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Original Articles

Anti-KL-6/MUC1 monoclonal antibody reverses resistance to trastuzumab-mediated antibody-dependent cell-mediated cytotoxicity by capping MUC1

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A B S T R A C T

Polymorphic epithelial mucin (MUC1) is generally overexpressed on the surface of most adenocarcinomas including breast cancer. MUC1 is associated with chemotherapeutic resistance and immune evasion of cancer cells; however, the association between MUC1 and trastuzumab-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) remains unclear. In this study, using six breast cancer cell lines with differing expression levels and MUC1 distribution, the present results show that cells with MUC1 overexpression and uniform surface distribution were resistant to trastuzumab-mediated ADCC. Importantly, trastuzumab resistance was reversed upon siRNA-mediated MUC1 knockdown and by using anti-KL-6/MUC1 monoclonal antibody (mAb). Additionally, we visually confirmed that anti-KL-6/MUC1 mAb induced capping of MUC1 molecules on the cell surface, resulting the in death of these cells. These results suggest that not only the quantity but also the cell-surface distribution of MUC1 affects the sensitivity of breast cancer cells to trastuzumab-mediated ADCC.

1. Introduction

Polymorphic epithelial mucin (MUC1) is a single-pass type I transmembrane protein with a heavily glycosylated extracellular domain that comprising 20-amino acid core tandem repeats and extends up to 200–500 nm from the cell surface [1]. It is normally expressed and localized at the apical surface of glandular epithelial cells in numerous tissue types, including breast, lung, and ovary. In adenocarcinoma cells, which develop from such glandular epithelial cells, the expression of MUC1 is up-regulated and is expressed not only on the apical surface, but also on the entire cell surface. MUC1 overexpression is associated with a poor prognosis in several different types of carcinomas [2–5]; however, the exact mechanism underlying this association is unclear. One possible mechanism might be the involvement of MUC1 in immune escape of cancer cells. We previously reported that MUC1 overexpression in breast cancer cell lines was associated with escape from immune effector cells such as lymphokine-activated killer (LAK) cells [6]. Importantly, capping and localization of MUC1 induced by a monoclonal antibody to Krebs von den Lugen-6 (KL-6), which is one of sialylated carbohydrate antigens on the N-terminal domain of MUC1 [7], enhanced tumor cell death by LAK cells [6]. Up-regulation of MUC1 helps protect cancer cells from immune-mediated killing via a mechanism depending on particular glycosylation steps in MUC1 [8]. These findings strongly support a role for MUC1 in immune evasion mechanisms of cancer cells.

Trastuzumab (Herceptin®) is a humanized monoclonal antibody that recognizes an epitope in the extracellular domain of the human epidermal growth factor receptor 2 (HER2)/erbB2 receptor and is used to treat breast cancers overexpressing HER2. Trastuzumab inhibits tumor cell growth and induces apoptosis by binding to HER2, thereby regulating cell signal transduction [9]. Additionally, it promotes tumor cell death by evoking antibody-dependent cell-mediated cytotoxicity (ADCC) [10,11]. However, a significant fraction of patients with HER2-expressing breast cancer exhibit or develop trastuzumab resistance

Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; HER2, human epidermal growth factor receptor 2; LAK, lymphokine-activated killer; PBMCs, peripheral blood mononuclear cells

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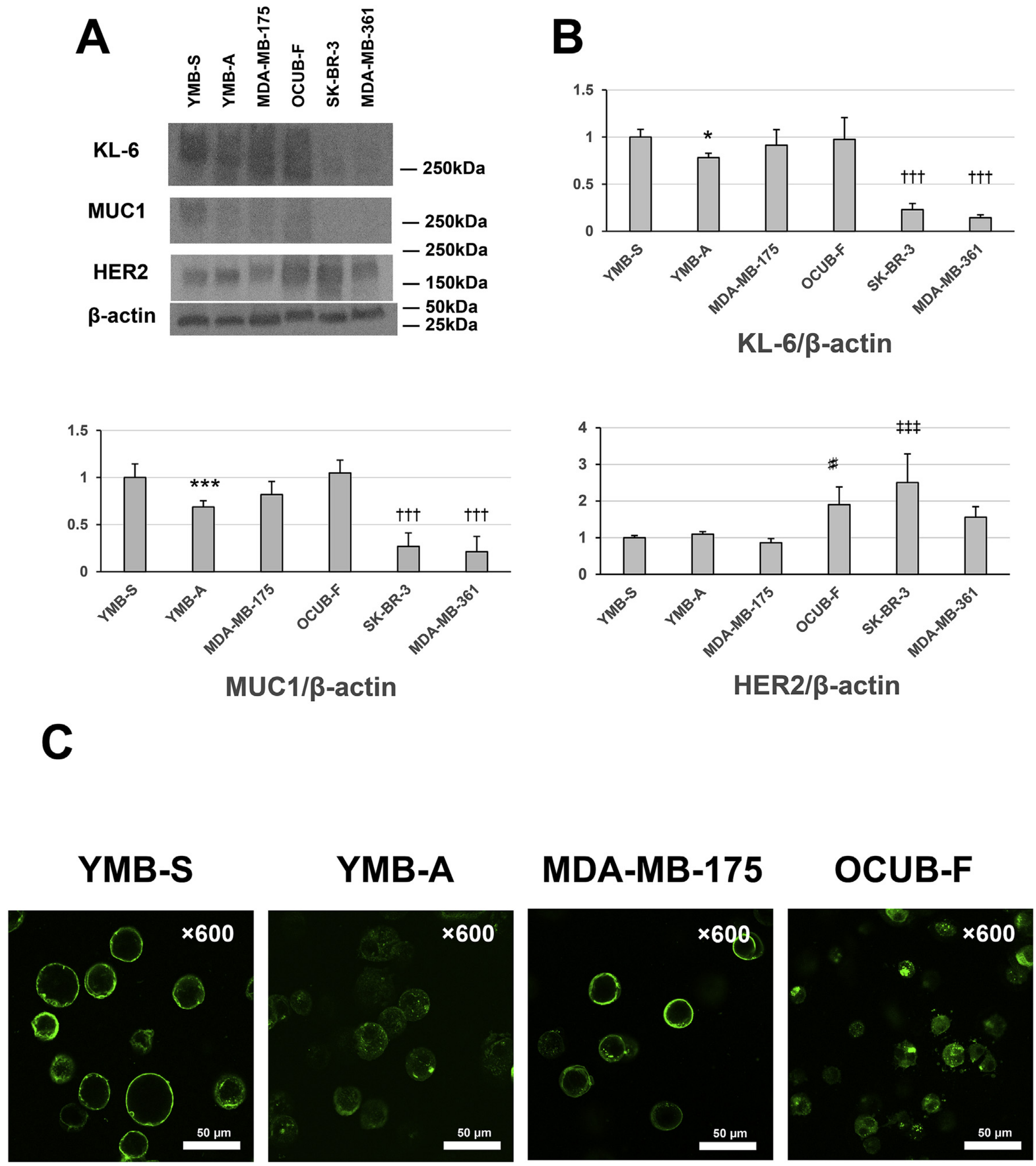

