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Research Article

Relationship between ganglioside expression and anti-cancer effects of a plant-derived antibody in breast cancer cells

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Abstract Production of therapeutic monoclonal antibodies (mAbs) using a plant platform has been considered an alternative to the mammalian cell-based production system. A plant-derived mAb CO17-1AK (mAb^P COK) can specifically bind to various types of cancer cell lines. The target protein of mAb^P COK is the epithelial cell adhesion molecule (EpCAM) highly expressed in human epithelial cancer cells, including breast and colorectal cancer cells. It has been hypothesized that its overexpression supports tumor growth and metastasis. A ganglioside is extended well beyond the surfaces of the various cell membranes and has roles in cell growth, inflammation, differentiation, and carcinogenesis. However, the regulation of EpCAM gene expression in breast cancers and the role of gangliosides in oncogenesis are unclear. Here, the purpose of this study was to determine the effects of mAb^P COK on human breast cancer cell proliferation, apoptosis, and ganglioside expression patterns. Our results show that treatment with mAb^P COK suppressed the growth of breast cancer cells and induced apoptotic cell death. It also upregulated the expression of metastasis-related gangliosides in breast cancer cells. Thus, treatment with mAb^P COK may have chemo-preventive therapeutic effects against human breast cancer.

Keywords Anti-cancer, Apoptosis, Breast carcinoma, Ganglioside, Plant-derived antibody CO17-1A

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Introduction

Breast cancer is the most common cancer affecting women, with an estimated 1.38 million new cases reported per year worldwide (Eccles et al. 2013). In addition, the worldwide incidence rate of breast cancer has increased steadily (Kim et al. 2010). Despite advances in therapeutic treatments, about 40% of patients still suffer from this disease mainly due to metastasis (Siegel et al. 2012).

Epithelial cell adhesion molecule (EpCAM), also known as KS1/4, gp40, GA733-2, 17-1A, and TROP-1, is one of the most well-known target antigenic proteins of human cancers (Baeuerle and Gires 2007; Strnad et al. 1989; Trizpis et al. 2007). It is a 40 kDa type I membrane glycoprotein that serves as a homotypic adhesion molecule extravagantly expressed in primary tumors (Balzar et al. 1999; Lintvinov et al. 1994) and up-regulated on actively proliferating epithelial tissues, during adult liver regeneration, and on many epithelial cell-derived carcinoma (Schnell et al. 2013; de Boer et al. 1999). It is closely related to metastasis of many epithelial tumors, particularly of adenocarcinoma (Spizzo et al. 2011; Went et al. 2004). EpCAM is highly expressed in cancer

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stem cells of pancreatic and colorectal adenocarcinomas as well as breast carcinomas (Dalerba et al. 2007; Li et al. 2007; Al-Hajj et al. 2003).

Various anti-EpCAM antibodies have been recently investigated in clinical studies as immunotherapeutic agents that mediate antibody-dependent cell-mediated cytotoxicity (ADCC) and induce complement-mediated cytotoxicity (CDC) (Münz et al. 2010; Moldenhauer et al. 2012). Despite the demand for therapeutic mAbs, current mammalian cell-based production systems provide only limited amounts (Brodzik et al. 2006). Plants offer several advantages for mAb production such as absence of contamination by pathogens affecting humans and relatively low cost of cultivation (Gomord et al. 2004; Ma et al. 2003). Therefore, these plant systems have been regarded as alternatives for recombinant mAb production (Daniell et al. 2001).

Meanwhile, recombinant anti-colorectal cancer mAb CO17-1A has been produced in tobacco plants with stable gene insertion through seedlings (Koprowski and Yusibov 2001; Verch et al. 1998). The plant-derived mAb (mAb^P) CO17-1A can uniquely recognize and bind to various colorectal carcinoma and effectively inhibit xenotransplanted colorectal cancer cell tumor growth in nude mice to the same extent as does the mammalian mAb CO17-1A (Ko et al. 2005).

Gangliosides are a subclass of acidic glycosphingolipids carrying one or more sialic acid residues in the carbohydrate moiety (Todeschini and Hakomori 2008). They can regulate signal transduction pathways involved in cell-cell adhesion, differentiation, cell proliferation, and recognition (Furukawa 1996; Julien et al. 2013; Ju et al. 2005; Lee et al. 2010). Sialyated glycosphingolipids from the ganglio-series are normally classified into four series from 0- to c-series and shed actively into the tumor microenviroment (Svennerholm 1980; Kudo et al. 2003). Human normal tissues express various gangliosides of 0- and a-series (Ji et al. 2000; Yamashita et al. 1999) and its complex expression is increased under manifold pathological disorders (Ariga et al. 2008), immune diseases (Shahrizaila and Yuki 2011), and cancer (Bobowski et al. 2012). GD3 has been considered a cell death effector because of its ability to interact with mitochondria-mediated apoptosome activation and subsequent apoptosis based on death ligands (Paris et al. 2002). In support, GD3 can induce apoptosis of human colorectal cancer cells and human breast cancer cells (Basu et al. 2004). It has been reported that GD1b can induce apoptosis of human breast carcinoma SKBR3 cells (Ma et al. 2004) and GD1a has an important role in the growth and differentiation of tissues in carcinogenesis (David and Michael 2005). However, the underlying role of GD1b in inducing apoptosis of tumor cells still remains unclear. In breast cancer, the effects of mAb^P CO17-1A on expression patterns of ganglioside and apoptotic cell death have not yet been reported.

