (T-DM1)[2] for human epidermal growth factor receptor 2 (Her-2)–positive breast cancer and brentuximab vedotin (SGN-35)[3] for relapsed or refractory CD30-positive lymphoproliferative disorders are now in phase III clinical trials as effective ADCs[4]. ADCs will have an important role in overcoming some types of refractory cancers, and will contribute to the field of tumor vascular targeting[5].

An essential property of ADCs is that the mAb should be efficiently internalized into the cell where the cytotoxic effects of anti-cancer drugs occur[1]. The isolation of mAbs with high cell-internalizing activity (cell-internalizing mAbs) is a limiting factor in the development of ADCs. The discovery of potent cell-internalizing mAbs, however, requires labor-intensive screening of a massive number of candidates and therefore the development of phage display-based methods to identify these candidates is highly desirable[6,7]. In the phage display-based method, a phage antibody library is added to the desired cells, and then phages bound to the cell surface are removed. Only internalized phages are rescued from the intracellular compartment. Even with this method, however, the internalizing activities of individual antibody candidates must be assessed, because the concentrated phage pool comprises a "polyclonal" population. To address this issue, we used

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high-throughput screening methods to estimate "monoclonal" cell-internalization activities using a protein synthesis inhibitory factor (PSIF)[8], which provided a breakthrough in reducing the time-consuming screening of the cell-internalizing activity.

PSIF is a fragment of a bacterial exotoxin derived from *Pseudomonas aeruginosa*[9]. PSIF lacks its cell binding domain, and its cytotoxic portion is used in a recombinant immunotoxin[10]. Upon entry into the cell, PSIF has strong cytotoxicity by inducing ADP-ribosylation of elongation factor-2, which is essential for protein synthesis[11]. Our group previously accelerated the identification of cell-internalizing novel protein transduction domains (PTDs) by expressing PTD-PSIF fusion proteins in the supernatant of *Escherichia coli*[8]. Using this system, we successfully discovered superior HIV-Tat PTD mutants by simultaneously estimating the cell-internalizing activities of several hundred monoclonal PTD-PSIF fusions[8]. Therefore, we expect this method to contribute to the identification of mAbs with high cell-internalizing activity (cell-internalizing mAbs) by expressing single-chain antibody Fv (scFv)-PSIF fusion proteins to estimate the cell-internalizing activities of a very large number of antibodies.

Roundabout homolog 4 (Robo4) is a potential tumor angiogenesis marker[12]. Robo4 expression is restricted to areas of *in vivo* angiogenesis[13,14] and the subpopulation of hematopoietic stem cells localized in the bone marrow[15]. At angiogenic sites, Robo4 is present in the endothelial lining of blood vessels in the developing embryo[16], placenta[14], and tumors[17]. We previously confirmed the endothelial cell-specific expression of Robo4 using transgenic mouse lines[18,19]. Robo4 acts as a receptor that modulates vascular endothelial growth factor A (VEGF) – VEGF receptor (VEGFR)

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signaling[20-23]. Therefore, Robo4 is a potential marker for tumor vascular targeting because angiogenesis is only activated in tumors in the adult[24], with the exception of some pathological states[25,26]. Another potential tumor angiogenesis marker is VEGFR2, a well-established tumor endothelial marker[27]. The VEGF-VEGFR2 signaling pathway has a crucial role in angiogenesis and anti-VEGF mAbs and small molecule inhibitors against VEGFR are approved for various types of cancers[28]. Anti-VEGFR2 mAbs are also used for tumor vascular targeting[29]. Although VEGFR2 is strongly expressed in active angiogenic sites, its expression is also observed in normal tissues[30]. Hypertension and proteinuria are common side effects of anti-VEGF therapy because VEGF-VEGFR signaling is also inhibited in normal tissue, including the kidney, heart, and resistance vessels[31-33].

Here we applied the PSIF system to search for novel cell-internalizing mAbs from an immune phage antibody library. Application of this method to Robo4 and VEGFR2 led to the successful discovery of anti-Robo4 and anti-VEGFR2 cell-internalizing mAbs, as well as mAbs with low cell-internalizing activity (low-internalizing mAbs) to be used as controls. Comparing mAbs with different cell-internalizing activities revealed that higher cell-internalizing activity enhanced the tumor targeting potency of mAbs. Furthermore, comparative studies with anti-Robo4 and anti-VEGFR2 cell-internalizing mAbs *in vivo* indicated that Robo4 was superior to VEGFR2 in terms of the therapeutic window. This is the first report demonstrating the benefits of cell-internalizing mAbs in tumor vascular targeting. Further, these findings demonstrate the potential of Robo4 as a target for further development of novel ADCs against tumor blood vasculature.

