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Design of a prodrug bispecific antibody masked by a functional molecule for lymphocyte activation for cancer therapy

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Abstract

Although T cells engaging bispecific antibodies (T-bsAbs) have shown great benefits, their use in treating solid tumors is challenging because of the minimal infiltration of T-cells. We fused an agonistic single-chain variable fragment (scFv) that induces a T cell co-stimulatory signal to the T cell-binding domain of T-bsAb via a linker containing a cancer-specific protease recognition site. With this antibody format, unexpected cytotoxicity to the surrounding normal tissue would be reduced and tumor-specific T cell activation would occur. The scFv-masked T-bsAb was cleaved by collagenase with intrinsic cancer-specific protease activity, releasing agonistic scFv without unwanted fragmentation and restoring the binding ability of the scFv-masked bsAbs to T cells. Compared to the original bsAb, a detectable enhancement of the T cell proliferation and cancer cytotoxicity was observed after the incubation with collagenase or protease-secretory cancer cells, which was suggested to be due to the modest co-stimulation by the released agonistic scFv. Our results provide important insights into an ideal T-bsAb prodrug format, precisely engineered to reduce side effects and exert high cancer cytotoxicity for solid tumor precision medicine.

Keywords Bispecific antibody, Prodrug, Co-stimulation, Single-chain variable fragment, Cancer therapy

Introduction

Immunotherapies are emerging as powerful therapeutic agents to fight cancer alongside surgery, chemotherapy, and radiotherapy [1]. They induce anti-tumor immune responses, including the activation of T and natural killer (NK) cells, and inhibit immune checkpoints, such as programmed cell death 1 (PD-1)/programmed cell death ligand 1 (PD-L1). Among the various cancer immunotherapy modalities, T cells engaging bispecific antibodies (T-bsAbs) are one of the most effective formats. T-bsAbs are designed to cross-link two antigens, one on T cells and the other on cancer cells, to kill cancer cells effectively [2]. We previously reported humanized T-bsAbs [3] that can target the epidermal growth factor receptor (EGFR) on cancer cells, CD3 on T cells, (Ex3) and its human Fc fusion format (Ex3-Fc and Ex3-scDb-Fc)

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[4]. T-bsAbs have occasionally been successful in clinical studies [5]. However, serious side effects such as cytokine release syndrome (CRS) remain concerning, especially the effects of T cell overactivation by the binding of T-bsAbs [6]. Additionally, the antigens on cancer cells are also expressed on normal cells, decreasing the efficacy and safety of T-bsAbs [7]. Therefore, providing T-bsAbs with effective cytotoxicity and regulating minimal side effects related to excess cytokine release are important.

The current T-bsAb drug approved for treating hematological cancers is blinatumomab, which targets CD19 on cancer cells and CD3 on T cells [8]. However, T-bsAb-approved drugs for the treatment of solid cancers remain elusive [9], mainly because of the limited number of tumor-infiltrating lymphocytes (TILs) within solid cancers [10]. The lack of TILs leads to insufficient T cell activation and cytokine release. Additionally, successive stimulation of T cells and cytokine release gradually decrease effector functions, leading to TIL exhaustion [11] because TILs are intermittently stimulated by cytokines such as tumor necrosis factor-alpha (TNF-α) from surrounding cancer cells [12].

T cell co-stimulation plays an important role in restoring exhausted TILs. CD137, also known as 4-1BB, is one of the co-stimulatory proteins. Since the activation of 4-1BB can induce the cytotoxicity of TILs by secretion of interferon-gamma (IFN-γ) [13], agonistic antibodies, including urelumab, were investigated. Overstimulation of co-stimulatory proteins results in CRS and hepatotoxicity [14], and clinical trials of urelumab have been terminated because of its side effects [15]. Utomilumab, another agonistic antibody against 4-1BB, showed reduced toxicity in clinical trials but had limited clinical activity [16, 17]. Therefore, strict regulation of the agonistic activity of antibodies is required when targeting co-stimulatory proteins in combination with T cell activation.

Paratope masking is an effective strategy to prevent nonspecific binding of antibodies during the development of prodrugs [18]. Antigen-based molecules, affinity peptides for paratopes [19, 20], or bulky molecules—such as variable domains [21] and coiled-coil peptides [22]—have been used as paratope-masking molecules. These masking molecules function through considerable steric hindrance to block antibody binding to antigens on the cells. Recently, our group reported a steric hindrance-based programmed masked T-bsAb constructed by inserting a matrix metalloprotease (MMP) recognition sequence. The sequence was placed between a repeated polypeptide comprising Pro, Ala, and Ser (PAS) and the anti-CD3 domain of Ex3-scDb-Fc and was activated by MMP-2 and -9 overexpression in cancer cells [23]. This showed a lowered binding ability to T cells and recovery of selective binding to T cells after MMP digestion than unmasked Ex3-scDb-Fc. As the steric hindrance of bulky polypeptides inhibits antibody binding, we hypothesized that the alternative use of a functional protein, such as an agonistic antibody with lymphocyte activation, would provide a more promising prodrug than the use of the bulky polypeptide.