Thus, this research is designed to determine the anticancer effects of mAb^P COK on cancer cell growth, proliferation, apoptosis, and the pattern of gangliosides in breast carcinoma. In the results, we showed that anti-colorectal cancer mAb^P COK not only suppress and inhibit cancer cell growth and proliferation but also induce apoptosis in breast cancer cells. For the first time, we found that the target gangliosides, such as GD1a and GM1, were expressed in human breast cancer after treatment with mAb^P COK.

Material and Methods

Preparation of plant material

The cDNA fragments encoding a heavy chain (HC) fused to the ER retention signal KDEL (HCK) and light chain (LC) of anti-colorectal cancer mAb CO17-1AK have been successfully cloned to a pBI COK vector (Fig. 1A) (Song et al. 2018). *Agrobacterium*-mediated plant transformation was conducted to generate transgenic *Arabidopsis* lines expressing mAb CO17-1AK as previously described (Paris et al. 2002). Transgenic seedlings were selected on MS media containing kanamycin (50 μg/mL), transplanted, and grown in fresh soil.

Purification of anti-colorectal cancer monoclonal antibody

Transgenic *Arabidopsis* leaves (200 grams) were freshly harvested and homogenized in an 800 mL extraction buffer (37.5 mM Tris-HCl pH 7.5, 50 mM NaCl, 15 mM EDTA, 75 mM sodium citrate, 0.2% sodium thiosulfate) using a HR2094 grinder (Philips, Seoul, Korea). The homogenized leaf extract was centrifuged at 8,800 × *g* for 30 min at 4°C and the supernatant was filtered through a Miracloth (Biosciences, La Jolla, CA, USA). After centrifugation at 10,200 × *g* for 30 min, supernatants were filtered through a Miracloth. The filtered solution was adjusted to pH 7.0 with 3 M Tris-HCl and mixed with ammonium sulfate to a concentration up to 8%. After centrifugation at 8,800 × *g* for 30 min at 4°C the supernatant was mixed with ammonium sulfate to a concentration of 24% and incubated overnight at 4°C. After centrifugation, the pellet was resuspended in one-twelfth of the volume of the starting extraction buffer. Then, the solution was centrifuged. The

