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Tumor-targeting CTL expressing a single-chain Fv specific for VEGFR2

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ABSTRACT

Cytotoxic T lymphocytes (CTL) are critical effector cells in tumor immunity. Adoptive transfer therapy with *in vitro*-expanded tumor-specific CTL is a promising approach for preventing cancer metastasis and recurrence. Transferred CTL are not effective in clinical trials, however, due to inadequate tumor-infiltration. Therefore, the development of functionally modified CTL, such as tumor-targeting CTL, is widely desired. Here, we designed the tumor-targeting CTL expressing a single-chain antibody fragment (scFv-CTL) specific for vascular endothelial growth factor receptor 2 (VEGFR2/flk1) by transducing the CTL with a retroviral vector. The scFv-CTL bound to VEGFR2/flk1-expressing cells and retained their cytotoxic activity against tumor cells. In addition, adoptive transfer of scFv-CTL into tumor-bearing mice effectively suppressed tumor growth due to the augmented accumulation of the transferred CTL in the tumor tissue. These findings indicate that the creation of CTL capable of targeting tumor vascular endothelial cells by scFv-expression technique is considerably promising for improvement of efficacy in adoptive immunotherapy.

Introduction

Efficient accumulation of immune cells, particularly cytotoxic T lymphocytes (CTL), into the tumor induces tumor regression [1–3]. Studies in both patients and animal models indicate that CTL can injure tumor cells upon recognizing antigenic peptides presented via major histocompatibility complex molecules on the tumor cells through their tumor-specific T cell receptor (TCR) [4,5]. Cancer immunotherapy approaches are aimed at activating CTL as the major effector cells in anti-tumor immune responses. Adoptive T cell therapy, which involves the *ex vivo* selection and expansion of antigen-specific CTL, augments antigen-specific immunity without the *in vivo* constraints that can accompany vaccine-based strategies [6,7]. TCR gene-modified T cells were generated previously [8,9]. The TCR gene transfer approach is a convenient method to produce tumor-specific T cells further allowing that an individualized therapy will be available for a mass of patients. CTL that recognize and disrupt tumor cells *in vitro*, however, are often ineffective in clinical trials of adoptive immunotherapy, due to their insufficient distribution to the tumor sites [10,11]. To establish a more effective adoptive immunotherapy for cancer, we developed a method to enhance the accumulation of transferred tumor-specific CTL in the tumor tissue.

Although tumor-specific CTL induce anti-tumor effects, their infiltration into the tumor tissue is inefficient due to the requirement of their extravasation from the bloodstream. On the other hand, tumor vessel-targeting CTL are more likely to efficiently accumulate in the tumor tissue, because intravenously transferred CTL are directly accessible to endothelial cells. Additionally, because tumor-endothelial target structures are commonly expressed in almost all solid tumors, such a tumor vessel-targeting approach would be broadly applicable. Neovascularisation occurs more often in tumor tissue than in normal tissue. Tumor angiogenesis is necessary to provide oxygen and nutrients for solid tumor progression and metastasis. Many molecules involved in tumor angiogenesis have been selected as markers for tumor-targeting. Vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2, also known as flk1 in mouse), a major receptor for crucial pro-angiogenic VEGF, is selectively expressed on endothelial cells and overexpressed on growing endothelial cells in tumor vasculature [12,13]. Therefore, flk1 is a candidate target molecule for tumor-selective delivery of various anti-cancer drugs

Abbreviations: CTL, cytotoxic T lymphocyte; FBS, fetal bovine serum; gp, glycoprotein; IL, interleukin; mAb, monoclonal antibody; OVA, ovalbumin; PBS, phosphate-buffered saline; scFv, single-chain variable fragment; scFv-CTL, CTL expressing anti-flk1 single-chain variable fragment; TCR, T cell receptor; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2; V_H, variable region of the heavy chain; V_L, variable region of the light chain

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[14] and monoclonal antibodies (mAbs) against flk1 (anti-flk1 mAb) may be promising as a selective-targeting agent.

CTL expressing anti-flk1 mAb are difficult to generate, however, due to the large size of the antibody and its tendency to form dimers. On the other hand, antigen binding activity is determined by the sequences and conformation of the amino acids of the complementarity-determining regions that are located on the light and heavy chains of the variable fragment (Fv) of the antibody. To overcome the limitations of the large mAb molecules, smaller engineered mAb-based molecules were developed. The 25-kDa single-chain Fv (scFv) molecule, composed of a variable region of the light chain (V_L) and a variable region of the heavy chain (V_H) joined via a short peptide spacer sequence, is the smallest antibody fragment developed to date with potential clinical applications [15,16]. The scFv can be used for genetic engineering techniques in place of mAb. In the present report, we used genetic engineering techniques to generate CTL expressing anti-flk1 scFv (scFv-CTL) and investigated their efficacy for adoptive immunotherapy.

