In conclusion, we provide evidence, for the first time, that an oncolytic adenovirus induces autophagic cell death in human cancer cells through induction of *miR-7* upregulation via enhancement of E2F1 expression and through suppression of oncogenic EGFR expression. An understanding of oncolytic adenovirus-mediated modulation of the cellular miRNA network would provide novel insights into the antitumor mechanism of oncolytic virotherapy.

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ORIGINAL ARTICLE

Mechanism of resistance to trastuzumab and molecular sensitization via ADCC activation by exogenous expression of HER2-extracellular domain in human cancer cells

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Abstract Trastuzumab, a humanized antibody targeting HER2, exhibits remarkable therapeutic efficacy against HER2-positive breast and gastric cancers; however, acquired resistance presents a formidable obstacle to long-term tumor responses in the majority of patients. Here, we show the mechanism of resistance to trastuzumab in HER2-positive human cancer cells and explore the molecular sensitization by exogenous expression of HER2-extracellular domain (ECD) in HER2-negative or trastuzumab-resistant human cancer cells. We found that long-term exposure to trastuzumab induced resistance in HER2-positive cancer cells; HER2 expression was downregulated, and antibody-dependent cellular cytotoxicity (ADCC) activity was impaired. We next examined the hypothesis that trastuzumab-resistant cells could be re-sensitized by the transfer of non-functional HER2-ECD. Exogenous HER2-ECD expression induced by the stable transfection of a plasmid vector or infection with a replication-deficient adenovirus vector had no apparent effect on the signaling pathway, but strongly enhanced ADCC activity in low HER2-expressing or trastuzumab-resistant human cancer cells. Our data indicate that restoration of HER2-ECD expression sensitizes HER2-negative or HER2-downregulated human cancer cells to trastuzumab-mediated ADCC, an outcome that has important implications for the treatment of human cancers.

Keywords HER2 · Extracellular domain · Trastuzumab · ADCC · Adenovirus

Abbreviations

HER2	human epidermal growth factor receptor 2
ADCC	antibody-dependent cellular cytotoxicity
NK	natural killer
ECD	extracellular domain
MOI	multiplicity of infection
IGF-1R	insulin-like growth factor-1 receptor
Ad5	adenovirus type 5
tzb	trastuzumab

Introduction

Human epidermal growth factor receptor 2 (HER2) is a member of a receptor family associated with tumor cell proliferation, adhesion, migration, and differentiation [1]. Trastuzumab, a humanized monoclonal antibody that targets HER2, inhibits the HER2-mediated signaling pathway [2, 3] and also induces antibody-dependent cellular cytotoxicity (ADCC) [4–7]. The randomized clinical trial that led to the approval of trastuzumab for clinical use was conducted in combination with standard cytotoxic chemotherapy [8]. Subsequent trials have confirmed the utility of

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trastuzumab in HER2-overexpressing breast cancer in various clinical scenarios [9]. Recently, the efficacy and safety of adding trastuzumab to chemotherapy in HER2-positive advanced gastric cancer was evaluated, and the combination therapy was found to be significantly superior to chemotherapy alone [10]. However, HER2 is overexpressed only in approximately 20 % of primary breast and gastric cancers [8, 11–13]. Moreover, even if the HER2 status is positive, the majority of patients that initially respond to trastuzumab eventually develop resistance [9, 14, 15]. Thus, more effective treatments against HER2-overexpressing cancer require a deeper understanding of the mechanisms of resistance to trastuzumab.

Several mechanisms for trastuzumab resistance have been proposed, including the truncation of the HER2 receptor into a constitutively activated form (p95HER2) [16, 17], increased cellular signaling through alternative receptor tyrosine kinases [18–20], and altered intracellular signaling involving the loss of PTEN [21–23], which increases Akt activity. However, the biochemical nature of the resistance mechanism is confusing and controversial. Although the activation of ADCC is an important antitumor mechanism of trastuzumab, few studies have examined the role of ADCC in trastuzumab resistance. ADCC relies on the binding of antigen–antibody complexes to Fcγ receptors expressed on immune cells, and it is mainly attributable to the activation of natural killer (NK) cells. In fact, ADCC and overall NK cell activity were found to correlate with responses to trastuzumab [24]. Tumor cells potentially avoid ADCC attack from therapeutic antibodies by various mechanisms, such as insufficient recruitment of effector cells into tumors and the reduction or elimination of antigen expression on tumor cells [25].

Several strategies have been proposed to re-sensitize resistant tumor cells to therapeutic antibodies. In particular, the modification of heterogeneous or decreased antigen expression in resistant tumor cells might overcome resistance by enhancing ADCC activity. HER2 contains an extracellular ligand-binding domain, a short hydrophobic transmembrane region, and a cytoplasmic tyrosine kinase domain, which is crucial for downstream signaling [26]. Therefore, we hypothesize that truncated HER2 without an intracellular domain could be used as a non-signaling target for ADCC.

In the present study, we analyzed the HER2 surface expression and ADCC susceptibility of HER2-positive human cancer cells following repeated exposure to trastuzumab. We found that the surviving cells had reduced HER2 expression and were consequently less susceptible to ADCC. Moreover, we explored the effect of exogenous overexpression of the extracellular domain (ECD) of HER2, which lacks an intracellular signaling fragment, in HER2-negative and trastuzumab-resistant human cancer cells.

Materials and methods

Cell lines and cell cultures

Three human mammary gland adenocarcinoma cell lines, SKBR3, BT474, and MCF7, were obtained from American Type Culture Collection. SKBR3 was cultured in McCoy's 5A medium. BT474 was cultured in Leibovitz's medium. MCF7 was cultured in DMEM supplemented with 2 mmol/ml L-glutamine. The human gastric adenocarcinoma cell lines MKN1 and MKN28 were obtained from Human Science Research Resources Bank and cultured in RPMI1640. Penicillin (100 units/ml), streptomycin (100 μg/ml), and 10 % fetal bovine serum were added to the medium for each cell line.

Construction of plasmids and establishment of stable cell lines

Complementary DNAs of human full-length HER2 (HER2-wt) and truncated HER2 containing extracellular and transmembrane regions (HER2-ECD) were kindly provided by Dr. Mien-Chie Hung (M. D. Anderson Cancer Center). HER2-wt and HER2-ECD cDNAs were subcloned into the multi-cloning sites of the pcDNA3 vector. MCF7 breast cancer cells were transfected with the vectors expressing HER2-wt or HER2-ECD. For selection of stably transfected cells, cells were maintained in medium containing 0.2 mg/ml geneticin (G418), and single colonies were isolated.

Recombinant adenovirus

Replication-deficient adenoviral vector expressing the extracellular and transmembrane domains of HER2 (Ad-HER2-ECD) was constructed. Briefly, the HER2-ECD expression cassette that contains the human cytomegalovirus promoter, HER2-ECD cDNA, and the SV40 early polyadenylation signal was inserted between the XbaI and ClaI sites of pXCJL.1. The HER2-ECD shuttle vector and the recombinant plasmid pJM17 were cotransfected into 293 cells (Ad5-transformed human embryonic kidney cell line). The culture supernatant of 293 cells showing the complete cytopathic effect was collected and used for subsequent infections. This virus was purified by ultracentrifugation in cesium chloride step gradients, and its titer was determined by a plaque-forming assay using 293 cells. Replication-deficient E1A-deleted adenovirus (dl312) was used as control adenovirus. The viruses were stored at −80 °C before use.

Establishment of trastuzumab-acquired auto-resistance in HER2-positive cancer cells

To establish the trastuzumab-resistant cells, human breast cancer cell lines SKBR3 and BT474 expressing HER2 were

exposed to increasing concentrations of the anti-HER2 monoclonal antibody trastuzumab (Chugai Pharmaceutical Co.) for more than 3 months. Briefly, HER2-positive cancer cells were initially exposed to 50 mg/ml trastuzumab for 1 month followed by 100 mg/ml trastuzumab for 2 months. Trastuzumab was administered twice a week. Trastuzumab-resistant cells established by continuous exposure to trastuzumab were maintained in medium with 100 mg/ml trastuzumab. The trastuzumab-resistant cancer cells were cultured in medium without trastuzumab for 5 days before each experiment.

Western blotting analysis

Primary antibodies against HER2-ECD (Thermo Scientific), β-actin (Sigma Chemical, Co.); PTEN (Santa Cruz), HER2-intracellular domain, IGF1-R, pAkt, pmTOR, pHER3, Akt, and mTOR (Cell Signaling Technology) and peroxidase-linked secondary antibodies (Amersham) were used. Proteins were electrophoretically transferred to Hybond-polyvinylidene difluoride transfer membranes (GE Healthcare Life Science) and incubated with primary antibody, followed by peroxidase-linked secondary antibody according to the manufacturer's protocol. The Amersham ECL chemiluminescence system (GE Healthcare Life Science) was used to detect the peroxidase activity of the bound antibody. In experiments with replication-deficient adenoviral vector, cells were infected with Ad-HER2-ECD or dl312 at a multiplicity of infection (MOI) of 20 for 36 h.

Flow cytometric analysis

In experiments to measure the affinity to trastuzumab, tumor cells were pretreated with 100 μg/ml of trastuzumab for 60 min at 37 °C. Tumor cells were fixed with 4 % paraformaldehyde in PBS for 10 min and then washed with PBS containing 0.5 or 1.5 % BSA. The cells were labeled with APC-conjugated rabbit monoclonal anti-HER2-ECD antibody (R&D Systems Inc.) or APC-conjugated Affini-Pure F(ab')2 fragment goat monoclonal anti-human IgG + IgM (H + L) antibody (Jackson ImmunoResearch Laboratories, Inc.) at room temperature for 45 min and analyzed by FACSAria instrument (BD Biosciences). The cell population was gated on forward scatter and side scatter. The intensity of staining was determined by the BD-FACS Software. In experiments with replication-deficient adenoviral vector, cells were infected with Ad-HER2-ECD at an MOI of 20 for 36 h.

Cell viability assay

Parental or trastuzumab-resistant human breast cancer cells were seeded on 96-well plates at a density of 1×10^3 cells/well for 24 h. Then, trastuzumab was added to every well at the indicated concentration for 5 days. Cell viability was determined 5 days after trastuzumab treatment by using the Cell Proliferation Kit II (Roche Molecular Biochemicals) with the sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) assay, according to the manufacturer's protocol.

Cell proliferation assays

Cells were trypsinized and re-plated in 24-well plates at a density of 1×10^4 cells/well. Parental MCF7 cells, mock vector-treated MCF7 cells, MCF7-HER2-wt cells, and MCF7-HER2-ECD cells were analyzed. Cells were incubated for 12 h to allow for attachment, after which the zero time point was determined. In experiments with adenoviral vector, trastuzumab-resistant SKBR3 or BT-47 cells with downregulated HER2 expression or low HER2-expressing breast and gastric cancer cells were infected with replication-deficient adenovirus (20 MOI) 1 day after the zero time point.