In this study, we used a co-stimulatory molecule as a masking molecule to design a novel T-bsAb prodrug format. We fused the anti-4-1BB agonistic single-chain variable fragment (scFv) to the terminus of the CD3 binding domain of Ex3-scDb-Fc using a peptide linker containing an MMP recognition sequence. We expected a reduced binding ability of Ex3 to T cells based on the steric hindrance of anti-4-1BB scFv in normal tissue. Once the linker was cleaved by MMP after reaching the solid tumor, a high anti-tumor effect was observed by the synergy of T cell-recruiting cytotoxicity of Ex3-scDb-Fc, and the co-stimulatory effect on TILs by the released anti-4-1BB agonistic scFv could be exerted (Fig. 1).

Materials and methods

Construction of expression vectors

The anti-4-1BB scFv gene (clone 94G1) [24] was synthesized (VH–VL region order), and expression vectors of the scFv-masked bsAbs, including an MMP recognition sequence for a mammalian expression system, were constructed as described previously [23]. The human rhinovirus (HRV) 3C protease recognition sequence, instead of the MMP recognition sequence, was used as a control. The amino acid sequences of these proteins are summarized in Figure S1.

Recombinant production of the antibodies

ScFv-masked bispecific antibodies were prepared using the Expi293 Expression System (Thermo Fisher Scientific, Waltham, MA, USA). They were purified via affinity chromatography using an rProtein A column (Cytiva, Tokyo, Japan), according to the manufacturer's protocol, and gel filtration chromatography. All protein concentrations were determined by measuring the absorbance at 280 nm, and purity was evaluated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Cleavage of the scFv-masked bispecific antibodies by collagenase type 4

Collagenase type 4 (Abnova, Taipei, Taiwan) was added to be at a final concentration of 16 U/mL to a solution containing 250 nM of the scFv-masked bispecific antibodies and incubated in a cleavage buffer (50 mM TES containing 0.36 mM $CaCl_2$ (pH 7.5)) at 37 ℃. One unit was defined as the amount of enzyme that cleaved 500 pmol of substrate per minute per microgram of enzyme. The cleavage reaction was terminated by adding ethylenediaminetetraacetic acid (EDTA) at a final