Materials and methods

Cell lines and mice. Murine melanoma expressing ovalbumin (OVA) B16-OVA cells (H-2^b) were kindly provided by Professor Y. Nishimura (Kumamoto University, Kumamoto, Japan) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 50 μM 2-mercaptoethanol, and 200 μg/ml hygromycin. Murine melanoma B16BL6 cells (H-2^b) were obtained from the JCRB cell bank (Tokyo, Japan) and cultured in minimum essential medium supplemented with 7.5% FBS. Murine islet endothelial cells (H-2^b), MS1 cells, and OVA cDNA-transfectants of EL4 murine thymoma cells (H-2^b), E.G7-OVA cells, were purchased from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS and in RPMI 1640 medium supplemented with 10% FBS, 50 μM 2-ME, and 400 μg/ml G418, respectively. PLAT-E cells [17], a helper cell line for retrovirus-propagation, were kindly provided by Professor T. Kitamura (Tokyo University, Tokyo, Japan) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 1 μg/ml puromycin, and 10 μg/ml blasticidin. C57BL/6 mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). OT-I mice and pmel-1 mice, transgenic mice whose CD8-positive T cells recognize the $OVA_{257–264}$ (SIINFEKL) peptide and the glycoprotein (gp) $100_{25–33}$ (KVPRNQDWL) peptide, respectively, in the context of H-2^b on a C57BL/6 background, were purchased from Jackson Laboratory (Bar Harbor, ME). Animal experimental procedures were in performed in accordance with the Osaka University guidelines for the welfare of animals in experimental neoplasia.

Construction of retroviral vectors expressing anti-flk1 scFv. Total RNA was extracted from Avas12α1 hybridoma cells [18], which were kindly provided by Professor S. Nishikawa (RIKEN, Kobe, Japan), and first-strand cDNA was synthesized with the Super-Script™ III First-Strand Synthesis System for reverse transcription-polymerase chain reaction (Invitrogen, Carlsbad, CA). The V_H-region and V_L-region genes of the antibody were amplified from the cDNA by PCR (94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min; 35 cycles) using their respective specific primers (V_H-region: forward 5′-ggagccgccgccgcc ggatccaccaccacctgaagagacagtgaccagagtgccttgg-3′, including G4S linker; reverse 5′-ctgtcatctcaccttgctcctgccccgcggccgtttcagctccagctc cagttggtcccaggtc-3′, including SfiI site; V_L-region: forward 5′-ctgtcat ctcaccttgctcctgccggcccagccggcccaggtacagctacagcaatcag-3′, including SacII site; reverse 5′-ggcggcggcggctccggtggtggtggttctgacatccaga tgacccagtct-3′, including G4S linker). The cDNAs of the V_H and V_L chain were assembled by 3 cycles of PCR (94 °C for 1 min, 63 °C for 30 s, 58 °C for 50 s, and 72 °C for 1 min) using a DNA linker fragment. The assembled scFv fragment was reamplified using both the V_H reverse and V_L forward primers. The resulting fragment was digested with SfiI and SacII and ligated into the pDisplay vector (Invitrogen). Subsequently, the scFv fragment, including the signal peptide and the transmembrane region of the platelet-derived growth factor receptor derived from the pDisplay vector were reamplified using primers to introduce the EcoRI site at the 5′-end and the NotI site at the 3′-end. The resulting fragment was digested with EcoRI and NotI and ligated into the pMXs-IG [19] (kindly provided by Professor T. Kitamura), a retroviral plasmid carrying green fluorescent protein. The PLAT-E cells were transfected with these expression vectors using FuGENE 6 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's recommendations. The medium was changed 1 day after the transfection, and retroviral vectors were harvested 48 h after the transfection, as previously described [17,19].