Fig. 1. Expression of KL-6, MUC1, and HER2 in six human breast cancer cell lines. (A) Western blot analysis of KL-6, MUC1, HER2, and β-actin in six human breast cancer cell lines (YMB-S, YMB-A, MDA-MB-175, OCUB-F, SK-BR-3, and MDA-MB-361). Expression of MUC1 was assessed using anti-KL-6 mAb and anti-MUC1 mAb (HMFG1). (B) Levels of KL-6, MUC1 and HER2 normalized to β-actin are shown as fold change compared to YMB-S. Bars represent the mean ± standard deviation (n = 3–5). Statistical analysis was performed using the Tukey-Kramer test. $^{*}P < 0.05$ vs YMB-S; $^{***}P < 0.001$ vs YMB-S; $^{\dagger\dagger\dagger}P < 0.01$ vs YMB-S, YMB-A, MDA-MB-175, and OCUB-F; $^{\ddagger\ddagger\ddagger}P < 0.001$ vs YMB-S, YMB-A, MDA-MB-175, OCUB-F, and MDA-MB-361. (C) YMB-S, YMB-A, MDA-MB-175, and OCUB-F cells were incubated with 1 μg/mL of FITC-conjugated anti-KL-6 mAb for 10 min at 4 °C, and fixed. Cells were visualized using a confocal laser scanning microscope (green). Bar, 50 μm.

[12]. MUC1 has been proposed to be involved in trastuzumab resistance. MUC1 expression levels reportedly affect the antitumor effect of trastuzumab [13,14] and anti-MUC1 antibodies help overcome trastuzumab resistance [15,16]. However, these studies have examined the effect of MUC1 on trastuzumab itself but not on trastuzumab-mediated ADCC.

Having established that MUC1 is involved in immune escape by cancer cells, we hypothesized that MUC1 overexpression would affect the intensity of trastuzumab-mediated ADCC. To test this hypothesis, we analyzed the relationship between the level of MUC1 and the degree of ADCC activity in several breast cancer cell lines. We also investigated the effect of siRNA-mediated knockdown of MUC1 on the intensity of trastuzumab-mediated ADCC. In addition, we determined whether anti-KL-6 monoclonal antibody (mAb)-induced capping of MUC1 affected the degree of ADCC.

2. Materials and methods

2.1. Cell culture

Human breast cancer cell lines MDA-MB-175, SK-BR-3 and MDA-MB-361 were obtained from the American Type Culture Collection