Antibody-dependent cellular cytotoxicity (ADCC) assay

Peripheral blood mononuclear cells (PBMCs) were separated from peripheral blood obtained from healthy donors by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. After centrifugation, PBMCs were washed three times with PBS and suspended with medium containing 10 % FBS. Target cells were labeled with 50 μCi (1.85 MBq) of Na51 Ci (Parkin Elmer, Waltham, MA, USA) for 60 min. Then, target cells (1×10^4/well) and effector cells at various effector/target ratios were co-incubated in 200 μl of X-VIVO medium in a 96-well U-bottomed plate for 4 h at 37 °C with trastuzumab (2 μg/well; Chugai Pharmaceutical co.) or control antibody, rituximab (2 μg/well; Chugai Pharmaceutical Co.). After 4 h of incubation, the radioactivity of the supernatant (100 μl) was measured with a γ-counter. The percentage of specific lysis = 100 × (experimental count per minute (cpm) − spontaneous cpm)/(maximum cpm − spontaneous cpm). In experiments with replication-deficient adenovirus, target cells were infected with Ad-HER2-ECD or dl312 at an MOI of 20 for 36 h (MCF7 and MDA-MB-231 cells) or 24 h (trastuzumab-resistant SKBR3 or BT474, MKN1, and MKN28 cells) before the ADCC assay was performed.

Statistical analysis

A comparison of continuous variables between two groups for in vitro assays was performed with the two-sided Student's t test. At least three independent experiments were

performed. The differences between groups were considered to be statistically significant when the p values were <0.05. Means and 95 % confidence intervals are reported, unless otherwise indicated. All data were analyzed with the statistical software SPSS 15.0 (SPSS, Inc, Chicago, IL, USA).

Results

Continuous exposure to trastuzumab downregulates cell-surface HER2 expression and impairs trastuzumab-mediated ADCC in HER2-positive human cancer cells

To elucidate the molecular mechanism of developing resistance to trastuzumab, we continuously treated HER2-positive SKBR3 and BT474 breast cancer cells with trastuzumab. Exposure to trastuzumab for 1 month reduced extracellular HER2 levels in both cell lines, and this downregulation was maintained for at least 2 months. HER2 downregulation did not recover following 5 days of incubation in the absence of trastuzumab (Fig. 1a). Intracellular HER2 expression was also reduced by long-term exposure to trastuzumab, although p95HER2 could not be detected (Supplemental Fig. 1). Flow cytometric analysis demonstrated that trastuzumab-exposed SKBR3 and BT474 cells showed decreased HER2 expression as compared with parental lines, leading to reduced affinity to trastuzumab (Fig. 1b).

SKBR3 and BT474 cells exposed to trastuzumab for 3 months were apparently more resistant to trastuzumab-mediated growth suppression in vitro (Fig. 1c). Western blotting analysis for assessment of the HER2-related signaling pathway demonstrated that phosphorylated Akt and mTOR expression were downregulated in these resistant cell lines. In contrast, insulin-like growth factor-1 receptor (IGF-1R) expression was notably enhanced following trastuzumab exposure in SKBR3 cells and constitutively high in BT474 cells without trastuzumab treatment (Fig. 1d). These results suggest that the development of resistance to trastuzumab at least partially depends on upregulation of an alternative signaling pathway downstream of other receptor tyrosine kinases such as IGF-1R. We further examined trastuzumab-mediated ADCC against parental and trastuzumab-resistant SKBR3 and BT474 cells by using PBMCs from healthy volunteer donors. Although apparent ADCC activity was observed in parental SKBR3 and BT474 cells, long-term exposure to trastuzumab significantly reduced this activity in both cell lines (Fig. 1e). Thus, impaired ADCC activity might be another possible mechanism contributing to acquired trastuzumab resistance.

Effects of exogenous HER2-ECD expression on in vitro growth and signaling pathways in human cancer cells

To investigate the effects of exogenous expression of HER2 protein on trastuzumab-mediated antitumor activity, we transfected human full-length HER2 (HER2-wt) and truncated HER2 c DNA containing extracellular and transmembrane regions (HER2-ECD) into low HER2-expressing MCF7 human breast cancer cells. HER2 overexpression contributes to breast cancer carcinogenesis, and studies have indicated that transfection of HER2-wt into mammary epithelial cells induces oncogenic transformation [27]. Indeed, stable HER2-wt-expressing MCF7 cells showed accelerated cell growth compared to parental MCF7 cells, whereas the growth pattern of HER2-ECD-transfected MCF7 cells was similar to that of parental MCF7 cells (Fig. 2a). Furthermore, transfection of HER2-wt, but not HER2-ECD, led to an increase in phosphorylated Akt and mTOR expression in the presence of HER3 ligand, HRG-β; these results suggest that exogenous HER2-ECD expression did not trigger the signaling pathways of HER2/HER3, which is the most potent combination of receptors in human breast cancer cells [28, 29] (Fig. 2b).

We next explored whether exogenous expression of HER2-wt or HER2-ECD was altered by long-term exposure to trastuzumab. The expression of 185-kDa full-length HER2 protein was slightly reduced in the presence of trastuzumab, whereas 3-month treatment with trastuzumab resulted in a slight increase in 100-kDa HER2-ECD expression (Fig. 2c). However, flow cytometric analysis demonstrated that neither cell-surface HER2 expression nor trastuzumab binding affinity changed following long-term trastuzumab exposure (Fig. 2d). Furthermore, the ADCC activity of trastuzumab against MCF7 cells transfected with HER2-wt or HER2-ECD was maintained even after a 3-month exposure to trastuzumab (Fig. 2e). These results indicate that exogenous overexpression of HER2-wt or HER2-ECD could overcome trastuzumab-mediated downregulation of endogenous HER2 expression.

Exogenous HER2-ECD expression enhances trastuzumab-mediated ADCC activity in low HER-2-expressing human cancer cells

We conducted a standard 4-h ^{51}Cr release assay with PBMCs from healthy volunteer donors to test the hypothesis that trastuzumab-mediated ADCC activity could be enhanced by exogenous overexpression of HER-ECD. With 5, 10, or 20 μg/ml of trastuzumab, ADCC activity was significantly increased in both HER2-ECD- and HER2-wt-expressing MCF7 cells as compared to MCF7 cells transfected with control pcDNA3 vector. A low concentration of trastuzumab (1 μg/ml) failed to enhance ADCC (Fig. 3a).

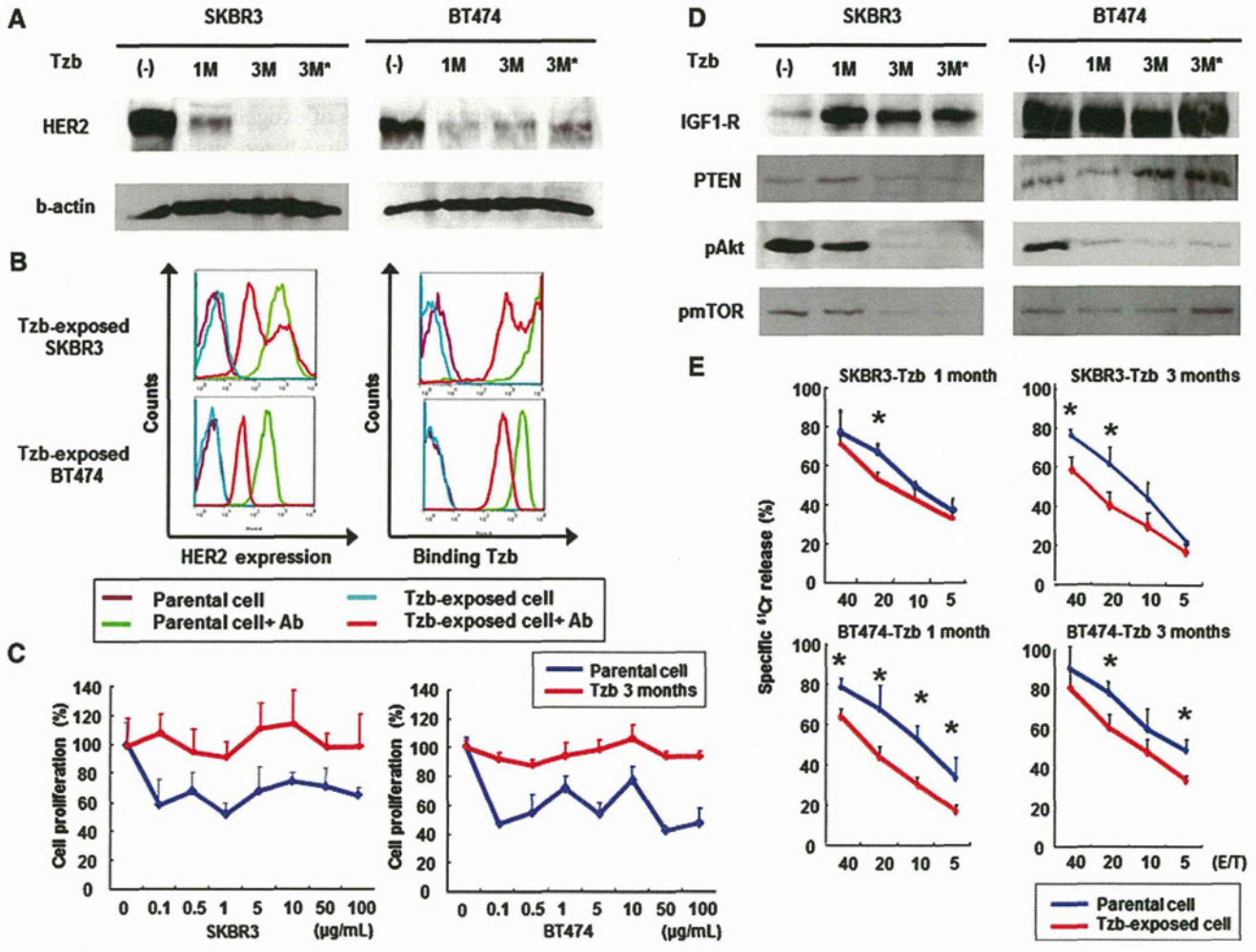

Fig. 1 Effects of continuous exposure to trastuzumab in HER2-overexpressing breast cancer cells. **a** Western blot analysis of HER2 expression. Human breast cancer SKBR3 and BT474 cells were initially incubated with 50 mg/ml trastuzumab (Tzb) for 1 month followed by 100 mg/ml trastuzumab for 2 months. *Cells were cultured in the absence of trastuzumab for 5 days before analysis. Equivalent amounts of protein from whole cell lysates were loaded into each lane. Blots were probed with anti-HER2-ECD antibody and visualized by using an ECL detection system. Equal loading of samples was confirmed by stripping each blot and reprobing with anti-β-actin antibody. **b** Flow cytometric analysis of HER2 expression and trastuzumab binding. Parental or trastuzumab-exposed cells were stained with APC-conjugated anti-HER2-ECD antibody to measure cell-surface HER2 expression or treated with trastuzumab followed by incubation with APC-conjugated anti-human antibody to measure the amount of bound trastuzumab. **c** Parental or trastuzumab-exposed cells were further treated with the indicated doses of trastuzumab for 5 days, and cell viability was assessed by XTT assay. **d** Western blot analysis for assessment of HER2-related signaling pathway. Blots were probed with anti-IGF1-R, anti-PTEN, anti-phosphorylated Akt, or anti-phosphorylated mTOR antibody. **e** ADCC activity of trastuzumab-exposed SKBR3 or BT474 cells. Parental or trastuzumab-exposed cells were incubated with PBMCs from healthy donors in the presence of 10 μg/ml of trastuzumab, and the cytotoxic activity was assessed by a 4-h standard ^{51}Cr-release assay. *Data* represent the mean ± SD of 3 wells at four different effector-to-target (E/T) ratios. *$p < 0.05$