Gene transfer to CTL. T cells were purified from OT-I or pmel-1 splenocytes as nylon wool non-adherent cells. CD8-positive cells were then purified using a CD8-isolation kit and Auto MACS (Miltenyi Biotec, Auburn, CA) according to manufacturer's specifications. Dendritic cells were prepared as previously reported [20,21]. The dendritic cells were incubated with 10 μM epitope peptide, $OVA_{257–264}$ (SIINFEKL) peptide, or $gp100_{25–33}$ (KVPRNQDWL) purchased from Sigma–Aldrich (St. Louis, MO). For prestimulation, OT-I or pmel-1 CD8-positive T lymphocytes were activated by co-culture with epitope peptide-pulsed dendritic cells for 48 h in RPMI 1640 supplemented with 10% FBS, 50 μM 2-ME, and 10 U/ml interleukin (IL)-2 (PeproTech EC Ltd., London, UK). Non-treated 6-well plates were coated with recombinant fibronectin fragment CH-296, RetroNectin® (TaKaRa Bio Inc., Ohtsu, Japan) according to the manufacturer's instructions. The viral supernatant was loaded on the RetroNectin® and incubated for 4 h. The virus-coating procedure was repeated twice. Before infection, the viral supernatant was washed away and the activated CD8-positive cells (i.e., CTL) were added to the virus-coating plate. Cells were cultured for 48 h. The gene transduction efficiency to the CTL was 10–15%. Gene-transduced CTL were sorted by FACSAria™ cell sorter (BD Biosciences, San Jose, CA), and cultured for amplification in complete medium with IL-2 (10 U/ml) for 3 days, which resulted in CTL expressing anti-flk1 scFv.

Evaluation of gene-transduced CTL activity. Cytotoxic specificity was determined using standard ^{51}Cr-release assays. Target cells (B16-OVA, MS1, B16BL6, and E.G7-OVA) were ^{51}Cr-labeled and incubated with gene-transduced CTL or mock CTL for 4 h at 37 °C. Cytolytic activity was determined using the following formula: (% of lysis) = [(experimental ^{51}Cr-release − spontaneous ^{51}Cr-release)/(maximum ^{51}Cr-release − spontaneous ^{51}Cr-release)] × 100. The spontaneous ^{51}Cr-release of the target cells was less than 10% of the maximum ^{51}Cr-release induced by detergent.

Flow cytometric analysis for CTL expressing anti-flk1 scFv. scFv-CTL were incubated with 100 μl staining buffer (PBS containing 0.1% bovine serum albumin and 0.01% NaN_3) containing recombinant mouse flk1/human Fc chimera (R&D Systems Inc., Minneapolis, MN) labeled with Zenon™ technology using R-phycoerythrin Human IgG labeling reagent (Invitrogen), according to the manufacturer's instructions. After incubation for 30 min, the cells were washed with staining buffer, and then 30,000 events of the stained cells were acquired on a FACSCalibur™ flow cytometer (BD Biosciences), and analyzed for anti-flk1 scFv protein expression using FlowJo™ software (Treestar, Inc., San Carlos, CA).

Binding assay. CTL labeling with PKH26 (Sigma–Aldrich) was performed according to the manufacturer's instructions. CTL were cultured on fixed MS1 cells, flk1-expressing cells, with the indicated cell number for 30 min. After washing off non-binding CTL five times with PBS containing 1 mM EDTA, the number of CTL bound to MS1 cells was counted under fluorescent microscopy. The number of CTL that were bound to MS1 cells was calculated by multiplying the numbers of PKH26-positive cells in each microscopic field by the ratio of the plate area to microscopic field area.

Accumulation of transferred CTL in tumor tissues. C57BL/6 mice were intradermally inoculated with 10^6 B16-OVA cells into the right flank. Seven days later, mice bearing tumors with diameters of 5.5–6.5 mm were intravenously injected with 10^6 CTL labeled with PKH26 dye in 100 μl PBS. The tumors were harvested 2 days after CTL transfer, and then were chopped into small pieces with a razor blade before incubation with a mixture of collagenase (1 mg/ml, Wako Pure Chemical Industries, Ltd., Osaka, Japan) dissolved in Hanks' balanced salt solution for 60 min at 37 °C. The cells were passed through a 70-μm nylon strainer to remove any debris, recovered by centrifugation, and resuspended in complete medium. The frequency of PKH26-positive CTL was assessed by flow cytometric analysis acquiring 100,000 events. The number of CTL that accumulated in the tumor was calculated by multiplying the PKH26-positive frequency by the total number of isolated tumor cells.

Evaluation of tumor growth. C57BL/6 mice were intradermally inoculated with 10^6 B16-OVA cells or 4×10^5 B16BL6 cells into the right flank. Seven days later, mice bearing tumors with diameters of 5.5–6.5 mm were intravenously injected with CTL in 100 μl PBS. Tumor growth was monitored two or three times a week by measuring the major and minor axes of the tumors using microcalipers, and tumor volume was calculated by the following formula: (tumor volume; mm^3) = (major axis; mm) × (minor axis; $mm)^2 \times 0.5236$. The mice were euthanized when one of the two measurements was greater than 20 mm.