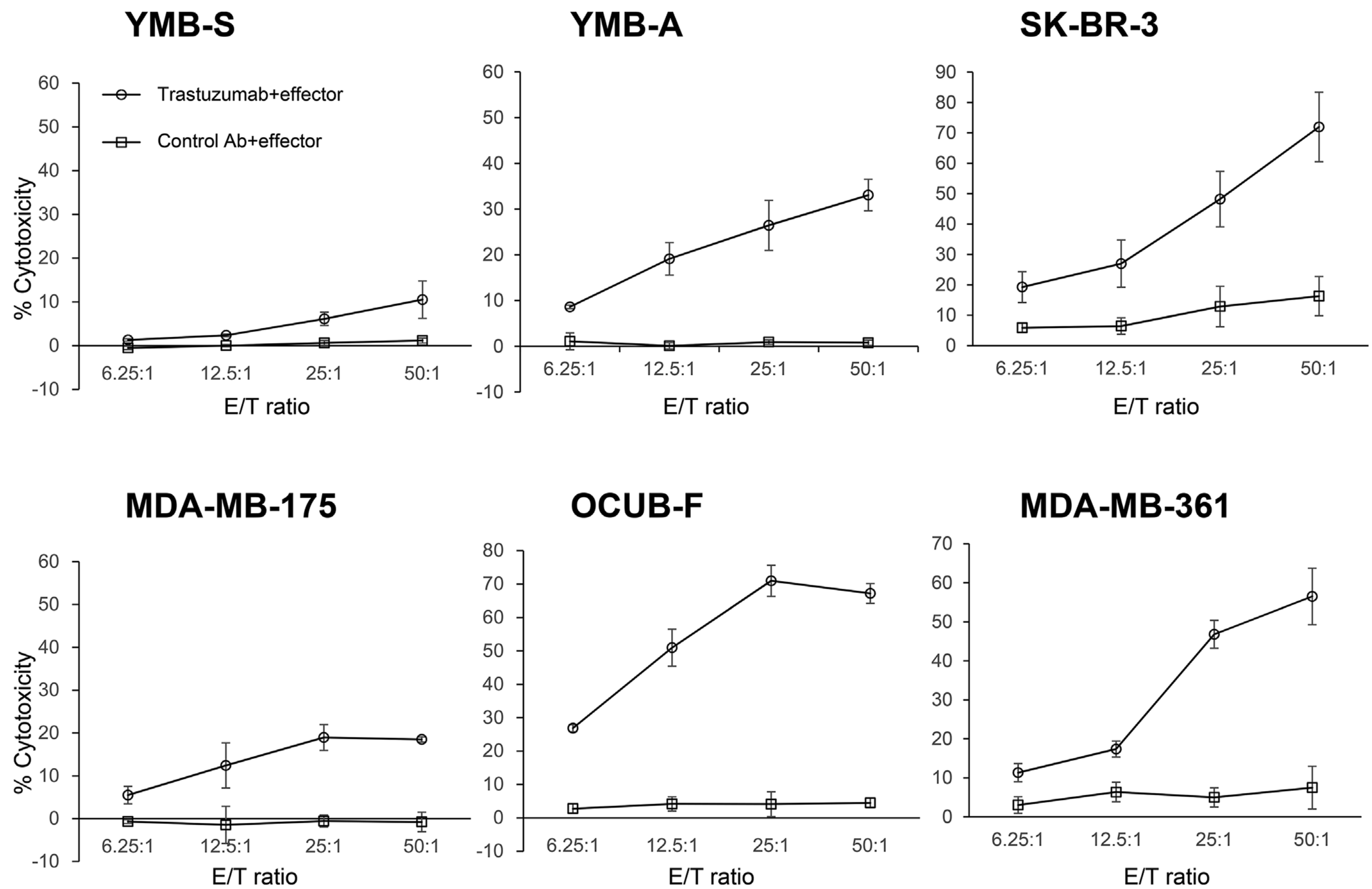

Fig. 2. YMB-S and MDA-MB-175 cells are resistant to trastuzumab-mediated antibody-dependent cell-mediated cytotoxicity (ADCC). ^{51}Cr-release ADCC assays were performed using six breast cancer cell lines as target cells and human peripheral blood mononuclear cells as effector cells. The target cells and effector cells were co-incubated with 40 μg/mL of trastuzumab (open circles) or control antibody (open squares) at various effector-to-target cell (E/T) ratios during a 4-h ^{51}Cr-release assay. The Y-axis indicates the percentage of cytotoxicity. All experiments were performed in triplicate wells. Values represent means ± standard deviations.

(Mannassas, VA, USA). Human breast cancer cell line OCUB-F was obtained from RIKEN Cell Bank (Ibaraki, Japan). Floating cells and adherent cells of human breast cancer cell line YMB-1 [17] were separately cloned as non-adherent YMB-S cells [18] and adherent YMB-A cells [19], respectively, and stored in our laboratory. These cell lines were cultured in RPMI-1640 (Thermo Fisher Scientific, Waltham, MA, USA) or DMEM (Thermo Fisher Scientific) with 10% fetal bovine serum (Thermo Fisher Scientific), penicillin G (100 IU/mL), streptomycin (100 μg/mL) and 10 mM HEPES (Sigma-Aldrich, St Louis, MO, USA) in an incubator with a 5% CO_2 atmosphere at 37 °C.

2.2. Antibodies

Anti-KL-6 mAb, a mouse IgG_1 mAb that recognizes a sialylated sugar chain of MUC1 [7], was purified from ascites of mice bearing KL-6 antibody-producing hybridoma using a protein A affinity column (Affi-Gel Protein A MAPS II Kit; Bio-Rad Laboratories, Hercules, CA, USA) in accordance with the manufacturer's protocol [20]. Anti-MUC1 mAb (HMFG1 (aka 1.10.F3); recognizing a peptide epitope (PDTR) within the tandem repeats region of the extracellular domain of MUC1) was obtained from Abcam (Cambridge, UK). Trastuzumab (Herceptin®), a humanized anti-Her2 receptor mAb, was obtained from Chugai Pharmaceutical Co (Tokyo, Japan). IgG from human serum (no. 14506; Sigma-Aldrich) and mouse IgG_1 (X093101-2; Agilent, Santa Clara, CA, USA) were used as control antibodies.

2.3. Western blot analysis

Total cell lysates were prepared using lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris-HCl) supplemented with 1% protease inhibitor cocktail (P8340; Sigma-Aldrich). The lysate was centrifuged at 12,000 rpm for 10 min at 4 °C. Protein concentration was determined using the supernatant and the BCA protein assay kit (Thermo Fisher Scientific). Protein extracts were loaded on 4–15% gradient SDS-PAGE gels (Mini-PROTEAN® TGX™ Precast Gels; Bio-Rad Laboratories) at 10 μg protein per lane. After electrophoresis, proteins were electrotransferred onto a polyvinylidene fluoride membrane (GE Healthcare UK Ltd, Little Chalfont, UK). The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline plus 0.5% Tween-20 for 1 h, and incubated overnight with anti-KL-6 mAb, anti-MUC1 mAb (HMFG1), rabbit polyclonal anti-HER2/ErbB2 antibody (no. 18299-1-AP; Proteintech group, Chicago, IL, USA), or rabbit β-actin antibody (#4967; Cell Signaling Technology, Beverly, MA, USA). The membrane was then washed and incubated with a secondary antibody conjugated with horseradish peroxidase (NA934; GE Healthcare UK Ltd). After further washing, the membrane was incubated with chemiluminescent substrate (SuperSignal West Dura; Thermo Fisher Scientific) and exposed using the WSE-6100 LuminoGraph I (ATTO, Tokyo, Japan). Band intensity was analyzed densitometrically using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