Furthermore, effector cells showed significantly increased ADCC against HER2-ECD- or HER2-wt-expressing MCF7 cells as compared to pcDNA3-transfected cells at the effector/target cell ratios of 5:1, 10:1, 20:1, and 40:1 (Fig. 3b). These results indicate that exogenous overexpression of HER2-ECD may be an appropriate strategy to sensitize human cancer cells with low or reduced expression of HER2 to trastuzumab. Thus, we next examined the most efficient tool for gene transfer.

Efficient HER2-ECD overexpression in human cancer cell lines by a recombinant replication-deficient adenovirus vector

Modified adenovirus type 5 (Ad5) vectors have been used as a platform to deliver genes of interest into various types of human cells. We constructed a replication-deficient adenoviral vector containing a gene that encodes the extracellular domain of HER2 plus the transmembrane domain

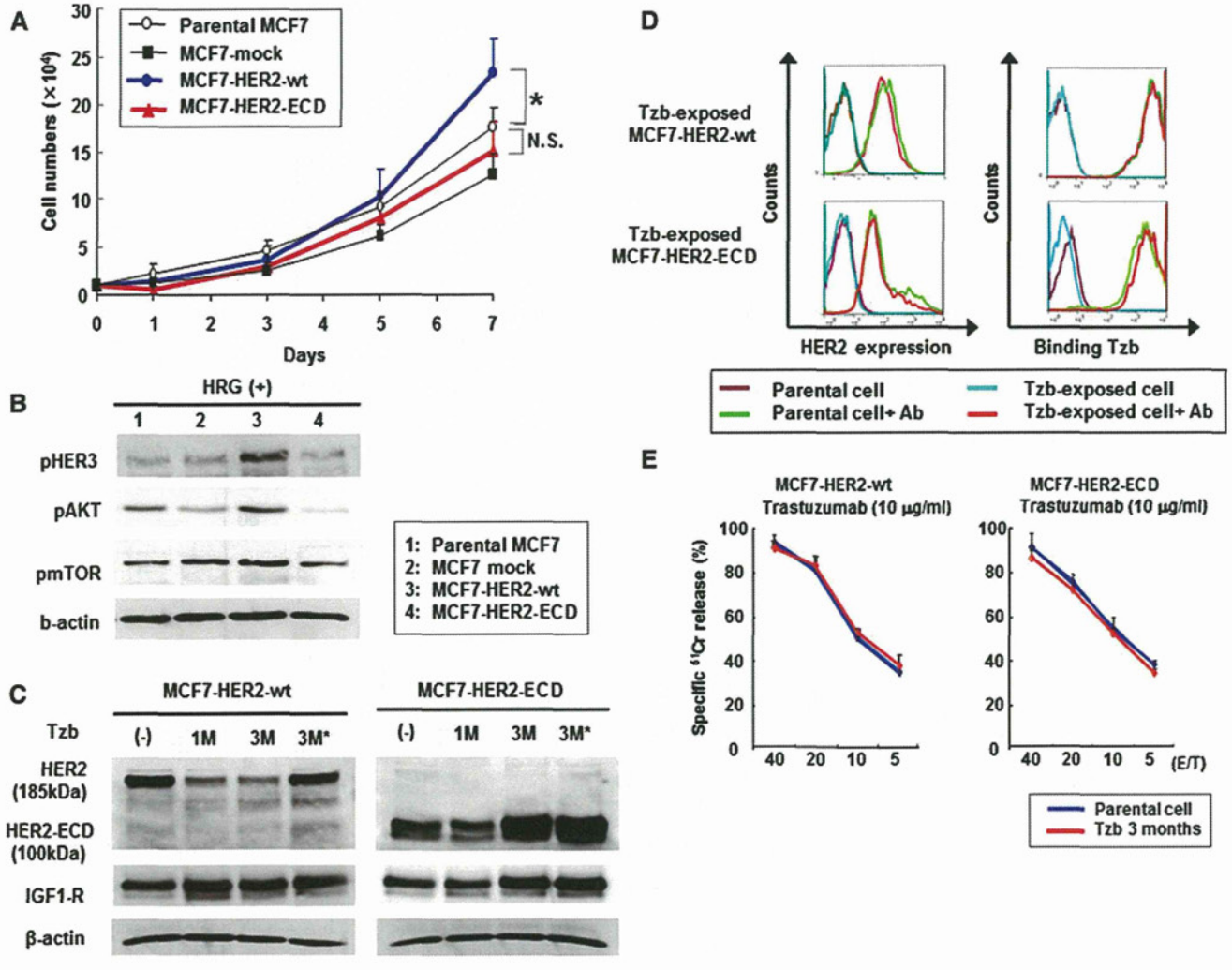

Fig. 2 Effects of exogenous HER2-ECD expression in low HER2-expressing MCF7 cells. **a** MCF7 human breast cancer cells were transfected with a vector expressing human full-length HER2 (HER2-wt) or truncated HER2 containing extracellular and transmembrane regions (HER2-ECD), or empty vector (mock). The cell growth was assessed for parental cells and stable clones. *$p < 0.05$. **b** Western blot analysis of phosphorylated HER3, Akt, and mTOR. Cells were stimulated with HER3 ligand, heregulin-β. **c** Western blot analysis of MCF7 cells expressing HER2-wt (185 kDa) or HER2-ECD (100 kDa) after continuous exposure to trastuzumab for 1 month or 3 months. Cells were prepared as described in the legend for Fig. 1a. **d** Flow cytometric analysis of HER2 expression and the amount of bound trastuzumab on parental or trastuzumab-exposed MCF7 cells expressing HER2-wt or HER2-ECD. Cells were stained and subjected to the analysis as described in the legend to Fig. 1b. **e** ADCC activity of parental or trastuzumab-exposed MCF7 cells expressing HER2-wt or HER2-ECD. The cytotoxic activity of PBMCs was assessed in the presence of 10 μg/ml of trastuzumab by a 4-h standard ^{51}Cr-release assay. *Data* represent the mean ± SD of 3 wells at four different E/T ratios

(Ad-HER2-ECD). To assess the efficient exogenous HER2-ECD overexpression by Ad-HER2-ECD infection, we used trastuzumab-resistant and low HER2-expressing human breast and gastric cancer cell lines. Various HER2 tests have demonstrated that the levels of HER2 expression in both MKN1 and MKN28 cells are low and that MKN7 cells overexpress HER2 antigen (Table 1). Indeed, the degree of HER2 expression correlated well with trastuzumab-mediated ADCC activity (Supplemental Fig. 2).

As expected, Ad-HER2-ECD infection at an MOI of 20 for 36 h resulted in a marked increase in the expression of 100-kDa HER2-ECD protein in trastuzumab-resistant breast cancer cells (SKBR3 and BT474), low HER2-expressing breast cancer cells (MCF7 and MDA-MB-231), and low HER2-expressing gastric cancer cells (MKN1 and MKN28) as compared to mock- or control dl312-infected cells (Fig. 4a). Western blot analysis also demonstrated that Ad-HER2-ECD had no apparent effects on the HER2 signaling pathway such as Akt and mTOR expression as well as phosphorylated mTOR expression, although Ad-HER2-ECD and control dl312 induced phosphorylated Akt. Flow cytometric analysis confirmed the cell-surface expression of HER2-ECD in Ad-HER2-ECD-infected cells, which in turn leads to increased trastuzumab binding (Fig. 4b).