Results and discussion

To achieve the expression of anti-flk1 scFv in CTL, we compared the gene transduction efficiency and cytotoxicity between adenoviral vector, retroviral vector, lentiviral vector, and electroporation methods (data not shown), and then selected retroviral vector as suitable transduction system to CTL. The expression levels of anti-flk1 scFv on CTL were assessed by flow cytometric analysis. scFv-CTL bound to more flk1/Fc chimera compared with CTL treated with control vector (sham-CTL; Fig. 1A), indicating that retroviral gene transduction induced reproducibly high and stable scFv-expression on the CTL surface. Next, to prove the functional specificity of the anti-flk1 scFv, we assessed the cell-to-cell binding between scFv-CTL and flk1-expressing MS1 vascular endothelial cells. The numbers of scFv-CTL binding to MS1 cells increased in a dose-dependent manner compared with sham-CTL (Fig. 1B), suggesting that anti-flk1 scFv on CTL could recognize native flk1 on cell surface. Moreover, to

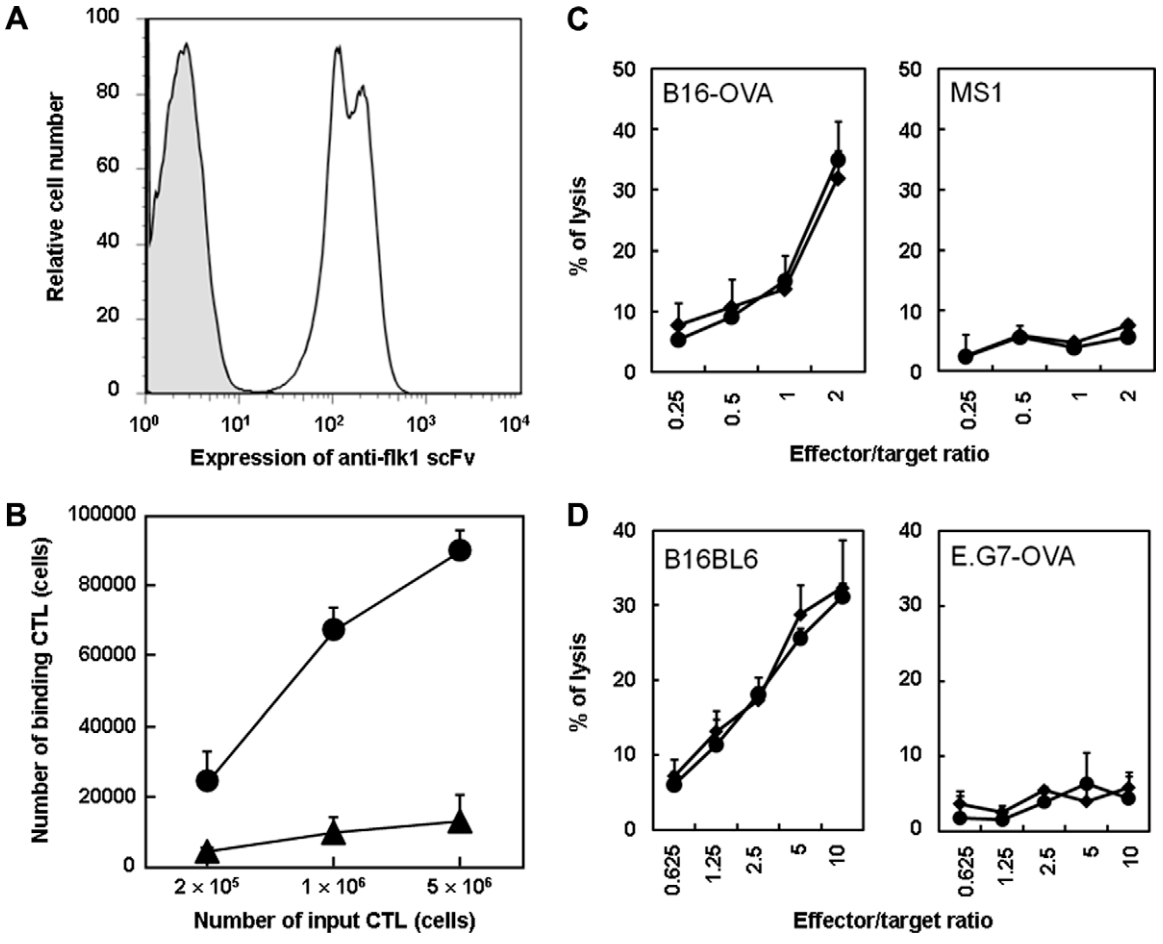

Fig. 1. Characterization of CTL expressing anti-flk1 scFv. CTL were purified from TCR transgenic mice and transduced with anti-flk1 scFv expressing-retroviral vectors as described in Materials and methods. (A) Anti-flk1 scFv gene-transduced CTL (scFv-CTL) or control vector treated-CTL (sham-CTL) were incubated with recombinant mouse flk1/Fc chimera labeled with a fluorescent molecule. Thirty minutes later, the expression level of anti-flk1 scFv on scFv-CTL (solid line) or sham-CTL (filled area) was analyzed by flow cytometry. (B) CTL labeled with PKH26 were cultured on fixed MS1 cells, flk1-expressing cells, with the indicated number of cells for 30 min, and non-binding CTL were removed by washing five times with PBS containing 1 mM EDTA. The numbers of scFv-CTL (●) or sham-CTL (♦) that bound to the MS1 cells on a 6-well plate was calculated by multiplying the numbers of PKH26-positive cells in a microscopic field by the ratio of plate area to microscope field area. Data are presented ±SD of results from 3 wells. (C, D) CTL derived from OT-I (C) or pmel-1 (D) mice as effector cells were co-cultured with B16-OVA cells or MS1 cells (C), or B16BL6 cells or E.G7-OVA cells (D) as target cells at varying effector-to-target cell ratios. Four hours later, the cytotoxic activity of scFv-CTL (●) or non-transduced CTL (▲) was determined using a standard ^{51}Cr-release assay. Each point represents the mean ± SD of three independent cultures.