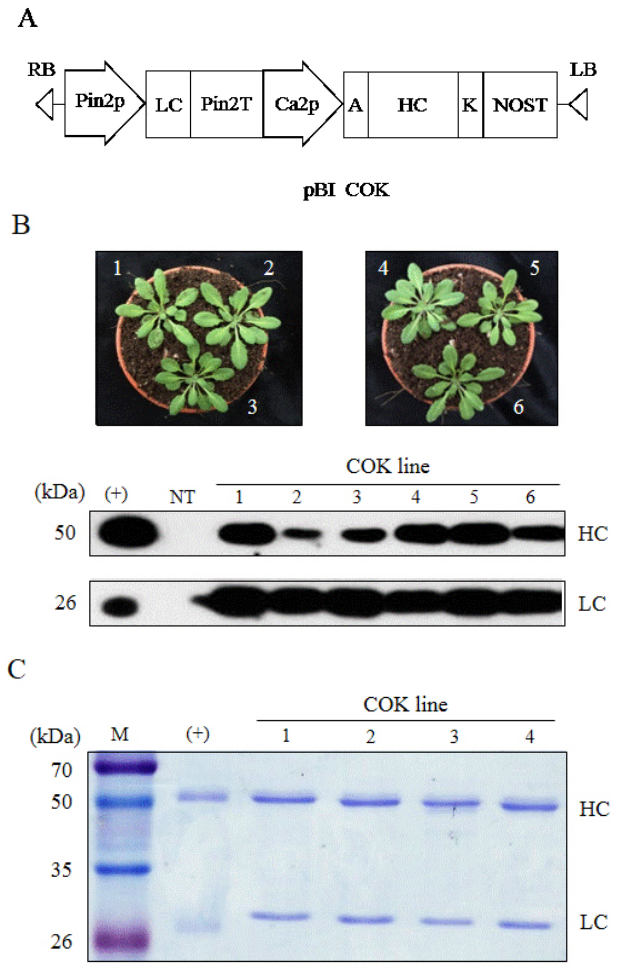

Fig. 1 Expression and purification of mAb COK from the transgenic *Arabidopsis* plant
(A) Schematic diagram of a plant expression cassette for mAb COK HC and LC for the transformation of *Arabidopsis* plant. Pin2p, promotor of Pin2 gene from potato; and Ca2p, cauliflower mosaic virus 35S promotor; K, KDEL, the 3' endoplasmic reticulum retention motif; A, an alfalfa mosaic virus untranslated leader sequence of RNA4; Pin2T, terminator of Pin2 gene from potato; NOST, terminator of nopaline synthase gene. (B) T_1 transformed shoots survived under kanamycin selection were transplanted to a soil pot and grown in the growth chamber (upper). Western blot analysis of T_1 generation plants to confirm HC and LC gene expression. HC and LC were detected with horseradish peroxidase-conjugated goat anti-murine IgG Fc- or IgG F(ab')$_2$-specific antibodies, respectively (lower). (+), positive control: mammalian-derived anti-EpCAM mAb; NT, non-transgenic plant; Lane 3-8, T_1 transformants expressing anti-colorectal cancer mAb COK. (C) SDS-PAGE analysis of purified samples from transgenic *Arabidopsis* expressing mAb COK (lanes 1, 2, 3, and 4). M, protein marker; (+), anti-EpCAM mAb; Lanes 3-6, purified after protein A affinity purification process

supernatant was filtered through a Miracloth and applied to an affinity column (GE Healthcare, Piscataway, NJ, USA) as described at manufacturer's instruments. The eluted mAb COK protein was dialyzed with 1 × PBS (pH 7.4) twice at 4°C for 3 h. Protein concentration was determined with a nano-drop (Biotek, Highland, VT). For further study, the dialyzed mAb COK was stored at -80°C deep freezer.

Cell culture

Human breast cancer MCF-7 cell was kindly provided from the Korean Cell Line Bank (KCLB, Seoul, Korea) and maintained in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL each of penicillin and streptomycin, at 37°C in 5% CO_2 incubator. RAW264.7 cells were also provided from the KCLB and maintained in Dullbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) FBS, 100 U/mL each of penicillin and streptomycin, at 37°C in 5% CO_2 incubator.

Cell viability (MTS) assay

CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS) was conducted to investigate the effects of the mAb COK on cell viability (Promega, Beijing, China) as described at manufacturer's recommendations. For 24 h, MCF-7 cells were seeded into a 96-well plate and treated with mAb COK (0-16.0 μg/mL), RAW264.7 cells, or RAW264.7 cells and mAb COK (0-16.0 μg/mL) for 8 h. Then MTS reagent (20 μL) was applied to each well followed by incubation in 5% CO_2 humidified air at 37℃ for 3 h. The absorbance at 490 nm was recorded using a TECAN Sunrise™ absorbance reader (Tecan Group Ltd, Männedorf, Switzerland).

Immunocytochemistry

To detect specific ganglioside GD1a, MCF-7 human breast cancer cells were blocked 5% (v/v) bovine serum albumin in PBS (BSA/PBS) and incubated with mAb COK at 4°C overnight. Probed cells were reacted with a fluorescence-conjugated secondary antibody and FITC-conjugated anti-murine IgG for the mAb COK at a 1:500 dilutions. Stained cells were visualized using a confocal microscope (Carl Zeiss Gmbh, Jena, Germany).

Immunoblot analysis

Leaves of T_1 plants were sampled for immunoblot analysis to confirm the expression of mAb HC and LC proteins. The leaf samples (50 mg) were frozen in liquid nitrogen and crushed immediately, and then suspended in 100 μL of sample buffer (1 M Tris-HCl, 50% glycerol, 10% SDS,

5% 2-mercaptoethanol, 0.1% bromophenol blue). The samples were boiled for 10 min and cooled on ice. Total soluble proteins were separated by 10% SDS-PAGE and electro-transferred to a nitrocellulose membrane (Millipore, Billerica, MA, USA). The membranes were incubated with 5% skimmed milk (Sigma, St. Louis, MO, USA) overnight at 4°C. The membranes were incubated with HRP-conjugated goat anti-mouse IgG Fcγ and anti-mouse IgG F(ab)'$_2$ to recognize the HC and LC of mAb COK, respectively. After washing three times with 1 × PBST buffer (1 × PBS plus 0.5% Tween-20, v/v, 10 min for each wash), proteins were detected with using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA) and X-ray film (Fuji, Tokyo, Japan). Leaves of wild type *Arabidopsis* were used as a negative control.

To investigate diverse protein expression of MCF-7 breast cancer cells by immunoblotting, whole-cell lysates were prepared using a radioimmunoprecipitation assay (RIPA) buffer (Sigma) containing mammalian protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Protein concentrations were determined using the Bradford method. Extracted proteins samples (40 μg) were separated by 10% or 12% SDS-PAGE and then transferred to membranes, which were incubated overnight at 4°C with specific antibodies. The protein bands were visualized after horseradish peroxidase-conjugated secondary antibody incubation, using a Priece ECL Western Blotting Substrate Plus (Thermo scientific, Rockford, IL, USA).