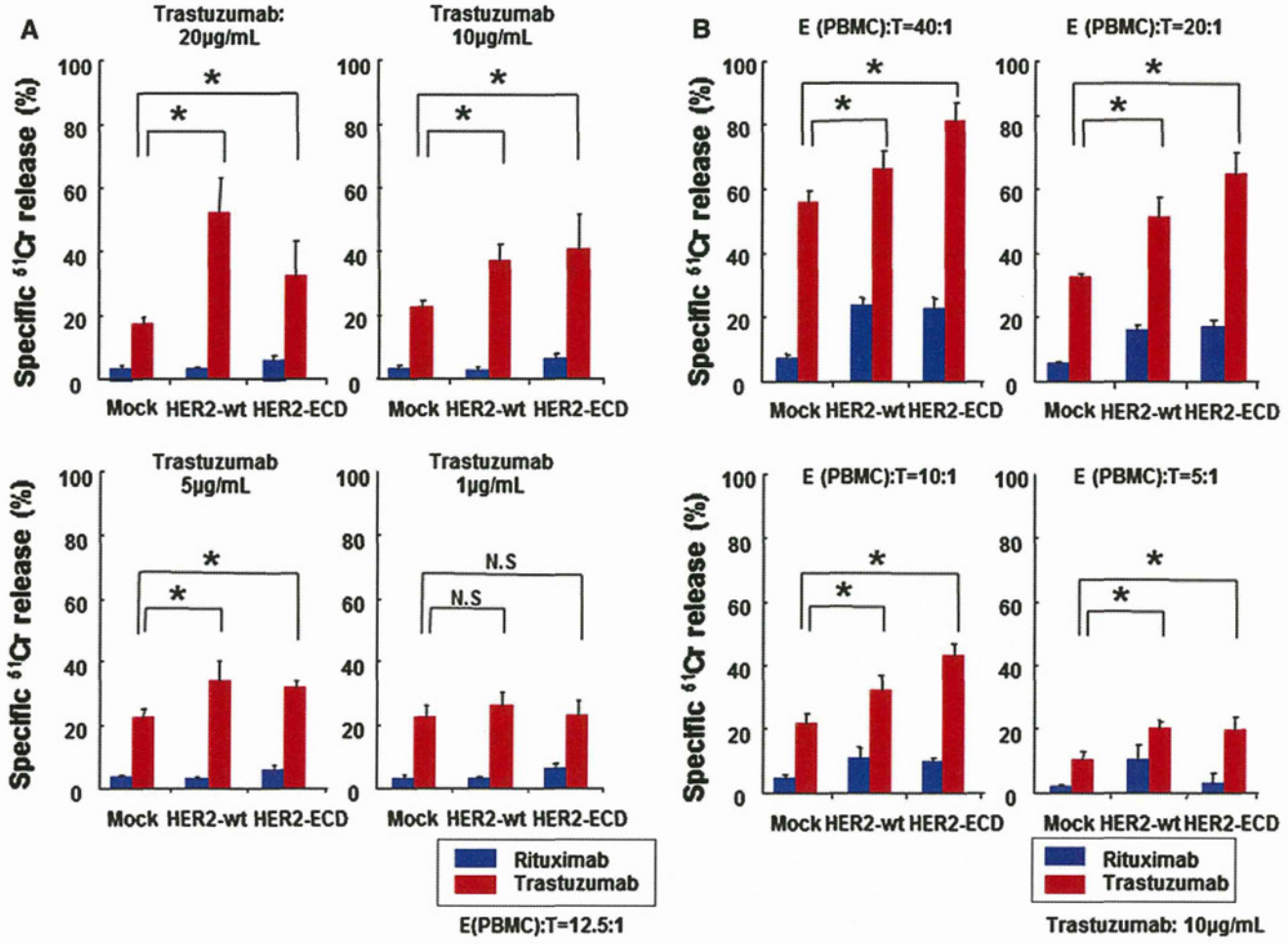

Fig. 3 Trastuzumab-mediated ADCC activity on HER2-ECD-expressing MCF7 cells. **a** The cytotoxic activity against MCF7 human breast cancer cells transfected with vector expressing HER2-wt or HER2-ECD or empty vector (mock) was assessed by a 4-h standard ^{51}Cr-release assay in the presence of the indicated doses of trastuzumab or control rituximab. Data represent the mean ± SD of 3 wells at an E/T ratio of 12.5:1. *$p < 0.05$. **b** A 4-h ^{51}Cr-release assay was also performed against MCF7 cells expressing HER2-wt or HER2-ECD, or mock-treated MCF7 cells in the presence of 10 μg/ml of trastuzumab or control rituximab. *Data* represent the mean ± SD of 3 wells at four different E/T ratios. *$p < 0.05$

Table 1 HER2 expression status of gastric cancer cell lines

Cell lines	HER2 status		
	FACS (MFI)	HercepTest	Western blotting
MKN1	13	–	Weak
MKN7	106	3+	Strong
MKN28	30	–	Negative
MKN45	23	1+	Weak
NUGC3	9	–	Negative
KATO-III	30	2+	Medium

HER2-expressing status of six different gastric cancer cell lines measured by flow cytometry, immunocytochemistry (HercepTest), and Western blotting analysis

MFI mean fluorescence intensity, *FACS* fluorescence-activated cell sorting

Direct antitumor effects of Ad-HER2-ECD on trastuzumab-resistant or low HER2-expressing human cancer cells

Next, we assessed the cell growth pattern of trastuzumab-resistant SKBR3 and BT474 human breast cancer cells and low HER2-expressing MKN1 and MKN28 human gastric cancer cells following Ad-HER2-ECD infection. MCF7 cells that were stably transfected with the HER2-ECD plasmid showed a growth pattern similar to that of parental or control vector-transfected MCF7 cells (Fig. 2a). However, adenovirus-mediated overexpression of HER2-ECD unexpectedly induced a significant suppression of in vitro growth in all cell lines as compared to uninfected cells or cells infected with control dl312 (Fig. 5). These results suggest that Ad-HER2-ECD had a slight but significant direct

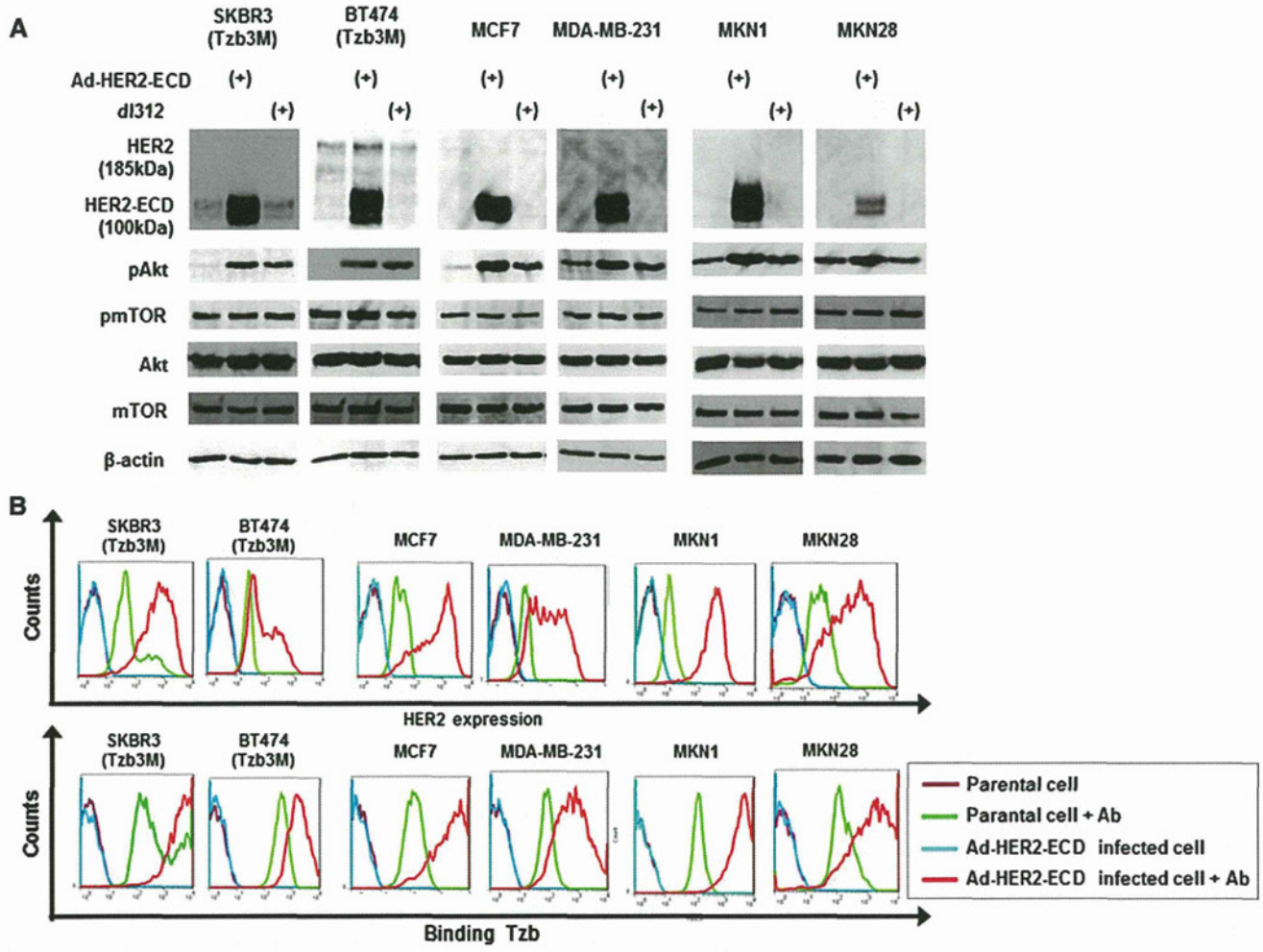

Fig. 4 Efficient HER2-ECD overexpression in human cancer cell lines by a recombinant replication-deficient adenovirus vector. **a** Western blot analysis of HER2-wt (185 kDa), HER2-ECD (100 kDa), and representative HER2-related signaling proteins in various types of human cancer cells. Trastuzumab-resistant breast cancer cells (SKBR3 and BT474), low HER2-expressing breast cancer cells (MCF7 and MDA-MB-231), and low HER2-expressing gastric cancer cells (MKN1 and MKN28) were infected with replication-deficient adenoviral vector expressing exogenous HER2-ECD (Ad-HER2-ECD) or replication-deficient control adenovirus (dl312) at an MOI of 20 for 36 h. **b** Flow cytometric analysis of HER2 expression and the amount of bound trastuzumab in cells 36 h after Ad-HER2-ECD infection at an MOI of 20

antitumor effect on trastuzumab-resistant and low HER2-expressing human cancer cell lines in vitro.

Adenovirus-mediated HER2-ECD overexpression sensitizes trastuzumab-resistant or low HER2-expressing human cancer cells to trastuzumab-mediated ADCC

Finally, we examined whether Ad-HER2-ECD infection could overcome acquired resistance to trastuzumab-mediated ADCC in SKBR3 and BT474 human breast cancer cells. Enhancement of ADCC activity by Ad-HER2-ECD infection was also assessed in low HER2-expressing human breast and gastric cancer cell lines. Following Ad-HER2-ECD infection, trastuzumab-resistant (Fig. 6a) as well as low HER2-expressing cells (Fig. 6b, c) were more efficiently killed by ADCC, and a significant difference was detected at all effector/target ratios in all cell lines, except trastuzumab-resistant SKBR3 cells, as compared to mock- or control dl312-infected cells. Thus, Ad-HER2-ECD-mediated exogenous expression of HER2-ECD could sensitize trastuzumab-resistant HER2-downregulated cells or low HER2-expressing cells to trastuzumab through ADCC activation in vitro.