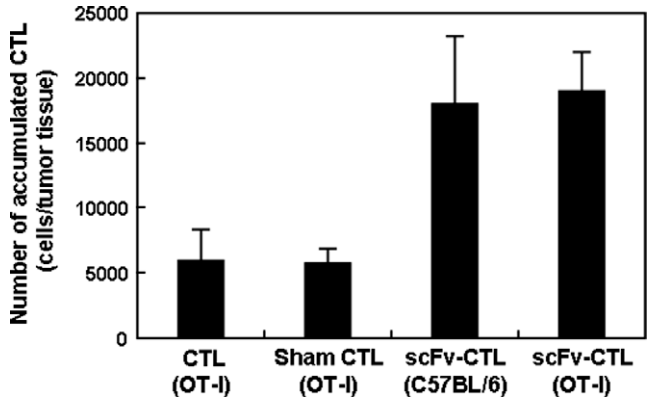

Fig. 2. Accumulation of transferred CTL expressing anti-flk1 scFv in tumor. PKH26-labeled scFv-CTL or sham-CTL derived from OT-I mice or wild-type C57BL/6 mice intravenously injected into B16-OVA tumor-bearing C57BL/6 mice. Two days later, tumors were removed for preparation of single-cell suspensions, as described in Materials and methods. The abundance of PKH26-positive CTL was assessed by flow cytometric analysis acquiring 100,000 events. The number of CTL that accumulated in the tumor was calculated by multiplying the PKH26-positive frequency by the total number of isolated tumor cells. Data are presented ±SD of results from 3 mice per experimental group.

investigate the effects of scFv-expression on CTL activity, we performed a cytolytic assay using scFv-CTL prepared from CTL derived from OT-I mice (scFv-CTL [OT-I]) against OVA-expressing cells. scFv-CTL (OT-I) as well as non-transduced CTL (OT-I), exhibited high cytotoxic activity against B16-OVA cells, whereas MS1 cells, which do not express OVA, were not injured (Fig. 1C). Likewise, scFv-CTL prepared from gp100-specific CTL, which were derived from pmel-1 mice (scFv-CTL [pmel-1]), retained the specificity of lytic activity against gp100-expressing B16BL6, which is a melanoma cell line (Fig. 1D). Taken together, these findings demonstrated that anti-flk1 scFv expressed on CTL possessed sufficient ability to recognize the flk-1 molecules on the vascular endothelial cells, and that the transduction procedures and the expression of scFv did not affect original cytolytic activity of CTL.

We next performed *in vivo* experiments in which B16-OVA tumor-bearing mice were adoptively transferred with fluorescently labeled scFv-CTL. Two days after CTL transfer, tumors were removed and single-cell suspensions were prepared for flow cytometry. The number of fluorescently labeled sham-CTL (OT-I) detected in tumors was equal to that of non-transduced CTL (OT-I), whereas that of scFv-CTL was greater than that of control CTL (Fig. 2). In addition, the number of tumor-accumulated scFv-CTL (OT-I) were comparable that of scFv-CTL prepared from CTL of wild-type C57BL/6 mice, which has no antigen-specific TCR. This finding indicated that the tumor-targeting activity of scFv-CTL was not due to OVA-specific TCR, but anti-flk1 scFv on the CTL. Therefore, scFv-CTL efficiently bound to flk1-expressing tumor vascular endothelial cells *in vitro* and *in vivo*, and scFv was useful for controlling the biodistribution of the CTL.

