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Expression and *in vitro* function of anti-cancer mAbs in transgenic *Arabidopsis thaliana*

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The anti-colorectal cancer monoclonal antibody CO17-1A (mAb CO), which recognizes the tumor-associated antigen EpCAM, was expressed in transgenic *Arabidopsis* plants. PCR and western blot analyses showed the insertion and expression of heavy chain (HC)/HC fused to the KDEL ER retention modif (HCK) and light chain (LC) of mAb CO and mAb CO with HCK (mAb COK) in *Arabidopsis* transformants. Both plant-derived mAb^P CO and mAb^P COK were purified from a biomass of approximately 1,000 seedlings grown in a greenhouse. In sandwich ELISA, both mAb^P CO showed a slightly higher binding affinity for the target, EpCAM, compared to mAb^M CO. In cell ELISA, both $mAbs^P$ COs showed binding affinity to the human colorectal cancer cell line SW480. Furthermore, mAb^M CO, mAb^P CO, and mAb^P COK exhibited dose and time-dependent regression effects on SW480 cells *in vitro*. In summation, both mAb^P CO and mAb^P COK, expressed in *Arabidopsis*, recognized the target antigen EpCAM and showed anti-proliferative activity against human colorectal cancer cells. [BMB Reports 2020; 53(4): 229-233]

INTRODUCTION

Colorectal cancer, presenting as an abnormal growth of malignant cells in the inner layer of the colon and rectum, is a very common, lethal disease, comprising 9 percent of all cancers worldwide (1). The demand for anti-colorectal cancer antibody reagents is steadily increasing. Immunotherapeutic recombinant proteins such as antibodies have been produced via fermentation systems using yeast, bacteria, and mammalian cells (2-5). However, these systems are known to have certain drawbacks related to bulk production, quality, and safety (6-9). Plants are considered as a promising alternative bioreactor source materials for the production of recombinant bio-pharmaceutical proteins via *in vivo* whole plant or *in vitro* plant cell platform techniques (6, 10-13). Production of anti-colorectal cancer mAbs in transgenic plants offers a promising avenue for providing their large quantities with comparatively free of human and animal contaminants at a low cost (9, 14). Therefore, the plant-derived recombinant products have been tested in early phase clinical trials to monitor safety and efficacy in use (15, 16).

Among diverse plant platforms, *Arabidopsis thaliana* plant has several strengths such as a relatively short life span, high total soluble protein (TSP) yields, and cost-effective transformation methods (17-19). The endoplasmic reticulum (ER) retrieval motif has been fused to the C-terminus of the heavy chain (HC) of mAb thereby accumulation in ER retention signal peptide for high yields of anti-colorectal cancer mAb (4, 13, 20).

In this study, anti-colorectal cancer mAb^Ps (mAb^PCO and mAb^PCOK) were expressed in *Arabidopsis*. The expression level and *in vitro* anti-cancer activities of the antibodies were compared between mAb^PCO and mAb^PCOK in *Arabidopsis* and mammalian-derived mAb CO17-1A (mAb^MCO) as a parental antibody. This is the first report that discussed the expression of functional anti-colorectal cancer antibodies mAbCO, and mAbCOK in *Arabidopsis* plants.

RESULTS

Generation of T_1 transgenic *Arabidopsis* plants to express mAb^PCO and mAb^PCOK

To investigate the effect of the ER retention motif (ERRM) on the expression and *in vitro* function of anti-colorectal cancer mAbs, both plant binary vectors, pBI CO17-1A (21) and pBI CO17-1AK (22), were delivered via *Agrobacterium tumefaciens* GV3101 to *Arabidopsis* to express the anti-colorectal cancer mAb^PCO and mAb^PCOK, respectively (Fig. 1A). The ERRM was added to the C-terminus of HC in pBI CO17-1AK in order to retain mAb CO in ER, thereby enhancing its accumulation in the plant cells. The expression levels of transgenic plants expressing mAb^PCO (CO) and mAb^PCOK (COK) were compared.