Extraction and purification of gangliosides

Gangliosides were extracted from cells as previously described (Lee et al. 2007). Total lipids were extracted with chloroform/methanol (1:1, v/v). Subsequently, neutral lipids were filtered off with 20 mL of chloroform/methanol/H_2O (15:30:4, v/v) by applying DEAE Sephadex A25 column (Sigma), and then acidic lipids were extracted with 15 mL chloroform/methanol/0.8 M sodium acetate (15:30:4, v/v). The eluted samples were dried with N_2 gas at 30°C, then dissolved in chloroform/methanol (1:1, v/v), neutralized with 12N NH_4OH overnight at room temperature. After the neutralized samples were dried again with N_2 gas at 30°C, dried samples were dissolved in distilled water, and the salt was removed with a Sep-Pak C18 cartridge (Millipore) to obtain gangliosides. Finally, eluted gangliosides were dried with N_2 gas at 30°C for 4 h. Dried samples were stored at -80°C until the next use.

High-performance thin-layer chromatography (HPTLC)

HPTLC analysis was performed to analyze ganglioside profile, as described previously (Lee et al. 2007). The eluted gangliosides with chloroform/methanol (1:1, v/v) were run on HPTLC plates and the plates developed with chloroform/methanol/0.25% $CaCl_2 \cdot H_2O$ (50:40:10, v/v). The developed gangliosides were stained with resorcinol solution (HCl, 0.1M $CuSO_4 \cdot 5H_2O$, resorcinol, distilled warter). Monosialoganglioside Mixture (Matreya LLC, State College, PA, USA) and disialoganglioside Mixture (Matreya LLC) were used as standard markers for individual ganglioside species.

Data analysis

Results were presented as mean ± standard deviation of at least three independent experiments performed in triplicates. Data were analyzed for statistical significance using one-way analysis of variance. *P*-value of less than 0.05 was considered statistically significant.

Results

Purification of plant-derived monoclonal antibody CO17-1AK (mAb^P COK) from transgenic *Arabidopsis*

Arabidopsis transgenic plants were obtained by *Agrobacterium*-mediated transformation with a plant expression vector carrying the cDNA encoding the heavy chain (HC) and light chain (LC) of mAb CO17-1AK (Fig. 1A, B). The presence of HC and LC genes was confirmed in genomic DNA isolated from transgenic plants by reverse transcription PCR (data not shown). Immunoblot analysis confirmed the expression of both HC (50 kDa) and LC (26 kDa) of mAb^P COK in transgenic plants. The binding activity of mAb^P COK to human colon cancer cells was confirmed by Cell ELISA (Ko et al. 2005). In SDS-PAGE analysis, the purified samples fraction #1, 2, 3, and 4 from transgenic lines showed two major bands (50 and 26 kDa, respectively) similar to anti-EpCAM mAb (Fig. 1C).

Effect of mAb COK on the viability of MCF-7 breast cancer cells

Increasing immuno-stimulatory activation of macrophages targeting cancer has been recognized as a new immunotherapeutic target (Klimp et al. 2002), in particular activated macrophages are well known to act as mediator of tumor