Discussion

The nature of acquired resistance to trastuzumab is an area of active research in both the laboratory and the clinic. In the present study, we exposed HER2-positive breast cancer cells to trastuzumab continuously in vitro to induce resistance against this antibody and investigate the mechanisms

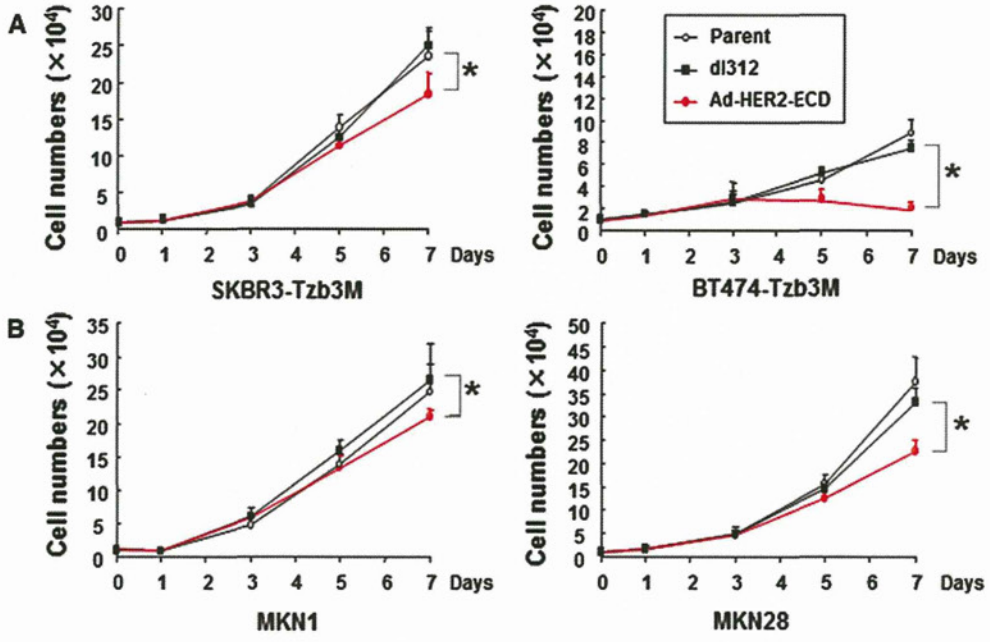

Fig. 5 Antitumor effects of Ad-HER2-ECD on trastuzumab-resistant or low HER2-expressing human cancer cells. Trastuzumab-resistant SKBR3 and BT474 breast cancer cells (**a**) and low HER2-expressing MKN1 and MKN28 gastric cancer cells (**b**) cultured as a monolayer were infected with Ad-HER2-ECD or control dl312 at an MOI of 20. The cell growth was determined by counting cell numbers each day after infection. The mean ± SD of three different wells is shown. $^{*}p < 0.05$

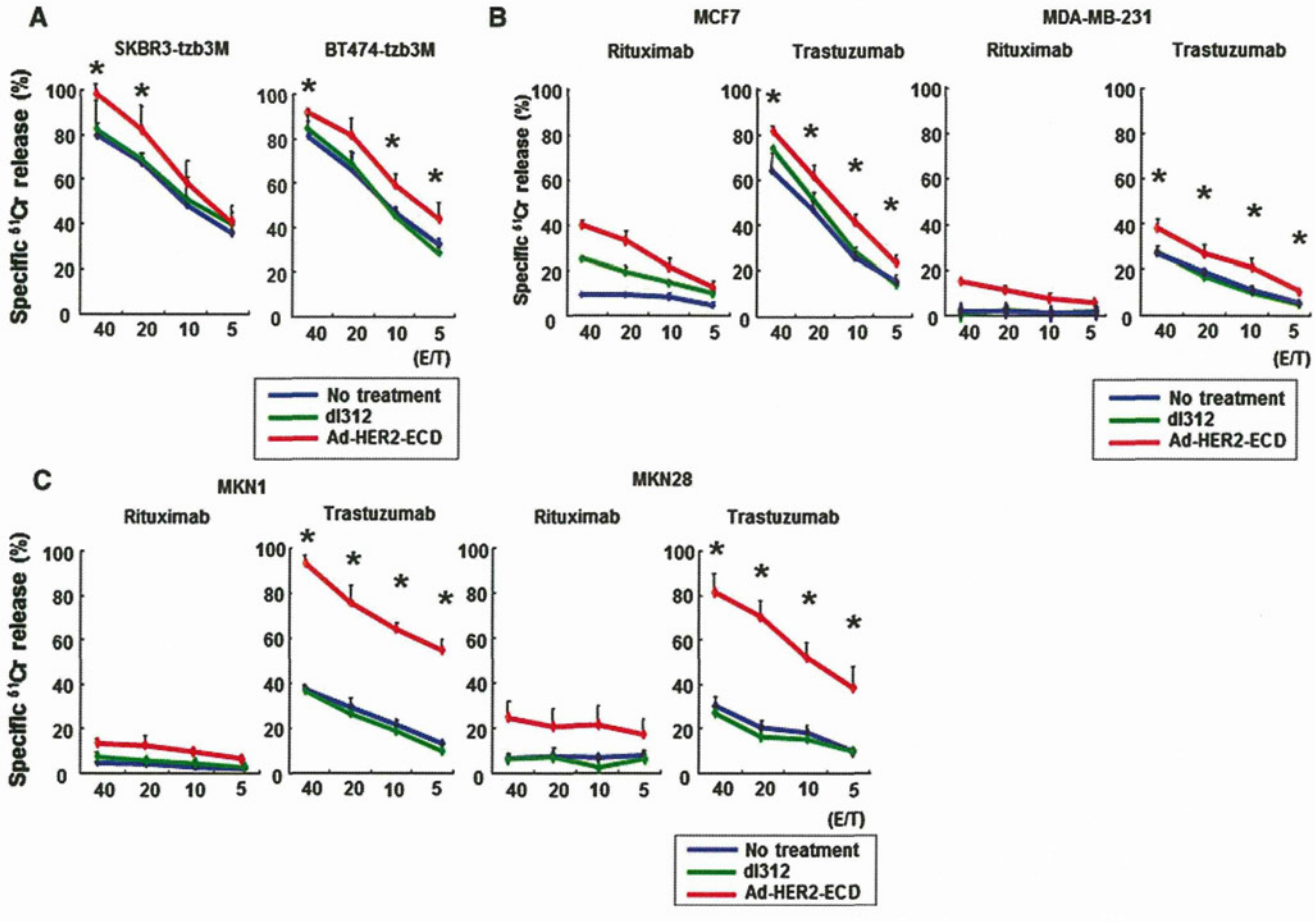

Fig. 6 Molecular sensitization of human cancer cells to trastuzumab by Ad-HER2-ECD-mediated exogenous expression of HER2-ECD. The cytotoxic reactivity of PBMCs against HER2-downregulated SKBR3 or BT474 cells (**a**), low HER2-expressing MCF7 or MDA-MB-231 human breast cancer cells (**b**), or low HER2-expressing MKN1 or MKN28 human gastric cancer cells (**c**) was assessed after Ad-HER2-ECD or dl312 infection in the presence of 10 μg/ml of trastuzumab or control rituximab by a 4-h ^{51}Cr-release assay. *Data* represent the mean ± SD of 3 wells at four different E/T ratios

responsible for this resistance. Some studies indicated that trastuzumab treatment does not alter the cell-surface HER2 expression status [30, 31]. However, we have demonstrated that continuous exposure to trastuzumab results in HER2 downregulation in HER2-overexpressing breast cancer cell lines in vitro. Previous studies also showed that alternative receptor tyrosine kinase signaling may play a role in trastuzumab resistance [18–20]. In fact, trastuzumab-exposed SKBR3 cells exhibited upregulated IGF-1R expression, suggesting that an alternative signaling pathway was enhanced to protect cells from trastuzumab-mediated HER2 signaling inhibition.

We also found that trastuzumab-exposed HER2-overexpressing breast cancer cells developed impaired trastuzumab-mediated ADCC activity in vitro. The ability of trastuzumab to mediate ADCC activity is strictly related to HER2 density [7]. In addition, Mimura et al. [32] previously reported that the HER2 status determined by flow cytometry is well correlated with trastuzumab-mediated ADCC activity in esophageal squamous cell carcinoma cell lines in vitro. Taking into account these reports, we conclude that the impaired trastuzumab-mediated ADCC activity in trastuzumab-exposed HER2-positive human cancer cells was due to the downregulation of HER2 expression on the cell surface. These results led us to examine whether exogenous expression of the HER2 receptor on the cell surface could re-sensitize HER2-downregulated human cancer cells to trastuzumab via ADCC re-activation.

HER2 overexpression is a significant prognostic factor in terms of nodal status, tumor grade, overall survival and probability of relapse in breast cancer patients [33, 34]. Although reports are conflicting, some studies have suggested that HER2-positive status in gastric cancer is associated with poor outcomes and aggressive disease [12, 13]. As expected, human cancer cells transfected with the full-length functional HER2 showed accelerated cell growth as compared to parental cells, whereas the cell growth pattern of HER2-ECD-transfected low HER2-expressing human cancer cells was similar to that of parental cells. Furthermore, we showed that HER2-ECD transfection of low HER2-expressing human cancer cells did not enhance the HER2/HER3 signaling pathway, which is the major oncogenic signal in HER2-overexpressing breast tumors [35, 36]. Although transfection of HER2-ECD-expressing plasmid did not influence cell growth, adenovirus-mediated exogenous HER2-ECD expression significantly suppressed the tumor cell growth in vitro, suggesting that the growth inhibition associated with HER2-ECD overexpression might be due to its levels on the cell surface. Therefore, Ad-HER2-ECD infection showed slightly enhanced cytotoxic activity against some types of human cancer cells even with the control antibody rituximab in the ^{51}Cr release assay. The mechanism of Ad-HER2-ECD-mediated cell growth inhibition is unclear; however, it is likely to be caused by the restriction of other HER family receptors through the formation of heterodimers with exogenously expressed HER2-ECD that lacks the downstream signaling pathway.

Some previous studies demonstrated that primary or acquired resistance to trastuzumab often results from preventing the binding of antibody to the HER2 protein by proteins such as membrane-associated glycoprotein mucin-4 [37, 38]. In our study, even after a long-term exposure to trastuzumab, trastuzumab-mediated ADCC activity on stably HER2-ECD-expressing MCF7 cells was significantly enhanced compared to mock-treated MCF7 cells, and, furthermore, HER2-downregulated or low HER2-expressing human cancer cells could be re-sensitized to trastuzumab via re-activation of trastuzumab-mediated ADCC. These results indicate that the degree of antibody-mediated ADCC activity is likely to be correlated with the cell-surface expression levels of HER2. These results suggest that the HER2-downregulated or low HER2-expressing human cancer cells exogenously overexpressing HER2-ECD is hard to develop resistance to trastuzumab in terms of the importance of ADCC activity in antitumor effects of this antibody.

A previous study has demonstrated that heterogeneity and incomplete membranous immunoreactivity for HER2 were more common in gastric cancer than in breast cancer [39], suggesting that the gastric tumors diagnosed as HER2-positive by immunohistochemistry or fluorescent in situ hybridization are more likely to be residual and regrow under trastuzumab treatment. Therefore, molecular sensitization to trastuzumab through the expression of HER2-ECD is thought to be effective even against HER2-positive gastric cancer. We would like to examine whether the ADCC activation by exogenous HER2-ECD expression functions in vivo; however, since murine NK cells do not recognize trastuzumab, which is a humanized antibody, the in vivo experiments are hard to be performed. The genetically engineered fluorescent tumor cells as well as the whole-body fluorescent imaging technology may be available for such kinds of in vivo studies [40, 41].