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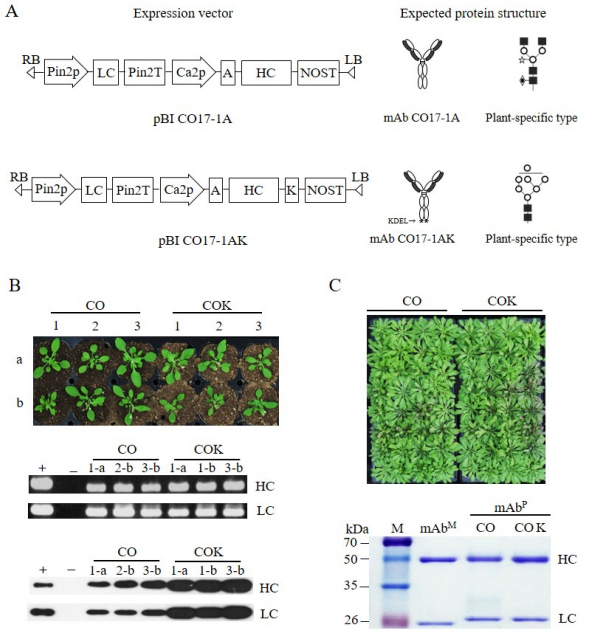

Fig. 1. Generation of transgenic *Arabidopsis* plant expressing anti-colorectal mAbs CO and COK, and purification of plant-derived mAb (mAb^P). (A) Schematic diagram of the mAb^{P}CO17-1A (mAb^{P}CO) and mAb^{P}CO17-1AK (mAb^{P}COK) gene expression cassette construction in a plant expression vector pBI121 used for the *Agrobacterium* floral dip transformation. The promoters Pin2p and Ca2p regulate the light and heavy chains, respectively. KDEL is the 3′ endoplasmic reticulum (ER) retention motif. Pin2p, promoter of *Pin2* from potato; Ca2p, cauliflower mosaic virus 35S promoter; A, an alfalfa mosaic virus untranslated leader sequence of RNA4; Pin2T, terminator of *Pin2* from potato; NOST, terminator of *nopaline synthase* (NOS). (B) Generation and identification of T_1 transformants expressing mAb^{P}CO and mAb^{P}COK using antibiotic selection, soil growth, PCR, and western blotting. Soil growth of transformants after T_1 seedlings was selected on MS media containing kanamycin (upper). Surviving seedlings were transferred to a pot and placed in a growth chamber with 16 hr of light and 8 hr of darkness at 23°C. Rosette leaves were sampled from T_1 seedlings to confirm target gene insertion using PCR (middle) and protein expression level using western blotting (bottom). (C) SDS-PAGE gel (bottom) to confirm purity of mAb^{P}CO and mAb^{P}COK, purified from transgenic *Arabidopsis* plant biomass (upper).

For *Arabidopsis* transformation, *Agrobacterium* was introduced to flowering plants using the floral-dip method (23), resulting eventually in mature seeds. Transgenic seedlings with green true leaves (20-30) were then selected from approximately 1,000 seeds germinated on *in vitro* germination media containing kanamycin. Most seeds sown in kanamycin-containing media germinated, but failed to produce true leaves and roots that were not transformants (Data not shown). In Agrobacterium-floral dip transformations with both pBI CO17-1A and pBI CO17-1AK expression vectors, the transformation rates were 1.8 and 2.1%, respectively. All putative, surviving seedlings with true leaves of CO (21) and COK (24) were grown in soil pots (Fig. 1B, upper). PCR detected HC and LC bands of the expected size in all tested CO and COK transgenic plants (Fig. 1B, middle). T_2 plants obtained from T_1 plants with high protein expression levels were used for bulk production of anti-colorectal cancer mAb from transgenic plants.

Expression and purification of mAb^{P}CO and mAb^{P}COK in *Arabidopsis*

HC and LC expression levels of both mAb CO and mAb COK in CO and COK *Arabidopsis* transgenic plants, respectively, were compared (Fig. 1B bottom). All seedlings with true leaves and PCR bands did not exhibit HC and LC expression in both CO and COK transgenic plants (data not shown). COK expression was significantly higher than that of CO (Fig. 1B bottom). HC and LC bands were not detected in non-transgenic *Arabidopsis* plant (−) (Fig. 1B bottom). Quantitative western blotting and nanodrop protein analyses indicated that the relative expression levels of CO and COK were 0.52 and 2.42, respectively (Data not shown). T_2 seeds of both transgenic *Arabidopsis* plants highly expressing anti-colorectal cancer mAb CO and mAb COK were sown, and T_2 seedlings were produced (Fig. 1C upper). Purified mAb^{P}CO and mAb^{P}COK were obtained from 300 g of fresh biomass containing both transgenic CO and COK plants. The amounts of purified mAb^{P}CO and mAb^{P}COK were 750 μg and 3,400 μg, respectively. HC and LC bands of purified mAb^{P}CO and mAb^{P}COK detected via SDS-PAGE were at the expected sizes of 50 and 25 kDa, respectively. The LC band sizes of both mAb^{P}CO and mAb^{P}COK were slightly higher than those of the LC of counterpart mAb^{M}CO (Fig. 1C bottom).

