RESEARCH ARTICLE

A Novel Monoclonal Antibody Induces Cancer Cell Apoptosis and Enhances the Activity of Chemotherapeutic Drugs

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Abstract

A novel monoclonal antibody (mAb), known as AC10364, was identified from an antibody library generated by immunization of mice with human carcinoma cells. The mAb recognized proteins in lysates from multiple carcinoma cell lines. Cell cytotoxicity assays showed that AC10364 significantly inhibited cell growth and induced apoptosis in multiple carcinoma cell lines, including Bel/fu, KATO-III and A2780. Compared with mAb AC10364 or chemotherapeutic drugs alone, the combination of mAb AC10364 with chemotherapeutic drugs demonstrated enhanced growth inhibitory effects on carcinoma cells. These results suggest that mAb AC10364 is a promising candidate for cancer therapy.

Keywords: Monoclonal antibody - chemotherapy - cancer - apoptosis - drug synergy

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Introduction

Antibody-based therapy for cancer has become established over the last few decades and achieved considerable success in recent years. Bevacizumab (Avastin), an anti-vascular endothelial growth factor antibody, cetuximab (Erbitux), an anti-epidermal growth factor antibody, rituximab (rituxan), an anti-CD20 antibody (Grillo-López et al., 2002), and Trastuzumab (Herceptin), an anti-HER2/neu antibody (Yip and Ward, 2002) have been approved by the FDA and become widely used. Targeted immunotherapy with mAbs has become critical for the successful treatment of many forms of cancer and has been proved to increase survival of patients in clinics. This is exemplified by rituximab, a chimeric anti-CD20 Ab, which has revolutionized the treatment of several B cell malignancies such as follicular lymphoma (Maloney, 2003; Estevez et al., 2007).

mAbs have selective tumor-targeting potential associated with relatively low toxicity. mAbs deliver chemotherapeutic agents, target signaling molecules, and induce complement-dependent cytotoxicity (CDC) or Ab-dependent cellular cytotoxicity (ADCC) (Saltz et al., 2004). Many studies concentrated on mAbs-induced apoptosis, killing or death of cancer cells (de Weers et al., 2011; Crescence et al., 2012; Willingham et al., 2012). These recent exciting results provide optimism for the development of mAbs that bind novel targets and possess novel mechanism of action. One major still exists with mAbs. That is that most mAbs do not directly induce cell death but have to rely on immune effector mechanism for efficacy. However, most tumors have already developed a variety of mechanisms to escape immune attack. Antibodies that induce cell death without the need for immune effector mechanisms may be very important in the treatment of solid tumors that have involved complex mechanisms to protect themselves from CDC and ADCC (Gorter and Meri, 1999). Erbitux does not just rely on ADCC and CDC but can directly inhibit cell proliferation, angiogenesis and survival by blocking EGF binding (Saltz et al., 2004).

In the search for more effective cancer therapies, recent studies have focused on combining immune-based drugs, such as therapeutic mAbs, with chemo-therapeutic agents (Attard et al., 2007; Gallamini et al., 2007). The outcome of such chemoimmunotherapy would be more profound once the use of antibody directly contributed to the cancer cell growth inhibition or cell death. In this study, we described the development of a novel mAb, which could not only induce carcinoma cell apoptosis but also sensitizes cancer cells to chemotherapeutic drugs.

Materials and Methods

Cell lines

The cell lines A2780, AGS, HCT-8, NCI-N87, HeLa, 293T, Hs 746T, KATO-III, SNU-1 and human dermal fibroblasts (Fibroblasts) were obtained from the American Type Culture Collection (ATCC) and cultured according to ATCC protocols. Bel/fu was a present from Dr. Wan-Zhou

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Zhao (Sino-EU Biomedical Innovation Center, Nanjing, China). Hs 746T cells were grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, USA)/10% fetal bovine serum (FBS) (Hyclone, USA), other cells were grown in RPMI 1640 (BasalMedia, China)/10% FBS at 37°C with 5% CO_2.