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Materials and Methods

Cell culture

MS1 immortalized murine endothelial cells were cultured in Dulbecco's Modified Eagle Medium containing 5% fetal bovine serum (FBS) and 1% Antibiotic-Antimycotic Mixed Solution. B16BL6 murine melanoma cells were cultured in Minimum Essential Medium containing 10% FBS and 1% Antibiotic-Antimycotic Mixed Solution at 37°C. These cells were maintained at 37°C under a humidified 5% CO_2 atmosphere.

B16BL6 tumor-bearing mice

B16BL6 cells (1×10^6 cells/100 μl) were inoculated intracutaneously into 6-week-old female C57BL6 mice (Japan SLC Inc., Japan) (Day 0). Biodistribution was analyzed on the day that the tumor width reached 10 mm. The therapy experiment was started on Day 3. As a validation of the model, we confirmed the expressions of VEGFR2 and Robo4 on the tumor endothelium, based on the immunofluorescence against B16BL6 tumor sections.

Antigens

Human VEGFR2 (hVEGFR2) and mouse VEGFR2 (mVEGFR2) were commercial recombinant proteins (Merck Chemicals, Inc., Germany, or R&D Systems, Inc., Minneapolis, MN). Human Robo4 (hRobo4) and mouse Robo4 (mRobo4) were produced as described previously[34].

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Immune phage antibody libraries

Phage antibody libraries were constructed from the spleen and bone marrow cells of immunized mice as previously described[35,36]. Our phage antibody library comprised single chain Fv fragment (scFv) fused with pIII phage coat protein. Four rounds of affinity panning were performed against hVEGFR2 and mVEGFR2 for the anti-VEGFR2 immune library, and against hRobo4 and mRobo4 for the anti-Robo4 immune library. Anti-FLAG panning was followed by each panning to concentrate the scFv-displaying phages, as described previously[36].

ELISA & cytotoxicity assay using TG1 supernatant

Plasmids were extracted from TG1 cells after the 4th panning against mVEGFR2 or mRobo4. These "enriched" scFv gene libraries were cloned into a PSIF-fusion expression vector derived from pCANTAB5E[8]. Monoclonal scFv-PSIF protein was induced in the TG1 supernatant, as previously described[8]. mVEGFR2 or mRobo4 was immobilized on an immunoassay plate and blocked with 4% skim milk in PBS (4% MPBS) at 37°C for 2 h. TG1 supernatant containing 2% MPBS was reacted with antigens at room temperature for 1 h. Bound scFv-PSIFs were detected by anti-FLAG-horseradish peroxidase (HRP; M2, Sigma-Aldrich Corporation, St. Louis, MO). For the cytotoxicity assay, MS1 cells were seeded on a 96-well plate at 1.0×10^4 cells/well. After incubation at 37°C for 24 h, TG1 supernatant was diluted in MS1 culture medium, and then added to the MS1 cells. After incubation at 37°C for 24 h, cell viability was assessed using a WST-8 assay (Dojindo Molecular Technologies, Inc., Japan). The viability of non-treated MS1 and completely killed

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MS1 with 1 mM cycloheximide were defined as 100% and 0%, respectively.

Expression and purification of scFv, dscFv, and scFv-PSIF recombinant protein

The isolated scFv gene with 15 amino acids linker (VL-GGGGSGGGGSGGGGS-VH) was cloned into modified pET15b vector, resulting in the scFv fused by FLAG-tag and His×6 tag at the C-terminus. A scFv gene with a 5 amino acid linker (VL-GGGGS-VH) was also cloned into modified pET15b, resulting in a non-covalent scFv dimer (dscFv) fused by FLAG-tag and His×6 tag at the C-terminus. An anti-His scFv gene was also cloned but only a FLAG-tag was fused at the C-terminus. A scFv gene with a 15 amino acid linker was cloned into pYas-PSIF vectors[37]. ScFvs, dscFvs and scFv-PSIFs were purified from inclusion bodies in *E. coli* according to the previously described methods[37]. The binding affinity of each recombinant protein was assessed by surface plasmon resonance using BIAcore3000 (GE Healthcare UK Ltd., United Kingdom).