Binding activity of mAb^{P}CO and mAb^{P}COK to recombinant EpCAM molecules

Sandwich ELISA was conducted to reconfirm binding affinity to EpCAM as detailed in the schematic diagram (Fig. 2 right). Sandwich ELISA results indicated that both mAb^{P}CO and mAb^{P}COK had higher absorbance values compared to that of mAb^{M}CO, the positive control. Both mAb^{P}CO and mAb^{P}COK showed a higher absorbance value than the positive control, mAb^{M}CO. Absorbance values of both mAb^{P}CO and mAb^{P}COK were similar to EpCAM-associated ELISA results (Fig. 2).

Binding activity of purified mAb^{P}CO and mAb^{P}COK to SW480

Cell ELISA was performed to assess the binding affinities of mAb^{P}CO and mAb^{P}COK to SW480 (Fig. 3A). Among the 4 mAbs (mAb^{M}CO as a positive control, mAb^{P}SO as a negative control) mAb^{M}CO showed the highest absorbance values. Compared to mAb^{P}CO, mAb^{P}COK showed slightly higher binding affinity to SW480 cells. The absence of an absorbance value for mAb^{P}SO indicated the absence of binding affinity. To investigate the region of SW480 to which mAb^{P}CO, mAb^{P}COK, and mAb^{M}CO bind, immunocytochemical analysis (ICC) was performed (Fig. 3B). ICC results showed that mAb^{M}CO was

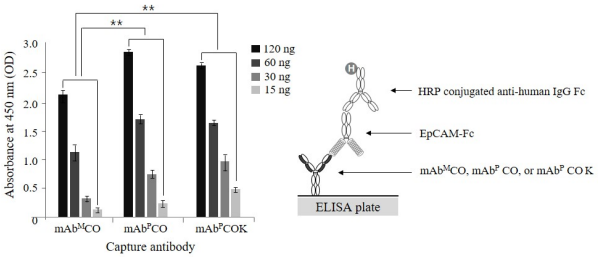

Fig. 2. ELISA analysis to confirm binding process of mAb^{M}CO, mAb^{P}CO, and mAb^{P}COK against the antigenic protein EpCAM-Fc. Sandwich ELISA to determine protein binding activity of anti-colorectal cancer mAbs to the EpCAM-Fc antigen molecule. Antibodies (mAb^{M}CO, mAb^{P}CO, and mAb^{P}COK) (15, 30, 60, and 120 ng gradient) were coated on Maxisorp 96-well microplates (Nunc, Roskilde, Denmark), and the epithelial cell adhesion molecule (EpCAM-Fc) antigen was placed in each well. HRP-conjugated goat anti-human IgG Fc fragment-specific antibody and TMB solution (KPL) were used to detect absorbance values. Absorbance at 450 nm was read using a UV-Vis microplate spectrophotometer (Biotek).