Antibody preparation and selection

Three female Balb/c mice, at least 6 weeks of age, from STAFF, Frederick National Laboratory for Cancer Research (USA), were injected intraperitoneally with 1×10^7 Bel/fu cells on days 1, 15 and 29, respectively. The mice were housed in an animal facility accredited by BSL-2, and all of the procedures were approved by the local ethical committee (Georgia Regents University, USA). At day 7 after the third immunization, an intravenous injection with 1×10^7 AGS cells was performed to boost the immune response. The spleen cells were harvested and fused with sp2/0 myeloma cells (ATCC, USA) using polyethylene glycol (Roche, USA). The fused cells were seeded into 96-well microplates and cultured continually with 10% FBS DMEM containing hypoxanthine-aminoprerin-thymidine (HAT) (invitrogen, USA) for 2 weeks. Hybridoma supernatants were screened by indirect immunofluorescence assay (IFA) and flow activated cell sorter analysis (FACs) using Bel/fu cells and other carcinoma cells, or 293T cells. Briefly, following the 48 h incubation in 96-wells plates, the cells were washed with phosphate-buffered saline (PBS) and fixed using -20°C methanol for 10 min at 4°C. Hybridoma supernatants were incubated with the cells for 1 h at 37 °C. After washes (3×5 min) with PBS, the cells were incubated with goat anti-mouse CY3-labeled secondary antibody (Sigma, USA) diluted in PBS for 45 min at 37 °C. The cells were washed three times and examined under a fluorescence microscope (Nikon, Japan). The positive clones were detected by FACs to confirm the reactivity on the cells membrane. Briefly, cells (1×10^5 cells/tube) were washed in 200μl PBS at 500 rpm, incubated with 200μl hybridoma supernatant for 60 min at room temperature. After three washes, cells were incubated with goat anti-mouse CY3-labeled secondary antibody (Sigma, USA) at 4°C for 30 min in the dark. After three washes, cells in 200μl PBS were analyzed on a FACScaliburTM flow cytometer with CELL QuestTM software (Becton Dickinson, Franklin Lakes, NJ, USA). Five to ten thousand events were acquired per sample with fluorescence measured on logarithmic scales. Forward and side light scatter gates were set to exclude dead cells, debris, and clumps of cells. Autofluorescence was removed from the samples by setting gates on unstained controls.

The mAbs production and purification

Ascites fluid was produced by intraperitoneal injection of 1×10^6 cloned hybridoma cells into Babl/c mice, using a protocol that was approved by the animal care committee of the animal facility (Georgia Regents University, USA). Ascites fluid was cleared of cells and cell debris, and IgG was purified using Protein G (Invitrogen, USA). Furthermore, analysis of mAbs subtype was performed with standard procedures illuminated by the protocol of Mouse Monoclonal Antibody Isotyping kit (Roche, USA). Purified antibody was stored at -20°C.

Cytotoxicity assay

After choosing candidate mAbs, the effects of the mAbs on carcinoma cells proliferation were performed. Cell viability was determined using cell counting kit-8 (CCK-8, Dojindo Laboratories, Japan). In brief, carcinoma cells were seeded into 96-well plates at a density of 3×10^3 (50μl) per well, then incubated for 24h at 37°C. After incubation, according to mAbs concentration, the mAbs were serially diluted in medium from 200 μg/ml, respectively. Then, 50μl mAbs were mixed with cells at an equal volume. After incubation for 72h at 37°C, cells were incubated with 3μl CCK-8 for 4h at 37°C. Then, the absorbance was measured at 490nm by use of a microplate reader (Thermo). Wells with untreated cells or 293T cells were used as controls. Growth inhibition curves were plotted as a percentage of untreated control cells according to standard curves and IC_{50} values were calculated. Based on the results, we chose the mAb named AC10364 for more detailed characterization.

Western Blotting (WB)

1×10^7 cells were collected. After centrifugation at 500 rpm for 5 min, the pellet was lysed with P-MER cell lyse buffer (1ml) (Thermo Scientific, USA) containing 1% Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, USA) for 30 min on ice. The supernatant was collected after 10 min of centrifugation, equaled by spectrophotometer, denatured with sample loading buffer for 10 min at 95°C and stored at 4°C for future use. Proteins were separated by 10% SDS-polyacrylamide gels and transferred to PVDF membrane (Bio-Rad, USA), and immune-blotted with mAb AC10364 at 4°C overnight. The appropriate horseradish-conjugated secondary antibody (Santa Cruz biotechnology, USA) at a dilution of 1:5000 in blocking buffer (1% BSA-TBST) for 1h at room temperature.