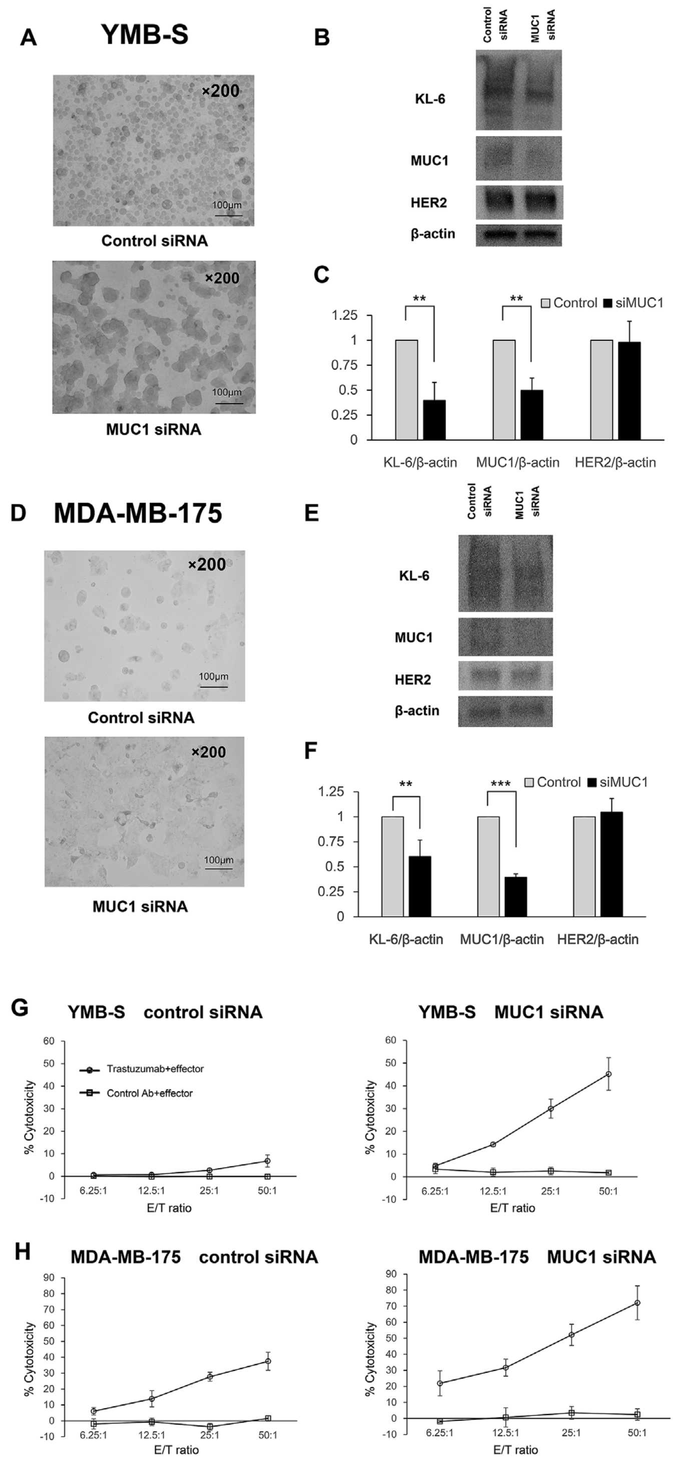

Fig. 3. Down-regulation of MUC1 by siRNA shifts breast cancer cells from resistant to susceptible to trastuzumab-mediated antibody-dependent cell-mediated cytotoxicity (ADCC). YMB-S and MDA-MB-175 cells were transfected with siRNA against MUC1 or with control siRNA for 4 h. Four days later, the experiments were performed. (A and D) Cells were visualized with a Keyence BZ-9000 microscope (magnification 200 ×). Bar, 100 µm. (B and E) Cell lysates were prepared and levels of KL-6, MUC1, HER2, and β-actin were evaluated via western blot analysis. (C and F) Levels of KL-6, MUC1, and HER2 divided by β-actin levels are shown as fold change compared to control siRNA. Bars represent the means ± standard deviations ($n = 4$). Statistical analysis was performed using a paired Student's *t*-test. $^{**}P < 0.01$; $^{***}P < 0.001$. (G and H) ^{51}Cr-release ADCC assays were performed using siRNA-transfected YMB-S and MDA-MB-175 cells as target cells, human PBMCs as effector cells, and 40 µg/mL of trastuzumab (open circles) or control antibody (open squares) at different effector-to-target cell (E/T) ratios (E/T ratio = 6.25:1, 12.5:1, 25:1, 50:1). The Y-axis indicates the percentage of cytotoxicity. All experiments were performed in triplicate wells. Values represent means ± standard deviations.

2.4. ADCC assay

Peripheral blood samples were obtained from healthy donors after obtaining informed consent in writing. Peripheral blood mononuclear cells (PBMCs) were separated from peripheral blood via centrifugation with Ficoll-Paque Plus (GE Healthcare UK Ltd). After target cells (T) were labeled with 200 µCi of ^{51}Cr (Perkin-Elmer Life and Analytical Sciences, Boston, MA, USA) for 60 min, they (1×10^4 cells per well) and PBMCs (E) (5×10^5 cells per well) were incubated at various effector-to-target (E/T) ratios in 200 µL of RPMI 1640 in a 96-well U-bottomed plate in triplicate. The mixture was incubated in the presence of 10 µg/mL of anti-KL-6 mAb, 40 µg/mL of trastuzumab, and/or control antibodies. After 4 h of incubation, radioactivity levels in the supernatant were measured using a gamma counter (ARC-370M; Hitachi Aloka Medical, Ltd, Tokyo, Japan). The percentage of cytotoxicity was calculated using the formula: percentage specific cytolysis = 100 × (experimental release – spontaneous release)/(maximum release – spontaneous release).

2.5. siRNA transfection

Transient MUC1-knockdown YMB-S and MDA-MB-175 cells were obtained via RNA interference. The synthetic, ready-to-use pooled siRNA against MUC1 (Hs MUC1 4 Flexitube siRNA) and non-specific control siRNA against GFP (GFP-22 siRNA) were obtained from Qiagen (Hilden, Germany). After YMB-S or MDA-MB-175 cells were seeded in 6-well plates (2×10^5 cells/well), cells were transfected with siRNA at a concentration of 200 pmol using 10 µL of Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific) in Opti-MEM (Thermo Fisher Scientific) for 5 h. Transfected cells were incubated for 4 days at 37 °C and visualized using a microscope (BZ-9000; Keyence, Osaka, Japan) at 200 × magnification and analyzed via western blotting.

2.6. Immunofluorescence staining

Anti-KL-6 mAb, anti-MUC1 mAb (HMFG1), and trastuzumab were conjugated with fluorescein isothiocyanate (FITC), using a fluorescein labeling kit (Dojindo Laboratories, Kumamoto, Japan) in accordance with the manufacturer's instructions. YMB-S, YMB-A, MDA-MB-175, and OCUB-F cells (all, 1×10^5 cells/mL) were incubated with 1 µg/mL of FITC-conjugated anti-KL-6 mAb for 10 min at 4 °C and fixed with 1% paraformaldehyde for 3 min and washed with PBS. Thereafter, the cells were visualized using a confocal laser-scanning microscope (FLUOVIEW FV1000; Olympus, Tokyo, Japan) with a 600 × magnification objective. The images were subsequently processed using Olympus Micro FV10-ASW software.

2.7. Capping formation

YMB-S and MDA-MB-175 cells (both, 5×10^5 cells/mL) were preincubated with 1 µg/mL of FITC-conjugated anti-KL-6 mAb (or anti-MUC1 mAb) for 10 min at 4 °C, and then incubated with 10 µg/mL of unconjugated anti-KL-6 mAb (or anti-MUC1 mAb) for 30 min at 4 °C or 37 °C. Thereafter, cells were fixed with 1% paraformaldehyde for 3 min and washed with PBS. Finally, cells were visualized using a FLUOVIEW FV1000 confocal laser scanning microscope at 600 × magnification. Capping formation was defined as a large aggregate of MUC1 glycoproteins at one pole of the cell, occupying less than 50% of the cell surface, as reported previously [21]. Quantification was achieved via