Although the strategy for molecular sensitization to trastuzumab via ADCC activation by using an adenoviral vector is considered to be effective, some limitations exist; for example, there are variations in the efficiency of viral infection and the expression levels of exogenous HER2-ECD. As we used a replication-deficient adenovirus vector, the viral spread might be less than ideal after intratumoral administration. We previously developed a telomerase-specific oncolytic adenovirus that causes cell death in human cancer cells with telomerase activities. These oncolytic viruses engineered to replicate in tumor cells but not in normal cells could be used as tumor-specific vectors carrying

therapeutic genes such as HER2-ECD. Moreover, ADCC activity of PBMCs from cancer patients is likely to be impaired due to immunosuppression and NK cell dysfunction, as previously reported for gastric cancer patients [42, 43]. The immunosuppressive state is associated with immunosuppressive cytokines such as IL-10 and TGF-β. These cytokines are produced within the tumor microenvironment and suppress the activity of NK cells, monocytes, and T cells [43–46]. Therefore, to sufficiently enhance the effect of trastuzumab-mediated ADCC activity in cancer patients, supportive immunotherapy such as the administration of immune-stimulating cytokines may be required.

In conclusion, our data demonstrate that HER2 downregulation and impaired ADCC activity may be one mechanism of trastuzumab resistance. We also show that exogenous overexpression of non-signaling HER2-ECD could sensitize HER2-downregulated or HER2-negative human cancer cells via ADCC activation, an outcome that has important implications for the treatment of human cancers.

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Conflict of interest All authors state that they have no potential conflicts of interest.

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Synergistic Interaction of Telomerase-Specific Oncolytic Virotherapy and Chemotherapeutic Agents for Human Cancer

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Abstract: Replication-selective tumor-specific viruses present a novel approach for treatment of neoplastic disease. These vectors are designed to induce virus-mediated lysis of tumor cells after selective viral propagation within the tumor. Telomerase activation is considered to be a critical step in carcinogenesis through the maintenance of telomeres, and its activity correlates closely with human telomerase reverse transcriptase (hTERT) expression. We constructed an attenuated adenovirus 5 vector, in which the hTERT promoter element drives expression of E1 genes, OBP-301 (Telomelysin). Since only tumor cells that express telomerase activity would activate this promoter, the hTERT proximal promoter allows for preferential expression of viral genes in tumor cells, leading to selective viral replication and oncolytic cell death. OBP-301 alone exhibited substantial antitumor effects both in animal models and in clinical trials; data regarding combination therapy with OBP-301 and chemotherapeutic agents are preliminary but encouraging. This article reviews synergistic interaction of virotherapy and chemotherapy, and illustrates the potential application for the treatment of human cancer.

Keywords: Telomerase, adenovirus, virotherapy, docetaxel, histon deacetylase inhibitor, gemcitabine.

INTRODUCTION

Gene and vector-based molecular therapies for cancer encompass a wide range of treatment types that all use genetic material to modify cancer cells and/or surrounding tissues to exhibit antitumor properties. One of the most common approaches to emerge from the concept of gene therapy is the introduction of foreign therapeutic genes into target cells. A number of genes of interest with different functions such as tumor suppressor genes [1, 2], proapoptotic genes [3, 4], suicide genes that cause cellular death with prodrugs [5, 6], and genes that inhibit angiogenesis [7] have been proposed for this type of therapy. In fact, we and others have completed clinical trials of a replication-deficient adenoviral vector that delivers normally functioning *p53* tumor suppressor gene to cancer cells (Ad5CMV-p53, Advexin). It has been reported that multiple courses of intratumoral injection of Ad5CMV-p53 are feasible and well tolerated in patients with advanced head and neck squamous cell carcinoma and non-small cell lung cancers, and appear to provide clinical benefits [8-12].

Another rapidly growing area of molecular therapy for cancer is the use of oncolytic vectors for selective tumor cell destruction. Since viruses infect cells and then induce cell lysis through their propagation, they can be used as anticancer agents by genetic engineering to replicate selectively in cancer cells while remaining innocuous to normal tissues [13]. One of the targeting strategies has involved the use of tissue-specific promoters to restrict gene expression or viral replication in specific tissues. A large number of different tissue-specific promoters have been used for virotherapy applications; for targeting tumors derived from various tissues, however, tumor-specific, rather than tissue-specific, promoters would be more advantageous. For example, the promoter of human telomerase reverse transcriptase (hTERT) is highly active in most tumor cells but inactive in normal somatic cell types. We previously developed an adenovirus vector that drives *E1A* and *E1B* genes under the hTERT promoter, OBP-301 (Telomelysin) [14-16], and showed its selective replication as well as a profound cytotoxic activity in variety of human cancer cells. Although the development of OBP-301 as a monotherapy is currently underway clinically based on promising results of preclinical studies, multi-modal strategies to enhance antitumor efficacy *in vivo* is essential for successful clinical outcome.

This review looks at recent developments in this rapidly evolving field, cancer therapeutic approach using the hTERT promoter, and highlights some very promising advances in the combination of oncolytic virotherapy with chemotherapeutic drugs.

TELOMERASE ACTIVITY FOR TRANSCRIPTIONAL CANCER TARGETING

One of the hallmarks of cancer is unregulated proliferation of a certain cell population, which eventually affects normal cellular function in the human body, and this almost universally correlates with the reactivation of telomerase. Tumor cells can maintain telomere length predominantly due to telomerase, and its activity is detected in about 85% of

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malignant tumors [17], whereas telomerase is absent in most normal somatic tissues [18], with a few exceptions including peripheral blood leukocytes and certain stem cell population [19, 20]. There is also a gradient increase in telomerase activity between early and late stage tumors. The strong association between telomerase activity and malignant tissue suggests that telomerase can be a plausible target for the treatment of cancer [21].

The enzyme telomerase is a ribonucleoprotein complex responsible for the addition of TTAGGG repeats to the telomeric ends of chromosomes, and contains three components: the RNA subunit (known as hTR, hTER, or hTERC) [22], the telomerase-associated protein (hTEP1) [23], and the catalytic subunit (hTERT) [24, 25]. Both hTR and hTERT are required for the reconstitution of telomerase activity *in vitro* [26] and, therefore, represent the minimal catalytic core of telomerase in humans [27]. However, while hTR is widely expressed in embryonic and somatic tissues, hTERT is tightly regulated and is not detectable in most somatic cells. Thus, the hTERT promoter region can be used as a fine-tuning molecular switch that works exclusively in tumor cells.

TELOMERASE-SPECIFIC ONCOLYTIC ADENOVIRUS FOR CANCER THERAPEUTICS

Structure of hTERT Promoter-Driven Oncolytic Adenovirus

The use of modified adenoviruses that replicate and complete their lytic cycle preferentially in cancer cells is a promising strategy for treatment of cancer. One approach to achieve tumor specificity of viral replication is based on the transcriptional control of genes that are critical for virus replication such as *E1A* or *E4*. As described above, telomerase, especially its catalytic subunit hTERT, is expressed in the majority of human cancers and the hTERT promoter is preferentially activated in human cancer cells [17]. Thus, the broadly applicable hTERT promoter might be a suitable regulator of adenoviral replication. Indeed, it has been reported previously that the transcriptional control of E1A expression via the hTERT promoter could restrict adenoviral replication to telomerase-positive tumor cells and efficiently lyse tumor cells [28-31].

The adenovirus *E1B* gene is expressed early in viral infection and its gene product inhibits E1A-induced p53-dependent apoptosis, which in turn promotes the cytoplasmic accumulation of late viral mRNA, leading to a shut down of host cell protein synthesis. In most vectors that replicate under the transcriptional control of the *E1A* gene including hTERT-specific oncolytic adenoviruses, the *E1B* gene is driven by the endogenous adenovirus E1B promoter. However, Li *et al.* [32] have demonstrated that transcriptional control of both *E1A* and *E1B* genes by the α- fetoprotein (AFP) promoter with the use of IRES significantly improved the specificity and the therapeutic index in hepatocellular carcinoma cells. Based on the above information, we developed OBP-301 (Telomelysin), in which the tumor-specific hTERT promoter regulates both the *E1A* and *E1B* genes Fig. (**1**). OBP-301 is expected to control viral replication more stringently, thereby providing better therapeutic effects in tumor cells as well as attenuated toxicity in normal tissues [14].

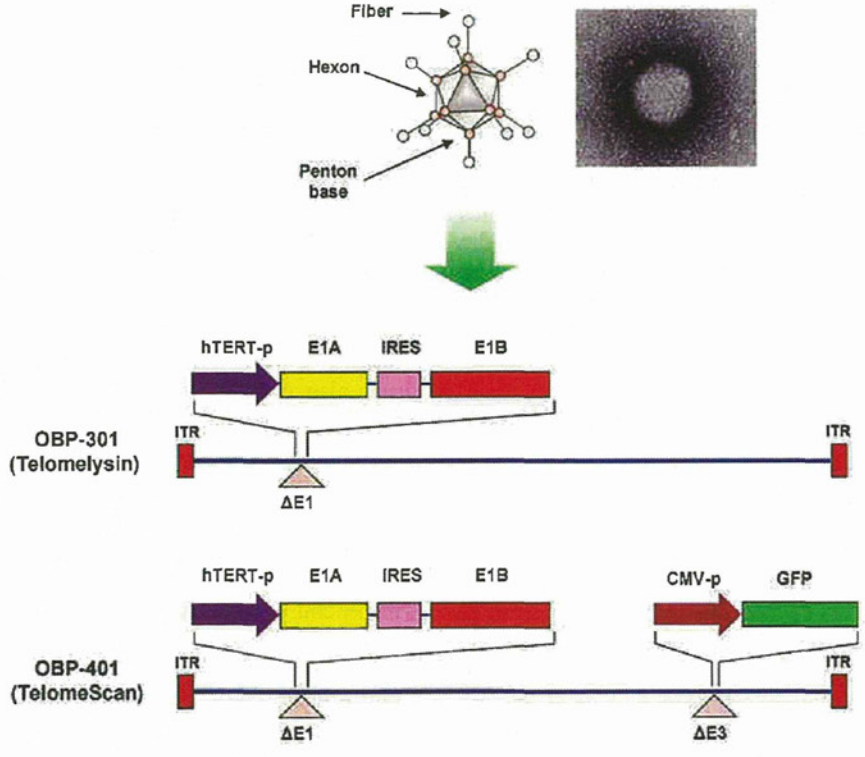

Fig. (1). Structures of telomerase-specific oncolytic adenovirus. OBP-301, in which the hTERT promoter element drives the expression of *E1A* and *E1B* genes linked with an IRES. OBP-401 (TelomeScan) is a telomerase-specific replication-competent adenovirus variant, in which *GFP* gene is inserted under cytomegalovirus (CMV) promoter into E3 region for monitoring viral replication. *Upper panel*, schematic representation depicting major structural components of OBP-301 (hexon, penton base, and fiber) and transmission electron microscopy image.