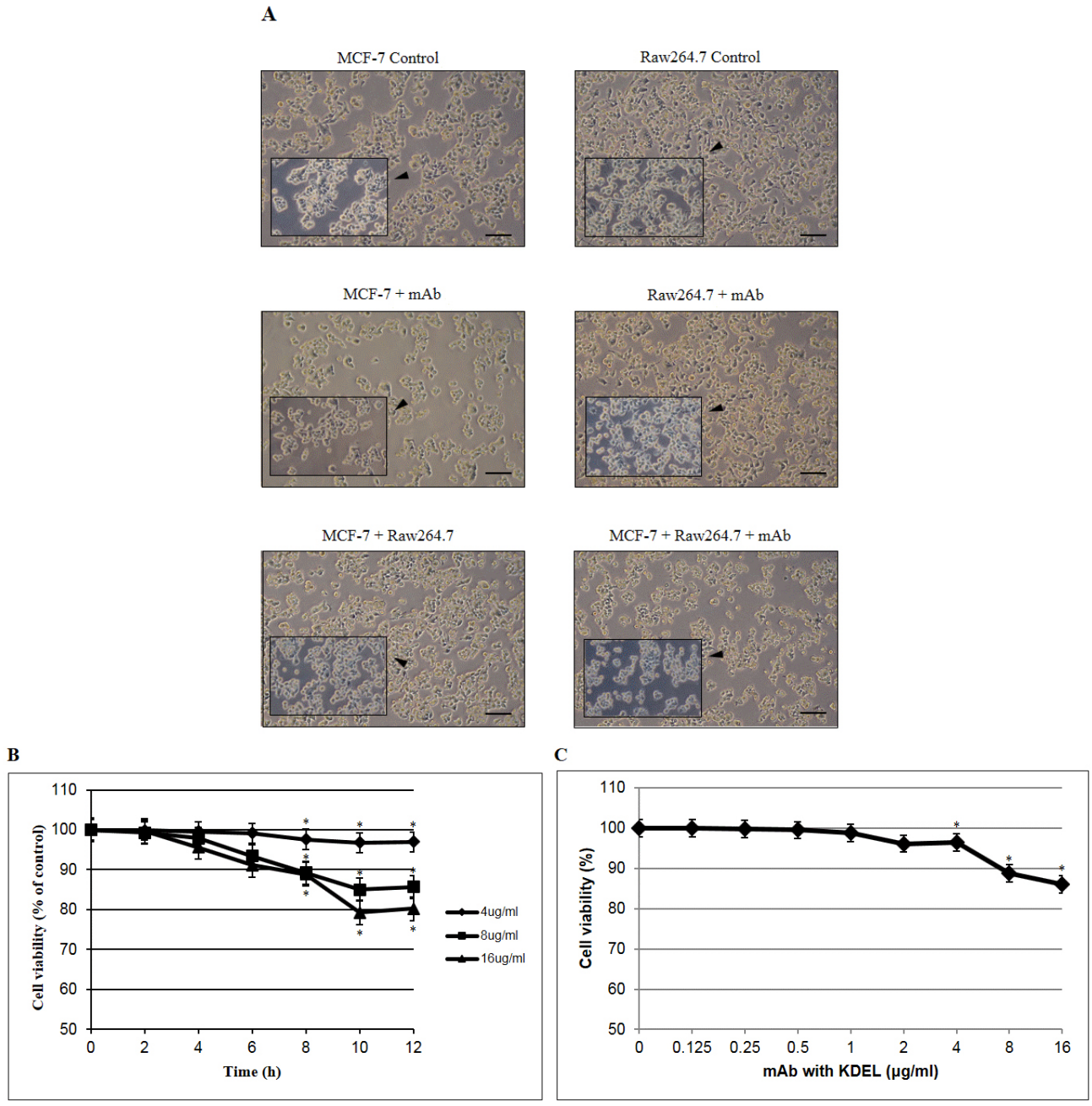

Fig. 2 Cytotoxicity of mAb COK to MCF-7 breast cancer cells
Morphological changes in MCF-7 human breast cancer cells treated with both mAb COK and RAW264.7 cells. (A) Morphology of MCF-7 human breast cancer cells. (B) Viability of MCF-7 human breast cancer cells treated with mAb COK (0, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 μg/mL) and RAW264.7 cells for 8 hours. The treatment ratio of MCF-7 human breast cancer cells to RAW264.7 cells was 1:10. (C) MTS assay for cell viability was performed with increasing concentrations of mAb COK and different time courses. Values are represented as mean ± standard deviation (SD) of at least three independent experiments. *$P < 0.05$. *Scale bar*, 100 μm

cell death (Keller et al. 1990). To assess whether immunoreaction of mAb COK with RAW264.7 cells is affected to cancer cell growth, the inhibitory effect of mAb COK on MCF-7 breast cacner cell growth was performed and the inhibition of cell proliferation by the immunoreaction of both treatments was observed (Fig. 2A). As shown in Figure 2A, no significant change of cell growth was confirmed for MCF-7 breast cancer cell treated with RAW264.7 cell. However, the growth of the MCF-7 breast cancer was remarkably decreased in the mAb COK treatment group. It is indicated that mAb COK induces immune function activity in RAW264.7 cells against breast cancer cells.