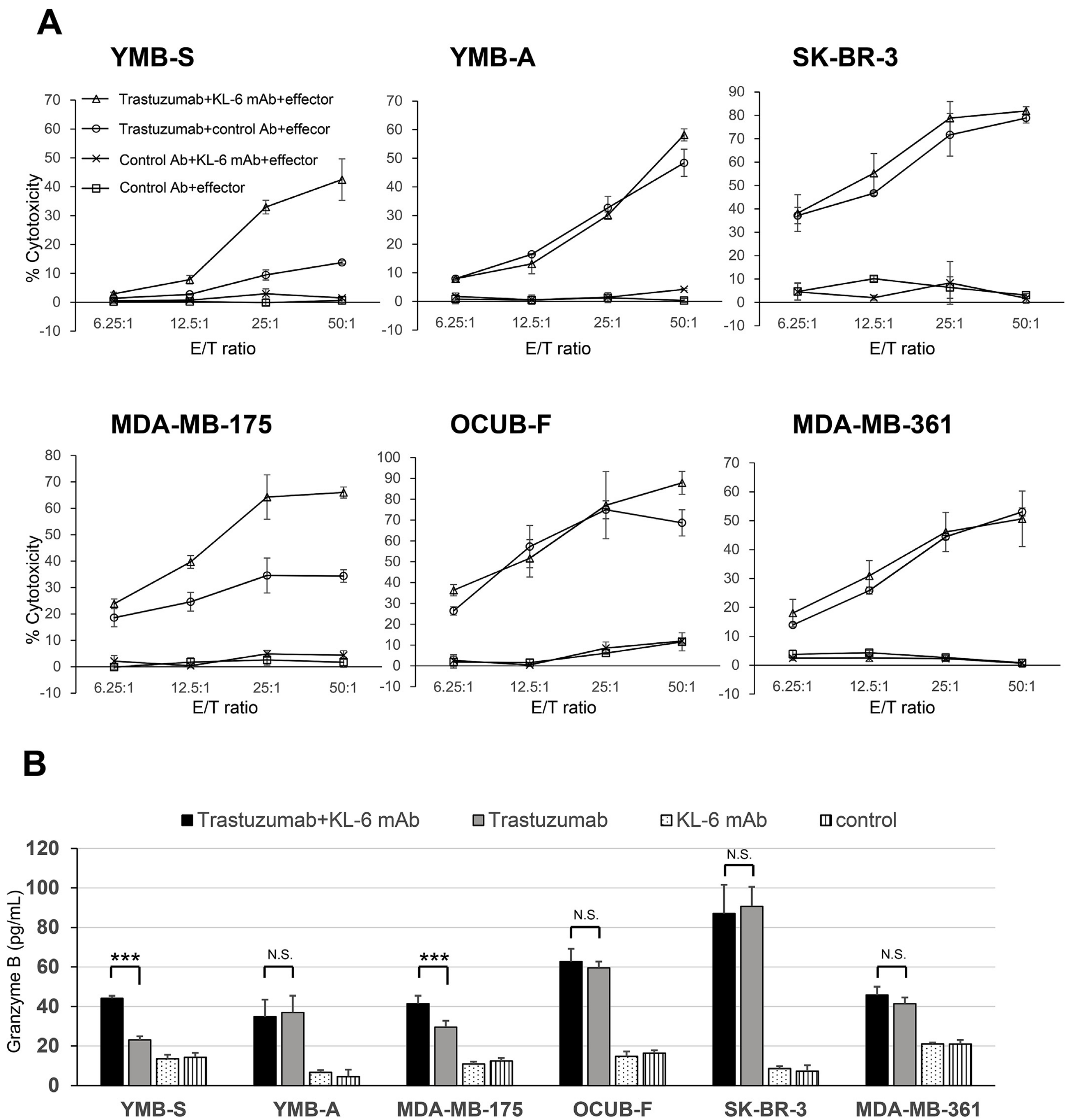

Fig. 4. Anti-KL-6 monoclonal antibody shifts breast cancer cells from resistant to susceptible to trastuzumab-mediated antibody-dependent cell-mediated cytotoxicity (ADCC). (A) ADCC assays were performed using six breast cancer cell lines as target cells and human PBMCs as effector cells. Target and effector cells were co-incubated with 40 μg/mL of trastuzumab or control antibody, and 10 μg/mL of anti-KL-6 mAb or isotype control antibody at various effector-to-target cell (E/T) ratios during a 4-h ^{51}Cr-release assay. The Y-axis indicates the percentage of cytotoxicity. All experiments were performed in triplicate wells. Values represent means ± standard deviations. (B) Concentration of granzyme B in the supernatant of culture medium during ADCC assay was measured using an ELISA kit. Values represent the means ± standard deviations (n = 4). Statistical analysis was performed using the Tukey-Kramer test. $^{***}P < 0.001$.

examination of the fluorescence distribution on at least 100 cells. The number of cells displaying capping formation is expressed as a percentage of the number of cells.

2.8. Fluorescence-activated cell sorting (FACS)

YMB-S and MDA-MB-175 cells (both, 5×10^5 cells/mL) were incubated with 100 μg/mL of FITC-conjugated trastuzumab for 10 min at 4 °C. Cells were washed, pelleted and resuspended in buffer (1 × PBS containing 0.5% BSA and 2 mM EDTA). FACS was performed using a BD FACS Verse (BD Biosciences, San Jose, CA, USA). Data were analyzed using FlowJo software version 7.6.5 (Tree Star, Ashland, OR, USA).