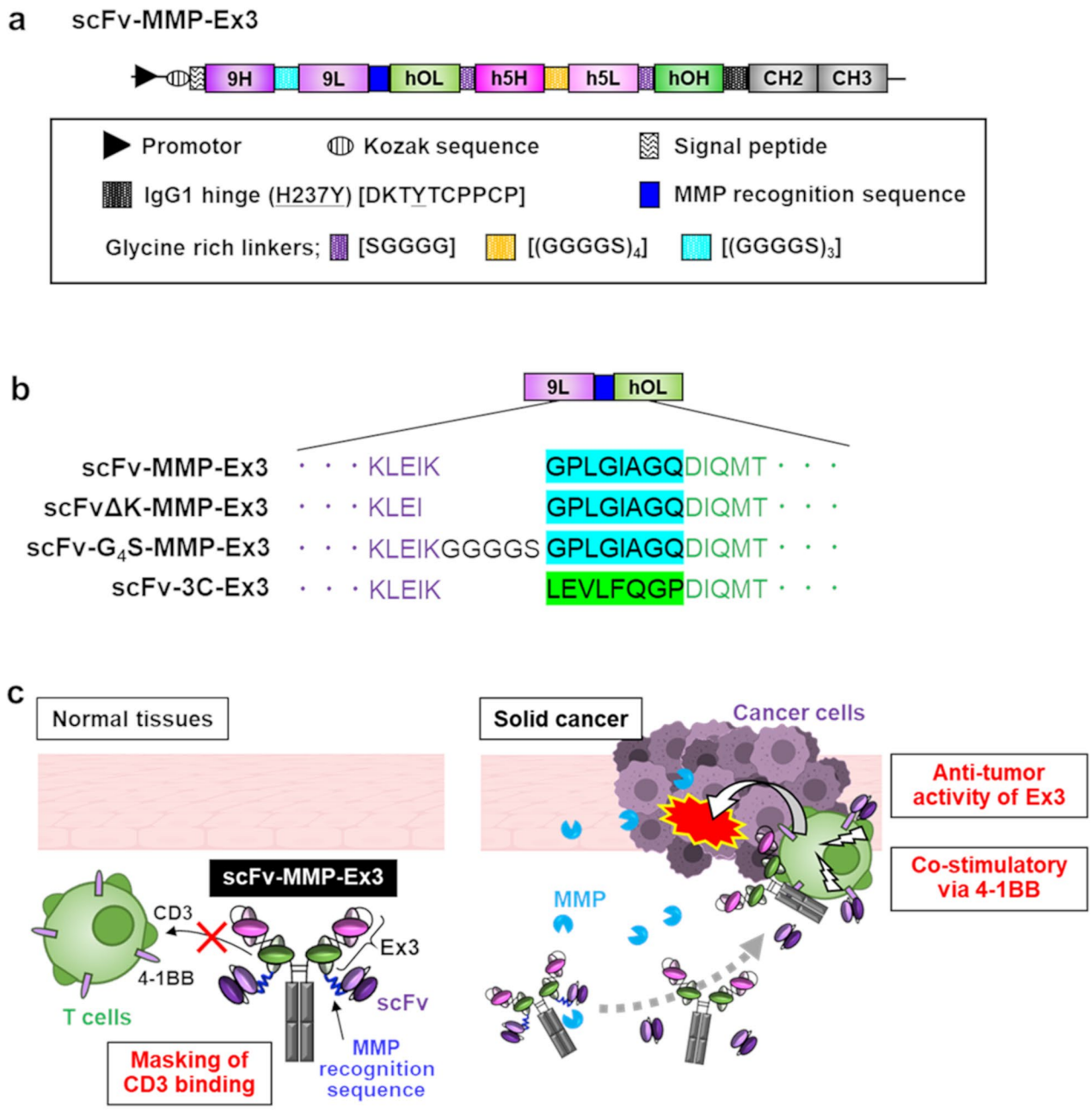

Fig. 1 Schematic illustrations of (**a**) the gene construction of scFv-MMP-Ex3 as a representative and (**b**) the amino acid sequences of the linker containing the matrix metalloprotease (MMP) recognition sequence. 9H and 9L represent the VH and VL regions of anti-4-1BB scFv (clone: 94G1). h5H and h5L show the VH and VL regions of the humanized anti-epidermal growth factor receptor (EGFR) antibody 528, and hOH and hOL represent the VH and VL regions of the humanized anti-CD3 antibody OKT3. (**c**) A schematic representation of the working mechanism of scFv-MMP-Ex3 prodrug to exert the tumor-specific activation upon the cleavage by MMP highly expressed in cancer cells

concentration of 1 mM. Antibody cleavage was evaluated using SDS-PAGE analysis.

Cell lines

Human bile duct carcinoma cells (TFK-1) were used as the cancer cell line [25]. These were cultured in Roswell Park Memorial Institute 1640 (RPMI) medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Lymphokine-activated killer cells with a T-cell phenotype (T-LAKs) were used as effector cells. T-LAK cells were prepared from peripheral blood mononuclear cells (PBMCs) cultured at a density of 1.0×10^6 cells/mL in a medium supplemented with

100 IU/mL recombinant human interleukin-2 (Shionogi Pharmaceutical Co., Osaka, Japan) for 48 h in a culture flask (A/S Nunc, Roskilde, Denmark). It was coated with anti-CD3 monoclonal antibody at a concentration of 10 μg/mL [26]. Human sarcoma (HT1080) and epidermoid carcinoma (A431) cell lines were used to evaluate antibody cleavage by MMPs secreted by cancer cells.

Binding ability analysis using flow cytometry

The binding abilities of the antibodies before and after cleavage were evaluated using flow cytometry with TFK-1 and T-LAK as cancer and effector cells, respectively. Approximately 5.0×10^5 cells were diluted in phosphate-buffered saline (PBS) and incubated with 250 nM antibodies on ice for 30 min. After washing twice with PBS, the cells were incubated with fluorescein isothiocyanate (FITC)-labeled anti-human IgG antibody (Sigma-Aldrich) on ice for 30 min and then washed twice with PBS. Finally, 500 μL of PBS was added and well-suspended, and the stained cells were analyzed using a flow cytometer (BD Accuri C6; Becton Dickinson, San Jose, CA, USA).

T-LAK cell proliferation assay and cytotoxicity assay

The T-LAK cell proliferation assay was performed as previously described [23]. Briefly, 5.0×10^4 of T-LAK cells were seeded in a 96-well plate (Costar, Cambridge, MA, USA) and cultured overnight at 37 ℃ in a humidified atmosphere containing 5% CO_2. Various concentrations of the scFv-masked bispecific antibody treated with or without the collagenase were added and incubated at 37 ℃ for 48 h in the same condition. After adding 10 μL of a water-soluble tetrazolium salt WST-8 solution (Cell Counting Kit-8; Fujifilm Wako Pure Chemical Co., Osaka, Japan) to each well, the plate was incubated at 37 ℃ for 18 h. Finally, the absorbance at 450 nm was measured using a microplate reader (Multiskan GO; Thermo Fisher Scientific).

An in vitro cytotoxicity assay was performed as previously described [27]. Briefly, approximately $5.0 \times 10^3$of TFK-1 cells were cultured in a well of the 96-well plate at 37 ℃ overnight in a humidified atmosphere containing 5% CO_2. After removal of the medium, 2.5×10^3 of T-LAK cells and various concentrations of the scFv-masked bispecific antibody treated with or without the collagenase were incubated at 37 ℃ for 20 h in the same condition. After removal of the medium and washing with PBS three times, the growth inhibition of the cancer cells was evaluated by measuring the absorbance at 490 nm using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 H-tetrazolium (MTS) assay kit (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay; Promega, Madison, WI, USA).

Cleavage of the scFv-masked bispecific antibody by MMP secreted from cancer cells

Three cell lines, HT1080, A431, and TFK-1, were seeded in a 96-well plate at 5×10^3 cells per well and cultured overnight in RPMI medium supplemented with 10% FBS at 37 ℃ in a humidified atmosphere containing 5% CO_2. The culture supernatant was collected by centrifuging at 300 × *g* for 5 min. The supernatant of each cell line was incubated with 100 nM of the scFv-masked bispecific antibodies overnight at 37 ℃. The proteins were analyzed by SDS-PAGE and western blotting using horseradish peroxidase (HRP)-conjugated anti-human IgG as the detection antibody (Abcam, Cambridge, UK).