Expression and purification of IgG recombinant protein

IgG recombinant proteins were expressed using an OptiCHO antibody expression kit (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's instructions. IgGs were purified from cell culture supernatant with protein G column (GE Healthcare). Eluted fractions were further purified with Superdex 200 column (GE Healthcare). Anti-FLAG[IgG] (anti-FLAG M2 antibody) was purchased from Sigma-Aldrich.

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Preparation of IgG-NCS

NCS was kindly provided by Kayaku Co, Ltd., Japan. NCS was thiolated by incubating it with 10 molar excess 2-iminothiolane (Thermo Fisher Scientific Inc., Waltham, MA) for 1 h at room temperature. IgG recombinant proteins were reacted with 10 molar excess of SPDP crosslinker (N-succinimidyl 3-[2-pyridyldithio]-propionate; Thermo Fisher) for 30 min on ice. SPDP-modified IgGs and thiolated NCS were separately purified using NICK^{TM} columns (GE Healthcare). They were then mixed for 8 h at room temperature. IgG-NCS were purified with a Superdex 200 column. Modification efficiency was quantified after sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a Gel DOC EZ system and Image lab software (Bio-Rad Laboratories, Inc., Hercules, CA).

Labeling of purified mAbs

For fluorescent labeling, mAbs were labeled using Cy5.5-NHS (GE Healthcare). For ^{125}I labeling, 100 μg mAbs in 0.4 M phosphate buffer were labeled with 0.2 mCi Na^{125}I (PerkinElmer, Inc., Waltham, MA) based on the Chloramine-T method[38]. For biotin labeling, mAbs were biotinylated using EZ-Link Sulfo-NHS-Biotin (Thermo Fisher). Each mAb was purified using a NICK^{TM} desalting column (GE Healthcare).

Flow cytometry

Cy5.5-labeled mAb (mAb$^{Cy5.5}$; 4 μM) was incubated with 5.0×10^5 cells of MS1 cells in 6 well plates for 2 h at 4°C. After washing three times, the cells were incubated for an additional 0.5 to 8 h at 37°C. At each time point, cells were collected in 2 mM EDTA/PBS. Bound mAbs were digested using 0.1% trypsin/PBS

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at 37°C for 20 min (trypsinized group) or PBS (non-trypsinized group). Cellular fluorescence was measured by Gallios^{TM} flow cytometer (Beckman Coulter, Inc., Miami, FL). The ratio of internalization was calculated using the following formula: internalization (%) = {internalized mAb$^{Cy5.5}$} / {total bound mAb$^{Cy5.5}$} × 100 (%) = {(MFI of mAb)$_T$ – (MFI of anti-His[mAb])$_T$} / {(MFI of mAb)$_N$ – (MFI of anti-His[mAb])$_N$} × 100 (%). MFI indicates mean fluorescence intensity. "T" and "N" indicate trypsinized and non-trypsinized group, respectively.

In vivo biodistribution

dscFvs^{125I} (0.2 nmol) was intravenously injected into B16BL6 tumor-bearing mice. At 2 h and 24 h after injection, the radioactivity of each organ was counted using the Wizard 2480 gamma-ray counter (PerkinElmer). %ID/g tissue was calculated using following formula: %ID/g tissue = (count/g tissue) / (total injected count) × 100 (%). Two individual experiments were combined for the final data (total 11 mice/group).

Immunofluorescence of the tissue sections

B16BL6 tumor-bearing mice were administered 2 nmol of dscFvs^{Bio}. At 2 h after the injection, the tumors, kidneys, and hearts were embedded in OCT compound (Sakura Finetek, Inc., Torrance, CA) and frozen in liquid nitrogen. Thin sections (7-μm thick) were prepared using a Cryostat CM1850 (Leica Microsystems GmbH, Germany) and fixed in 4% paraformaldehyde. DscFvs^{Bio} in the sections were stained with streptavidin phycoerythrin conjugate (eBioscience Inc., San Diego, CA) in Dako REAL^{TM} Antibody Diluent (DAKO Corporation, Carpinteria, CA). CD31+ vascular endothelial cells were stained with rat anti-CD31 antibody (MEC13.3;

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Becton Dickinson and Company, Franklin Lakes, NJ) in Dako REAL^{TM} Antibody Diluent and Alexa488 conjugated anti-rat IgG (A11006; Invitrogen). The samples were embedded with Prolong Gold anti-fade reagent with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) and observed with a fluorescence microscope FSX100 (Olympus Corporation, Japan).