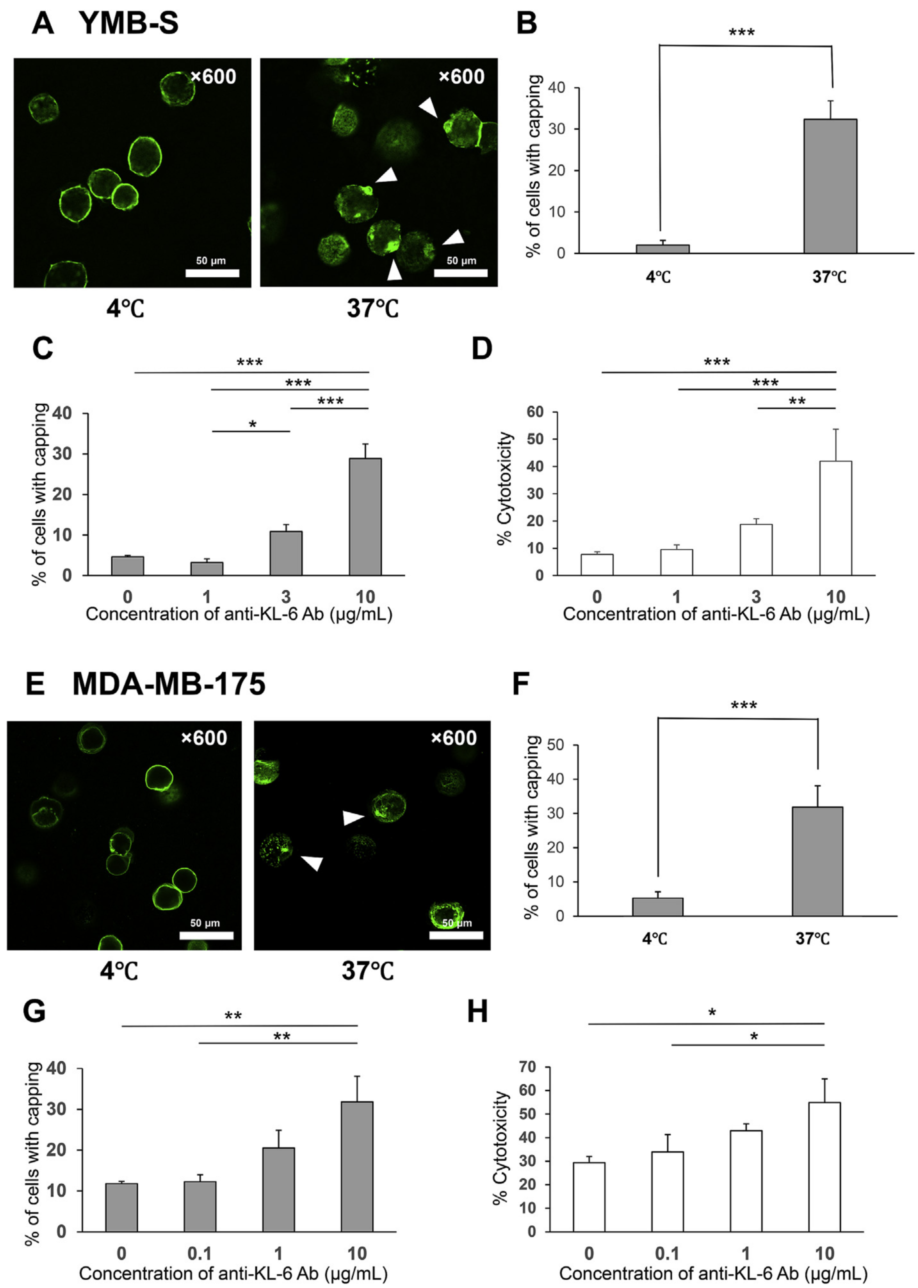

Fig. 5. Anti-KL-6 monoclonal antibody induces capping of MUC1 on the breast cancer cell surface. (A, B, E and F) YMB-S cells (A and B) and MDA-MB-175 cells (E and F) were preincubated with 1 μg/mL of fluorescein isothiocyanate (FITC)-conjugated anti-KL-6 mAb for 10 min at 4 °C, and then incubated with 10 μg/mL of unconjugated anti-KL-6 mAb for 30 min at 4 °C or 37 °C. After incubation, cells were fixed and visualized using a confocal laser scanning microscope (green). A white arrowhead indicates capping of MUC1 on the cancer cell membrane. The percentages of cells with capping were obtained by enumerating YMB-S (B) and MDA-MB-175 cells (F) with capping of MUC1 on at least 100 cells. Values represent the mean ± standard deviation ($n = 3$). Statistical analysis was performed using a paired Student's *t*-test. *P < 0.05; ***P < 0.001. Bar, 50 μm. (C and G) YMB-S cells (C) and MDA-MB-175 cells (G) were preincubated with 1 μg/mL of FITC-conjugated anti-KL-6 mAb for 10 min at 4 °C, and then incubated with 0, 1, 3, or 10 μg/mL of unconjugated anti-KL-6 mAb for 30 min at 37 °C. After incubation, cells were fixed and analyzed via microscopy. Values represent the mean ± standard deviation ($n = 3$). Statistical analysis was performed using a paired Student's *t*-test. *P < 0.05. (D and H) ^{51}Cr-release ADCC assay with trastuzumab and YMB-S cells (D) or MDA-MB-175 cells (H). Target cells and peripheral blood mononuclear cells were co-incubated with 40 μg/mL of trastuzumab and 0, 1, 3, or 10 μg/mL of anti-KL-6 mAb at an effector-to-target (E/T) ratio of 50:1. The Y-axis indicates the percentage of specific cytolysis. Experiments were performed in triplicate wells. Statistical analysis was performed using a paired Student's *t*-test. *P < 0.05; **P < 0.001; ***P < 0.001.

2.9. Live imaging of ADCC

Live imaging of ADCC was performed using a computer-assisted fluorescent microscopy system (LCV110; Olympus). Target cells were preincubated with 1 μg/mL of FITC-conjugated anti-KL-6 mAb for 10 min at 4 °C. Thereafter, target cells and PBMCs were incubated at an E/T ratio of 50:1 with 30 μg/mL of anti-KL-6 mAb and 40 μg/mL of trastuzumab in a glass base dish (AGC techno glass, Shizuoka, Japan) at 37 °C. The dish was imaged at several predetermined locations. Cell death was detected via a drastic cellular morphological change. Images were acquired at 400 × magnification every 10 min for 4 h.

2.10. Enzyme-linked immunosorbent assay (ELISA)

Target cells and PBMCs were incubated at an E/T ratio of 50:1 with 30 μg/mL of anti-KL-6 mAb or isotype control antibody, and 40 μg/mL of trastuzumab or control antibody in 200 μL of RPMI 1640 in a 96-well U-bottomed plate in quadruplicate. After 4 h of incubation, the concentration of granzyme B in the supernatant was determined using a Granzyme B Human ELISA kit (Thermo Fisher Scientific).

2.11. Statistical analysis

All experiments were performed at least in triplicate. Data are expressed as the mean ± standard deviation. Statistical analysis was carried out using JMP pro® 13 software (SAS Institute Inc, Cary, NC, USA). Statistical comparisons were performed using the Tukey-Kramer test for multiple comparisons and paired Student's *t*-test for two comparisons. A p-value < 0.05 was considered to indicate statistically significant data.