Preclinical Studies of hTERT Promoter-Driven Oncolytic Adenovirus

The majority of human cancer cells acquire immortality and unregulated proliferation by expression of the hTERT [17] and, therefore theoretically, hTERT-specific OBP-301 can possess a broad-spectrum antineoplastic activity against a variety of human tumors [14, 16]. OBP-301 induced selective E1A and E1B expression in cancer cells, which resulted in viral replication at 5-6 logs by 3 days after infection; on the other hand, OBP-301 replication was attenuated up to 2 logs in cultured normal cells [14, 16]. *In vitro* cytotoxicity assays demonstrated that OBP-301 could efficiently kill various types of human cancer cell lines including head and neck cancer, lung cancer, esophageal cancer, gastric cancer, colorectal cancer, breast cancer, pancreas cancer, hepatic cancer, prostate cancer, cervical cancer, melanoma, sarcoma, and mesothelioma in a dose-dependent manner [33]. These data clearly demonstrate that OBP-301 exhibits desirable features for use as an oncolytic therapeutic agent, as the proportion of cancers potentially treatable by OBP-301 is extremely high.

The *in vivo* antitumor effect of OBP-301 was also investigated by using athymic mice carrying xenografts. Intratumoral injection of OBP-301 into human tumor xenografts resulted in a significant inhibition of tumor growth and enhancement of survival [14, 16]. Macroscopically, massive ulceration was noted on the tumor surface after injection of high-dose OBP-301, indicating that OBP-301 induced intratumoral necrosis due to direct lysis of tumor cells by virus replication *in vivo* [34]. Head and neck cancer is characterized by locoregional spread, and it is clinically accessible, making it an attractive target for intratumoral virotherapy. Thus, an orthotopic nude mouse model of human tongue squamous cell carcinoma was also used to explore the *in vivo* antitumor effect of OBP-301. Intratumoral injection of OBP-301 significantly shrunk the tongue tumor volumes, which in turn increased the body weight of mice by enabling oral ingestion [35]. Since the body weight loss due to a feeding problem in this orthotopic tongue cancer model resembles the disease progression in head and neck cancer patients, the finding that OBP-301 increased the body weight of mice suggests that telomerase-specific virotherapy could potentially improve the quality of life in advanced head and neck cancer patients [36].

Clinical Application of Telomerase-Specific Oncolytic Adenovirus

Preclinical models suggested that OBP-301 could selectively kill a variety of human cancer cells *in vitro* and *in vivo* via intracellular viral replication regulated by the hTERT transcriptional activity. Pharmacological and toxicological studies ranging from 10^6 to 10^{12} virus particles (VP) in mice and cotton rats demonstrated that none of the animals treated with OBP-301 showed signs of viral distress (e.g., ruffled fur, weight loss, lethargy, or agitation) or histopathological changes in any organs at autopsy. These promising data led us to design a phase I clinical trial of OBP-301 as a monotherapy.

The protocol "A phase I dose-escalation study of intratumoral injection with telomerase-specific replication-competent oncolytic adenovirus, Telomelysin (OBP-301) for various solid tumors" sponsored by Oncolys BioPharma, Inc. is an open-label, phase I, 3 cohort dose-escalation study [37]. The trial commenced following approval of the US Food and Drug Administration (FDA) in October, 2006. The study has completed to assess the safety, tolerability, and feasibility of intratumoral injection of the agent in patients with advanced solid cancer. The doses of OBP-301 were escalated from low to high VP in one log increment. Patients were treated with a single dose intratumoral injection of OBP-301 and then monitored over one month.

All patients received OBP-301 without dose-limiting toxicity. Additionally, it was demonstrated tat circulating OBP-301 viral genome became detectable in the plasma within 24 hours after injection in 13 of 16 patients. This dose-dependent initial peak in circulating virus was followed by a rapid decline; however, 3 patients showed a second peak of circulating viral DNA on days 7 and 14, suggesting OBP-301 replication in primary tumors. Clinical trials of CG7870, a replication-selective oncolytic adenovirus genetically engineered to replicate preferentially in prostate tissue, also demonstrated a second peak of the virus genome in the plasma [38,39], suggesting similar active viral replication and shedding into the bloodstream. One of the 3 "second peak" patients also had disappearance of the injected malignant lesion and loco-regional un-injected satellite nodules, fulfilling a definition of complete response at day 28. Seven patients fulfilled RECIST definition for stable disease day 56 after treatment. Thus, OBP-301 is well-tolerated and warrants further clinical studies for solid cancer.

SYNERGISTIC INTERACTION OF ONCOLYTIC VIROTHERAPY AND CHEMOTHERAPEUTIC AGENTS

Preclinical studies provided experimental evidence for effectively killing of cancer cells by oncolytic viruses [40-42]. In animal models, however, established xenograft tumors are rarely eliminated despite existence of persistently high viral titers within the tumor. Total elimination of solid tumor requires higher doses of oncolytic viruses, which might be toxic or lethal. The efficacy of virotherapy combined with anticancer drugs has been reported previously in preclinical studies. A replication-selective adenovirus, ONYX-015 combined with 5-fluorouracil or CDDP produced greater effect than each individual modality and prolonged survival [43, 44]. Furthermore, synergistic efficacy was also observed in the combination of a tumor-specific HSV mutant (HSV-1716) with chemotherapeutic agents in human non-small cell lung cancer [45].

Most of the clinical trials for oncolytic viruses have been also conducted in combination with chemotherapy or radiotherapy [46-49]. Disappointingly, a clinical trial of Onyx-015 showed no clinical benefit in the majority of patients, despite the encouraging biological activity [50]. Tumor progression was rapid in most patients, even though substantial necrosis was noted in the tumors after treatment [51, 52]. Therefore, multi-disciplinary therapy composed of oncolytic virotherapy combined with low-dose chemotherapeutic agent may provide capacity to enhance the antitumor efficacy. Moreover, the combination of two agents may allow the use of reduced dosage of each agent, and lessen the likelihood of adverse effects.

Enhanced Antitumor Efficacy of Telomerase-Selective Oncolytic Adenovirus with Docetaxel

Taxanes are novel antimicrotubule agents that promote the assembly of microtubules from tubulin dimers and stabilize microtubules by preventing depolymerization. This stability results in the inhibition of the normal dynamic reorganization of the microtubule network, which is essential for vital interphase and mitotic cellular functions, resulting in cell arrest in G2 and M phases [53, 54]. Apoptotic cell death is induced subsequently, but this does not inhibit DNA synthesis of host cells. Therefore, taxanes are promising for combination with virotherapy. It was reported previously that paclitaxel had a synergistic or an additive effect in several cancer models when combined with adenovirus vector-mediated p53 gene therapy [55]. It has been also demonstrated that CV787, a prostate cancer-specific adenovirus, exhibited synergistic antitumor effect when combined with taxanes [56].

Infection with OBP-401 (GFP-expressing OBP-301 was used as an alternative to OBP-301 in some experiments) alone or followed by treatment with docetaxel (Taxotere), a chemotherapeutic agent, resulted in a profound *in vitro* cytotoxicity in various human cancer cell lines originating from different organs (lung, colon, esophagus, stomach, liver, and prostate), although the magnitude of the antitumor effect varied among the cell types Fig. (2) [57]. Quantitative real-time PCR analysis demonstrated that docetaxel did not affect viral replication. For *in vivo* evaluation, mice xenografted with human lung tumor received intratumoral injection of OBP-301 and intraperitoneal administration of docetaxel. Analysis of growth of implanted tumors showed a significant, therapeutic synergism, while each treatment alone showed modest inhibition of tumor growth [57]. The antitumor effect of the combination therapy was likely additive *in vitro;* there might be, however, some particular interactions between OBP-301 and docetaxel to produce a synergistic effect *in vivo*. It has been reported that metronomic chemotherapy, which refers to long-term administration of comparatively low doses of cytotoxic drugs at close, regular intervals, has an antiangiogenic basis [58]. Like our approach, the potent antiangiogenic properties of drugs administered in a metronomic fashion find favor in a number of *in vivo* preclinical studies; to prove this efficacy by *in vitro* experiments is, however, technically difficult. There are some possible explanations for the superior *in vivo* antitumor activity in our experiments. Systemically administered docetaxel may attack the vascular endothelial cells at the tumor site, which in turn can block the escape of locally injected OBP-301 into the peripheral circulation. Another possibility is that OBP-301 itself may inhibit the vascular supply by killing endothelial cells.

Enhanced Antitumor Efficacy of Telomerase-Selective Oncolytic Adenovirus with Histon Deacetylase Inhibitor

FR901228 (depsipeptide, FK228) is a novel anticancer agent isolated from the fermentation broth of *Chromobacterium violaceum*. FR901228 has been identified as a potent histone deacetylase (HDAC) inhibitor. Histone deacetylation is an important component of transcriptional control, and FR901228 increases Coxsackie's-adenovirus receptor (CAR) gene expression in various cancer cell lines [59-62]. Moreover, FR901228 is known to increase viral and transgene expression following adenovirus infection [59]. Indeed, FR901228 treatment upregulated CAR levels on target tumor cells, which in turn increased the amount of cellular OBP-301 replication, thereby promoting a synergistic antitumor effect [63]. These data indicate that FR901228 may be an appropriate partner for OBP-301 because it does not affect the virus life cycle. Two phase I clinical trials involving ad-