In vivo therapy experiments

Activities of scFv-PSIFs and IgG-NCSes were confirmed by WST-8 assay as described above. B16BL6 cells were inoculated intracutaneously into C57BL6 mice (Japan SLC) on Day 0. Mice were intravenously injected with 15 pmol scFv-PSIFs and 10 pmol IgG-NCSs on day 3, 5, 7, 9, and 11 (7 mice/group). The volume of the tumor was calculated according to the following formula: tumor volume (mm^3) = {major axis of tumor (mm)}×{minor axis of tumor (mm)}^{2}×0.4.

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Results

High-throughput screening for cell-internalizing mAbs

To identify cell-internalizing mAbs, we applied the phage immune scFv library to high-throughput screening of cell-internalizing molecules based on the PSIF system[8] (Fig. 1). Our anti-Robo4 or anti-VEGFR2 phage library comprised approximately 3.0×10^8 or 5.0×10^8 independent scFvs, which was validated by sequence analysis. To assess the qualities of the libraries, affinity panning was performed against each antigen. During the panning, output phages were increased, suggesting that the desired scFvs were enriched in the library (Supplemental Fig. S1a-b,e-f). After the 4^{th} panning, over 40% of monoclonal scFvs showed specific binding in enzyme-linked immunosorbent assay (ELISA) (Supplemental Fig. S1c-d,g-h).

To validate the efficacy of cell-internalizing mAbs in a mouse model, we selected libraries enriched against murine antigens (mRobo4 and mVEGFR2) and chose MS1 murine endothelial cells for the screening of cell-internalizing mAbs because we confirmed the expressions of both mRobo4 and mVEGFR2 in MS1 cells using RT-PCR. For the screening, scFv genes obtained after the 4^{th} round of panning were cloned into the PSIF expression vector. Monoclonal scFv-PSIFs were expressed in TG1 supernatants (315 clones per library). For anti-Robo4s, 178 of 315 clones bound to mRobo4 in ELISA and 20 of these 178 binders were cytotoxic against MS1 cells (Fig. 2a). In a similar manner, for anti-VEGFR2s, 156 of 315 clones bound to VEGFR2 and 17 of the 156 binders were positive in the ELISA and cytotoxicity assays (Fig. 2b). Sequence analysis to omit redundant clones revealed that these clones comprised 1 anti-Robo4 cell-internalizing mAb, 2 anti-Robo4

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low-internalizing mAbs, 2 anti-VEGFR2 cell-internalizing mAbs, and 14 anti-VEGFR2 low-internalizing mAbs. For anti-Robo4s, only one anti-Robo4 cell-internalizing mAb was named "R4-13i" and a low-internalizing mAb with high affinity and low cytotoxicity was named "R4-16". In a similar manner, "V2-05i" and "V2-02" were selected as an anti-VEGFR2 cell-internalizing mAb and a low-internalizing mAb, respectively. After purification of the recombinant proteins, both anti-Robo4 scFvs bound to hRobo4, similar to mRobo4. On the other hand, anti-VEGFR2 scFvs bound to mVEGFR2, but not to hVEGFR2. We also confirmed using competitive ELISA that the mAbs did not share their antigen-binding epitopes (Supplemental Fig. S2).

Characterization of mAbs

We purified scFvs, dimerized scFvs (dscFvs), IgGs, and scFv-PSIF as recombinant proteins. IgGs conjugated with neocarzinostatin (IgG-NCSes) were also prepared for *in vivo* experiments. NCSes were confirmed to be conjugated to IgG molecules in the purified IgG-NCS fraction, and the efficiencies of the NCS modifications were similar in all IgG-NCSes (1.6~1.8 NCSes per a single IgG). Surface plasmon resonance analysis revealed that cell-internalizing mAbs and low-internalizing mAbs had similar affinities against antigens in all antibody forms (Table 1).