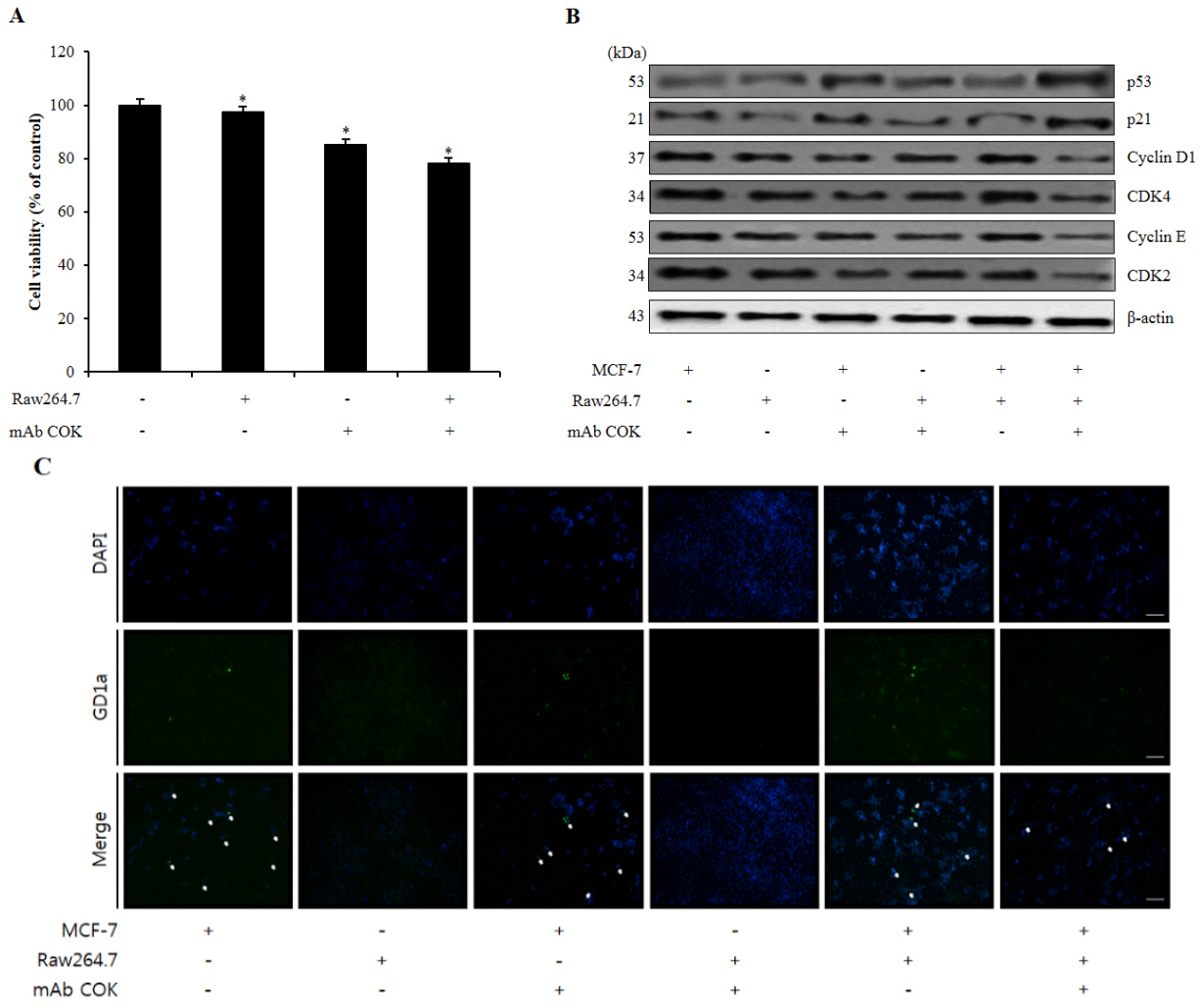

Fig. 3 Effect of mAb COK on cell proliferation and expression level of cell cycle proteins in MCF-7 cancer cells
(A) MCF-7 cells were seeded into plates at a cell density of 5.0×10^4 cells/well and treated with mAb COK and RAW264.7 cells for 8 hours and cell viability was determined by 96-well MTS assay. (B) Levels of p53, p21, and G1 to S phase-related proteins in MCF-7 human breast cancer cells were examined by western blot analysis using specific antibodies. (C) Changes of ganglioside expression in cancer cells were stained by double immunofluorescence. Treatment with anti-EpCAM mAb (10 μg/mL) for 8 h. Values are represented as mean ± SD of at least three independent experiments. $*P < 0.05$. β-actin was used to indicate the amount of loading protein

Then, MTS assay was performed to investigate the effect of mAb COK on the viability of breast cancer cell line (Fig. 2B, C). First, the concentration of mAb COK required to obtain a significant inhibitory effect on the viability of MCF-7 breast cancer cells was determined by incubating the cells with 0 to 16.0 μg/mL of mAb COK. Its IC_{50} value for inhibiting the viability of MCF-7 human breast cancer cells was 10.0 μg/mL. Significant apoptotic effects on MCF-7 breast cancer cells were observed at a concentration of 8.0 to 16.0 μg/mL. Results showed that the viability of MCF-7 breast cancer cells was decreased by mAb COK in a concentration- and time-dependent manner. To observe morphological changes, MCF-7 cells were treated with 4.0 - 16.0 μg/mL of mAb COK for 8 h. Results showed that cell size was decreased after treatment with mAb COK. After treatment, mAb COK, MCF-7 breast cancer cells steadily adopted a little round shape.

Effect of mAb COK on the proliferation of MCF-7 breast cancer cells

MCF-7 human breast cancer cells were treated with mAb COK, RAW264.7 cells, or RAW264.7 cells and mAb COK for 8 h to determine whether mAb COK inhibit the proliferation of MCF-7 breast cancer. Results showed that treatment with mAb COK resulted in reduced cell proliferation (Fig. 3A). We investigated the possible mechanisms involved in the inhibitory effect of mAb COK on the