We therefore investigated whether scFv-CTL could exhibit more powerful anti-tumor effect due to the ability to target neovascular endothelial cells. B16-OVA tumors in mice systemically transferred with sham-CTL (OT-I) showed a slight growth delay compared to those treated with PBS (control; Fig. 3A). The sham-CTL (OT-I) treatment is considered a standard model of adoptive immunotherapy. In comparison with the sham-CTL (OT-I) transfer groups, however, mice transferred with scFv-CTL (OT-I) exhibited marked tumor growth suppression. In addition, the anti-tumor effect of 10^5 scFv-CTL (OT-I) transfer was almost the same as that of 5×10^5 CTL transfer. Tumor-infiltrating immune cells have been reported to positively correlate with prognosis [22]. Here, we demonstrated that the tumor-infiltration of CTL directly correlated to

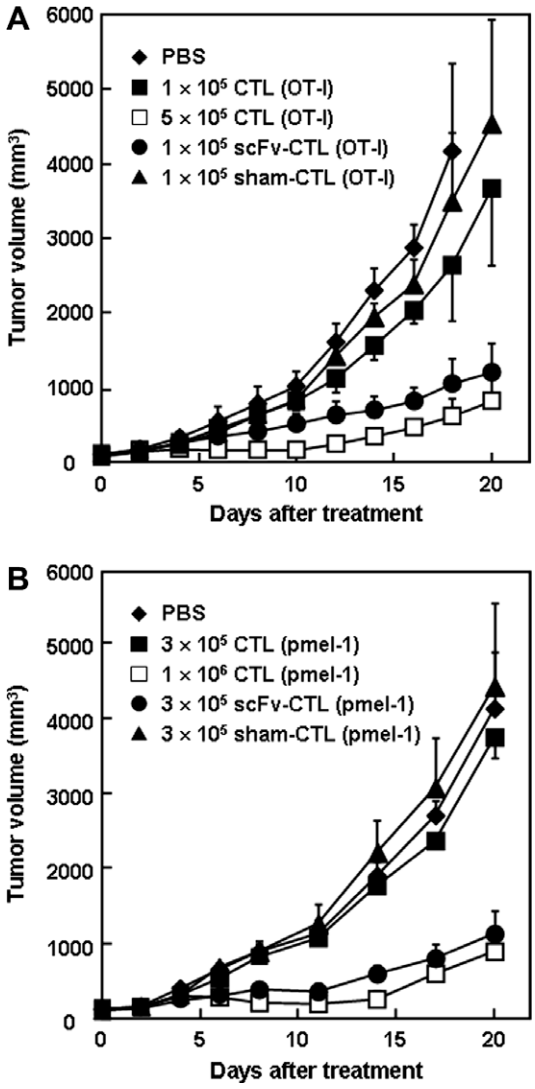

Fig. 3. Anti-tumor efficacy of transferred CTL expressing anti-flk1 scFv in tumors. CTL derived from OT-I mice (A) or pmel-1 mice (B) was intravenously injected into B16-OVA tumor (A) or B16BL6 tumor (B)-bearing C57BL/6 mice. Likewise, PBS was intravenously injected into tumor-bearing mice. The tumor volume was calculated after measuring the major and minor axes of the tumor at indicated points. Each point represents the mean ± SD from 6 mice.

the anti-tumor effect, because, as shown in Fig. 2, the number of scFv-CTL that accumulated in the tumor was almost four times higher than that of sham-CTL. In a melanoma model, adoptive transfer of scFv-CTL (pmel-1) into B16BL6 tumor-bearing mice more effectively suppressed tumor growth compared with the transfer of sham-CTL (pmel-1), and the anti-tumor effect of 3×10^5 scFv-CTL (pmel-1) transfer was almost the same as that of 10^6 CTL (pmel-1) transfer (Fig. 3B). These findings indicated that expression of anti-flk1 scFv in CTL was effective not only against a model antigen (OVA) but also a tumor-associated antigen (gp100 of melanoma cells). Moreover, our strategy is suitable for adoptive immunotherapy against various tumors, because tumor vascular endothelial cells are common to all solid tumor tissues.

Most T cell antigens targeted on tumor cells are normal self proteins. Therefore, immunologic tolerance to these self antigens may limit the T cell repertoire, which in turn would limit the host's ability to mount an effective anti-tumor immune response. Generally, tumor-specific CTL are expanded from tumor-infiltrating cells or peripheral blood mononuclear cells by stimulation with tumor-antigen peptide plus IL-2 or dissociated tumor lesions plus IL-2

[23,24]. It is difficult, however, to obtain large numbers of antigen-specific CTL from tumor-infiltrating cells or peripheral blood mononuclear cells. The strategy is not applicable to all cancer patients because a sufficient tumor mass is necessary for CTL expansion and the defined tumor-antigen peptide essential for expansion is limited in specific human leukocyte antigen-types (HLA-A2 and A24). Additionally, CD28-specific superagonistic mAb reportedly caused a life-threatening side effect, "cytokine storm", in healthy volunteers in clinical trials [25,26]. Transfer of a large number of activated lymphocytes (CTL) is considered to induce side effects due to a cytokine imbalance in the body. Therefore, our approach to add the flk1-targeting function to CTL will be useful for adoptive immunotherapy because, due to their tumor specificity, fewer CTL are necessary.