To quantify the internalization, flow cytometry analysis was performed with Cy5.5-labeled mAbs ($scFv^{Cy5.5}$, $dscFv^{Cy5.5}$, and $IgG^{Cy5.5}$; Fig. 3a,c). After $mAbs^{Cy5.5}$ bound to the cell surface, internalization was induced by incubation at 37°C for 2 h. By removing cell-surface $mAbs^{Cy5.5}$ with trypsinization, the internalized $mAbs^{Cy5.5}$ were quantified by flow cytometry. At 2-h, approximately 30% of cell-internalizing

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mAbs remained after trypsinization, whereas most of the low-internalizing mAbs were degraded (Fig. 3a,c). This result clearly suggested that the internalization efficiencies differed between cell-internalizing mAbs and low-internalizing mAbs, even among the three different mAb forms. In a similar manner, a time-shift analysis revealed that over 40% of cell-internalizing mAbs were internalized after 8 h incubation (Fig. 3b,d). These findings indicate that only cell-internalizing mAbs were efficiently internalized into the cells, although low-internalizing mAbs had affinities similar to those of cell-internalizing mAbs (Table 1).

Intracellular localization

The intracellular behaviors of cell-internalizing mAbs were analyzed with a confocal laser-scanning microscope (CLSM). In MS1 cells, intracellular fluorescence derived from $scFv^{Cy5.5}$ was observed with cell-internalizing scFvs, but not with low-internalizing scFvs (Supplemental Fig. S3a,d,e,h). Fluorescence was suppressed, however, under the inhibition of energy-dependent endocytosis (Supplemental Fig. S3b,c,f,g). These results suggested that cell-internalizing scFvs entered into the cells *via* energy-dependent endocytosis.

For in-depth analysis of the intracellular behavior, CLSM analysis was performed with immunostaining of endosome markers (Supplemental Fig. S3i-ab). After $scFvs^{Cy5.5}$ were bound to the cell-surface, the cells were incubated for an additional 1 to 8 h at 37°C. The early endosome marker, early endosome antigen 1 (EEA1), and the late endosome marker, lysosomal-associated membrane protein 1 (LAMP1), were visualized using Alexa488-conjugated antibodies. Colocalization with EEA1+ early endosomes decreased over time (Supplemental Fig. S3i-m,s-w), whereas

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colocalization with LAMP1+ late endosomes increased (Supplemental Fig. S3n-r,x-ab). These findings suggested that cell-internalizing scFvs were encapsulated in EEA1+ early endosomes at an early stage, and eventually accumulated in LAMP1+ late endosomes. This is thought to be a typical endocytotic molecular sorting pathway[39].

Influence of cell-internalizing activity on biodistribution

To assess the biodistribution of cell-internalizing mAbs, ^{125}I-labeled dscFvs (dscFv^{125I}) were intravenously injected into B16BL6 tumor-bearing mice. In this experiment, we selected the dscFv form because dscFv has superior *in vivo* tumor-targeting potency compared with scFv[40]. At 2-h, the tumor distribution of anti-Robo4 and anti-VEGFR2 dscFvs^{125I} was similar, but significantly higher than that of a negative control dscFv^{125I} (anti-His[dscFv]^{125I}; Fig. 4a,b). This finding suggested that the anti-Robo4 and anti-VEGFR2 dscFvs had tumor-targeting properties. Anti-Robo4 dscFvs^{125I} also accumulated in the kidney, indicating a nonspecific distribution of dscFvs for their elimination[41,42], because no significant difference was observed between anti-Robo4 dscFvs^{125I} and anti-His[dscFv]^{125I} (Fig. 4a). Importantly, the accumulation of anti-VEGFR2 dscFvs^{125I} in the kidney was significantly greater than that of anti-His[dscFv]^{125I} (Fig. 4b). A similar accumulation of anti-VEGFR2 dscFvs^{125I}, but not anti-Robo4 dscFvs^{125I} (Fig. 4a), was observed in the heart (Fig. 4b).