Caspase 3/7 activity

3×10^3 Bel/fu cells were seeded into 96-well plates, incubated with mAb (500μg/ml) at 37°C for 10h. The caspase 3/7 Green Detection Reagent (Life Technologies, USA) was diluted for 1000 times with medium, added 30μl into wells, incubated for 30min. The cells were examined under a fluorescence microscope (Nikon, Japan) as previous description.

Chemotherapeutic drugs combined with the mAb

Chemotherapeutic drugs such as Vincristine (VCR) (SIGMA, USA) and Doxorubicin (ADM) (SIGMA, USA) were selected for this study. In brief, 3×10^3 carcinoma cells were seeded into 96-well culture plates, then mAb AC10364 (50μl) was added at four different concentrations, 2000μg/ml, 400μg/ml, 100μg/ml, 0μg/ml. After incubation for 24h at 37°C, drugs (50μl) were mixed with cells. After incubation for 72 h at 37°C, cells were incubated with 3μl of CCK-8 for 4h at 37°C. In addition, after incubation with AC10364 (100 μg/ml, 50 μl) for 10h, Bel/fu or A2780 cells were cultured with

Table 1. Influence of mAb AC10364 on IC_{50} of VCR and ADM Against Cancer Cells

IC_{50} (nM)	VCR				ADM			
	1mg/ml	200μg/ml	50μg/ml	0	1mg/ml	200μg/ml	50μg/ml	0
Bel/fu	0.2	30.7	33.7	65.3	0.46	7.8	28.8	104.8
A2780	0.9	1.3	1.4	2.7	10.1	15.8	27.4	88.4
AGS	374.3	390.6	580.2	674.6	2.7	6	7.1	9.9

*Four mAb concentrations (2000, 400, 100 and 0μg/ml) added to cell cultures for 24 h at 37°C and chemotherapeutic drugs were added. Inhibition was measured at 72h after drug treatment

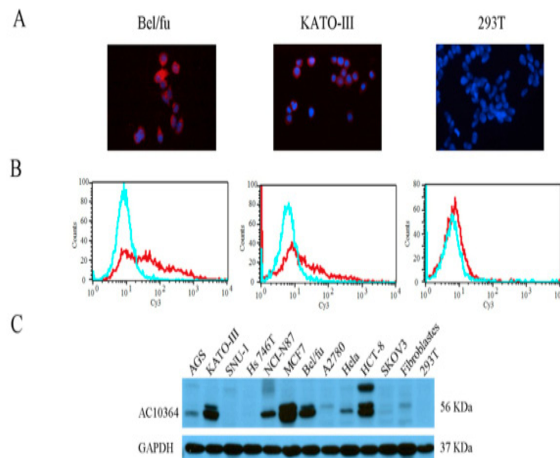

Figure 1. Screening of Monoclonal Antibody Library and Selection of mAb AC10364. A) IFA of three cell lines using mAb AC10364. Strong reactivity was observed for Bel/fu and KATO III cells but not with 293T cells (RED: CY3-labeled second antibody). Cell nucleus appeared as blue fluorescence stained with DAPI (magnification 100×). **B)** FACS analysis of cell surface. Red: AC10364 staining, Teal: isotype control). **C)** Western-blotting using AC10364. Cells lysates were used as antigens