Results and discussion

Design and preparation of scFv-masked bispecific antibodies

First, we designed scFv-masked bispecific antibodies using a protease recognition sequence. ScFv-MMP-Ex3 is a basic scFv-masked bispecific antibody (Fig. 1a) in which the linker between scFv and Ex3 comprises a conventional flexible amino acid sequence and an MMP recognition sequence (Fig. 1b). We designed the scFvΔK241 and connected it to Ex3 with the same linker (scFvΔK-MMP-Ex3) to increase the proximity of the scFv to Ex3 and enhance the masking effect on Ex3 to T cells. We prepared an scFv-masked bispecific antibody with an elongated linker with G_4S to increase digestion efficiency and accommodate MMP (scFv-G_4S-MMP-Ex3). Finally, an scFv-masked bispecific antibody containing another protease, human rhinovirus 3C (HRV-3C), was used to evaluate specific cleavage by MMP as a control (scFv-3C-Ex3). As shown in Figure S2, while certain engineered antibodies resulted in the formation of multimers [28], all scFv-masked bispecific antibodies were prepared as monomers. The fractionated monomers were used for further investigation with high yields of 6.0, 7.9, 4.2, and 6.6 mg/L culture of scFv-MMP-Ex3, scFvΔK-MMP-Ex3, scFv-G_4S-Ex3, and scFv-3C-Ex3, respectively.

Evaluation of cleavage of scFv-MMP-Ex3 by collagenase type 4

Collagenase type 4, containing intrinsic MMP-2 and MMP-9 activities, was used to cleave the MMP recognition sequence [29]. First, we investigated the time to cleavage. The results indicated that the band at the theoretical molecular weight of Ex3 (Mw: 78 kDa) increased time-dependently and the band corresponding to scFv (Mw: 27 kDa) was observed after cleavage (Figure S3a, S3b), suggesting that the linker containing the MMP recognition sequence was cleaved by the collagenase. In contrast, the cleavage of scFv-G_4S-MMP-Ex3 resulted in short fragmentation (Figure S3c). This was caused by the cleavage of the linker in Ex3. In addition, the 3C protease

recognition sequence was unexpectedly cleaved by adding the collagenase due to the non-specific cleavage by the collagenase. However, its cleavage efficiency was lower than that of scFv-MMP-Ex3 (Figure S3d). We constructed model structures of the scFv-masked bispecific antibodies using the AlphaFold3 web tool [30]. As shown in Figure S4, the positions of scFv to Ex3 differed depending on the linker, suggesting that differences in the tertiary structure of the scFv-masked bispecific antibodies caused differences in collagenase cleavage efficiency. The accessible surface area (ASA) was calculated using the model structures and the GETAREA web tool [31]. The parameters are summarized in Table S1. The average ASA of the middle linker of scFvΔK-MMP-Ex3 was smaller than that of scFv-MMP-Ex3, indicating that the linker of scFvΔK-MMP-Ex3 was less exposed to the solvent. This suggested that the deletion of a single amino acid may contribute to be the proximity of scFv to Ex3. Nevertheless, the cleavage efficiency of scFvΔK-MMP-Ex3 was superior to that of scFv-MMP-Ex3, as shown in Figure S3a and S3b, because the band intensities corresponding to Ex3 after cleavage differed from that after 1 h incubation with the collagenase. This could be explained by the larger ASA per residue of scFvΔK-MMP-Ex3 than that of scFv-MMP-Ex3. This suggested that the deletion changed the tertiary structure of scFvΔK-MMP-Ex3 and loosened it to be exposed to the solvent, accommodating the collagenase. The same is true for the fragmentation of scFv-G_4S-MMP-Ex3 via collagenase cleavage. In addition, because the collagenase type 4 extracted from a bacterium is a crude sample [32], it was cleaved non-specifically by the middle linker containing the 3C protease during incubation. Collectively, the fusion protein containing the MMP recognition sequence in the linker was successfully cleaved by the collagenase, which has intrinsic MMP-like activity. Additionally, there is a possibility of cleavage of only a single recognition site in a molecule at shorter incubation times. However, even if only a single site of the antibody was cleaved and the scFv was released, the crosslinking between T cells and cancer cells by Ex3 would occur, and the activation of T cells by the co-stimulation induced by the released scFv and some degree of cancer growth inhibition would begin at that point. Based on these results, the cleavage time was optimized to 4 h to fully cleave the antibody while preventing non-specific cleavage by the collagenase.