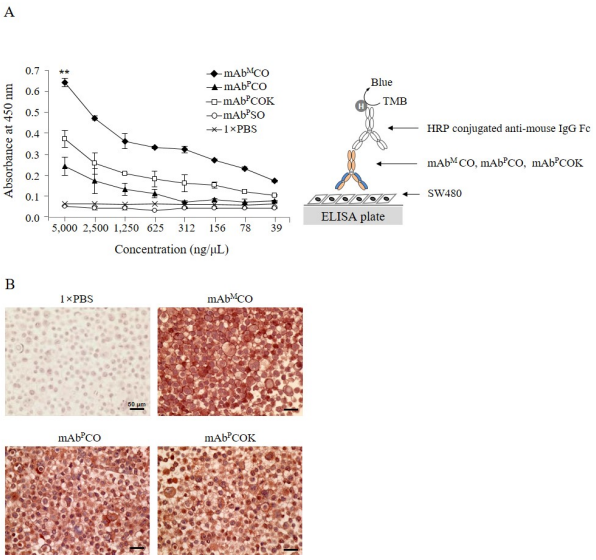

Fig. 3. Cell ELISA and immunocytochemistry to determine the binding affinity of mAb^{M}CO, mAb^{P}CO, and mAb^{P}COK for SW480 cells. (A) mAb^{M}CO, mAb^{P}CO, mAb^{P}COK, and the plant-derived anti-rabies mAbSO57 (mAb^{P}SO), serially diluted to a range from 5,000 ng · μl^{-1} to 39 ng · μl^{-1} were applied to ELISA plates coated with human SW480 cells showing high EpCAM (epithelial cell adhesion molecule) expression. The negative control was used 1×PBS. Absorbance at 450 nm was measured using the Epoch Microplate Reader (Biotek). (B) Immunocytochemistry was used to determine the affinity of mAb^{P}s (mAb^{P}CO and mAb^{P}COK for binding to SW480. SW480 cells were fixed in 10% formalin for 2 hr and processed for paraffin embedding. mAb^{M}CO, mAb^{P}CO, and mAb^{P}COK were used as primary antibodies. Samples were visualized with an HRP-conjugated goat anti-mouse/rabbit antibody and NovaRED (Dako) and counterstained with Mayer's hematoxylin (Muto Pure Chemicals CO., Tokyo, Japan). Positive control was mAb^{M}CO [magnification, X 400; BX53F (Olympus)]. mAb^{P}CO and mAb^{P}COK are shown where asterisks indicate significant differences ($P < 0.05$).

mainly bound to the surface membrane of SW480 cells, whereas both mAb^{P}CO and mAb^{P}COK were bound throughout SW480 cells (Fig. 3B). Both mAb^{P}CO and mAb^{P}COK exhibited similarities in the pattern of binding to SW480 cancer cells. The negative control groups (1 × PBS) did not bind to SW480 cells.

Dose- and time-dependent effects of mAb^{P}CO and mAb^{P}COK on cell growth inhibition of colorectal cancer cell line SW480

A dose-dependent tumor cell regression assay indicated that the number of intact live cancer cells was not significantly decreased by mAb^{M}CO, mAb^{P}CO, or mAb^{P}COK below the concentration of 250 ng (Fig. 4A, C). However, at concentrations above 500 ng per well, both mAb^{P}CO and mAb^{P}COK decreased the number of intact, live SW480 cells, to a value similar to that of parental mAb^{M}CO (Fig. 4A, C). A time-dependent tumor cell regression assay (Fig. 4B, D) indicated that inhibition of cell growth appeared approximately 4 hr after the anti-cancer antibody treatment (mAb^{M}CO, mAb^{P}CO, and mAb^{P}COK) (Fig. 4B, C). The lowest number of cells was observed at 6 hr after inoculation with the three anti-cancer mAbs. After 6 hr, the cancer cells appeared to proliferate again. 1× PBS treatment, as a negative control did not reduce the number of cancer cells.

DISCUSSION

The current study explored the expression and *in vitro* function of anti-colorectal cancer mAbs produced in transgenic *Arabidopsis* plants. The *HC*/*HCK* and *LC* genes of the recombinant therapeutic protein mAb CO, which recognizes the target antigen, EpCAM, highly expressed in human colorectal cancer cells (SW480) were expressed in *Arabidopsis* plants, which have high total soluble protein levels (19) Transgenic *Arabidopsis* expressing anti-colorectal cancer mAb^{P}CO and mAb^{P}COK (CO and COK, respectively) were obtained via *Agrobacterium*-mediated transformation. The expression and amount of purified mAb^{P}COK were approximately two times higher than that of mAb^{P}CO (4, 24). We hypothesized that the ER retrieval motif enabled retention of proteins in the intracellular organelle of plant cells, resulting in higher expression levels. These results were consistent with previous reports, indicating that production levels of recombinant mAbs and vaccines tagged with the KDEL ER retention signal were significantly higher than those tagged without KDEL and the high mannose *N*-glycan structure (4, 5, 12, 20, 25, 26).