To confirm this phenomenon, the localization of dscFvs in the tissues was analyzed by immunofluorescence studies (Fig. 4e-s). Biotin-labeled dscFvs (dscFvs^{Bio}) were intravenously administered to B16BL6 tumor-bearing mice and the tumors, kidneys,

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and hearts were extracted 2 h after injection. The dscFv^{Bio} and vascular endothelial cells were stained by streptavidin-AP and anti-CD31 antibody, respectively. In the tumor sections, all of the anti-Robo4 and anti-VEGFR2 dscFvs^{Bio} were clearly detected with CD31+ tumor blood vasculature, whereas anti-His[dscFv]^{Bio} was not detectable (Fig. 4e,h,k,n,q). This finding suggested that both anti-Robo4 and anti-VEGFR2 dscFvs recognized tumor endothelial cells *in vivo*, which contributed to their accumulation in the tumor. Interestingly, in the kidney and heart sections, signals around CD31+ blood vasculature were detectable only with the anti-VEGFR2 dscFvs^{Bio}, and not with anti-Robo4 dscFvs^{Bio} or anti-His[dscFv]^{Bio} (Fig. 4f-g,i-j,l-m,o-p,r-s). This finding was compatible with the biodistribution results (Fig. 4a,b). These results suggested that anti-VEGFR2 dscFvs recognized VEGFR2 on normal blood vessels because VEGFR2 plays an important role in normal tissues, including the kidney and heart[31-33]. No specific accumulation of anti-Robo4 dscFvs was observed in normal tissues, suggesting that the anti-Robo4 mAbs were useful for specific tumor vascular targeting.

Comparison of the cell-internalizing mAbs and low-internalizing mAbs revealed a significantly greater accumulation of cell-internalizing dscFvs^{125I} in the tumors compared to low-internalizing dscFvs^{125I} at 24-h (Fig 4c,d), whereas no differences were observed at 2-h (Fig. 4a,b). This finding suggested that cell-internalizing mAbs were retained in the tumor for a longer time than the low-internalizing mAbs. This phenomenon was also observed in the kidney and heart with the anti-VEGFR2 dscFvs (Fig. 4d). This retention might be due to the mAb internalization, which allowed the mAb to escape from the blood stream and accumulate in the tumor blood endothelial cells. Taken together, these results suggest that mAb internalization into

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the tumor endothelium improves mAb-based drug-delivery *in vivo*.

Enhanced anti-tumor effect depends on the cell-internalizing activity

To assess the anti-tumor potencies of the cell-internalizing mAbs, we selected the scFv-PSIF and IgG-NCS forms. Both forms were suitable models of ADCs because both drugs are used clinically as successful anti-cancer medicines[10,43]. First, the *in vitro* cell-killing activities of scFv-PSIFs and IgG-NCSes were estimated by a cytotoxicity assay with MS1 cells (Fig. 5a-d). Both forms of cell-internalizing mAbs showed approximately 10-fold higher cytotoxicity than the low-internalizing mAbs. These findings clearly suggest that internalization enhanced the delivery of conjugated drugs into the cells because our cell-internalizing mAbs and low-internalizing mAbs had similar affinities against antigens (Table 1).

As the therapy experiment *in vivo*, scFv-PSIFs and IgG-NCSes were intravenously injected into B16BL6 tumor-bearing mice once every 2 days for a total of 5 injections (Fig. 5e-h). All cell-internalizing mAbs significantly suppressed tumor growth, whereas the anti-tumor effects of the low-internalizing antibodies were similar to those of the negative controls (anti-His[scFv]-PSIF and anti-FLAG[IgG]-NCS). The anti-tumor effects of R4-13i and V2-05i were similar in both ADC forms. These findings strongly suggest that the cell-internalizing activity of the mAbs was essential to maximize the delivery of the conjugated drug into the target cells, which significantly enhanced the anti-tumor effect of the ADCs.

Interestingly, the group of mice administered V2-05i[scFv]-PSIF had a significant loss of body weight, whereas the other groups did not (Fig. 5i-l). As a preliminary result, 6 of 7 mice died in the V2-05i[scFv]-PSIF group with a similar protocol but

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with a 4-fold higher dosage (60 pmol/mouse), perhaps due to the disruption of VEGFR2-positive cells in normal tissues by V2-05i[scFv]-PSIF, as shown in Fig. 4. This side-effect was not observed in the V2-05i[IgG]-NCS group. Therefore, we also hypothesized that the toxicity of NCS in normal cells was weak because NCS inhibits DNA synthesis in growing cells, such as tumor cells[44]. At a higher dosage, however, V2-05i[IgG]-NCS carries the risk of side effects. With regard to this point, none of the anti-Robo4 ADCs induced a loss of body weight; therefore, we concluded that Robo4 is a potential target for tumor vascular targeting with ADC.