In conclusion, the findings of the present study demonstrated that the expression of anti-flk1 scFv on CTL resulted in a powerful anti-tumor effect due to both enhanced binding activity to flk1-expressing cells *in vitro* and the accumulation of these cells in tumor tissue *in vivo*. In addition, our findings suggest that scFv gene transduction to immune cells will greatly contribute to establish an innovative concept, a "cell delivery system" based on the "drug delivery system", which allows for better control of the tracking and biodistribution of immune cells by applying genetic engineering techniques. Our technology and methodology also will likely have far-reaching implications for hundreds of other types of cell therapies. At present, to improve adoptive immunotherapy, we are now working to develop tumor vessel-damaging CTL that are engineered to express chimeric immunoreceptors using scFv specific for flk1.

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References

[1] N. Kanagawa, M. Niwa, Y. Hatanaka, Y. Tani, S. Nakagawa, T. Fujita, A. Yamamoto, N. Okada, CC-chemokine ligand 17 gene therapy induces tumor regression through augmentation of tumor-infiltrating immune cells in a murine model of preexisting CT26 colon carcinoma, Int. J. Cancer 121 (2007) 2013–2022.

[2] J.Q. Gao, T. Tsuda, M. Han, D.H. Xu, N. Kanagawa, Y. Hatanaka, Y. Tani, H. Mizuguchi, Y. Tsutsumi, T. Mayumi, N. Okada, S. Nakagawa, NK cells are migrated and indispensable in the anti-tumor activity induced by CCL27 gene therapy, Cancer Immunol. Immunother. 58 (2009) 291–299.

[3] J.Q. Gao, N. Kanagawa, Y. Motomura, T. Yanagawa, T. Sugita, Y. Hatanaka, Y. Tani, H. Mizuguchi, Y. Tsutsumi, T. Mayumi, N. Okada, S. Nakagawa, Cotransduction of CCL27 gene can improve the efficacy and safety of IL-12 gene therapy for cancer, Gene Ther. 14 (2007) 491–502.

[4] T. Boon, P.G. Coulie, B.J. Van den Eynde, P. van der Bruggen, Human T cell responses against melanoma, Annu. Rev. Immunol. 24 (2006) 175–208.

[5] L. Zitvogel, A. Tesniere, G. Kroemer, Cancer despite immunosurveillance: immunoselection and immunosubversion, Nat. Rev. Immunol. 6 (2006) 715–727.

[6] L. Gattinoni, D.J. Powell Jr., S.A. Rosenberg, N.P. Restifo, Adoptive immunotherapy for cancer: building on success, Nat. Rev. Immunol. 6 (2006) 383–393.

[7] L. Fang, A.S. Lonsdorf, S.T. Hwang, Immunotherapy for advanced melanoma, J. Invest. Dermatol. 128 (2008) 2596–2605.

[8] H.J. Stauss, M. Cesco-Gaspere, S. Thomas, D.P. Hart, S.A. Xue, A. Holler, G. Wright, M. Perro, A.M. Little, C. Pospori, J. King, E.C. Morris, Monoclonal T-cell receptors: new reagents for cancer therapy, Mol. Ther. 15 (2007) 1744–1750.

[9] M.A. de Witte, A. Jorritsma, A. Kaiser, M.D. van den Boom, M. Dokter, G.M. Bendle, J.B. Haanen, T.N. Schumacher, Requirements for effective antitumor responses of TCR transduced T cells, J. Immunol. 181 (2008) 5128–5136.

[10] A.K. Eerola, Y. Soini, P. Pääkkö, A high number of tumor-infiltrating lymphocytes are associated with a small tumor size, low tumor stage, and a favorable prognosis in operated small cell lung carcinoma, Clin. Cancer Res. 6 (2000) 1875–1881.

[11] Y. Naito, K. Saito, K. Shiiba, A. Ohuchi, K. Saigenji, H. Nagura, H. Ohtani, CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer, Cancer Res. 58 (1998) 3491–3494.

[12] L.F. Brown, B. Berse, R.W. Jackman, K. Tognazzi, E.J. Manseau, H.F. Dvorak, D.R. Senger, Increased expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in kidney and bladder carcinomas, Am. J. Pathol. 143 (1993) 1255–1262.