The relative binding affinity of each mAb to target intact SW480 cells from the EpCAM positive cell line was quantitatively analyzed via Cell ELISA. Compared to mAb^{M}CO, mAb^{P}CO showed a slightly weaker binding affinity to the target cancer cell. Whereas mAb^{M} bound to entire cells, mAb^{P} mainly bound

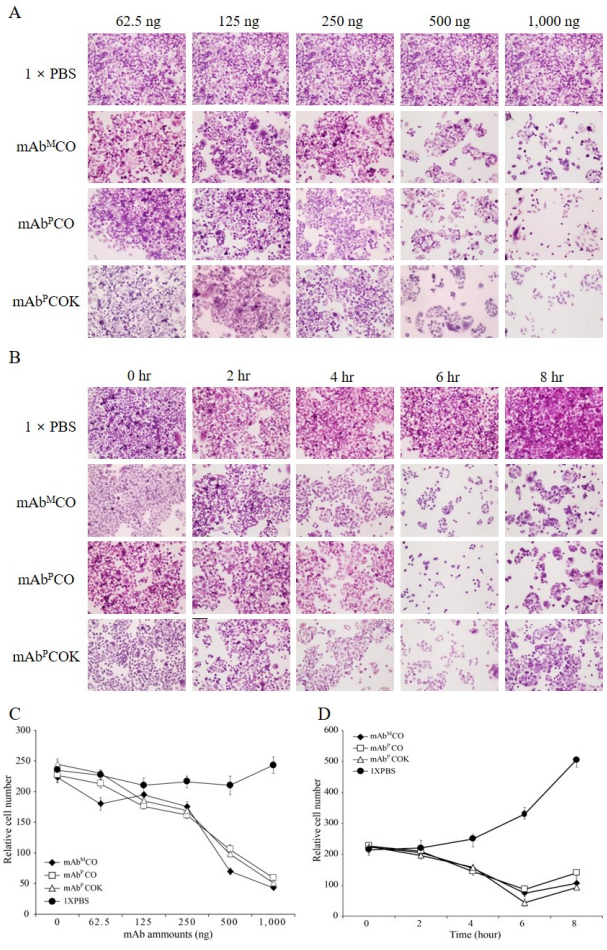

Fig. 4. Dose and Time-dependent effects of $mAb^{M}CO$, $mAb^{P}CO$, and $mAb^{P}COK$ on human colorectal cancer SW480 cell growth. SW480 cells (1×10^5 cells per well) were seeded on the coverslips of 24 well cell culture plates. To investigate (A) the dose-dependent and (B) time-dependent effects of antibodies, $mAb^{M}CO$, $mAb^{P}CO$, and $mAb^{P}COK$ (1,000, 500, 250, 125, and 62.5 ng) were applied to the SW480 cells. (A) Antibody of $mAb^{M}CO$, $mAb^{P}CO$, and $mAb^{P}COK$ (1,000, 500, 250, 125, and 62.5 ng) were applied to the SW480 cells at concentration of 1,000, 500, 250, 125, and 62.5 ng. (B) $mAb^{M}CO$, $mAb^{P}CO$, and $mAb^{P}COK$ were added to each well, where SW480 cells were seeded (1×10^5 cells per well) and incubated for 2, 4, 6, and 8 hr. Positive control was used $mAb^{M}CO$. The cells were incubated under conditions of 37°C and 5% CO_2, for 2 hr. Positive control used was ^{M}CO, respectively. Two randomly selected areas of each plate were photographed, and the cell numbers were counted. The slides were observed under a microscope [magnification, ×200; BX53F (Olympus)]. (C) The relative SW480 cell number was counted after adding serially diluted antibodies to each well at 2 hr. These experiments were performed in duplicate. (D) Relative cell numbers were determined after incubating for 2, 4, 6, and 8 hr with the antibodies mentioned above. These experiments were performed in duplicate, and error bars are shown on the graph.

to the surface membrane of cells. These results were anticipated since the regions of epitopes that mAb^{M} and mAb^{P} bind to may be different. The origin sequences of $mAb^{P}CO$ and $mAb^{P}COK$ were specifically selected to recognize the extra cellular region of EpCAM proteins. The $mAb^{M}CO$ (Anti-EpCAM antibody) recognized the epitopes of both extracellular and intracellular EpCAM proteins.

Cell regression assay indicated that the intact SW480 tumor cell population was efficiently decreased in a dose- and time-dependent manner in the $mAb^{P}CO$ treatment groups, as well as in the $mAb^{M}CO$ treatment groups. These observations suggest that interaction between the target antigen, EpCAM, and antibodies may induce apoptotic signaling to cancer cells without the addition of complement and serum (29, 30). Therefore, these assays may help determine optimal dosages and administration frequencies required for efficient anti-cancer therapy.

In summation, our research indicates that *Arabidopsis* may be an alternative platform for producing therapeutic proteins such as antibodies and vaccines because these proteins demonstrate a level of biological efficacy that is very similar to that of mammalian-derived monoclonal antibodies.

MATERIALS AND METHODS

See Supplementary information.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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