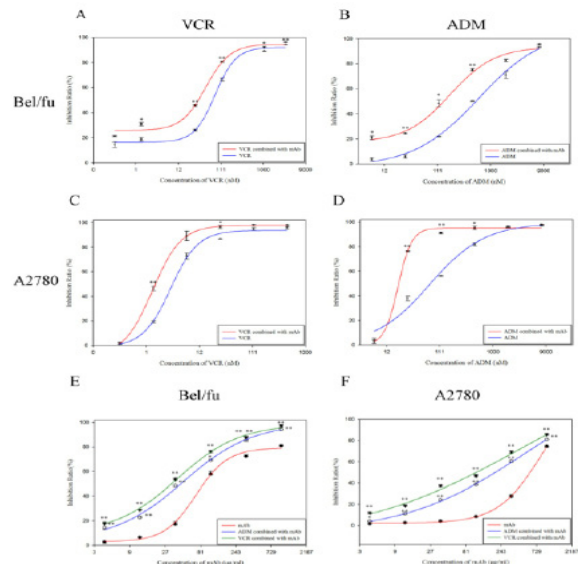

Figure 2. Therapeutic Effect of AC10364. A) Inhibition curves on six cell lines. Inhibition was estimated with the CCK-8 kit after 72h exposure to AC10364. Data are inhibition (%) mean±SD of three independent experiments. *$p<0.05$, **$p<0.01$. **B)** Morphological changes in Bel/fu, KATO III and A2780 cells upon incubation with mAb (500μg/ml) observed under a phase-contrast microscope. **C)** Caspase 3/7 staining (green signal) with IFA at 10h after treatment with AC10364 (500μg/ml) treatment. Bel/fu cells without mAb treatment were used as controls

chemotherapeutic drugs (VCR/ADM, 50 μl) at different concentrations. Then, IC_{50} of VCR/ADM against Bel/fu or A2780 was calculated as previous description after incubation with AC10364 at final concentration of 50μg/ml. Moreover, after incubation with chemotherapeutic drugs (VCR/ADM) (50μl, 20nM) for 1h, Bel/fu or A2780 cells were cultured with AC10364 (50μl) at different concentrations. Then, IC_{50} of AC10364 against Bel/fu or A2780 was calculated after incubation with VCR/ADM at final concentration of 10 nM.

Statistical analysis

All data are presented as mean±SD. Significant differences among the concentrations were determined by unpaired Student t test. The P values <0.05 were considered significant.

Results

Hybridoma library screening using IFA and FACs

From a library of approximately 2000 hybridomas, 76 were identified by IFA to strongly react with cells. Five of the 76 hybridomas also had strong reactivity with Bel/fu cell surface by FACs analysis. These clones were further screened against several other carcinoma cell lines and normal cell lines by IFA and FACs. Ultimately, one clone (AC10364) was selected because it strongly reacted with multiple carcinoma cells and weakly reacted with normal cells (293T). Figure 1A and 1B shows the reactivity of AC10364 on Bel/fu and KATO III cells. Ig subclass typing indicated that AC10364 belonged to the IgG1 subtype.

Western blot analysis

As shown by Western blot in Figure 1C, AC10364 recognized proteins in cell lysates from multiple carcinoma cell lines including AGS, KATO-III, NCI-N87, MCF-7, Bel/fu, A2780, HeLa and HCT-8. In some cell lines, multiple strong bands were observed, suggesting that there are potentially multiple isoforms of the protein. Three cancer lines (SNU-1, Hs746T and SKOV3) are negative or have very weak reactivity against AC10364. Two normal cell lines (Fibroblast and 293T) are also negative or have very weak reactivity.

AC10364 inhibits cancer cell growth

The in vitro anti-tumor activity of AC10364 was evaluated using a proliferation/ inhibition assay with six different cell lines. As shown by the inhibition curves in Figure 2A, Bel/fu is the most sensitive cell line to AC10364 with an IC_{50} value of 78.7μg/ml. However, the maximum inhibition for Bel/fu only reaches 80% at the concentration of 1000μg/ml. Compared to Bel/fu, KATO-III and A2780 cells have weaker sensitivity to AC10364