Evaluation of the binding ability of the scFv-masked bispecific antibodies after the cleavage

The masking effect on the binding to the antigen on cells and its recovery after cleavage by the collagenase were evaluated using flow cytometry. The binding ability of scFv-masked bispecific antibodies to T-LAK cells was lower than that of the original Ex3 before cleavage (Fig. 2a). In contrast, after the cleavage, they recovered to the same level as in Ex3. The binding of scFv-3C-Ex3 to T-LAK cells was also observed after cleavage. This could be explained by the nonspecific cleavage of the linker containing the 3C protease recognition sequence by collagenase, as shown in Figure S3d. The binding ability of the scFv-masked bispecific antibodies to TFK-1 cells was not influenced by the fusion of scFv or treatment with the collagenase (Fig. 2b). We previously evaluated the difference and recovery of the affinities of the masked antibodies using the MMP recognition sequence as the linker [23]. This demonstrated that the fusion of scFv to Ex3 inhibited the binding of Ex3 to T-LAK cells through a masking effect, and that the cleavage of the linker containing the MMP recognition sequence by the collagenase released scFv from Ex3. The masking effect of the scFv-masked bispecific antibodies was specifically exerted upon binding to T-LAK cells, maintaining their binding ability to TFK-1 cancer cells. As there were no major differences in the masking effect and the recovery of the binding ability, and it showed high cleavage efficiency by the collagenase, scFvΔK-MMP-Ex3 was used for further experiments

Effects on T cell activation and cancer growth inhibition

T cell activation and the cancer growth inhibition of scFvΔK-MMP-Ex3 after the cleavage by the collagenase were evaluated. The ability to activate T cells of scFvΔK-MMP-Ex3 was lower than that of Ex3 (Fig. 3a). Despite the successful binding of Ex3 to the surface proteins of T cells, which induces proliferation, the ability to activate T cells is weakened owing to the masking effect of scFv. In contrast, the ability to activate T cells was increased rather than restored after the cleavage and release of scFv from Ex3. The expression of 4-1BB in T-LAK cells used in this study was confirmed (Figure S5), suggesting that the released scFv functions as a co-stimulatory molecule to enhance T cell proliferation. This was elucidated when anti-4-1BB agonistic scFv was externally added to Ex3, resulting in increased T cell proliferation and cancer growth inhibition (Figure S6). Similarly, the cancer growth inhibition ability of scFvΔK-MMP-Ex3 was lower than that of Ex3, and significantly high growth inhibition was observed after cleavage by the collagenase (Fig. 3b). Additionally, adding collagenase did not affect cancer growth inhibition (Figure S7). No significant increase in cancer growth inhibition was observed at low antibody concentrations when scFvΔK-MMP-Ex3 was used, whereas T cell proliferation was increased at these concentrations. This was due to insufficient T cell activation for cancer killing. If cancer cells are added and co-incubated with T cells, they may become more activated, which can exert higher killing efficiency. These results demonstrated that the fusion of scFv to Ex3 induced a