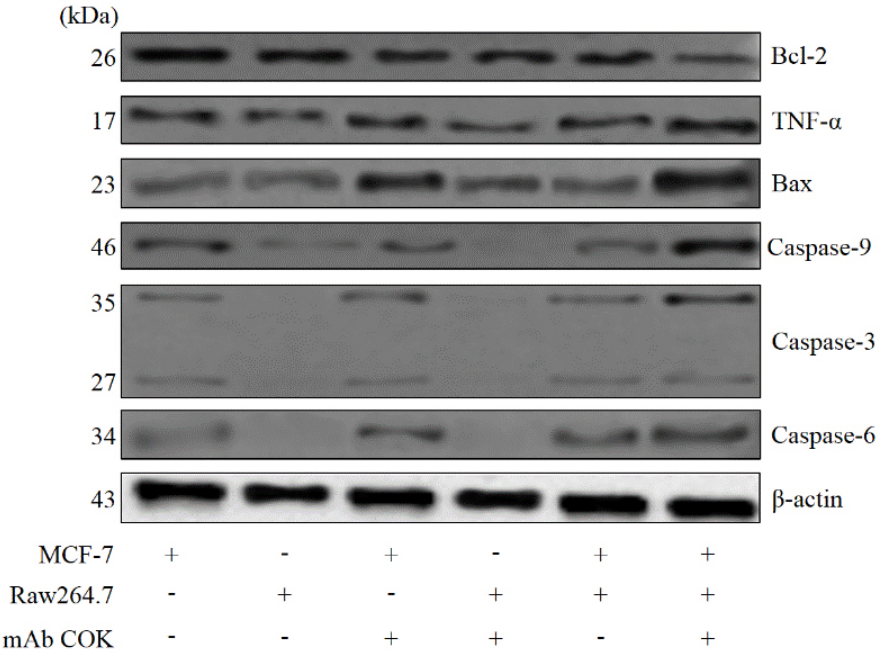

Fig. 4 Effect of mAb^P COK on the regulation of apoptosis in MCF-7 cells
Apoptotic regulatory protein expression in MCF-7 human breast cancer cells treated with both treatments. The level of anti-apoptotic protein and pro-apoptotic proteins were analyzed by immunoblot analysis using specific antibodies. β-actin was used to indicate the amount of loading protein. This experiment was repeated three times with similar results

proliferation of MCF-7 human breast cancer cells. Since cellular proliferation depends on the progression of the cell cycle, the effect of plant-derived mAb COK on cell cycle-related protein expression was determined by immunoblot analysis (Fig. 3B). MCF-7 cells treated with both treatments showed that a significant increase in p53 and p21 expression but reduction in the expression of proteins related with cell cycle control. These results suggest that plant-derived mAb COK can inhibit the growth of MCF-7 human breast cancer cells by inducing p53 and p21 while blocking the specific phase in cell cycle. In addition, the binding of mAb^P COK to GA733-2 antigen was determined by immuno-fluorescence. As shown in Figure 3C, the expression of ganglioside GD1a was found to be significantly decreased in MCF-7 human breast cancer cells treated with mAb^P COK and RAW264.7 cells, compared to untreated cells.

Effect of mAb COK on apoptotic regulatory proteins

To examine whether the inhibition of MCF-7 human breast cancer cell growth by anti-GA733-2 mAb could induce apoptosis, the effects of mAb^P COK treatment on the expression of apoptotic regulatory proteins were determined. We confirmed that treatment of MCF-7 cells with both treatments resulted in reduced expression of anti-apoptotic protein compared to untreated MCF-7 cells. On the contrary, expression levels of pro-apoptotic proteins such as TNF-α, Bax, caspase-3, caspase-6, and caspase-9 increased after treatment with plant-derived mAb COK and RAW264.7 cells (Fig. 4). Moreover, no effect of itself mAb^P COK on the activity of those proteins on cell apoptosis has been found (data not shown). These data indicate that the mAb^P COK treatment regulates the cell apoptosis proteins on human breast cancer cells.

Effect of mAb COK on ganglioside expression

Whether mAb COK corresponds to be altered ganglioside expression was determined by HPTLC in six different groups: untreated MCF-7 breast cancer cells (lane 3), untreated RAW264.7 cells (lane 4), MCF-7 breast cancer cells treated with mAb^P COK (lane 5), RAW264.7 cells treated with mAb^P COK (lane 6), MCF-7 breast cancer cells treated with RAW264.7 cells (lane 7), and MCF-7 breast cancer cells treated with both mAb^P COK and RAW264.7 cells (lane 8). As shown from HPTLC in Figure 5, the expression level of two other gangliosides, GD1a and GM1, was well detected in cells treated with both treatments (lane 8). No expression of ganglioside GD2 or GT1b was observed in any group. These data suggest that gangliosides GD1a and GM1 are related to mAb^P COK-mediated anti-proliferation and apoptotic cell death.

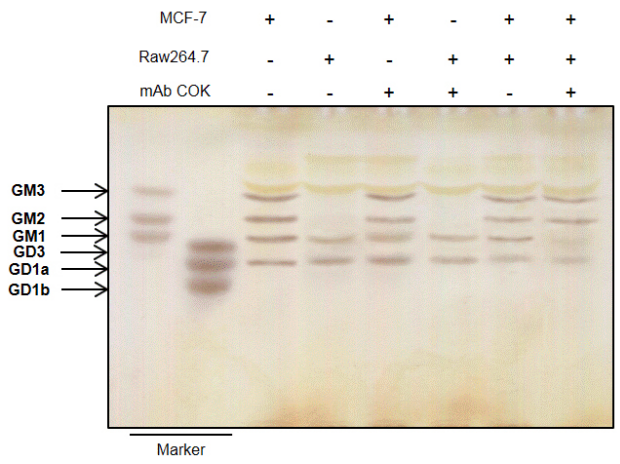

Fig. 5 HPTLC profiles of gangliosides in MCF-7 human breast cancer cells treated with mAb^P COK
Quantitative analysis of extracted glycosphingolipid in MCF-7 human breast cancer cells was performed by resolving them on TLC silica gel plates with chloroform/methanol/0.25% $CaCl_2 \cdot H_2O$ (50:40:10, v/v). The gangliosides were visualized using resorcinol spraying. Lanes 1 and 2, ganglioside standard markers; lane 3, untreated MCF-7 breast cancer cells; lane 4, untreated RAW264.7 cells; lane 5, MCF-7 breast cancer cells treated with mAb^P COK; lane 6, RAW264.7 cells treated with mAb^P COK; lane 7, MCF-7 breast cancer cells treated with the RAW264.7 cells; lane 8, MCF-7 breast cancer cells treated with both treatments