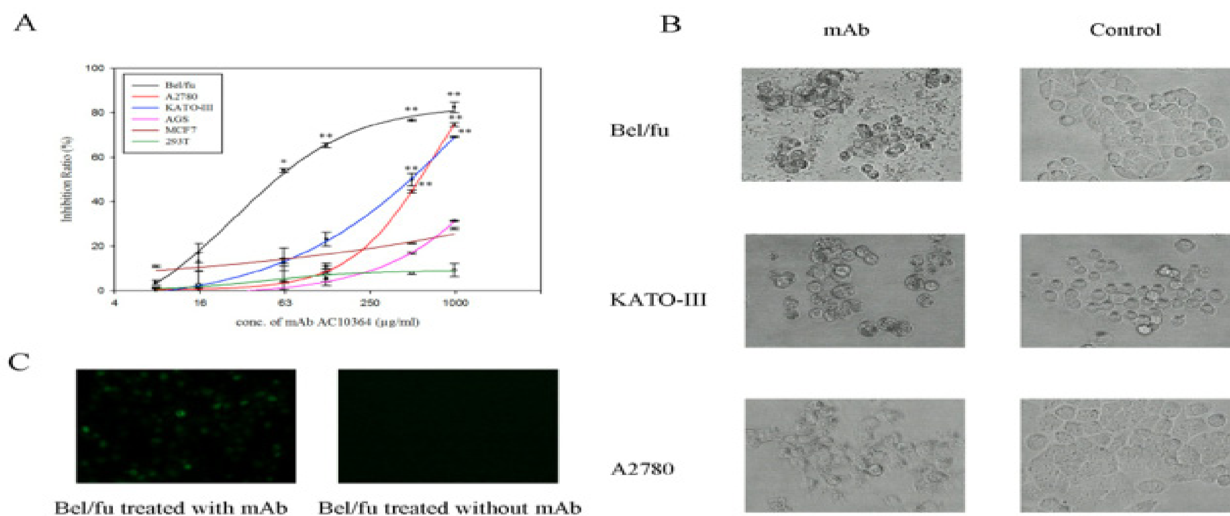

Figure 3. Combinatorial Effects of AC10364 and Chemotherapeutic Drugs. Data are inhibition (%) mean±SD of three independent experiments. **p*<0.05, ***p*<0.01. **A, B, C & D**: Final concentration of AC10364 is at 50μg/ml. **E & F**: VCR or ADM at final concentration of 10nM

with IC_{50} values of 420.2, 453.5μg/ml, respectively ($p<0.05$). Inhibition of KATO-III and A2780 cells at low Ab concentration (~100μg/ml) is very weak but increases at high Ab concentrations (above 300μg/ml), reaching 75% and 62% at 1000μg/ml, respectively. Inhibition of MCF-7 and AGS cells is very weak, reaching a maximum inhibition of approximately 30% inhibition. No inhibition was observed on the normal cell line 293T.

Figure 2B shows the morphological changes in Bel/fu, KATO-III and A2780 cells after treatment with mAb AC10364 (500μg/ml) as observed under a phase-contrast microscope. Cell growth actually was halted 24h after mAb treatment. Moreover, mAb AC10364 induced apoptosis of Bel/fu cells at high Ab concentration (500μg/ml) as shown by the green staining indicating caspase 3/7 activity that is observable 10h after mAb treatment (500μg/ml) (Figure 2C).

Sensitization of cancer cells to chemotherapeutic drugs by AC10364

To evaluate the potential of AC10364 to increase the sensitivity of cancer cells to chemotherapeutic drugs, AC10364 was used in combination with two commonly used anti-cancer drugs (Figure 3). The IC_{50} of VCR or ADM alone against Bel/fu cells were 65.3 and 104.8nM, respectively (Table 1 and Figure 3A, B). Pretreatment of Bel/fu with mAb AC10364 (50μg/ml) significantly lowered the concentrations of both VCR and ADM to achieve the same levels of inhibition (Figure 3A, B) ($p<0.05$). With 1mg/ml of AC10364, the IC_{50} value decreased from 65.3nM to 0.2nM for VCR and from 104.8nM to 0.46nM for ADM (Table 1). Similarly, AC10364 treatment (50μg/ml) sensitizes A2780 cells to VCR and ADM (Figure 3C, D) ($p<0.05$). Most interestingly, AC10364 can dramatically increase the efficacy of ADM at lower concentration (Figure 3D) ($p<0.05$). With ADM alone, a concentration of 9000nM is required to achieve the maximum inhibition of close to 100%, while in combination with AC10364 (50μg/ml) approximately 50nM of ADM can achieve maximum inhibition for A2780. This is approximately a 200-fold reduction of ADM concentration to achieve the maximum inhibition. Since AC10364 alone has less than 10% inhibition at 50μg/ml (Figure 2A) and ADM alone has less than 30% inhibition at 50nM (Figure 3D), the combination data suggest a synergistic effect between AC10364 and ADM on A2780 cells. IC_{50} value of mAb AC10364 against Bel/fu or A2780 cells was decreased after combination with VCR or ADM (10nM) ($p<0.05$; Figure 3E, F). Compared with mAb or chemotherapeutic drugs used alone, the combination of mAb AC10364 with chemotherapeutic drugs demonstrated enhanced growth inhibitory effect on carcinoma cells.