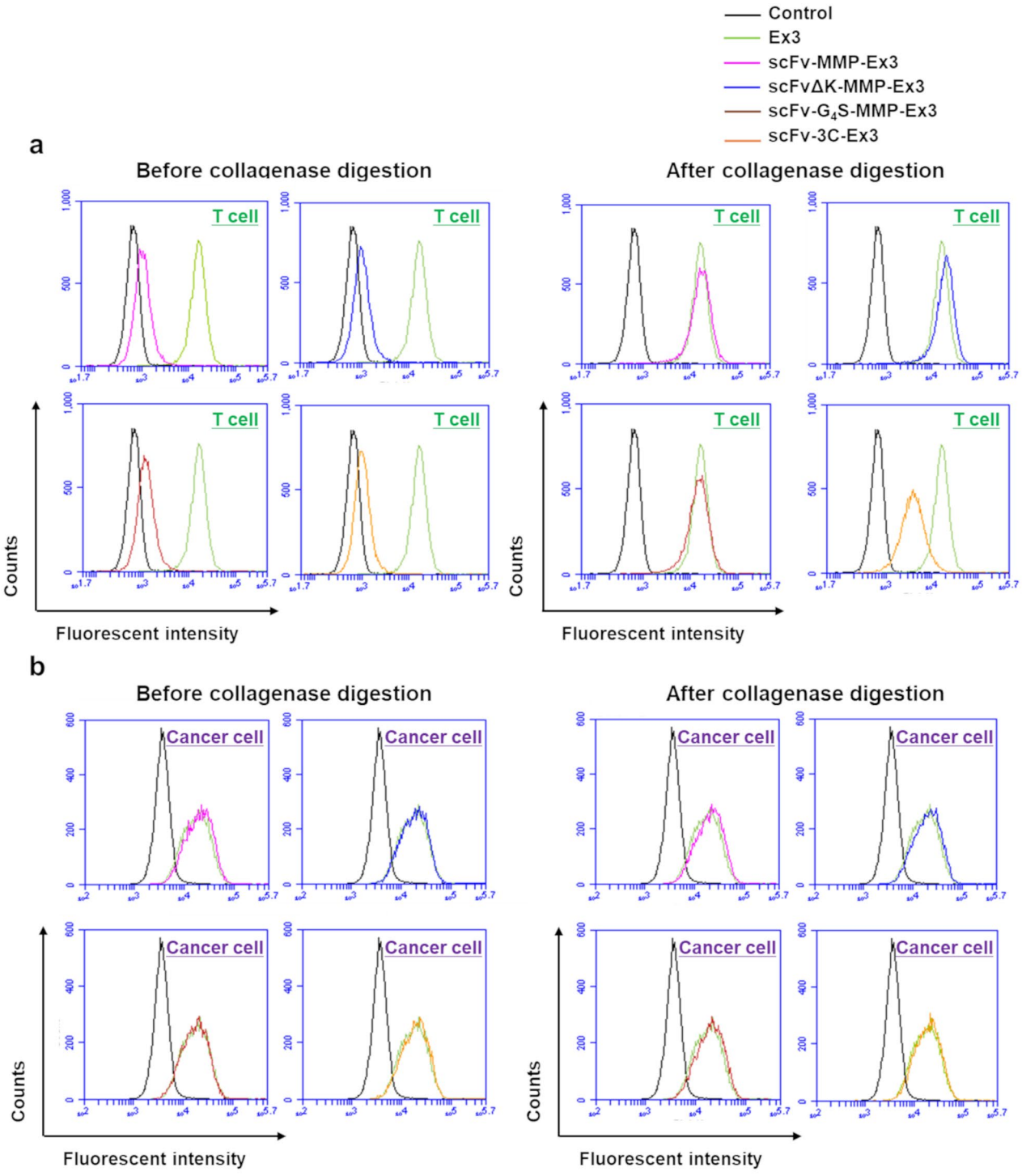

Fig. 2 Binding ability analysis of the scFv-masked bispecific antibody against (**a**) T cells and (**b**) cancer cells before and after collagenase digestion. Phosphate-buffered saline (PBS) was used as a negative control in each assay, and each antibody was incubated with CD3-positive lymphokine-activated killer cells with a T-cell phenotype (T-LAK) cells or epidermal growth factor receptor (EGFR)-positive TFK-1 cells. The cells were stained with a fluorescein isothiocyanate (FITC)-labeled anti-human antibody after incubation

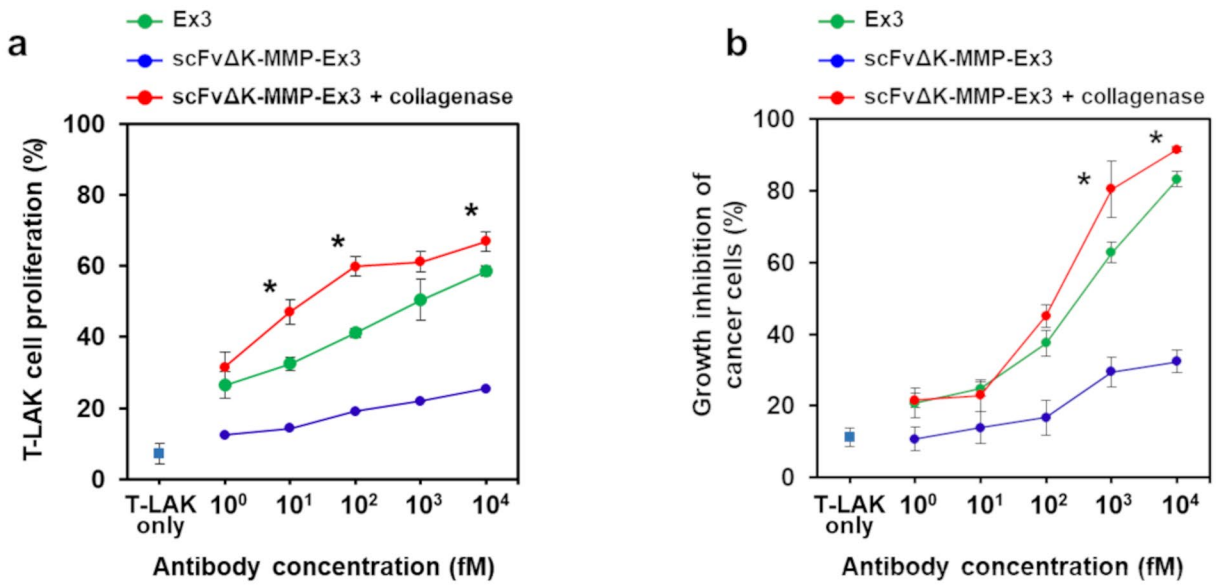

Fig. 3 (**a**) T cell proliferation and (**b**) the cancer cell cytotoxicity analyses of Ex3, scFvΔK-MMP-Ex3, and scFvΔK-MMP-Ex3 treated with the collagenase. (**b**) T-LAK cells were added to TFK-1 cells at a ratio of 0.5:1 ($n = 4$). Data are expressed as mean ± SD. The significance of the results was analyzed using one-way ANOVA and followed by Bonferroni's post hoc test (*$p < 0.05$; Ex3 vs. scFvΔK-MMP-Ex3 + collagenase)

masking effect and that the scFv released after cleavage was used as a co-stimulator to enhance the ability to activate T cells and inhibit the growth of TFK-1 cells. However, the agonistic effect of released scFv was relatively small, especially at lower antibody concentrations. The anti-4-1BB scFv used in this study was constructed by connecting the heavy and light chains via a flexible linker from the reported amino acid sequence [24], which may have altered the functionality compared to that of the reported formats, i.e., Fab and IgG. In addition, agonist activity varies widely depending on the antibody clone [33]; therefore, using another clone of anti-4-1BB scFv may improve agonistic efficiency. Future studies should explore the scFv clone for the application of scFv-masked bispecific antibodies to in vivo analysis, which may exert further agonistic effects.