[13] K.H. Plate, G. Breier, B. Millauer, A. Ullrich, W. Risau, Up-regulation of vascular endothelial growth factor and its cognate receptors in a rat glioma model of tumor angiogenesis, Cancer Res. 53 (1993) 5822–5827.

[14] S. Ramakrishnan, T.A. Olson, V.L. Bautch, D. Mohanraj, Vascular endothelial growth factor-toxin conjugate specifically inhibits KDR/flk-1-positive endothelial cell proliferation in vitro and angiogenesis in vivo, Cancer Res. 56 (1996) 1324–1330.

[15] L. Mohr, A. Yeung, C. Aloman, D. Wittrup, J.R. Wands, Antibody-directed therapy for human hepatocellular carcinoma, Gastroenterology 127 (2004) S225–S231.

[16] S. Shen, A. Forero, A.F. LoBuglio, H. Breitz, M.B. Khazaeli, D.R. Fisher, W. Wang, R.F.. Meredith, Patient-specific dosimetry of pretargeted radioimmunotherapy using CC49 fusion protein in patients with gastrointestinal malignancies, J. Nucl. Med. 46 (2005) 642–651.

[17] S. Morita, T. Kojima, T.. Kitamura, Plat-E: an efficient and stable system for transient packaging of retroviruses, Gene Ther. 7 (2000) 1063–1066.

[18] H. Kataoka, N. Takakura, S. Nishikawa, K. Tsuchida, H. Kodama, T. Kunisada, W. Risau, T. Kita, S.I. Nishikawa, Expressions of PDGF receptor alpha, c-Kit and Flk1 genes clustering in mouse chromosome 5 define distinct subsets of nascent mesodermal cells, Dev. Growth Differ. 39 (1997) 729–740.

[19] T. Kitamura, Y. Koshino, F. Shibata, T. Oki, H. Nakajima, T. Nosaka, H. Kumagai, Retrovirus-mediated gene transfer and expression cloning: powerful tools in functional genomics, Exp. Hematol. 31 (2003) 1007–1014.

[20] N. Kanagawa, R. Koretomo, S. Murakami, F. Sakurai, H. Mizuguchi, S. Nakagawa, T. Fujita, A. Yamamoto, N. Okada, Factors involved in the maturation of murine dendritic cells transduced with adenoviral vector variants, Virology 374 (2008) 411–420.

[21] N. Okada, Y. Masunaga, Y. Okada, S. Iiyama, N. Mori, T. Tsuda, A. Matsubara, H. Mizuguchi, T. Hayakawa, T. Fujita, A. Yamamoto, Gene transduction efficiency and maturation status in mouse bone marrow-derived dendritic cells infected with conventional or RGD fiber-mutant adenovirus vectors, Cancer Gene Ther. 10 (2003) 421–431.

[22] A.G. Menon, C.M. Janssen-van Rhijn, H. Morreau, H. Putter, R.A. Tollenaar, C.J. van de Velde, G.J. Fleuren, P.J. Kuppen, Immune system and prognosis in colorectal cancer: a detailed immunohistochemical analysis, Lab. Invest. 84 (2004) 493–501.

[23] A.B. Bakker, G. Marland, A.J. de Boer, R.J. Huijbens, E.H. Danen, G.J. Adema, C.G. Figdor, Generation of antimelanoma cytotoxic T lymphocytes from healthy donors after presentation of melanoma-associated antigen-derived epitopes by dendritic cells in vitro, Cancer Res. 55 (1995) 5330–5334.

[24] Y. Kawakami, S. Eliyahu, C. Jennings, K. Sakaguchi, X. Kang, S. Southwood, P.F. Robbins, A. Sette, E. Appella, S.A. Rosenberg, Recognition of multiple epitopes in the human melanoma antigen gp100 by tumor-infiltrating T lymphocytes associated with in vivo tumor regression, J. Immunol. 154 (1995) 3961–3968.

[25] M. Takita, T. Matsumura, M. Kami, Cytokine storm and an anti-CD28 monoclonal antibody, N. Engl. J. Med. 355 (2006) 2593.

[26] R. Stebbings, L. Findlay, C. Edwards, D. Eastwood, C. Bird, D. North, Y. Mistry, P. Dilger, E. Liefooghe, I. Cludts, B. Fox, G. Tarrant, J. Robinson, T. Meager, C. Dolman, S.J. Thorpe, A. Bristow, M. Wadhwa, R. Thorpe, S. Poole, "Cytokine storm" in the phase I trial of monoclonal antibody TGN1412: better understanding the causes to improve preclinical testing of immunotherapeutics, J. Immunol. 179 (2007) 3325–3331.