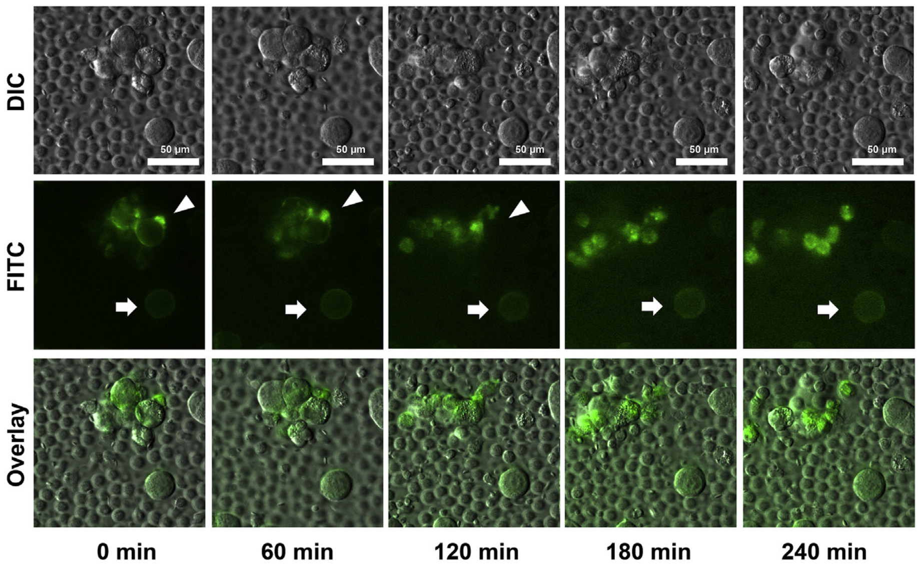

Fig. 6. Capping of MUC1 induces cell death of breast cancer cells during trastuzumab-mediated antibody-dependent cell-mediated cytotoxicity. YMB-S cells were preincubated with 1 µg/mL of fluorescein isothiocyanate (FITC)-conjugated anti-KL-6 mAb at 4 °C. Then, target cells and peripheral blood mononuclear cells (PBMCs) were incubated at an effector-to-target (E/T) ratio of 50:1 with 30 µg/mL of anti-KL-6 mAb and 40 µg/mL of trastuzumab for 240 min at 37 °C. Live imaging showed that only target cells with/without capping were stained with FITC (green); however, PBMCs were not. PBMCs, whose diameter is 10 µm or less, were smaller than target cells. A white arrowhead indicates capping of MUC1 on a target cell membrane. A white arrow indicates a target cell without capping of MUC1. DIC, differential interference contrast. Bar, 50 µm.

3. Results

3.1. *Breast cancer cells with MUC1 overexpression and uniform surface distribution are resistant to trastuzumab-mediated ADCC*

To investigate the association between the expression level of MUC1 and trastuzumab-mediated ADCC, we first evaluated the expression levels of KL-6 and MUC1 in six breast cancer cell lines (YMB-S, YMB-A, MDA-MB-175, OCUB-F, SK-BR-3, and MDA-MB-361). As shown in Fig. 1A and B, KL-6 and MUC1 were abundantly expressed in YMB-S, YMB-A, MDA-MB-175, and OCUB-F cells; however, they were scarcely expressed in SK-BR-3 and MDA-MB-361 cells. HER2 was sufficiently expressed in each cell line, albeit at a significantly higher level in OCUB-F and SK-BR-3 cells than in other cell lines.

Second, we observed the distribution of MUC1 on the YMB-S, YMB-A, MDA-MB-175, and OCUB-F cells, individually, each of which abundantly expressed MUC1. MUC1 was evenly distributed on the entire cell surface of YMB-S and MDA-MB-175 cells; however, patchy distribution and polarization were observed on the cell surface of YMB-A and OCUB-F cells (Fig. 1C).

We then analyzed the activity of trastuzumab-mediated ADCC in the six breast cancer cell lines. SK-BR-3 and MDA-MB-361 cells with low MUC1 expression were very sensitive to trastuzumab-mediated ADCC. YMB-A and OCUB-F cells, both with abundant MUC1 expression and patchy membrane distribution, were also susceptible to trastuzumab-mediated ADCC. Interestingly, YMB-S and MDA-MB-175 cells, both with abundant MUC1 expression and even membrane distribution, appeared resistant to trastuzumab-mediated ADCC (Fig. 2).

3.2. *MUC1 down-regulation makes trastuzumab-mediated ADCC-resistant cancer cells susceptible*

Initially, we focused on the association between the amount of MUC1 and resistance to trastuzumab-mediated ADCC. To determine whether reduction of MUC1 expression in cells resistant to trastuzumab-mediated ADCC affected its activity, YMB-S and MDA-MB-175 cells were transfected with siRNA targeting MUC1. As shown in Fig. 3, transfection with siRNA successfully down-regulated KL-6 and MUC1 in both cell lines; however, it did not affect HER2 protein levels (Fig. 3B, C, E and F). Interestingly, knockdown of MUC1 converted non-adherent YMB-S cells to adherent cells and increased adhesiveness of inherently adherent MDA-MB-175 cells, whereas transfection with control siRNA did not cause any morphological alteration (Fig. 3A and D). In addition, knockdown of MUC1 caused YMB-S and MDA-MB-175 cells to shift from being resistant to being susceptible to trastuzumab-mediated ADCC (Fig. 3G and H). We also confirmed the absence of a significant difference in the degree of trastuzumab binding between cells transfected with siRNA against MUC1 and control siRNA upon flow cytometry (Supplementary Fig. S1).

3.3. *Anti-KL-6 mAb makes trastuzumab-mediated ADCC-resistant cancer cells susceptible*

Based on our previous observation that addition of anti-KL-6 mAb enhanced the susceptibility of breast cancer cells to LAK [6], we attempted to determine whether reaction with anti-KL-6 mAb affected activity of trastuzumab-mediated ADCC in six breast cancer cell lines. As shown in Fig. 4 and Supplementary Fig. S2, reaction with anti-KL-6 mAb converted YMB-S and MDA-MB-175 cells from being resistant to being sensitive to trastuzumab-mediated ADCC in a dose-dependent manner. In contrast, anti-KL-6 mAb did not affect trastuzumab-mediated ADCC in YMB-A, OCUB-F, SK-BR-3, and MDA-MB-361 cells, each of which was originally susceptible to it. We also confirmed that, in the absence of trastuzumab, ADCC was not induced in any of the breast cancer cell lines.

To investigate whether the perforin/granzyme pathway is involved in trastuzumab-mediated ADCC, granzyme B in culture supernatants was measured via ELISA. Granzyme B released during trastuzumab-mediated ADCC significantly increased upon addition of anti-KL-6 mAb in YMB-S and MDA-MB-175 cells. In contrast, in YMB-A, OCUB-F, SK-BR-3, and MDA-MB-175 cells, granzyme B sufficiently released and did not increase upon addition of anti-KL-6 mAb (Fig. 4B). Furthermore, to investigate whether perforin/granzyme B pathway was crucial for trastuzumab-mediated ADCC, we used concanamycin A, which blocks perforin-dependent cell-mediated cytotoxicity without affecting the Fas-based cytolytic pathway [22]. Trastuzumab-mediated ADCC was completely inhibited by concanamycin A (Supplementary Fig. S3).