Cleavage by MMP secreted from cancer cells and the effect on cancer growth inhibition

Cancer cells that secrete MMPs, HT1080 cells and A431 cells, were used as model cancer cells to evaluate whether the linker of scFvΔK-MMP-Ex3 can be cleaved [34]. The culture supernatant of each cell was incubated with scFvΔK-MMP-Ex3 overnight, and the cleavage of the linker was evaluated using western blotting. As shown in Fig. 4a, chemiluminescence at the theoretical molecular weight of Ex3 was observed after incubation with each culture supernatant, whereas scFv-3C-Ex3 was not cleaved, unlike when the collagenase was used for cleavage (Figure S3). These results supported that the middle linker of Ex3 and the 3C protease linker were non-specifically cleaved by the collagenase and demonstrated that the linker of scFvΔK-MMP-Ex3 was specifically cleaved by MMP secreted by cancer cell lines.

Finally, we evaluated cancer growth inhibition using A431 and HT1080 cells. As a result, scFvΔK-MMP-Ex3 showed higher cytotoxicity than scFv-3C-Ex3 in both cancer cell lines (Fig. 4b, c), consistent with scFvΔK-MMP-Ex3 cleavage by the collagenase (Fig. 3). In contrast, no significant differences were observed when TFK-1 cells were used as cancer cells (Figure S8). The MMP secretion level of HT1080 cells was higher than that of A431 cells [34], suggesting higher cancer growth inhibition at the lowest antibody concentration (10 fM) was observed when using HT1080 cells. These results suggested that the co-stimulatory effect of the released agonistic scFv enhanced cancer growth inhibition while evaluating the binding of the released scFv by MMP cleavage to 4-1BB expressed on cancer cells is challenging. However, investigations such as long-term stability analysis, establishing a suitable in vivo model, and the cleavage manner by MMPs are needed for further applications, including in vivo study to understand the efficacy, safety, pharmacokinetics, and dynamics of MMPs cleavage. For such investigations, fine-tuning through protein engineering is required in the future. Taken together, scFvΔK-MMP-Ex3 was cleaved by MMP secreted by cancer cells, and the combination of a bispecific antibody, Ex3, and co-stimulation molecule, scFv, induced the cancer growth inhibition ability. This ultimately demonstrated that scFvΔK-MMP-Ex3 could be a useful prodrug format for solid cancer treatment.