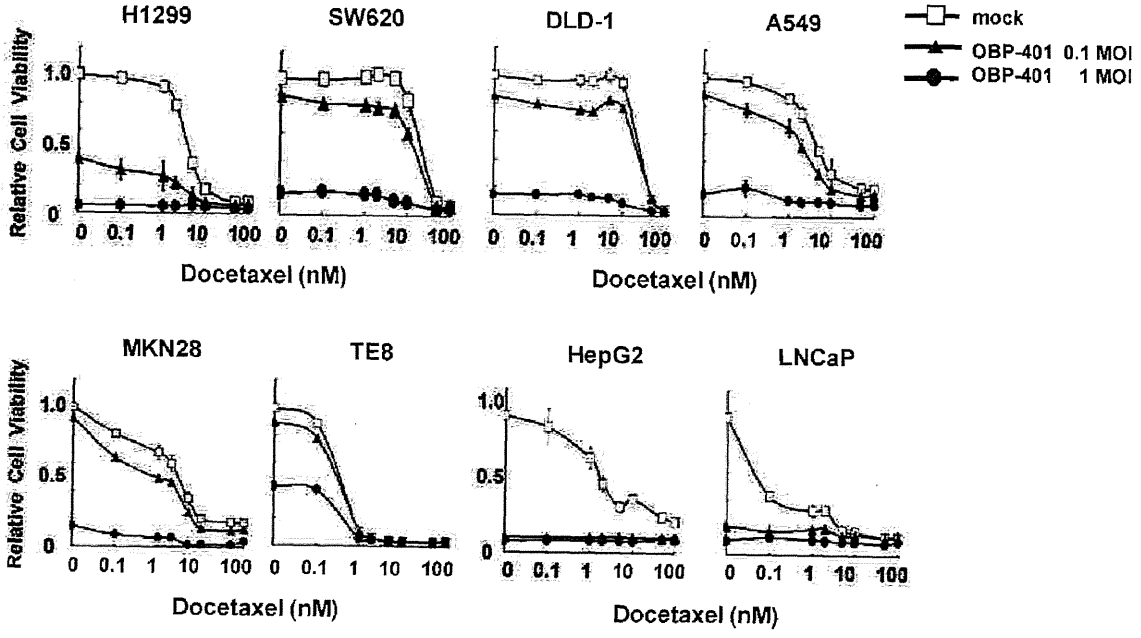

Fig. (2). Combination effect of OBP-401 and docetaxel on human cancer cell lines. Cells were infected with 0.1 or 1 MOI of OBP-401, and then exposed to docetaxel at the indicated concentrations at 24 hours after infection. Cell viability was assessed by XTT assay at 5 days after OBP-401 infection. The results of H1299 (lung), SW620 (colon), DLD-1 (colon), A549 (lung), MKN28 (stomach), TE8 (esophagus), HepG2 (liver), and LNCaP (prostate) cells were shown as representative of 10 cell lines. Bars, standard deviation (SD).

vanced cancer and leukemia have shown that FR901228 can be safely administered without life-threatening toxicity, including cardiac toxicity, although the appropriate administration schedule has to be further examined [64, 65].

Enhanced Antitumor Efficacy of Telomerase-Selective Oncolytic Adenovirus with Gemcitabine

Gemcitabine (2,2-difluorodeoxycytidine) is a third-generation agent that has been developed in the past decades. Gemcitabine is a deoxycytidine analogue that has shown efficacy as a treatment for many solid tumors and is now extensively used in the treatment of patients with various tumor types [66, 67], but inherent and acquired resistance has resulted in low response rates. Adenovirus therapy combined with gemcitabine has been reported in the treatment of pancreatic cancer. Halloran *et al.* reported that incubation of Panc-1 cells with either 5-FU or gemcitabine followed by adenovirus-mediated overexpression of $p16^{INK4A}$ resulted in a substantial reduction in cell viability under conditions where the drugs alone had minimal cytotoxicity [68]. It has been also reported that the type 5 adenoviral E1A sensitizes hepatocellular carcinoma cells to gemcitabine [69]. These observations support the notion that oncolytic adenoviruses combined with gemcitabine is a rational modality for the treatment of human cancer.

The antitumor efficacy of OBP-301 was found to be enhanced when combined with gemcitabine in human lung cancer cells *in vitro* and *in vivo* [70]. Gemcitabine is a deoxycytidine analogue and the incorporation of gemcitabine triphosphate into DNA causes chain termination, which is the major mechanism underlying the cytotoxicity of gemcitabine [71]. Although there was concern over whether gemcitabine would interrupt the viral replication of OBP-301, quantitative real-time PCR analysis showed that intracellular replication of OBP-301 was not affected by gemcitabine. The cytotoxic mechanisms of OBP-301 are distinct from those of gemcitabine and, therefore, combination effects could be observed provided that gemcitabine does not inhibit viral replication.

It has been reported that many DNA viruses can drive quiescent cells through G1 into S phase by the expression of viral proteins [72-74]. During the early phase of the adenovirus infection, the host cell is transformed into an efficient producer of the viral genome. The first gene that is transcribed in the viral genome is E1A, which can bind to numerous cellular proteins and acts as a multi-functional protein. Our data demonstrated that OBP-301 infection increases the phosphorylation of Akt, as well as E2F-1 expression Fig. (**3**). These effects are thought to be due to adenoviral E1A protein expression, as the dl312 adenovirus lacking the E1 genes did not phosphorylate Akt. As cells progress into the cell cycle, cyclin-dependent kinases phosphorylate retinoblastoma (Rb), freeing E2F and allowing it to directly transactivate genes required for S phase entry [75]. In fact, replication-deficient adenovirus-mediated *E2F-1* gene transfer into human cancer cells resulted in accumulation of an S-phase cell population. Thus, OBP-301 infection expressed E1A protein, which in turn upregulated the expression of phosphorylated Akt and E2F-1, leading to cell cycle promotion and S phase entry presumably by the deactivation of Rb (Table **1**). The accumulation of the tumor cells in S phase increases the cytotoxicity of gemcitabine, which kills cells in S phase.

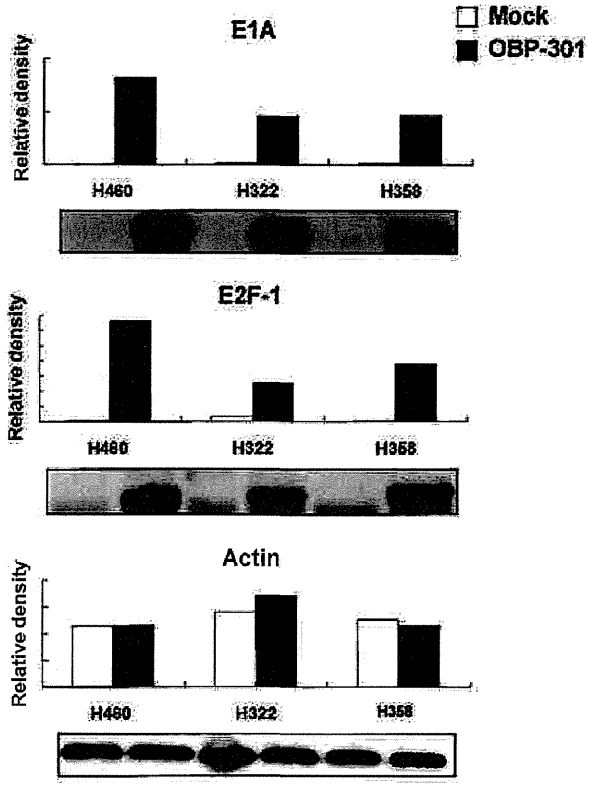

Fig. (3). Changes in cell cycle regulator protein expression following OBP-301 infection. H460, H322, and H358 cells were either mock-infected or infected with OBP-301 at an MOI of 40, 100 and 80 MOI, respectively. Following the removal of virus inocula, cells were collected at 24 h after infection, and were subjected to analysis. Equivalent amounts of protein obtained from whole cell lysates were loaded into each lane, probed with primary antibodies, and then visualized using an ECL detection system. Equal loading of samples was confirmed by reprobing with anti-actin antiserum. Protein expression was quantified by densitometric scanning using NIH Image software. Expression levels of adenoviral E1A and E2F1 greatly increased after OBP-301 infection as compared with the mock-infected controls in all three cell lines.

CONCLUSIONS AND PERSPECTIVES

There have been very impressive advances in our understanding of the molecular aspects of human cancer and in the development of technologies for genetic modification of viral genomes. Transcriptional targeting is a powerful tool for tumor selectivity in cancer therapy and diagnosis, and the hTERT-specific oncolytic adenovirus achieves a more strict targeting potential due to the amplified effect by viral replication. Several independent studies that used different regions of the hTERT promoter and different sites of adenoviral genome responsible for viral replication, have shown that the hTERT promoter allows adenoviral replication as a molecular switch and induces selective cytopathic effect in a variety of human tumor cells [14, 16, 28-30, 33]. Among these viral constructs, to the best of our knowledge, OBP-301 seems to be the first hTERT-dependent oncolytic adeno-

Table 1. Cell Cycle Analysis after OBP-301 Infection in Human Lung Cancer Cells

Cell Lines	Treatment	Cell Cycle		
		G1 (1%)	S (%)	G2 (%)
H460	Mock	43.54	43.85	8.61
	OBP-301	10.91	56.41	32.54
H322	Mock	40	46.72	10.85
	OBP-301	27.49	67.09	3.23
H358	Mock	45.89	38.22	14.29
	OBP-301	28.93	57.67	11.45

H460, H322 and H358 cell lines were treated with OBP-301 at 40 MOI, 100 MOI, and 80 MOI, respectively. Cells were then subjected to cell cycle analysis at 24 h after treatment by the FACS method. The percentages of cells in the G1, S and G2 phases are shown. The number of cells in S phase increased as compared with mock0infected cells after OBP-301 infection in all cell lines tested, although there was no increase in the sub-G0/G1 population indicating apoptotic cell death.

virus that has been used in a clinical trial based on preclinical pharmacological and toxicological studies. Thus, telomerase-specific targeted oncolytic adenovirus holds promise for the treatment of human cancer.

A future direction for OBP-301 includes combination therapy with conventional therapies such as chemotherapy, radiotherapy, surgery, immunotherapy, and new modalities such as antiangiogenic therapy. This review emphasized the synergistic interaction of OBP-301 with various types of chemotherapeutic agents. Since clinical activities observed by intratumoral injection of OBP-301 suggest that even partial elimination of the tumor could be clinically beneficial, the combination approaches may lead to the development of more advanced biological therapy for human cancer. We recently confirmed that OBP-301 infection and ionizing radiation mutually modulate their respective biological effects and thereby potentiate each other, profoundly enhancing *in vivo* antitumor activity [76]. Moreover, we demonstrated that preoperative delivery of OBP-301 into primary tumors prevented the exacerbation of lymph node metastasis by surgical procedures [77], suggesting that OBP-301 may be also valuable as adjuvant therapy in areas of microscopic disease to prevent recurrence or regrowth of tumors.

The field of targeted oncolytic virotherapy is progressing considerably and is rapidly gaining medical and scientific acceptance. Delineating specific virus/drug combinations tailored to be particularly effective in human cancer could potentially improve the already encouraging results seen in the field of oncolytic virotherapy.

CONFLICT OF INTEREST

None declared.

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