Discussion

We have previously shown that mAb^P COK expressed in tobacco plants suppressed cancer cell proliferation and changed the expression pattern of certain gangliosides, such as GD1a and GM1, in colorectal cancer cells (Ryu et al. 2013). A previous study has reported that anti-cancer mAbs produced by transgenic *Arabidopsis* plants specifically recognize the tumor-associated antigen GA733-2 (Song et al. 2018). In this study, we investigated the bioactivity of mAb^P COK expressed in transgenic *Arabidopsis* plants on anti-cancer effects and the expression change of glycosphingolipid in breast carcinoma, not colorectal carcinoma.

The GA733-2 has been considered as a major gastrointestinal tumor-associated protein amply expressed in colorectal cancers (Wengi et al. 2009). GA733-2 expression levels are also correlated with intestinal cell proliferation and reversely with differentiation (Zhang et al. 2016). Because of this, GA733-2 has been elucidated as a marvelous candidate for anti-cancer therapeutic agent due to its potential clinical significance (Staib et al. 2001). Ryu et al (2013) have suggested that transgenic plant-derived mAb COK and mAb^M COK have a remarkable similarity in binding human colorectal cancer cells. The mAb COK is as effective as mAb^M COK in suppressing the proliferation and tumor growth in *in vivo* (Ko et al. 2005).

Various chemotherapeutic agents on cancers are regarded as the suppression of cell growth by affecting cell cycle (Alfonso et al. 2009; Xiao et al. 2005). In present study, we investigated the effects of mAb^P COK cell growth suppression and focused on the distribution cells to the various phases of the cell cycle. Treatment of mAb^P COK to MCF-7 cells resulted in decreased expression on proteins of the first of four phases in cell cycle proteins. Cyclin D1, known to induce cell migration, is essential at diverse phases of the cell cycle (Lapenna and Giordano 2009; Neumeister et al. 2003; Satyanarayana and Kaldis 2009). Immunoblot results suggested that expression levels of proteins, such as CDKs and cyclins, involved in cell cycle regulation, were downregulated in MCF-7 cells after treatment with both mAb^P COK and RAW264.7 cells in Figure 3. Besides p21, which functions as a regulator of specific cell cycle, was induced by treatment with both treatments.

Our findings in Figure 4 indicated that the mAb^P COK significantly and effectively induces apoptosis of MCF-7 cells. In MCF-7 cells treated with both mAb^P COK and RAW264.7 cells, caspase-3, caspase-6, and caspase-9 were significantly activated. Numerous anti-apoptotic and pro-apoptotic proteins are up- and down-regulated in many colorectal cancer (Coffey et al. 2002; Schoemaker et al. 2002). Zha et al (1996) demonstrated that Bax, an apoptosis regulator, plays a role in the mitochondrial apoptotic process and prevents inhibition of apoptosis in cancer cells. The researchers revealed that the apoptosis regulator also regulates cell death by families of protease enzymes (Seol et al. 2001). Thus, our results suggest that mAb^P COK efficaciously act as inducing apoptotic mechanisms.

Remarkably, HPTLC analysis indicated that both ganglioside GD1a and GM1 expression were highly decreased in MCF-7 cells treated with mAb^P COK and RAW264.7 cells. Shah et al (1996) have previously shown that specific gangliosides have anti-cancer activities on immunoreactions and tumor progression. Interestingly, researchers have showed that specific gangliosides has suppression effects on the metastasis of the colorectal carcinoma in *in vivo* (Vogel et al. 1996). Hyuga et al (1999) demonstrated that GD1a to regulate metastasis of cancer cells. Therefore, Figure 5 indicated that the ganglioside GD1a and GM1 expression can be decreased in MCF-7 cells treated with *Arabidopsis*-derived mAb^P COK.

As the final outcome, we found that the plant-derived mAb COK has various biological activities, including the regulation of gangliosides expression. Further clinical inves-

tigation is needed to examine the potential capabilities of mAb^P COK in treating human breast cancer.

Conclusions

Our current study suggests that the plant-derived anti-cancer monoclonal antibody COK has significant anticancer activities and works by inducing apoptosis, blocking the transition from the G1 to S phase of the cell cycle, and changing the expression pattern of gangliosides GD1a and GM1. *Arabidopsis* plant might be another alternative plant expression system for the production of anti-cancer therapeutic mAb.

Conflict of Interest

The authors have no conflict of interest.

Acknowledgments

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