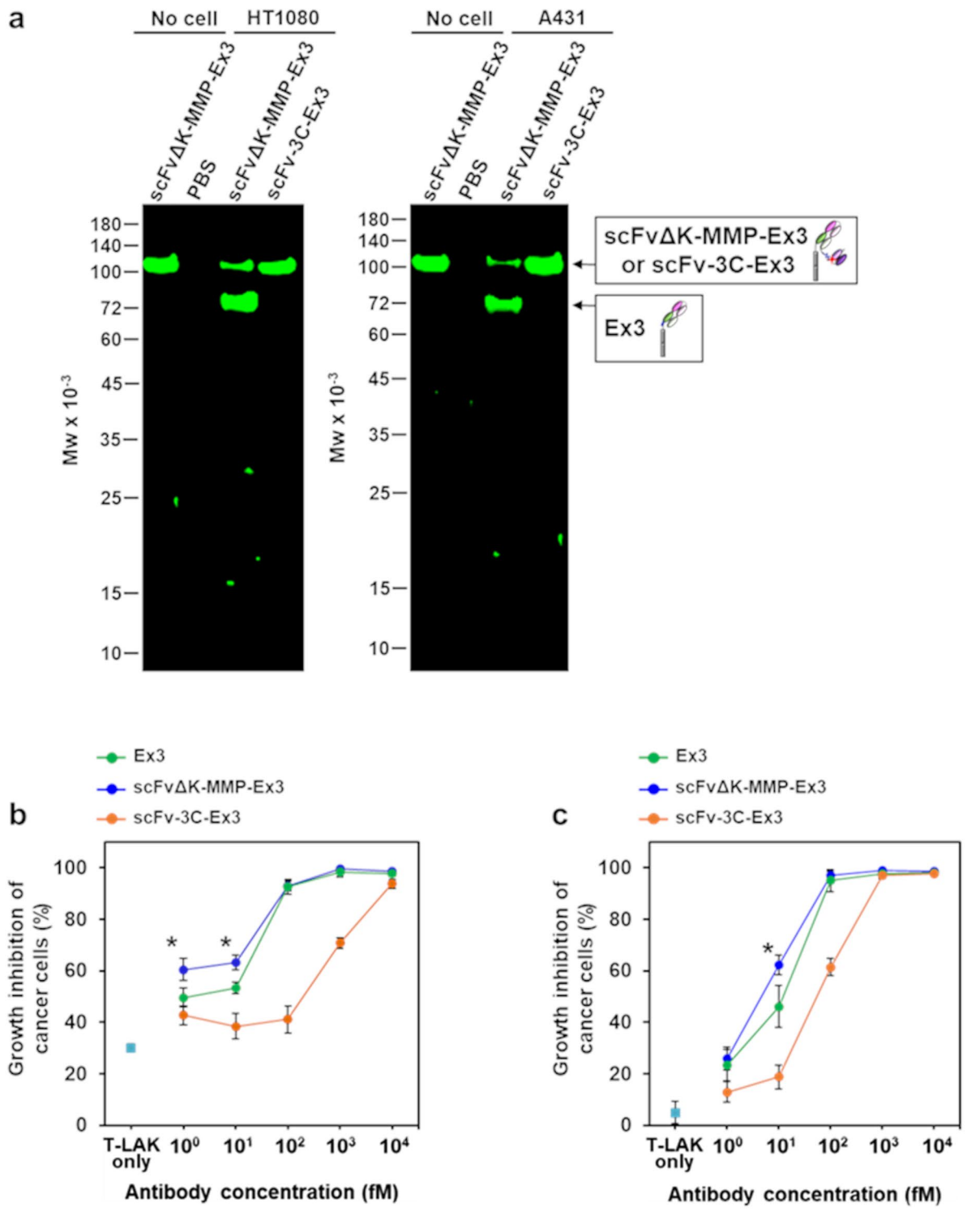

Fig. 4 (**a**) Confirmation of the cleavage of scFvΔK-MMP-Ex3 using the culture supernatant of MMP-expressing cancer cell lines, HT1080 and A431, using western blotting. Ex3 was detected by horseradish peroxidase (HRP)-conjugated anti-human antibody, and the chemiluminescence was imaged. (**b**, **c**) Cancer cell cytotoxicity analysis of Ex3, scFvΔK-MMP-Ex3, and scFv-3C-Ex3 as a negative control. As cancer cell lines, (**b**) HT1080 and (**c**) A431 were used, respectively. T-LAK cells were added to cancer cells at a ratio of 5:1 ($n = 4$). Data are expressed as mean ± SD. The significance of the results was analyzed using one-way ANOVA and followed by Bonferroni's post hoc test (*$p < 0.05$; Ex3 vs. scFvΔK-MMP-Ex3)

Several strategies have been proposed to avoid CRS after using T-bsAbs, such as changing the bsAb design [35, 36] or decreasing their affinity to the antigens [37]. Paratope masking is an effective alternative for modulating the binding ability of the T cell-binding domain of T-bsAbs. However, appropriate and specific activation of exhausted TILs is required to achieve effective cytotoxicity against solid tumors. We focused on a collaborative approach based on these two opposing strategies by designing masked T-bsAbs using an agonistic scFv fused to a cancer-specific protease recognition sequence. To the best of our knowledge, this is the first study to use agonistic scFv as a masking molecule for T-bsAbs.

Conclusion

In this study, we designed a T-bsAb prodrug by incorporating an agonistic anti-4-1BB scFv into an Ex3 T-bsAb fused via the MMP recognition sequence next to the T cell-binding domains. Suitable linker compositions were effectively cleaved by the collagenase with MMP activity without any unwanted cleavage. The scFv-masked bispecific antibody showed reduced binding ability to T cells before cleavage, which was restored after cleavage. It was suggested that the released agonistic scFv contributed to the enhancement of T cell proliferation and effective cancer cytotoxicity owing to co-stimulation. These phenomena have also been observed in MMP-secretory cancer cell lines, where specific cleavage of the linker was also confirmed. The co-stimulatory effect of the agonistic scFv was relatively weaker than expected, and it is necessary to perform pharmacokinetic analysis including the cleavage manner in the tumor in detail. However, our results can provide important insights into the development of an ideal T-bsAb prodrug format with a precise design to exert cancer cytotoxicity by co-stimulating the agonistic scFv.

Abbreviations

T-bsAbs	T cells engaging bispecific antibodies
scFv	Single-chain variable fragment
NK	Natural killer
PD-1	Programmed cell death 1
PD-L1	Programmed cell death ligand 1
EGFR	Epidermal growth factor receptor
CRS	Cytokine release syndrome
Ex3	T-bsAb targeting EGFR and CD3
TILs	Tumor-infiltrating lymphocytes
TNF-α	Tumor necrosis factor-alpha
IFN-γ	Interferon-gamma
MMP	Matrix metalloprotease
HRV	Human rhinovirus
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
T-LAK	Lymphokine-activated killer cells with a T-cell phenotype
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
FITC	Fluorescein isothiocyanate
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 H-tetrazolium
HRP	Horseradish peroxidase

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13036-025-00517-9.

Supplementary Material 1

Acknowledgements

This is not applicable.

Author contributions

Y.K., M.Y., I.K., and R.A. contributed to the conception and design of the study; D.M., Y.K., M.Y., R.A. contributed to the acquisition of data and/or analysis, and interpretation of data; R.A. contributed to acquisition of funding; D.M. and R.A. contributed to preparation of the manuscript draft; all authors contributed to the revision of the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

This is not applicable.

Consent for publication

This is not applicable.

Competing interests

The authors declare no competing interests.

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