Discussion

Therapeutic anticancer mAbs currently used in clinics usually recognize extracellular or cell surface proteins, which constitute only a small fraction of the cellular proteins and are not tumor-specific (Chao et al., 2010; Scott et al., 2012; Weiner et al., 2012). Trastuzumab is clinically efficient for advanced chemotherapy of refractory breast cancer (Yip and Ward, 2002) and rituximab for treatment of non-Hodgkin's lymphoma (Grillo-Lopez et al., 2002). In cancer immunotherapy, effective therapeutic Abs are typically thought to require both cytostatic and cytotoxic abilities (Parren and van de Winkel, 2008). In this study, we screened a mAb library against carcinoma cells by IFA and FACs and identified mAbs that had strong reactivity with multiple carcinoma cells and weak reactivity with normal cells. Ultimately, we identified one mAb that significantly inhibited growth of multiple carcinoma cells in vitro.

A number of recently developed mAbs can kill target cells by a mechanism called oncosis (Loo et al., 2007; Zhang et al., 2010; Hernandez et al., 2011). Oncosis, as a mechanism of killing, requires a far higher concentration of mAb than is typi¬cally required for ADCC and CDC. Cell death by apoptosis leads to necrosis with karyorrhexis; however, cell death by oncosis may result

in necrosis with karyolysis (Majno and Joris, 1995). Our mAb AC10364 was able to inhibit carcinoma cell growth at high concentration of 1mg/ml. At the concentrations more than 400μg/ml, the inhibition rates on A2780 and KATO-III cells were up to 50% except AGS and MCF-7 cells. Fortunately, the mAb has no significant effect on normal cells, such as 293T, at high concentration of 1mg/ml.

The activation of caspases leads to irreversible biochemical and morphological changes in cells. Caspase-3 has been reported to contribute mainly to the characteristic morphologic changes in apoptotic cells including membrane blebbing, chromatin condensation and DNA fragmentation (Thornberry and Lazebnik, 1998). It is the first of all effector caspases activated for amplifying downstream apoptotic process. The activation of caspase-3 is a very rapid process in the cell death process (Tyas et al., 2000) which is associated with mitochondrial membrane permeabilization (Giansanti et al., 2011). In the study, apoptosis was induced in Bel/fu cells in as little as 10h after mAb treatment (Figure 2C). Furthermore, combination treatment with mAb and chemotherapeutic drugs was more effective than either agent alone. Jiang et al. (2013) examined the effect of embelin on the sensitivity of the A549 (non-small cell lung cancer cell line) to tumor necrosis factor-related apoptosis-inducing ligand receptor 2 (TRAIL R2) mAb, and concluded embelin could enhance TRAIL-induced apoptosis in A549 cells and the synergistic effect of the combination treatment might be due to modulation of multiple components in the TRAIL receptor-mediated apoptotic signaling pathway. These biological effects provide a basis for mAb/chemotherapeutic drug combination therapy, and also help explain the underlying mechanisms of combined efficacy (Xu et al., 2011). These results suggest mAb/chemotherapeutic drug combination therapy might be used as a new strategy for cancer therapy, and further studies are clearly required to explore this possibility.

In conclusion, we developed a novel mAb which significantly inhibited cell growth in multiple carcinoma cell lines and directly induced carcinoma cell apoptosis. Most importantly, the sensitization and synergistic effect of mAb AC10364 with chemotherapeutic drugs indicates that the mAb is an excellent candidate for cancer therapy.

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