3.4. *Anti-KL-6 mAb induces "capping" of MUC1 molecules on the surface of breast cancer cells resistant to trastuzumab-mediated ADCC*

We then focused on the association between the change in the cell-surface distribution of MUC1 and resistance to trastuzumab-mediated ADCC. We attempted to determine whether antibodies against MUC1 affected the distribution of MUC1 on the surface of YMB-S and MDA-MB-175 cells (Fig. 5). When these cells reacted with anti-KL-6 mAb at 4 °C, MUC1 remained distributed on the entire cell surface (Fig. 5A and E); however, at 37 °C, anti-KL-6 mAb caused MUC1 to localize at one pole of the cell surface, a phenomenon called "capping," in numerous cells (Fig. 5A, B, E and F). Anti-KL-6 mAb increased the number of cells with capping of MUC1 and increased trastuzumab-mediated ADCC in a dose-dependent manner (Fig. 5C, D, G and H). When YMB-S cells reacted with another anti-MUC1 mAb, HMFG1, at 37 °C, capping of MUC1 on the cell surface could be induced only in a few YMB-S cells (Supplementary Figs. S4A and B). As shown in Supplementary Fig. S4C, HMFG1 could convert YMB-S cells from being resistant to being susceptible to trastuzumab-mediated ADCC, but not as efficiently as anti-KL-6 mAb (Fig. 4). However, HMFG1 did not induce capping of MUC1 and affect trastuzumab-mediated ADCC in MDA-MB-175 cells (data not shown).

To visualize the association between MUC1 cell-surface distribution and trastuzumab-mediated ADCC, live imaging of ADCC was performed. Time-lapse imaging revealed cell death in the form of drastic cellular morphological changes only in cells with MUC1 capping, whereas, cells without MUC1 capping did not die (Fig. 6 and Video). Finally, we also confirmed the absence of a significant difference in trastuzumab binding between cells treated with anti-KL-6 mAb and control mAb via flow cytometry analysis (Supplementary Fig. S5).

4. Discussion

In the present study, we report that cells with abundant and even MUC1 distribution on the entire cell surface were resistant to trastuzumab-mediated ADCC, and knockdown of MUC1 in these cells overcame such resistance. Treatment with anti-KL-6/MUC1 mAb induced the capping of MUC1 molecules on the cell surface, converting these cells from resistant to susceptible to trastuzumab-mediated ADCC. We also visually confirmed that cell death via trastuzumab-mediated ADCC was only induced in cells with MUC1 capping.

We clearly demonstrated that in YMB-S and MDA-MB-175 cells, MUC1 overexpression is associated with resistance to trastuzumab-mediated ADCC and knockdown of MUC1 reverses this resistance. This result confirms the important role played by MUC1 in acquiring resistance to immune cell-mediated cytotoxicity, similar to trastuzumab-mediated ADCC. Moreover, previous studies already reported that forced expression of MUC1 in MUC1-null cancer cells made these cells less sensitive to cytotoxic T lymphocyte-mediated killing [23]. Moreover, the level of MUC1 is inversely correlated with the sensitivity to ADCC- and cytotoxic T lymphocyte-mediated cell death [8]. However, these studies failed to note that down-regulation of MUC1 expression enhanced recognition of cancer cells by immune effector cells. This study revealed that knockdown of MUC1 by siRNA reversed the immune evasion of cancer cells in trastuzumab-mediated ADCC.

An interesting finding of the present study is that YMB-A and OCUB-F cells, both abundantly expressing MUC1, were susceptible to trastuzumab-mediated ADCC, similar to SK-BR-3 and MDA-MB-361 cells, both of which scarcely expressed MUC1. To elucidate the mechanism underlying this phenomenon, we performed two experiments. In the first experiment, MUC1 distribution on the cell surface was observed in MUC1-overexpressing breast cancer cells. MUC1 was evenly distributed on the entire cell surface of YMB-S and MDA-MB-175 cells, which were resistant to trastuzumab-mediated ADCC; however, patchy distribution and polarization of the cell surface of YMB-A and OCUB-F cells was observed among those sensitive to trastuzumab-mediated ADCC. In the second experiment, we observed that anti-KL-6/MUC1 mAb induced capping and localization of MUC1 molecules on the cell surface. Following reaction with anti-KL-6 mAb or HMFG1, evenly distributed MUC1 was shown to be capped on the cell surface in a proportion of cells. The distribution of capped MUC1 on the cell surface of YMB-S and MDA-MB-175 cells resembled that of MUC1 on the cell surface of YMB-A and OCUB-F. These results potentially imply that the cells with originally patchy MUC1 distribution and localization were susceptible to trastuzumab-mediated ADCC, and capping MUC1 on the cell surface by anti-KL-6/MUC1 mAb made trastuzumab-mediated ADCC-resistant cancer cells susceptible. In addition, we visually confirmed that cells with MUC1 capping died during trastuzumab-mediated ADCC, whereas those with MUC1 evenly distributed on the cell surface did not. These results strongly suggest that MUC1 distribution on the cell surface, rather than its expression level, is an important factor in determining sensitivity to trastuzumab-mediated ADCC.

We further showed that MUC1 inhibited the recognition of cancer cells by immune effector cells in trastuzumab-mediated ADCC. Anti-KL-6 mAb significantly increased granzyme B released from effector cells during trastuzumab-mediated ADCC only in YMB-S and MDA-MB-175 cells with even cell surface distribution of MUC1. In addition, we demonstrated that trastuzumab-mediated ADCC were completely suppressed by inhibiting perforin/granzyme pathway. These results suggest that perforin/granzyme is a crucial pathway in trastuzumab-mediated ADCC as reported previously [24] and anti-KL-6 mAb inducing capping of MUC1 promotes the recognition of cancer cells by immune effector cells.

In conclusion, breast cancer cells whose MUC1 was abundantly expressed and distributed over the entire cell surface were resistant to trastuzumab-mediated ADCC. Both siRNA-mediated knockdown of MUC1 and the capping of MUC1 on the cell surface by anti-KL-6/MUC1 mAb converted these cells from the resistant state to susceptible to trastuzumab-mediated ADCC. The present results thus indicate that not only the amount but also the distribution of MUC1 on the cell surface affect recognition of cancer cells by immune effector cells.

Conflicts of interest

No potential conflicts of interest were disclosed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2018.10.037.

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